CONTROLE INTEGRE DE LA QUALITE DANS L'AMELIORATION GENETIQUE DU PORC

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RÉSUMÉ

Les saisies de cœur, foie et poumons opérées dans le cadre de l 'abattage de plus de 30.000 ont été étudiées. Une étude statistique descriptive, suivie d 'une analyse de l 'association entre les divers types de saisies et la relation entre les pourcentages de saisies et les caractères de production ont été réalisées.

L 'impact économique des saisies et donc d 'un mauvais niveau sanitaire dans les exploitations d 'engraissement a ainsi pu être estimé.

La saisie des poumons entraîne une augmentation de 5 jours de la durée d'engraissement, ce qui correspond à une perte de plus de 100 FB/porc, en ne comptant que les coûts alimentaires et d'occupation des locaux. Les poumons sont saisis dans presque 10 % des abattages.

Les pathologies pulmonaires ont donc un impact considérable sur la rentabilité de la production porcine. Les saisies du cœur et du foie a également été étudiée.

1. INTRODUCTION

La production porcine en Belgique comme chez nos voisins se focalise depuis de nombreuses années sur les caractères de production tels que le Gain Quotidien Moyen, l 'Indice de Consommation ou le pourcentage de viande maigre.
Depuis quelques années l 'attention du secteur s 'est portée sur d 'autres caractères jusqu 'ici peu étudiés tel que; la résistance aux maladies; la qualité d 'un animal n 'est plus uniquement de grandir vite et bien, mais elle est aussi qualité sanitaire.
Les saisies de coeur, foie et poumons opérées dans le cadre de l 'abattage de plus de 30.000 porcs à l 'abattoir Detry à Aubel ont été étudiées. Une étude statistique descriptive, suivie d 'une analyse de l 'association entre les divers types de saisies et la relation entre les pourcentages de saisies et les caractères de production ont été réalisées.
L 'impact économique des saisies et donc d 'un mauvais niveau sanitaire dans les exploitations d 'engraissement a ainsi pu être estimé.

2. MATÉRIEL ET MÉTHODE

2.1. Données

Les données proviennent de la zone d 'expertise de l'abattoir Detry à Aubel. La base de données en plus des saisies d'organes contenait l 'identification des animaux et de leurs parents, le poids à l'abattage, la date de naissance. Ces données nous ont permis d 'estimer le GQM et l'âge à 100 kg.

2.2. Modèle mathématique

Des tables de contingence ont été construites afin d 'évaluer l 'association entre les divers types de saisies.

Afin d 'estimer l 'influence des saisies sur les paramètres de production, le modèle linéaire suivant a été ajusté:

$$\text{Age à 100 kg} = \square + \text{race} + \text{verrat(race)} + \text{éleveur} + \text{engraisseur} + \text{lignée truie} + \text{saisie cœur} + \text{saisie foie} + \text{saisie poumon} + \text{sexe} + b_1\ \text{pourcentage de viande} + b_2\ \text{pH} + e$$

Ensuite plusieurs modèles de régression logistique ont été ajustés aux données. Le modèle suivant a été choisi comme modèle de travail:

$$\text{logit}[p(\text{saisie}^*)=1] = a_0 + a_1 \text{ race du verrat} + a_2 \text{ lignée de la truie} + a_3 \text{ engraisseur} + a_4 \text{ GQM} + a_5 \text{ poids à l'abattage} + e$$

*: saisie du foie, du coeur, des poumons.

3. RÉSULTATS

Sur l'ensemble du matériel, 2,10 % des coeurs, 1,63 % des foies et 9,59 % des poumons sont saisis. Il y a 60 fois plus de chances que le foie soit saisi si le coeur l'est. Il y a 7,34 fois plus de chances de voir les poumons saisi si le coeur l'est. Si le foie est saisi, les poumons ont 52 fois plus de chance de l'être.

La saisie des poumons ou du coeur allonge respectivement de 3 et 5 jours la durée d'engraissement, la saisie du coeur et du foie de 8 jours et la saisie des trois organes de 10 jours.

L'engraisseur influe significativement sur les pourcentages de saisies, ainsi que la race du verrat et la lignée de la truie.

4. DISCUSSION

Les associations entre saisies sont assez fortes, une mauvaise hygiène augmente aussi bien les problèmes infectieux que parasitaires. Cette étude met en évidence l'impact économique des saisies. La saisie des poumons à elle seule, augmente de 5 jours la durée d'engraissement, ce qui correspond à une perte de plus de 100 fb par porc, en ne comptant que les pertes alimentaire et d'occupation des locaux.

La race du verrat et la lignée de la truie ont une influence significative sur le pourcentage de saisies, ce qui invite à penser à la présence d'une variation génétique et donc de possibilité de sélection. L'établissement d'engraissement influe également sur le pourcentage de saisie, cette influence résulte du niveau d'hygiène dans l'exploitation.

5. REMERCIEMENT

Cette étude a été rendue possible grâce au soutient du Ministère de l'Agriculture et de la Ruralité de la Région Wallonne.

THE BELGIAN PCB/DIOXIN INCIDENT : ANALYSIS OF THE FOOD CHAIN CONTAMINATION AND HEALTH RISK EVALUATION

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INTRODUCTION

In January 1999, Belgium was the theatre of an unprecedented food crisis caused by the contamination of animal feeds with polychlorobiphenyls (PCBs) and dioxins (PCDD/Fs). The first signs of contamination were observed early February in several poultry farms. The first symptom was a sudden drop in eggs production, followed a few weeks later, by a marked reduction in egg hatchability, a reduced weight gain and an increased mortality of chicks. These birds presented ascites, subcutaneous edema of the neck and neurological disturbances (ataxia). Histology revealed degenerative changes of the skeletal and cardiac muscles. Such lesions resembled the classical manifestations of the " chick-edema disease " which was described in the 1950s-70s in outbreaks of poultry poisoning by polyhalogenated hydrocarbons (1).

These observations led to suspect dioxins as the causative agent, an hypothesis which was confirmed in April when exceptionally high levels of dioxins (more than 1,000 pg TEQ/g fat) were found in feed, meat and eggs of affected poultry. In June, additional analyses revealed that these dioxins, as suggested by their patterns, originated from a contamination by PCB oil accidentally introduced in a tank of recycled fats used in the manufacture of animal feeds. When it appeared that the contaminated fat could have been sold to nine manufacturers of animal feeds which in turn supplied a total 2,500 farms, it became impossible to trace the contamination in the Belgian food chain. This prompted the Belgian authorities to implement a large PCB monitoring program to identify contaminated foods. Initially this program was intended to detect contaminated products from suspected farms but rapidly it was extended to all farms over the country as a result of the embargo imposed on all Belgian food products. At the end of 99, the database contained the results of more than 50,000 PCB and 500 dioxin analyses performed on virtually all types of food products.

In the present study, we analyzed the levels and the patterns of PCBs and dioxins in animal feeds and in food products which were effectively contaminated (poultry, pigs) or suspected of having been contaminated by the incident (bovines). Using the PCB/dioxin fingerprints and the PCBs:dioxins ratio to trace the contamination in the food chain, we determined the time course of the incident, the source and amount of PCB oil as well as the transfer of the contamination into farms animals and their products. By estimating the fraction of the food chain really affected by the contamination, we then assessed the likelihood this incident may have increased the body burden of the general population.

RESULTS

Levels of PCBs and PCDD/Fs in the contaminated food chain

Table 1 gives the geometric means and ranges of PCB and dioxin concentrations in the most contaminated samples discovered after the PCB incident. Samples of chicken feed produced end of January showed the highest levels of contamination, with PCB and dioxin concentrations reaching 450 µg/g fat and 11,140 pg TEQ/g fat, respectively. The PCB concentrations in poultry products were at least one order magnitude lower with means ranging from 0.84 to 8.2 µg/g fat and maximal values between 1.63 and 56.8 µg/g fat. The mean dioxin concentrations in poultry products ranged from 3.8 to 255 pg TEQ/g fat and the maximal values from 19.6 to 2,613 pg TEQ/g fat. The highest concentrations of PCBs (> 50 µg/g fat) and dioxins (> 1,000 pg TEQ/g fat) were found in birds with signs of intoxication (reduced fertility and deformed chicks). In pig farms no sample of the originally contaminated feed manufactured in January could be retrieved, explaining why in Table 1 concentrations of PCBs appear lower in pig

than in poultry feed. Pig farms however have presumably received feeds as contaminated as feeds delivered to chicken farms as evidenced by the PCB concentrations in pig and poultry animals. Surprisingly, despite similar levels of PCBs, pigs showed much lower dioxin concentrations than chickens, the highest values not exceeding 40 pg TEQ/g. No abnormality which could be related a PCB contamination was observed in pig farms. In the bovine livestock, the few samples positive for the PCB test corresponded to meat of dairy and culled cows. The concentrations of PCBs and dioxins in these samples were however much lower than in contaminated pig and poultry animals. In milk, PCB and dioxin concentrations were still lower and were within the range of normal background levels in Belgium and Europe. These observations indicate thus that dairy cows and dairy products were spared by the incident. The concentrations of dioxin-like PCBs were determined in five contaminated samples, two of eggs (dioxin values : 53 and 18 pg TEQ/g fat) and three of chicken feed (dioxin values : 301, 288 and 315 pg TEQ/g fat). The addition of dioxin-like PCBs increases the TEQ values of the egg samples on average by 5.3 and that of feeds by 2.9.

*PCBs and PCDD/Fs in animal feeds***.** The PCB contamination peaked in feeds produced end of January attaining a mean PCB level of about 300 µg/g fat. Between February and March 1999, PCB level in feed rapidly dropped and mid-April, no sample of feed exceeded the PCB tolerance level of 1 µg/g fat. During this period, PCB and dioxin profiles in all contaminated feeds delivered to chicken or pig farms were remarkably similar. It is also interesting to note that the proportions of the PCB 52 and 101 congeners were fairly constant. Since these two congeners are labile in the livestock, this allowed us to rule out the possibility of secondary contamination due to the recycling of fats from contaminated animals. The concentrations of dioxins and PCBs in feeds were very closely correlated, with an average PCBs:dioxins ratio of about 50,000. No difference was found between poultry and pig feeds in their mean PCBs:dioxins ratio. The PCB profiles in poultry and pig feeds were matched to a mixture of Aroclors 1260/1254 (or of commercial PCBs with similar compositions) in the proportion of 75/25 %. Because of its high content in penta- and hexa-chlorinated congeners (more than 60 %), this mixture should have a resinous/waxy appearance, similar to that of a frying oil.

Amounts of PCBs and dioxins at the origin of the contamination. The total amounts of PCBs (sum of 7 markers), dioxins (17 PCDD/Fs congeners) and of dioxin-like PCBs accidentally mixed with the recycled fat in January were estimated at about 50 Kg, 1 g TEQ and 2 g TEQ, respectively. These figures were obtained by extrapolating the mean concentration of PCBs and dioxins in the fat of the most contaminated poultry feeds (end of January) to the total volume of contaminated fat (60 tons), assuming a two-fold dilution of the original PCB and dioxin concentrations with the vegetable fat used in the production of feeds. For estimating the amount of dioxin-like PCBs, we used the ratio of three as derived from the analysis of three contaminated feed samples (see above). Since the sum of the seven PCB markers represents theoretically 30 % of the total weight of all PCB congeners in a mixture 75/25 % of Aroclors 1260/1254 (or of commercial PCBs with similar compositions), we estimated the total amount of PCBs at the origin of the incident at approximately 150 kg (sum of all congeners), which corresponds to a volume of about 100 liters of PCB oil (density 1.60 g/cm3).

Poultry. The patterns of PCBs were very consistent in all poultry products, the fingerprint being transferred almost unchanged from hens to eggs and from eggs to chicks. Compared to the original pattern in feed, the patterns in poultry were characterized by the disappearance of the PCB 52 and 101 congeners as a result of the preferential biotransformation of these lower chlorinated congeners. The PCDD/Fs patterns in poultry feed, chickens, hens, chicks and brooded eggs were almost indistinguishable, indicating a very limited biotransformation of dioxins in these birds. As for PCBs, the dioxin patterns were transferred almost intact from the hens to the eggs and then to the chicks. Laying hens and consumption eggs presented by contrast quite different dioxin profiles with a predominance of OCDD and OCDF. These profiles, especially those in consumption eggs, exhibited a typically environmental fingerprint which was unaltered by the PCB incident. The concentrations of dioxins and PCBs in poultry meat and in eggs were highly correlated, with slopes or PCBs:dioxins ratios almost identical to that in feeds. These correlations allowed to validate the PCB test used to trace the contamination in the food chain.

Pig products. By comparison with the pattern in the contaminated feed, the metabolic alteration of PCB patterns in pigs was still more pronounced than in poultry, leading to the disappearance of PCB 118 in addition to the PCB 52 and PCB 101 congeners. Of the seven PCB markers measured, only the three most chlorinated congeners were still present in significant proportions in pig meat. The dioxin patterns in pigs were very different from that in feeds, containing only a few persistent congeners (OCDD and 1,2,3,4, 7, 8- HxCDF). If one compares these profiles with

those reported in the literature in pigs exposed to the background pollution, the only difference reminiscence of a PCB contamination was the presence of HxCDF in a relatively high proportions. The concentrations of dioxins and PCBs in pigs were significantly correlated but through a non-linear relationship contrasting sharply with the perfectly linear relationships observed in contaminated feeds and in poultry products. The concentration of dioxins in pig meat increased with that of PCB up to value PCB level of 1 µg/g fat. Above this value, the dioxin concentrations leveled off despite very high concentrations of PCBs. As a corollary, the PCBs:dioxins ratio in pigs showed extremely variable values extending from 50,000 up to 10,000,000.

Bovine products. The patterns of PCBs in the contaminated bovine meat (dairy or culled cows) from suspected farms were similar to found in PCB-positive samples of bovine meat (dairy cows) originating from unsuspected farms. Dioxin patterns in dairy cow meat were not different between suspected and unsuspected farms and they exhibited fingerprints corresponding to the background environmental contamination. The dioxin profile characterizing the PCB incident was found only in a few meat samples from culled cows. These cows also had the highest levels of PCBs presumably because of the lack of milk production. The PCB patterns in the few milk samples exceeding the tolerance level were indistinguishable from the patterns in samples with normal PCB levels and also from the patterns reported previously for human and cow's milk in Belgium. The proportion of PCDFs in these samples was increased but this change cannot be interpreted as a reflection of PCB contamination since the same profile was found also in about 60 % of PCB-negative milk samples. At the exception of culled cow meat, the bovine livestock appears thus to have been largely spared by the PCB incident since the levels and patterns of PCBs in bovine products from suspected farms were not different from those associated with the background environmental pollution by these substances.

PCBs:dioxins ratio in the Belgian food chain. The PCBs:dioxins ratio in the PCB oil at the origin of the Belgian incident has been estimated at 50,000. This is a relatively low value reflecting an important thermal degradation of the oil. This ratio was found in all contaminated feeds delivered to pig or poultry farms, suggesting an unique source of contamination since of course biotransformation is unlikely to occur in recycled fat. By contrast, the PCBs:dioxins ratio presented substantial variations between animal species. The ratio was fairly constant in most contaminated poultry products, varying between 47,848 in chicks and 71,563 in brooded eggs. A somewhat higher ratio was observed in consumption eggs (182,238) probably because these products were unaffected by the incident. In all pig animals, by contrast, the PCBs:dioxins ratio was dramatically increased, reaching values more than to 100 times higher than that found in the feed.

Risk assessment. PCBs and dioxins are cumulative toxins which may produce toxic effects when critical concentrations are reached in target tissues. To assess the potential health risks, we have thus calculated the increase of body burden that might be achieved from the consumption of the most contaminated products observed during the incident. Of the whole database, the highest PCB concentrations in foodstuff were found in samples of poultry, and in particular in chicken meat (56.9 µg/g fat) and in chicks with edema disease (47.1 µg/g fat) (Table 1). The worst case scenario would thus consist to regularly consume foods with such levels of contamination. For a reliable estimate, we used the arithmetic means of the three highest concentrations of dioxins and PCBs found in chicken meat (50 µg/g fat and 1,000 pg TEQ/g fat, respectively). If one considers the case of a person eating 200 g of chicken (5 % fat taking into account the loss of fat during cooking), this leads to an intake of about 500 µg PCBs and 10,000 pg TEQ dioxins. Assuming that the average body burden of PCBs and dioxins by the young adult population in Belgium is respectively around 5 mg and 200,000 pg TEQ, this intake would result in a doubling of the PCB and dioxin body burden after 10 and 20 contaminated meals, respectively. Such a doubling of the body burden would mean going back to the levels of PCBs and dioxins which were those in the 80s and a further increase by a factor 3 to 4 would bring the body burden to the levels probably prevailing in the 70s or of populations eating regularly contaminated fish from polluted seas.

The probability that some individuals could have experienced this worst case scenario depends both on the duration of the contamination episode and on the proportion of the food chain really contaminated during that period. As shown by the time course of PCB levels in animal feeds, the contamination has been limited to the period of January 20 to March 15. After that time, the most contaminated part of the food chain (chickens) had probably been consumed and only pigs or their offspring could still contain elevated levels PCBs (but not of dioxins which had been largely eliminated). According to the veterinary inspector (Dr Destickere), about 40,000 reproduction hens and one million chickens were contaminated in farms affected by the incident. This estimate is in good accordance with

the maximal number of chickens (also around one million) which theoretically can be contaminated to a PCB level of 50 µg/g fat and a dioxin level of 1,000 pg TEQ/g fat with a total of 50 kg PCBs (seven markers) and 1 g dioxins (of which 80% were delivered to chicken farms). These numbers represent 2 % of the total number of chickens produced in Belgium in February-March, an estimate which fits well with the proportion of chicken farms where samples positive for the PCB test were discovered (1.98 %). Under these conditions, it appears extremely improbable that a person could have consumed these most contaminated chickens a sufficient number of time to significantly increase his body burden. The only situation in which such a scenario could be conceivable is that of individuals who would have consumed contaminated products from a single farm but even in that case, the time factor allows to exclude any serious health effects even in pregnant women who are the subjects the most at risk.

DISCUSSION

The Belgian PCB incident was almost an exact replica of the poultry poisoning episodes which repeatedly occurred in the 50s and 60s in the USA and Japan (1). Like in these earlier incidents, the alert was given by laying hens which showed a sudden drop in egg production and a few weeks later by the chicks which developed the clinical manifestations of the chick edema disease. The incident most probably would have never been detected if the contaminated fat had been used only in the production of feeds for pigs or bovines. These animals indeed did not show any sign of intoxication and the levels of dioxin in cow's milk (the only foodstuff regularly monitored for dioxins in Belgium at that time) were not increased by the incident. The possibility of a dioxin contamination was envisaged only in March after having tested unsuccessfully a series of other hypotheses. In April, when high concentrations of dioxins were discovered in dead birds and their feed, most of the contaminated poultry had already been destroyed or consumed. End of May, when the incident was revealed to the public triggering a major political and food crisis, saws and their offspring were the only animals to contain still elevated levels of PCBs. These animals, however, had already eliminated most of their dioxin body burden. Paradoxically thus, the dioxin contamination was almost over when the dioxin-contaminated food scare broke out in Belgium and spread all over Europe and even the world.

The PCB oil at the origin of the accident was inadvertently introduced in the food chain via the recycling of oils and fats collected in public containers parks, a practice which was forbidden in Belgium in June 1999. The total volume and the characteristics of this PCB oil could be determined with a certain precision thanks to the analysis of originally contaminated feed samples which could be retrieved in chickens farms. By extrapolating the PCB and dioxin concentrations in these samples to the volume of the contaminated tank, we estimated the total amount of PCBs mixed with animals feeds to about 50 kg PCBs (seven markers) or about 150 kg total PCBs, which corresponds to about 100 liters of PCB oil. The pattern of PCB congeners in the contaminated fat was matched to a mixture of Aroclors 1260/1254 (or of similar commercial PCBs) in the proportions of 75/25. In view of the widespread use in the past of a few commercial PCB mixtures, one can conceive that different sources of PCB oil could by chance present the same profiles of PCBs but it is extremely unlikely that these sources could also present exactly the same extent of thermal degradation as reflected by the PCBs:dioxins ratio. The consistency of both the PCB profiles and the PCBs:dioxins ratio in all contaminated feeds allowed us to conclude that the incident was caused by an unique source of PCB oil which peaked at the end of January and progressively resolved Mid-March 1999. The relatively slow disappearance of PCBs from animal feeds most likely results from the well-known phenomenon of carry-over and memory effect in the transportation (trucks) and production (tanks) facilities. The possibility that the contamination could be have been perpetuated by the recycling of fats from contaminated animals can be formally ruled out on the basis of the PCBs patterns in feeds which showed fairly constant proportions of the labile PCB 52 and 101 congeners during the whole contamination period.

The study of PCB and dioxins patterns revealed interesting differences in the metabolism and elimination of these compounds by farm animals. The patterns of both contaminants were remarkably preserved all along the poultry chain from the hens to the eggs and from the eggs to the chicks. The incapacity of birds to metabolize these polyhalogenated hydrocarbons probably explains the great sensitivity of their embryos which during brooding are exposed to increasing concentrations of these lipophilic pollutants. The most interesting observation however was made when comparing the fates of PCBs and dioxins in pigs. These animals which were killed more than six months after the incident had still elevated levels of PCBs but surprisingly they were almost free of dioxins. Since the PCB fingerprints and the PCBs:dioxins ratios in pig feeds were identical to that in poultry feeds, the only logical way to

explain this discrepancy was to postulate a faster elimination of dioxins by the pigs, which could manifest itself more easily as these animals or their offspring were sacrificed several months after the peak of exposure. Several observations support this interpretation : (i) the different shapes of the relationships between PCBs and dioxins between the two species. In pigs, the PCBs/dioxins relationship was not linear but evoked a saturation kinetics highly suggestive of a faster elimination of dioxins than PCBs. Since PCB mixtures (e.g. Aroclors 1254 and 1260) are classical inducers of the xenobiotics biotransformation and in particular of the glucuroconjugaison, one attractive hypothesis would be that the biotransformation of dioxins in pigs has been progressively induced by the rise of the PCB body burden, explaining the shape of the relationship between both contaminants (20); (ii) as reported by Liem and Theelen (9), the liver to fat ratio of dioxins is about one order of magnitude higher in pigs than in chickens, suggesting also a more efficient hepatic metabolism of dioxins in pigs compared to chickens ; (iii) the alterations of patterns of PCBs and dioxins were much more pronounced in pigs than in chickens, confirming again the greater metabolic potential of mammals compared to birds.

The health risk has been a matter of a hot debate in Belgium during the crisis and even after when several research teams proposed to the authorities to undertake large scale epidemiological studies on the possible health outcomes of the incident. The concern was particularly focused on pregnant women in view of the poorly characterized developmental effects of dioxin-like PCBs. The present analysis indicates that the contamination has not only been limited in time but also has affected a very small fraction of the food chain. This dispersion of the contamination in the whole food chain has made almost impossible the identification of individuals who have consumed contaminated foods. The risk assessment is also further complicated by the lack of information on the PCB and dioxin body burden of the general population of Belgium before the incident. In view of these uncertainties, we have limited the risk assessment to the estimate of the likelihood that some individuals could have increased their PCB and dioxin body by consuming the most contaminated products. We calculated that, in the worst case scenario, a doubling of the PCB and dioxin body burden could have been reached after having consumed respectively 10 and 20 meals of the most contaminated chickens. In the case of PCBs, this estimate is valid whether PCBs are expressed as total PCBs, the seven PCB markers or as dioxin-like PCBs. In proceeding with the analysis, we realize now that this scenario, which was envisaged in the heart of the dioxin crisis, was quite improbable at the population level in view of the proportion of chickens on the food market which could really be contaminated (around 2 %). Such a scenario was conceivable only for individuals like farmers who would have consumed contaminated products from a single farm. But even in this case, a doubling of their PCB or dioxin body burden would have brought them at contamination levels similar to that of fish consumers or of people who were living in the 80s. This conclusion is in accordance with other evaluations and also with the view shared by most scientists and international bodies that for cumulative toxins like dioxins or PCBs, a short excursion above the TDI like that observed in Belgium is not consequential provided the integrated dose remains largely below the critical body burden.

After the development of the bovine spongiform encephalopathy in the UK, the PCB/dioxin contamination of poultry and pig products in Belgium is the second major food crisis in Europe due to recycling practices upstream the food chains. If these practices are beneficial for the environment and the energetic yield of the agriculture, they may accidentally or chronically contaminate the food chains by a variety of polyhalogenated hydrocarbons and other persistent lipophilic contaminants. There is thus an urgent need for food safety and public health to carefully evaluate the risks inherent to such practices and to implement monitoring programs for animal feeds. Another important lesson learned from the Belgian incident is the need to carefully analyze the fingerprints of dioxins and PCBs when a case of contamination is discovered. This analysis may indeed provide crucial information to identify the source of the contamination and also to predict its transmission along the food chain and its possible impact on public health.

REFERENCES AND NOTES

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Table 1 Concentrations of PCBs and dioxins (PCDD/Fs) in PCB-positive samples originating from farms suspected of having received contaminated feeds during the Belgian PCB incident.

Type of sample	N (PCBs)	PCBs (ng/g fat) [range]	N (PCDD/Fs)	PCDD/Fs (pg TEQ/g fat) [range]
Pig feed	11	4,258 [809-14,154]	3	180.2 [73-301.4]
Poultry feed	20	14,996 [832-452,836]	12	231.5 [20.1-11,143]
Chicken	15	3,409 [1,010-56,856]	4	255.4 [15.8-2,613]
Laying hen	14	889 [234-3,868]	3	3.7 [2.6-6.95]
Chick	5	8,160 [2,721-47,101]	5	170.5 [54.4-965.4]
Hen	13	5,434 [2,549-22,637]	1	463.3
Brooded eggs	23	2,852 [510-38,890]	9	44.9 [1.0-713.1]
Whole Eggs	23	839 [515-1,631]	17	3.8 [1.2-19.6]
Pig	94	2,928 [1,188-15,080]	48	0.9 [0.03-36.25]
Young pig	9	2,957 [1,040-25,472]	4	0.8 [0.38-2.7]
Saw	60	6,688 [654-17,271]	40	2.1 [0.08-23.82]
Bovine [1]	12	487 [246-1,060]	9	5.81 [3.6-13.2]
Milk	55	25 [6-160]	54	2.07 [1.09-6.0]
TOTAL	355		209	

[1] includes dairy and culled cows.

PREVALENCE OF PARATUBERCULOSIS (JOHNE'S DISEASE) IN THE BELGIAN CATTLE POPULATION

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ABSTRACT

The national paratuberculosis seroprevalence in the Belgian cattle population was determined by a serosurvey that was conducted in the winter of 1997-1998. In a random sample of herds (N=556), all adult cattle ≥ 24 months of age (N=13,317) were tested for the presence of antibodies using a commercially available ELISA. The paratuberculosis herd seroprevalence was 18% (95% confidence interval=14-21).

The methodological calculation of the true paratuberculosis herd prevalence revealed that the test specificity has a dramatic effect on the estimation; assuming a test sensitivity of 45% and a true within-herd prevalence of 7%, the true herd prevalence estimation decreased from 36 to 0.8% if the test specificity decreased from 99.9 to 99% respectively. For this reason we augmented the herd specificity for herds with larger adult herd size (> 5). This approach resulted in our best estimation of the true herd prevalence of 6%.

SAMENVATTING

In de winter van 1997-1998 werd de nationale paratuberculose seroprevalentie in de Belgische rundveestapel geschat door een serologisch survey onderzoek. Hiervoor werden in een steekproef van toevalsgewijs gelote beslagen (N=556), alle runderen ≥ 2 jaar (N=13.317) getest voor aanwezigheid van serumantistoffen met een in de handel verkrijgbare ELISA. De paratuberculose beslag seroprevalentie was 18% (95% betrouwbaarheidsinterval=14-21).

De methodologische berekening van de ware beslag prevalentie toonde aan dat de test specificiteit de schatting in zeer hoge mate beïnvloedde; bij een test gevoeligheid van 45% en een ware binnenbeslag prevalentie van 7% daalde de schatting van de ware beslag prevalentie van 36 tot 0.8%, wanneer de test specificiteit respectievelijk daalde van 99.9 tot 99%. Om deze reden verhoogden we de beslag specificiteit voor beslagen met >5 volwassen runderen. Dit leverde onze beste schatting van de ware beslag prevalentie van 6%.

1. INTRODUCTION

Paratuberculosis (PTB), or Johne's disease, is a chronic infectious disease of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis*. It is an enzootic disease on the B List of the 'Office International des Epizooties' (O.I.E.), and is characterized by chronic, granulomatous degenerative enteritis that causes intermittent but persistent diarrhea, progressive weight loss, and eventually, death. The disease is untreatable and slowly progressive. Paratuberculosis is probably the most widespread infectious disease of domestic animals and causes important economic losses in ruminants, particularly cattle, worldwide (Buergelt and Duncan, 1978; Chiodini et al., 1984; Chiodini and Van Kruiningen, 1986; Benedictus et al., 1987). Expanded efforts to control this disease, including regulatory programs in some countries, may lead to future market restrictions.

In Belgium PTB is not a notifiable disease, and hence no official control or eradication program is executed. Vaccination has been recommended in heavily, clinically infected herds. However, vaccination precludes the serodiagnosis of PTB-infected cattle, and is administered under the authority of the Veterinary Inspection since it interferes with the diagnosis of bovine tuberculosis.

When dealing with infectious diseases, the group of animals which is of epidemiological importance in terms of the transmission and maintenance of infection - and therefore of disease control and eradication - is the herd (Thrusfield, 1995). This is particularly true for PTB because the control and eradication measures implicate the herd - not the animal - (O.I.E., 1998). Therefore, in this survey, the sampling units were defined as the cattle herds.

To date, few methodological serological surveys have been organized to estimate the PTB prevalence at the regional or national levels. Moreover, these surveys are affected by the nature of the study design (sample or census surveys), the study population (subclinical or clinical), the type of prevalence parameters studied (herd, individual animal or within-herd prevalence), the diagnostic test used, and the age of the tested animals. Comparison of these survey results is virtually impossible. Moreover, only very few studies adjust the seroprevalence for factors such as test sensitivity and specificity to calculate the true prevalence, creating further difficulty in comparison across studies.

Estimations of the herd seroprevalence in the USA range from 50% in Wisconsin (Collins et al., 1994) to 74% in Missouri (Thorne and Hardin, 1997) for dairy herds. In Louisiana beef herds, Turnquist et al. (1991) found a herd seroprevalence of 30%, whereas Thorne and Hardin (1997) estimated it to be 40%. At the individual-animal level, serological surveys results range from 7.3% in Wisconsin (Collins et al., 1994) to 17.1% in Florida (Braun et al., 1990) for dairy cattle, and from 1.2% in Finland (Hintikka, 1998) to 25.2% in Texan beef cattle (Alexander et al., 1993). In Belgium, a regional survey in Southern Belgium found 12% of the cattle seropositive to PTB (Vannuffel et al., 1994).

Published estimations of the true prevalence are only available for dairy herds. The true herd prevalence is estimated to range from 1.3% in England, United Kingdom (Cetinkaya et al., 1998) to 34% in Wisconsin (Collins et al., 1994). The true individual-animal prevalence in dairy cattle is estimated to range from 4.8% in Wisconsin (Collins et al., 1994) to 6.1% in Ontario, Canada (NcNab et al., 1991). Hardly any data exist on the true within-herd prevalence of PTB. Estimations range from 5% (Obasanjo et al., 1997) based on whole herd examination by fecal culture, to 20% based on sample surveys by absorbed ELISA (Collins et al., 1994).

To investigate PTB prevalences in the Belgian adult cattle population, a pilot survey was conducted from December 1997 to March 1998 in all the provinces of Belgium. The goal of this survey was first to provide an unbiased estimate of the national herd-level seroprevalence of *M. paratuberculosis* infected dairy, mixed and beef herds, by random selection of herds to sample, and second to calculate the true national PTB herd prevalence.

2. MATERIAL AND METHODS

2.1. Survey design

The survey was organized using the co-ordinates for the cattle herds registered in SANITEL-Cattle, the central computerized database for the identification and registration of the Belgian cattle population (Ministry of Small Enterprises, Traders and Agriculture, Belgium). SANITEL-Cattle constitutes a permanent basis for efficient organized disease control. By law, all Belgian cattle keepers have to be registered in SANITEL-Cattle and have the duty to report all the necessary data that are needed for making up their herd and cattle-movement inventories. This information is updated daily in SANITEL-Cattle by the Regional Veterinary Investigation Centers. In SANITEL-Cattle, a herd is defined as a stock of cattle kept in a geographical entity - containing one or several buildings with adjacent premises - that makes up a clear and distinct unit on the basis of epidemiological bounds set by the Veterinary Inspection. Therefore, in this survey, the sampling units were defined as these cattle herds.

The survey was conducted on herds of all types from December 1997 to March 1998. A stratified random sample design was followed. The total number of herds to be sampled was set at 1% of the total number of Belgian cattle herds. The sample was stratified by province. The number of herds to be sampled in each province was determined by proportional allocation (Thrusfield, 1995). Herds were randomly selected from SANITEL-Cattle using a software random generator function of Visual Basic 3.0 (Microsoft Corp., 1993). In the selected herds, all of the adult herd, i.e. all cattle over 24 months of age were blood sampled. A herd was defined to be PTB-seropositive if at least one PTB-seropositive adult bovine was present.

2.2. Collection of samples and herd and management characteristics

The blood samples were taken by the veterinary practitioners and sent to the Veterinary and Agrochemical Research Center. The age of the cattle was known by the SANITEL-Cattle herd inventories. By means of a questionnaire, the veterinary practitioners also interviewed the farmer concerning the following herd and management characteristics: herd type (dairy herd, mixed herd or beef herd), herd size (number of cattle on the premises), whether the farmer vaccinated yearlong against PTB, and whether there was historical evidence of PTB (previous diagnosis and/or clinical signs).

2.3. Serological testing

The serum samples were tested for antibodies to *M. paratuberculosis*, using a commercially available Absorbed ELISA (HerdChek®, IDEXX, France). All samples were tested using one batch of test kits, according to the manufacturer's instructions. Sera with corrected optical density (OD)-values < 0.2 and ≥ 0.3 were considered negative and positive, respectively. Intermediate OD-values were considered doubtful and classified as negative in the data analysis.

2.4. Data analysis

The inclusion criteria were as follows: (1) the samples had to be obtained from adult cattle; (2) the samples had to be obtained from herds that never vaccinated against PTB. Data originating from herds with all sampled cattle outside the required age category or that ever vaccinated against PTB were excluded from the analysis. The prevalences were analyzed per herd type to allow comparison with other published PTB prevalence figures. Data originating from herds without herd type specification were excluded from the analysis.

2.5. Statistical methods used to calculate the true herd prevalence for unvaccinated herds

The overall and herd type specific true within-herd prevalence (TPWH) were estimated based on the survey results from the PTB seropositive herds, assuming that non-reactor herds were non-infected. This consisted in calculating the median of the estimations of the TPWH for each of the PTB seropositive herds. The TPWH for each of the PTB seropositive herds was estimated according to the standard equation of Marchevsky (1974).
The true individual-animal prevalence (TAP) was calculated according to:

$$TAP = \frac{\sum_{i=1}^{n} d_i}{N}$$

whereby d_i is the number of infected animals that was estimated for each seropositive herd by multiplying the sample size by the TPWH, and whereby N was total number of adult animals held in the unvaccinated herds.

Estimation of the true herd prevalence of *M. paratuberculosis* infection should incorporate factors, such as test sensitivity and specificity, true within-herd prevalence, sample size and the cut-off number of reactors required to call a herd truly positive, that lead to uncertainty in the observed herd prevalence (Martin et al, 1992). First, the following assumptions found in the literature concerning the intrinsic properties of the absorbed ELISA were made: an overall diagnostic test sensitivity (SENS) and specificity (SPEC) of respectively 45% and greater than 99% (Collins, 1996). Second, true within-herd prevalence (TPWH) for each of the PTB seropositive herds was estimated as described above. Third, the infected herd detectability (IHD) was calculated based on the following exact probabilities formula (Boelaert et al., 2000):

$$IHD = 1 - [(1-SENS)^{m \times TPWH} * (SPEC)^{m \times (1-TPWH)}]$$

whereby m is the median sample or adult herd size because all adult cattle present were sampled.

This formula is the equivalent of the Herd Sensitivity formula developed by Martin et al. (1992), adapted for sampling of all adult animals present in the herds. The overall and herd-type-specific IHD were calculated as the median IHD of the PTB seropositive herds and the median of the seropositive dairy, mixed and beef herds respectively. Fourth, the herd-level specificity (HSPEC) was calculated according to Martin et al. (1992);

$HSPEC = (SPEC)^m$

whereby m is the median sample or adult herd size because - as for the IHD - all adult cattle present were sampled.

Fifth, based on the calculated IHD and HSPEC, the true herd-level prevalence was estimated according to the standard equation of Marchevsky (1974). The IHD, the HSPEC, and herd true prevalence were also estimated according to a range of test sensitivities and specificities of respectively 25 - 55% and 99.0 - 99.9%.

Apart from the above method of true herd prevalence calculation, we used another approach to augment the HSPEC for herds with more than 5 adult cattle since for these herds the HSPEC drops below 95% if the SPEC is 99% (Martin et al. 1992). This approach consisted in increasing the cut-off number of positive cattle required to classify a herd truly positive, as described by Jordan (1996), and adding herds with one positive test result if there was historical evidence of PTB (previous diagnosis and/or clinical signs) on the farm.

3. RESULTS

3.1. General features of the target and study population

In 1997 there were 3,242,600 cattle and 58,811 cattle herds in Belgium. The average herd size was 55.
The sample consisted of 594 randomly selected herds (Table 1). There were 83 nonresponding herds (14% of the 594 herds) from which no samples were received and for which no replacement occurred either. The major reasons for no response were: (1) the farmer had ceased his activities (53 herds, 8.9%); (2) no adult cattle were present (26 herds, 4.4%); and (3) no cattle were blood sampled in due time, due to lack of coordination between different project partners (4 herds, 0.7%). A total of 14,699 adult cattle from 511 herds (86.0%) were tested for *M. paratuberculosis* during this survey. There were 47 tested herds that did not meet the inclusion criteria for data analysis. The reasons for this were: (1) no complete information was available about the PTB vaccination scheme (29 herds, 4.9%); (2) no questionnaire was send in (14 herds, 2.4%); and (3) the herd was vaccinated against PTB (4 herds, 0.7%). A total of 13,317 adult cattle from 464 (78.1%) herds that did not vaccinate against PTB, met the inclusion criteria. The median, the average and the range of the herd size were 38, 55, and 1-326. The study population was made up of 98 (21%) dairy herds, 101 (22%) mixed herds and 259 (56%) beef herds. At the animal-level the total numbers of animals held in dairy, mixed and beef herds were 7,775 (31%), 9,137 (36%) and 8,303 (33%), respectively. The median and the range of the herd size of herds were: 81, 2-238 for dairy herds; 72, 4-252 for mixed herds; 14, and 1-326 for beef herds.

Table 1. National seroprevalence of paratuberculosis in Belgium, 1998

	Number of herds		Number of cattle ≥24 months of age	
	N	%	N	%
Total [a]	58,811		3,242,600	
To be sampled	594	100	15,635	100
Actually sampled	511	86	14,699	94
Actually sampled non-vaccinated herds	464	78	13,317	85
Actually sampled non-vaccinated herds, with herd type specification	458	77	13,150	84
PTB seroprevalence, [95% CI [b]]	82	18 [14.2 , 21.1]	116	0.87 [0.71 , 1.03]

[a] SANITEL-Cattle, 1997. Ministry of Small Enterprises, Traders and Agriculture, Belgium
[b] confidence intervals

3.2. PTB seroprevalence in unvaccinated herds

The PTB overall herd and individual-animal seroprevalence (95% confidence interval) for unvaccinated herds were respectively 18% (14.2-21.1) and 0.87% (0.71-1.03) (Table 1). The distribution of the herd test results is depicted

in Table 2. Seventy three percent of herds testing positive (60/82) had only one single positive test result. The overall median (quartiles) and average within-herd seroprevalence were respectively 2.9% (1.6-5.6) and 7.1%. The frequency distribution of the PTB within-herd seroprevalence is shown in Figure 1. Of the positive herds, 90% had a maximum within-herd seroprevalence of 10%. The herd-type-specific seroprevalence parameters are summarized in Table 3.

Table 2. Distribution of test results of non-vaccinated herds with adult cattle seropositive to paratuberculosis in Belgium, 1998

a. Number of adult cattle tested for *M. paratuberculosis* per herd :

Number of cattle sampled	Number of herds	
	N	%
5 or fewer	129	27.8
6-25	132	28.4
26-50	106	22.8
51-75	57	12.3
76-100	26	5.6
101-250	14	3.0
Total	464	100

Average: 29; minimum:1; first quartile:5; median:19; third quartile:43; maximum:213.

b. Number of *M. paratuberculosis*-seropositive adult cattle per herd :

Number of test positive cattle	Number of herds	
	N	%
0	382	82.3
1	60	12.9
2	17	3.7
2	2	0.4
4	1	0.2
5	1	0.2
6	0	0.0
7	1	0.2
Total	464	100

Average: 1.4; minimum:1; first quartile:5; median:1; third quartile:2; maximum:7.

Table 3. National seroprevalence of paratuberculosis in non-vaccinated herds, per herd type, Belgium, 1998

	Within-herd seroprevalence (%)		Individual-animal seroprevalence		Herd seroprevalence	
	median (quartiles)	average	N	Npos	N	Npos
Dairy herd	2.2 (1.7-4.9)	3.3	4,497	52 (1.16%)	98	31 (32%)
Mixed herd	2.9 (1.7-4.7)	4.4	4,643	40 (0.86%)	101	30 (30%)
Beef herd	4.2 (1.1-17.5)	18.4	4,010	21 (0.52%)	259	18 (7%)

3.3. PTB true prevalence

As a randomized survey design was followed, and adult cattle of 24 months of age or older were sampled, a test sensitivity of 45% and a test specificity of 99% were assumed, according to Sweeney et al. (1995) and Sockett et al. (1992).

The overall median (quartiles) and average within-herd prevalence were respectively 7% (4-12) and 13%. The overall frequency distribution of the PTB true within-herd prevalence is shown in Figure 1; of the positive herds, 93% had a maximum true within-herd prevalence of 30%. The true overall individual-animal prevalence was 2%.

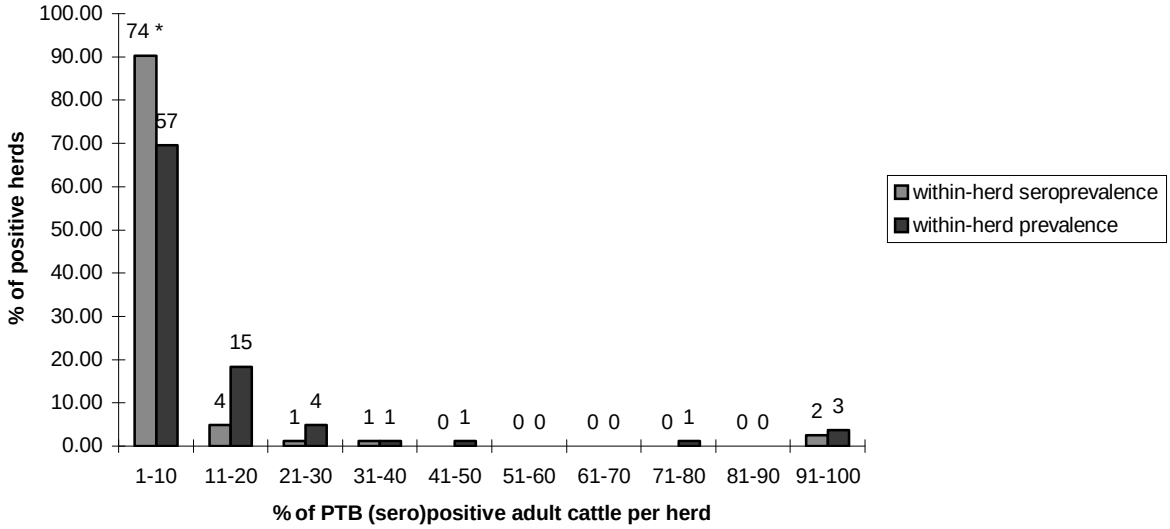

Figure 1. Frequency distribution of the within-herd seroprevalence and prevalence of adult cattle seropositive to paratuberculosis in Belgium, 1998 [a]

* Number of positive herds
[a] Assuming a test sensitivity of 45%, and a test specificity of 99%,

Based on the aforementioned parameters, the overall IHD and the overall HSPEC were both 83%. Consequently, the overall true herd prevalence was 0.8%.

Figure 2 depicts the calculated IHD, HSPEC, and herd true prevalences according to a range of test sensitivities and specificities of respectively 25 - 55% and 99.0 - 99.9%, a median adult herd sample size of 19 animals, and a PTB overall true within-herd prevalence of 7%. It shows that, for a test sensitivity of 45%, the true herd prevalence estimation decreased from 36 to 0.8% if the test specificity decreased from 99.9 to 99% respectively.

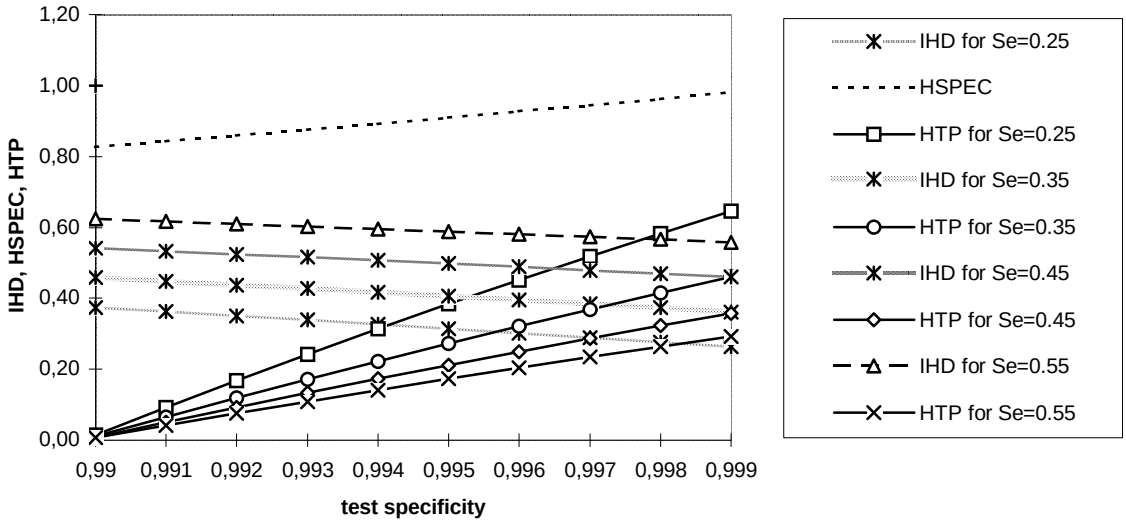

Figure 2. Sensitivity analysis of the paratuberculosis infected herd detectability, herd specificity and herd true prevalence in non-vaccinated herds, Belgium 1998 [a]

[a] Assuming a true within-herd prevalence of 7%

The alternative approach consisting in using the cut-off of two positive test results, led to a true herd prevalence estimation of 4.7% (22/464) (Table 2). In the group of 60 herds with only one positive test result there was one herd with historical evidence of PTB, and five other herds with small adult herd size (≤ 5). Consequently, the estimated true herd prevalence is 6% (28/464).

The herd-type-specific true prevalence parameters are summarized in Table 4.

Table 4. Estimates of the national prevalences of paratuberculosis in non-vaccinated herds, per herd type, Belgium, 1998

	Within-herd prevalence (%) [a]		True individual-animal prevalence [a]			True herd prevalence (%) [b]	
	median (quartiles)	average	N	Npos		N	Npos
Dairy herd	5 (4-11)	8	4,497	209	(5%)	98	10 (10%)
Mixed herd	7 (4-10)	10	4,643	159	(3%)	101	11 (11%)
Beef herd	9 (3-36)	27	4,010	76	(2%)	259	7 (3%)

[a] Assuming (1) a median sample size of 46 for dairy herds; 39 for mixed herds; and 6 for beef herds, (2) a test sensitivity of 45%, and a test specificity of 99%, and (3) non-reactor herds non-infected,
[b] Including herds with ≥ 2 positive animals, herds with one positive test result if there was historical evidence of PTB (previous diagnosis and/or clinical signs) on the farm, and herds with small adult herd size (≤ 5) with one positive test result

4. DISCUSSION

4.1. PTB seroprevalence

The present survey aimed to provide an unbiased estimate of the true national PTB herd prevalence by random selection of herds to sample. Because the percentage of nonresponding herds was low (14%), this random sample of herds can be considered to be representative for the Belgian cattle population (Thrusfield, 1995). This was also evidenced by the fact that there was no difference in the average herd size of the target and study population.

When comparing the apparent prevalences to published figures of seroprevalence, the following observations can be made. The average Belgian PTB within-herd seroprevalence of dairy herds, 3.3% was lower than the average figure of 20% found by Collins et al. (1994). At the individual-animal level, the Belgian figures for dairy and beef cattle, 1.16 and 0.52% respectively, are lower than analogue figures for other countries published so far, ranging from 7.3% in Wisconsin (Collins et al., 1994) to 17.1% in Florida (Braun et al., 1990) for dairy cattle and from 1.2% in Finland (Hintikka, 1998) to 25.2% in beef cattle in Texas (Alexander et al., 1993). Also the Belgian dairy and beef herd seroprevalences, 32 and 7% respectively, are lower than analogue figures for other countries published so far, ranging from 50% in Wisconsin, USA (Collins et al., 1994) to 74% in Missouri (Thorne and Hardin, 1997) for dairy herds, and from 30% in Louisiana (Turnquist et al., 1991) to 40% in Missouri (Thorne and Hardin, 1997) for beef herds.

4.2. PTB true prevalence

The aforementioned calculations assume a perfect test sensitivity and specificity of 100%. Because no test is perfect, the testing procedure could also have been a source of information bias.

Considering that in the selected herds all adult animals were tested, the reactor herds provided data without sampling bias for estimation of the true within-herd prevalence, compared to studies with a within-herd sample-based design. The median true within-herd prevalence of PTB seropositive herds was 7%. This estimation assumed non-reactor herds to be non-infected, which is a potential bias, because the use of tests of poor sensitivity to attempt to substantiate freedom from diseases of low within-herd prevalence is extremely difficult (Cameron and Baldoc, 1998). Consequently, the present estimated PTB within-herd prevalence, based on seropositive herds, could be an overestimation.

Corrected for testing procedures the overall true within-herd prevalence and the overall true individual-animal prevalences increased to 7 and 2% respectively. The Belgian true PTB within-herd prevalence of dairy herds, 5% was comparable with the figure of 5% found by Obasanjo et al. (1997) based on whole herd examination by

fecal culture. The Belgian true individual-animal prevalence in dairy cattle, 5%, was in line with the estimation of 4.8% in Wisconsin (Collins et al., 1994) and of 6.1% in Ontario (NcNab et al., 1991).

When true herd prevalence calculations were applied to PTB, problems arose because of the poor sensitivity of the available diagnostic tests, the low within-herd prevalence of infection, and clustering of false positives within a herd (Jordan, 1996). In the case of PTB, animals usually become infected as calves and develop clinical disease as adults several years later (Chiodini et al., 1984). Antibodies to *M. paratuberculosis* appear to occur late in the course of the infection, albeit before the onset of clinical signs. Thus, the pathobiology of PTB somewhat limits the ability of tests for serum antibodies to detect animals in the early stages of a *M. paratuberculosis* infection (Collins, 1996). The absorbed ELISA is, at present, the most sensitive and specific test for serum antibodies to *M. paratuberculosis* (O.I.E., 1996). Ridge et al. (1991) found an absorbed ELISA to have a sensitivity of 88.3% in clinical cases, and 48.8% in subclinical cases; whereas the specificity was 99.8%. Sweeney et al. (1995) showed that the sensitivity in low-level fecal shedders could be as low as 15%. Although no published data of sensitivity and specificity of the absorbed ELISA kit, used in this survey, exist, the overall sensitivity and specificity of absorbed ELISA's are considered to be respectively 45% and greater than 99% (Collins, 1996). The probability of false positives created problems in classifying seropositive herds as being infected herds, especially those with only one single positive test result. This classification problem was particularly important in this study because 73% of herds testing positive had only one single positive test result. The lack of test specificity has a dramatic effect on the estimation of the true herd prevalence; some decimal changes in test specificity result in a true herd prevalence being 2, 3 or more times higher or lower, for constant test sensitivity, true within-herd prevalence and sample size. For instance, assuming a test sensitivity of 45% and a true within-herd prevalence of 7%, the true herd prevalence estimation decreases from 36 to 0.8% if the test specificity of the absorbed ELISA decreases from 99.9 to 99% respectively. Lack of test sensitivity leads to higher estimations of the true herd prevalence, with a greater impact at higher specificity levels. The true herd prevalence calculations revealed the implications of the aforementioned parameters, as depicted in Figure 2. This sensitivity analysis showed that the practical limits of the accuracy of the used screening test jeopardize the estimation of the true herd prevalence within reasonable confidence intervals.

For this reason we used an approach that increased the herd specificity. If the herd specificity was less then 95%, we raised the cut-off number of positive cattle required, as described by Jordan (1996), and we included herds with one positive test result if there was historical evidence of PTB (previous diagnosis and/or clinical signs) on the farm. The latter was an attempt to use available information from the herds to correctly identify herds that have only one positive test as truly positive herds. If the herd specificity was at least 95%, i.e. if the adult herd size was at maximum 5, we assumed that herds were truly infected even if they had only one positive test result. Consequently, our best estimate of the true herd prevalence of *M. paratuberculosis* infection is 6%. The Belgian true dairy herd prevalence, 10%, is higher than in England, 1.3% (Cetinkaya et al., 1998) and lower than in Wisconsin, 34% (Collins et al., 1994).

This pilot study provides estimates regarding the PTB prevalence in the Belgian dairy, mixed and beef cattle population. A risk factor study considering all herd and management characteristics possibly associated with the PTB herd prevalence would be extremely beneficial.

5. ACKNOWLEDGMENTS

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ENVIRONMENTAL PCB CONTAMINATIONS

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During the dioxin- or PCB-crisis of 1999, a very large number of samples of feed, poultry, eggs, meat and milk have been analyzed for the presence of PCB. As regards bovines, only a few cases of contamination were found, and these were obviously not connected with the Verkest case. The herds were destroyed, but the problem remained : as long as the origin of the contamination on these farms was not identified, there could be no certainty that new cattle would not become contaminated too. We present here the results of our investigations on three such cases.

Warisoulx-Courrière :

In the Warisoulx case, a spot of contaminated ground was finally found, not in the vicinity of the farm, but on a pasture rented by the farmer in Courrière, some 20 km further South. Further sampling and analyses showed that the contaminated area is only a few square meters, but with spots where the soil concentration is very high : up to 4000 ng/g (dry weight) in the surface layer, and even 66000 ng/g in the sub-soil. The water-solubility of PCBs is so low that they are not taken up by the plant roots, but their slow evaporation from the soil leads to transfer through the gas phase, and contamination of the grass over an area finally much larger than that of the soil contamination. This is especially important in the spring, and, more than 30 meters away from the center of the contaminated zone, a point was found with only 10 ng/g in the soil but over 600 ng/g (dry weight) in the grass.

The origin of this contamination was clearly the transformer on a pylon at the edge of the pasture, reported to have been struck by lightning a few years ago. This was confirmed by the presence of PCB-oil on the bark of a young tree directly underneath the pylon.

The PCBs found in all those samples were almost exclusively the three heavier congeners 138, 153 and 180. This allows the use of ternary diagrams to study the finer variations in the PCB patterns in soils, grass and bovine fat. This plot shows that the (PCB138:PCB153) ratio is almost constant (1:3), whereas the PCB180 fraction varies between 25% and 75%. This is clearly a consequence of weathering through volatilization : PCB138 and PCB153 are both hexa-chlorinated, and have therefore the same molecular weight and approximate vapor pressure, whereas PCB180 is hepta-chlorinated, heavier, and four times less volatile. There is thus a tendency towards relative enrichment in PCB138 and 153 for the grass far away from the original contamination spots, and in PCB180 for the topsoils in these spots, progressively depleted in the lighter congeners.

It must be pointed out that the area where the soil is significantly contaminated is so small that finding it is like finding a needle in a haystack. Of course the grass, being contaminated over a larger area, may serve as a sampling device, but only at certain times of the year, and only if it is not mixed with too much non-contaminated grass from other areas of the pasture.

Soumagne :

In Soumagne also the PCBs found were almost exclusively the congeners 138, 153 and 180. A factory situated 500 m North-West from the farm had been accused of being responsible by using PCB-contaminated oils as fuel. One argument was that the congener pattern of a sample of smoke from that factory resembled that of some of the contaminated bovines. However this did not fit with the very localized character of the contamination, which apparently did not affect the farms situated on the other side of the factory, and thus downwind. Here again, it was finally found that the sediments in the spring bordering one of the pastures contained up to 20000 ng/g (dry weight) of PCBs. Later another spot was discovered, 1 km further South, at the foot of the slag heap of an abandoned coal mine. It was then found that, on another farm, the grass of the pastures closest to this spot also contained up to 60 ng/g (dry weight), and that the milk produced in that farm was often close to the present norm of 100 ng/g (fat).

It is not yet known whether these PCBs come from mining equipment abandoned in the slag heap, or from the illegal disposal of waste after the closing of the mine.

Again the ternary diagrams show that the PCB138:PCB153 ratio (2:3) changes little, while the PCB180 fraction varies between 20 and 60%. However the range of PCB180 fraction values is smaller than in the case of Courrière, which could indicate that the contamination is more recent, or that for some reason the weathering of the congener pattern is slower.

Ghent area :

In another case near Ghent, the origin of the contamination also seems to be local, but has not yet been found. Once again the PCBs found are almost exclusively (>90%) the three heavier congeners PCB138, 153 and 180. The PCB138:PCB153 fraction is slightly higher (1:1), and the PCB180 fraction more constant (32%) than in Courrière and Soumagne.

CONCLUSIONS

One of the difficulties involved with the search for local contaminations of the environment is that the contaminated areas are often so small that thay can be found only through luck and/or a very fine-meshed sampling program. Another is that the congener pattern is much more variable than in the Verkest case, where no weathering took place, and that therefore conclusions based on congener pattern resemblances or differences must be taken with caution. For example one of the best fits for the pattern found in the smoke from the Soumagne factory is actually the Verkest-contaminated feeds, even if there is obviously no possible connection between the two.

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TRANSMISSION OF FOOT-AND-MOUTH DISEASE VIRUS TO CONTACT SHEEP AND CONTACT PIGS: DETECTION OF INFECTED ANIMALS BY VIRUS ISOLATION AND RT-PCR-ELISA.

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ABSTRACT

Two non-vaccinated sheep were experimentally infected with foot-and-mouth disease virus (FMDV) and one day later 8 other sheep were placed in contact. Based on clinical signs, serology, viral RNA detection with a sensitive RT-PCR-ELISA and virus isolation it was concluded that only 3 out of 8 contact animals were infected. The time from exposure to onset of seroconversion in the contact sheep was longer than in the experimentally infected sheep. The presence of virus in mouth and nose swabs of the contact sheep was very rare suggesting that sheep excreted small amounts of virus. The reduced transmission rate to other sheep over time could explain the disappearance of the disease in some outbreaks in sheep flocks. Although the infective dose of FMDV excreted by contact sheep was very low, it was enough to spread the virus to contact pigs. This confirms that sheep can be important for the transmission of FMDV. These infected contact pigs excreted an infective dose of virus capable of infecting new contact sheep. The time for onset of seroconversion in these sheep was shorter indicating a larger infective dose. Interspecies contact between sheep and pigs could be an important trigger during a FMD epidemic and in an area with high pig density precipitate a major epidemic.

1. INTRODUCTION

The outbreaks of foot-and-mouth disease (FMD) in 1996 in the European countries Bulgaria, Greece, Turkey, Albania and Macedonia prove that there is a permanent risk of importing foot-and-mouth disease virus (FMDV) consequent to the more extensive and free trade promoted by the WTO and as a result of free movement of livestock within trade blocks [13]. In particular sheep can be efficient disseminators of disease. The disease is usually mild in sheep and if the animals are not closely observed can go unrecognised. The danger of this species acting as maintenance host and asymptomatic transmitter of FMD is emphasised in several reports [for an overview see 14]. Contact with infected sheep or goats is the most probable cause of the severe FMD outbreak in Tunisia 1989. However, it was noticed during the Greek outbreak in 1994 that FMD could fade out in a sheep population even without the implementation of the recommended control measures.

In a previous study [2] we have shown that contact sheep did not develop clinical signs of FMD but serology and virus recovery indicated that all sheep had been infected. The excretion of virus from these contact animals was very low and viral RNA could only be detected by a sensitive RT-PCR-ELISA for a short period of time. Virus isolation (VI) and standard RT-PCR were frequently negative, although there was some evidence that these infected contact animals were capable of infecting two sentinel pigs. The time from exposure to the onset of seroconversion in the contact sheep was longer than in the experimentally infected sheep suggesting a relationship between incubation period and amount of challenge virus.

As contact between animals is the predominant way of FMDV transmission [18] this study further investigated the transmission of FMDV to contact sheep and the transmission from contact sheep to other species, such as pigs. The latter could be of importance when subclinically infected sheep are moved into an area of high pig density. Virus genome detection was performed using RT-PCR-ELISA and FMDV was detected by VI. Additional confirmation of infection was obtained from serology and clinical signs of the disease.

2. MATERIALS AND METHODS

2.1. Experimental design and sampling

The Romanian animal facilities consists of 7 separate, disease secure units in star form accessible trough a central hall. Two non-vaccinated sheep (1 & 2) were inoculated in the epithelium of the tongue (0.6 ml), in the lip (0.4 ml) and intranasally (1 ml) with FMDV O_1 BFS at 10^7 $TCID_{50}$. At 24 h post infection 8 additional sheep (3-10) were put in contact with the experimentally infected sheep. At 3 different time intervals two of the contact sheep (groups a-c) were removed from that unit and brought into contact with two pigs of 3 months old housed in another isolation unit, following decontamination in the central hall:

- group a: 6 days post contact (dpc) sheep 8 & 9 were put in contact with pigs 5 & 6. After 6 days the pigs were removed and introduced to two non-vaccinated sheep (11 & 12), indicated as group d.
- group b: 12 dpc sheep 4 & 6 were put in contact with pigs 1 & 2.
- group c: 16 dpc sheep 3 & 5 were put in contact with pigs 3 & 4.

Samples of nasal and mouth secretions taken on cotton swabs and of blood were collected prior to infection or contact and then from 3 dpc. Samples were collected daily for 10 days and then twice a week up to the day of slaughter from groups a, b and c and in sheep 7 & 10. Group d was sampled twice a week. At every sampling the sheep were examined for clinical signs of FMD. All samples were stored at -80°C.

2.2. Sample preparation prior to RNA extraction or virus isolation

Nose and mouth swabs were hydrated in 1 ml PBS buffer and 100 µl was used for RNA extraction. Samples for VI were treated with 1,1,2-trichlorotrifluoroethane (Genetron 113, Fluka) to dissociate possible immune complexes [5]. Equal volumes of sample and Genetron 113 were blended with a X10 dispersal agent (Ystral) at 16000 rpm for 3 min on ice. After centrifugation for 8 min at 13000 g, 1% yeast extract was added to the supernatant for blocking possible toxic effects of residual Genetron 113 on cells.

2.3. Serology

Serum samples were tested for the presence of antibodies against FMDV O_1 by the liquid-phase blocking ELISA [8,7]. All positive results were confirmed by virus neutralisation test, using flat bottom microtitre plates [8] and SK-6 cells [12].

2.4. RNA extraction

Total RNA was extracted with the Purescript RNA isolation kit of Gentra Systems, Inc. as described by the manufacturer. The purified RNA thus obtained, was re-suspended in 20 µl hydration solution [3]. The quality of the extracted RNA was controlled by performing RT-PCR for actin mRNA [15].

2.5. RT-PCR-ELISA

A sensitive RT-PCR-ELISA including several controls was used as previously described in detail [2]. The digeoxigenin-labelled FMD amplicons were detected using the PCR-ELISA kit from Boerhinger Mannheim.

2.6. Virus isolation and antigen-detecting ELISA

Virus isolation (VI) was carried out by inoculating 300 µl of the pre-treated swab sample on to a monolayer of secondary foetal lamb kidney (FLK) cells [11] in 24 well plates. After 30 min incubation at 37°C the inoculum was replaced by Minimal Essential Medium (MEM). Plates were checked for cytopathic effect (CPE) from 48 h to 72 h. Positive CPE due to FMDV was confirmed by indirect sandwich ELISA [16]. All samples were tested in duplicate. In case of a negative result, 2 blind passages were performed using FLK cell suspensions.

3. RESULTS

3.1. Clinical signs

All animals had a fever of up to 40.5 - 41°C, except for pigs 3 & 4. At 3 days post infection (dpi) the experimentally infected sheep showed increased salivation and sheep 2 developed an erosion on the tongue. No clinical signs of FMD were detected on the contact sheep except for sheep 4 which showed some increased salvation and sheep 8 which developed a vesicular lesion on the upper lip at 4 dpc. Of the pigs only pigs 5 & 6 developed clinical signs of FMD. At 6 dpc these pigs showed increased salivation and disturbance of the respiration. Pig 6 developed foot lesions and lameness.

3.2. Serology

In the two experimentally infected sheep antibodies against the structural proteins could be detected, from 4 to 5 dpi. Of the contact sheep only 3 out of the 8 animals seroconverted (sheep 6 from 6 dpc and sheep 8 & 9 from 8 dpc) (Table 1 & 2). After 4 days of contact with sheep 8 & 9 antibodies were present in pig 6 and at a low level in pig 5 (Table 1). The other sentinel pigs remained seronegative. Sheep 11 & 12 developed antibodies 4 days after exposure to the infected contact pig 5 & 6 (Table 3). The results of group c and sheep 7 & 10 are not shown as all samples were negative.

3.3. FMDV-genome and FMDV detection in sheep

FMDV-genome and FMDV was found in mouth swabs of the 2 experimentally infected sheep. Viral RNA was present from 1 to 3 dpi for sheep 1 and from 1 to 6 dpi for sheep 2. FMDV was found by VI at 2 dpi for sheep 1 and from 2 to 4 dpi for sheep 2. In the contact sheep exposed to the experimentally infected sheep, virus genome and live virus could only be detected in sheep 8, 9 and 6 (Table 1 & 2). Moreover the detection was very limited and intermittent between 3 and 10 dpc. Mouth swabs were a better source of virus and viral genome than nasal swabs.

The excretion of virus from the infected contact pigs was clearly higher. In mouth and/or nose swabs from pig 5 & 6 viral RNA could regularly be detected until 15 dpc with the contact sheep (Table 1 & 3). The last positive sample was found at 36 dpc. The recovery of viral RNA was higher in nose swabs compared to mouth swabs and was only successful in one serum sample (pig 6 at 3 dpc). Live virus could only be found by VI in pig 6 at 4 dpc in a mouth swab.

In sheep 11 & 12, exposed to pigs 5 & 6, the detection of FMDV-genome was very sporadic. Only two mouth samples of sheep 12 were positive (Table 3). VI remained negative.

4. DISCUSSION

Antibody positive contact sheep without clinical signs present a danger for the transmission of FMDV and constitute a threat to the trade of animals with regions free of FMD. These animals can easily be considered as FMDV-free after examination using classical laboratory methods [2]. In this study the transmission of FMDV from experimentally infected sheep to contact sheep was studied using more sensitive techniques. It was possible to show the further transmission of the virus from these contact sheep to pigs, and then from these to sheep. In addition, the period of virus excretion was examined.

Based on a combination of clinical signs, serology, viral RNA and virus detection it was concluded that only 3 out of the 8 contact sheep became infected. This limited transmission of FMDV from two experimentally infected sheep to contact sheep suggests that a low amount of virus was excreted although the infection load was high. The number of infected contact sheep was lower than in a previous similar experiment [2] where all contact sheep became subclinically infected. Based on the lesion rates, the percentage of infected sheep in a flock can vary from 78 % [19] to as low as 7 % [1]. This variation maybe caused by a difference in pathogenicity of the virus strain [14]. In this study the sheep were infected with FMDV O_1 BFS and in the previous experiment it was FMDV O_1 Romania, which is a very similar strain. A more likely difference is that the experimentally infected animals in this study excreted a lower amount of virus and for a shorter period. Gibson and Donaldson [5] published that the percentage of infected sheep depends on the exposure dose of virus. An aerosol dose range of 1 to 50 $TCID_{50}$ is sufficient to infect 58 % of the sheep, while 100 % of the sheep are infected when the animals are exposed to more than 100 $TCID_{50}$.

The detection of the FMDV-genome or FMDV in the contact sheep brought in contact with the experimentally infected sheep was intermittent. However, viral RNA and virus was found in all the animals that seroconverted. It was noticed that the mean number of days of sequential virus excretion was reduced from 4.5 days in the experimentally infected sheep to 1.7 days in the contact sheep. The limited recovery of FMDV genome and the fact that most sheep failed to infect contact pigs, indicates that there was little FMDV transmission among the contact sheep. A further decrease in virus transmission could be expected if a second series of contact sheep in place of pigs was used as sheep excrete less virus than pigs [4] and have a low air sampling rate [17]. A decrease in the excretion of infective virus is one of the parameters that can lead to a decrease in transmission rate, reducing the basic reproductive number (R_0). R_0 must be greater than one to establish an infectious disease in a population [10]. A R_0 value < 1 could explain why a FMD epidemic in sheep could fade out, as seen in Greece during the 1994 outbreaks. Even in the lambing season the disease did not re-establish itself. However,

the severe outbreak in Tunisia (1989) causing widespread disease with high morbidity in sheep and mortality in lambs, indicates that other parameters than virus excretion play a role in the spread of a FMDV epidemic. Haydon and colleagues [9] described that exceptionally good conditions for wind-borne spread of virus during the beginning of the FMD epidemic in the UK 1976 was responsible for the high R_0 value. The parameters leading towards a widespread severe disease or a fading out epidemic in sheep require further research using larger numbers of animals.

The role of contact sheep in the spread of the disease to pigs has been investigated. In group a, sheep 8 & 9 succeeded in transmitting virus to pigs 5 & 6, although evidence of FMDV in the secretions of the contact sheep was rare. The infection of these pigs was demonstrated by the presence of clinical signs, seroconversion, viral RNA and virus detection. Unlike viral RNA detection, FMDV could only be detected in one mouth swab. In group b the contact sheep 4 & 6 failed to transmit virus to pigs. The sheep were introduced to the pigs at 12 dpc and at that moment no virus or viral RNA could be detected in the secretions of the sheep. In our previous experiment [2] there was some evidence of transmission of virus to pigs even at 24 dpc, and at that time viral RNA could still be found in nose and mouth secretions of the contact sheep. Unfortunately group c (contact sheep introduced to pigs at 16 dpc) contact sheep were not infected.

Additional confirmation of active excretion of virus from the pigs came from the transfer of virus from contact pigs 5 & 6 to contact sheep 11 & 12 (group d). The detection of virus in these sheep was at a low level. Only two mouth swabs of sheep 12 were positive for FMDV-genome although both sheep seroconverted after 4 days of contact with the infected pigs. The time from exposure to onset of seroconversion was comparable with that observed in the experimentally infected sheep and shorter than the period in the contact sheep 8, 9 and 6. There appears to be a correlation between the exposure dose of virus and the onset of seroconversion. However, this had no influence on the clinical signs nor on the frequency of virus detection. This aspect requires further study.

From these experiment it can be concluded that the detection of FMDV-genome and FMDV in contact sheep and pigs requires highly sensitive techniques performed on multiple samples and for sheep, preferably mouth swabs. It was also noticed that the frequency of virus detection decreased in time. Pigs can be infected by sheep as long as a trace of virus can be found in the sheep. The dose of virus sufficient to infect pigs appears to be lower than the dose needed to infect sheep. These infected pigs were capable of transmitting the infection to contact sheep and could therefore amplify the amount of FMDV circulating and establish an epidemic in a susceptible population.

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Table 1: Serology and detection of FMDV-genome and FMDV in two contact sheep and in two pigs. The sheep were moved in contact with the pigs 6 days post contact with the experimentally infected sheep (group a).

dpc sheep		0	3	4	5	6	7	8	9	10	11	12	16	21	26
dpc pigs						0	1	2	3	4	5	6			
sheep 8	Serology	-	-	-	-	-	-	+	+	+	+	+	+	+	+
	PCR M	-	-	-	-	+	-	-	+	-	-	-	na	-	-
	VI/ELISA M	-	-	-	-	+	-	-	-	-	-	-	na	-	-
	PCR N	-	-	-	-	-	-	-	-	-	-	-	na	-	-
	VI/ELISA N	-	-	-	-	-	-	-	-	-	-	-	na	-	-
	PCR serum	-	-	+	+	-	-	-	-	-	-	-	na	-	-
sheep 9	Serology	-	-	-	-	-	-	+	+	+	+	+	+	+	+
	PCR M	-	-	na	-	-	-	-	-	-	-	-	na	-	-
	VI/ELISA M	-	-	na	+	-	-	-	-	-	-	-	na	-	-
	PCR N	-	-	-	-	+	-	-	-	+	-	-	na	-	-
	VI/ELISA N	-	-	-	-	-	-	-	-	-	-	-	na	-	-
	PCR serum	-	-	-	-	-	-	+	+	-	-	-	na	-	-
pig 5	Serology					-			±	±	+	±			
	PCR M					-			na	-	+	-			
	VI/ELISA M					-			na	-	-	-			
	PCR N					-			na	+	+	+			
	VI/ELISA N					-			na	-	-	-			
	PCR serum					-			na	-	-	-			
pig 6	Serology					-			-	+	+	+			
	PCR M					-			na	+	+	-			
	VI/ELISA M					-			na	+	-	-			
	PCR N								na	+	+	+			
	VI/ELISA N					-			na	-	-	-			
	PCR serum					-			+	-	-	-			

dpc sheep: number of days post contact with the experimentally infected sheep
dpc pigs: number of days post contact with the contact sheep
PCR: RT-PCR-ELISA [3]
VI/ELISA: virus isolation on foetal lamb kidney cells/confirming antigen detecting ELISA
M: mouth swab
N: nasal swab na: not available

Table 2: Serology and detection of FMDV-genome and FMDV in two contact sheep and in two pigs. The sheep were moved in contact with the pigs 12 days post contact with the experimentally infected sheep (group b).

dpc sheep		0	3	4	5	6	7	8	9	10	11	12	16	21	26
dpc pigs												0	4	9	14
sheep 4	Serology	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR M	-	-	-	-	-	-	-	-	-	-	-	na	-	-
	VI/ELISA M	-	-	-	-	-	-	-	-	-	-	-	na	-	-
	PCR N	-	-	-	-	-	-	-	-	-	-	-	na	-	-
	VI/ELISA N	-	-	-	-	-	-	-	-	-	-	-	na	-	-
	PCR serum	-	-	-	-	-	-	-	-	-	-	-	na	-	-
sheep 6	Serology		-	-	-	+	+	+	+	+	+	+	+	+	+
	PCR M	-	+	-	+	+	+	-	-	-	-	-	na	-	na
	VI/ELISA M	-	+	+	+	-	-	-	-	-	-	-	na	-	na
	PCR N	-	-	-	+	-	-	-	-	-	-	-	na	-	na
	VI/ELISA N	-	-	-	-	-	-	-	-	-	-	-	na	-	na
	PCR serum	-	-	-	-	+	-	-	-	-	-	-	-	-	nd
pig 1	Serology											-	-	-	-
	PCR M											-	-	-	-
	VI/ELISA M											-	-	-	-
	PCR N											-	-	-	-
	VI/ELISA N											-	-	-	-
	PCR serum											-	-	-	-
pig 2	Serology											-	-	-	-
	PCR M											-	-	-	-
	VI/ELISA M											-	-	-	-
	PCR N											-	-	-	-
	VI/ELISA N											-	-	-	-
	PCR serum											-	-	-	-

dpc sheep: number of days post contact with the experimentally infected sheep
dpc pigs: number of days post contact with the contact sheep
PCR: RT-PCR-ELISA [3]
VI/ELISA: virus isolation on foetal lamb kidney cells/confirming antigen detecting ELISA
M: mouth swab na: not available
N: nasal swab nd: not done

Table 3: Serology and detection of FMDV-genome and FMDV in two contact pigs and two contact sheep. The pigs were moved in contact with two sheep 6 days post contact with contact sheep 8 and 9, mentioned in table 1 (group d).

dpc pigs		6	7	10	13	15	20	26	31	36	42	48	54				
dpc sheep		0	1	4	7	9	14	20	25	30	35	40	46	61	66	71	76
pig 5	Serology		-	-		-	-	-	-	-							
	PCR M		-	-	+	-	-	-	-	-							
	VI/ELISA M		-	-	-	-	-	-	-	-							
	PCR N		+	-	+	-	-	-	-	+							
	VI/ELISA N		-	-	-	-	-	-	-	-							
	PCR serum		-	-		-	-	-	-	-							
pig 6	Serology		+	+		+	+	+	+	+							
	PCR M		-	-	+	+	-	-	-	-							
	VI/ELISA M		-	-	-	-	-	-	-	-							
	PCR N		+	-	+	na	-	-	-	-							
	VI/ELISA N		-	-	-	na	-	-	-	-							
	PCR serum		-	-		-	-	-	-	-							
sheep 11	Serology	-		+		+	+	+	+	+	+	+	+	+	+	+	+
	PCR M	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	VI/ELISA M	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	PCR N	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	VI/ELISA N	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	PCR serum	-		-		-	-	-	-	-	-	-	-	-	-	-	-
sheep 12	Serology	-		+		-	+	+	+	+	+	+	+	+	+	+	+
	PCR M	-	-	+		+	-	-	-	-	-	-	-	-	-	-	-
	VI/ELISA M	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	PCR N	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	VI/ELISA N	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
	PCR serum	-		-		-	-	-	-	-	-	-	-	-	-	-	-

dpc pigs: number of days post contact of the pigs with the contact sheep 8 & 9
dpc sheep: number of days post contact of the sheep with the contact pigs
PCR: RT-PCR-ELISA [3]
VI/ELISA: virus isolation on foetal lamb kidney cells/confirming antigen detecting ELISA
M: mouth swab
N: nasal swab
na: not available

O157 AND OTHER VEROTOXINOGENIC *E. COLI* STRAINS ON BELGIAN CATTLE FARMS

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ABSTRACT

Verotoxin producing *Escherichia coli* (VTEC) strains have been responsible for outbreaks of diarrhea and hemorrhagic colitis. Two major verotoxin types, VT1 and VT2, have been described. The corresponding genes are located on lambdoid bacteriophages.
We collected bovine VTEC strains in slaughterhouses and on cattle farms. Eight selected cattle farms were repeatedly sampled. We compared human and bovine strains by studying the diversity of the toxins and the phages. We observed differences in morphology and polymorphism of phage DNA among bovine and human *E. coli* O157 phages. Different phage-specific replication genes and antiterminator genes have been identified and restriction analysis of PCR fragments revealed significant differences. These results all demonstrate a high level of genetic heterogeneity among VT-converting phages.
We also studied the *vt2* genes of VTEC strains of human and bovine origin belonging to different serotypes. A PCR-RFLP scheme was developed. Our results showed a high diversity and led to the identification of new VT2 subtypes.

SAMENVATTING

Verotoxine producerende *Escherichia coli* (VTEC) stammen zijn verantwoordelijk voor uitbraken van diarree en hemorragische colitis. Twee verotoxine types, VT1 en VT2 werden reeds beschreven. De corresponderende genen zijn gelegen op lambdoïde bacteriofagen.
In deze studie werden boviene VTEC stammen verzameld in slachthuizen en op rundveebedrijven. In 8 geselecteerde rundveebedrijven werd herhaaldelijk stalen genomen. Verotoxine coderende fagen werden geïsoleerd uit O157 VTEC stammen van boviene en humane oorsprong. Verschillen in homologie en polymorfismen in faag DNA werden geobserveerd. Daarnaast werd het genoom van de VTEC stammen geanalyseerd via PCR met een verotoxine specifieke primer in combinatie met een primer specifiek voor het faag genoom. Verschillende faag-specifieke replicatie genen en antiterminator genen werden gevonden en restrictieanalyse van de PCR fragmenten toonde eveneens significante verschillen aan. Al deze resultaten duiden op een zeer hoge genetische heterogeniciteit van de circulerende VT-converterende fagen.
Daarnaast werden ook de *vt2* genen van VTEC stammen van humane en boviene oorsprong en behorende tot verschillende serotypes bestudeerd, via PCR-RFLP. Onze resultaten toonden een grote diversiteit aan en leidden tot de identificatie van nieuwe VT2 subtypes.

1. INTRODUCTION

Verotoxin producing *Escherichia coli* (VTEC) is an important cause of gastrointestinal disease in humans (mostly young children and elderly persons) including bloody diarrhoea, hemorrhagic colitis and eventually leads to renal failure (haemolytic uremic syndrome) and death. Infection occurs via consumption of contaminated food or by direct transmission of VTEC from infected humans or animals (9). Undercooked beef, roast beef, dry minced salami and raw milk have often been implicated in foodborne infections (9, 20) but vegetables (1) and apple cider (3) can also be a source of infection.

VTEC strains produce two types of verotoxins, i.e. those that are immunologically similar to the Shiga toxin produced by *Shigella dysenteriae* (VT1) and a second group that are not (VT2) (14, 25). Severe clinical symptoms were found to be more frequently associated with *E. coli* producing VT2 than with those producing VT1 (5, 15).

The genes encoding the verotoxins in several VTEC strains are located on lambdoid bacteriophages (13). Recent studies have revealed some genomic variation within *vt*-encoding bacteriophages from *E. coli* O157 isolates (6, 22) and an even larger variation among *vt2*-encoding phages derived from different VTEC serotypes (27).

Verotoxins are composed of a single enzymatic A subunit and a pentamer of receptor-binding B subunits (15, 7). Very little DNA sequence variation has been observed for the VT1 encoding genes whereas different variants of VT2 have been described. The most prominent VT2 variants found in human clinical isolates are VT2 (12) and VT2c (24). Other variants occurring in human or bovine VTEC strains are VT2vha and VT2vhb (11), VT2d (P19), VT2f (23) and VT2vO111, VT2vOX392 and VT2vOX392 (16, 17). VT2e (4), one of the first described VT2 variants, is mainly found in isolates causing oedema disease in pigs and a human variant, designated VT2ev (8), is rarely detected in human clinical isolates.

In this study, we started a longitudinal survey on 8 selected Belgian cattle farms and checked for the presence of VTEC strain in faecal samples, in dust and in feed. To study Belgian cattle as a possible source of infection, we examined the diversity of verotoxins and of verotoxin-converting phages present in human and bovine strains.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Bovine strains were isolated during longitudinal studies or from faecal samples collected in slaughterhouses. Human clinical isolates and additional bovine isolates were selected from the collection of the VTEC reference laboratory based on the presence of *vt2* or on the O157 serotype.

2.2 Longitudinal study of cattle farms

Selected farms were visited monthly. Each time, 10 % (maximum 20) of healthy animals between 6 months and 2 years were individually sampled. In addition, available feed and dust in the stable were analysed for VTEC.

2.3 Bacteriological isolation

Faeces was homogenized in modified Trypticase Soy Broth plus novobiocin and incubated at 37° C. Enrichment culture was transferred to MacConkey agar plates. Plates were incubated aerobically at 37° C overnight. Twenty lactose-fermenting colonies that exhibited *E. coli* growth characteristics were randomly pooled (5 colonies per pool). Bacterial DNA for PCR analysis was prepared by boiling the bacterial pellet for 10 min.

2.4 Phage preparation, isolation of lysogens

To isolate spontaneously induced bacteriophages, the VTEC strains were grown overnight in LB medium without NaCl (O157 medium) and were centrifuged to spin down cells. The supernatant was filtered through a 0,2 µm filter. The presence of bacteriophages was revealed by spotting the undiluted filtrate and dilutions onto a bacterial layer of an overnight culture of *E. coli* host cells. Plaques were observed after overnight incubation at 37°C and assayed for the presence of *vt1* or *vt2* genes by PCR or plaque hybridisation.

2.5 Electron microscopy

Phages were allowed to adhere to Formvar-coated grids and were negatively stained with 1% uranylacetate before observation by transmission electron microscopy.

2.6 Isolation of genomic and plasmid DNA

Genomic DNA from the bacteria was prepared using the CTAB method (31). Plasmid DNA was prepared using the QIAGEN Plasmid Mini Kit.

2.7 PCR amplification

PCR amplifications were done on purified bacteriophage DNA and on total DNA extracted from lysogens and from the original isolates. PCR products were separated by 0,8 % agarose gel electrophoresis, stained with ethidium bromide and observed under UV light.

2.8 Endonuclease restriction of PCR products

DNA was incubated with the restriction enzyme in the buffer provided by the manufacturer (Gibco BRL overnight at 37°C. Restriction fragments were separated by 1.5% agarose gel electrophoresis.

3. RESULTS

3.1 Longitudinal study of VTEC in 8 cattle farms

The presence of VTEC strains was studied on selected cattle farms. Farms were followed for a period of several months. Six farms were contaminated with *E. coli* O157, belonging to different pathotypes. In two farms *E. coli* O157 was also isolated from feed and/or dust samples. It is not clear whether feed or dust were a source of infection on these farms. One farm contaminated with *E. coli* O103 was studied. *E. coli* O103 was isolated from faecal samples, dust and feed. We also followed a *E. coli* O145 farm. Our results show that VTEC strains belonging to different serotypes and different pathotypes are present on Belgian cattle farms. The isolated types are not animal specific but can also be found in humans.

3.2 Isolation of verotoxin-encoding bacteriophages

Twelve human and 14 bovine O157 VTEC strains were tested for bacteriophage production. Verotoxin-encoding bacteriophages could be isolated from 5 of the human strains and 2 of the bovine VTEC strains. The isolated bacteriophages were further characterized. Morphologically, the phages fell into two groups. Restriction endonuclease digestion of phage DNA provided further evidence of their diversity.

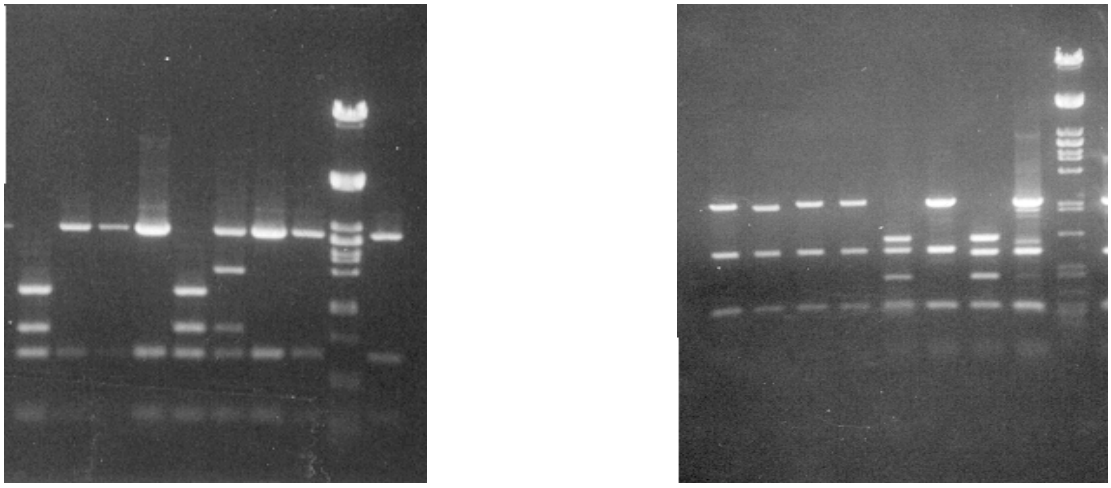
3.3 Study of the phage specific genes

As described above, plaque-forming phages could only be isolated from a limited number of strains. To further characterize the 29 human and 24 bovine VTEC strains, PCR analysis for the presence of different phage-specific regions was done. PCR was carried out using a common *vt*-specific primer and one specific for the replication genes of two types of phages. Both types of replication genes were equally present. Different sizes of amplification products were detected. Some of the strains possessed both replication genes. To determine the presence of phage-encoded antiterminator genes, a *vt*-specific primer was used in combination with respectively primers specific for the antiterminator genes from 4 different phages. Two types were frequently detected; the two other types were rarely detected. Some strains were shown to contain more than one *Q* gene, which confirmed previous results obtained for the replication gene demonstrating the presence of more then one phage in some of the O157 VTEC strains.

3.4 RFLP of other phage genomic regions

The gene *roi* is a common gene in lambdoid phages. A *vt* specific primer in combination with a *roi*-specific primer amplifies the *vt-roi* region of lambdoid phages. PCR fragments were obtained for a high number of the strains tested. Restriction analysis of the amplification product revealed different restriction profiles. The most prevalent profile is found in both human and bovine O157 VTEC strains. One profile was only observed in the bovine strains.
A second phage specific region is the region L0105-*R*. Amplification of the genomic region L0105-*R* resulted in fragments of different sizes in the majority of the strains tested. Restriction analysis revealed different patterns. Some profiles are dominant both in human and bovine VTEC strains while the prevalence of other band patterns is low. One profile was only found in bovine *E. coli* O157 isolates.

Examples of results of PCR-RFLP analysis of the *vt*-phage specific regions are given in figure 2.



A **B**

Figure 1: PCR-RFLP analysis of the regions specific for verotoxin-encoding phages.

3.5 vt2 subtyping of human VTEC strains

A *vt2* subtyping scheme allowing to distinguish different *vt2* subtypes was developed. *vt2* genes were amplified by PCR and restriction analysis with four restriction enzymes. By means of the proposed PCR-RFLP scheme the distribution of different *vt2* subtypes in a collection of human and bovine strains was studied. 37 human clinical isolates were analysed. Only 4 different *vt2* subtypes were found. The most prominent *vt2* subtypes found were *vt2* and *vt2c*. The *vt2* genes present in three strains did not belong to previously described subtypes and were designated according to their restriction profile. Some strains harboured more than one *vt2* variant.
We also studied 37 bovine isolates. The majority of the *vt2* subtypes were different from the described variants and were named according to their restriction profiles. Some strains contained several *vt2* genes, which hampered the identification of the variants. Figure 3 shows *vt2* RFLP profiles.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

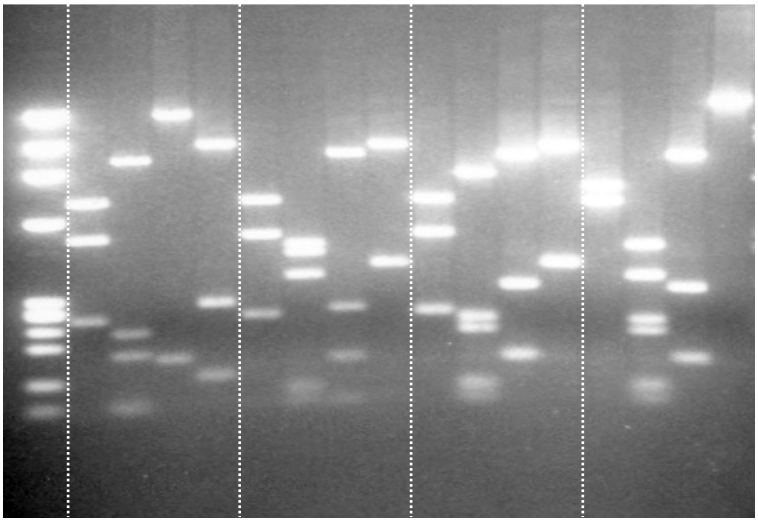


Figure 2: *vt2* RFLP profiles of four strains: lane 2-5, lane 6-9, lane 10-13 and lane 14-17. Restriction enzymes were used in the following order: *Acc*I, *Hae*III, *Hinc*II and *Pvu*II. Lane 1 shows the molecular marker.

4. DISCUSSION

Verotoxinogenic *E. coli* were first described in 1977 (9) and their prevalence in humans and animals has steadily increased since then. VTEC are commonly isolated from the intestines of healthy cattle and share common virulence factors with EHEC strains pathogenic for humans (28). The majority of the clinical manifestations of VTEC infection observed in the latter originate from the production of one or both types of verotoxins, VT1 and VT2. Although both VT1 and VT2 have the same mode of action, their effect *in vitro* and *in vivo* differs considerably.
Bacteriophages have played an important role in the evolution of many bacterial pathogens. Integration of phages in the bacterial cell often changes the properties of the host, for example by acquisition of virulence factors. In VTEC isolates, toxin production is coupled with phage release during lytic growth since the toxin genes are part of a late phage transcript (21).

Our longitudinal study of 8 selected cattle farms demonstrated the presence of VTEC strains in Belgian cattle farms. Different serotypes and different pathotypes can circulate on the same farm. We studied Belgian cattle as a possible source of infection. Therefore we need to compare human and bovine strains. In our approach we used molecular methods to study the diversity of verotoxins and of verotoxin-converting phages present in human and bovine strains.

First we studied the characteristics of *vt* converting phages. Although we confirmed the presence of *vt*-sequences in VTEC strains from human and animal sources by PCR, most of the isolates failed to produce verotoxin-encoding phages. It is possible that these VTEC strains harboured defective VT phages that lost parts of the bacteriophage genome essential for the formation of phage particles. Alternatively, the appropriate conditions for release or plating of phages or the appropriate receptor strain may not have been found. We studied the morphology and restriction enzyme patterns of the genomes of the phages we isolated and detected clear differences.
However, since it is not possible to produce verotoxin-encoding from all VTEC strains, another approach must be taken. We characterized the genomic DNA of human and bovine O157 VTEC strains by PCR analysis with combinations of phage specific and toxin specific primers. In all the O157 *E. coli* tested, there was a close linkage between the *vt2*-encoding genes and phage encoded genes implicating that these *vt2* genes are phage-encoded. Our findings also revealed that the DNA regions flanking the *vt2* gene showed significant variation. We studied the distribution of known replication genes among VTEC strains. Not in all strains studied, the replication gene could be identified by our PCR systems, indicating that other phage replication genes might be present. Several different antiterminator genes from lambdoid phages have been described. Group-specific primers were designed and used in PCR in combination with a *vt2*-specific primer. Both in human and bovine strains, we detected different antiterminator genes. In some strains more than one phage antiterminator gene is present, indicating the presence of more than one VT-converting phage. Obviously, our PCR-RFLP analysis scheme provides a tool for typing of O157 VTEC strains. Interpretation of DNA fingerprints obtained for different bacteriophages present in bovine and human VTEC strains may contribute to a better understanding of their heterogeneity and of their potential to cause disease in humans.
A second molecular approach was *vt2* typing. In contrast to VT1, VT2 toxins form a diverse group with many antigenically different variants (12, 24, 19, 10, 8, 16, 17, 10). A *vt2* subtyping scheme was designed by Tyler *et al*. (26). However, this typing scheme is not able to discriminate between all types (2). In our study, we subtyped VT2 encoding genes from human and bovine VTEC isolates by PCR-RFLP applied to the whole *vt2* operon. All *vt2* variants detected in human strains were also found among the bovine strains, which confirms that cattle might be a reservoir for human VTEC infections. Two subtypes that were previously described for human EHEC strains (18) were also found among the bovine VTEC strains. The number of different VT2 variants observed amongst the bovine isolates was significant higher than those detected in human isolates. The latter only formed a subset of the variants found in the bovine VTEC population. In addition, we found several new variants whose RFLP patterns of the PCR products, did not fit the predicted patterns for the variants described.
With the PCR-RFLP scheme we were able to detect more than one *vt2* gene in a VTEC isolate. The presence of such closely related sequences in the same strain suggest that the strong variation of *vt2*-related genes may be linked to recombinational events.
Our results indicate that the PCR-RFLP scheme for subtyping *vt2* genes is an interesting tool to follow the epidemiological evolution of VTEC in cattle farms and might also be applied in epidemiological studies trying to determine the source of human VTEC infections.

5. ACKNOWLEDGEMENTS

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1. INTRODUCTION

Verotoxin producing *Escherichia coli* (VTEC) is an important cause of gastrointestinal disease in humans (mostly young children and elderly persons) including bloody diarrhoea, hemorrhagic colitis and eventually leads to renal failure (haemolytic uremic syndrome) and death. Infection occurs via consumption of contaminated food or by direct transmission of VTEC from infected humans or animals (9). Undercooked beef, roast beef, dry minced salami and raw milk have often been implicated in foodborne infections (9, 20) but vegetables (1) and apple cider (3) can also be a source of infection.

VTEC strains produce two types of verotoxins, i.e. those that are immunologically similar to the Shiga toxin produced by *Shigella dysenteriae* (VT1) and a second group that are not (VT2) (14, 25). Severe clinical symptoms were found to be more frequently associated with *E. coli* producing VT2 than with those producing VT1 (5, 15).

The genes encoding the verotoxins in several VTEC strains are located on lambdoid bacteriophages (13). Recent studies have revealed some genomic variation within *vt*-encoding bacteriophages from *E. coli* O157 isolates (6, 22) and an even larger variation among *vt2*-encoding phages derived from different VTEC serotypes (27).

Verotoxins are composed of a single enzymatic A subunit and a pentamer of receptor-binding B subunits (15, 7). Very little DNA sequence variation has been observed for the VT1 encoding genes whereas different variants of VT2 have been described. The most prominent VT2 variants found in human clinical isolates are VT2 (12) and VT2c (24). Other variants occurring in human or bovine VTEC strains are VT2vha and VT2vhb (11), VT2d (P19), VT2f (23) and VT2vO111, VT2vOX392 and VT2vOX392 (16, 17). VT2e (4), one of the first described VT2 variants, is mainly found in isolates causing oedema disease in pigs and a human variant, designated VT2ev (8), is rarely detected in human clinical isolates.

In this study, we started a longitudinal survey on 8 selected Belgian cattle farms and checked for the presence of VTEC strain in faecal samples, in dust and in feed. To study Belgian cattle as a possible source of infection, we examined the diversity of verotoxins and of verotoxin-converting phages present in human and bovine strains.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Bovine strains were isolated during longitudinal studies or from faecal samples collected in slaughterhouses. Human clinical isolates and additional bovine isolates were selected from the collection of the VTEC reference laboratory based on the presence of *vt2* or on the O157 serotype.

2.2 Longitudinal study of cattle farms

Selected farms were visited monthly. Each time, 10 % (maximum 20) of healthy animals between 6 months and 2 years were individually sampled. In addition, available feed and dust in the stable were analysed for VTEC.

2.3 Bacteriological isolation

Faeces was homogenized in modified Trypticase Soy Broth plus novobiocin and incubated at 37° C. Enrichment culture was transferred to MacConkey agar plates. Plates were incubated aerobically at 37° C overnight. Twenty lactose-fermenting colonies that exhibited *E. coli* growth characteristics were randomly pooled (5 colonies per pool). Bacterial DNA for PCR analysis was prepared by boiling the bacterial pellet for 10 min.

2.4 Phage preparation, isolation of lysogens

To isolate spontaneously induced bacteriophages, the VTEC strains were grown overnight in LB medium without NaCl (O157 medium) and were centrifuged to spin down cells. The supernatant was filtered through a 0,2 µm filter. The presence of bacteriophages was revealed by spotting the undiluted filtrate and dilutions onto a bacterial layer of an overnight culture of *E. coli* host cells. Plaques were observed after overnight incubation at 37°C and assayed for the presence of *vt1* or *vt2* genes by PCR or plaque hybridisation.

2.5 Electron microscopy

Phages were allowed to adhere to Formvar-coated grids and were negatively stained with 1% uranylacetate before observation by transmission electron microscopy.

2.6 Isolation of genomic and plasmid DNA

Genomic DNA from the bacteria was prepared using the CTAB method (31). Plasmid DNA was prepared using the QIAGEN Plasmid Mini Kit.

2.7 PCR amplification

PCR amplifications were done on purified bacteriophage DNA and on total DNA extracted from lysogens and from the original isolates. PCR products were separated by 0,8 % agarose gel electrophoresis, stained with ethidium bromide and observed under UV light.

2.8 Endonuclease restriction of PCR products

DNA was incubated with the restriction enzyme in the buffer provided by the manufacturer (Gibco BRL overnight at 37°C. Restriction fragments were separated by 1.5% agarose gel electrophoresis.

3. RESULTS

3.1 Longitudinal study of VTEC in 8 cattle farms

The presence of VTEC strains was studied on selected cattle farms. Farms were followed for a period of several months. Six farms were contaminated with *E. coli* O157, belonging to different pathotypes. In two farms *E. coli* O157 was also isolated from feed and/or dust samples. It is not clear whether feed or dust were a source of infection on these farms. One farm contaminated with *E. coli* O103 was studied. *E. coli* O103 was isolated from faecal samples, dust and feed. We also followed a *E. coli* O145 farm. Our results show that VTEC strains belonging to different serotypes and different pathotypes are present on Belgian cattle farms. The isolated types are not animal specific but can also be found in humans.

3.2 Isolation of verotoxin-encoding bacteriophages

Twelve human and 14 bovine O157 VTEC strains were tested for bacteriophage production. Verotoxin-encoding bacteriophages could be isolated from 5 of the human strains and 2 of the bovine VTEC strains. The isolated bacteriophages were further characterized. Morphologically, the phages fell into two groups. Restriction endonuclease digestion of phage DNA provided further evidence of their diversity.

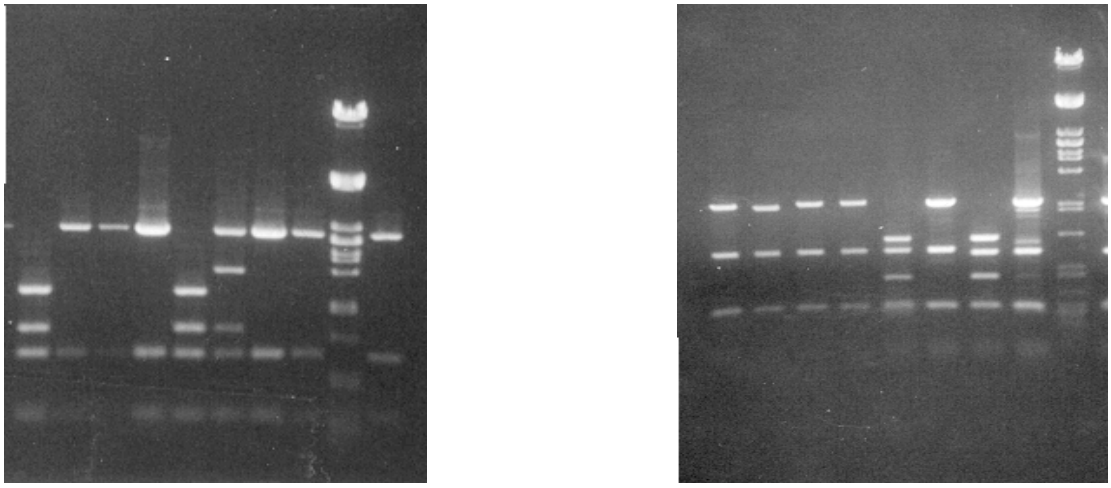
3.3 Study of the phage specific genes

As described above, plaque-forming phages could only be isolated from a limited number of strains. To further characterize the 29 human and 24 bovine VTEC strains, PCR analysis for the presence of different phage-specific regions was done. PCR was carried out using a common *vt*-specific primer and one specific for the replication genes of two types of phages. Both types of replication genes were equally present. Different sizes of amplification products were detected. Some of the strains possessed both replication genes. To determine the presence of phage-encoded antiterminator genes, a *vt*-specific primer was used in combination with respectively primers specific for the antiterminator genes from 4 different phages. Two types were frequently detected; the two other types were rarely detected. Some strains were shown to contain more than one *Q* gene, which confirmed previous results obtained for the replication gene demonstrating the presence of more then one phage in some of the O157 VTEC strains.

3.4 RFLP of other phage genomic regions

The gene *roi* is a common gene in lambdoid phages. A *vt* specific primer in combination with a *roi*-specific primer amplifies the *vt-roi* region of lambdoid phages. PCR fragments were obtained for a high number of the strains tested. Restriction analysis of the amplification product revealed different restriction profiles. The most prevalent profile is found in both human and bovine O157 VTEC strains. One profile was only observed in the bovine strains.
A second phage specific region is the region L0105-*R*. Amplification of the genomic region L0105-*R* resulted in fragments of different sizes in the majority of the strains tested. Restriction analysis revealed different patterns. Some profiles are dominant both in human and bovine VTEC strains while the prevalence of other band patterns is low. One profile was only found in bovine *E. coli* O157 isolates.

Examples of results of PCR-RFLP analysis of the *vt*-phage specific regions are given in figure 2.



A **B**

Figure 1: PCR-RFLP analysis of the regions specific for verotoxin-encoding phages.

3.5 vt2 subtyping of human VTEC strains

A *vt2* subtyping scheme allowing to distinguish different *vt2* subtypes was developed. *vt2* genes were amplified by PCR and restriction analysis with four restriction enzymes. By means of the proposed PCR-RFLP scheme the distribution of different *vt2* subtypes in a collection of human and bovine strains was studied. 37 human clinical isolates were analysed. Only 4 different *vt2* subtypes were found. The most prominent *vt2* subtypes found were *vt2* and *vt2c*. The *vt2* genes present in three strains did not belong to previously described subtypes and were designated according to their restriction profile. Some strains harboured more than one *vt2* variant.
We also studied 37 bovine isolates. The majority of the *vt2* subtypes were different from the described variants and were named according to their restriction profiles. Some strains contained several *vt2* genes, which hampered the identification of the variants. Figure 3 shows *vt2* RFLP profiles.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

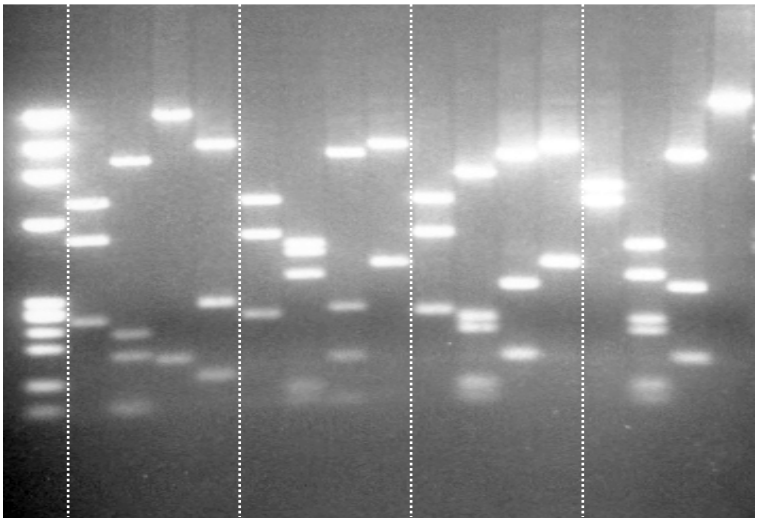


Figure 2: *vt2* RFLP profiles of four strains: lane 2-5, lane 6-9, lane 10-13 and lane 14-17. Restriction enzymes were used in the following order: *Acc*I, *Hae*III, *Hinc*II and *Pvu*II. Lane 1 shows the molecular marker.

4. DISCUSSION

Verotoxinogenic *E. coli* were first described in 1977 (9) and their prevalence in humans and animals has steadily increased since then. VTEC are commonly isolated from the intestines of healthy cattle and share common virulence factors with EHEC strains pathogenic for humans (28). The majority of the clinical manifestations of VTEC infection observed in the latter originate from the production of one or both types of verotoxins, VT1 and VT2. Although both VT1 and VT2 have the same mode of action, their effect *in vitro* and *in vivo* differs considerably.
Bacteriophages have played an important role in the evolution of many bacterial pathogens. Integration of phages in the bacterial cell often changes the properties of the host, for example by acquisition of virulence factors. In VTEC isolates, toxin production is coupled with phage release during lytic growth since the toxin genes are part of a late phage transcript (21).

Our longitudinal study of 8 selected cattle farms demonstrated the presence of VTEC strains in Belgian cattle farms. Different serotypes and different pathotypes can circulate on the same farm. We studied Belgian cattle as a possible source of infection. Therefore we need to compare human and bovine strains. In our approach we used molecular methods to study the diversity of verotoxins and of verotoxin-converting phages present in human and bovine strains.

First we studied the characteristics of *vt* converting phages. Although we confirmed the presence of *vt*-sequences in VTEC strains from human and animal sources by PCR, most of the isolates failed to produce verotoxin-encoding phages. It is possible that these VTEC strains harboured defective VT phages that lost parts of the bacteriophage genome essential for the formation of phage particles. Alternatively, the appropriate conditions for release or plating of phages or the appropriate receptor strain may not have been found. We studied the morphology and restriction enzyme patterns of the genomes of the phages we isolated and detected clear differences.
However, since it is not possible to produce verotoxin-encoding from all VTEC strains, another approach must be taken. We characterized the genomic DNA of human and bovine O157 VTEC strains by PCR analysis with combinations of phage specific and toxin specific primers. In all the O157 *E. coli* tested, there was a close linkage between the *vt2*-encoding genes and phage encoded genes implicating that these *vt2* genes are phage-encoded. Our findings also revealed that the DNA regions flanking the *vt2* gene showed significant variation. We studied the distribution of known replication genes among VTEC strains. Not in all strains studied, the replication gene could be identified by our PCR systems, indicating that other phage replication genes might be present. Several different antiterminator genes from lambdoid phages have been described. Group-specific primers were designed and used in PCR in combination with a *vt2*-specific primer. Both in human and bovine strains, we detected different antiterminator genes. In some strains more than one phage antiterminator gene is present, indicating the presence of more than one VT-converting phage. Obviously, our PCR-RFLP analysis scheme provides a tool for typing of O157 VTEC strains. Interpretation of DNA fingerprints obtained for different bacteriophages present in bovine and human VTEC strains may contribute to a better understanding of their heterogeneity and of their potential to cause disease in humans.
A second molecular approach was *vt2* typing. In contrast to VT1, VT2 toxins form a diverse group with many antigenically different variants (12, 24, 19, 10, 8, 16, 17, 10). A *vt2* subtyping scheme was designed by Tyler *et al*. (26). However, this typing scheme is not able to discriminate between all types (2). In our study, we subtyped VT2 encoding genes from human and bovine VTEC isolates by PCR-RFLP applied to the whole *vt2* operon. All *vt2* variants detected in human strains were also found among the bovine strains, which confirms that cattle might be a reservoir for human VTEC infections. Two subtypes that were previously described for human EHEC strains (18) were also found among the bovine VTEC strains. The number of different VT2 variants observed amongst the bovine isolates was significant higher than those detected in human isolates. The latter only formed a subset of the variants found in the bovine VTEC population. In addition, we found several new variants whose RFLP patterns of the PCR products, did not fit the predicted patterns for the variants described.
With the PCR-RFLP scheme we were able to detect more than one *vt2* gene in a VTEC isolate. The presence of such closely related sequences in the same strain suggest that the strong variation of *vt2*-related genes may be linked to recombinational events.
Our results indicate that the PCR-RFLP scheme for subtyping *vt2* genes is an interesting tool to follow the epidemiological evolution of VTEC in cattle farms and might also be applied in epidemiological studies trying to determine the source of human VTEC infections.

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IDENTIFICATION AND SEQUENCE ANALYSIS OF NEW *VT2* VARIANTS OF VEROTOXINOGENIC ESCHERICHIA COLI.

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ABSTRACT

Verotoxin producing *Escherichia coli* (VTEC) strains are responsible for outbreaks of diarrhea and hemorrhagic colitis. Moreover, VTEC infections are frequently associated with hemolytic uremic syndrome in young children and can lead to systemic disorders and eventually to death. Two major verotoxin types, VT1 and VT2, have been described. In the present study, VTEC strains of different serotypes from human and bovine origin were characterized by analysis of their *vt2* genes. Predicted restriction profiles of earlier described *vt2* variants were compared to the band patterns of the *vt2* genes isolated in this study. This led to the identification of new *vt2* subtypes. A PCR-RFLP scheme was developed to subtype the different *vt2* genes found, and a rational nomenclature was proposed. A phylogenetic tree was calculated for the whole *vt2* operon.

1. INTRODUCTION

Verotoxin producing *Escherichia coli* (VTEC) strains are responsible for outbreaks of diarrhea and hemorrhagic colitis. Moreover, VTEC infections are frequently associated with hemolytic uremic syndrome, especially in young children and elderly persons, and can leadto systemic disorders and eventually to death.
VTEC strain share the ability to produce verocytotoxins (VT), named after their cytotoxicity for Vero cells. Verotoxins play a prominent role in the pathogenesis. Two major verotoxin types have been described: VT1 and VT2. Verotoxins consist of a single catalytic A subunit and a pentameric binding subunit B. They exert their cytotoxic effect by inhibiting eukaryotic protein synthesis. Several variants of the *vt2 gene* have been described, both from human and bovine origin, while not much variation has been observed for the *vt1* gene.
We have characterized VTEC strains of different serotypes from human and bovine origin by analysis of the *vt2* genes. We identified new *vt2* subtypes and developed a PCR-RFLP scheme to subtype the different *vt2* genes found. A rational nomenclature was proposed. A phylogenetic tree was calculated for the whole *vt2* operon.

2. MATERIALS AND METHODS

2.1 Bacterial strains and plasmids
The strains ATCC35150 (VT2), E32511 (VT2c) and ATCC 51435 (VT2vha and VT2vhb) were a kind gift of Dr. D. Piérard.
All other strains were isolated from faeces of bovine origin, collected from living animals, from carcasses in the slaugtherhouse or from clinical human isolates.
The plasmids PHP, O31Q, 343, O31D and 544 were a kind gift of Dr. J. Paton and carry respectively the folowing *vt2* variants: $vt2_{OX393}$, $vt2_{OX392}$, $vt2_{vha}$, $vt2_{vO111}$,and $vt2_{vhb}$.

2.2 PCR
Primers were selected to amplify a 1405-base pair fragment of the VTEC genome containing the *vt2* gene and his flanking regions:
oli321: 5'-GGGATCCTGAATTGTGACACAGATTACACTTGTTAC-3'
oli320b: 5'-GGTCACTGGTTCGAATCCAGTAC-3'
PCR amplification was performed as follows:
after an initial denaturation at 94°C for 2 minutes, 30 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 1 minute and extending at 72°C for 90 seconds are performed.
PCR was carried out in a 50µl reaction mixture containing polymerase buffer, 10 ng bacterial DNA, 2µM dNTP's, 2.5 units of Pfu DNA polymerase, and 0.4µM of each primer.

2.3 Endonuclease restriction of PCR products
PCR products were dialyzed against sterile water during 2 hours using Isopore 0.025 µm membranes. Ten microliters of dialyzed PCR product was incubated for 16 hours with ten units of restrictionendonuclease, in a final volume of 20 µl. The enzymes *Acc*I, *Hae*III, *Hinc*II and *Pvu*II were used. Restriction fragments were separated by 1.5% agarose gel electrophoresis.

2.4 Cloning and DNA sequencing of vt2 genes.
New *vt2* variants were cloned into pUC18 using the SureClone Ligation kit. Recombinant plasmids were checked by endonuclease restriction analysis.
The sequence of the new *vt2* variants was determined using a DyeDeoxy Terminator Cycle Sequencing kit. Sequence analysis was done on both strains.

2.5 Phylogenetic comparison of vt2 sequences.
A phylogenetic tree was constructed according to sequence relatedness across the interval of nucleotides 1 to 960 of the gene encoding the A subunit of the verotoxin and of nucleotides 1 to 267 of the gene encoding the B subunit of the verotoxin using the NEIGHBOR-JOINING method as implemented in the computer program NEIGHBOR. A dendrogram was plotted using the program Treeview.

3. RESULTS

3.1 Analysis of published vt2 sequences, and PCR-RFLP analysis.
Examination of eleven sequences of described *vt2* variants for endonuclease restrictionsites predicted five different *Acc*I patterns (A1-5), seven different *Hae*III patterns (H1-7), six different *Hinc*II patterns (I1-6) and four different *Pvu*II patterns (P1-4). Combination of these individual profiles can be used to identify *vt2* subtypes, and allows a rational nomenclature for the variants. PCR-RFLP analysis on the corresponding control strains confirmed the predicted AHIP patterns. The sequences *vt2ev* and *vt2f* (subtype A2H4I4P1) are too closely related to be discriminated since they differ in only two nucleotides.

3.2 vt2 subtyping of human and bovine VTEC strains.
VTEC strains from bovine origin and from human clinical isolates were analyzed by means of PCR-RFLP.
A number of strains contained two or more *vt2* variants. The *vt2* genes of these strains were cloned in a pUC18 vector, sequenced and the AHIP pattern was calculated.
In human VTEC strains only four *vt2*-variants were detected: vt2c, vt2, $vt2_{OX392}$ and a new *vt2* variant $vt2_{A1H3I2P2}$.
In bovine VTEC strains more *vt2* variants were detected: besides the *vt2* variants found in the human VTEC strains, other known and unknown *vt2* variants were present in the bovine VTEC strains.

3.3 Phylogenetic analysis of new vt2 variants.
Sequences of published and new *vt2* variants were imported into the computer, extending from the first ATG codon of the A subunit to the stop codon of the B subunit.
Sequence analysis shows that there is more variation in the A-subunit than in the B-subunit. Variations in the nucleotide sequence result only in a limited number of cases in aminoacid substitutions.
The phylogenetic tree shows that the overall identity between the *vt2* nucleotidesequences is more than 70%. furthermore, most of the *vt2* variants detected in the human and bovine VTEC strains during our study are more than 97% identical.

4. CONCLUSION

Our results show that the variability among the bovine VTEC strains is higher than among the human VTEC strains. Moreover, VTEC strains that are pathogenic for humans were found to be present in the bovine VTEC population.

HELICOBACTER SPECIES FROM ANIMALS: A ZOONOTIC RISK?

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SUMMARY

Since the isolation of *Helicobacter pylori* in humans, many new *Helicobacter* species have been isolated from the gastrointestinal tract of animals. Morphological, epidemiological and genotypic data strongly suggest the involvement of *Helicobacter* species from domestic animals in gastric ("*H. heilmannii*"), enteric (*H. cinaedi*, *H. fennelliae*, *H. pullorum*, "*Flexispira rappini*") and hepatic disease (*H. bilis*, *H. pullorum*, "*Flexispira rappini*") in humans. In this paper, a review of the literature addressing the current knowledge about epidemiology, diagnosis, pathogenesis and therapy of these infections is given.

SAMENVATTING

Sinds de isolatie van *Helicobacter pylori* bij de mens begin jaren tachtig, werd ook een groot aantal *Helicobacter* species geisoleerd vanuit het maagdarmstelsel van dieren. Morfologische, genetische en epidemiologische gegevens duiden op een mogelijke betrokkenheid van *Helicobacter* soorten van huisdieren in maagziekten ("*H. heilmannii*"), darmziekten (*H. cinaedi*, *H. fennelliae*, *H. pullorum*, "*Flexispira rappini*") en leveraandoeningen (*H. bilis*, *H. pullorum*, "*Flexispira rappini*") bij de mens. Met dit artikel willen we een overzicht geven van de huidige kennis in verband met de epidemiologie, diagnose, pathogenese en behandeling van deze infecties.

INTRODUCTION

The isolation of *Helicobacter pylori* from the human stomach in 1984 (1) and its subsequent identification as a gastric pathogen, ushered a new era both in gastroenterology as in microbiology. Today, *H. pylori* is considered the primary cause of chronic gastritis, peptic ulceration and gastric neoplasia in humans (2-4). The increasing number of *Helicobacter* species identified from a wide variety of animals and from different types of ecological niches along the gastro-intestinal tract, created an awareness within the research community that the impact of these organisms is far wider than that of the human gastric environment (5). To date, the genus *Helicobacter* includes 18 validly named *Helicobacter* species and several more formally unnamed closely related organisms.

In veterinary medicine, the role of *Helicobacter* species as potential pathogens in a diverse range of pathologies is starting to be recognized. Host-specific gastric helicobacters from dogs, cats, cheetahs, ferrets, pigs and non-human primates have been associated with chronic gastritis. In pigs, the involvement of "*Candidatus* Helicobacter suis" in the onset of ulceration of the non-glandular "pars oesophagea" of the stomach is under investigation. Recently, *Helicobacter mustelae* and *Helicobacter felis* have been identified as potential gastric carcinogens. In mice, chronic hepatitis and hepatocellular neoplasia have been linked with the presence of *Helicobacter hepaticus*, while acute outbreaks of diarrhoea infections in immunodeficient mouse colonies have been attributed to *Helicobacter bilis* and *Helicobacter rodentium*. In sheep, "*Flexispira rappini*", an intestinal helicobacter species, has been isolated from cases of abortion and acute hepatic necrosis in foetuses.

During the last decade, evidence has emerged, suggesting a possible transmission of animal helicobacters to humans. Some of these infections have been associated with different kinds of disease entities in humans. The epidemiological background ando the pathogenic pathways by which these organisms act, is still largely unknown. In the following sections, an overview of the literature, addressing the current knowledge about epidemiology, diagnosis, pathogenesis and therapy of these infections, is given.

GASTRIC PATHOLOGY

"Helicobacter heilmannii"

GENERAL

Soon after the discovery of *H. pylori*, gastroenterologists world-wide started to report a second type of spiral-shaped organism in the gastric (6-16) and duodenal mucosa of humans (17;18) These organisms were observed

as long, tightly coiled , Gram negative bacteria, 0.4-0.9 µm in diameter, 4-10 µm in length, with 4-8 spiral turns and multiple bipolar flagellae (7;15). In the stomach, they are mostly found in the antral region where they may occur as single organisms or in small groups, located between the mucus layer and the surface cells, or deep within the lumen of the foveolae. In some cases, they have even been observed within the canaliculi of the parietal cells (13;15;19) . They are less numerous than *H. pylori* and have a patchy distribution.

Originally, these long tightly coiled bacteria were thought to belong to a genus other than *Helicobacter* and were named "*Gastrospirillum hominis"* (8). However, Solnick and coworkers (20).later identified these organisms as belonging to the genus *Helicobacter*, based on phylogenetic analysis of bacterial 16S rRNA coding genes, cloned from different *"G. hominis"*-infected stomachs. Their data also suggested that at least two different species were involved. As these bacteria were yet uncultivated, a formal characterization following the guidelines of the International Committee on Systematic Bacteriology was not possible and as such these organisms were provisionally renamed "*Helicobacter heilmannii*" type 1 and "*Helicobacter heilmannii*" type 2 respectively, in remembrance of Konrad Heilmann, the German histopathologist who described the first large series of "*G. hominis*"-infected patients.

PATHOLOGY

Infection of the human stomach with these organisms is almost always accompanied by an active chronic gastritis, generally less severe than in *H. pylori*-infected tissues, and is presented with dyspeptic symptoms such as postprandial discomfort, epigastric pain, vomiting, heartburn and dysphagia (15;21). Glandular atrophy or intestinal metaplasia is not as common as in *H. pylori*-infections (21). Non-*H. pylori*-like spiral organisms have also been identified as a possible cause of acute gastric and duodenal ulceration, independent from NSAID use (22-27). Both types of gastric pathology resolve with clearance of these non-*H. pylori* infections, indicating a possible aetiopathogenic role. A similar finding was observed in a patient suffering from primary gastric low grade lymphoma (stage E1) (28;29), confirming earlier reports linking non-*H. pylori*-like organisms to gastric neoplasia (30-32). Experimental long-term infection of 294 mice with these bacteria, induced a lymphocytic infiltration which in 83.6 % of the cases was characterized by the presence of lympho-epithelial lesions. In 13.6 % of the animals these lesions progressed to a low grade MALT lymphoma and in 4.1 % to a high grade MALT lymphoma (33).

ZOONOSIS

Based on 16S rRNA comparative data, it was shown that *"H. heilmannii"* type 2 was indistinguishable from *H. felis*, *H. salomonis* and *H.* bizzozeroni, three species isolated from cats and dogs (34;35). The "*H. heilmannii*" type 1 sequence was identical to that of "*Candidatus* Helicobacter suis", a porcine helicobacter (36) and to that of helicobacter-like organisms detected in the stomach of non-human primates (37). In 1996, a "*H. heilmannii*"-like strain was cultured for the first time *in vitro* from an infected human by Andersen and collegues (38). Very recently, this strain was characterized as *H. bizzozeronii* based on phenotypic analysis, 16S rRNA, DNA-DNA hybridization and whole-cell protein profiling data (39). It was the first study to provide conclusive evidence that the organisms characterised as "*H. heilmannii*" were not a new human-specific *Helicobacter* species but were existing *Helicobacter* species, most probably transmitted through animals.

In a recent epidemiological survey, 802 patients infected with either *H. pylori* or "*H. heilmannii*"-like organisms (HHLO) were questioned about their contact with animals. Subsequent logistic regression analysis, identified cats, dogs and pigs as reservoirs in the transmission of HHLO (40). For cats and dogs this came as no surprise as many other studies had reported similar findings (7;13;41-47). Other studies demonstrated the presence of *H. felis* in the human stomach either by genotyping (48) or by electronmicroscopic analysis (12).

Additional evidence linking pets directly to human "*H. heilmannii*"-infections was given by Dieterich *et al.* in a comparative study of urease coding gene sequences (*ureB*) determined from HHLO, derived from the stomachs of a *"H. heilmannii"*-infected patient and his two cats (132). Although *Helicobacter* ureases are encoded by conserved genes, intra-species gene heterogeneity has been shown (49). Despite this variability, one cat sequence was found identical to one of the human-derived sequences, while another cat *ureB* gene sequence was a perfect match of that of *"H. heilmannii"* type 2 (50).

EPIDEMIOLOGY

How these feline and canine helicobacters are transmitted is still unclear but a prolonged close contact is indicative. In one case report, intensive licking of a 12-year old girl suffering from chronic active gastritis by her symptomatic dog, was suggested as a possible transmission route.

In addition to pet carnivores, pigs are believed to be another source of non-*H. pylori* infections in humans, based on morphological similarities and a 99.5 % 16S rDNA sequence homology between "*Cand.* H. suis" and "*H. heilmannii*" type 1 (36;51-53). Fecal-oral contact probably accounts for these infections, especially since most pigs seem to be colonised by "*Cand.* H. suis" (54).

The prevalence of HHLO-infections in adult humans is rather low when compared to *H. pylori* and ranges between 0.1 and 1.1 % with an average of 0.3 % (Table 1). However, in one study, a 6.2 % prevalence was reported in a series of 257 Thai individuals (55). Children can also be infected by HHLO. Infections have been reported between the age of 1.5 and 19 years with a 0.3-0.4 % frequency range (19;41;56-60). This indicates that

an age-dependent increase in prevalence as observed in *H. pylori* epidemiology is not present in HHLO infections. Nevertheless, it has been shown that HHLO are capable to survive for long periods (15). Mixed infections of HHLO and *H. pylori* are uncommon (11;14;15;61;62), suggesting competitive colonization.

DIAGNOSIS

In vitro cultivation of HHLO both in humans as in animals is extremely difficult or even impossible, excluding microbiology as a diagnostic tool. In general, microscopical detection of HHLO either in histological sections or in smears, is the most widely used diagnostic method. However, Fawcett and coworkers described recently an experiment in which *H. pylori* assumed a morphological appearance indistinguishable of that of HHLO, indicating morphology not to be a reliable diagnostic parameter (63). A similar finding was also reported for *H. felis* (35). Recently, an ELISA-test using antigens extracted from the human *H. bizzozeronii* strain was developed and tested with serum samples of 281 Turkish blood donors, determining a prevalence as high as 6%. However, the specificity and sensitivity of this test was not validated (64). 16S rRNA-based PCR methods have recently been developed for the detection of *H. felis, H. bizzozeronii* and *H. salomonis* as a group (De Groote D., *et al.* unpublished data) and the specific detection of "*Cand.* H. suis" in gastric biopsy specimens (36). In a preliminary experiment performed in our laboratory, animal helicobacters could be detected in human HHLO-infected tissues with these techniques (De Groote D., *et al.*unpublished data). These studies could help resolve the epidemiological background of these infections.

PATHOGENESIS

The pathogenic pathway by which HHLO act, is still largely unknown and depends on the species involved. Experimental infection of mice with HHLO-infected human tissues produces a chronic gastritis (65;66). With the isolation of *H. felis* from cats, a mouse model was developed to study host-pathogen interactions. In a series of studies, it was shown that *Helicobacter felis*-induced gastritis is a cell-mediated, host-dependent process. Experimental infection of specific mouse strains (SJL, CrH/He, DBA/2, C57BL/6) with *H. felis* induced a severe to moderate gastritis in contrast to other mouse lineages (BALB/c, CBA) in which no or only a very mild gastritis was produced (67;68). Recently, evidence was provided, suggesting host T-cell response to be a critical mediator in the onset of *H. felis*-associated gastric pathology (69). In addition, cellular adhesion has been identified as an essential factor in gastric colonization. Mutant *H. felis* strains lacking genes *flaA* and *flaB*, coding for flagellar structures, are not capable to colonize the stomach in a mouse model (70). In another study, infection of rats with either *H. felis* or HHLO did not result in any significant output of gastrin or any inflammatory response. It was concluded that an increase of gastrin levels as seen in *H. pylori* was not mediated through the effects of the urease enzyme but probably caused by inflammation (71). In *H. pylori*, the *vacA* gene and genes located on the *cag* pathogenicity island (*cag* PAI) have been identified as important genes regulating and coding for virulence-enhancing processes (72-75). In pigs infected with HHLO resembling "*Cand.* H. suis", the presence of the *vacA* gene was demonstrated by PCR using *H. pylori*-derived primers (76). Further research is needed to detect and characterize these virulence genes in all HHLO-related *Helicobacter* species.

TREATMENT

Different chemotherapeutic regimens have been used with success to eradicate HHLO-infections in humans using different combinations of antibiotics and acid reducing drugs (Table 2). In an experimental study in which antibiotics were screened in "*H. heilmannii*"-infected mice, amoxycillin, tetracycline and clindamycine were found effective against HHLO (77). However, as it has become clear that different *Helicobacter* species are involved in these infections, the efficiency of species-specific therapies should be evaluated. In a study by Dick-Hegedus *et al.*, *H. felis*-infected mice were treated orally using either a mono or a triple – based therapy during 4 weeks (133). Only 25% of the mice were cleared with either bismuth subcitrate or erythromycin, 47% with metronidazole, 0% with tetracycline, and 70% with amoxycillin. In contrast, triple therapy with metronidazole, amoxycillin, and bismuth subcitrate resulted in 80% eradication, whereas triple therapy with metronidazole, tetracycline, and bismuth subcitrate eradicated *H. felis* from all the animals (78). Recently, the experimental *H. felis* mouse model has been used by a number of laboratories to investigate the feasibility of immunotherapy to prevent and/or cure *Helicobacter* infection. Oral vaccination with either native or recombinant *Helicobacter pylori* urease (*rUre*) has been shown to confer long-term protection against challenge with *Helicobacter felis* in mice when co-administered with cholera-toxin (CT) or heat labile enterotoxin (LT) of *Escherichia coli* (79;80).

ENTERIC PATHOLOGY

During the last decade, an increasing number of helicobacters has been isolated from the lower intestinal tract from a variety of mammals and birds (81-84) Some of these *Helicobacter* species have been associated with enteric disease and sepsis in humans raising questions about the zoonotic significance of these species.

Helicobacter cinaedi* and *Helicobacter fennelliae

Initially, *H. cinaedi* and *H. fennelliae* were isolated from the distal part of the bowel of human immunodeficiency virus (HIV) – infected homosexual men suffering from colitis and proctitis and were first

classified as *Campylobacter cinaedi* (CLO-1A) and *Campylobacter fennelliae* (CLO-2) (85;86). Although most additional strains have since been isolated from immunocomprised patients, *H. cinaedi* and *H. fennelliae* were also recovered from immunocompetent men, women and children. In an experimental study using infant pig-tailed macaques, the pathogenic potential of these organisms was confirmed, as a diarrheal illness and bacteremia was consistently observed in infected animals (87). *H. cinaedi*-associated bacteremia and fever, accompanied by leucocytosis and thrombopenia has also been reported in natural cases involving immunocomprised humans (88-90;90-94). Although less frequently, similar cases have also been observed in association with *H. fennelliae* infections (88;89;95;96). Other reports linked *H. cinaedi* with recurrent cellulitis and arthritis (90;97-99). Successful treatment of these infections has been reported with tetracyclines, aminoglycosides (100) and ampicillin (96).

Beside from humans, *H. cinaedi* and *H. fennelliae* have also been cultured from feces of asymptomatic hamsters, dogs, cats and a macaque in whom they seem to represent normal intestinal flora.(101;102).

Recently, scientific interest has focused on the possible zoonotic significance of these animal strains. In a case report a *H. cinaedi*-strain was recovered from the blood and cerebrospinal fluid of an eight-day old neonate suffering from septicaemia and meningitis (95). During the first two trimesters of her pregnancy, the mother was exposed to hamsters. It was suggested by the authors that the neonate was infected during the birth process by the mother who may have acquired *H. cinaedi* through contact with hamsters as *H. cinaedi* strains were recovered both from the mother and the hamster. No further characterization was performed on these strains. The importance of such typing has recently been demonstrated by Kiehlbauch *et al.* (101). Using ribotyping techniques, human-derived *H. cinaedi* strains could be clearly distinguished from animal isolates. In addition, it was shown that hamster isolates exhibited a ribotype pattern different from that seen with dog and cat strains, establishing the existence of different host-specific biotypes (101). Further studies will be necessary to determine whether these different biotypes also exhibit different virulence properties and if transmission can occur between animals and humans.

Helicobacter pullorum

A group of campylobacter-like organisms (CLO) were isolated from the caeca of asymptomatic poultry and from the livers and intestinal contents of laying hens presented with hepatic lesions performing polyphasic taxonomical analysis, these were identified as a new helicobacter species and were named *Helicobacter pullorum* (81). In the same study, 6 CLO- strains, isolated from different patients suffering from mild to severe diarrhoea, were also characterized as *H. pullorum* strains, indistinguishable from those isolated from poultry, suggesting a possible zoonosis. Both immunocompetent and immunocomprised individuals were involved (103). Recently, a possible transmission route was identified by Atabay *et al.* as they recovered *H. pullorum* from the carcasses of poultry with a prevalence as high as 60% (104). The fact that *Campylobacter jejuni*, a close relative of *H. pullorum*, has been identified as a major food-borne pathogen and more *H. pullorum* associated cases are described (105), raises questions about the importance of *H. pullorum* in human gastrointestinal disease .

"Flexispira rappini"

Originally, "*Flexispira rappini*" was isolated from aborted sheep fetuses (106;107). It has been classified as a *Helicobacter* species based of 16S rRNA sequence analysis (108;109), although a formal description of these organisms has never been published. In humans, it has been isolated from patients suffering from either mild chronic diarrhoea (110), bacteremia (111;112) or pneumonia (113) involving both immunocompetent and immunocomprised individuals. Dogs, cats and rabbits were identified as possible sources for these infections (111;113;114). In one case, "*Flexispira rappini*" was recovered from the puppy of an infected patient (110), while in two other reports the patients disease coincided with the introduction of a puppy in the household (111;113). Treatment with either erythromycin (113), meropenem (111) or a combination of gentamicin and imipenem (115) resulted in eradication of "*Flexispira rappini*" and subsequent clearance of symptoms.

Recent phylogenetic analysis of 28 "*Flexispira rappini*" strains showed that they represented 9 different species (F. Dewhirst, J. Fox, B. Paster; unpublished observations). This finding illustrates the importance of a well-defined charachterization of such strains, if we want to learn more about the significance of these organisms.

HEPATIC PATHOLOGY

In recent years, new *Helicobacter* species have been isolated from the livers of a wide variety of animals and have been associated with hepatic disease. *H. hepaticus*, which consistently colonizes the cecum and colon of mice, has been associated with liver tumors in A/JCr mice as well as hepatitis in other susceptible inbred mouse strains (83;116;117). Local urease-induced ammonia (118), the production of cytolethal toxins (119) and the involment of autoimmune mediated processes (116;120) have been suggested as possible pathogenic pathways.

In addition to *H. hepaticus*, other *Helicobacter* species can colonize the hepatobiliary tract of animals. "*Flexispira rappini*" has been shown to cause hepatic necrosis in fetuses of sheep and guinea pigs (107;114;121). *H. bilis* is originally associated with hepatitis in aged inbred strains of mice (84), but has also been isolated from

the gastrointestinal tract of asymptomatic gerbils, dogs and cats (F. Dewhirst, J. Fox, B. Paster; unpublished observations). Other species linked to hepatitis are *H. canis* and *H. pullorum* in dogs and chickens respectively (81;122). In hamsters, cholangiofibrosis and centrilobular pancreatitis are believed to be caused by *H. cholecystus* (123). A common characteristic observed in these species is their ability to grow in the presence of bile in contrast to other non-hepatic helicobacters.

In a recent study by Fox *et al.,* 16S rDNA sequences of three different *Helicobacter* species could be determined from bile and gallbladders of patients suffering from chronic cholecystitis (118). *In vitro* cultivation was unsuccessful. Five sequences represented strains of *H. bilis*, two of "*Flexispira rappini*" and one strain of *H. pullorum*, suggesting a possible role of animals as reservoirs for these kind of infections. Indirectly, these results were confirmed by two other studies in which DNA of *Helicobacter*-like organisms was sequenced from the biliary tract of humans (124;125). Although these sequences were believed to originate from *H. pylori*, this latter finding was questioned by Fox *et al.* (118).

These results strongly indicate the involvement of *Helicobacter* species in biliary tract related disease. However, the evidence provided is still circumstantial and needs further clarification (126). Further studies are also required to determine the origin of the different *Helicobacter* species observed in the human biliary tract.

DISCUSSION

Review of the literature learns that *H. pylori* is not the only member of the genus *Helicobacter* threatening human health. A growing number of different helicobacters have been identified from the human gastrointestinal tract in association with disease.

In gastric pathology, the involvement of *Helicobacter* species other than *H. pylori* has been clearly demonstrated. The recent isolation of *H. bizzozeronii* from a "*H. heilmannii*" infected patient strongly suggests pet carnivores to be a possible source for these infections but needs further clarification. More studies are equally required to assess the risk of porcine gastric helicobacters as potential food-borne pathogens.

The recent recovery of different *Helicobacter* species from both immunocomprised as immunocompetent patients suffering from enteric and hepatic disease raises questions about the impact and the origin of these infections. Most of the *Helicobacter* species characterized in these cases have also been identified in a variety of animals. In addition, close contact with animals was reported in some cases. However, evidence linking animals directly to these enteric infections has not yet been provided.

Better methods to subtype the different strains isolated from both humans and animals are required to identify the source of these infections. In the same sense, an increased awareness among gastroenterologists and the implication of better and adapted diagnostic methods could help resolve the epidemiological background and identify critical points of entry in animal-to-human relationships.

TABLES

Table 1: "*Helicobacter heilmannii*" prevalence

Author	**%**	**Series**
Dent *et al.*,1987 (6)	0.2	3/1300
Dye *et al.*, 1989 (7)	0.5	2/400
Queiroz *et al.*, 1990 (11)	0.3	1/315
Fisher *et al.*, 1990 (10)	0.7	4/600
Heilmann *et al.*, 1991 (15)	0.25	39/15180
Wegmann *et al.*, 1991 (13)	0.3	5/1551
Mazzucchelli *et al.*, 1993 (129)	1.1	2/175
Debongnie *et al.*¸1994 (130)	0.45	17/3800
Monno *et al.*, 1995 (131)	0.07	2/2781
Hilzenrat *et al.*, 1995 (62)	0.5	4/1223
Zhang *et al.*, 1998 (55)	6.2	16/257

Table 2: Therapeutic regimens used for "*Helicobacter heilmannii*" treatment

Author	**Therapy**	**Weeks**
Dye *et al.*, 1989 (7)	1. Bismuth subsalicylate 30 ml, *qid* (D0-D21) 2. Amoxycilin 500mg, *qid* (D7-D21) 3. Metronidazole 500 mg *tid* (D18-D21)	3 w
Morgner *et al.*, 1999 (28)	1. Omeprazole (40mg *tid*) 2. Amoxycillin (750mg *tid*)	2 w
Thomson *et al.*, (41)	1. Bismuth compound 2. Omeprazole 3. Amoxycillin	6 w
Oliva *et al.*, 1993 (59)	1. Bismuth compound 2. Amoxycillin 3. Ranitidine	4 w
Oliva *et al.*, 1993 (59)	1. Bismuth compound 2. Amoxycillin	2 w
Michaud *et al.*, 1995 (127)	1. Ranitidine 2. Metronidazole 3. Amoxycillin	4 w
Tanaka *et al*, 1994 (128)	1. Cimitidine 2. Tetracycline	NA
Alhimyary *et al.*, 1994 (26)	1. Sucralfate 2. Omeprazole	NA

NM = Not available

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SOMATIC CELL COUNTS IN DAIRY HEIFERS DURING EARLY LACTATION

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ABSTRACT

This paper describes the distribution of the first somatic cell counts (SCC), measured between 5 and 14 days of lactation, during a one year period (1999) of 12994 dairy heifers on 3221 herds in Flanders (Belgium). Somatic cell counts ≤ 150 x 10^3 cells/ml were allocated to class 1. Somatic cell counts between 151 x 10^3 and 300 x 10^3 cells/ml, between 301 x 10^3 and 1,000 x 10^3 cells/ml and > 1,000 x 10^3 cells/ml were allocated to classes 2, 3, and 4, respectively. The prevalence per class was 65.4, 15.6, 12.9 and 6.1% for the classes 1, 2, 3, and 4, respectively. The within-herd prevalence per SCC class was calculated for 137 herds where at least 10 SCC were available. The within-herd prevalence for class 1 varied from 27.3 - 100% with a median of 66.7%. For the classes 2, 3, and 4, the median within-herd prevalence was 15.4 (range: 0.0 - 50.0%), 10.0 (0.0 - 54.5%), and 0.0% (0.0 - 30.0%), respectively.

1. INTRODUCTION

Mastitis is the most common and expensive disease in dairy cattle (13). Current mastitis control practices are focusing on lactating cows. Little attention has been paid to intramammary infections (IMI) in primigravid dairy heifers and the inspection of their udders is usually restricted to palpation shortly before or even after freshening. As their nonlactating udders have traditionally been regarded as uninfected (Trinidad and others 1989) it took a long time before it was realised that IMI in dairy heifers were present in far greater numbers than previously accepted (Munch-Petersen 1970, Oliver and Mitchell 1983, Daniel and others 1986, Boddie and others 1987, Sobiraj and others 1988, Hirsch 1988, Trinidad and others 1989, Trinidad and others 1990, Pankey and others 1991, Fox and others 1995, Nickerson and others 1995). The importance of udder health in heifers upon entering the milking herd can however not be overstated because heifers have an impact on future milk yield and quality in the herd.

Somatic cell count of milk samples is an important instrument for monitoring udder health in lactating cows (Trinidad and others 1990) and is used worldwide as an indicator of subclinical (Laevens and others 1997) and clinical (Trinidad and others 1989) mastitis. However, while SCC is available as a useful tool for the farmer to detect and control udder health on his farm, it is in the meantime used to penalize this farmer if he doesn't produce milk according to SCC treshhold levels. As the major factor affecting SCC is IMI (Harmon 1994, Laevens and others 1998), SCC of heifers in early lactation reflect the IMI status at the start of their first lactation. These IMI either originate from the prepartum period or arise at the time of calving.

The aim of this study was to describe the distribution of the first lactational SCC from dairy heifers in early lactation during a one year period (1999) by allocating them to 4 classes. The period defined as "early lactation" was kept short, extending from day 5 to day 14 post partum.

2. MATERIAL AND METHODS

2.1. *Data collection:*
The individual SCC of all lactating heifers and multiparous cows from the year 1999 from all herds participating in the Dairy Herd Improvement (DHI) program in Flanders (Belgium) were made available as one dataset by the Flemish Cattle Breeding Association. This dataset contained the following information:

- Herd Identification
- Cow Identification
- Breed Code

- Parity
- Days In Milk (DIM)
- Cumulative Milk Production
- Date of SCC Measurement
- SCC: measured at monthly intervals of every cow that was at least 5 DIM. Composite milk samples were therefore collected from two successive milkings and were analysed with the Fossomatic 5000 (Foss Electric®).

2.2. *Data processing:*
Only SCC from heifers measured between day 5 and day 14 day post partum were selected. A classification was done:

- Class 1: SCC ≤ 150 x 10^3 cells/ml
- Class 2: SCC between 151 x 10^3 and 300 x 10^3 cells/ml
- Class 3: SCC between 301 x 10^3 and 1000 x 10^3 cells/ml
- Class 4: SCC > 1000 x 10^3 cells/ml.

The time dependent variation of SCC during the observed time interval was assessed by fitting a regression line through the observed $\log_e$-transformed SCC (lnSCC) using SPSS 9.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

The within-herd prevalence per class was calculated when at least 10 SCC were available per herd.

3. RESULTS

The first SCC of in total 12994 heifers from 3321 herds were included. From 137 herds at least 10 SCC were available.

The distribution of SCC of classes 1, 2, 3, and 4 was 65.4, 15.6, 12.9 and 6.1%, respectively (Table 1).

The majority of the SCC were measured during the months October, November and December (n=4414), showing a calving pattern with most heifers calving during autumn (Table 1).

A seasonal variation was observed as shown in Table 1. The prevalence of class 1 SCC was lowest in the months April, May and June (55.9%), whereas it was highest in the months October, November and December (71.7%). The prevalences of the classes 2,3 and 4 were just the opposite. They were highest in the months April, May and June (19.6, 16.6, and 7.8%, respectively) and lowest in the the months October, November and December, except for class 4 that had the lowest prevalence in the months July, August, September (11.8, 10.5 and 5.5%).

A time dependent variation was observed. Somatic cell counts decreased with increasing DIM (lnSCC=5.47-0.081*DIM).

Table 1: Overall and seasonal prevalence per SCC class.

Period	**Prevalence**				
	n (%)	Class 1	Class 2	Class 3	Class 4
January-February-March	3177 (24)	62.5%	17.2%	14.1%	6.2%
April-May-June	1468 (11)	55.9%	19.6%	16.6%	7.8%
July-August-September	3935 (30)	64.0%	17.1%	13.3%	5.5%
October-November-December	4414 (35)	71.7%	11.8%	10.5%	5.9%
Overall	12994 (100)	65.4%	15.6%	12.9%	6.1%

The within-herd prevalence for each class is shown in Figure 1. For the SCC of class 1 the within-herd prevalence varied from 27.3 to 100% with a median of 66.7%. For the SCC of classes 2, 3, and 4 the median within-herd prevalence was 15.4 (range: 0.0 - 50.0%), 10.0 (0.0 - 54.5%), and 0.0 (0.0 - 30.0%), respectively.
If only the SCC measured on day 5 post partum were selected (n=1055), 48.3 % (n=510) belonged to class 1, 20% (n=211), 21.9% (n=231), and 9.8% (n=103) belonged to classes 2, 3, and 4 respectively.

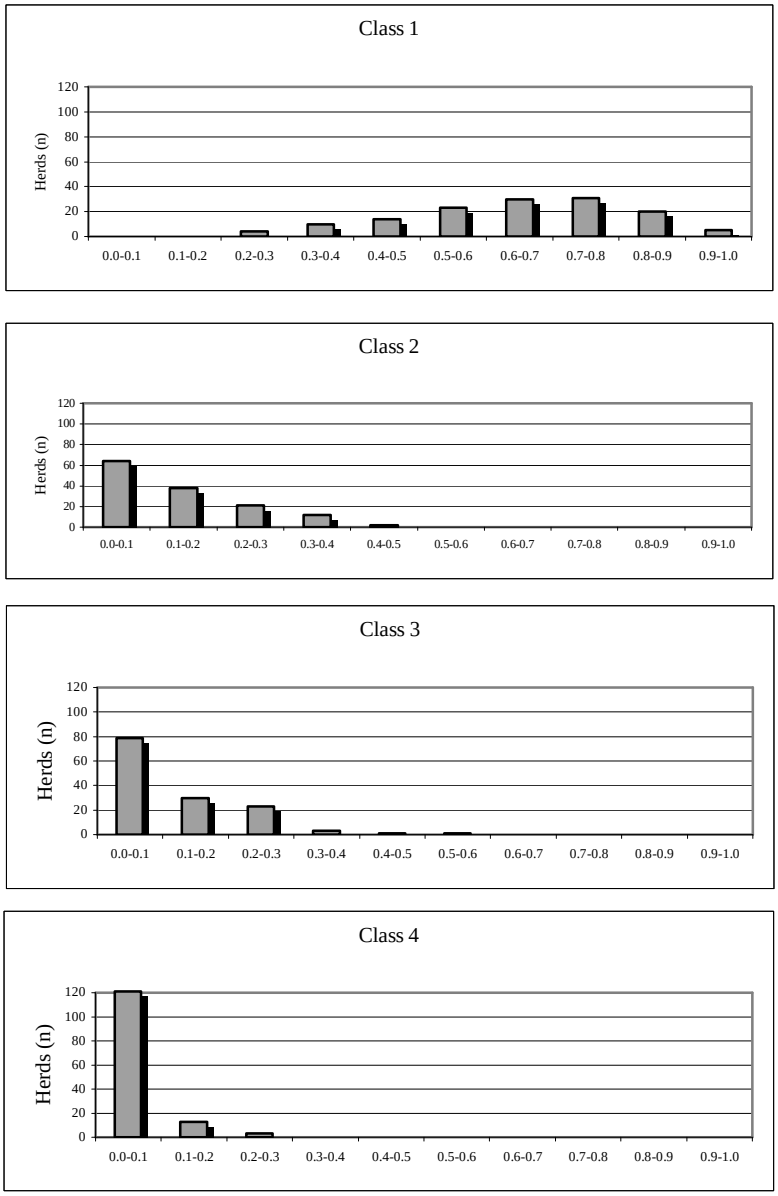

Figure 1: Within-herd prevalence per SCC class.

4. DISCUSSION

In this study nearly 35 % of all heifers had a first lactational SCC, measured between day 5 and day 14 post partum, > 150 x 10^3 cells/ml, and 19 % had a first lacational SCC > 300 x 10^3 cells/ml . This is high, considering the fact that a heifer is expected to have a SCC of 100 x 10^3 cells/ml or lower (O'Rourke and Blowey 1992) and considering the results from(Laevens and others 1997)(7) who found that 95% of the heifers which were bacteriologically negative during their first lactation, had a SCC ≤ 150 x 10^3 cells/ml during the first month of lactation.

Interpretation of the results in this study, in order to get an idea of IMI in heifers in early lactation, should however be done with precaution as it is known that SCC are physiologically high during the first week of lactation {O'Rourke & Blowey 1992 68 /id}10). Therefore Dohoo *et al.* (1993)(3) recommended not to consider all individual SCC measured during the first 9 DIM, not to have an upwards bias. (Barkema and others 1999)(1) however stated that quarter-milk SCC was applicable as of day 2 post partum to determine IMI in an udder quarter.

The observed seasonal variation of SCC might be explained by the fact that the heifers have been indoors during winter, creating a higher risk for IMI and is in correspondence with the study by (Fox and others 1995)(4). In contrast, (Klaas and others 1998)(6) have found the lowest prevalence of IMI in heifers between August and September.

Besides the seasonal variation there was also a large between-herd variation. This suggests an important role of heifer management in the prevention of IMI. Prevention of prepartum IMI is currently based on controling flies during the summer period, using individual calf hutches to avoid suckling among calves and segregating pregnant heifers from dry cows (Trinidad and others 1989) and applying prepartum treatment (Shearer and Harmon 1993). However, further investigations have to be done to determine risk factors associated with increased SCC in early lactation and IMI in primigravid dairy heifers.

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An Experimental Infection with Classical Swine Fever Virus in Pregnant Sows: Transmission of the Virus, Course of the Disease, Antibody Response and Effect on Gestation.

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ABSTRACT

An experimental infection with classical swine fever (CSF) virus in twelve conventional gilts, housed in a sow-box housing system, was conducted in order to evaluate horizontal transmission, clinical, virological and serological response, and the effect on gestation. Two out of the twelve gilts, of which 10 were pregnant, were experimentally inoculated. They became viraemic for the first time 6 days post inoculation (dpi). Contact gilts became viraemic between 18 and 21 days post inoculation. Based on the virological findings and the martingale estimate of R_0 (25.1) It can be concluded that all contact gilts got infected by the two experimentally inoculated gilts, although random contacts between gilts were not possible.
A present CSF infection could be diagnosed earlier and during a longer period when leukocyte count or PCR was used in comparison with virus isolation in whole blood ($p<0.05$). The observed clinical symptoms were atypical and highly variable between the gilts, which hampered clinical diagnosis. The pregnant gilts got infected between day 43 and 67 of gestation. In all cases vertical virus transmission occurred. This resulted partially in abortion and/or mummification.

1. INTRODUCTION

Classical swine fever (CSF) is known as a highly contagious pig disease causing considerable economic losses. In 1980 the European Union (EU) adopted an eradication strategy for CSF (Council Directive 80/217, EU). Since the control of CSF in the EU is based on a policy of non-vaccination and stamping-out. This policy has resulted in an eradication of the disease in most of the member states of the EU.

However, recent outbreaks have shown that CSF epidemics in densely populated pig areas are difficult to control and can have dramatic consequences (Elbers et al., 1999). In the 1997-1998 CSF epidemic in the Netherlands, it once again has been proven that the early detection of the primary CSF infected herd is crucial to minimize the size of an outbreak. The longer CSF remains undetected, the larger the opportunities are for the virus to become widespread (Horst et al., 1998; Elbers et al., 1999). The most important hindrance to detect a present CSF infection in an early stage is the appearance of atypical clinical symptoms and the relatively large chance to miss an infection if only a limited number of blood samples are taken (Koenen et al., 1996).

In order to design a surveillance system which maximises the possibility of detecting a present infection, it is essential to have detailed information on the clinical picture and on the dynamics of the infection. Moreover, information of the within-herd virus spread is of great importance to assess the risk of between-herd virus spread.
The spread of CSF in weaner and slaughter pigs has already been investigated (Laevens et al., 1998; Laevens et al., 1999). Similar experiments in sows housed in a sow-box housing system have not yet been conducted.

In the presented study the transmission of CSF virus among gilts housed in a sow-box housing system was examined. Furthermore, the virological and serological response, the clinical symptoms, and the effect on gestation, following a CSF infection are described.

2. MATERIALS AND METHODS

2.1. Animals

Twelve conventional gilts, 8 months of age, originating from a selection herd and controlled on the absence of bovine viral diarrhoea (BVD) and CSF antigen and antibodies were used.

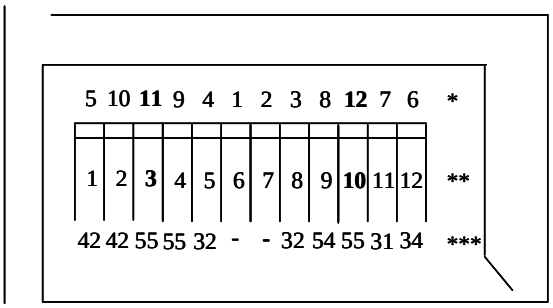
2.2. Virus

The isolate used for the experimental inoculation was originally obtained from the first CSF-infected herd of the 1993-1994 Belgian epizootic. The isolate was verified to be free of African swine fever virus and BVD virus. By using monoclonal antibodies, it was characterised to be similar to an isolate known as the 'souche Lorraine' (Koenen and Lefebvre, 1995). Virus infectiousness was 10^3 median tissue culture infective dose ($CTID_{50}$ / ml).

2.3. Experimental design

Upon arrival, gilts were housed in individual sow boxes where oestrus detection was carried out on a daily basis. Within a range of 24 days oestrus was observed in all gilts. During oestrus, gilts were inseminated twice. Twenty-five days after the last insemination gilts were chequed on pregnancy using ultrasound. The number of days of gestation on the day of experimental inoculation are shown in Figure 1. After pregnancy diagnose, gilts were transferred to an isolation unit where they were again housed in individual sow-boxes. The two gilts that were inseminated first (longest period of gestation) were housed in boxes 3 and 10, respectively. The two gilts that were not pregnant were housed in the middle boxes (6 and 7). The remaining 8 gilts were randomly allocated to the remaining boxes (Figure 1). Direct nose-to-nose contact was only possible between neighbouring pigs.

Figure 1: Map of the isolation unit, length of gestation and visiting procedure



* : Visiting order
** : Box identification order
*** : Number of days of gestation on the day of experimental inoculation
(- : not pregnant)

Following a ten day acclimatisation period after arrival at the isolation unit, two gilts, housed in box 3 and box 10, were experimentally inoculated with CSF virus through deep intramuscular injection (2 ml) plus intranasal inoculation (2 ml). After experimental inoculation, sows were not released from the boxes until the end of the experiment or at the moment of death. All gilts that survived the infection were slaughtered one week before the end of gestation.

In a 75-days post-inoculation period, the boxes were visited following a strict route starting as far as possible from the experimentally inoculated gilts and moving towards the sources of infection (Figure 1). By applying this visiting procedure, it was ensured that the virus was not transferred from infected to uninfected gilts through the during sample collection. Additionally, all materials necessary for blood sampling and rectal temperature monitoring were provided for each box separately.

2.4. Sample collection and clinical examination

Clotted and heparinized blood samples were collected from all gilts upon arrival. Again, blood samples were taken upon arrival at the isolation unit and two days prior to inoculation. During the post-inoculation period, blood samples were collected from all gilts every 3 days until 54 days post inoculation (dpi), and every 6 days between 54 and 75 dpi. Additionally, swabs of nasal secretion and faeces were collected from the experimentally inoculated gilts every 3 days during the first 30 dpi. Simultaneously with sample collection, all gilts were examined clinically. The following symptoms were recorded: liveliness (apathy), body condition (cachexy),

coughing, conjunctivitis, diarrhoea, ataxia, and erythema. Rectal temperature, feed intake and mortality were recorded daily.

From every pig that died or had to be euthanatized, tissue samples (tonsil, muscles of shoulder and rump, mesenterial, ileocecal and maxillary lymph node, kidney, spleen, heart, lung, liver, brain, eye fluid, blood, faeces, urine) were collected. After dead or after abortion, blood and tissue samples (tonsils, kidney, spleen, heart, and lung) were collected from the foetuses.

2.5. Sample analyses

For virus isolation (VI) in blood, 100 µl whole blood was inoculated in duplicate onto a non-confluent monolayer of PK_{15} cells cultured in multiwell plates (24 wells / plate). After 48 hours, the cells were fixed with isopropanol and stained with a polyclonal fluorescein-conjugated anti-CSF immunoglobulin. Additionally, a single tube RT-nPCR test (McGoldrick et al., 1999) was used to detect viraemia in serum. The same single tube RT-nPCR test was used to detect CSF virus in nasal secretion and faeces of the experimentally inoculated pigs.
For antibody detection in serum, the virus neutralisation (VN) test and the CTB-ELISA (Ceditest) (Wensvoort et al., 1988) were used. Leukocyte count was carried out using the Coulter-Counter ZM (Analis).

2.6. Data analyses

The basic reproduction ratio (R_0), a measure of transmission of infection, and defined as the mean number of new infections arising from one typical infectious case introduced in a totally susceptible population, was calculated using the martingale and the maximum likelihood estimator.
The martingale estimator is defined as:

$$R_{0mrt} = \frac{N}{C - Z} \sum_{i=S_t+1}^{S_0} \frac{1}{i}$$

where N is the total number of animals at the beginning of the outbreak, C is the total number of cases that occurred during the observation period, Z is the sum of fractions of infectious periods that were spent at the time when no susceptibles remained, S_0 is the number of susceptibles at the beginning of the observation period, and S_t is the number of susceptibles at the end of the observation period (de Jong and Kimman, 1994). To calculate Z, the day of infection was estimated for all gilts and it was assumed that the gilts were infectious during their entire viraemic period. The "SIR" (Susceptible-Infective-Removed) model was used to describe the final size distribution in terms of R_{0mrt} (de Jong and Kimman, 1994). Statistical test of R_{0mrt} were performed as described by Kroese and De Jong (in preparation) (H_0: R_0 Ω 1).

The maximum likelihood estimator is calculated numerically from:

$$R_{0mle} = \max \prod_{i=1}^{n} F\left\langle X_i, R_0 \,\middle|\, N, S_0, I_0 \right\rangle$$

where $F(X_i,R_0|N,S_0,I_0)$ is the likelihood function for the observed value X_i. X is the total number of pigs that become infected, N, S_0, and I_0 are the total number of animals, the number of susceptible animals and the number of infectious animals at the beginning of the outbreak, respectively (Bouma et al., 1996).

Fever was defined as a rectal temperature > 39.0°C. This is the one-sided upper 95% confidence limit (CL) calculated on the average rectal temperature of each gilt during the last three days before experimental inoculation. Leucopenia was defined in a similar way and the one-sided lower 95% CL limit was equal to 11,500 cells/ml.
Periods during which a given clinical symptom occurred started with the first of at least two subsequent observations of a given clinical symptom and ended with the first of at least two subsequent observations for which the given clinical symptom was absent. Periods of positive VI, PCR and leucopenia were defined in a similar way.

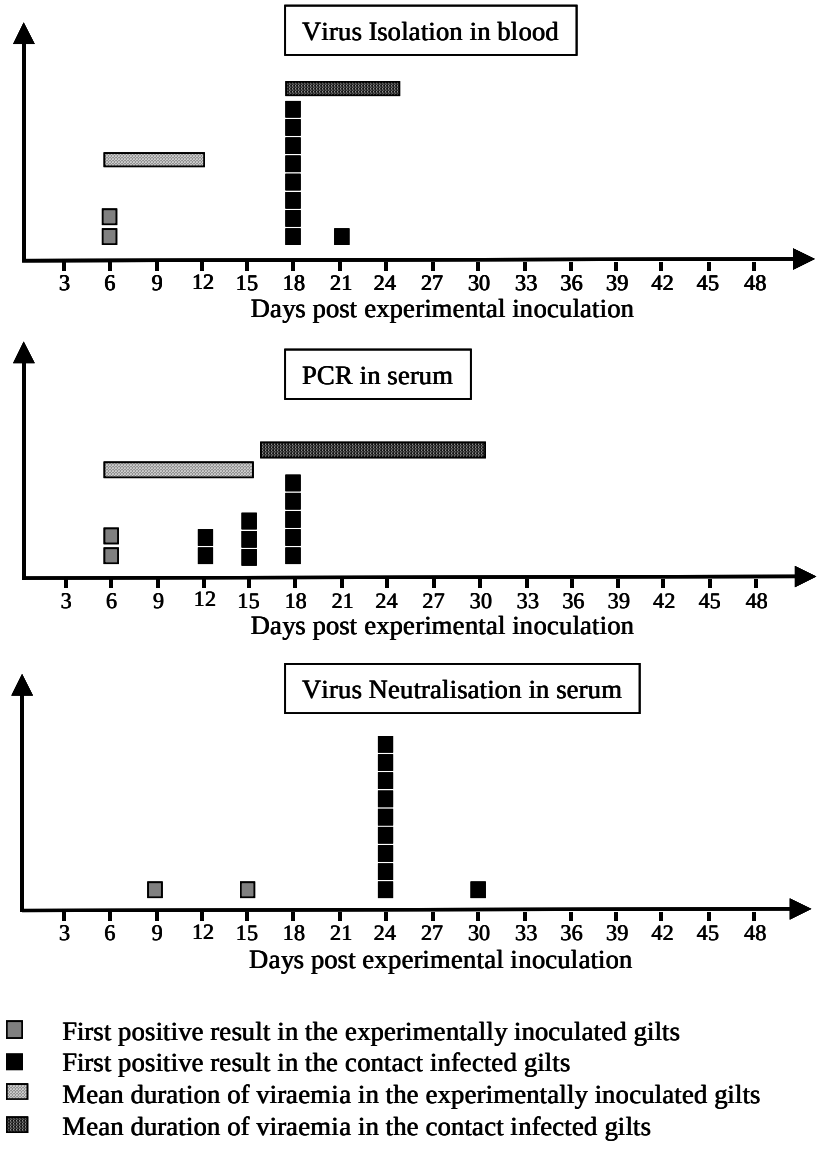
The time to first leucopenia, positive PCR and positive serology was compared with the time to first positive VI in blood using a paired sample T-Test (SPSS). Also the period during which leucopenia was present and during

which PCR was positive, was compared with the period during which VI in blood was positive using a paired sample T-Test (SPSS).

3. RESULTS

Both experimentally inoculated gilts were first detected positive for CSF on VI at 6 dpi. At the same time virus was also detected (PCR) for the first time in the nasal secretion and faeces of these gilts. The number of gilts with first positive VI, PCR and VN test at each time point is shown in Figure 2. In gilt 7 no viraemia was detected using VI, yet PCR and VN were positive.

Figure 2: Virological and serological response after infection



- First positive result in the experimentally inoculated gilts
- First positive result in the contact infected gilts
- Mean duration of viraemia in the experimentally inoculated gilts
- Mean duration of viraemia in the contact infected gilts

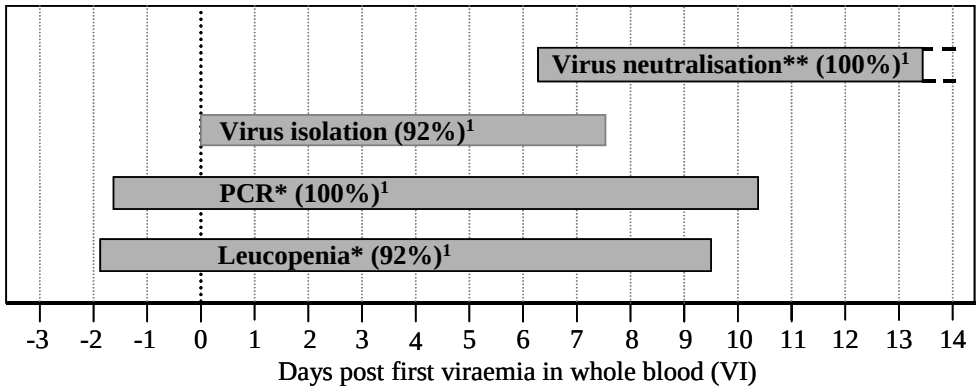
Based on the results of VI in the experimentally inoculated gilts, the moment of infection of the contact infected gilts was estimated to be two observations (6 days) before the first positive VI.

Since there was no positive VI in gilt 7 the moment of infection of gilt 7 was estimated based on the results of the PCR in serum. The first positive PCR in serum occurred on average 1.64 days before the first positive VI (Figure 3). Therefore the day of infection was estimated to be 4.36 days (6 – 1.64) before the first positive PCR. However, since there were only observations every three days, the estimated day of infection of gilt 7 was equal to one observation (3 days) before the first positive PCR.

The martingale estimate of R_0 was calculated to be 25.1 (H_0: R_0 Ω 1; $p<0.01$). Since no susceptible gilts remained at the end of the experiment, the maximum likelihood estimate of R_0 was $+\infty$. The lower boundary of the 95% CI of the R_{0mle} was 1.24.

In Figure 3 the diagnostic techniques are compared, with VI in whole blood as reference. Both leucopenia (1.8 days) and positive PCR in serum (1.6 days) occurred significantly ($p<0.05$) earlier than positive VI. Antibodies (VN test) were detected on average 6.3 days after the first positive VI ($p<0.01$). The average period during which leucopenia was present (10.5 days) and PCR was positive (12 days) was also significantly longer ($p = 0.015$ and $p = 0.049$, respectively) in comparison with the period during which VI in whole blood (7 days) could be observed.

Figure 3: Different diagnostic methods, using virus isolation (VI) as reference



* significantly earlier and longer than virus isolation ($p < 0.05$)
** significantly later than virus isolation ($p < 0.01$)
1 percentage of gilts with positive test

Figure 4: Evolution of temperature and leukocyte count

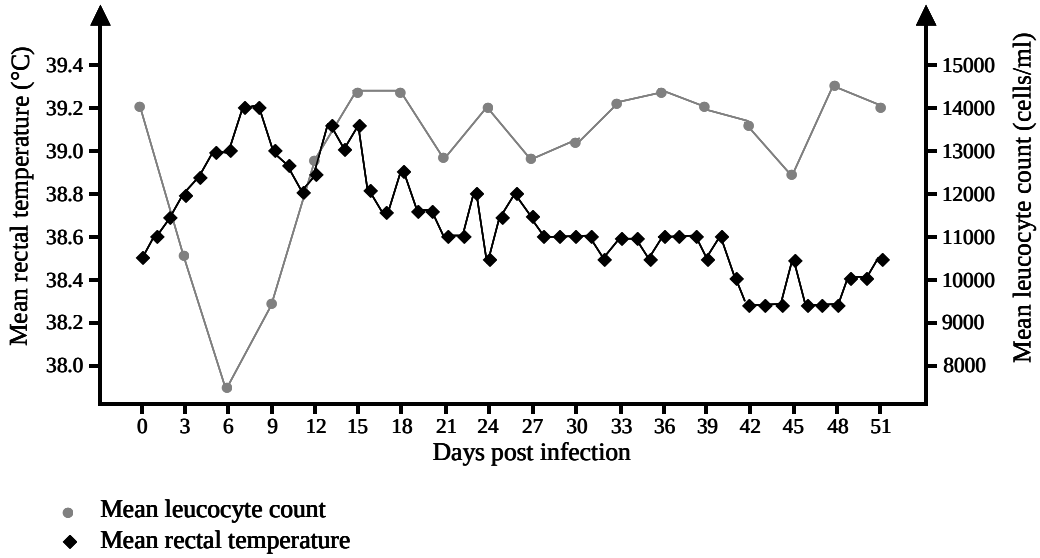
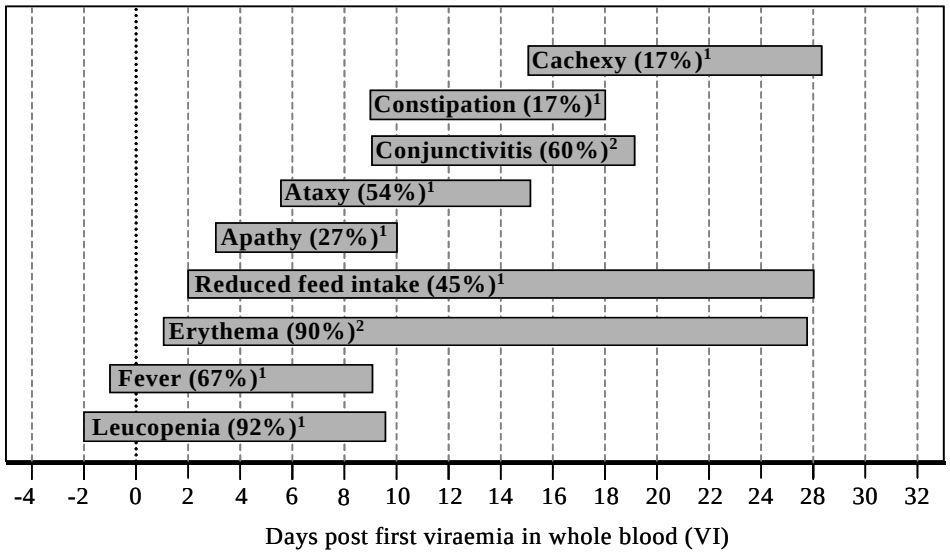


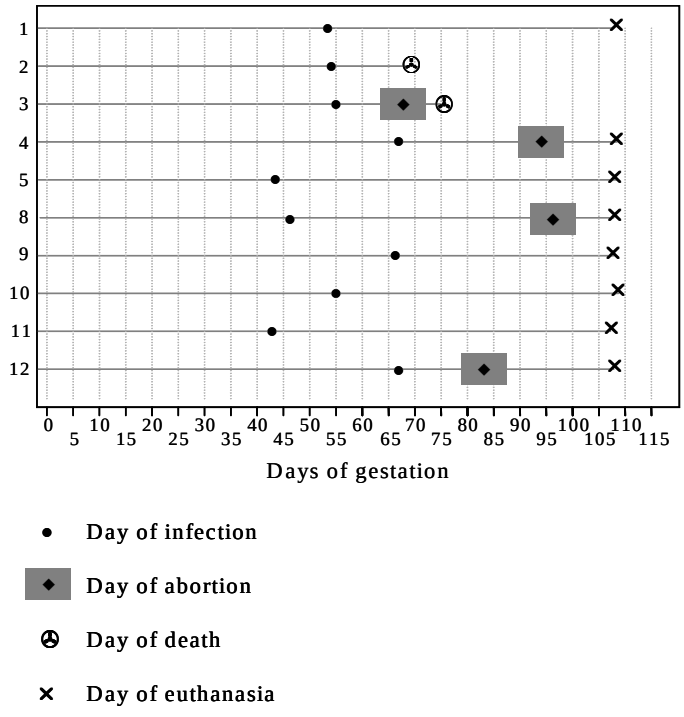
Figure 5: Different clinical symptoms, using beginning of viraemia (VI) as reference



[1] percentage of gilts with positive test, based on observations of 11 gilts, since no viraemia (VI) was detected in gilt 7

[2] percentage of gilts with positive test, based on observations of only 10 gilts, since gilt 12 showed already erythema and conjunctivitis before the day of experimental inoculation

Figure 6: Moment of infection in relation to the day of gestation



On the day of experimental inoculation the inoculated gilts were both on day 55 of gestation. The other gilts were between day 31 and 55 of gestation (Figure 1). All gilts got infected between day 43 and 67 of gestation (Figure 6). Four out of the 10 pregnant gilts aborted. The abortions occurred between 13 and 49 days after infection. In all pregnant gilts (aborted + euthanatized) the offspring was at least partially infected (between 45 and 100%). In four out of the five gilts that did not die nor abort before the end of the experiment, the offspring was partially mummified (between 20 and 73%). None of the infected offspring had seroconverted against CSF.

The clinical symptoms are summarized in Figures 4 and 5. Eight out of the twelve gilts showed fever (>39.0 °C). Fever appeared on average 5 days after infection, varying from 1 to10 days. The duration of fever varied between 2 and 31 days. The occurrence and the duration of the other clinical symptoms were also highly variable. For example gilt 6 remained without any clinical symptom during the whole observation period, although leucopenia and fever were observed, whereas gilts 8, 9, and 10 showed conjunctivitis and erythema without having fever. Gilts 2 and 3 died 15 and 20 days after infection, respectively (Figure 6). They both showed severe clinical illness before dying.

4. DISCUSSION

During the 1997 epidemic in The Netherlands 322 out of 429 outbreaks were detected based on the presence of clinical signs (Elbers et al., 1999). This illustrates the importance of regular clinical examinations during an outbreak. However, detecting a present CSF infection by clinical examination seems to be more difficult in breeding herds than in fattening herds. In fact, during the 1993-1994 epidemic in Belgium it was found that the time between the first occurrence of clinical signs and the reporting of CSF suspicion was longer when the disease was introduced in sows, boars or suckling piglets as compared with fattening pigs (Koenen et al., 1996).

The extended time between the detection of the first clinical symptoms and the suspicion of a CSF infection in breeding herds compared to fattening herds may be the result of a combination of factors.
First, the clinical symptoms in sows, following a CSF infection, are atypical and discrete and do not incline immediately CSF suspicion, unless the fact that the farmers spent more time in a sow unit whish makes the inspection of the sows more intense
(Elbers et al., 1999). Secondly, in a sow-box housing system, virus spread may proceed much slower, since it is generally assumed that direct contact between infected and susceptible pigs is the principal way of virus transmission (Edwards, 2000).

The atypical and discrete clinical symptoms and the low mortality rate following a CSF infection in sows are probably the most important factors causing a delayed diagnosis. In this experiment the first clinical symptoms that could be observed were fever and leucopenia. Other clinical symptoms (apathy, ataxy, conjunctivitis, constipation, cachexy) occurred later on and in a variable number of gilts. The symptoms are comparable with observations in the field during outbreaks (Koenen et al., 1996; Elbers et al., 1999). In comparison with experimental infections with the same strain in weaner and slaughter pigs (Laevens en al., 1998; Laevens et al., 1999) clinical symptoms were less severe in gilts. This is in agreement with previous studies where it was found that the clinical course of the infection is influenced by the age of the infected animal (Depner et al., 1994; Koenen and Lefebvre, 1995; van Oirschot, 1999). It should however be emphasised, that a large individual variability in the occurrence of the clinical symptoms was observed.

The effect of the infection on the gestation as observed in this experiment is comparable to what is described in literature (Terpstra, 1988). The “carrier-sow” syndrome remains important in the epidemiology of CSF, especially at the beginning and the end of an outbreak when the control measures are less strict.

The second possible explanation for the delayed diagnosis of a CSF infection in sows is the slower virus transmission in sows, especially in sow-box housing systems. The dynamics of a CSF infection in sows may differ from an infection in weaner or slaughter pigs because of the difference in age and housing system. The relation between age and the severity of the clinical symptoms has been discussed previously. However, the effect of age on the virus transmission has not been fully explained yet.

In this experiment it was found that both experimental inoculated gilts became viraemic between 3 (last negative response on VI) and 6 (first positive response on VI) dpi. These results are consistent with previous experimental inoculations in weaner and slaughter pigs (Depner et al., 1994; Laevens et al., 1998; Laevens et al., 1999; Dewulf et al., 2000) and indicate that age has no major effect on the time between infection and viraemia.

The calculated R_{0mrt} (25.1), which is larger than what has been found in previous experiments for slaughter pigs (13.7) (Laevens et al., 1999), and the observation that the two experimentally inoculated gilts infected all contact gilts, indicates that the virus spread in gilts proceeds relatively fast. These results also demonstrate clearly that CSF virus spread is indifferent to direct nose-to-nose contact. Therefore, airborne virus transmission may be more important in a sow-box housing system than previously accepted.

In view of the atypical and variable clinical symptoms, confirmation of a suspected infection should be done by diagnostic tests. It has been shown that leukocyte count and PCR are the two techniques that respond first, on average 2 days before the VI. Leukocyte count is a fast and easy technique that is sensitive however not at all specific. PCR on the other hand is sensitive and specific but it is labour intensive and expensive. To limit the workload, a first selection of the samples based on leukocyte count followed by a PCR on the samples with leukopenia may be preferred. An additional advantage of leukocyte count and PCR is that viraemia can be detected during a longer period compared to VI. The serology is of little use for an early detection, it is of great importance for screening purposes, due to the large number of samples that can be processed and due to the long detectable period.

In conclusion it can be stated that there is no major difference in the dynamics of a CSF infection between breeding and fattening pigs. Therefore, the late clinical detection of a present CSF infection is mainly due to the atypical and discrete clinical symptoms. As a preventive measure it may be recommended that in the presence of an unknown disease in sows, with atypical clinical symptoms as described, blood samples should be taken for CSF diagnosis. Leukocyte count with PCR as confirmation test is very suitable for an early diagnosis.

5. ACKNOWLEDGEMENTS

This study was supported by grants of the Belgian Ministry of Agriculture, Research Foundation and the Fund for health and quality of animals and derived products.

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EVOLUTION OF *SALMONELLA* AND *CAMPYLOBACTER* CONTAMINATION IN BROILER FLOCKS

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ABSTRACT

Eighteen broiler flocks were examined for *Salmonella* and *Campylobacter* from the hatchery until the slaughterhouse. In the hatchery, samples were taken during the collection of the one-day-old broiler chicks. The cleaning of the poultry house was tested, as were the paper tray liners of the transport boxes on arrival in the farm. During the rearing period the flocks (overshoes, caecal drops) and the environment were sampled at weeks 2, 4 and 6. In the slaughterhouse, faecal material of the transport crates and the chicken hides after cooling of the carcasses were tested.
In the hatchery and during transport of the chicks, in 1 and 2 flocks *Salmonella* was isolated, respectively. In 4 houses, *Salmonella* was found after cleaning, although molecular typing indicated that these strains did not always persist in the next flock. In the farm environment, a high contamination was established for *Salmonella* and *Campylobacter*, which clearly transferred to the animals in the poultry house. In 10 and 8 flocks, a contamination with *Salmonella* or *Campylobacter* was detected, respectively. The highest shedding for *Campylobacter* occured during 4 till 6 weeks rearing. The carcasses of 13 and 12 flocks were contaminated (>10%) with *Salmonella* or *Campylobacter*, respectively. For *Salmonella*, no relation was found between the contamination during rearing and after slaughtering. The identity of the slaughterhouse seemed to be the determining factor for the contamination of the end product. For *Campylobacter* the results of the living phase and the end product related better.

1. INTRODUCTION

Salmonella and *Campylobacter* are important foodborne pathogens causing gastroenteritis in humans. In spite different food products can cause foodborne infections, poultry products are frequently associated with *Salmonella* and *Campylobacter* infections.

Contamination of poultry may occur throughout the whole production chain. *Campylobacter* and *Salmonella* are spread in flocks by horizontal transmission. Different risk factors for such transmission are described in the literature: 1/ inadequate cleaning and desinfection of the rearing houses (4), 2/ poor level of hygiene (3), 3/ contamination of feed (2) and 4/ litter, beetles in the house and rodents on the farm (1). Moreover, for invasive *Salmonella* serotypes also vertical transmission is possible. Slaughtering of contaminated flocks leads to the contamination of the carcasses. Monitoring of foodborne pathogens in Belgium indicated that poultry products are highly contaminated with both pathogens.

Since a few years different measures are developed in Belgium to reduce the contamination of the end product: 1/ parent flock are systematically controlled on the presence of *S.* Enteritidis and *S.* Typhimurium, 2/ all flocks (layers and broilers) have to be examined before sending to the slaughterhouse and 3/ at the slaughterhouse logistic slaughtering must be applied. The two latter measures have no direct effect on the horizontal contamination during rearing of the broilers. Only prevention of the cross contamination during slaughtering can be obtained.

A significant reduction of contaminated poultry products can only be achieved by an integrated approach. This is only possible based on the knowledge of risk factors in the Belgian poultry sector. This present article describes the results of an epidemiological study on contamination sources of *Salmonella* and *Campylobacter* during the production of broilers.

2. MATERIALS AND METHODS

Eighteen broiler flocks were followed from the hatchery to the slaughterhouse. In the hatcheries, different samples (chicks, shells and environment) were taken during the collection of the one-day-old chicks. Before arrival of the chicks at the farm, swab samples were taken from poultry house the environment and feed and water. After the arrival of the chicks, tray liners of the transport boxes were collected. During the rearing period, the farm was visited three times (day 14, 28 and 42) and following samples were taken: inside the poultry house, caecal drops, different pairs of overshoes, feed and drinking water. Also the environment of the farm was sampled: faeces from other animals, water from surrounding brooklets, barrels with dead animals, footwear of the farmer, etc. Birds were about 42 days old when slaughtered. In the slaughterhouse (at about day 42), the following samples were taken: faeces from the transport containers, intestines and carcasses after cooling. All samples were put into plastic bags and boxes, placed in cooling boxes and immediately transported to the laboratory.

Following samples were tested for *Salmonella*: all samples from the hatchery, the farm, the faeces from the transport containers, livers and refrigerated carcasses. After preenrichment in Buffered Peptone Water (BPW), the following enrichment media were used: Rappaport-Vassiliadis (RV), Diassalm and Modified Semi-solid Rappaport-Vassiliadis medium (MSRV). After incubation these media were streaked onto Xylose Lysine Desoxycholate medium. Presumptive colonies were identified using biochemical confirmation tests or PCR.

For *Campylobacter*, the following samples were tested: a limited number of samples in the hatchery, all samples from the farm, the faeces from the transport containers, cecum content and refrigerated carcasses. Faecal material and cecum content were inoculated directly on the selective plate CCDA. These samples and all other samples were also enriched in Preston broth before selective plating on CCDA.

3. RESULTS AND DISCUSSION

The status of a flock was determined using the results of the analyses performed on caecal droppings and overshoes. If minimum one sampling yielded *Salmonella* or *Campylobacter* in the caecal drops or the overshoes, the flock was considered positive. The status for *Salmonella* during rearing of the broilers was clearly determined most sensitively by the 'overshoe method'. No extra positive flocks were found by testing caecal drops. Moreover on different sampling days less than 50% of the collected overshoes were found *Salmonella* positive. These results indicated that more than 1 or 2 pairs of overshoes has to be examined in order to determine the presence of *Salmonella* in a flock. In contrast nearly all caecal drops from *Campylobacter* contaminated flocks were found positive.

Ten of the 18 flocks yielded positive overshoes and received the status *Salmonella* positive (table 1). *Salmonella* was present in samples from 1 hatchery (*S.* Enteritidis) and from paper tray liners of transport boxes of 2 flocks (1x *S.* Enteritidis and 1x *S.* Hadar). In the 2 cases of *S.* Enteritidis contamination the flocks became also contaminated with this serotype. *Salmonella* was found after cleaning in 4 poultry houses. From 2 of the flocks reared in these houses the same serotype could be isolated. Four feed samples were *Salmonella* positive. In 2 of the cases, the contamination (with *S.* Mbandaka) of the feed did not lead to the contamination of the chickens. In the 2 other cases, the same serotype was found in the overshoes. However, in one flock the serotype was already isolated from the overshoes 14 days before. This result suggests that this feed was contaminated at the farm. In the environment of the farm, a high contamination level was detected (11 positive farms out of 18). On 3 of these farms, the environment must be considered as the source of contamination because the same serotypes isolated from the overshoes were already found in the environment of the farm at the arrival of the one-day-old chicks. On 4 other farms the same serotypes were only isolated in the environment after these were found on the overshoes from the flocks. Consequently, the role of the environment in the contamination is not clear. Probably the environment was contaminated by the contaminated flock. This hypothesis is supported by the fact that the environment (4 flocks) not always led to the contamination of a flock.

Table 1: Prevalence of *Salmonella* and *Campylobacter* in 18 flocks of broilers

	+ flocks

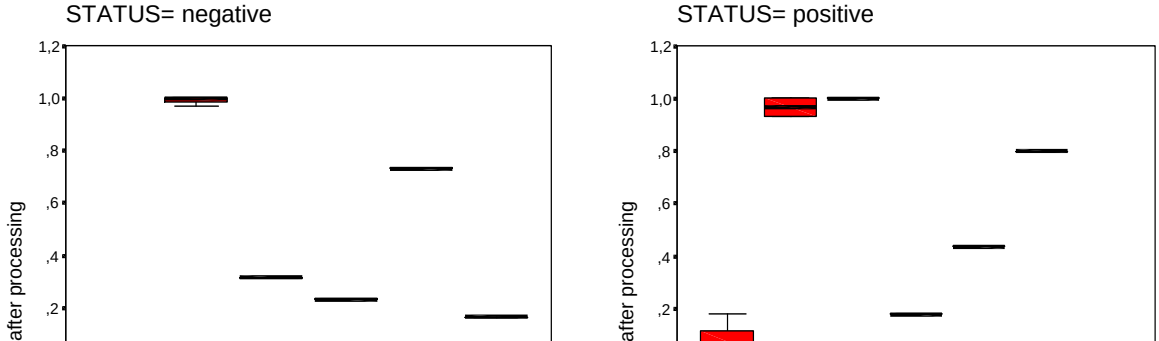
		Salmonella	*Campylobacter*
Hatchery		1	0
Transport		2	ND
Farm	hygiene house	4	0
	feed	4+/115 samples	ND
	environment house	11	13/15
	status animals	10	8
Slaughterhouse	faecal material	12/16	ND
	cecum content	ND	14/17
	neck skin	13 (>10% + samples)	12 (>10% + samples)

ND: not determined

Eight flocks were contaminated with *Campylobacter*. This pathogen was not present in samples collected from the hatcheries and the houses after cleaning and desinfection. However the flocks became contaminated during rearing especially at day 24 and 42. All flocks, with the exception of one flock, were contaminated with *Campylobacter jejuni*. The environment of the broiler houses was very frequently contaminated with *Campylobacter*. In most cases, *Campylobacter* could be isolated from the environment before the animals were found positive. This suggests that the source of contamination is mainly the environment. However, further genetic typing of isolated strains is necessary to confirm this.

At the slaughterhouse level, carcasses of more flocks became contaminated, especially with *Salmonella*. For *Salmonella* no relation was found between the contamination during the rearing period and the contamination found after slaughtering. The identity of the slaughterhouse seemed to be the determining factor for the contamination of the end product (figure 1). Hereby the applied evisceration method and the time of slaughter during the day did not have an apparant influence. During rearing contaminated flocks were frequently contaminated with 2 different serotypes. At the slaughterhouse up to 2 and 7 different serotypes were isolated from faecal material of the transport containers and the carcasses respectively. Moreover, the same serotypes could only be identified from the faecal material and the carcasses from 3 flocks. These data clearly showed that transport and slaughtering are important sources of contamination for *Salmonella*. Slaughtering of contaminated flocks resulted in a high *Campylobacter* contamination level of the carcasses. Carcasses of 4 flocks found negative during rearing were also found to be contaminated. The latter data suggest that cross contamination of *Campylobacter* can occur during transport and slaughtering of flocks.

Figure 1: The isolation of *Salmonella* positive carcasses in function of the status in the poultry house and the identity of the slaughterhouse



From the results of this study the following conclusions can be drawn:
1/ The overshoe method was shown to be the most sensitive sampling method for the detection of a *Salmonella* contamination. However different pairs of overshoes must be taken to obtain reliable results. For *Campylobacter*, caecal drops can be collected.
2/ For *Salmonella* vertical transmission and the hygiene of the broiler house are still important.
3/ Because the environment can be an important source of contamination for *Salmonella* and *Campylobacter* more attention must be given to the correct use of the hygiene gate in the broiler houses.
4/ Contamination during rearing is not significantly correlated with the contamination of the end product, especially for *Salmonella*. The identity of the slaughterhouse is significant for the carcass quality obtained. Further research has to be carried out to determine the effect of transport on the contamination and to find the contamination sources during slaughtering.

4. ACKNOWLEDGEMENTS

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COMPARISON OF THE BOVINE HERPESVIRUS TYPE1 GLYCOPROTEIN E SEROPREVALENCE IN DAIRY AND DAIRY/BEEF MIXED HERDS

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ABSTRACT

The distribution of the seroprevalence of the glycoprotein E of the bovine herpesvirus type 1 was determined at animal level in January 1997, in 34 dairy herds and 32 dairy/beef mixed herds.
Animals were classified by production type in 5 age classes in females and in 4 classes in males. To avoid bias due to the differences in population structures, the statistical analysis using a generalised linear model with Poisson error distribution was performed after stratification on sex.
In females, in both pure dairy and dairy/beef production types, the seroprevalence was related to the age. In animals younger then 6 months, a high level of seroprevalence was due to maternal immunity. In older animals, seroprevalence increased with increasing age. A significant interaction between age classes '6-12 months' and '1 to 2 years' was observed. The seroprevalence therefore calculated to be higher in mixed herds than in dairy herds.
In males, the prevalence was also function of age, following the same pattern: increased seroprevalence in the youngest age group, attesting the presence of maternal immunity then increasing with increasing age. In this group the effect of age and production type were significant but no interaction between production type and age class was demonstrated.
The major conclusions of the analysis were that the type of production determined the characteristics of the cattle population and that sex and age of animals explained the differences in the gE seroprevalence observed in dairy herds and dairy/beef mixed herds.

1. INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) is the etiological agent of two major clinical entities: *i.* the respiratory form affects the upper respiratory tract causing infectious bovine rhinotracheitis (IBR) and *ii.* the genital form causes infectious pustular vulvo-vaginitis (IPV) or balanoposthitis. BHV-1 is responsible for important economic losses due to respiratory disease, abortion (Wyler et al., 1989) and decreased milk production (Hage et al.1998).

The production of BHV-1 marker vaccine (Kaashoek et al., 1994), deleted in the glycoprotein E (gE) gene, and the development of his companion gE blocking Elisa's test kit that allows to make the distinction between naturally infected and vaccinated animals (Van Oirschot et al.,1997), made available a powerful tool for IBR control. Indeed, after BHV-1 infection, animals remain lifelong latent carriers (Wyler et al., 1989) and reactivations stimulate host immune response that allows the persistence of high antibody titres. Any gE seropositive animal may be therefore considered as latently infected (Kaashoek et al., 1996).

BHV-1 seroprevalence varies among European countries from 0% for certified IBR-free countries such as Denmark or Sweden, to high positive level: 67% for Belgium (Boelaert et al., 2000a) and 84% for The Netherlands Van Wuyckhuize et al., 1998).

Epidemiological studies analysed BHV-1 circulation (Hage et al. 1996) and seroprevalence in farms housing dairy cows (Hartman et al., 1997; Van Wuykhuize et al., 1998) but few information exists about the BHV-1 seroprevalence in farms combining dairy and beef productions or in farms specialised in beef production (de Wergifosse et al., 1997; Boelaert et al. 2000a). In none of these studies the effect of production type on gE seroprevalence was pointed out.

As production type has an influence on the length of animal's production life, replacement rate and housing, it can also influence the BHV-1 seroprevalence. As 79 % of the Belgian herds are concerned with beef production (Boelaert et al., 2000b) and as the Belgian Government analyses the feasibility of an official BHV-1control plan approved by the EU, the present data are of major interest.

The aim of the present work is to compare the age structure, sex ratio and the differences in age related BHV-1 gE seroprevalence between production types, in 34 dairy herds (DH) and 32 dairy/beef mixed herds (DBMH), in January 97.

2. MATERIAL AND METHODS

2.1. Sampling method

Two pools of farmers willing to participate in a longitudinal field study were recruited by veterinary surgeons: one for the study of pure dairy herd, the other for the study of dairy/beef mixed herds. In each pool, a group of 36 farms was drawn at random. In pure dairy herds 34 farmers satisfied the inclusion criteria until the end of the study, for 32 in dairy/beef mixed herds. The inclusion criteria were: herd size about 100 animals, at least 3 gE seropositive animals at the start of the study, adherence to the blood sampling calendar and type of production.
Blood was collected from all animals (total number = 6,784), over one month of age, early January 1997 and analysed at the VAR.

2.2. Collected data

Animal and herd characteristics were obtained from SANITEL-cattle, the central computerised database for the identification and registration of the Belgian cattle population (Ministry of Agriculture, Belgium).
Individual animal data were recorded: date of birth, sex, date of blood sampling, result of serological analysis with the ELISA HerdChek* IBR gE diagnose kit (IDEXX). The results were expressed as blocking percentage. All results under 30 % were considered as negative. As no individual information on previous vaccination was available no difference was made between the seropositivity due to the presence of maternal antibodies, to vaccination with non-marker vaccines or to wild type BHV-1 infection (Lemaire et al., 1997).

2.3. Age related distribution of seroprevalence

For each production type, females were grouped in 5 classes according to age: (1) 0-6 months: possible presence of maternal antibodies (Menanteau-Horta et al., 1985), (2) 6-12 months, (3) 1-2 years: possibility of first contact with bulls or with dry cows (Hanzen, 1994) belonging to the highly gE+ group, (4) 2-4 years: expected age at first and second calving and (5) animals older than 4 years: animals that can be considered as indicators of the herd infection history (IHIH).

For each production type, males were grouped in 4 classes according to age. The three first classes were the same as those hereabove described for classes 1 to 3 and class 4 corresponded to all animals older than 2 years.
Individual serological analyses were performed and the gE seropositivity was analysed by means of a generalised linear model using the following explanatory variables: age category, production type and sex.
Statistical analysis was carried out using STATA ^{tm} 6.0 (StataCorp 1999) and threshold for alpha error fixed at 0.05.

3. RESULTS

3.1. BHV-1 gE seroprevalence

Because of the differences observed between production types with respect to numbers and age distribution of male animals, a statistical analysis was performed after stratification by sex.
A generalised linear model with Poisson error, i.e. a log linear model, was retained.
In females significant differences in seroprevalence were found between different age groups but these differences were not the same in the 2 types of production.

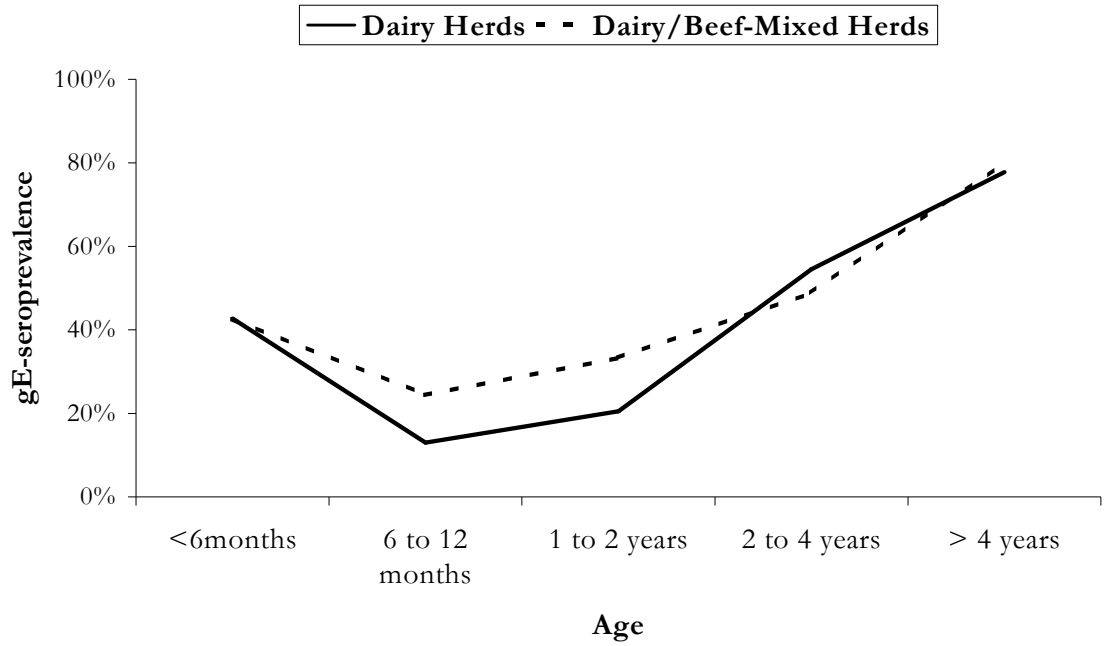

Figure 1: gE-seroprevalence in function of age by production type in female cattle animals

The Figure 1 presents the seroprevalence in function of age groups in females for the 2 production types. No difference in seroprevalence between production types was found in females younger than 6 months of age (43%). The seroprevalence at animal level is decreased between 6 months and one year and also in the group '1 - 2 years',compared to the the group 0-6 months of age, but this decrease was significantly smaller in DBMH (24 % and 33%, respect.) than in DH (13% and 20% respect.). The seroprevalence increased after 2 years of age and no significant difference persisted between production types in these older animals. For the two last age categories the seroprevalence was calculated to be 55% and 78% in DH and to be 49% and 80% in DBMH.

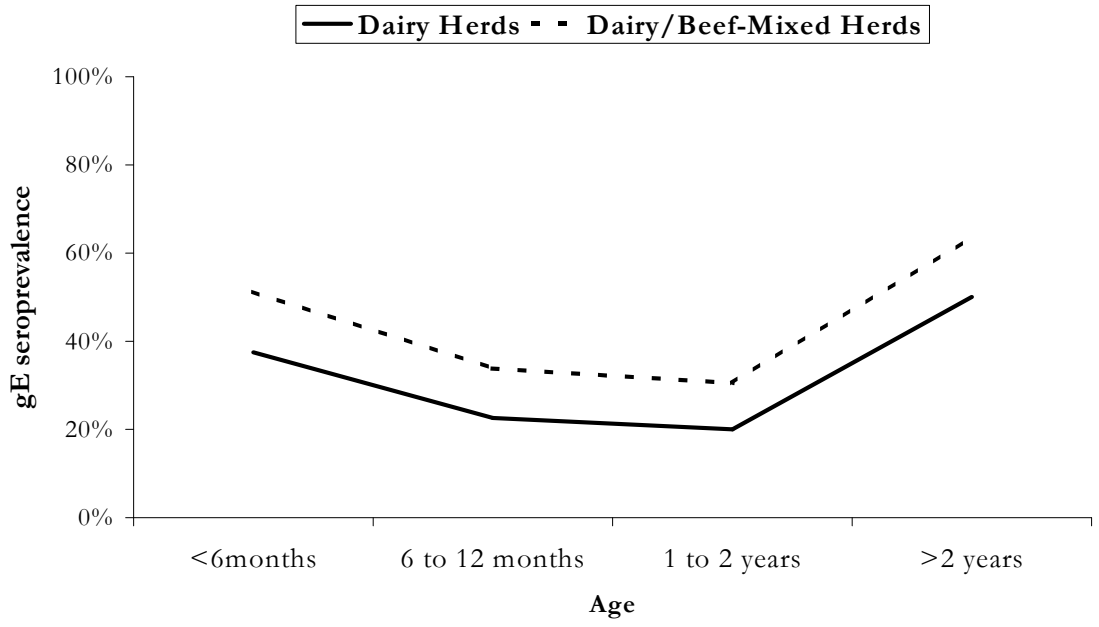

Figure 2: gE-seroprevalence in function of age by production type in male cattle animals.

A similar model was applied in males, presented in Figure 2, but no significant interaction existed between age and production type.

No significant difference between seroprevalence was found between males and females but the low number of males has to be taken into consideration.

The seroprevalences by age classes, presented in Figure 2, were calculated to be 37%, 23%, 20% and 50% in the dairy group and were calculated to be 51%, 34%, 30% and 64% in the dairy/beef mixed group.

The relative importance of these levels of gE seropositivity have to be weight by the number of animal in each age class.

The figure 3 and 4 present the number of females and males, respectively, by age classes, in dairy herds and dairy/beef mixed herds.

The Figure 3 presents the number of female animals by production type. Animals were classified in 5 age categories.
In the dairy group the count of animals, by growing age, was calculated to be 398, 254,747,1066 and 982. It was 183, 328, 572, 928 and 631 in the dairy/beef mixed group.

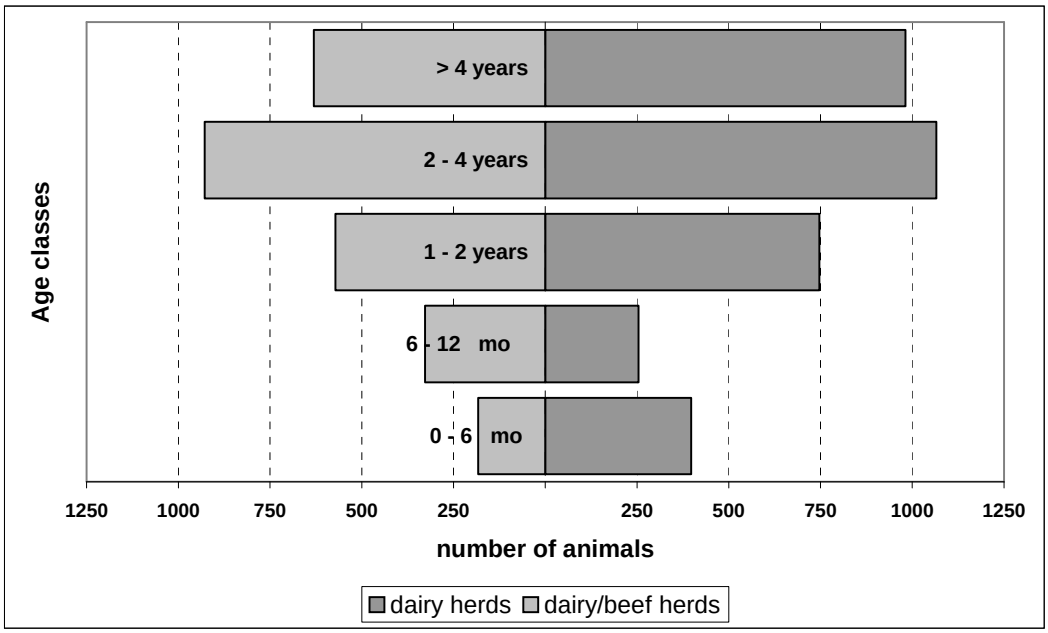

Figure 3: Distribution of females by age classes in Dairy and Dairy/Beef mixed herds

The Figure 4 presents the number of males by production type, classified in 4 age categories. In the dairy group the count of animals, by growing age was 21, 55, 26 and 5. In the dairy/beef mixed group it was recorded to be 131, 256, 161 and 40.

4. DISCUSSION

This study compares the gE seroprevalence by age classes in dairy herds and dairy/beef mixed herds, after stratification on sex, in january 1997, at the start of a 18 month long field study.

The herds were selected assuming that: i. the chosen size allows the farmer to live with the single income of his production and to avoid confounders linked with herd size (van Schaik et a., 1998) which is often considered as risk factor for BHV-1 infection (Van Wuykhuise et al. 1998) and ii. the goodwill of the farmer was ensured for the whole length of the study.

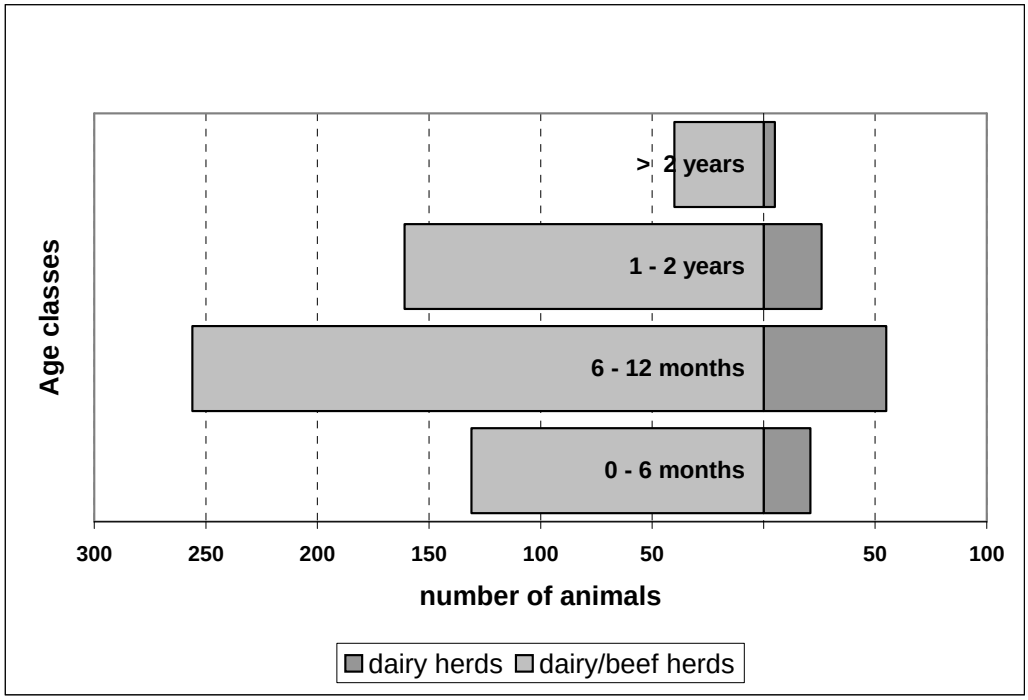

Figure 4: Distribution of males by age classes in Dairy and Dairy/Beef mixed herds

Because of the differences existing in age and sex related distributions of animals in the two production groups, the gE seroprevalences in males and females were analysed separately.
The seroprevalence in the group of female animals younger than 6 months shows the combination of early infections and maternal immunity.
In the next age category the seroprevalence is lower: animals have lost their maternal antibodies. The seroprevalence increases in the third age class for 4 possible reasons: **i.** vaccination with non marker vaccine, **ii.** first contacts with dry cows (possible shedders of the highly gE seropositive group), **iii.** spontaneous virus reactivation in animals which were infected when protected by a passive immunity (Lemaire et al. 2000a, 2000b) and **iv.** infection linked with sexual activity.
In the group of animals " 2 to 4 years" of age the level of gE seropositivity increases when the contact rate increases with animals having cumulated more vaccinations or infections risk. In the last group, the gE seropositivity reaches a maximum of 80%. This group has cumulated the maximum risk of being infected by a wild type virus strain and being vaccinated with a conventional vaccine.

The herd management clearly influences the levels of gE seroprevalence. The differences of serological profiles between DBMH and DH can most likely be explained by the closer contact between calves and dams in beef production where suckling calves are present. These contacts allow earlier infection in DBMH. In opposite, in DH, if the IHIH group is considered as an inner reservoir of BHV-1, the longer life expectancy of dairy cows could act as brake on BHV-1 cleaning up.

In males, the seroprevalence is higher in the DBMH, in all age classes. The difference with DH, although not significant, was explained by **i.** the higher contact rate between animals in feedlots **ii.** the fact that animals of different farms may be brought together after transport (Thiry and al., 1987).
The cattle populations of both production type are different in age related distribution : in DH males are rare, while they represent almost 25% of the animal number in DBMH. The relative number of females in the group '0-6 months' of age is higher in the DH, than in DBMH, but the proportion is inverted in the next age class. There are proportionally more females in the oldiest age class in DH than in DBMH. This age distribution is the reflection of the wish to obtain a higher milk production in the winter period and a longer production life in the DH.

No significant effect of sex was demonstrated, although the seroprevalence is higher in males in the first two age categories (0-6 months and 6-12 months). The influence of sex could be underestimated because of the difference in animal numbers (see figures 3 and 4).

Herd management has an important impact on BHV1 infection levels (Ackerman et al.,1990; Wentinck et al.1993; van Schaik et al.,1998). All the risks factors of infection linked with production type will be further considered and analysed in a longitudinal study. The longer production period in DH could have a direct influence on the herd's sanitary status: the majority of adult animals are seropositive and may be virus reservoirs, maintaining a constant level of prevalence by recurrent episodes of virus reactivation and shedding after stress conditions (Thiry et al. 1985). In DBMH, the high purchase rate, the transport (Thiry et al. 1987) of young animals in order to insure proper feedlots throughout the year, and the extended contacts between suckling calves and dams could represent different risk factors for BHV-1 circulation.
In conclusion, we demonstrated that significant difference exists in BHV-1 seroprevalence between the 2 production types. The major conclusions of the analysis are that the type of production determines the characteristics of the cattle population in the farm and that sex and age of the animals could explain the differences in the seroprevalence observed in the 2 types of production.

5. ACKNOWLEDGEMENTS

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IS THERE A RELATIONSHIP BETWEEN CROHN'S DISEASE IN HUMANS AND PARATUBERCULOSIS

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ITG

ABSTRACT

Reports on the role of *Mycobacterium avium* subsp. *paratuberculosis* (MPTB) as an etiological agent of Crohn's disease (CD) have been conflicting. Despite this the interest in the association of MPTB with CD is still high, especially with recent reports that worldwide incidence estimates of bovine paratuberculosis (PTB) may double with newer diagnostic techniques and that MPTB may survive pasteurisation. There are clinical and pathological similarities of PTB to CD. Comparison between epidemiological data available for CD and PTB do not provide evidences for causal association between the geographical incidence and prevalence of both diseases. Attempts to recover MPTB by culture from patients with CD have only rarely succeeded. Reports on the role of MPTB in CD that used PCR-IS900 are numerous and particularly controversial. Serological studies have failed to demonstrate antibody specific to MPTB in patients with CD and attempts to show cell-mediated immunity were also unrewarding. Immunocytochemistry studies and experimental infection of animals also failed to establish the link. Controlled studies of the treatment of CD with antimycobacterial agents have generally resulted in no improvement, and most studies that have shown a positive response include a broad spectrum antibiotics. In conclusion there is no sufficient evidence to support an etiological role for MPTB in CD. The priority of MPTB research is clearly the development of tools for eradication of PTB in domestic animals.

Crohn disease (CD).

In 1932, Crohn and co-workers described a chronic disease in humans which was characterised by fever, diarroea, progressive anemia and loss of weight. The pathological lesions consisting of ulcerative granulomas and fistulas are mainly in the terminal ileum and colon but may also occurs in any part of the gastrointestinal tract.from the mouth to the anus. It is a life long disease and has no cure.
A multicenter European study has calculated an incidence rate for CD of 5.6 per 100,000 per year. Considerable local differences are observed. It is estimated that over 200,000 people are affected by CD into the European Union.

The pathogenesis of CD probably involves an interaction of genetic and environmental factors. The mechanisms that is responsible for initiation of chronic intestinal inflammation is not known.Four hypotheses are currently considered: (1) reaction to a persistent infection (2) defective mucosal barrier to luminal antigens (3) dysregulated immune response to ubiquitous antigens (4) breakdown of tolerance against microbial flora of the gut.

As yet, the cause of Crohn's disease (CD) remains unknown, but a multi factorial etiology including environmental, genetic, immunologic, and microbial factors have been suggested.
This speech will focus on a critical examination of previously published data about the possibility that Crohn's disease is caused by of *Mycobacterium avium* subsp. *paratuberculosis* (MPTB). MPTB has been implicated as one of the etiologic factors of CD. Numerous bacteria and viruses have also been investigated.

Mycobacterium avium subsp. *paratuberculosis* (MPTB)

In 1895, Johne and Frotingham demonstrated the presence of acid fast bacilli in affected animals They named the disease paratuberculosis (PTB), later known as Johne's disease, because they thought that it was an atypical form of tuberculosis.
Paratuberculosis is a contagious and enzootic disease of ruminants caused by the multiplication of *Mycobacterium avium subsp. paratuberculosis* (MPTB) in the mucous membrane of the intestine. MPTB can also infects a wide range of domestic and wild animals. Transmission can occur by the fecal-oral route.

The symptoms consist of diarrhoea, progressive anemia and weight of loss. The lesions are usually restricted to the ileum. The pathological lesions (as in CD) consist of granulomatous inflammation, without caseation necrosis.
The main characteristics of MPTB are its slow growth and dependency on exogenous mycobactin (an iron chelating agent). Currently available evidences points out homogeneity within MPTB subspecies. To date the use of molecular markers has not been able to demonstrate clear association of MPTB subtypes with the animal of origin.

Association of MPTB with CD

In 1984, Chiodini et al., isolated MPTB from 3 out of 14 patients with CD. Since then reports on the role of MPTB as an etiological agent of CD have been conflicting. The interest in the association of MPTB with CD is high, especially with recent reports that worldwide incidence estimates of bovine PTB may double with newer diagnostic techniques and that MPTB may survive pasteurisation of milk. Increasing concern about the transmission of infectious diseases from animal to man has also contributes to refocus the attention on the possible association of MPTB with CD.

Pathological and clinical similarity between CD and PTB

Clinical and pathological similarities of bovine PTB to CD led to the hypothesis that CD was caused by MPTB. The absence of visible acid fast bacilli in CD tissues and the lack of important extraintestinal manifestations in PTB are important differences. However acid fast bacilli are also scarce or absent in lymphocytic/paucibacillary form of PTB and most publications are comparing CD in humans with PTB in ruminants. Comparison with MPTB infections in other animals may be relevant.

Epidemiological data do not support the hypothesis MPTB-CD

Although PTB is a contagious enzootic disease infecting a wide range of domestic and wild animal species, and infected animals shed MPTB in large numbers, no case of zoonotic transmission has been recorded. Moreover, higher incidence of CD has not been observed in occupational groups (farmers, veterinarians and abattoir workers) coming in contacts with infected animals.
Comparison between epidemiological data available for CD and PTB do not provide evidences for causal association between the geographical incidence and prevalence of both diseases suggesting that CD is not caused by simple contact and infection with MPTB.
Time lag before clinical signs, multifactorial nature of both diseases, dietary habits in humans and influence of migrations are considered by scientists supporting the association MPTB-CD as the main factors responsible for this lack of evidence.

Attempts to culture mycobacteria from CD tissues area rarely successful

Attempts to recover MPTB by culture have only rarely succeeded and the significance of spheroplasts that appear more frequently on culture is seriously in question.
Investigators who support the role for M.PTB in CD have argued that culturing the organism from tissues of CD patient is extremely difficult, as is the ability to histologically demonstrate the presence of MPTB in the affected tissue. Some have suggested that detection is difficult because MPTB may exist as cell wall deficient spheroplast in tissues.
PCR method should easily overcome these problems.

Reports on detection of MPTB DNA in CD tissues by PCR are controversial

The mobile genetic element IS900 is a DNA sequence considered so far as the best target for detection of MPTB DNA by the polymerase chain reaction (PCR). Reports on the role of MPTB that used PCR-IS900 are numerous and particularly controversial.
BJ Jayarao in1999 made available a review of scientific papers published between 1990 and 1998. The papers were analysed using meta-analytic techniques to determine the association of MPTB with CD. Meta analysis of 20 scientific papers (458 patients with CD, 584 other) exploring the association CD-MPTB with the help of IS900-PCR suggested that MPTB was more likely to be detected in patients with CD (Odds ratio 2.35:1). However, the study reveals that odds of detecting MPTB in CD patients were strongly influenced by the geographical location and the date of the study. The five most recent polymerase chain reaction attempts to find

MPTB DNA were uniformely negative. BM Jayarao concludes: “Our review suggests that MPTB does not play an etiological role in CD.”

Serological and CMI studies were unrewarding

Serological studies have failed to demonstrate antibody specific to MPTB and attempts to show cell-mediated immunity (CMI) were also unrewarding. These studies suffer from the fact that a considerable proportion of the population has been exposed to *M. avium* subspecies other than MPTB. It is actually not expected to detect differences in serological or CMI reactivity between patients with CD and other people having developed immunity to other *M. avium* with the antigenic preparations used so far.

Results of antimycobacterial drug trials in treating patients with CD are not conclusive

If MPTB is an etiological factor of CD, specific chemotherapy may help in reducing the symptoms. Controlled studies of the treatment of CD with antimycobacterial agents have generally resulted in no improvement, and most studies that have shown a positive response are either uncontrolled or include a broad spectrum antibiotics that may be acting on pathogens other than mycobacteria.

Other strategies…

Five immunocytochemistry studies have failed to find mycobacterial antigens in diseased tissue. Inoculation of experimental animal models with CD tissue has failed to induce PTB.
Inoculation of various animals species with MPTB has equally failed to result in CD.

CONCLUSIONS

A careful conclusion would be to leave the question open, writing: “while the evidence does not support an etiological role for mycobacteria in CD, newer techniques may change this conclusion” (Hubbard and Surawicz, 1999).
A more pragmatic conclusion for the mycobacteriologists looking for funding would be the one from the EC report (Badiola et al, 2000): “The current available evidence is unsufficient to confirm or disprove that MPTB is a causative agent of at least some cases of CD in man. There are sufficient grounds for concern to warrant increased and urgent research activity to resolve the issue”.
I would keep for my own conclusion what is common to the two previous citations: “there is no sufficient evidence to support an etiological role for MPTB in CD”. I believe that the additional comments from both reviews are not relevant. Of course the present conclusion can be modified by new experimental results obtained with newer techniques. It is useless to specify it. By definition conclusions from experimental sciences can be modified by new results.
If the problem is to attract funding for MPTB research may I suggest that the priority is to eradicate MPTB from animals. There are numerous evidences that it would greatly contribute to the quality of meat and milk and the welfare of farmers.

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SALMONELLA: FOOD-BORNE INFECTIONS AND SANITARY MEASURES IN POULTRY

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ABSTRACT

Food-borne infections due to *Salmonella* are frequently described, both in Belgium and abroad. Often eggs, egg products and chicken meat are identified as source of infection. Throughout the production chain from layers and broilers to the consumer, it is clear that at almost all points *Salmonella* contamination may occur. It is obvious that all partners involved in food production need to collaborate to control these infections.

Samenvatting

Voedseltoxi-infecties ten gevolge van *Salmonella* worden zeer frequent beschreven, zowel in het buitenland als in België. Vaak kunnen eieren, eiproducten en kippenvlees als bron van infectie aangetoond worden. Bij het overlopen van de productieketen van eieren en kippenvlees van op het kippenbedrijf tot bij de consument is het duidelijk dat op zowat alle stadia mogelijke contaminatie met *Salmonella* kan optreden. Het is dus evident dat alle betrokken partijen moeten samenwerken om deze infecties te bestrijden.

INTRODUCTION

Salmonella and also *Campylobacter* infections are among the most frequently registered food-borne infections in many European countries and even worldwide. Salmonellosis is frequently associated with gastrointestinal illness: mild to sever diarrhea, nausea, eventually headache and fever. Especially the very young, the old, pregnant women and immunocompromised people are affected. Septicaemia and arthritis may occur also. In case of infection with multidrug resistant *Salmonella*, treatment with antimicrobial drugs may even be unsuccessful. Beside medical treatment (visit to general practitioner, medicines) absence from work determine the economic losses due to infection with *Salmonella*.

Salmonellosis is a zoonosis, a communicable disease from animals to man, often via the food chain (both from animal and vegetable origin). Alternatively, man may be infected directly through contact with infected animals. The primary reservoir of *Salmonella* is the intestinal tract of vertebrates, and the bacterium can survive for months in the environment, protected from sunlight and embedded in organic material. Therefore, *Salmonella* is widespread in nature, and almost impossible to get rid of.

A recent example of food-borne infections due to *Salmonella* is the contamination of ice cream with *S.* Enteritidis, served at a wedding in Arlon in June 2000 (12). A total of 95 persons, of which 60 reported ill, attended the wedding. An epidemiological study identified locally prepared ice cream as the source of infection. Two layer farms were recognised as the possible origin of the eggs with which the ice cream was prepared.

The Flemish Community registered in 1998 26 community food-borne infections outbreaks, of which most were due to *S.* Enteritidis infection (4). Often, raw egg products were involved: ice cream, chocolate mousse, pastry or desserts, and mashed potatoes. In the same year 31 outbreaks of gastroenteritis with more than 2 cases were registered. These figures for 1999 rose to 54 and 60, respectively. Also the national network of sentinel microbiology laboratories, an electronic surveillance system of the scientific Institute of Public Health – Louis Pasteur, registered for 1998 and 1999 an increasing number of gastrointestinal infections due to *S.* Enteritidis, and the National Reference Laboratory located at the same institute analysed in 1999 10 385 *S.* Enteritidis and 3 278 *S.* Typhimurium strains from man.

As for figures from meat and meat products, the Institute for Veterinary Inspection organised from 1997 to 1999 official national random sampling plans at slaughterhouses and cutting plants to estimate contamination rates of, among other zoonotic agents, *Salmonella* (7). In that period, about 40% of broiler carcasses were found contaminated with *Salmonella*, whereas for laying hen carcasses an incidence of about the double was found.

At the European level each year a report (8) is made in accordance to the Zoonosis Directive 92/117/EEC (1), which aims at centralising incidence rates of zoonoses and zoonotic agents among feed, the living animal, food and human. Although these data are not comparable due to significant differences in sampling plans and in analytical techniques in the various European countries, it can be concluded that salmonellosis is an important food-borne infection, together with campylobacteriosis. About 51 cases per 100 000 inhabitants were recorded

in 1998, with large geographic differences (1,9 to 136). Eggs, egg products and chicken were most often reported as major source.

The epidemiology of Salmonella *infection*

Salmonella is an enteric pathogen that is mainly ingested via the faecal-oral route (horizontal transmission). The bacterium interacts with the epithelial cells of the gut, and may invade the submucosa, reach the macrophages and thus via the blood stream the inner organs. Both the enteric and the septicemic phase are important for the pathogenesis of salmonellosis, especially in poultry where hens can pass an infection on to the chicken via the infected egg (vertical transmission) (9, 10, 11)

In the period following the primary production phase, e.g. in the slaughterhouse, during processing and at retail or distribution level, and finally during preparation of the food infection may equally well occur. Therefore, not only the degree of infection within the living animal will determine if and to what extent *Salmonella* will reach the consumer, but also hygiene in the whole production and distribution chain. In what follows, an overview of some well-known critical stages within the poultry production chain is given (9, 11).

The poultry sector is an intensely integrated, fairly well structured industry with a pyramidal configuration. The top of the pyramid is composed of few elite or grandparent flocks. The reproduction breeders produce hatching eggs that give rise to day-old-chickens for layers (table eggs) or broilers (poultry meat production). Although the sector is relatively small in Belgium, our country exports hatching eggs (mainly for broilers), shell eggs, egg products and poultry meat (5). A consequence of the strongly integrated structure is the thorough planning of the activities which, in case of misfortune, has a consequence on all activities downstream in the chain.

Especially in modern poultry farms feed stuffs are often heat-treated and therefore are less frequently contaminated with *Salmonella*. However, additional feeding with on-farm non heat-treated feed from vegetable origin may occur. Also cross-contamination in feed mills and compound feeds on farms is known. A recent study of the Belgian Ministry of Agriculture showed that *Salmonella* infection of primary feed stuffs and compound feeding stuffs from vegetable origin is limited, although not absent (6).

Since 1993 a continuous surveillance programme among poultry breeders is in place in Belgium, under the responsibility of the Veterinary Services. In 1997, some supplementary samplings were added, resulting in a relatively intense programme that allows the monitoring of breeder birds for *Salmonella*, at least on a yearly basis. All day-old breeder chickens are sampled during arrival at the farm, for bacteriological and serological examination. Breeder hens and cocks, raised abroad and therefore not subject to the Belgian surveillance system, are also examined (bacteriology only). Since 1993, all breeder flocks from 16 weeks on are followed by means of bacteriological analysis, each six weeks. As a consequence, the prevalence of *Salmonella* among breeders is well known. Actually, no similar plan is available for layers or broilers.

Following the sanitary qualification decrees (2) all flocks, independent of the category of poultry (breeders or commercial birds), must be sampled for *Salmonella* when sent to slaughter. Thus, it is clear that the authority has at its disposal an efficient way to follow up the possible contamination rate of the primary production as close as possible to the consumer. However, since sampling before slaughter depends on the herd's dimension, not all poultry farms are sampled in the same way.

The level of *Salmonella* found at the slaughterhouse or in the processing plants is a consequence of the contamination level of the animals submitted for slaughter in addition to the general hygiene at the abattoir. Almost inevitable is the increased excretion of *Salmonella* during transport and stress before entering the slaughterhouse. Only fasting before presenting the animals for slaughter may circumvent this. Carcasses may be contaminated during almost the entire process: scalding, defeathering, evisceration and chilling. The implementation of a well-structured HACCP plan is one way to lower the contamination with *Salmonella* at the end of the slaughterline.

As for table eggs, contamination can occur via the oviduct (vertical transmission) since *Salmonella* bacteria may spread intracellularly in macrophages throughout the body. Invasion depends on the serotype, e.g. *S.* Enteritidis probably is more invasive than other serotypes. It is not clear how many eggs are contaminated in a flock of infected laying hens, and how many *S.* Enteritidis penetrate into a single egg. Clearly, via the upper region of the oviduct only very few bacteria reach the albumen or the yolk membrane, and under favourable conditions these bacteria may multiply and reach levels above the infectious dose for humans. In the 24 hours period immediately after laying the number of *Salmonella* in the contaminated egg remains rather stable due to the limited iron accessibility in the egg white. Especially when temperature is not very constant, the vitelline membrane looses its integrity and thus iron may leak through, leading to multiplication of *Salmonella* bacteria. Eggs may also be spoiled through superficial faecal contamination. Conditions favourable for shell penetration are high relative humidity, shell damage and quick decrease in temperature.

During processing of poultry meat (cooling, transportation, cutting, packing) cross-contamination may occur. Obviously, the general hygiene and the routine practices in the processing plants are determining. Not all those

that manipulate food are possible causes of contamination, but manipulation by people with diarrhoea should absolutely be prevented. Some *Salmonella* serovars (e.g. *S.* Enteritidis PT4) probably give more raise to food contamination, due to their invasiveness (septicaemia) and penetration in the meat during handling.
The main risk factors at the final stage in the preparation of foods that contribute to outbreaks of salmonellosis are well known, i.e. preparation of food more than 12 hours before consumption, inadequate cooling and re-heating, simultaneous manipulation of fresh (contaminated) meat and cooked food. Obviously, proper kitchen hygiene both in catering holdings and in households can prevent erroneous manipulations and thus infection with food-borne agents.

Control measures at the primary production

At the European level the Zoonosis Directive 92/117/EEC enforces the monitoring and control for the presence of *Salmonella* in poultry breeding flocks (1). This directive, which is currently under revision, is transposed to a Belgian decree (3). Although the Veterinary Services have not submitted a monitoring plan to the European Commission that is completely in agreement with this directive, a continuous bacteriological surveillance programme of breeder flocks is in place since 1993 (see above). In addition, all flocks for slaughter are monitored through the sanitary qualification decrees (2). Mandatory general conditions for infrastructure and for operation aim at the improvement of the hygiene level at all poultry farms. A hygiene barrier, the separate storage of feed (especially for feed with a withdrawal period), requirements for the site of loading and unloading of animals and dead birds that should be easy to clean and disinfect, and a place for the storage of dead birds are structural measures that help improve the separation of clean and possibly soiled areas at the farm. In addition, management practices such as limited access for visitors, technical personnel or veterinarians to the herd, rodent and insect control, adequate cleaning and disinfections, the provision of feed from recognised suppliers, and re-population from certified herds help in keeping a flock *Salmonella* free. The categorisation of poultry herds may assist to reach this goal.
However, imposing sanitary measures on industry without the parallel implementation of control activities by a competent authority is likely to be inefficient and may not necessarily lead to an improved product quality. For instance, it should be checked that the mandatory sampling in the official monitoring programmes is exact (number of samples taken, methodology of sampling) and that samples are analysed in agreement with quality controlled laboratory methods. An infected flock of breeders or laying hens should not be treated with antimicrobial drugs, but if no sufficient financial compensation is made available, contaminated eggs or young chickens may still enter the production chain, leading to *Salmonella* in the food.
A specific problem constitutes chickens that are infected with *Salmonella* but that do not excrete these bacteria with their faeces. These carrier animals are not detected by routine bacteriological analysis and represent a serious problem for the identification of contaminated flocks. Consequently, the control of the infection in the corresponding herd is impaired. Probably improved diagnostic tests are needed to identify these flocks.

GENERAL CONCLUSIONS

From the above, it is clear that food-borne infections in general, and of salmonellosis specifically, represent a serious problem for the whole food chain. A global approach of all partners involved, from primary producers to the consumer, seems necessary. Although the authorities occupy a difficult position between the industry and the consumer, clear and efficient sanitary and hygiene measures are unavoidable to reduce the possibility that consumers get infected via their food.

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BOVINE AND HUMAN ENTEROHAEMORRHAGIC *ESCHERICHIA COLI* BELONGING TO THE SAME O SEROGROUPS.

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ABSTRACT

O157:H7 enterohaemorrhagic *Escherichia coli* infections (EHEC) in humans have been associated, directly or indirectly, with foodstuffs of ruminant origin, which are asymptomatic carriers. Epidemiological data on human infections with EHEC belonging to other serotypes (026, 0103, 0111, 0118…) are scarce. There is thus a need to develop epidemiological tools to compare them and also to define their host specificity, if any. Typing tools for EHEC can target the *stx* genes, coding for the verocytotoxins, and their carrying phages, the LEE (Locus for Enterocyte Effacement)-located genes coding for the AE (Attaching and Effacing) lesions, the hly_{EHEC} genes, coding for the enterohaemolysin, and their pO157 carrying plasmids, or the whole genome. The purpose of this study was thus to compare (i) the *eae*, *tir*, *espA*, *espB*, and *espD* gene variants present on the LEE of; (ii) the production of an enterohaemolysin and the presence of the hly_{EHEC} genes on a plasmid in; and (iii) the resistance to tellurite compounds by bovine and human EHEC; and to correlate the typing results to the EHEC serotype. The following results have been obtained so far: bovine and human EHEC strains belonging to the same serogroup O can (i) give identical PCR amplification results of the LEE-located genes; (ii) produce an enterohaemolysin and carry hly_{EHEC} genes on a plasmid; and (iii) resist to tellurite compound. Typing of the *stx* genes by PCR and, if necessary, of whole-DNA by Pulse-Field Gel Electrophoresis will complete the characterization and comparison of bovine and human EHEC belonging to identical serogroups.

1. INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) belong to the attaching and effacing *Escherichia coli* (AEEC) group. These cause a characteristic intestinal lesion called attaching and effacing (AE) lesion (6) because of the intimate attachment of the bacteria to the enterocyte and the local effacement of the microvilli. In addition, EHEC produce verotoxins (vt) or shiga toxins (Stx).

EHEC are widespread among humans and cattle (4, 7). They can cause diarrhea and dysentery in 2 to 8-week-old calves but they are also recovered from healthy bovines (2). O157:H7 enterohaemorrhagic *Escherichia coli* infections in humans have been associated, directly or indirectly, with foodstuffs of ruminant origin, which are asymptomatic carriers. Other serotypes (O26, O103, O111, O118…) may have a similar importance. There is thus a need to develop epidemiological tools to compare them and to define their host specificity, if any.

Typing tools for EHEC can target some of the virulence factors : the *stx* genes, coding for the verocytotoxins, the hly_{EHEC} genes, coding for the enterohaemolysin and their pO157 carrying plasmids and the LEE (Locus for Enterocyte Effacement)-located genes coding for the AE lesions. The LEE locus is a 35 kb chromosomal fragment which contains the *eae* and *tir* genes, coding for the intimin protein and its receptor, Tir, responsible of the intimate adherence to the enterocyte, and the *esp* genes coding for the secreted proteins EspA, EspB and EspD involved in the signal tranduction which leads to the effacement of the microvilli (5).

2. MATERIAL AND METHODS

2.1 Bacterial strains

The 29 human strains were isolated from patients with clinical diarrhea. All the bovine strains were isolated at the slaughterhouse excepted 8 O118 strains which were isolated from calves with diarrhea.

2.2 PCR reactions

PCR reactions for *eae*, *tir*, *espABD*, *vt* and *hly* genes and for the location of the LEE in *selC* were performed as described previously (3, 5, 8). The human EPEC strain E2348/69 (O127 :H6), the human EHEC strain

ATCC43888 (O157 :H7) and the rabbit EPEC strain RDEC-1 (O15) were used as positive controls. The *E. coli* K12 HB101 was the negative one.

2.3 DNA plasmid hybridization
The plasmid DNA was extracted according to a modified Kado and Liu method (1) and was hybridized with a probe specific of the enterohaemolysin (hly_{EHEC}) gene.
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2.4 Tellurite resistance and production of enterohaemolysin
The strains were grown on Gassner-tellurite (10^{-4}M), CT-SMAC (Mast Diagnostics) and sheep blood agar (Oxoid) plates, respectively.

3. RESULTS

Table 1: Pathotype of human EHEC and bovine EHEC and EPEC strains.

Serogroup	Origin	*eae/tir/ espABD*	Verotoxin (vt)	hly_{EHEC} / hly_{EHEC} on plasmid	*selC* disrupted by LEE
O157	human (10)	γ/γ/γ (10)	vt2 (9) vt1-vt2 (1)	+ (10) / + (10)	yes (8) NA (2)
	bovine (19)	γ/γ/γ (19)	vt2 (12) vt1-vt2 (5)	+ (10) / + (10)	yes (15) no (3) NA (1)
O103	human (9)	ε/β/β (9)	vt1 (9)	+ (9) / ND	no (9)
	bovine (5)	ε/β/β (5)	vt1 (3)	ND	no (5)
O118	human (4)	β/β/β (4)	vt1 (4)	+(4) / ND	ND
	bovine (12)	β/β/β (8) γ/α/α (4)		ND	ND
O145	human (6)	γ/γ/γ (5) β/β/β (1)	vt1 (4)	+ (6) /ND	ND
ATCC43888 (O157:H7)	human	γ/γ/γ	vt1-vt2	+	yes
E2348/69 (O127:H6)	human	α/α/α	-	ND	yes
RDEC-1 (O15)	rabbit	β/β/β	-	ND	no
HB101		NA	-	-	no

NA: no amplification; ND: not done

Table 2: Phenotype of bovine and human O157 *E. coli*.

	O157	
	bovine	human
enterohaemolysin	+ (19/19)	+ (10/10)
Te^R	R (19/19)	R (10/10)

4. DISCUSSION

Bovine and human EHEC strains belonging to the same O serogroup can (i) give identical results for PCR amplification of the LEE-located genes; (ii) produce an enterohaemolysin and carry the hly_{EHEC} gene on a plasmid; and (iii) resist to tellurite compounds. Study of whole-DNA by Pulse Field Gel Electrophoresis will complete the characterization and comparison of bovine and human EHEC strains. Thus, for the moment, nor the serotype, nor the pathotype of the bacteria can't help us to predict the zoonotic risk.

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ACTINOBACILLUS PLEUROPNEUMONIAE SEROVARS 2, 3 AND 9: SERO-EPIDEMIOLOGICAL CHARACTERISTICS IN SLAUGHTER PIGS FROM FARROW-TO-FINISH PIG HERDS

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ABSTRACT

This study was conducted in 150 farrow-to-finish pig herds to investigate descriptive epidemiological characteristics of infections with three different serovars of *Actinobacillus pleuropneumoniae*, and to identify risk factors for the within-herd seroprevalences of these serovars. Different farm characteristics were examined as potential risk factors for the percentage of pigs with antibodies against serovars 2, 3 and 9. The median within-herd seroprevalences were 95%, 100%, and 35% for serovars 2, 3, and 9, respectively. The within-herd seroprevalence of serovar 2 was significantly higher in farms that purchased gilts from ≥2 origin herds and in farms with poor biosecurity measures. The proportion of pigs seropositive for serovar 3 was significantly higher when tested pigs were slaughtered in May-August and in November-December, in herds without a growing unit, and in herds with a direct air-entry into the finishing unit. The within-herd seroprevalence of serovar 9 increased significantly in herds with poor biosecurity measures.

1. INTRODUCTION

Porcine pleuropneumonia is a contagious respiratory disease caused by the gram-negative bacterium *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*). The disease is distributed worldwide and causes severe economic losses to the pig industry. Because antibiotic therapy is effective only during the initial phases of the disease (Desrosiers, 1986), control measures rather than treatment of clinically affected animals should be pursued. The control can be accomplished in different ways namely by the use of antimicrobials, by vaccination, and by improvements of management practices and housing conditions (Taylor, 1999). Continuous or intermittent use of antimicrobials during the fattening period should not be instituted for a long time because of the increased risk for antibiotic resistance (Vaillancourt *et al.*, 1988) and the presence of antibiotic residues in the slaughter pigs. Strategic medication appears to work very well in some herds but it can only be used when the periods of risk are known. A wide range of vaccines against pleuropneumonia has been developed. The most recent ones provide a high protection against the disease but like the use of antimicrobials, they cannot prevent colonisation of *A. pleuropneumoniae* organisms in the respiratory tract nor eliminate the infection from a herd. Improvement of management practices and housing conditions are considered to be very effective in reducing the risk for clinical outbreaks and economic consequences of pleuropneumonia (Fenwick and Henry, 1994).

There is insufficient knowledge about specific risk factors associated with *A. pleuropneumoniae* infections in swine farms. Some studies focused on descriptive sero-epidemiologic characteristics during the grow-finishing period (Andreasen *et al.*, 2000), on the interaction with other respiratory pathogens (Van Til *et al.*, 1991; Elbers *et al.*, 1992), or on the lung lesions associated with *A. pleuropneumoniae* infections (Mousing *et al.*, 1990). Besides identifying different farm characteristics as risk factors, it is also important to quantify the impact of each risk factor. The present study was conducted to investigate descriptive epidemiological characteristics of infections with three different serovars of *A. pleuropneumoniae*, and to identify risk factors for the within-herd seroprevalences of these serovars in 150 farrow-to-finish (FTF) pig herds. Serovars 2, 3 and 9 were included because preliminary data of diagnostic labs indicated that these serovars were frequently isolated from Belgian pigs.

2. MATERIALS AND METHODS

2.1. Study population and herd factors

The study population comprised 150 FTF pig herds with more than 50 sows. The herds were randomly selected from all FTF pig herds located in West-Flanders and in an adjacent small region of East-Flanders. This is an area with a surface of 11% of Belgium, and in which 54% of the Belgian pig population is located. Each herd was visited once to collect different farm characteristics as potential risk factors for respiratory disease. Therefore, we used a questionnaire with precise definitions of the data to be recorded. The data were obtained through inspections of the pigs and the pig units, and through face-to-face interviews of the pig farmers. Information pertained to herd size, month of slaughter, pig and pig herd density in the municipality, type of breed of the sows, management practices, housing conditions, disease prevention procedures, hygienic measures within and outside the unit (biosecurity measures). Management practices included purchase policy of gilts, stocking densities in the finishing units, number of pigs per pen and per compartment, and type of production. Housing conditions referred to presence of a growing unit in which pigs are raised from approximately 70 until 120 days of age, compartmentalisation, number of compartments in the finishing unit, type of ventilation system and type of floor in the finishing unit. All these data were contemporary for the pigs that were examined at slaughter. More details about the study population and the different potential risk factors are described in a previous paper (Maes *et al.*, 2000). Vaccination against *A. pleuropneumoniae* was not practised in any of the selected swine farms.

2.2. Slaughterhouse inspection and serological testing

From each herd, a group of 60 to 150 pigs was sent to the slaughterhouse, and a blood sample was taken from 25 pigs per herd. Pigs were selected systematically for blood sampling, and 20 out of the 25 samples were tested for the presence of serum antibodies against *A. pleuropneumoniae* serovars 2, 3 and 9. The sampling procedure permitted to detect with 95% certainty at least one positive pig from a group of 150 pigs when the minimum within-herd prevalence was 14 %. An indirect ELISA that was developed according to a previous study published by Trottier *et al.* (1992), was used with some modifications. The test was based on heat-stable antigens of *A. pleuropneumoniae*, and 1/400 dilutions of sera were made. ELISA titers were expressed as the reciprocal of the highest serum dilution with an OD-value higher than the calculated cut-off level. Under experimental conditions, all infected pigs were tested positive whereas all control pigs remained negative. No or very minimal cross-reactions were observed with other serovars. Consequently, the ELISA tests used in the present study are considered to have a high sensitivity and specificity.

2.3. Statistical analyses

The farm characteristics were examined as potential risk factors for the percentage of pigs with antibodies against *A. pleuropneumoniae* serovars 2, 3 and 9. Therefore, logistic regression analyses (SAS 6.12, Proc Genmod) were performed at the herd-level with the proportion of seropositive pigs being the dependent variable, and the different herd factors being the independent variables. Overdispersion due to the non-independence between pigs of the same herd (McDermott *et al.*, 1994) was taken into account by including a scale parameter in the logistic regression model (Collett, 1996). A forward stepwise procedure was used to select independent variables that were significantly associated with the different seroprevalences (Neter *et al.*, 1990). The goodness of fit of the final models was assessed by calculating the root mean squared errors (RMSE) using the following formula (Mittlböck and Schemper, 1996): $\sqrt{\frac{1}{n}\sum(P_{observed} - P_{fitted})^2}$ with n denoting the number of herds, and P denoting the within-herd seroprevalence. Potential values for RMSE range between 0% and 100%, corresponding to perfect predictability or complete lack of predictability by the model, respectively. Odds ratios and their 95% confidence intervals were calculated from the final logistic regression models. More details about the statistical analyses are explained in a previous paper (Maes *et al.*, 2000).

3. RESULTS

The within-herd seroprevalences of *A. pleuropneumoniae* serovars 2, 3 and 9 and the 95% confidence intervals are presented in Table 1. The median within-herd seroprevalences were 95% (range: 0-100%), 100% (range: 10-100%), and 35% (range: 0-100%) for serovars 2, 3, and 9, respectively.

Table 1. Within-herd seroprevalences of *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) serovars 2, 3 and 9 in slaughter pigs from 150 farrow-to-finish pig herds

	Within-herd seroprevalences		
	Serovar 2	Serovar 3	Serovar 9
Minimum	0 [0-14] [a]	10 [2-29]	0 [0-14]
Median	95 [78-100]	100 [86-100]	35 [14-59]
Mean	81 [59-93]	90 [71-98]	39 [20-63]
Maximum	100 [86-100]	100 [86-100]	100 [86-100]

[a] Between brackets: 95% confidence intervals

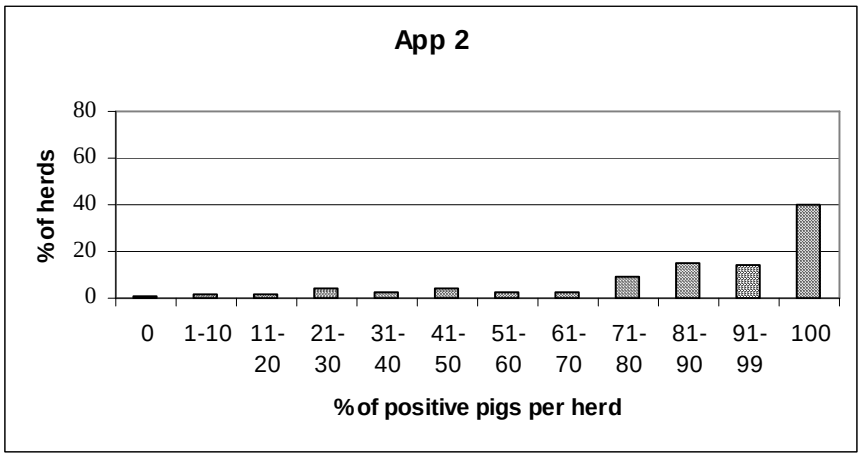
The distributions of the within-herd seroprevalences of the different serovars are shown in Figure 1. The distributions of the within-herd seroprevalences of serovar 2 and 3 were both skewed with a longer tail towards the lower seroprevalences (left skewed), whereas the within-herd seroprevalence of serovar 9 was skewed with a longer tail towards the higher seroprevalences (right skewed). In only one herd, all the tested pigs were seronegative for serovar 2 and in another herd, all tested pigs were seronegative for serovar 9. All herds were seropositive for *A. pleuropneumoniae* serovar 3.

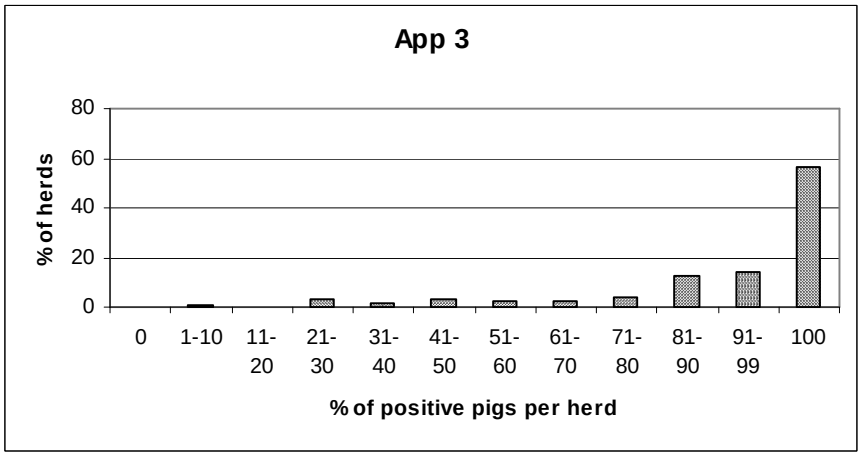
The risk factors for the within-herd seroprevalences of *A. pleuropneumoniae* serovars 2, 3 and 9 are presented in Table 2. The within-herd seroprevalence of serovar 2 was significantly higher in farms that purchased gilts from ≥2 herds (OR=2.33), and in herds with poor biosecurity measures (OR=4.62). The proportion of pigs seropositive for serovar 3 was significantly higher when tested pigs were slaughtered in May-August and in November-December (OR=5.96), in farms without a growing unit (OR=1/0.38=2.63), and in farms with a direct air-entry ventilation system in the fattening unit (OR=1.92). The within-herd seroprevalence of serovar 9 increased significantly in herds with poor biosecurity measures (OR=1.76). The interaction terms and the squared terms of the continuous independent variables in the intermediate and final models were not significant. RMSE values for the final models were 25%, 17% and 22% for serovars 2, 3, and 9, respectively.

4. DISCUSSION

In this cross-sectional study, different sero-epidemiological characteristics of infections with *A. pleuropneumoniae* in slaughter pigs from Belgian farrow-to-finish pig farms were investigated. Since the farms were selected randomly, the results may be generalized to other farrow-to-finish swine farms located in regions with a high pig density. Based on the observed seroprevalences, it appeared that infections with *A. pleuropneumoniae* serovars 2, 3 and 9 were very common in the selected herds. Almost all of them were infected with each of the three serovars. The seroprevalences were particularly high for serovars 2 and 3, and to a lesser extent for serovar 9. Elbers *et al.* (1990) also found higher seroprevalences for serovar 2 (55%) than for serovar 9 (4%) in slaughter pigs from herds in the Southern part of the Netherlands. Our results further corroborate with other studies (Bossé *et al.*, 1990; Falk and Lium, 1991; Wallgren *et al.*, 1993; Habrun *et al.*, 1998) showing that multiple serovars may exist within the same farm. In contrast to a Danish study (Andreasen *et al.*, 2000), we did not observe farms to be infected with one dominant serovar of *A. pleuropneumoniae*. Serovars 2, 3 and 9 were considered in the present study because preliminary information from diagnostic labs indicated that these serovars were frequently isolated from Belgian pigs. Although each of the three serovars may be implicated in clinical outbreaks of porcine pleuropneumonia (Marsteller and Fenwick, 1999; Hoflack *et al.*, 2000), the serologic data provided convincing evidence that most swine farms with intensive rearing systems are subclinically infected with *A. pleuropneumoniae*. According to Mousing *et al.* (1990) and Beskow *et al.* (1993), infections with serovar 2 are a very important cause of chronic pleuritis in slaughter pigs from Scandinavian countries.

Figure 1. The distribution of the within-herd seroprevalence of *Actinobacillus pleuropneumoniae* serovar 2 (A), serovar 3 (B), and serovar 9 (C) in slaughter pigs from 150 farrow-to-finish pig herds





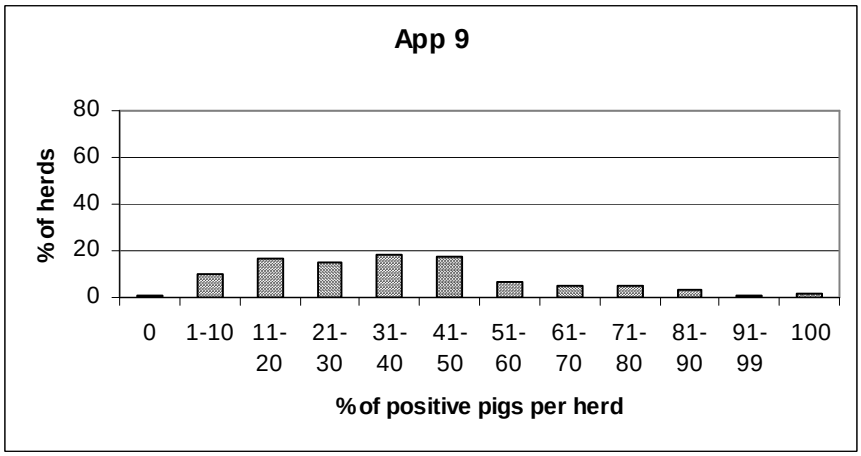


Table 2. Risk factors for the within-herd seroprevalences of *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) serovars 2, 3 and 9 in slaughter pigs from 150 farrow-to-finish pig herds: parameters in the final logistic regression models, coefficients with standard errors, *P*-values and odds ratios with 95% confidence intervals (CI)

Final model for *A. pleuropneumoniae* serovar 2 (deviance 1194; RMSE 25%)			
Parameters	Coefficients ± standard error	*P*-value	Odds ratio [95% CI]
Intercept	0.8494 ± 0.3208	0.008	-
Purchase of gilts			
≥2 herds	0.8475 ± 0.3840	0.027	2.33 [1.10-4.95]
from one herd	-0.0790 ± 0.3619	0.827	0.92 [0.45-1.88]
no purchase	0.0000 ± 0.0000	-	-
Biosecurity measures			
poor	1.5303 ± 0.6330	0.016	4.62 [1.34-15.9]
moderate	0.3947 ± 0.3450	0.253	1.48 [0.75-2.92]
good	0.0000 ± 0.0000	-	-
Scale parameter	2.9146 ± 0.0000	-	-

Final model for *A. pleuropneumoniae* serovar 3 (deviance 694; RMSE 17%)			
Parameters	Coefficients ± standard error	*P*-value	Odds ratio [95% CI]
Intercept	2.7519 ± 0.3878	<0.001	-
Month of slaughter			
May-Aug and Nov-Dec	1.7857 ± 0.3942	<0.001	5.96 [2.75-12.91]
other months	0.0000 ± 0.0000	-	-
Presence of growing unit			
yes	-0.9736 ± 0.3428	0.005	0.38 [0.19-0.74]
no	0.0000 ± 0.0000	-	-
Ventilation system			
direct air-entry	0.6527 ± 0.2953	0.027	1.92 [1.08-3.43]
indirect air-entry	0.0000 ± 0.0000	-	-
Scale parameter	2.2687 ± 0.0000	-	-

Final model for *A. pleuropneumoniae* serovar 9 (deviance 675; RMSE 22%)			
Parameter	Coefficients ± standard error	*P*-value	Odds ratio [95% CI]
Intercept	-0.8079 ± 0.2032	<0.001	-
Biosecurity measures			
poor	0.5651 ± 0.2776	0.0418	1.76 [1.02-3.03]
moderate	0.3753 ± 0.2240	0.0938	1.46 [0.94-2.26]
good	0.0000 ± 0.0000	-	-
Scale parameter	2.0562 ± 0.0000	-	-

A significantly higher proportion of slaughter pigs with antibodies against serovar 2 was observed in herds that purchased gilts from ≥2 origin farms. There was however no difference between herds with on-farm raising of gilts and farms that purchased the gilts from one origin farm. The higher risk to be seropositvefor serovar 2 in herds that purchased gilts from ≥2 origin farms may be attributed to the increased risk of introducing the pathogen by subclinically infected (carrier) gilts. Although we have not investigated the gilts directly, it is reasonable to assume that many of them have been exposed previously to *A. pleuropneumoniae* infections (Levonen *et al.*, 1996) and that

they have become carriers of the organism. Carrier pigs usually harbor the bacteria in the tonsils and/or in necrotic lung lesions, less frequently in the nasal cavity (Kume *et al.*, 1984). Carrier gilts may shed *A. pleuropneumoniae* organisms to other pigs in the herd, including their offspring (Marsteller and Fenwick, 1999). The results further demonstrated that poor biosecurity measures significantly increased the risk of seropositivity for infections with serovar 2 (OR=4.62) and 9 (OR=1.76). The variable biosecurity measures in the present study pertained to prevention of disease entry by trailers carrying pigs, feed or manure, presence of a sanitary room, use of farm specific boots, clothes and protective head-gear. The variable was classified as being good, moderate or bad when these measures were always, sometimes or never practiced in the herd. Biosecurity issues are becoming more important in the modern swine industry, especially in farms with a high health status (Amass and Clark, 1999). Unfortunately, only a few studies have assessed the importance of biosecurity measures in swine farms to prevent *A. pleuropneumoniae* infections. Gutierrez *et al.* (1995) showed that the organism is sensitive to a wide range of commonly used disinfectants, and they advocated that the use of disinfectants might be very helpful in the adoption of a control program against porcine pleuropneumonia.

The farm characteristics identified as risk factors for the within-herd seroprevalences of *A. pleuropneumoniae* serovar 2 were different from those associated with serovar 3. There was some seasonal pattern in the seroprevalence of serovar 3 with more than 5 times higher risks for seropositivity for pigs slaughtered in May through August and in November and December. It is not clear why the risk to be seropositive at slaughter was higher in these periods, especially for the pigs slaughtered during the summer months. The average within-herd seroprevalence of the herds investigated in May through August and in November and December was approximately 13% higher than the within-herd seroprevalence of herds investigated in the other months. Maybe, the worst herds were selected during these periods. It was also shown that pigs raised in herds with a growing unit were at lower risk (OR=0.38) to be positive for serovar 3. In a growing unit, pigs are raised from approximately 70 days (when they leave the nursery unit) until 120 days of age (when they are moved to the finishing unit). The presence of a growing unit has the advantage that younger pigs do not share the same airspace with older finishing pigs. Horizontal transmission of serovar 3 organisms may have been lower in these herds or the infection may have been delayed to a later time in the finishing unit, resulting in a lower seroprevalence at slaughter age. The risk to be seropositive for serovar 3 was almost twice as high in herds with a direct air-inlet in the fattening unit. Such a ventilation system allows the outside air to enter the units and the pig area directly without being warmed up first. Consequently, such a system will more likely be associated with wider variations in temperature, relative humidity and other climatic parameters in the pig units. These factors are generally considered to be important in the development of porcine pleuropneumonia (Taylor, 1999). Thus, the climatic conditions were probably better or remained more constant in the finishing units with an indirect air-entry.

The within-herd seroprevalence of slaughter pigs was used to assess the presence and spread of three serovars of *A. pleuropneumoniae* in the herds. Since the presence of antibodies reveals a physiological response of the pig resulting from infection with these serovars, it is an indirect measure of the infection itself. Measuring the presence of antibodies, like most other tests currently available for porcine pleuropneumonia, is not a perfect tool to detect infection in living pigs. It has been shown that *A. pleuropneumoniae* organisms can remain undetected in some seropositive pigs, whereas some seronegative pigs can be infected (Møller *et al.*, 1993; Sidibé *et al.*, 1993). However, serologic data of slaughter pigs may be useful to assess the infection status of the herds with regard to porcine pleuropneumonia. Recording clinical disease or lung lesions at slaughter is less informative for this purpose. Measuring clinical disease is less useful because in most endemically infected herds, subclinical infections rather than clinical outbreaks are the rule (Fenwick, 1994). In addition, precise measurements of clinical respiratory disease are difficult to obtain in practice and in many instances, multiple pathogens are involved. Focusing on slaughter lesions has the disadvantage that no etiological diagnosis can be established and that the results only reflect the end-stage of a particular pathological process. Consequently, lung lesions resulting from *A. pleuropneumoniae* infections occurring early in the fattening period may be healed at slaughter age (Mousing, 1988). Using serology, clinical as well as subclinical infections can be detected (Nielsen, 1988). Clinical outbreaks of pleuropneumonia cause substantial economic losses to the pig industry, especially because of the high mortality. However, the economic losses incurred by pig producers due to subclinical infections are also very high. According to Rohrbach *et al.* (1993), seropositive, but subclinically infected pigs require 5.6 additional days to reach market weight.

A cross-sectional design was used in this study. This implies that the risk factor and the disease outcome were measured at one point in time (Rothman and Greenland, 1998). Therefore, the significant risk factors may not be considered as being causal factors but they should be interpret as factors that contribute significantly to a higher

seroprevalence in the herds. Because we used serology as disease parameter, the risk factors are not necessarily the same as those associated with clinical disease or with lung lesions caused by *A. pleuropneumoniae*. The RMSE values of the final logistic regression models appeared to be fairly low, ranging from 17 to 25%. These values correspond to a moderate to high predictability of the final models (Mittlböck and Schemper, 1996).

In conclusion, this study documented that infections with *A. pleuropneumoniae* serovars 2, 3 and 9 were common in slaughter pigs from FTF pig herds. It was also shown that epidemiologic characteristics like overall seroprevalence, distribution of within-herd seroprevalence among farms and risk factors associated with the within-herd seroprevalence showed some variation across the three serovars. Additional research, preferably including a higher number of different serovars and using longitudinal studies, is warranted to further investigate the epidemiological characteristics of *A. pleuropneumoniae* infections in pig herds.

5. ACKNOWLEDGEMENTS

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The list of references can be obtained from the first author.

ACTINOBACILLUS PLEUROPNEUMONIAE SEROVARS 2, 3 AND 9: SERO-EPIDEMIOLOGICAL CHARACTERISTICS IN SLAUGHTER PIGS FROM BELGIAN FATTENING PIG HERDS

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ABSTRACT

This study aimed to investigate sero-epidemiological characteristics of infections with *Actinobacillus pleuropneumoniae* serovars 2, 3 and 9, and to identify risk factors for the within-herd seroprevalences of these serovars in 50 randomly selected fattening pig herds. Different herd factors as potential risk factors for respiratory disease were obtained by means of a questionnaire. At slaughter, 25 blood samples were collected from each herd, and the proportion of positive pigs per herd was assessed. The median within-herd seroprevalences of serovars 2, 3 and 9 were 58%, 53% and 35%, respectively. The within-herd seroprevalence of serovar 2 was significantly associated with the pig herd density in the municipality and with no use of preventive medication at the start of the fattening period. No significant risk factors were found for serovar 3. The percentage of pigs positive for serovar 9 was significantly associated with slaughter date in June and with the absence of compartmentalization.

1. INTRODUCTION

Pig farmers are currently faced with a narrowed income margin, and are changing from a curative to a more preventive health approach. Consequently, it is imperative for them to be aware of the risks and costs of diseases. Epidemiological research may play a key-role to attain and sustain a preventive-based pig production, and identifying risk factors associated with the spread of infectious agents may be the first step towards appropriate adaptations in management practices and housing conditions. This is especially true for respiratory diseases in grow-finishing pigs, which are economically very important, and which are well known to be of a multifactorial nature (3).

This cross-sectional study focused on some descriptive and analytical epidemiological aspects of infections with *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) in slaughter pigs. This gram-negative bacterium is the primary cause of porcine pleuropneumonia, a contagious respiratory disease causing substantial economic losses to the pig industry. Currently, there is insufficient knowledge about specific risk factors associated with *A. pleuropneumoniae* infections. At least twelve different serovars of *A. pleuropneumoniae* have been described (12). However, it is not known whether the spread and epidemiology are similar across these serovars. The aims of the present study, which was conducted in fattening pig herds, were to investigate descriptive epidemiological characteristics of infections with *A. pleuropneumoniae* serovars 2, 3 and 9, and to identify risk factors for the within-herd seroprevalences of these serovars. Because major differences in management exist between different types of pig farms, only fattening pig farms were selected for this study. Serovars 2, 3 and 9 were included because preliminary data indicated that they were frequently isolated from Belgian pigs. The study was carried out in addition to a previous study that investigated sero-epidemiological aspects of infections with 4 other respiratory pathogens in slaughter pigs (4).

2. MATERIALS AND METHODS

2.1. Study population and herd factors

The study population consisted of 50 fattening pig herds that were randomly selected from all herds located in the North-Western part of Belgium. In the selected herds, pigs were raised from approximately 23 kg until slaughter weight. Approximately one third of the Belgian pig herds consists of this type of herd (10). The farm characteristics were obtained through a questionnaire that was mailed to the pig farmers. Additional telephone interviews with the farmer and the herd health veterinarian were performed when the questionnaire was not received within three weeks, and to address any other deficiencies. The integrity of the returned questionnaires was checked by a herd visit in 10

(20%) randomly selected herds. The data of the questionnaires were in accordance with the situation present in the herd. The collected information pertained to herd size, month of slaughter, pig (herd) density in the municipality, management practices, housing facilities, feeding system, and disease prevention procedures. All data refer to the pigs that were examined at slaughter. More details about the study population and the different risk factors are described in a previous paper (4). Vaccination against pleuropneumonia was not practised in any herd at the time of the study.

2.2. Slaughterhouse inspection and serological testing

From each herd, a group of 60 to 150 pigs was sent to the slaughterhouse, and a blood sample was taken from 25 pigs per herd. The blood samples were selected systematically, and 20 out of the 25 samples were tested for the presence of serum antibodies against *A. pleuropneumoniae* serovars 2, 3 and 9. The 20 blood samples permitted to detect with 95% certainty at least one positive pig from a group of 150 pigs for a minimum within-herd prevalence of 14 %. We used an indirect ELISA that was developed according to a previous study published by Trottier *et al.* (14), but some modifications were implemented. The test was based on heat-stable antigens of *A. pleuropneumoniae*, and 1/400 dilutions of sera were made. ELISA titers were expressed as the reciprocal of the highest serum dilution with an OD-value higher than the calculated cut-off level. Under experimental conditions, all infected pigs were tested positive whereas all control pigs remained negative. No or very minimal cross-reactions were observed with other serovars. Consequently, the ELISA tests used in the present study are considered to have a high sensitivity and specificity.

2.3. Statistical analyses

The farm characteristics were examined as potential risk factors for the percentage of pigs with antibodies against *A. pleuropneumoniae* serovars 2, 3 and 9. Therefore, logistic regression analyses (SAS 6.12, Proc Genmod) were performed at the herd-level with the proportion of seropositive pigs being the dependent variable, and the different herd factors being the independent variables. Overdispersion due to the non-independence between pigs of the same herd (6) was taken into account by including a scale parameter in the logistic model (2). A forward stepwise procedure was used to select independent variables that were significantly associated with the different seroprevalences (8). The goodness of fit of the final models was assessed by calculating the root mean squared errors (RMSE) using the following formula (7): $\sqrt{\frac{1}{n}\sum(P_{observed} - P_{fitted})^2}$ with n denoting the number of herds, and P denoting the within-herd seroprevalence. Potential values for RMSE range between 0% and 100%, corresponding to perfect predictability or complete lack of predictability by the model, respectively. Odds ratios and their 95% confidence intervals were calculated from the final logistic regression models. More details about the statistical analyses are explained in a previous paper (4).

3. RESULTS

The within-herd seroprevalences of *A. pleuropneumoniae* serovars 2, 3 and 9 and the 95% confidence intervals are presented in Table 1. The median within-herd seroprevalences of *A. pleuropneumoniae* serovar 2, 3 and 9 were 58% (range: 0-100), 53% (range: 10-95) and 35% (range: 5-100), respectively.

Table 1. Within-herd seroprevalences of Actinobacillus pleuropneumoniae *(*A. pleuropneumoniae*) serovars 2, 3 and 9 in slaughter pigs from 50 fattening pig herds*

	Within-herd seroprevalences		
	Serovar 2	Serovar 3	Serovar 9
Minimum	0 [0-14] [a]	10 [2-29]	5 [0-22]
Median	58 [32-79]	53 [29-75]	35 [14-59]
Mean	57 [32-79]	54 [29-75]	41 [21-65]
Maximum	100 [86-100]	95 [78-100]	100 [86-100]

[a] Between brackets: 95% confidence intervals

Table 2. Risk factors for the within-herd seroprevalences of *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) serovars 2 and 9 in slaughter pigs from 50 fattening pig herds: parameters in the final logistic regression models, coefficients with standard errors, *P*-values and odds ratios with 95% confidence intervals (CI)

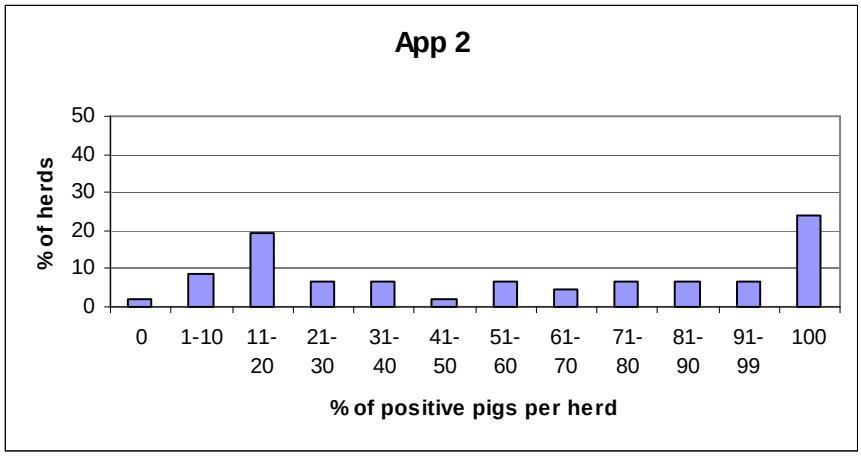
Final model for *A. pleuropneumoniae* serovar 2 (deviance 517; RMSE 39%)			
Parameters	Coefficients ± standard error	*P*-value	Odds ratio [95% CI]
Intercept	-1.4160 ± 0.7221	0.0499	-
Herd density	0.4728 ± 0.2202	0.0318	1.60 [a] [1.04-2.47]
Preventive medication			
no	1.0173 ± 0.5900	0.0847	2.77 [0.87-8.79]
yes	0.0000 ± 0.0000	-	-
Scale parameter	3.2750 ± 0.0000	-	-

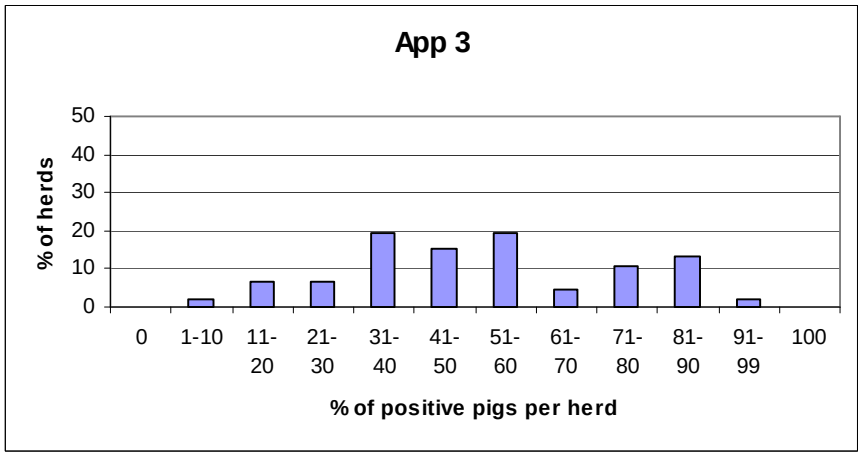
Final model for *A. pleuropneumoniae* serovar 9 (deviance 241; RMSE 24%)			
Parameters	Coefficients ± standard error	*P*-value	Odds ratio [95% CI]
Intercept	-0.6183 ± 0.6027	0.3050	-
Month of slaughter			
March-April-May-July	-0.8320 ± 0.4277	0.0518	0.44 [0.19-1.01]
June	0.0000 ± 0.0000	-	-
Compartmentalization			
no	1.0936 ± 0.5308	0.0394	2.99 [1.05-8.45]
yes	0.0000 ± 0.0000	-	-
Scale parameter	2.2517 ± 0.0000	-	-

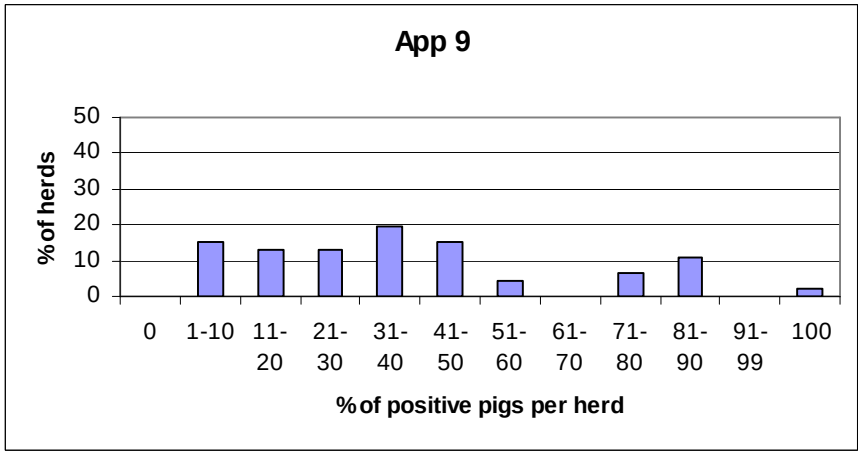
[a] per increase of one herd / km^2

The distributions of the within-herd seroprevalences of the different serovars are shown in Figure 1. In only one herd, all the tested pigs were seronegative for serovar 2. All herds were seropositive for serovars 3 and 9. The percentages of herds in which all investigated pigs tested positive for serovars 2, 3 or 9 were 24%, 0% and 2%, respectively.

Figure 1. The distribution of the within-herd seroprevalence of *Actinobacillus pleuropneumoniae* serovar 2 (A), serovar 3 (B), and serovar 9 (C) in slaughter pigs from 50 fattening pig herds







The risk factors for the within-herd seroprevalences of *A. pleuropneumoniae* serovars 2, 3 and 9 are presented in Table 2. The within-herd seroprevalence of serovar 2 was significantly associated with the pig herd density in the municipality (OR=1.60 per increase of one herd/km^2; $P<0.05$) and with no use of preventive medication at the start of the fattening period (OR=2.77; $P<0.10$). There were no significant risk factors for the within-herd seroprevalence of serovar 3. The percentage of pigs positive for serovar 9 was significantly associated with slaughter date in June (OR=2.27; $P<0.10$) and with the absence of compartmentalization in the fattening unit (OR=2.99; $P<0.05$). The interaction terms and the squared terms of the continuous independent variables in the intermediate and final models were not significant. RMSE values for the final models were 39% and 24%, respectively.

4. DISCUSSION

Sero-epidemiological aspects of infections with *A. pleuropneumoniae* serovars 2, 3 and 9 were investigated in 50 randomly selected fattening pig herds. The random selection of the herds together with the acceptable participation rate after the first selection (80%) enhance the external validity of the study, and facilitate the extrapolation of the results to other fattening pig herds. Since the validity of the data is of paramount importance in observational studies, special attention was paid to the construction of the questionnaire (13). The number of questions was restricted (n=13), and only closed questions for which different answer categories are provided, were included. The integrity of the returned questionnaires was further confirmed by a herd visit in 20% of the herds. The applied serological tests to detect antibodies against the three serovars are considered to have a high specificity and sensitivity. Due to the moderate number (50) of herds involved in this study, risk factors were considered significant at the 0.10 level. The RMSE values of the final logistic models corresponded to a moderate predictability of these models (7).

This study concerned risk factors associated with the spread of these 3 serovars as measured by their within-herd seroprevalence. These risk factors are not necessarily the same as those found to influence clinical disease within the herd. We did not select clinical signs as outcome variable because these are difficult to describe and quantify, and may vary considerably within a short time-span. Serological testing can be performed in an objective manner, and has the advantage that clinical as well as subclinical infections can be detected (9). From an epidemiologic perspective, greater success at preventing disease occurrence may be realized if one concentrates on how infections occur and persist in the absence of disease, rather than focusing solely on clinical disease as the outcome of interest.

It appeared that infections with serovars 2, 3 and 9 were common in the selected herds. Almost all of them were infected with each of the three serovars. However, the seroprevalences of serovars 2 and 3 were lower than those observed in slaughter pigs from Belgian farrow-to-finish pig herds (5). A direct explanation is not available. The seroprevalences of *Mycoplasma hyopneumoniae*, porcine influenza viruses and Aujeszky disease virus were somewhat higher in slaughter pigs from fattening pig herds than in those from farrow-to-finish pig herds. The serologic data of the present study clearly demonstrated that most swine farms with intensive rearing systems are subclinically infected with *A. pleuropneumoniae*.

The within-herd seroprevalence of serovar 2 was observed in slaughter pigs from herds located in an area with a high pig herd density and from herds that did not use preventive medication at the start of the grow-finishing period. Two parameters were used to assess the pig density in the municipality: the number of pigs per km^2, and the number of pig herds per km^2. Both were correlated and significantly associated with the seroprevalence of serovar 2. However, the number of pig herds per km^2 was the most significant factor and was retained in the model. The higher prevalences in regions with a high pig herd density may be the result of increased exchange of *A. pleuropneumoniae* organisms between herds in such areas. Airborne transmission of *A. pleuropneumoniae* between herds must be considered, but until now, it has not yet been proven (12). In pig-dense regions, different contacts between herds e.g. by vehicles, clothing, birds, rodents and flies, may also increase and facilitate transmission of *A. pleuropneumoniae*. Preventive medication at the start of the fattening period implied the preventive application of broad-spectrum antibiotics against respiratory and enteric diseases in feed, or in drinking water. Although it has been shown that antimicrobials cannot prevent the establishment of *A. pleuropneumoniae* organisms in the respiratory tract, the preventive use of antimicrobials at the start of the grow-finishing period may reduce the infection pressure in pig farms.

No significant risk factors were found for the within-herd seroprevalence of serovar 3. There was some seasonal pattern in the seroprevalence of serovar 9 with a higher prevalence in pigs slaughtered in June, compared to pigs

slaughtered in March, April, May and July. It is not clear why the risk to be positive at slaughter was higher in June. In slaughter pigs from Belgian farrow-to-finish pig herds, higher seroprevalences of serovar 3 were observed during the summer months (5). The proportion of slaughter pigs with antibodies against serovar 9 was also higher (OR=3) in herds without compartmentalization. A compartment was defined as a subdivision of a stable, having an own ventilation system. It is generally accepted that compartmentalization, especially when combined with all-in/all-out production, is beneficial to prevent horizontal transmission of pathogens between different groups of pigs (1). When pigs of different ages share the same airspace, younger pigs can get infected easily by pathogens transmitted by the older pigs.

In conclusion, this study showed that almost all selected fattening pig herds were simultaneously infected with *A. pleuropneumoniae* serovars 2, 3 and 9. The risk factors that were associated with the within-herd seroprevalences differed across the serovars. The pig herd density in the municipality and the month of slaughter are risk factors that cannot be changed by the farmers. The use of preventive medication at the start of the grow-finishing period and the implementation of compartmentalization are factors that can be managed by the pig producers. Because of the cross-sectional design of the present study, more research should be performed to further elaborate on the epidemiology of *A. pleuropneumoniae* infections in pig herds.

5. ACKNOWLEDGEMENTS

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A COMPARATIVE STUDY BETWEEN THE PREVENTIVE USE OF TILMICOSIN PHOSPHATE (PULMOTIL PREMIX®) AND *MYCOPLASMA HYOPNEUMONIAE* VACCINATION IN A PIG HERD WITH CHRONIC RESPIRATORY DISEASE

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ABSTRACT

The objective of the study was to compare the effects of a preventive in-feed medication program using tilmicosin (Pulmotil® 200 premix, Elanco Animal Health) at 200 ppm with those of a *Mycoplasma hyopneumoniae* (Mh) vaccination program (Stellamune™ Mycoplasma, Pfizer Animal Health) in a pig herd with chronic respiratory disease. In total, 208 piglets were randomly allocated to either the medication (P) or the vaccination (V) group. Pigs of the P group received medicated feed during 3 weeks after weaning, and during 2 weeks at the start of the finishing period. The piglets of the V group were vaccinated twice, at 4 and 22 days of age. Both groups were compared by ADG and FCR (major variables) and by a coughing index, pneumonia lesions and serology for Mh (minor variables). No significant differences (P>0.05) were observed between both groups. In this type of herd, the preventive use of tilmicosin had similar beneficial effects compared to Mh vaccination.

1. INTRODUCTION

Although major efforts have been made to control respiratory disease in modern pig herds, they continue to be an important problem for the pig industry. Within the chronic respiratory disease complex, infections with *Mycoplasma hyopneumoniae* (Mh) are generally considered to play a key-role since they can render pigs more susceptible to infections with other respiratory pathogens (10). Control measures against respiratory disease problems usually aim to prevent clinical symptoms and to reduce the associated economic losses. Because of the high pig herd density in some areas in Belgium and the frequent contacts between herds, eradication programs to make and maintain herds free of certain respiratory pathogens like Mh are very seldom implemented in commercial pig farms. In most cases of respiratory problems in pig farms, some management practices and housing conditions should be improved such as the use of all-in/all-out (AIAO) production, compartmentalization, minimizing mixing and moving pigs, optimizing ventilation etc. However, some farms continue to suffer from respiratory problems although no major shortcomings can be detected in the management and housing. In such cases, vaccination or anti-microbial medication programs are frequently used.

Tilmicosin is a semi-synthetic macrolide antibiotic with excellent *in vitro* activity against Mh and many pathogenic bacteria found in the respiratory tract of pigs (1, 2, 8). The *in vivo* activity of this antibiotic for the prevention of respiratory disease has also been demonstrated in studies conducted under experimental (9) and field conditions (7). Mh vaccines are widely used for the control of enzootic pneumonia. Different studies have shown that Mh vaccination can significantly improve the performance of grow-finishing pigs and decrease the number of lung lesions at slaughter (5). The objective of the present study was to compare the effects of a preventive in-feed medication program using tilmicosin (Pulmotil® 200 premix, Elanco Animal Health) at 200 ppm with those of a Mh vaccination program (Stellamune™ Mycoplasma, Pfizer Animal Health) in a pig herd with chronic respiratory disease.

2. MATERIALS AND METHODS

2.1. *The production system and farm history*

The study was conducted in a 400-sow herd that was part of a closed production system. 'Van Gennip' hybrid sows were inseminated with semen of Pietrain boars. The sows were vaccinated against Aujeszky disease virus, Parvovirus, *Erysipelothrix rhusiopathiae*, atrophic rhinitis and *E. coli*. Piglets received an iron injection at three days of age and male piglets were castrated at about 7 days of age. The pigs were weaned at 22 days and

transferred into a nursery unit in which they were raised until 14 weeks (approximately 40 kg liveweight). Thereafter, they were moved into the finishing unit in which they were housed until slaughter age (6-7 months). The nursery and finishing unit were located at the same site approximately 4 km from the sow herd. During the nursery and finishing period, the pigs received a commercial feed containing 40 ppm salinomycin as a performance enhancer.
According to the farmer and the herd health veterinarian, the nursery-finishing herd had a history of chronic respiratory disease. This was confirmed by clinical, pathological and serological investigations prior to the start of the study. From a sample of 195 slaughter pigs, 52% and 28% had pneumonia or pleuritis lesions, respectively. Out of 10 randomly taken blood samples in pigs of 80 kg, 100% showed antibodies against Mh.

2.2. *Study population and experimental design*
In total, 208 piglets were selected for the trial. They were derived from 21 sows and were born within a timespan of one week. At three days of age, they were ear tagged and randomly allocated to either the medication (P) or vaccination (V) group. An equal number of vaccinated and control pigs per sow were selected (block randomisation per sow). Pigs belonging to the P group received a feed containing 200 ppm tilmicosin phosphate (Pulmotil® 200 premix, Elanco Animal Health) during three weeks, starting approximately one week after weaning, and during two weeks starting at 77 days of age. Pigs of the V group were vaccinated twice against Mh (Stellamune™ Mycoplasma, Pfizer Animal Health) according to label instructions, namely at 4 and 22 days of age. Preventive measures (castration, iron injection, tail docking) and other management practices were identical for both groups. During the nursery-finishing period, the two groups were housed in 2 separated, identical compartments. The 2 compartments consisted of 8 pens (13 pigs/pen) and were located in the same building. The ventilation and feeding system were identical. There was one feeder with two integrated drinking nipples per two pens.

2.3. *Major variables of comparison*

Average Live Weight (ALW) and Average Daily weight Gain (ADG)
The live weight of each pig was determined at five different ages: at 4 days (first vaccination), 22 days (at second vaccination), 70 days and 107 days of age and at slaughter (212 days of age). The ADG (g per pig per day) during the different production stages was computed as the difference between starting and final weight divided by the duration of that production stage.

Average daily Feed Consumption (AFC) and Feed Conversion Rate (FCR)
The AFC (g per pig per day) was estimated per 2 pens during the nursery and the finishing period. The FCR of each pen was estimated as the ratio of AFC to ADG.

Mortality rate
The percentage of pigs that died during the nursery and finishing period was compared for both groups. The weight and age of the pigs that died were recorded. All pigs that died during the trial were necropsied by the investigator to assess the possible cause of death. Where appropriate, the pigs were processed for further laboratory examination.

2.4. *Minor variables of comparison*

Serological testing
Thirty pigs of each group were randomly selected at pen level, and they were successively bled at the following ages: 70 days, 107 days, 167 days and at 212 days (slaughter). The blood samples were analyzed for presence of antibodies against Mh using the DAKO®Mh ELISA (DAKO, Glostrup, Denmark). Sera with Optical Density (OD)-values < 50% and ≥ 65% of the OD buffer control were considered positive or negative, respectively. Intermediate OD-values were considered doubtful. The sera from the 30 blood samples taken at slaughter were additionally tested for presence of antibodies against Influenza H1N1 and H3N2 viruses and *Actinobacillus pleuropneumoniae* (App) biotype 1 serotype 2 and 9. Fifteen out of the 30 blood samples taken at slaughter from each group were tested for the presence of antibodies against porcine reproductive and respiratory syndrome virus (PRRSV). A standard haemagglutination-inhibition test was used to detect antibodies against the Influenza viruses (Palmer et al., 1975), the Herd Check® PRRS ELISA (Idexx Laboratories, Westbrook, ME, USA) to detect PRRSV antibodies, and a complement fixation test to detect antibodies against the App serotypes. HI-titers ≥ 4 and ≥ 20 were considered positive for H1N1 and H3N2 viruses, respectively. For PRRSV sera with S/P-values > 0.4 and < 0.3 were considered positive or negative, respectively. CBR-titers ≥ 40 for the App serotypes were considered positive.

Coughing index
A coughing index was performed weekly by the investigator and compared for both groups. The pigs in each pen were observed for a period of ten minutes after they had been moved about for two minutes. The number of pigs that coughed was recorded and divided by the total number of pigs present in these pens.

Macroscopic lung lesions
The presence of pneumonia, interlobular fissures, abcesses, App-lesions and pleuritis was recorded at slaughter. The lungs were thoroughly palpated and sliced for inspection if necessary. The percentage of pigs with lesions was compared for the two treatment groups.

2.5. *Statistical analysis*
Variables were considered to be significant at the 0.05 level (two-sided). ALW, ADG, AFC and FCR were summarized across the pens for each treatment group and compared using two-sample t-tests. Mortality rate was analyzed using chi-square tests (with correction for continuity). Serological results and prevalences of pneumonia, interlobular fissures, abcesses, App-lesions and pleuritis were compared using chi-square tests. Fisher's exact tests were applied when small numbers were involved. Statistical analyses were performed using SAS.

3. RESULTS

At the start of the study, the ALW of the pigs in the P and V group were 2.66kg and 2.75kg, respectively (P = 0.81). Throughout the study, ADG and FCR were generally similar for both groups as shown in *table 1*.

Table 1. Results of average daily gain (ADG) (g/day) and feed conversion rate (FCR) in the medicated (P) and vaccinated (V) groups during different periods

Parameter	Period	P group	V group	Difference (P-V)	*P*-value
ADG	D7 - D22	225	214	11	0.77
	D23 - D70	370	354	16	0.68
	D71 - D107	648	696	-48	0.20
	D108 - D212	626	591	35	0.18
FCR	D22 - D97	1.30	1.32	-0.02	0.61
	D98 - D212	3.31	3.28	0.03	0.89

More pigs died in the P group (10%) than in the vaccination group (5%), but this difference was not statistically significant (P = 0.24).

All death pigs were necropsied. This led to diverse results concerning the causes of death. Bacteriological culture of lung tissue was performed for 2 pigs of the P group. This revealed the presence of *Streptococcus suis* spp.

As a result of vaccination, there was a significant difference ($P < 0.001$) in seroprevalence for Mh at 70 days of age. However, at 107, 167 and 212 days, there was no significant difference in seroprevalence for Mh. Likewise, there was no significant difference for Influenza H1N1 and H3N2, for PRRSV and for App biotype 1 serotype 2 and 9 at slaughter age (*Table 2).*

Table 2. Seroprevalence of *Mycoplasma hyopneumoniae* (Mh) throughout the study period, and of Influenza H1N1 and H3N2, PRRSV and App serotype 2 and 9 at 212 days of age (at slaughter) in the medicated (P) and vaccinated (V) group

	% of pigs with positive serum antibodies		
	P-group (n=30)	V-group (n=30)	*P*-value
Mh - 70 days	0	33	<0.001
Mh - 107 days	0	7	0.49
Mh - 167 days	25	52	0.07
Mh - 212 days	89	96	0.56
Influenza H1N1	91	82	0.67
Influenza H3N2	78	74	1.00
PRRSV	100	100	1.00
App serotype 2, 9	0	15	0.11

No significant differences were found between both groups in terms of prevalence of pneumonia, interlobular fissures, abcesses, App-lesions and pleuritis (*Table 3*).

Table 3. Prevalence of the different lung lesions at slaughter in the medicated (P) and vaccinated (V) groups

Type of lesion	Prevalence of lesion (%)		
	P-group (n=82)	V-group (n=96)	*P*-value
Pneumonia	28	35	0.32
Fissures	24	28	0.69
Abcesses	2	3	1.00
App-lesions	10	9	1.00
Pleuritis	18	19	1.00

The mean coughing index was generally similar for both groups throughout the study (*figure 1, see appendix).*

4. DISCUSSION

This study compared the effects of a preventive in-feed medication program using tilmicosin at 200 ppm with those of a Mh vaccination program in a pig herd with chronic respiratory disease. The results showed that in this herd infections with Mh were highly prevalent, mainly occurring during the second half of the finishing period (*Table 2*). The seroprevalence increased from 25% to almost 90% at slaughter age. The results of the necropsies and the additional serological results at slaughter indicated that infections with other bacterial and viral respiratory pathogens were also common in this herd. Approximately 80% of the pigs was seropositive for both Influenza viruses and all pigs were seropositive for PRRSV. These high percentages of seropositives at slaughter age are also frequently found in other pig farms in Flanders (4, 6).

The preventive medication with tilmicosin during 3 weeks after weaning and during 2 weeks at approximately 10 weeks conferred similar beneficial effects compared to a Mh vaccination program. Because of practical reasons, there was no negative control group. The improvement of performance parameters ADG and FCR

associated with Mh vaccination in chronically infected herds usually amounts to approximately 5% (3, 5). These 2 medication periods were selected because in closed pig herds, respiratory disease outbreaks usually occur at these times. During the first period, the pigs are weaned and usually mixed with other pigs to form homogeneous pens. In addition, a lot of other stress factors take place such as transferring pigs to the nursery unit, change of feed etc. The medication program was not initiated immediately after weaning because the feed intake might have been insufficient to obtain the persued body levels in these pigs. Therefore, the medication was postponed to the second week after weaning. The second medication period was initiated when the pigs were approximately 11 weeks old. At that time, pigs are usually placed in the finishing unit. Respiratory problems during the first month of the finishing period are very commonly encountered in pig farms.

The Mh vaccine was applied according to label directions namely at 4 and 22 days of age. Since the first seroconversion to Mh was observed at the earliest at day 167, vaccination at a later age would also have been successful in this herd. Moreover, since the sows were seropositive to Mh, the effect of vaccination would probably have been better when the pigs were vaccinated later. Although it is not yet fully understood, it is assumed that maternal antibodies may interfere to some degree with the efficacy of early Mh vaccination.

In conclusion, the study documented that in this chronically infected farm with its specific production system and infection pattern, the preventive use of tilmicosin during 3 weeks in the nursery unit and during 2 weeks from 11 weeks of age has similar beneficial effects compared to Mh vaccination. Further studies preferably including a larger number of pigs and pig herds should be conducted to confirm the results.

5. ACKOWLEDGEMENTS

This work was partially supported by a grant of Eli Lilly Benelux, Elanco Animal Health. The authors thank the personnel from the Regional Investigation Centre of East Flanders (Drongen) for the laboratory work and the herd owner for participation in this study.

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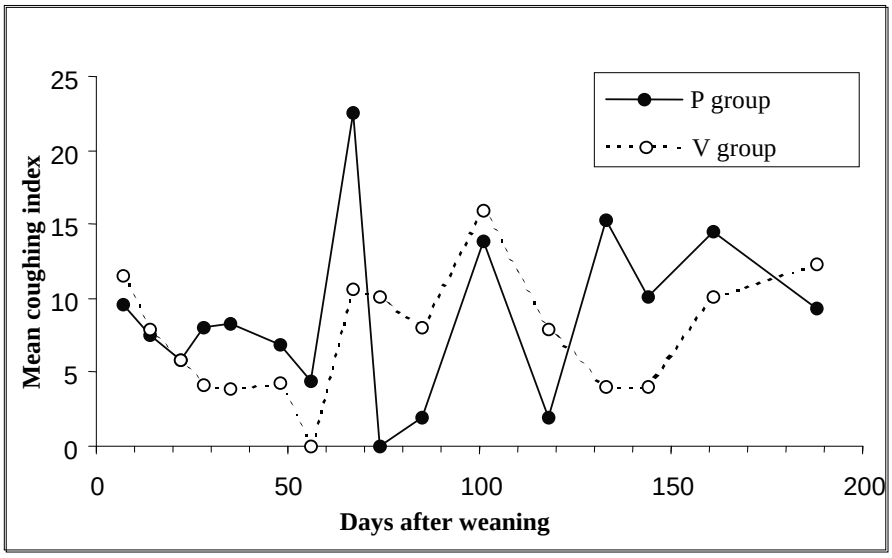
7. APPENDIX

Figure 1. Results of the coughing index in the medicated (P) and vaccinated (V) group throughout the entire study period



THE PREDICTION OF THE RISK FOR THE TRANSMISSION OF CLASSICAL SWINE FEVER VIRUS TO HERDS IN THE CLOSE NEIGHBOURHOOD OF AN INFECTED HERD IN BELGIUM

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ABSTRACT

This paper described the methodology behind the creation of a map, which displays the predicted risk for the occurrence of 'neighbourhood infection' of Classical Swine Fever in the different regions of Belgium. The risk is predicted based on a logistic regression model, which is the result of a risk factor analysis of the 1994 Classical Swine fever epidemic that occurred in the East-Flanders Province of Belgium. The model includes a bivariate kernel estimate of intensity of pig herds in an area with a one-kilometre radius surrounding a primary CSF-infected herd as only predictor variable.

The map displays areas, which incorporate a distinct higher risk for the occurrence of neighbourhood infections compared to other regions of Belgium and can be used as a 'decision support system' in case of Classical Swine fever epidemics and the assignment of concession for new pig farms

1 INTRODUCTION

In the past decades, certain areas of the European Union developed highly concentrated livestock populations because of their considerable economic advantages. These areas also appear to incorporate an increased risk for epizootic diseases. Therefore, despite the economical advantages, the areas may prove to be unsustainable over long term. It was in this framework that the Scientific Veterinary Committee of the European Commission urged the need to identify and classify the densely populated livestock areas (DPLA) of the EU.

The aim of this study was to classify the different regions in Belgium according to the risk for the occurrence of 'neighbourhood infections' (4) they incorporate in case of a Classical Swine Fever (CSF) epidemic. This risk is estimated by the predicted probability that pig herds become infected with CSF when located in an area with a one-kilometre radius surrounding a primary CSF-infected pig herd.

2. MATERIAL AND METHODS

2.1 Model

The logistic regression model, which was used for the prediction of the risk for the occurrence of 'neighbourhood infections', resulted from a risk factor analysis of a data set obtained during the control of the 1994 CSF-epidemic that occurred in the East-Flanders Province of Belgium (3). The model contains besides the intercept one predictor variable, i.e. a bivariate kernel estimate of intensity (2) of pig herds in an area with a one-kilometre radius surrounding a primary CSF-infected. As can be extrapolated from the parameter estimates of the model in table 1, a higher value for the kernel estimate results in a higher risk for the occurrence of 'neighbourhood infections'.

Table 1: parameter estimates the logistic regression model

Predictor variable	Parameter estimate
Intercept	-2.9580
Bivariate kernel estimate of intensity	0.2957

2.2 Data

The bivariate kernel estimate of intensity was calculated for every pig herd in Belgium, for which the exact location by geographical coordinates was available. The Belgian Veterinary Services obtained these coordinates in 1997 through a query in which the farmers were asked to locate their premises on a 1:10,000 scale topographic map.

The distance between the different herds was calculated using the geographical coordinates and the Pythagoras theorem. For every pig herd all other pig herds contained in the 'neighbourhood' (1 kilometre radius) were identified. In a next step the kernel estimate of intensity was calculated for every pig herd based on the location of the other herds in the 'neighbourhood'.

2.3 Graphical presentation of the predicted risk

The predicted risk for the transmission of neighbourhood infection was plotted on a vector map displaying the Belgian municipalities using a geographical information system (ArvView GIS 3.2a, ESRI Inc.). The point estimates of the predicted risk were smoothened and therefore transferred into a continuous surface variable using an Inverse Distance Weighted (IDW) interpolator (1)

3. RESULTS

For only 34 (0.003%) out of the 13,115 pig herds located in Belgium in 1997 no geographical coordinates were available. Of the remaining pig herds, 1,202 (11.9%) did not have other pig herds in their neighbourhood. For the pig herds with neighbouring herds within a one-kilometre radius, the kernel estimate of intensity varied from 7.7×10^{-8} to 13.022 (mean: 2.306; median: 1.817).

For the pig herds with no neighbouring herds within a one-kilometre radius, the risk for 'neighbourhood infections' was set to zero. For the pig herds with neighbouring herds the predicted risk varied from 0.05 to 0.71(mean: 0.11; median: 0.09). The smoothened predicted risk is mapped in Figure 1. Even after smoothening, areas with a potential higher risk, e.g. Tielt-Wingene', 'Kaprijke-Eeklo', can be noticed from the map.

4. DISCUSSION

The logistic regression model that was used for predicting the risk for neighbourhood infections is based on a data set collected during a real CSF outbreak in 1994. In this data set the kernel estimate of intensity varied from 0.426 to 7.203. For our study the model was used to predict a risk for neighbourhood infections based on a kernel estimate of intensity, which varied from 7.7×10^{-8} to 13.022. This means that the predicted risk for neighbouring spread for kernel estimates below 0.426 and above 7.203 will not necessarily be correct. Furthermore since the logistic regression model was not validated, the presented map should be seen as an illustration of a possible methodology for predicting the risk for CSF spread instead of an accurate estimate of the risk. Nevertheless, in some of the displayed areas of higher risk, CSF neighbourhood infections have occurred in the past, e.g. Tielt-Wingene (1993), 'Zomergem-Nevele-Kaprijke-Eeklo(1994), and Bocholt-Bree (1997).

One of the recent developments in the control and eradication of epizootic diseases is the implementation of decision support systems (DSS). The DSS allow the prediction, based on epidemiological models containing risk factors for the spread of the disease, of the evolution of a disease outbreak over shorter or longer terms. The presented map with underlying methodology can be used as a DSS as the map can be consulted in case a CSF infection is diagnosed and specific sanitary measures can be taken depending on the displayed risk for neighbourhood infections. The map can also be used in case concessions are to be assigned to new pig farms. The assignment can then be based on the predicted risk for neighbourhood infections or the kernel estimate of intensity.

5. ACKNOWLEDGEMENT

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FIELD EXPERIENCE WITH THE USE OF VACCINATION TO CONTROL SALMONELLA IN POULTRY

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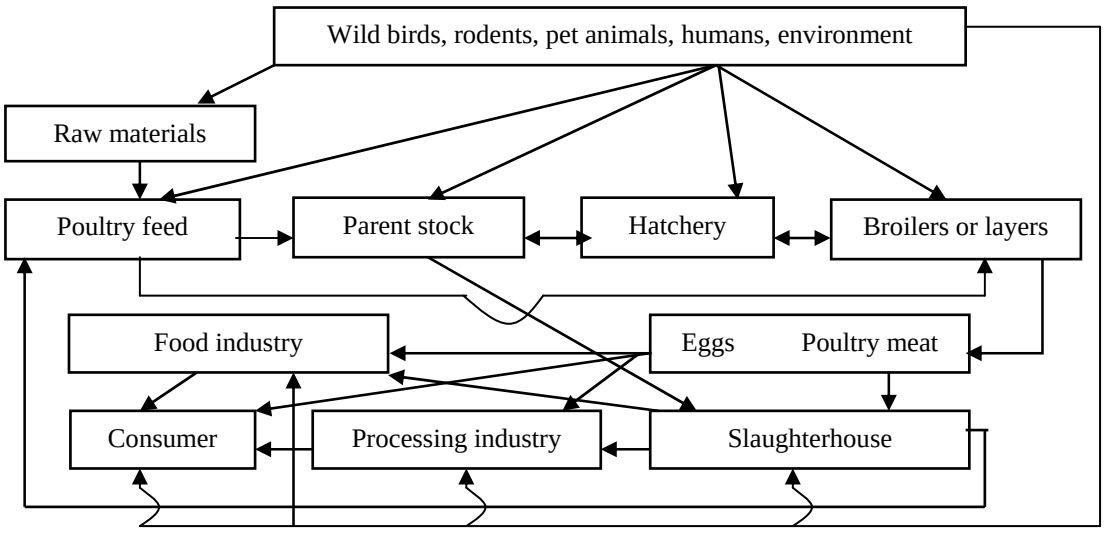
ABSTRACT

In recent years the topic of food safety in relation to products of poultry origin is mainly concentrated on Salmonella contamination. The poultry industry has implemented several instruments to reduce the level of Salmonella contamination during the different stages of production. This paper concentrates on the field experience obtained after the implementation of vaccination in layers in the United Kingdom and in broiler breeders in The Netherlands. Based on the experience in both countries it is concluded that the use of the inactivated Salmonella enteritidis vaccine Salenvac® clearly reduced the spread of Salmonella.

1. INTRODUCTION

The human health authorities and the poultry industry world-wide recognise the implications of Salmonella infections in humans and poultry. During the 1980s one particular type of Salmonella, *Salmonella enteritidis (S.e.)* became of major importance as it became a predominant source for human salmonellosis, originating from poultry products. Meanwhile this serotype has contaminated the environment and the poultry industry to a level that an eradication program is unrealistic. The contamination of the environment will stay a source for a continuous flow of new infections. The different routes of infection are schematically summarised in figure 1.

Figure 1. Infectionroutes of Salmonella in poultry production



Ref.: varied after Int. Poultry Production, Vol. 4, No.2, 1996

Since 1994 Salenvac® is used as a major tool in Salmonella Control Programs. The success of such programs does not solely depend on the use of vaccines. The more “hurdles”, that will decrease the spread of Salmonella, are incorporated, the more success may be expected.

This paper will concentrate on the experience with Salenvac® as a major contribution to prevent the spread of *S.e.* in poultry. Recently additional information became available on the field use of this vaccine in layers and broiler breeders.

It is of utmost importance to realise that the efficacy of a Salmonella Control Program does not solely depend on the preventive use of vaccines. Simultaneous implementation of a adequate monitoring program, in case of

observed contamination followed by corrective measures, and adequate biosecurity is essential. Another important, sometimes neglected phenomenon is the birds' own natural intestinal microflora that can provide protection against Salmonella infections (3).

2. MATERIALS AND METHODS

Salenvac® is an inactivated Salmonella enteritidis, phage type 4, vaccine. Aluminium hydroxide is used as adjuvant and it is therefor unlikely to cause harmful reactions, either in the vaccinated bird or in the operator, due to accidental self-injection. The vaccine is produced under iron restriction. Research has shown that growing bacteria in "laboratory conditions" can result in antigenic differences from bacteria growing in their natural environment, e.g. the chicken's intestine and other tissues. In the natural environment iron (Fe) is less available to the growing Salmonella-organism, because of iron binding to host proteins, normally present in intestines. In this environment *Salmonella's* and also other *Enterobacteriaceae*, react with the formation of iron-transfer mechanisms on their surface (2 and 4). These Iron Regulating outer membrane Proteins (IRP's) enable them to resorp iron actively. The bird also recognises them as antigens and antibodies will be produced. If a vaccine is produced under the conditions of restricted iron availability, the growing organisms will form these IRP's on their surfaces. The antibodies produced by the bird in response to such a vaccine are the same as those produced after a natural infection and will result in a better protection.
Vaccination by Salenvac® enables each individual bird to protect itself against a *S.e*.-infection. As this organism is the predominant source for human salmonellosis, it is included in the EC-zoonosis-directive (EC/92/117). The other *Salmonella* serovar included with regard to poultry production is *Salmonella typhimurium (S.t.).*
In 2000 a vaccine containing both organisms (Salenvac T®) is registered in UK. For Belgium the registration for this product may be expected in 2001.
After completion of the vaccination schedule (first vaccination of 0.5 ml i.m. at 12 weeks of age followed by again 0.5 ml i.m. at 16 weeks of age) the birds will react by producing antibodies. These antibodies will protect them against an infection. This protection is not restricted to *S.e*. in case of Salenvac or *S.e*. and *S.t*. in case of Salenvac T®. A broader protection against other serovars of the D-group and B and D-group of the Muller-Kaufmann scheme is proven for Salenvac® and Salenvac T® respectively.
These antibodies will be transferred to the offspring via the hatching egg and result in a protection during the first weeks of life. This is a crucial period, as it is known that then only a few *S.e*.-bacteria are sufficient for infection. The importance of protection during this period is furthermore underlined by the risk of an explosion of infection due to the logistic handling of hatching eggs and day old chickens before, during and after the incubation period.

3. RESULTS

3.1. Layers in United Kingdom

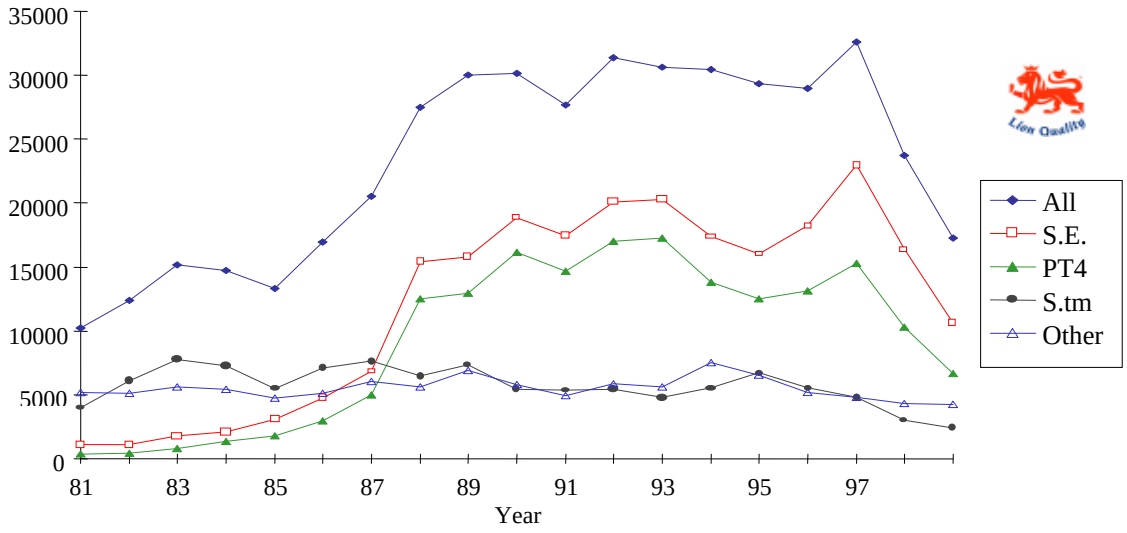
In 1997 3.2 Mio layers were vaccinated. A major integrator and a retailer followed the effect by sampling table eggs, as bought in the supermarket. All sampled eggs were produced by free range egg layers and could be traced to a vaccination or a non-vaccination origin. The analysis for Salmonella-contamination was performed by the Public Health Laboratory Service. The results are summarised in table 1.

Table 1. Salmonella-contamination in eggs originating from Salenvac®-vaccinated layers versus a control group of nonvaccinated origin.

	Number of eggs analysed	Contamination ratio
Vaccination-group	44.700	0
Control-group	42.642	1 : 1.100

After these results became public the beneficial reducing effect of vaccination on the spread of Salmonellosis originating from eggs was recognised by the British Egg Industry Council. They implemented the use of vaccination in their Code of Practice for Lion Quality Eggs. This program represents the vast majority (over 80%) of the UK table egg production and is an essential condition to be mentioned on the list of possible supplier for eggs for all UK retailers. After this general implementation of vaccination in the layer segment a steady decrease in the amount of human Salmonellosis in England and Wales is observed in the data of the official monitoring. These data are summarised in figure 2.

Figure 2. Trends in human Salmonellosis in England and Wales
(ref: website Public Health Laboratory Services)



3.2. Broiler breeders in The Netherlands

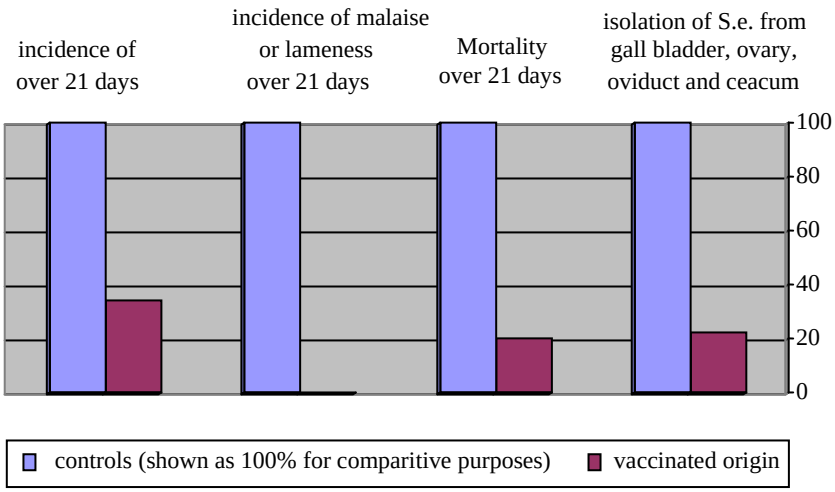
From August 1995 until the first half of 1996 the effect of adding a *Salmonella enteritidis* vaccination to a certified standardised biosecurity program in a situation of increased infection risk was examined by the Animal Health Service in a field trial. In this trial 1.100.000 broiler breeders with increased infection risk were vaccinated using Salenvac®. In the same period as the vaccinated flocks 608 nonvaccinated flocks were hatched. These flocks acted as the control group. *S.e.*-infections in both groups were assessed by monitoring according to the Dutch Salmonella Control Program. The results of this trial are reported by Feberwee et al. (1). It is concluded that the proportion of *S.e.*-infected flocks with a risk of reinfection in the Salenvac®-vaccinated group (0%) was significantly lower (P = 0.02) than in the nonvaccinated group (18%)

3.3. Maternal immunity

The level of Salmonella-infections in broiler production is dependent on the level of infection of the parent stock (vertical transmission) and on the amount of infections that is originating from the environment of the broilers (horizontal transmission). Vaccination has a direct effect on the level of vertical transmission as it protects the vaccinated parents against an infection. Vaccination of broiler breeders can also have a protective value for the corresponding broilers during the first weeks of age as maternal antibodies are transmitted to the offspring.

This effect is illustrated by a trial carried out at the Central Veterinary Laboratory in UK. In this trial day-old chickens from 57-week-old vaccinated parents or unvaccinated parents were placed in contact with an equal number of seven-day-old "seeder" birds which were infected by an oral dose of 1.3 x 10^5 *Salmonella enteritidis* at one day old. The results of this trial are summarised in figure 3.

Figure 3. Effect of maternal antibodies after introduction to seeder birds



4. DISCUSSION / CONCLUSIONS

Extensive field data confirm the efficacy of Salenvac®-vaccination as a major instrument to reduce the level of Salmonella-infections in poultry production.
Vaccination will not only limit the spread of *Salmonella enteritidis* at the level of the vaccinated birds themselves, but will also protect the offspring by maternal antibodies during the first weeks of life. The last phenomenon is of major importance in broiler production as broilers are extremely vulnerable during this period, due to the fact that the competitive exclusion mechanisms of a stable, mature intestinal microflora have not developed to their full capacity. The protection achieved by the use of Salenvac® is not limited to *Salmonella enteritidis*, but is extended to the other serovars of the D-group of the Muller-Kaufmann scheme.
It is of utmost importance to realise that the efficacy of a Salmonella Control Program does not solely depend on the preventive use of vaccines. Simultaneous implementation of an adequate monitoring program, in case of observed contamination followed by corrective measures, and adequate biosecurity is essential.

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THE USE OF FLAVOPHOSPHOLIPOL (FLAVOMYCIN®) TO CONTROL SALMONELLA IN POULTRY

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ABSTRACT

In recent years the topic of food safety in relation to products of poultry origin mainly concentrated on Salmonella contamination. This paper will concentrate on the results of an experiment in which the shedding pattern of *Salmonella enteritidis* after oral challenge of broilers fed Flavomycin® were compared with a zero-control group. In addition to this the effects of inclusion of this product in broiler feed on the level of Salmonella contamination in the slaughterhouse under USA-field conditions and the possible synergetic effect of Flavomycin® in broiler diets with the general use of Salmonella vaccines in broiler breeders will be discussed. The position of Flavomycin® in the current discussion on the use of antimicrobial growth promoters will also be reviewed.
It is concluded that the use of Flavomycin® clearly reduced the spread of Salmonella in broilers in an infectious environment without creating a risk for an increase in antibiotic resistance.

1. INTRODUCTION

The demand for poultry products is strongly influenced by the consumers' concern for healthy and safe food. The topic of food safety mainly concentrates on Salmonella contamination, but in recent years there are public concerns that the use of antibiotic feed additives in animals may give rise to bacterial resistance to human therapeutic drugs, aspecially those antibiotics that are closely related to human drugs (1 and 16).
The poultry industry implements an array of measures to prevent the infection by Salmonella and to reduce the level of contamination in all stages of the production.

Recently new data were published on the reducing effect on Salmonella shedding of inclusion of the feed additive flavophospholipol in broiler diets. This product may give an additional stimulation to the animals' natural protection against Salmonella colonisation by the intestinal microflora. The intestinal microflora has the ability to protect the animal against infection in a contaminated environment. This phenomenon is known as Competitive Exclusion and was first described by Nurmi en Rantala (10). The underlying mode of action(s) is (are) still not fully understood. Most probably it is a combination of several factors, among which:

- Production of agents by certain beneficial organisms that have a negative influence on the growth capacity of other harmful or at least unwanted organisms (2 and 4). Recently van der Wielen et al (15) concluded that volatile fatty acids (in the undissociated form) are responsible for the reduction in numbers of *Enterobacteriaceae* in the ceca of broiler chickens during growth;
- Competition for substrate;
- Competition for attachment sites;
- Immunomodulation.

As the routes for Salmonella infection are numerous, it is essential to realise that the efficacy of a Salmonella Control Program does not solely depend on the implementation of one measure exclusively. Simultaneous implementation of a adequate monitoring program, in case of observed contamination followed by corrective measures, adequate biosecurity and vaccination of broiler breeders is essential. Another important, sometimes neglected phenomenon is the birds' own natural intestinal microflora that can provide protection against Salmonella infections.

2. MATERIALS AND METHODS

2.1. The study on the reducing effect on Salmonella shedding of inclusion of flavophospholipol in broiler diets.
In the European Union a new registration procedure, known as the Fifth Amendment of the Feed Additive Directive (70/524/EEC) requires, among other data, the submission of information on the possible effect of feed additives on the excretion of food-borne pathogenic bacteria like *Salmonella* and *Campylobacter*. This study was conducted to satisfy the new EU requirement for the commercial product Flavomycin® and was recently published by Bolder et al. (3). For the complete experimental design the original publication is available on request. The experimental design can be summarised as follows. Commercial broiler chickens were orally challenged on Day 11 and 12 with 10^8 cfu *Salmonella enteritidis* of a chicken field isolate. Samples during the experimental period up to the fifth week consisted of a mixture of fecal and cecal material. In the sixth week on the day of slaughter the animals were killed and the ceca were removed and sampled.

2.2. The USA field study
Under field conditions broiler rations containing Flavomycin® were compared to rations containing other antimicrobial growth promoters. Intestinal samples were collected at the slaughterhouse and these were analysed for the numbers of Salmonella. A part of this work is published by Schleifer et al. (13).

2.3. Product characteristics of flavophospholipol
Flavophospholipol (Flavomycin®), belonging to the antibiotic-class of the phosphoglycolipids, is licensed as a digestive enhancing antibiotic by the regulatory authorities in the EU, the USA and most countries world-wide. Due to it's limited efficacy against human bacteria and it's poor pharmaceutical properties it is not related to any antibiotic currently in use or under development for the treatment of human or animal diseases. In May 1999 it was described by the EC Scientific Steering Committee on Antibiotic Resistance from the Directorate XXIV as posing "no known risk" to humans or animals with regard to resistance.
The very limited direct antibacterial activity is restricted to the gram-positives. The enzyme glycosyltransferase, that plays an essential role in the synthesis of the cell wall of this group, can not distinguish between Flavomycin® and the natural compound. This results in an instable cell wall, leading to the death of this cell. In gram-negatives glycosyltransferase plays no role, due to the different cell wall structure. However when a plasmid bridge (pylus) is formed it's biosynthesis is disrupted in a similar way, leading to the death of the donor-cell. This plasmid bridge plays an essential role during the transfer of genetic information on antibiotic-resistance from one bacterium to another. In this way Flavomycin prevents this genetic transfer and actually reduces the number of resistance carrying bacteria. This reducing effect of Flavomycin® on plasmid-bound antibiotic resistance has already been described since the early 70's (5, 6, 7, 9 and 14). Of more recent date is the publication of Riedl et al (12).

3. RESULTS

3.1. The study on the reducing effect on Salmonella shedding of inclusion of flavophospholipol in broiler diets.
The results of this study are summarised in figure 1 and 2.

Figure 1. Salmonella-shedding, group average

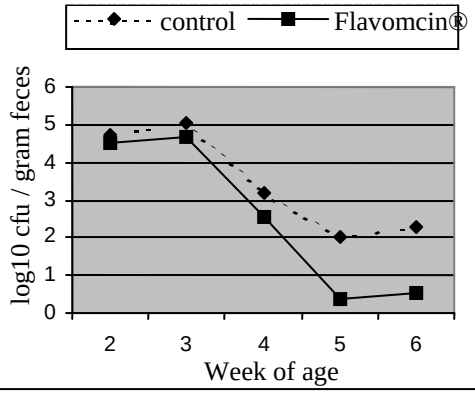
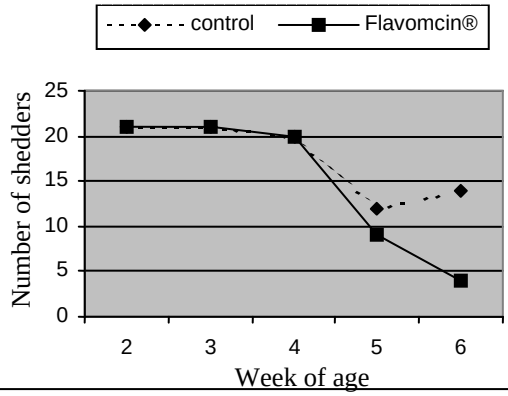


Figure 2. Number of broilers shedding Salmonella enteritidis



In this study Flavomycin®reduced significantly (P<0.05) the level of Salmonella-shedding at slaughter age. Less broilers in the Flavomycin®-group were Salmonella-positive after the initial oral infection at day 12 and 13 and mean fecal Salmonella cfu counts were significantly (P<0.05) lower in the Flavomycin®-group then those in the control group. These results confirm earlier studies in broilers (8). In this study an effect to the same extent was found on Clostridium perfringens shedding. The level and incidence of Campylobacter was not affected. Studies in pigs and calves (5 and 6) show identical results on the level of Salmonella-shedding.

3.2. The USA field study

The results of this study are summarised in table 1.

Table 1. Percentage of Salmonella-positive samples at Slaughterhouse-level under USA-field conditions

	Positive control	Flavomycin®-group
Integrator A	50 %	18 %
Integrator B	45 %	21 %
Integrator C	11 %	5 %
Integrator D	81 %	15 %

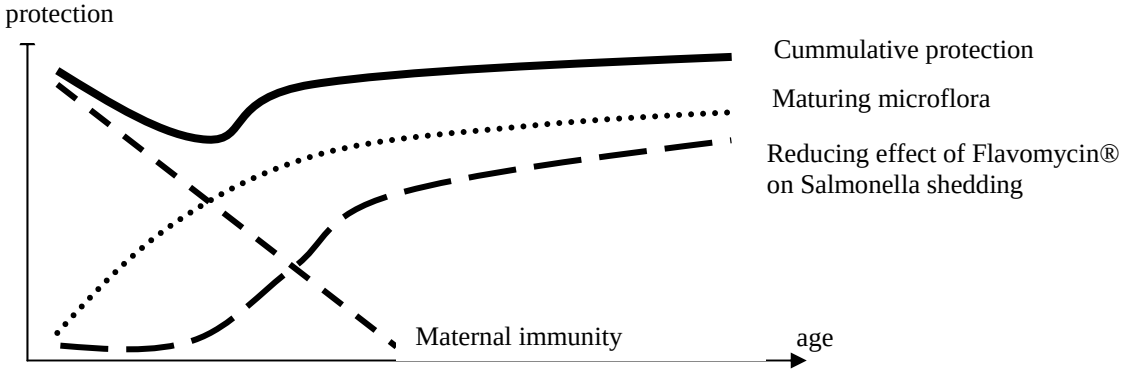
These data correspond with several millions of broilers processed every week, covering al major production regions in USA. The only difference between the positive control and the Flavomycin®-group is the used antimicrobial growth promoter. These data confirm the results of Bolder et al. under field conditions.

3.3. The possible synergetic effect of Flavomycin® in broiler diets with the general use of Salmonella vaccines in broiler breeders.

It is possible to combine the use of Salmonella vaccines at breeder level with the use of Flavomycin® in broiler diets. This concept will combine the benefits of both (11). The offspring of vaccinated breeders will start uninfected and will be protected by maternally derived antibodies during the first weeks. An existing infection pressure on a broiler farm will be countered by the inclusion of Flavomycin® in the feed. This mechanism will act as a barrier against infection by the time the protection by the maternal antibodies is absent.

It is known that due to the Competitive Exclusion of the normal intestinal flora the infectious dose necessary for a Salmonella infection decreases with increasing age. As Flavomycin® will not affect the equilibrium in the intestinal flora, the natural defence of the Competitive Exclusion mechanism will be an additional barrier against Salmonella-infection of the broilers. In figure 3 an idea is given how these protective mechanisms can be used in combination and can provide a useful concept for further development.

Figure 3. Effect of different protective mechanisms, that can be used in a Salmonella Control Program



4. DISCUSSION / CONCLUSIONS

The above mentioned data clearly indicate that inclusion of Flavomycin® in broiler diets is an efficacious instrument to reduce the level of Salmonella-infections in broiler production in an infectious environment. The impact thereof is underlined by the experience that all benefits in earlier stages of the production may be lost, due to the vulnerability of broilers to Salmonella-infection. The effect of Flavomycin® is most pronounced at slaughter age and processing. These are the stages in broiler production that come nearest to the consuming moment and are therefor of utmost importance.

Combination of the use of Salmonella vaccines at breeder level and the inclusion of Flavomycin® in broiler diets could be a synergetic beneficial instrument in a Salmonella Control Program in addition to an adequate monitoring program, in case of observed contamination followed by corrective measures, and adequate biosecurity.

However Salmonella is only one aspect of Food Safety. Another aspect is the level of antibiotic resistant organisms in animal production as this may be a risk that could influence the efficacy of human antibiotic treatments. Flavomycin® is supported by sufficient scientific evidence that it does not increase the level of antibiotic resistant organisms in animal production. In fact a reducing effect is more likely.

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ABATTOIR SURVEY FOR DETECTION OF PCBS OR DIOXINS IN BELGIAN CATTLE

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INTRODUCTION

In February 1999, a poisoning episode broke out in several industrial poultry farms in Belgium [1]. The source of this contamination was found to be a stock of recycled fat that had been delivered by the VERKEST-FOGRA group to several compound feed producers, between 19 and 31 January 1999. A fairly good correlation was observed between dioxins (PCDFs and PCDDs) and PCBs. Consequently a contamination mainly by PCBs was hypothesized. This finding made it possible to detect contaminated animals and animal products by dosage of the PCBs, more specifically the 7 congeners with numbers 28, 52, 101, 118, 138, 153 and 180 [1, 3 and 4]. The advantages of the dosage of PCBs compared to dioxins were its rapid test protocol and the fact that a larger number of laboratories could participate.

In the cattle sector, 409 herds were submitted to the risk of feed contamination. The methodology used to detect a PCBs/dioxins contamination in the Belgian cattle population that was not submitted to the VERKEST-FOGRA risk, is presented. This population is directly or indirectly destined for consumption. It consisted in the systematic sampling of all calve fattening stations and all lots of exported bovines, and in the random sampling of slaughter cattle. This methodology is compared to the approach described in directive 96/23/CE.

MATERIALS AND METHODS

Analysis of PCBs : includes the determination of a set of 7 'indicator' PCBs (IUPAC numbers 28, 52, 101, 118, 138, 153 and 180) and is expressed as the sum of these 7 marker congeners (usually in ng/g fat). The procedures for the PCB analyzes used by the different laboratories are described previously [2]. All applied methods were based on Gas Chromatography (e.g. GC-ECD).

Analysis of PCDDs and PCDFs : The procedures for PDDDs and PCDFs analyzes used by a limited number of laboratories consisted in Gas Chromatography - High Resolution Mass Spectrometry (GC-HRMS) after extraction of fat and after purification and concentration of samples. These procedures are previously described [6]. The standard method included the determination of the seventeen 2, 3, 7, 8-substitued congeners. Determination of these

congeners provides diagnostic information to evaluate the concentrations of the most toxic representative family of dioxins. The concentration in Toxic Equivalent Quantity (TEQ) was obtained by multiplying the concentration for each congener (usually expressed in pg/g fat) by a Toxic Equivalency Factor (I-TEF or WHO-TEF in this case).

Systematic sampling of all calve fattening centers : All lots of fattening calves were sampled (a lot is a center with a same feed producer). Three samples of 40 g fat that were taken from three animals were pooled. All samples were analyzed for PCBs by SGS-Agrilab (accredited from Beltest - norm EN-45004 type A). The sum of 7 congeners was determined.

Systematic sampling of all lots of bovines, before exportation: Between 2 August and 3 October '99 according to decision 99/449/CE as modified by decision 99/551/CE samples of all lots of bovines were tested before exportation (maximum one month before). The epidemiological unit for this sampling is an exported lot of animals. When the number of animals in a lot was lower than 7, all animals were submitted to a sampling of 100 g fat. When the number of animals in a lot was between 7 and 500, a random sampling of 7 animals was tested. This sampling was performed to account for a probability to accept a lot of 95 % and an acceptable quality level of 0.25. A total of 3409 bovines were submitted to PCBs analysis in different private laboratories that are to have an agreement by the Ministry. All samples were sent to private laboratories by the Veterinary Investigation Centers. During this period the percentage of exported bovines for slaughtering and fattening or breeding were respectively 62 % and 39 % (BNB-CEA, 1999).

Random sampling of slaughter cattle : In all cattle slaughterhouses random day during one week (between 23rd and 27th August '99) each official veterinary expert took a sample from one bovine per herd of origin. Each sample consisted in 100 g fat. A total of 1235 samples were collected. All samples were dispatched and stocked in the Veterinary Investigation Centers of Ciney. In Belgium among 17000 bovines were slaughtered on average weekly. We assumed that the sensitivity and the specificity of PCBs analysis is 100 %. The number of samples submitted to PCBs analyzes was calculated according to Martin *et al.* [5]. The confidence level was fixed at 95 % and the detectable prevalence in the samples was fixed at 1 %. This sample size was 296. We extended it to 299 (1 % of samples in surplus). The samples were standardized per province and per age of animals. All samples were analyzed for PCBs by SGS-Agrilab (accredited from Beltest - norm EN-45004 type A). The sum of 7 congeners was determined.

Epidemiological, food and environmental inquiry : For all positive PCBs results epidemiological, food and environmental investigations were performed in the herds of origin. These investigations were based on a checklist and a standard report of visit.

RESULTS

Herds of bovines submitted to the risk of feed contamination

In the cattle sector, 409 herds were submitted to the risk of feed contamination (0.81% of the total number of Belgian cattle herds). These herds were temporarily blocked for all commercial transactions. This measure was lifted on the basis of the results of feed and epidemiological investigations, on the basis of PCBs/dioxins analyses carried out on a representative sample of animal products originating from the concerned cattle herds (PCBs ≤ 100 ng/g milk fat, PCBs ≤ 200 ng/g body fat and/or dioxins ≤ 5 pg TEQ/g fat), or on the basis of stamping out and incineration of all cattle originating from the 6 herds with PCBs/dioxins analyses results above the fixed level.

A fairly good correlation was observed between PCBs and Dioxins concentration in bovine fat (r^2 = 0.731 ; p = 0.0006).

Systematic sampling of all calve fattening centers

At the moment of sampling a total of 386 calve fattening centers were active (presence of fattening calves). All samples presented negative results (range ; 11 - 39 ng/g body fat) except one station with an outlier of 277 ng PCBs

per gram fat. The counter-evaluation on the same sample was performed in another laboratory and gave a result of 166 ng/g fat, which is under the level of 200 ng/g fat. An epidemiological, food and environmental inquiry was immediately carried out in that center. Any risk factor in this center was identified. Two feed producers delivered feed products to this center : the first producer did not receive fat from the Verkest-Fogra group ; whereas the second producer did receive fat from the Verkest-Fogra group but not during the risk period. Nevertheless a feed monitoring was performed on this second producer. All results were negative. In addition, two samples in the concerned center and the three herds where the calves were born tested negative.

Systematic sampling of all lots of bovines, before exportation

Between 2 August '99 and 3 October '99, 3409 individual samples of body fat were collected and analyzed for PCBs in different private laboratories. In case of positive results the bovines were not exported, additional samples were collected, and an epidemiological, food and environmental inquiry was performed to determine the source of contamination. For these cases analyses of dioxins were also realized. Only 31 results with levels over 200 ng PCBs per gram of body fat were observed (0.91 %; confidence interval 95 %: 0.59 to 1.23 %); respectively : 20 samples with levels between 200 and 499 ng/g fat (0.59 %), 8 with levels between 500 and 999 ng/g fat (0.23 %) and 3 with results equal to or above 1000 ng/g fat (0.09 %). These positive results were found in animals originating on 15 different herds. Three of them held 9 bovines with results above 500 ng/g fat (including all results ≥ 1000 ng PCBS per gram fat which results of dioxins analysis were also above 5 pg TEQ/g fat). For these herds a clearly environmental source was determined (ironworks and dielectric failure). These herds were cleared by destruction of the animals and/or products (with fair compensation for the losses).

Random sampling of slaughter cattle

Only one out of 299 samples of body fat was found positive for PCBs : 837 ng/g body fat. The farm of origin of this bovine received feed products of a feed producer that was not delivered with fat by Verkest-Fogra group. Moreover, this feed producer was subjected to a feed monitoring. All results of this monitoring were negative for PCBs. The positive animal was a calve of 4 month of age and permanently infected (immunotolerant) for BVD. In the herd of origin of this calve an epidemiological, food and environmental inquiry was immediately carried out and 4 complementary samples of fat were analyzed for PCBs : results were also negative. No source of contamination was determined. The mother of this calve, which was 6 years old, was culled at the same time for BVD eradication. This cow was born in another farm but this primary farm was closed since three years. In this farm a potential industrial source of contamination could not be excluded (historically).

Consequently, the prevalence of Belgian slaughter cattle with a level of PCBs ≥ 200 ng/g fat in the body fat was 0.3 % (95 % superior confidence interval 95%: 1.59%). The probability density is a Poisson distribution. This is a left asymmetric distribution.

DISCUSSION

The prevalence of Belgian slaughter cattle with a level of PCBs ≥ 200 ng/g fat in the body fat was 0.3% (95 % superior confidence interval: 1.59%). This study demonstrated that surveying the national slaughter cattle population within a short time is technically feasible under a joint multidisciplinary collaborative effort. The results of the systematic sampling surveys confirmed that the incidence of the PCBs/dioxins contamination is low and that there are possible sources of environmental contamination that are common to all industrialized countries. The research of source of contamination requests a multidisciplinary collaborative effort.

Our results are objective data for reported to the reports for Standing Veterinary Committee. With these reports the bovine sector was carried off the application of decision 99/449/CE.

The approach developed in the directive 96/23/CE is based on a target sampling after a risk analysis of potential source of contamination in each Member state. Compared to this approach random sampling of slaughter cattle is indicated to estimate the PCBs-prevalence in a country. This approach allows for comparison prevalences between

countries. The validation of new tests in live animals (e.g. dosage of activity of Ah-receptor by ELISA) may be considered for a detector system and may give opportunities for future population studies.

After this PCBs/dioxins crisis Belgian authorities decided to create a federal agency for security of food chain (AFSCA). A new **CO**ntaminant **SU**rveillance **S**ystem (**CONSUM**) for a better control of the production chain was also installed. CONSUM is based on a group of actions : permanent monitoring of critical raw materials, random monitoring of feed producers, obligatory tracing in processing plants, random and target monitoring in all farms and introduction of a contaminant status, random monitoring of transforming and distribution plants and an emergency plan for contamination accidents.

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GEVOLGEN VAN DE DIOXINECRISIS OP DE BEWAKING VAN DE VOEDSELKETEN

J. Verhaeghe *IVK*

Lessen van de crisis :

1. algemeen : organisatorisch vlak

   - verbeterde samenwerking, coördinatie en communicatie tussen de verschillende betrokken diensten nodig voor ketenbewaking ;

   - wet van 4 februari 2000 houdende oprichting van het Federaal Agentschap voor de Veiligheid van de Voedselketen ;

   - beoogt samenbrengen van taken DG4, DG5, IVK, AEWI, (Farm. Insp).

2. specifiek : functioneel vlak

   - crisis : oplossing gezocht via tracabiliteit en laboratoriumanalyses
     - dieren, voeders en de grondstoffen of toevoegsels ;
     - producten : melk, eieren, vlees, voedingsmiddelen ;

     + certificering

   - conclusie : verruiming en verhoogde performantie nodig van preventief georganiseerde tracabiliteit evenals statistisch verantwoorde systemen voor bemonstering en analyse (monitoring).

Klassieke overheidsaanpak met 2 aspecten :

- regelgeving : normatieve en eerder repressieve aanpak

- preventieve bewakingsaanpak :

  2 elementen

  - monitoring : voornamelijk CONSUM

  - tracabiliteit : SANITEL-BELTRACE

'Uitwendig' doel :

* gezondheid consument beschermen

* vertrouwen herstellen in
  - Belgische producten ;
  - toezichthoudende overheid ;

* vertrouwen herstellen bij
  - consument ;
  - handelspartners ;
  - andere Lidstaten, EG, derde landen.

I. DE REGELGEVING : normen, handelsverboden, handelsverplichtingen

I.1. M.B. 17 april 2000 (B.S. 24.05.2000, ed. 2)

Ministerieel besluit tot wijziging van het ministerieel besluit van 12 februari 1999 betreffende de handel en het gebruik van stoffen bestemd voor dierlijke voeding

Landbouw

Wet 11 juli 1969, bestrijdingsmiddelen en grondstoffen in landbouw, tuinbouw, bosbouw en veeteeelt
K.B. 8 februari 1999, stoffen bestemd voor dierlijke voeding

Ongewenste stoffen en producten in diervoeding

MATRIX	**PCB-norm**
Mengvoeders	200µg/kg vet
Voedermiddelen van dierlijke oorsprong (vetgehalte hoger of gelijk aan 2%)	250µg/kg vet
Voedermiddelen van dierlijke oorsprong (vetgehalte lager dan 2%)	50 µg/kg product

Gehalte aan PCB op basis van de som van 7 toxische congeneren :
PCB 28 ; PCB 52 ; PCB 101 ; PCB 118 ; PCB 138 ; PCB 153 ; PCB 182

MATRIX	**Dioxinenorm**
Citruspulp (enig vermeld in M.B. voor wijziging)	0,5 ng WHO-TEQ/kg product
Dierlijk vet en andere van landdieren afkomstige producten (vetgehalte hoger of gelijk aan 25%)	2 ng WHO-TEQ/kg product
Andere van landdieren afkomstige producten (vetgehalte lager dan 25% vet)	0,5 ng WHO-TEQ/kg product
Visolie en vis, andere mariene dieren, producten en bijproducten daarvan (minder dan 25% vet)	6 ng WHO-TEQ/kg product
Plantaardige oliën en bijproducten	1 ng WHO-TEQ/kg product
Mengvoeders met uitzondering van visvoeders	0,75 ng WHO-TEQ/kg product
Visvoeders	3 ng WHO-TEQ/kg product

bind-, verdunnings- en stollingsmiddelen E516 tot E566 (vb. E559: kaolien houdende klei) (sommige producten van vulkanische oorsprong slechts tot 30/9/2000)	500 pg WHO-TEQ/kg product

WHO-TEQ : toxiciteitsequivalent volgens de Wereldgezondheidsorganisatie.
Besluit zelf vermeldt geen tabel met toxiciteitsequivalenten.
II.2. K.B. 19 mei 2000 (B.S. 31.05.00, ed. 2) (~~K.B. 28 dec. 99, BS 31.12.99~~)

Koninklijk besluit tot vaststelling van maximale gehaltes aan dioxines en polygechloreerde bifenylen in sommige voedingsmiddelen

Volksgezondheid

Wet 24 januari 77, bescherming gezondheid verbruiker van voedingsmiddelen

Voorgaand K.B. 23 april '98: omvatte alleen normen voor dioxines in melk, melkproducten en voedingsmiddelen waarin melk of melkproducten verwerkt.

K.B. 28 december '99 om procedureredenen vervangen

MATRIX (meer dan 2% vet)	**PCB-norm** (7 congeneren)
Varkensvlees	200ng/g vet
Rundvlees	200ng/g vet
Pluimveevlees	200ng/g vet
Melk	100ng/g vet
Kaas	100ng/g vet
Eieren	200ng/g vet
Eiproducten	200ng/g vet
Voedingsmiddelen op basis van eieren	200ng/g vet
Vetten/oliën van dierlijke oorsprong Visoliesupplementen (?)	200ng/g vet

MATRIX (meer dan 2% vet)	**dioxinenorm** (17 congeneren van TCDD en PCDF)
Varkensvlees	3 pg TEQ/g vet
Rundvlees	5 pg TEQ/g vet
Pluimveevlees	5 pg TEQ/g vet
Melk	5 pg TEQ/g vet
Kaas	5 pg TEQ/g vet
Eieren	5 pg TEQ/g vet
Eiproducten	5 pg TEQ/g vet
Voedingsmiddelen op basis van eieren	5 pg TEQ/g vet
Vetten/oliën van dierlijke oorsprong Visoliesupplementen (?)	5 pg TEQ/g vet

TEQ : toxiciteitsequivalenten van 2,3,7,8, TCDD (=WHO-TEQ);
besluit bevat tabel

Paardenvlees en vis (visolie?) als dusdanig niet van normen voorzien, hoewel opgenomen in bewakingsprogramma (PCB: aquacultuur 30 monsters IVK, visolie 15 monsters AEWI - Dioxine: paard 9 monsters, aquacultuur, zeevis en garnalen telkens 3 monsters IVK)(vis: sterke schommeling in vet% ngl. soort!)

Maatregelen: - handelsverbod (strafsanktie in personam)
- schadelijk verklaren van de producten: leidt tot beslag en vernietiging (sanktie in rem)

Verboden voedingsmiddelen of ingrediënten die niet voldoen te vermengen met andere met het oog op « verdunningseffect » !
II.3. K.B. 3 juni 1999 (B.S. 04.06.99, ed. 2)

Koninklijk besluit tot vaststelling van beschermende maatregelen met betrekking tot dioxineverontreiniging van voor dierlijke voeding bestemde dierlijke producten
(Landbouw - wet 11 juli 1969)

Art. 1, b) handels-, invoer- en gebruiksverbod voor dierlijke voeding van vetten die niet afkomstig zijn van dierlijk afval of niet direct afkomstig van de voedingsmiddelenindustrie

Maatregelen: - handelsverbod (strafsanktie in personam)
- beslag, leidend tot vernietiging wegens beletsels vanuit oogpunt volksgezondheid

II.4. K.B. 18 november 1999 (B.S. 02.12.99, ed. 2)

Koninklijk besluit tot wijziging van het koninklijk besluit van 30 oktober 1998 betreffende de erkenning en de registratie van fabrikanten en tussenpersonen in de sector dierenvoeding
(Landbouw - wet 11 juli 1969)

Voorafgaande ministeriële (niet overdraagbare!) toelating vereist voor :

- Operatoren actief in de sector voedermiddelen van dierlijke oorsprong of in de sector toevoegingsmiddelen (bindmiddelen, verdunnigsmiddelen, stollingsmiddelen) ;
- Handelaars in mengvoeder.

Verplichtingen voor de operatoren :

- elke partij bemonsteren (3x500g) ;
    - 1 exemplaar analyse (voedermiddelen: PCB ; bindmiddelen: dioxine) ;
    - 1 exemplaar aan eindgebruiker van de partij ;
    - 1 exemplaar 6 maand ter beschikking van bevoege autoriteit ;
- traceerbaarheidsgegevens bijhouden : aard, hoeveelheid, fabricatie- of invoerdatum, lotnummer, tank of silo, bestemmeling ;
- analyserapport bezorgen aan bestemmeling :
- aangifte van overschrijding der normen.

Verplichtingen fabricanten van mengvoeders :

- monster (500g) bewaren van elke partij aangewend voedermiddel of toevoegingsmiddel en gedurende 6 maanden ter beschikking bevoegde autoriteit houden ;
- monster van elke geproduceerde partij mengvoeder 3 maanden bewaren en ter beschikking bevoegde autoriteit houden ;
- traceerbaarheidsgegevens bijhouden :
    - leveranciers voedingsmiddelen, toevoegingsmiddelen en voormengsels ;
    - aard, hoeveelheid, leveringsdatum, lotnummer van voedingsmiddelen, toevoegingsmiddelen en voormengsels ;
    - aard, hoeveelheid, productiedatum, leveringsdatum, lotnummer en bestemmeling van de partijen mengvoeder

Verplichtingen voor de handelaars in mengvoeder :

gegevens bijhouden voor tracering : datum levering, gegevens van de koper, aard van het voeder, geleverde hoeveelheid, lotnummer (of fabricatiedatum).

II. CONSUM : Contaminant Surveillance System

Buitengewoon opsporingsprogramma voor PCB en dioxines in 2000.

'Inwendig' doel van deze aanpak :

- snelle opsporing van gevallen van besmetting
- snelle en efficiënte reactie op vastgestelde besmettingen

Uitgangspunt : Voornaamste (enige?) contaminatiemogelijkheid : de voederketen

Conclusies :

1. bewaking en controle in de diervoedersector versterken
   * risicogrondstoffen verboden : bv. frituurvet (KB 3/6/99, art. 1, b)
   * systematische analyse van kritische voedermiddelen (producten van dierlijke oorsprong) en toevoegingsmiddelen (bv. kaolienklei) ;
   * monitoring van mengvoeders en voormengsels ;
   * verbeteren van traceerbaarheid
     - registratie van producenten van kritische grondstoffen ;
     - registratie handelaars mengvoeders ;
     - handelaars in mengvoeders dienen verrichtingen te registreren ;
     - werken aan de invoering van een C-statuut voor gecontamineerde bedrijven.
   * annex : inspanningen op milieutoezicht (+ Gewesten)

2. steekproeven op eetwaren van dierlijke oorsprong : melk, eieren, vlees
   normale residuenprogramma's op grond 96/23/EG : frequentie verhoogd

Hoewel gezamenlijk concept, treden de diverse betrokken administraties of diensten op binnen het kader van hun « historisch » werkterrein.

- Vaste communicatieprocedure via CONSUM-gegevensbank :

Alarmformulier - Inlichtingenformulier - Opvolgingsformulier

- Geaccrediteerde laboratoria, Beltestmethode, Database ivm. capaciteiten

DE MONITORING

DG4 : Inspectie-generaal van grondstoffen en afgewerkte producten

Voormengsels en mengvoeders voor dierlijke voeding :

PCB :

- objectief : 12.000 monsters/jaar (10.000 binnenland, 2000 invoer) ;
- gebaseerd op loten per fabrikant ;
- betrouwbaarheid 99% bij incidentie 2% op niveau fabrikant ;
- betrouwbaarheid 95% bij incidentie 5% gefabriceerde loten ;
- ad random selectie, rekening houdend met diersoort van bestemming ;
- Rijksontledingslaboratoria Tervuren, Gent, Luik (resultaat 5 dagen) ;
- resultaat : 6 positief, waarvan 1 alarmprocedure (Feluy : aanvullend voeder kalveren, nadien ook varkensvoeder)(Arochlor, herkomst ?).

DIOXINE :

- aantal monsters vooropgesteld : 1000, in elk geval de + PCB-monsters ;
- resultaat : 4 positief, waarvan 1 behorend tot Feluy-incident.

DG5 :
Inspectie-generaal kwaliteit dierlijke producten

PCB :

- objectief : 200 monsters eieren legbedrijven,
  180 melkmonsters ophaalwagen ;
- geen positief.

DIOXINE :

- objectief : 10 monsters eieren,
  30 melkmonsters ophaalwagen,
  30 melkmonsters (gericht omgeving verbrandingsovens);
- 6 positief (gericht luik)

DG5 :
Inspectie-generaal veterinaire diensten

Geen monitoringprogramma ;
Gericht onderzoek op veebedrijf ingeval positief resultaat van andere dienst.

IVK :

Standaardprogramma algemene monitoring residuen :

- Basis : richtlijn 96/23/EG, Bijlage I, groep B3 : andere in het milieu aanwezige stoffen ('illustratief' onderdeel organische chloorverbindingen met inbegrip van PCB)(Lidstaten verdelen vrij het aantal monsters te verdelen binnen groep B3 volgens ervaring en inzicht) ;
- Jaarlijks een plan voorleggen aan EG + resultaten van voorgaand jaar
- Controleniveau volgens slachtvolume voorgaand jaar ;
- België enige Lidstaat waar sedert '99 dioxineanalyses (buiten crisis) !

PCB :

- objectief : pluimvee, varkens, runderen telkens 300 monsters/jaar
  + aquacultuur 30 monsters/jaar ;
  ('99 : resp. 30, 50, 50 in 'normale' 96/23/EG-toepassing) ;
- statistische basis : betrouwbaarheid 95% bij incidentie 1% ;
- resultaat : <u>1 alarmprocedure</u> (olievat in weide rundveehouder) ;
- daarnaast aanvullende onderzoeken op producten uit zee.

DIOXINE

- objectief : per diersoort enkele tot een paar tiental monsters ;
  ('99 : 96 monsters - 2000 : 105 monsters)
  + alle gevallen van positief PCB-onderzoek ;
- resultaat : geen positieven

Algemene Eetwareninspectie

Ook hier verhoogde monsterneming tov. 96/23/EG (eieren x3)
Verbreding van de te bemonsteren voedingsmiddelen

PCB :

- objectief : eieren (105), eiproducten (50), levensmiddelen op basis van eieren (20), vetten en oliën van dierlijke oorsprong (50), visoliesupplementen (15), kaas (20) ;
- resultaat : geen positieven.

DIOXINES :

- objectief : eieren (5), vetten en oliën (5), kaas (20) ;
- resultaat : geen positieven

III. TRACABILITEIT

III.1. Niveau veevoederproductie en –distributie (zie ook rubriek II.4)

Registratie (voorafgaande toelating) van

- producenten van kritische grondstoffen (voedermiddelen/toevoegingsmiddelen)
- handelaars in mengvoeders

- alle verrichtingen van handelaars in mengvoeders (zelf)
  = link tussen mengvoeder en veestapel (Sanitel)

III.2. C-statuut voor gecontamineerde veebedrijven (in voorbereiding)

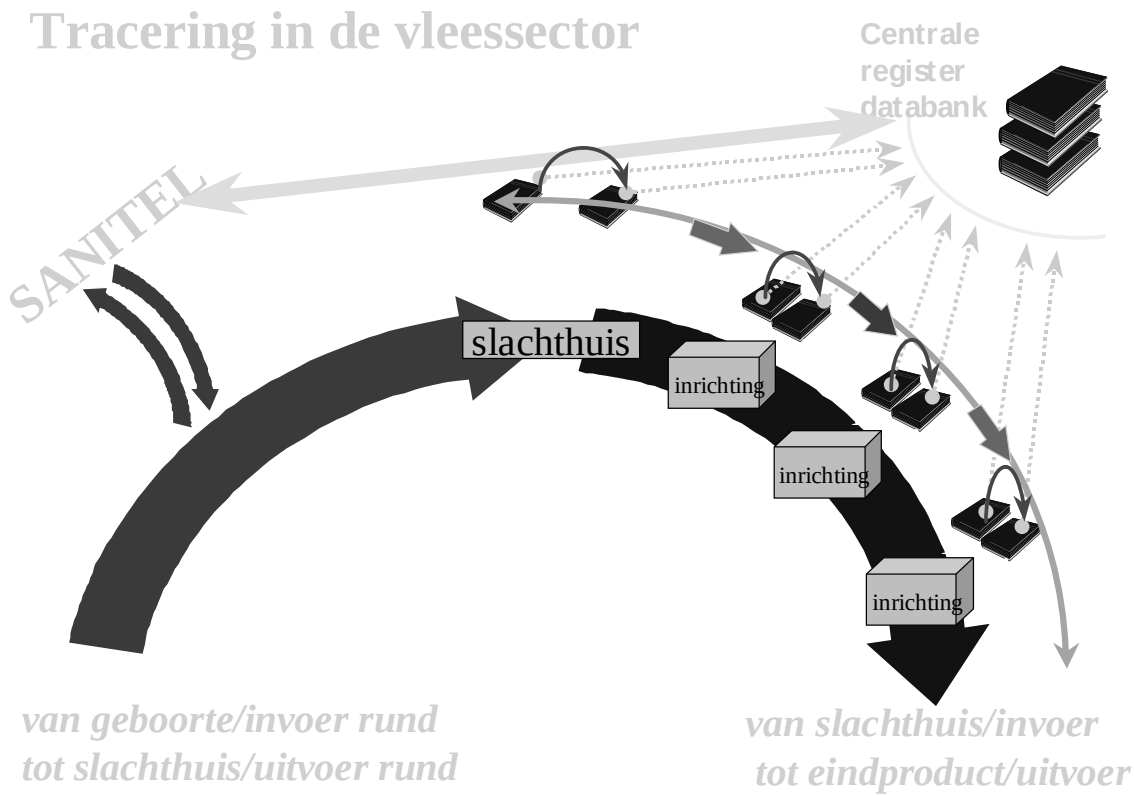
Aansluitend op of geïntegreerd in K.B. 8 sept. 1997 en M.B. 10 sept. 1997
= R- en H-statuut

Vermoeden of vaststellen van contaminatie :

➔ bewarend beslag : identificatiedocumenten of beslagvignetten ingehouden
= dieren mogen bedrijf niet verlaten
= afzonderlijke ophaling en verwerking van kadavers

III.3. Beltrace

Project tot doortrekken van de Sanitel-tracabiliteit tot de vleessektor
= electronisch ondersteund systeem van identificatie en traceerbaarheid



Het systeem verbindt verschillende initiatieven :

* Ministerie van Landbouw
    - snelle en sluitende afmeldingen in Sanitel
    - beheersing dierenziekten en incidenten
* IVK
    - tracering vleesketen
    - efficiëntere keuring
    - statistieken en parameters voor rechtenberekening
    - betrouwbaar certificeren
    - snelle en doelgerichte crisisinterventie
* IVB (=sektoren)
    - kwaliteitsgarantie Belgisch vlees (labels)
    - neutrale karkasclassificatie

IV. CONCLUSIE

1) België beschikt over reglementaire normen ivm. residuen PCB en dioxines in voedermiddelen, mengvoeders en voedingsmiddelen.

2) Monitoring gebaseerd op ervaring met dioxinecrisis ’99 en wetenschappelijke onderbouw werkt.

- administraties en laboratoria kunnen de planning waarmaken ;
- inspanningen geleverd zoals nergens ter wereld ;
- incidenten worden ontdekt en bezworen ;

Voor sommige species (paarden, visserijproducten) dringen verdere acties zich op (advies, studie en normering)(rol wetenschappelijke raad van FAVV).

Aanhouden van dergelijke werkwijze gewenst.

Uitbreiding naar andere contaminanten gewenst. (advies, studie en normering)(rol wetenschappelijke raad van FAVV).

3) Tracabiliteit in de vleessektor, hoewel veel complexer dan in de veeteelt, dient versneld ingevoerd en versterkt, ook om veel andere epidemiologische en economische redenenen.

4) Overleg met de Gewesten nodig om controle op de verspreiding van contaminanten in het milieu te versterken

ECONOMIC IMPACT OF THE DIOXIN CRISIS

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ABSTRACT

The occurrence of the dioxin crisis in May 1999 had major economic impacts on the food sector in Belgium. The total effect of the dioxin crisis on the Belgian economy is estimated at 0.2 to 0.25 %-points of the Gross National Product. Losses are attributed to product recall, destruction, access export market and worsening consumer confidence. Although the total bill of the crisis was quite substantive, recovery has rapidly been realised. After all, the major impact of the crisis is situated at the policy level, where new initiatives and co-operation throughout the food chain emerged in order to avoid a similar crisis in the future.

SAMENVATTING

Onderhavige paper geeft een overzicht van de economische impact van de dioxine crisis van mei 1999 op de voedselketen. De totale impact van de crisis wordt geschat op 0.2 tot 0.25 procent punten van het Bruto Binnenlands Product. Schade en verliezen waren te wijten aan product recall, vernietiging, moeilijker toegang tot exportmarkten en dalend consumentenvertrouwen. Ondanks de aanzienlijke rekening van de crisis, herstelde de voedingssector vrij snel van de crisis. Méér dan één jaar na de crisis, blijkt het belangrijkste effect van de crisis te maken te hebben met beleidsveranderingen. Deze omvatten een aantal nieuwe initiatieven en doorgedreven samenwerking tussen de verschillende schakels van de voedselketen, met het oog het vermijden van een gelijkaardige crisissituatie in de toekomst.

1. INTRODUCTION

With the occurrence of the dioxin scare of May-June 1999, the Belgian meat industry experienced another difficult time after the meat safety crises from previous years. Like the previous meat safety issues, the dioxin crisis received considerable attention from mass media, which actually brought the scare to the public on May 27, 1999. Media reports initially suggested a playing-down or cover-up operation by the Ministries of Agriculture and Public Health of human health risks resulting from a dioxin contamination in animal feed. This new crisis intervened with promotion efforts that aimed at re-establishing the image of meat in general and beef in particular after the hormone and BSE-crises from the second half of the nineties.

The cause of the dioxin problem dated back to the end of January 1999 and pertained to PCB and dioxin contaminated transformer oil that entered the food chain (Bernard, 2000; North, 2000). It initially led to abnormal laying hen mortality and sharply decreased egg hatchability. Consequent analyses indicated dioxin levels that 1,500 times exceeded legal standards applicable to e.g. chicken fat. The 28th of May, all chicken and eggs had to be removed from the Belgian retail shelves. Additionally, some of the contaminated broiler feed appeared to have been recycled into pig feed, thus also involving pork meat into the debate and product recall. The initial blocking of meat products in Belgium was soon followed by import bans of Belgian meat and egg products by other EU countries, backed up by the EU veterinary committee's decisions. Finally, any remaining restrictive measures have only been lifted by EU decision 2000/301 on April 18, 2000, which denoted the formal end of the crisis. Despite the elevated dioxin levels in animal feed and some meat tissues, consumer exposure to contamination appeared to have been very small thanks to dilution effects and limited doses in the more valuable meat cuts. Nevertheless, consumption levels of poultry and pork fell respectively to 69% and 93% in June 1999 as compared to their levels of June 1998.

The current paper focuses on the economic impact of the dioxin crisis. The economic impact pertains to direct and indirect costs. Direct costs are quantifiable and mainly result from product recall and destruction, compensatory payments and stop of activities. Indirect costs result from the loss of export markets and declining consumer confidence in Belgian meat and meat products. The total economic impact of the dioxin crisis has been estimated by

the Ministry of Finance at 25 milliard BEF. This includes compensatory payments, operational costs (analyses, destruction, transport, storage, slaughtering), market and processed product losses. The total effect of the dioxin crisis on the Belgian economy is estimated at 0.2 to 0.25 %-points of the Gross National Product. Several factors contributing to these economic impacts are reported and discussed in the following paragraphs. Three major sections are distinguished. The first focuses on the impact on the supply and processing side, the second on export markets, the third on domestic consumers' reactions.

2. SUPPLY AND PROCESSING LEVEL

The economic impact of the dioxin crisis on supply and processing companies is illustrated through presenting costs and losses for several affected sectors. A first item deals with destruction of **affected animals and products**. Table 1 indicates volumes and values of destroyed animals and products from the pork and poultry sectors. Values are based on the expected prices under normal market situations. The total value destroyed amounts to almost 1.3 milliard Belgian francs, of which 58 % is accounted for by the pork sector.

Table 1. Destruction within dioxin affected agricultural production

Product	Market value (BEF/unit)	Volume destroyed (tonnes or 1,000 units)	Value destroyed (million BEF)
Pigs	30.0	25,000 kg	750
Broilers	28.0	10,700 kg	300
Laying hens	61.0	1,460 units	89
Breeder hens	197.5	400 units	79
Eggs	25.4	590 kg	15
Hatchery eggs	6.0	10,800 units	64
Total	-	-	1,297

Source: CLE, 1999

During the crisis, primary **producer prices** for animal products dropped with about 11 BEF/kg for broilers, 25 BEF/kg for eggs, 18 BEF/kg for pigs and 520 BEF/100 kg for beef cattle. Compensatory payments have been established to account for a part of the losses of primary producers. In total, about 5 milliard BEF has been attributed by the federal Ministry of Agriculture in order to compensate for the losses at the primary producer level. Table 2 illustrates the situation for a poultry producers with 30,000 broilers, counted at 2 kg per unit. The normal expected labour income for one round amounts to 180,000 BEF. The data show the situation with and without compensatory payment for free and contract producers.

Table 2. Impact of the dioxin crisis on poultry producer labour income (farm size 30,000 units)

	Without dioxin crisis	Dioxin crisis		
		Free producer	Contract producer	Compensatory payment
Market price (BEF/kg)	28	10	20	+ 9
Total output per round (BEF)	1,680,000	600,000	1,200,000	+ 540,000
Cost (BEF/kg)	25	25	25	-
Total cost per round (BEF)	1,500,000	1,500,000	1,500,000	-
Labour income (BEF)	+ 180,000	- 900,000	- 300,000	+ 540,000

Source: Gellynck, 2000

The same exercise for a pork producer is reported in Table 3. Labour income is expressed in BEF/pig, which should have accounted to about 1,000 BEF under normal circumstances.

Table 3. Impact of the dioxin crisis on pork producer labour income

	Without Dioxin crisis	Dioxin crisis		
		Free producer	Contract producer	Compensatory payment
Market price (BEF/kg)	45	30	38	+ 3.8
Output per pig (BEF)	4,500	3,000	3,800	- 380
Cost (BEF/kg)	35	35	35	-
Cost per pig (BEF)	3,500	3,500	3,500	-
Labour income per pig (BEF)	+ 1,000	- 500	+ 300	- 380

Source: Gellynck, 2000

Besides effects at the primary producer side, major impacts pertained to the **food processing industry**. The index figure of production indicated a decrease of 14.3% for the food industry as a whole in June 1999. Second and third quarter figures indicate decreases with 6.6% and 5.5% as compared to the year before. The meat sector has clearly been affected with a production decrease of 42.2% in June 1999 as compared to June 1998.

Table 4 gives an overview of the losses for the different affected food sectors. The figures are estimates, including 15-20% estimated profit losses due to the crisis. The slaughterhouse sector was the most affected, with a total loss of about 8.6 milliard BEF. More than half of this loss is attributed to storage and retours. Although not directly affected by dioxin contamination, the dairy sector experienced losses of almost 3 milliard BEF. The direct affected sectors of poultry and meat processing experienced losses of 2.1 and 2.3 milliard BEF, respectively. None of these losses have been compensated in any way by the regional Flemish government. Clearly, the impact will fully be reflected in the 1999 balance sheets of the individual companies.

Table 4. Losses per food processing sector (1,000 BEF)

Sector	Loss
Slaughterhouses	8,649,221
Dairy processing	2,935,687
Meat processing	2,327,714
Poultry	2,085,134
Chocolate and biscuits	774,510
Animal feed	706,674
Eggs	686,919
Other food processing	657,820
Retailing	2,150,828
Total	20,974,507

Source: CRB, 2000

Besides the negative impact on production, also investments and employment in the food industry suffered from the dioxin crisis. At the height of the dioxin crisis, i.e. during the third quarter of 1999, food industry investments fell 12.9%. This decrease largely contrasts with the 10.9% increase of total industry investments during the same period. The situation even worsened during the fourth quarter of 1999. Food industry investments were 23.2% lower than the year before, while total industry investments were up 4.4%. With respect to employment in the food industry, a drop of 1,883 timely unemployed in June 1999 is noticed. From September on, employment levels of 1998 were re-established. After all, the traditional unemployment indicators do not show any effect of the dioxin crisis.

3. EXPORT MARKETS

A major problem and cause of economic loss during the crisis pertains to the situation on export markets. Food industry exports (including meat) increased 12.1% in 1997 as compare to 1996. This growth level fell to zero in 1998 and started to decrease during the first half of 1999. With the occurrence of the dioxin crisis, further dramatic declines were noticed. In June 1999, total food industry exports were 23.7% lower than in June 1998 (CRB, 2000). The immediate short term impact was strongest for meat and processed meat products, with losses of about 60% in June as compared to May 1999 (Table 5). With the exception of processed meats, most sector exports attained their 1998 level by the end of the third quarter of 1999.

Table 5. Evolution of export, index June-September 1999 with May as reference, in % of value

Product	June/May '99	July/May '99	August/May '99	Sept./May '99
Meat	- 61	- 29	- 30	- 6
Processed meat	- 60	- 49	- 53	- 48
Animal fat and oils	- 17	- 11	+ 16	+ 22
Dairy products	- 41	- 21	- 22	- 2
Eggs	- 46	- 45	- 34	+ 3
Chocolates	- 36	- 2	+ 27	+ 54
Confectionery and sweets	-21	- 13	- 4	+ 7
Live pigs	+ 4	+ 480	+ 290	+ 530

Source: CRB, 2000; CLE, 2000

During the months following the dioxin crisis outbreak, exports of live pigs boosted. With May as the reference, live exports of pigs increased fivefold during July and September. Overall, live exports almost tripled during the second half of 1999 as compared to the first half year.

4. DOMESTIC CONSUMPTION

Reactions at the consumer level include consumption decrease and loss of confidence in food and meat production. Changes in terms of consumption can be measured through household panel data. Loss of confidence is reflected by shifts in attitude and perception, as they have been measured in surveys with Belgian meat consumers in April 1998 (n=300) and April 2000 (n=200).

The at-home meat consumption data from the GfK consumer panel reveal a further, but small, decrease of total fresh meat consumption from 51.4 until 50.5 kg per capita for 1998 and 1999 respectively (GfK, 2000) (Table 6).

Table 6. At-home meat consumption evolution in Belgium, 1995-1999

Meat Type	1995	1996	1997	1998	1999	% 99 / 95
Beef and Veal	14,6	12,8	12,0	12,1	11,6	- 20
Pork	14,1	13,8	13,1	13,6	14,0	-/+
Poultry	12,1	10,9	11,1	11,8	12,0	-/+
Meat mixtures	10,4	10,8	12,6	11,5	10,3	-/+
Other fresh meat	3,1	2,5	2,5	2,4	2,6	- 16
Total fresh meat	54,3	50,8	51,2	51,4	50,5	- 7
Processed meat products	13,0	13,7	13,6	15,0	15,0	+ 15
Total meat	67,3	64,5	64,8	66,4	65,5	- 3

Source: GfK, 2000

Despite the timely drop of poultry meat consumption in June 1999, the balance for the whole year is slightly positive with an increase of 0.2 kg per capita as compared to 1998. Major losers are veal and meat mixtures. Within the total GfK sample, the percentage of fresh meat buyers dropped from 99.4% in 1998 until 98.2% in 1999, which is quite substantive. At first sight, the figures in Table 6 do not clearly reflect the occurrence of a major meat safety crisis in 1999. However, monthly consumption data for June and July clarify the situation during the height of the crisis (Figure 1).

During June 1999, poultry and meat mixture consumption fell considerably as compared to the same month of the previous year. A large part of this decrease can be attributed to empty shelves and out-of-stocks at the retail level. The impact for beef and pork was rather small, while gains were obtained for fish, horse and lamb meat. The same graph for the month July displays a totally different picture, i.e. a positive evolution for most animal protein products, including poultry. These findings point to a rapid re-establishment of consumption after the height of the crisis.

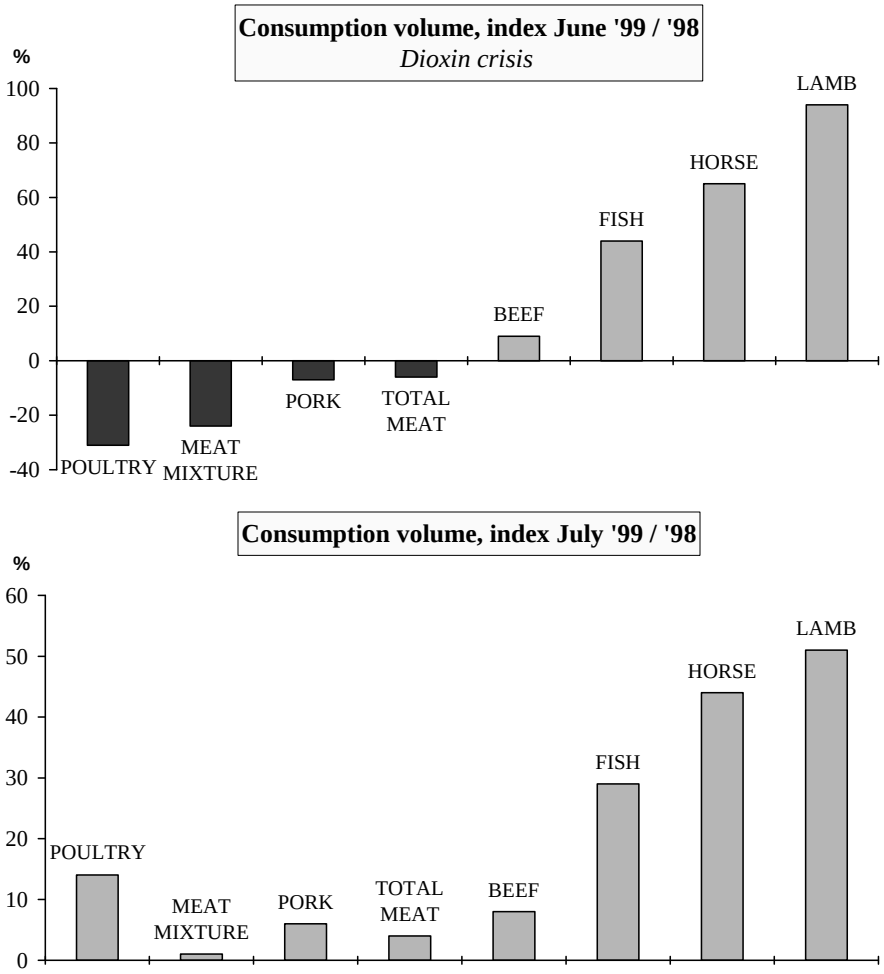

Figure 1. Short impact of the dioxin crisis, consumption index at-home meat consumption, June and July, 1998/1999
Source: GfK, 1999

Both in 1998 and 2000, consumer attitude to beef, pork and poultry has been assessed through investigating consumer perception on 16 fresh meat product attributes that were generated from qualitative exploratory research and literature review. In both surveys, the top five important attributes were “quality”, “taste”, “freshness”, “free of hormones”, and “healthiness”. However, within the top five “healthiness” apparently became more important over time with 10.2% of the respondents ranking this attribute at the most important in 2000, versus 5.0% in 1998. Reversibly, the number of respondents attaching top importance to “free of hormones” dropped from 19.5% in 1998 until 10.1% in 2000. Both findings corroborate expectations, first, given the occurrence of the dioxin crisis with a major perceived health threat, and second, given the absence of further hormone scandals and the efforts by the industry to re-establish the beef image.

A major focus in the analysis pertained to the perception profiles for fresh meats. Significant shifts in perception between the 1998- (n=300) and 2000-sample (n=200) were assessed through performing an independent samples t-test. All significant shifts, with their associated t- and p-values are displayed in Table 7.

Table 7. Significant shifts in attribute perception, comparison of mean scores (on 7-point scale ranging from -3 until +3) through independent samples t-test, 1998 (n=300) and 2000 (n=200)

	1998	2000	t-value / significance
BEEF			
With - Without hormones	-0.68	-0.20	-2.25**
Not healthy - Healthy	0.25	0.65	-2.54**
With - Without harmful substances	-0.77	-0.12	-3.63**
Not trustworthy - Trustworthy	-0.49	0.25	-4.14**
Tough - Tender	0.70	1.11	-2.51**
Not safe - Safe	-0.47	0.24	-3.93**
PORK			
With - Without hormones	0.43	0.08	2.22**
POULTRY			
Bad - Good quality	1.48	1.22	1.94*
With - Without hormones	1.33	0.94	2.51**
Not trustworthy - Trustworthy	0.92	0.66	1.87*
Not safe - Safe	1.10	0.60	1.96*

significance level: * = p<0.10, ** = p<0.0
Source: Verbeke & Viaene, 2000

Both the observed perceptual shifts and persistence corroborate expectations. Beef's perception on health and safety related attributes significantly improved, as could be expected from the absence of major negative press directly linked to beef in 1999-2000, combined with efforts by the industry to re-establish its image. Remarkably, beef perception on "tenderness" also improved along with its healthiness and safety perception. The overall quality, price and convenience attribute perception did not shift, herewith reinforcing the position in the perceptual mapping of 1998.

Pork and especially poultry were affected by the dioxin crisis which is reflected in their perceptual profiles. Apparently, respondents associated dioxins and PCBs with hormones, leading to significant shifts toward the "with hormones"-pole of the semantic differential scale for both meats. Additionally, poultry perception on "quality", "trustworthiness" and "safety" significantly worsened after the dioxin crisis. No other shifts of the pork and poultry perception profiles were discovered, which is reasonable in absence of substantial changes in sensory, price, convenience or animal welfare issues over the considered time interval.

Overall, the observed shifts and persistence lead to closer matching beef, pork and poultry perception profiles. The gaps between the worst and best ranked meat on safety issues have clearly narrowed. Major differences between the three meats remain on price and leanness perception. Both in terms of quality and taste, beef and poultry score almost similar and largely better than pork.

Apparently, decisions to decrease fresh meat consumption from the past are associated with higher degrees of importance attached to "safety" and "free of hormones", as well as with lower importance attached to "taste". Reversibly, intentions to decrease fresh meat consumption in the future are associated with a higher degree of importance attached to "safety", "healthiness" and "animal friendliness".

Further, significant associations between attribute ratings and claimed past and intended behaviour were assessed through independent samples t-tests for comparison of means. It is evidenced that the less consumers believed that beef has a good taste and is safe in a broad sense, the more they claimed to have decreased meat consumption. Similar conclusions can be drawn related to the perception of "taste", "leanness" and also "safety" of pork. The stronger the belief in these attributes, the less consumers claimed to have decreased their meat consumption. Before the dioxin crisis, no significant associations between claimed meat consumption decrease and poultry perception were found. However now, a year after the outbreak of the crisis, claimed decrease of

poultry meat consumption is found to be significantly associated with its perception on containing hormones. Those respondents who report to have cut consumption have a worse perception of poultry meat on this attribute. Again, it is assumed that in consumer's minds and terminology, the reference to "hormones" is used as a proxy for dioxins.

5. CONCLUSIONS

The economic impact of the dioxin crisis on the food industry typically showed up during June 1999. Key elements were decreasing industrial production and investments at the processing industry level, and immediate consumption declines for poultry. Despite the immediate effect, several economic indicators showed recovery of the food industry by the end of 1999 (APS, 2000). Both producer and consumer confidence (as measured by confidence indices) appeared to have been recovered to the level of the previous year. Despite a worsening consumer attitude to poultry and pork on some health and safety related attributes, consumption levels for the year 1999 do not show decreases that could have been expected from a major crisis. From September on, industry production reached back to its 1998 level. Also, exports of animal products, fats and oils recovered rapidly.

Although the total bill of the crisis was quite substantive, recovery has rapidly been realised. After all, the major impact of the crisis is situated at the policy level, where new initiatives and co-operation throughout the food chain emerged in order to avoid a similar crisis in the future.

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PATTERNS OF *MYCOPLASMA HYOPNEUMONIAE* INFECTION IN CLOSED PIG HERDS USING SEROLOGY AND NESTED PCR ON NASAL SAMPLES

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ABSTRACT

This study aimed to investigate patterns of *Mycoplasma hyopneumoniae* (Mh) infection in 7 closed pig herds that were clinically or subclinically infected with Mh. Therefore, serology and nested PCR on nasal samples were used in pigs of different age groups. The percentage of seropositive pigs, the OD-values and the number of pigs with positive PCR were compared between the two types of herds. The percentage of seropositive pigs and the OD-values were higher in the older age groups. Pigs from clinically infected herds tended to seroconvert earlier than pigs from subclinically infected herds. No major differences in the OD-values were observed between pigs of both types of herds. Pigs from herds with clinical symptoms appeared to excrete Mh at a younger age compared to pigs from subclinically infected herds. In addition, the percentage of pigs with positive PCR was more evenly distributed across the age groups in the subclinically infected herds.

1. INTRODUCTION

Enzootic pneumonia in pigs, caused by *Mycoplasma hyopneumoniae* (Mh) as primary agent, is a chronic respiratory disease that causes major economic losses to the pig industry worldwide. Previous epidemiologic studies have shown that almost all pig farms in Belgium are infected with Mh (8). The disease is mainly characterised by chronic coughing and reduced performance during the grow-finishing period. However, under practical circumstances, many Mh infections can occur without obvious clinical symptoms. The clinical course and the economic losses of the disease are largely influenced by the management practices and housing conditions in the herd.

The impact of different Mh strains on the clinical course of the disease is not known. Previous studies indicated that some differences exist across field strains of Mh (2, 4). At this moment however, very little is known about possible differences in pathogenicity and immunogenicity among Mh field strains. In addition, the spread of Mh organisms within pig herds is not yet fully understood. Previous studies have been conducted to estimate the seroprevalence of Mh in pig herds (5), to identify risk factors for increased seroprevalence in slaughter pigs (9) or for reinfection of SPF herds (17, 18). Other studies investigated the seroconversion and shedding patterns in herds with the same management practices (11, 14, 19). However, differences in seroconversion and nasal shedding between clinically and subclinically infected herds have not yet been studied.

The objective of this cross-sectional study was to determine patterns of Mh infection in Belgian closed pig herds that were either clinically or subclinically infected with Mh. Therefore, serology and nested PCR (nPCR) on nasal swabs of piglets of different age groups were used.

2. MATERIALS AND METHODS

2.1. Herds

Seven farrow-to-finish pig herds located in 4 different provinces in Flanders (Belgium) participated in the study. They were selected by contacting the herd health veterinarian. All herds were infected with Mh as demonstrated by previous laboratory data. Four herds were subclinically infected whereas the 3 other herds were clinically infected. Although there was some coughing in the subclinically infected herds, it was not considered to be a problem. In addition, the production parameters in the grow-finishing pigs reached target levels. In the clinically infected herds, typical symptoms of enzootic pneumonia namely chronic coughing and reduced production parameters in grow-finishing pigs were present. Clinical observations and laboratory investigations showed that the clinical problems were not due to infections with other respiratory pathogens such as *Actinobacillus*

pleuropneumoniae or swine Influenza virus. Interestingly, the subclinically infected herds used poor management practices and the housing conditions were unfavourable. In the clinically infected herds, however, the management and housing were good (compartmentalisation, indirect air-entry, all-in/all-out production). One clinically infected herd practised Mh vaccination at 1 and 4 weeks of age.

2.2. Nasal swabs and nPCR

In each herd, nasal swabs were collected from pigs belonging to 4 different age groups namely 6, 9, 12 and 15 weeks. Per age group, 5 pigs were randomly selected at pen-level. The pigs were housed in the nursery unit from 4 weeks until approximately 10 weeks of age. Thereafter, they were transferred to the grow-finishing unit. The swabs were 15 cm long and they were inserted into the nostrils as deep as possible. The nasal swabs were transported in 700 µL PBS with 0.1% Triton x-100. In the laboratory, they were vortexed and discarded. The transport medium was heated at 100°C for 5 min and stored frozen at –25°C until PCR was performed. Five µL of undiluted and 10-fold diluted sample was used in the nested PCR as previously described by Stärk *et al.* (1998). PCR products were analysed by electrophoresis on 1% agarose gels in TBE buffer and visualised under UV illumination after staining in Et Br.

2.3. Blood samples and ELISA

In each herd, blood samples were taken from 10 randomly selected pigs per age group. In total, pigs of 4 different age groups were selected namely pigs of 9, 12, 15 and 18 weeks. All pigs selected for nasal sampling were also blood sampled, except the 6 weeks old piglets. The blood samples were allowed to clot under refrigeration and they were processed by centrifugation at 3000 rpm for 10 min. The sera were stored at 4°C until analysed for presence of antibodies to Mh. A commercial ELISA (Checkit® Hyoptest II; from Dr. W.R. Bommeli, Switzerland) was used and applied according to the manufacturer's instructions. Selected positive standard serum was considered to have an OD-value of 100%. All the field samples were expressed as a percentage of this value. Values >30%, between 30% and 20%, and <20% were considered positive, doubtful and negative, respectively. Doubtful results were considered positive in the calculations. This ELISA-test does not differentiate between antibodies resulting from vaccination or infection.

2.4. Statistical analysis

Data were analysed with the statistical package Statistics for Windows. The Fisher's Exact Test was used to compare the proportions of ELISA positive pigs and nested PCR positive pigs per age group. The OD-values of the different age groups were compared using a two-sample t-test.

3. RESULTS

3.1. Nasal swabs and nPCR

The results of the nPCR on the nasal swabs in the clinically and subclinically infected herds are presented in Table 1. In both types of herds, positive pigs could be demonstrated by nPCR at 6 weeks of age, when the pigs were approximately 2 weeks in the nursery unit. The highest number of nPCR positive pigs was present in the age group of 9 weeks in both types of herds. Although no major differences could be observed between both types of herds, the percentage of pigs positive on nPCR appeared to be higher in the nursery period (6 and 9 weeks) of clinically infected herds compared to the subclinically infected herds. During the grow-finishing period (12 and 15 weeks), the percentage of positive pigs was lower in the clinically infected herds (7%) than in the subclinically infected herds (20 and 14%). The percentage of positive pigs was more evenly distributed among the different age groups in the subclinically infected herds. Similar percentages were obtained when the vaccinated clinically infected herd was excluded from the analysis (data not shown).

3.2. Blood samples and ELISA

In both types of herds, the percentage of seropositive pigs was higher in the older age groups (Table 1). The percentage of seropositive pigs in the nursery unit and the beginning of the grow-finishing unit (9 and 12 weeks) was higher in the clinically infected herds than in the subclinically infected herds, although the difference was not statistically significant. At 15 weeks of age the percentage of seropositive pigs was significantly higher in clinically infected pigs ($p=0.012$). At 18 weeks of age, no differences in percentage of seropositive pigs were

Table 1. The results of the nested PCR (nPCR) on nasal swabs and the ELISA on blood samples from pigs of different age categories in 6 farrow-to-finish pig herds that were clinically (n=2) and subclinically (n=4) infected with *Mycoplasma hyopneumoniae*

Age group	**% positive pigs in clinically infected herds**			**% positive pigs in subclinically infected herds**		
	nPCR[1]	ELISA[2]	OD-value[3](SD)	nPCR	ELISA	OD-value (SD)
6 W	7	–	–	5	–	–
9 W	40	23	34 (4)	25	2.5	33 (-)
12 W	7	30	37 (20)	20	0	–
15 W	7	47	79 (44)	14	7.5	77 (32)
18 W	–	57	68 (24)	–	50	78 (48)

[1]: 5 pigs per age category were selected in each herd for nPCR on nasal swabs
[2]: 10 pigs per age category were selected in each herd for ELISA on blood samples
[3]: Average optical density values (%) of seropositive pigs

observed between both types of herds. The OD-values of seropositive pigs were higher in the older age groups (Table 1). No remarkable differences were observed between both types of herds. Similar OD-values were observed when the Mh vaccinated herd was excluded from the calculations.

4. DISCUSSION

This study was conducted in order to compare the patterns of Mh infections between herds that were clinically or subclinically infected with Mh. The clinically infected herds used good management practices and housing conditions whereas the management and housing conditions were poor on the subclinically infected herds. Under practical conditions, clinical symptoms of enzootic pneumonia are more likely to occur in herds with poor management and unfavourable housing conditions because of the multi-factorial nature of the disease (13). This selection procedure was intentionally adopted because in further studies, possible differences between the Mh strains in both types of herds will be investigated. By using such a selection procedure, the chance of finding differences between Mh strains will be higher. Possibly, strain differences can allow us to ascribe the difference in clinical pattern in both herds to different characteristics of Mh strains.

The pattern of Mh infection was investigated using nPCR on nasal swabs and serology of blood samples. Nested PCR is a valuable technique in profiling herds infected with Mh and it is more useful than a simple PCR because of the increased sensitivity (3). Nevertheless, the significance of obtaining a positive result from a nasal swab is not quite clear. PCR is a qualitative, not a quantitative test, so we cannot assess the amount of Mh organisms that is excreted. PCR does not depend on viable bacteria. Consequently, a positive nPCR result can be due to either bacterial debris or active mycoplasma shedding or colonisation. On the other hand, a negative nPCR result can be due to negligible quantities of Mh organisms or to an excess of cellular debris present on the swab, which can inhibit PCR. Nested PCR is an expensive technique, which makes it less attractive for routine diagnostic use. Serological testing is a useful tool to diagnose Mh infections on a herd basis. Since antibodies are detected and not the presence of Mh, it is an indirect measure of infection. Because of the long time-span (2 to 8 weeks) between infection and seroconversion and the variability in time to seroconversion, serological testing is less appropriate to determine the age at which pigs become infected (6, 7, 11, 14).

In both types of herds nasal excretion of Mh could already be detected in some pigs of 6 weeks of age, and the largest number of nPCR positive pigs was observed at 9 weeks of age. Although the management and housing conditions were better in the clinically infected herds, the number of positive nursery pigs (6 and 9 weeks) was higher in the clinically infected herds compared to the subclinically infected herds. This may indicate that the spread of Mh organisms was more intensive in younger pigs in the clinically infected herds. Further research is warranted to find possible mechanisms responsible for this observation. During the grow-finishing period (12 and 15 weeks), a higher percentage of nPCR positive pigs was found in the subclinically infected herds. There was a more evenly distribution of nasal excretions across the age groups in the subclinically infected herds. Interestingly, there was no marked difference in number of nPCR positive pigs between the non-vaccinated herds and the Mh vaccinated herd. This finding is in agreement with previous studies (3, 13) indicating that Mh

vaccination does not prevent Mh from colonising the respiratory tract. However, the extent of Mh colonisation in the respiratory tract may be lower in vaccinated pigs. This could not be assessed with a qualitative PCR as used in the present study.

A higher proportion of pigs in the nursery unit (6 and 9 weeks) was positive using PCR testing compared to serology. This corroborates with the results of a previous study (3, 16) and indicates that PCR testing is more appropriate to assess the age of infection. The age of pigs seroconverting to Mh and excreting the Mh organisms varied between clinically and subclinically infected herds. In clinically infected herds, the highest proportion of pigs showed seroconversion between 12 and 15 weeks of age. In subclinically infected herds, most pigs showed seroconversion between 15 and 18 weeks. These ages of highest risk of seroconversion were the same as those observed by Yagihashi *et al*. (1993) and Morris *et al.* (1995). Nicolet *et al.* (1990) however found that most positive reactions occurred later, namely at 5 to 6 months of age. Even within clinically or subclinically infected herds, individual pigs start seroconverting at different ages. This was also observed by Yagihashi *et al*. (1993). In this respect, it is difficult in practice to assess 'the' age of infection. Because of the chronic nature of the disease, the number of infected pigs gradually increases towards the end of the finishing period. Thus, to get insight into the infection pattern in the herd, it is important to assess the age at which most of the pigs get infected. Although it cannot be ruled out with certainty, it is not likely that the positive ELISA titres in pigs of 9 weeks are due to maternal antibodies. Morris *et al*. (1994) showed that maternal antibodies persist as long as 9 weeks in pigs from sows with high serum titres. Since the OD-values tended to increase at this age, it is more likely that positive results were caused by active Mh infection.

There was no major difference in OD-values between pigs from clinically or subclinically infected herds. The highest OD-values were observed in pigs of 15 weeks old in clinically infected herds and in pigs of 18 weeks in subclinically infected herds. In addition, there was no obvious difference in OD-values between the vaccinated and non-vaccinated pigs in clinically infected herds. Pigs with doubtful serological results were considered as positive in this study because we assume that they were seroconverting at the moment of sample collection. Doubtful results are common in the early or late stages of Mh infection (15). Since the Tween 20 ELISA is specific, it is unlikely that doubtful outcomes are due to cross-reactions with other mycoplasmas which are frequently isolated from pigs, like *M. flocculare* (1, 15).

The sample sizes used in this study may not suffice to make accurate estimates of the prevalence of positive animals in each age group. However, they permitted to assess the dynamics of Mh infection and to elucidate differences in infection patterns between clinically and subclinically infected herds. The use of nPCR on nasal swabs combined with serological testing provided valuable insights into the epidemiology of infections with Mh. Further research will include more farms and will elaborate on possible differences among Mh strains from these farms.

6. ACKNOWLEDGEMENTS

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