ANTIMICROBIAL RESISTANCE IN BOVINE COMMENSAL NASAL PASTEURELLACEAE

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ABSTRACT
The presence of *Pasteurellaceae* in the nasopharynx of 57 (extensively housed) clinically healthy calves was investigated with special reference to the recent reclassification within this bacterial family and tetracycline resistance. A total of 40 strains belonging to the family *Pasteurellaceae* were isolated in 34 calves (59.6%) out of 10 herds (76.9%). Identification of the strains resulted mainly in *Pasteurella (P.) multocida* (n=35). Other bacteria were *P. canis* (1), *P. trehalosi* (1), *Mannheimia (M.) varigena* (2) and one untypable *Mannheimia* spp. *Mannheimia haemolytica senso stricto* was not isolated. Overall antimicrobial resistance was rare. In five tetracycline resistant *P. multocida* strains originating from the same farm no plasmid DNA was detected, suggesting a chromosomal localisation and a clonal spread of the underlying resistance determinants. In summary, antimicrobial susceptible *P. multocida* strains were the predominant *Pasteurellaceae* present in the nasopharynx of young extensively housed calves.

SAMENVATTING
In 57 gezonde en extensief gehuisveste kalveren werd het voorkomen van *Pasteurellaceae* en hun respectievelijke antibioticumresistentiepatronen onderzocht. Hierbij werd speciale aandacht besteed aan tetracyclineresistentie en aan de recente omvorming van *[Pasteurella] haemolytica* naar *Mannheimia haemolytica*. In totaal werden 40 *Pasteurellaceae* geïsoleerd in 34 kalveren (59,6%) op 10 bedrijven (76,9%). *Pasteurella multocida* werd het meest geïsoleerd (n=35), naast andere *Pasteurella* (n=2) en *Mannheimia* species (n=3). *Mannheimia haemolytica senso stricto* werd niet gevonden. Tetracyclineresistentie werd gedetecteerd in 5 *Pasteurella multocida* stammen afkomstig van hetzelfde bedrijf, maar kon niet worden geassocieerd met de aanwezigheid van plasmidair (horizontaal overdraagbaar) genetisch materiaal. Samenvattend werden voornamelijk antibioticumgevoelige *P. multocida* stammen geïsoleerd uit de neusflora van de extensief gehuisveste kalveren.

1. INTRODUCTION

Bovine respiratory disease (BRD) has been attributed to be the syndrome having the highest morbidity and mortality in calves [7]. *[Pasteurella] haemolytica* and *P. multocida*, both belonging to the family *Pasteurellaceae*, are known to act as primary or secondary (opportunistic) pathogen in the BRD-complex. Older studies have shown that both are present in the upper respiratory tract of healthy and infected calves [1]. In 1999, the reclassification of the trehalose negative *[Pasteurella] haemolytica* complex by means of an extensive polyphasic study has led to the new genus *Mannheimia*. Herein, *Mannheimia (M.) haemolytica* has been defined the type species, besides four so far designated other species [2]. The commensal behaviour of these organisms also implies that they can harbour resistance determinants which may be transferred horizontally into pathogenic bacteria and consequently cause therapy failure [4, 10].
The aim of the present study was to describe the presence and distribution of the so far known species within the genera *Pasteurella* and *Mannheimia* in the nasopharynx of cattle. Special reference has been made to the detection method and tetracycline resistance its determinants, since this has often been reported to be present within organisms of the *Pasteurellaceae* [8] and because tetracyclines are the most common antibiotics used in veterinary medicine [9].

2. MATERIALS AND METHODS

2.1. Animals and Sampling

A total of 57 Holstein, Belgian Blue and mixed breed calves, less than 4 months old and originating from 13 Flemish herds (extensively housed) were examined. On each herd, a maximum of 5 animals without a history of antimicrobial therapy prior to sampling (30 days) were included in the study. Sampling was performed in the period December 2002- March 2003, which is the season with the highest incidence of BRD in the region. Prior to sampling, the nostril was disinfected using alcohol 90%. A sterile swab was introduced in the nasal cavity (dorsal conchae, 15 cm depth) and rotated 360°. After sampling, each swab was inserted into a transportmedium (Venturi Transsystem®, Copan) and cooled (4-7°C) during transport. Bacteriological investigations were set up within 2 hours after sampling.

2.2. Bacteriological identification

The primary isolation of bacteria was done by directly streaking one side of each swab onto Columbia blood agar containing 5% sheep blood (Oxoid) to which 16 µg/ml bacitracin (Alpha Pharma) was added. Likewise the other side of the swab was streaked onto Columbia blood agar containing 5% sheep blood (Oxoid) to which bacitracin (Alpha Pharma) and oxytetracyline (Sigma) at a concentration of 16 µg/ml and 4 µg/ml, respectively, were added. The plates were used within three days after preparation. Bacteria grown aerobically at 37°C were selected by colonial morphology at 24h and 48h post incubation, followed by subculturing for another 24 h on Columbia blood agar containing 5% sheep blood (Oxoid). If no *Pasteurella* or *Mannheimia* species were isolated, the bacteriological investigation was considered negative. Colonies resembling *Pasteurella* or *Mannheimia* isolates were checked for purity, morphology was re-evaluated and bacteria were tentatively designated as *Pasteurella* or *Mannheimia* [3]. The identification of the organisms up to (sub)species level was molecularly confirmed by means of tDNA-PCR as described earlier [5].

2.3. Antimicrobial resistance analysis

Each identified *Pasteurella* or *Mannheimia* organism underwent susceptibility testing through the Kirby Bauer disc diffusion test by means of Neosensitabs® (Rosco) for following antimicrobials: tetracycline, sulphonamide/trimethoprim, ampicillin, amoxicillin+clavulanate, enrofloxacin, and ceftiofur. Testing was done according to NCCLS-guidelines with the exception that Colombia agar supplemented with 5% sheep blood was used as medium. *E. coli* ATCC 25922 was used as reference strain.

Bacteria harbouring consistent tetracycline resistance were investigated for the presence of plasmid DNA as described previously [8]. Shortly, plasmids were alkaline lysed, purified by affinity chromatography on Qiafilter® Midi columns (Qiagen), and seperated by agarose gel electroforese. The respective detected plasmids were directly transformed into *E. coli* TOP10 vectors by the TOPO® transformation procedure (Invitrogen). Tetracycline resistant transformants were selected during overnight incubation by 37°C under aerobic conditions on Luria-Bertani agar (LB) supplemented with 20 mg/ml oxytetracycline (Caesar & Lorenz GmbH). Confirmation of transformation was investigated by reinvestigation of the tetracycline resistance plasmid profiles in the transformant *E. coli* TOP10 organisms by alkaline lysis followed by agar gel electroforese. In case of a succesful transformation experiment, the respective plasmid DNA underwent PCR amplification in order to investigate the presence of *tet*(H). For plasmid analysis, the bacterial collection was extended with 5 tetracycline resistant *Pasteurellaceae* (4 *Mannheimia (M.) haemolytica* and 1 *Pasteurella (P.) multocida* isolates) isolated from calves suffering from BRD.

3. RESULTS

3.1. Bacteriological investigations

Thirty-five % of the bacteriological investigations were negative (n=20). A total of 40 *Pasteurellaceae* were isolated in 34 calves (59.6%) out of 10 herds (76.9%). Identification of the strains resulted mainly in *Pasteurella (P.) multocida* (n=35). Other bacteria were *P. canis* (1), *P. trehalosi* (1), *Mannheimia (M.) varigena* (2) and one untypable *Mannheimia* spp. *Mannheimia haemolytica senso stricto* was not isolated.

3.2. Antimicrobial resistance analysis

Antimicrobial resistance patterns of the strains identified as *Pasteurella multocida* (n = 35) revealed 100% susceptibility for ampicillin, amoxicillin+clavulanate, enrofloxacine, ceftiofur and the combination sulphonamides+trimethoprim. Five *P. multocida* isolates, all originating from the same herd, were resistant to tetracycline and 12 *P. multocida* strains were resistant (n=1) or intermediate susceptible (n=11) for tylosin. The three *Mannheimia* isolates and the *P. trehalosi* strain were only resistant for tylosine. The remaining organisms (n=22) were fully susceptible for the tested antimicrobials. In the tetracycline resistant *P. multocida* isolates from the clinically healthy calves no plasmid DNA was detected.

Of the additional *Pasteurellaceae* series originated out of pneumonic bovine lungs, two *M. haemolytica* and one *P. multocida* were found to contain plasmid DNA. Only from one *P. multocida* strain the tetracycline resistance was successfully transformed into the *E. coli* TOP10 acceptor bacteria. Two clones of the transformant *E. coli* demonstrated a plasmid profile similar to the donorstrain. PCR detection of the *tet*(H) gene in both the donorstrain and the transformant *E. coli* revealed the presence of this gene.

4. DISCUSSION

Since the aforementioned substantial reclassification within the *Pasteurellaceae*, no investigations have been carried out in order to describe the prevalence of these different species in the nasopharynx of calves. However, this knowledge is essential in order to evaluate nasal cultures for diagnostic and epidemiological purposes. Especially since recently a good correlation has been found between the bacteriological identification results of pared nasopharyngeal swabs and broncheoalveolar lavages in the individual pneumonic calf [6]. Based on unpublished preliminary studies, we emphasize the need for disinfection of the nostrils prior to sampling and the use of a selective medium (e.g. Columbia sheep blood agar + 16 µg/ml bacitracine) for a better detection rate of *Pasteurellaceae* in the nasopharynx. Remarkably, none of the detected *Mannheimia* strains was identified as *M. haemolytica*. This is of particular interest because older reports [1] described the presence of *[P.] haemolytica* in the upper respiratory tract of healthy cattle, and *[P.] haemolytica* has recently been reclassified into five new *Mannheimia* species with *M. haemolytica* being the type species [2].

Antimicrobial resistant strains were rare and in accordance with a previous report on pathogenic *Pasteurellaceae* out of the same region [3]. The limited use of antimicrobials and the extensive housing conditions in the investigated animals may have contributed to the overall low prevalence of antimicrobial resistant strains found. In the original series *Pasteurellacea*e, no plasmid DNA was detected in the 5 tetracycline resistant strains. The origin of the decreased tetracycline susceptibility is therefore probably clonally which is indicated by the fact that these strains all were isolated in the same herd. Of the additional *Pasteurellaceae* strains, one strain proved to harbour a plasmid mediated tetracycline resistance gene. The identified *tet*H gene, is the most encountered tetracycline resistance gene within the family of *Pasteurellaceae* [8]. Further investigations are necessary to explore the identity and localisation of the other antibiotic resistance genes involved.

In conclusion, antimicrobial susceptible *P. multocida* strains were the predominant *Pasteurellaceae* present in the nasopharynx of young extensively housed calves.

5. ACKNOWLEDGEMENTS

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DISTRIBUTIONAL CONSIDERATIONS IN SAMPLING INDICATOR BACTERIA FOR ANTIMICROBIAL RESISTANCE

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ABSTRACT
Rectal samples were taken from 20 healthy calves originating from the same herd. At each sampling occasion five *E. coli's* were analyzed for antibiotic susceptibility testing to seven antimicrobials using the Kirby Bauer disk diffusion method. An overall high percentage and a large between animal diversity were found. These data demonstrated that the distribution of antimicrobial resistance on herd level better fits the beta-binomial model than the widely used binomial model. The importance for the interpretation of resistance data and sampling strategies in monitoring programmes is discussed. As a conclusion, antimicrobial resistance trends mentioned in monitoring programmes should be interpreted with caution.

SAMENVATTING
In de mest van 20 gezonde kalveren van 1 bedrijf werden per dier vijf *E. coli*'s geïsoleerd waarop gevoeligheidsbepalingen werden verricht. Het percentage resistentie en de diversiteit tussen de dieren voor een gegeven antibioticum was hoog. Aan de hand van de dataset werd aangetoond dat antibioticumresistentiepatronen op een bedrijf beta-binomiaal verdeeld zijn en niet binomiaal. Het belang hiervan voor de interpretatie van resistentiegegevens en de bemonsteringsprocedure met betrekking tot monitoringsprogramma's wordt toegelicht. Hieruit volgt dat tendensen die opgemerkt worden in monitoring programma's voorzichtig dienen te worden geïnterpreteerd.

1. INTRODCUTION

The emergence of multi-antibiotic resistant bacteria in the commensal flora of men and animals in previously susceptible bacterial populations is considered as a major public health issue, because the resistance genes involved form a potential reservoir that can horizontally be transferred into pathogenic bacteria [1, 2]. To monitor the selection pressure exerted by antimicrobials, several countries examine antimicrobial resistance in commensal bacteria from different animal species. Mainly *Escherichia coli* and enterococci are isolated and screened for their susceptibility against various antimicrobials [6]. Mostly a limited number of isolates per herd are investigated and little attention is paid to variation of resistance patterns within and between animals. This is of importance in order to find a relevant sampling strategy for monitoring antimicrobial resistance. However, in order to evaluate antimicrobial resistance prevalence and trends, distributional considerations may be more appropriate prior to investigating different sampling strategies.
The objective of this preliminary study was to investigate if the widely used binomial assumption holds for antimicrobial resistance patterns or, on the other hand, the necessity to take into account overdispersion.

2. MATERIALS AND METHODS

2.1. Sampling

Out of one herd twenty healthy calves (mixed breeds) were randomly selected, aged one to three months and housed in individual boxes. Fresh faecal samples (approximately 15 g) were taken aseptically by means of disposable plastic gloves and placed in sterile tubes. Gloves were changed between samples. All tubes were cooled and transported to the laboratory for further processing, which was accomplished within 2 hours after collection.

2.2. *Bacteriological investigations*

To one gram of each faecal sample 9 ml PBS was added followed by vortexing for 30 s. Then, 1 µl of each suspension was grafted onto McConckey agar and incubated aerobically for 24 h by 37°C. After 18 hours incubation, from each plate five lactose positive (red) colonies were randomly selected and further identified as *Escherichia coli* using Kligler medium (Oxoid) and a positive indole test (Difco). Subsequently susceptibilities for tetracycline, trimethoprim/sulphonamides, ampicillin, chloramphenicol, ceftiofur, enrofloxacine, and gentamicin were determined using the Kirby Bauer agar diffusion method (Rosco) according to NCCLS-guidelines. The reference strain used was *E. coli* ATCC 25922.

2.3. *Statistical analysis*

To test the goodness of fit of the Binomial distribution (H_0) of antimicrobial resistance patterns against the overdispersed Beta-Binomial distribution (H_a) we used Tarone's *Z* statistic [6]. The null hypothesis was that resistance against a given antibiotic of faecal *E. coli*'s of calves followed a binomial distribution. The alternative hypothesis was the resistance of faecal *E. coli*'s of calves followed the overdispersed beta-binomial distribution.

3. RESULTS

3.1. *Bacteriological investigations*

Intermediate susceptible strains were rare (3%) and for the statistical analysis further classified as resistant. Overall resistance prevalence in *Escherichia coli* (n=100) on individual isolate level and on animal level is presented in table 1. Twenty-five percent of all isolates were fully susceptible to all antimicrobials. Single or double resistance patterns were only found in 18 % of the isolates with resistance to tetracycline being the most prevalent. Multiple antimicrobial resistance was present in 58%, of which almost all contained the resistance type ampicilline-tetracycline-sulphonamides/trimethoprim (n=51), often in combination with enrofloxacine resistance (n=44). Penta- or more resistance was present in 26% of the isolates. The results showed a total of 17 different resistance patterns, and a mean of 1.65 patterns per five colonies per calf with a range of 1-5 different patterns. The amount of resistance per 5 isolates in every individual calf is presented in table 2.

Table 1. Comparison of resistance prevalences (percentages) on animal and on isolate level.

Antibiotic	**TET[a]**	**SXT**	**CHLOR**	**AMPI**	**ENRO**	**CEFT**	**GENTA**
Animal Prev. (n=20)	80	55	35	70	45	10	20
Isolate Prev.(n=100)	73	51	33	64	45	4	6

[a]TET, oxytetracycline; SXT, Sulphonamides+Trimethoprim; CHLOR, chloramphenicol; AMPI, ampicillin; ENRO, enrofloxacine; CEFT, ceftiofur; GENTA, gentamicin.

Table 2. Antimicrobial resistance abundance per 5 isolates in the individual calves (5 isolates/sample).

Calf	**TET[a]**	**SXT**	**CHLOR**	**AMPI**	**ENRO**	**CEFT**	**GENTA**
1	5	5	5	5	0	0	2
2	0	0	0	0	0	0	0
3	2	0	0	0	0	0	0
4	0	0	0	0	0	0	0
5	5	0	0	5	0	0	0
6	4	0	0	1	0	0	0
7	5	5	0	5	5	0	0
8	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0
10	5	0	0	5	0	0	0
11	5	5	0	5	5	0	0
12	5	5	5	5	5	1	0
13	2	2	3	3	0	0	1
14	5	5	0	5	5	0	0
15	5	0	5	0	0	0	2
16	5	5	5	5	5	3	0
17	5	5	5	5	5	0	0
18	5	4	0	5	5	0	0
19	5	5	0	5	5	0	0
20	5	5	5	5	5	0	1

[a]TET, oxytetracycline; SXT, Sulphonamides+Trimethoprim; CHLOR, chloramphenicol; AMPI, ampicillin; ENRO, enrofloxacine; CEFT, ceftiofur; GENTA, gentamicin.

Testing the goodness of fit of the Binomial distribution (H_0) against the overdispersed Beta-Binomial distribution (H_a) resulted in a significant rejection of the H_0 for all antibiotics, with the exception of gentamicin.

4. DISCUSSION

Determination of the prevalence of antimicrobial resistance in bacteria in livestock in monitoring-surveillance programmes is dependent on the ability to accurately and efficiently measure resistance and can be strongly influenced by the sampling strategy [4]. However, a good sampling strategy for indicator bacteria on herd level, e.g. the number of animals and the number of isolates per animal that must be enclosed in these surveys, remains a point of discussion [2]. In the Danish DANMAP programme for instance, one herd is represented by one sample [3]. Comparison of these kind of data are classically done under the simple binomial model which assumes that the probability to obtain a success (a resistant bacteria) is the same for each trial. If this is not so and the probability of success is different between animals or herds, the variance of the estimated parameter will be inflated.

In the study reported here, we compared the prevalence of resistance to individual antimicrobials in order to evaluate the distributional behaviour of the within herd resistance prevalence found. The significant rejection of the null hypothesis with the Tarone's *Z* statistics proves that the data obtained within this experiment are overdispersed (beta binomial), which is the same as saying that the probability of isolating a resistant bacteria is animal dependent and that it can be assumed that these probabilities are, on their turn, beta distributed. Ignoring this inter animal variation (e.g. when demonstrating trends in monitoring programmes), leads to an underestimation of the variance of prevalence estimates and an increase of the type I error (e.g. falsely state that resistance prevalences are significantly different). Therefore, statistical analysis conducted on this kind of data should take into account overdispersion. Several methods like Generalized Mixed Models and Bayesian Monte Carlo Markov Chain models can be applied to tackle this problem though they require a more profound and complicated statistical approach.

In conclusion, by considering a correct distribution of resistance patterns on herd level, the chance on under- and overestimation of resistance prevalences and trends can be minimised. If no correction is established for the overdispersion, trends mentioned in antimicrobial resistance monitoring programmes should be interpreted with caution.

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ANTIBIOTIC SENSITIVITY TESTING

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1. THEORETICAL BACKGROUND

Bacterial resistance to antimicrobial compounds is not to be captured in a single definition. In order to differentiate between sensitivity and resistance, one may discern four types of criteria: a microbiological, genetic, pharmacological and clinical criterion. For the microbiological criterion, the species-specific sensitivity of the bacterium is taken into account: bacterial strains having the same sensitivity level as the type strain are considered sensitive; those strains exhibiting less sensitivity are classified as resistant. With the second criterion, that is the genetic criterion, a bacterial strain is classified as resistant if genes encoding resistance or mutations associated with resistance are present. According to the pharmacological criterion, the sensitivity level of the strain is compared with the anticipated blood or tissue level of the antibiotic: bacteria which are sensitive to concentrations lower than the blood or tissue level are considered sensitive; bacteria not sensitive to this level are resistant. Upon adopting the fourth criterion, a bacterium is classified as sensitive when the caused infections respond well to treatments with normal dose rates of the antibiotic in question. The microbiological, pharmacological and clinical criterion are discussed below.

1.1. Microbiological criterion

Every bacterial species displays a fairly typical sensitivity pattern to the various antibiotics. This is considered as the normal sensitivity or resistance of the species involved. Nevertheless, strains which, although belonging to the same species, display different sensitivity levels are frequently encountered. This is due to the fact that, by means of mutations or transfer of resistance genes, these strains have become less sensitive than what is to be expected for the species in question. This type of resistance is called acquired resistance, as opposed to the natural or intrinsic resistance as described above. Sensitivity levels are fairly easy to determine in vitro by quantitative dilution tests. These tests allow to determine the Minimum Inhibitory Concentration (MIC) of an antibiotic for a certain bacterial strain (see below).

When using the microbiological criterion, most strains carrying transferable resistance genes associated with presently known transfer mechanisms will be clearly distinguished from susceptible strains. Indeed, a bimodal distribution of sensitivity levels often is noted upon determining the sensitivity of a series of strains belonging to one and the same species. Even threemodal distributions of sensitivity levels may appear when more than one type of resistance genes are active within one bacterial species. However, it is recognized that strains with non-transferable non-inherent antimicrobial resistance caused by chromosomal mutations are not always readily distinguished from susceptible strains by studying the distribution of MICs.

The above described aberrant sensitivity levels do not necessarily have a practical (clinical) implication but they are a good criterion for detection of acquired resistance.

Combining the sensitivity level of the bacterial strain used to evaluate the clinical effect of an antibiotic, with that of strains which do or do not respond to treatment, these sensitivity levels can be compared to the clinical effects. In

this way, one may conclude that a certain bacterial species exhibits an intrinsic sensitivity or resistance to a certain antibiotic and other bacteria with a similar sensitivity pattern consequently may also be considered sensitive or resistant. One needs to keep in mind, however, that this is not a general rule in that a lot of infections caused by different bacterial species with a similar sensitivity level, do not respond uniformly negative or positive to the same type of antibiotic treatment. To illustrate this, infections caused by *Arcanobacterium pyogenes* or *Bordetella bronchiseptica* react to a lesser extent to antibiotics compared to infections by other bacteria exhibiting the same in vitro sensitivity. Likewise, the bacteriological in vivo response to treatments of different bacteria with the same antibiotic sensitivity, may differ markedly. For example, some antibiotics with an excellent in vitro activity against *Salmonella* hardly influence the colonisation of the gut whereas others exhibit a strong repressive (seldomly eliminative) effect.

1.2. Pharmacological criterion

According to this criterion, a bacterium is considered sensitive when the MIC value is lower than the blood level or the antibiotic concentration at the site of infection, achieved following normal dose rating. Other pharmacodynamic properties used to define this criterion include concentration-dependent (aminoglycosides, fluoroquinolones) or time-dependent killing (beta-lactams, macrolides, lincosdamides), first-exposure effect, post-antibiotic effect, sub-MIC post-antibiotic effect and post-antibiotic leukocyte enhancement effect. Concentration dependent killing means that as the concentration of the antibiotic increases above the MIC of the pathogen, there is increased killing of the pathogen. In this case, a strain is considered sensitive if peak serum or tissue concentrations of the antibiotic achieved following normal dose rating are at least 8-10 times MIC. This contrasts with time-dependent killing. In the latter case, serum or tissue concentrations of the antibiotic should be continuously above MIC.

For the systemic infections, the clinical results correlate well with the sensitivity level-blood level ratio. In case a bacterial strain is sensitive to concentrations below the blood level, one may reasonably expect the bacerium to be inhibited by the antibiotic under in vivo conditions, except when the bacterium finds itself in a state in which it is hardly influenced, e.g. when it is located intracellularly. Besides the pharmacological criterion, bacteriological and clinical findings however need the be included to be able to generate a full picture.

The pharmacological criterion has a number of important limitations. Firstly, it does not really stand for local infections which are of considerable importance in veterinary medicine. The antibiotic concentrations which are achieved in the gut following oral administration, in the udder (mastitis) following intramammary infusions, in the uterus (uterus infections) after intra-uterine infusions and in the skin following topical treatments, do not always have a direct correlation with blood levels. Likewise, the blood levels are not directly relevant in case one wants to treat urinary bladder infections, where the urine concentrations can be markedly higher or lower than the blood levels. The same goes for, although to a lesser extent, the concentrations in the airways and alveolae of the udder following systemic administration. In fact, different interpretation schemes should be set up for all these types of infections and antibiotic applications. These should be based upon the ratio between sensitivity level and local concentration. Except for the urinary tract infections, no such interpretation schemes are currently available. The local concentrations are too variable or not sufficiently known for actual use.

1.3. Clinical criterion

The clinical criterion is very important in veterinary practice. This criterion however is not always manageable as it is difficult to be measured and dependant on a number of additional factors.

It is rather difficult to actually measure the clinical outcome of antibiotic treatment since relatively large homogenous groups of patients are needed. In most cases, the latter can only be accomplished through experimental infection studies, in which the typical clinical signs or lesions caused by a specific bacterium are reproduced and consequently treated according to specific schemes. This however is not always possible, as some infections are difficult to reproduce or as experiments using certain animal species are expensive or ethically not justifiable.

In daily practice, the clinical outcome of antibiotic treatments depends on various factors which are diffcult to uniformize. Next to the sensitivity of the pathogen to the administered antibiotic, the following factors frequently

play a role: the presence or absence of primary or secondary viruses and other bacteria, the immune status of the animals, the time of treatment onset, ... These factors are often not known in practice. Therefore, clinical impressions and data resulting from practice should be interpreted with care.

2. SUSCEPTIBILITY TESTING METHODS

Two types of tests can be adopted: the dilution techniques and the diffusion techniques.

2.1. Quantitative dilution tests

The cultures to be tested are inoculated onto agar plates containing different concentrations of the antibiotic (agarplate dilution method), or in microtiter plates containing broth with different antibiotic concentrations (broth or microtiter dilution method). The MIC value is defined as the lowest concentration of antibiotic with no visible bacterial growth. The MIC value is expressed as ug/ml, mg/kg or ppm.

Studies involving the determination of MIC values only take into account the bacteriostatic activity. Using the broth dilution test, one may deduct the Minimal Bactericidal Concentration (MBC), by transferring those cultures exhibiting no growth after incubation onto broth devoid of antibiotic.

Antibiotics may also be effective below the MIC value. There are indications that bacteria are influenced in a negative manner at concentrations below the MIC value, for instance in their adhesive ability. The lowest concentration of the antibiotic which exerts antibacterial activity, is then assigned as the Minimal Antibacterial Concentration (MAC). These values are however difficult to determine in vitro and no standardised protocols are hitherto available.

2.2. Diffusion tests

- Qualitative diffusion tests

The disk diffusion test consists of preparing a uniform lawn of test bacterium on an agar plate and putting disks which are impregnated with an antibiotic, on the agar surface before incubation. Depending on the sensitivity of the strain to the antibiotic in question, the size of the inhibition zone around the disk following incubation will vary. The size of the inhibition zone can be correlated with the MIC value of the strain to the antibiotic in question. Strains with a low MIC will give zone sizes which are larger than those of strains with high MIC values. When a new antibiotic is launched, both types of strains will be included in the quantitative and qualitative tests, and the resulting data correlated. Following, a regression line may be drawn by means of which the MIC value corresponding to a specific size of inhibition zone can be determined. In view of the fact that this approach is indirect and less accurate, guidelines have been developed which indicate in a qualitative manner that a test result of e.g. 14 or 20 mm signifies resistance whereas an inhibition zone of e.g. 25 mm correlates with sensitivity. These values may fall within a transitional intermediate zone, resulting in the classification of the bacterium in question as being intermediately sensitive. These interpretation criteria are available and differ in between antibiotics and bacterial species.

- Quantitative diffusion tests

The concentration gradient strip (E test®) generates quantitative results. The test relies on the diffusion of a continuous concentration gradient of an antimicrobial agent from a plastic strip into an agar medium seeded with a pure culture of the test bacterium. From top to bottom, the plastic strip has a defined continuous concentration gradient of a stabilized dried drug on one side and a MIC interprative scale on the other. After incubation, the MIC is determined by reading the concentration on the strip where the zone of inhibition intersects the strip. Studies carried out in our laboratory demonstrated that results obtained with this test are very similar to results obtained in dilution tests.

3. PRACTICAL IMPLICATIONS

Following the identification of the bacterium which is to be considered of clinical importance, an antibiotic sensitivity test will mostly be performed. Whether or not the latter is done, may depend on the request of the clinician sending the sample, but primarily depends on the result of the bacteriological examination. The decision in the laboratory is made on the basis of a number of factors. The questions to be asked are the following. Is the isolated agent considered to be of relevance for the clinical case? Is there a lot of acquired resistance among the isolated bacterial species in the animal species in question? In the laboratory, one needs to be well acquainted with the various infectious diseases in animals in order to make these decisions. Reporting the sensitivity results of a bacterium which hardly plays a role or does not have any significance in the disease problem, is a frequently occurring mistake.

It is advisable to start from a pure culture from the bacteria under investigation. In case of contaminants being present, this may lead to very misleading results.

Some technical errors may also occur. The density of the bacterial inoculum needs to be standardised. Too thick bacterial suspensions may result in false resistance results whereas too thin suspensions may lead to false data of the bacterium being sensitive.

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PRESENCE OF MACROLIDE AND LINCOSAMIDE RESISTANCE AMONG ENTEROCOCCI AND STREPTOCOCCI ISOLATED FROM BROILERS

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Macrolide and lincosamide (ML) resistant enterococci and streptococci from cloacal swabs from 26 broilers originating from 13 different farms in Belgium were isolated on selective media containing erythromycin or lincomycin. Their ML resistance phenotypes and genotypes were determined by disk diffusion tests and PCR, amplifying the *erm*(B) gene, respectively.
A total number of 110 strains could be identified as known enterococcal or streptococcal species. A variety of enterococcal strains were obtained, with *Enterococcus faecium* and *E. gallinarum* as the predominant species, followed by *E. casseliflavus*, *E. faecalis*, *E. avium*, and *E. durans*. *Streptococcus alactolyticus* was the only streptococcal species present.
The majority of the strains (88%) were phenotypically resistant to erythromycin, tylosin, lincomycin and clindamycin, and 93% of these strains carried the *erm*(B) gene. Furthermore, from each of the 26 broilers, at least one strain carrying the *erm*(B) gene was isolated. Resistance against the lincosamides only was demonstrated in 10 strains (9%).
In conclusion, the *erm*(B) gene was found to be widespread among enterococci and streptococci from poultry.

Macrolide en lincosamide (ML) resistente enterokokken en streptokokken werden geïsoleerd uit cloacale swabs van 26 braadkippen afkomstig van 13 verschillende bedrijven in België. Hierbij werd gebruik gemaakt van selectieve media die erythromycine of lincomycine bevatten. Het ML resistentiefenotype en –genotype van deze stammen werden respectievelijk bepaald met disk diffusie testen en een PCR die het *erm*(B) gen detecteert.
In totaal konden 110 stammen geïdentificeerd worden als gekende enterokokken en streptokokken species. *Enterococcus faecium en E. gallinarum* waren de meest voorkomende species, gevolgd door *E. casseliflavus, E. faecalis, E. avium* en *E. durans. Streptococcus alactolyticus* was de enige streptokokken soort die werd teruggevonden.
De meerderheid van de stammen (88%) was resistent tegenover erythromycine, tylosine, lincomycine en clindamycine, en 93% van deze stammen was drager van het *erm*(B) gen. Verder werd uit elk van de 26 braadkippen tenminste één stam geïsoleerd die drager was van het *erm*(B) gen. Resistentie tegenover enkel de lincosamiden werd aangetoond in 10 stammen (9%).
Het *erm*(B) gen is bijgevolg wijd verspreid bij enterokokken en streptokokken afkomstig van pluimvee.

1. INTRODUCTION

Macrolide, lincosamide and streptogramin (MLS) antibiotics are important in veterinary and human medicine. Although there appears to be a high level of MLS resistance in bacteria originating from chickens (3,10), few studies were done to actually determine the mechanisms of resistance. Moreover, nothing is known about the spread of the resistance genes among the floral bacteria of poultry. A resistant flora may constitute a reservoir for resistance genes, which might be transferred to pathogenic bacteria.

The purpose of this study was to determine the occurrence of acquired resistance against MLS antibiotics in enterococci and streptococci from broilers and to identify the genes encoding this resistance.

2. MATERIAL AND METHODS

2.1. Isolation and identification of bacterial strains

Cloacal swabs were collected from 26 healthy, three to five week old broilers originating from thirteen different farms. The swabs were inoculated on Columbia agar supplemented with 5% sheep blood, colistin and aztreonam (CA) to inhibit the Gram-negative flora, and Slanetz and Bartley (SL) agar. Both agars were supplemented with 1 µg/ml erythromycin or 10 µg/ml lincomycin for the selective isolation of streptococci and enterococci resistant to MLS antibiotics. Four plates were inoculated for each swab and these plates were consequently incubated at 37°C in a 5% CO_2-enriched environment for 24h (CA-plates) and 48h (SL-plates). All colony types were purified and identified using "tRNA intergenic length polymorphism PCR" (tDNA-PCR) (1,2).

2.2. Resistance phenotype

The collected bacterial strains were inoculated on Columbia agar supplemented with 5% sheep blood (Columbia blood agar) and incubated overnight at 37°C in a 5% CO_2 atmosphere. Bacterial suspensions were prepared in 0.9% NaCl to a 0.5 McFarland standard and were inoculated on Columbia blood agar for streptococci and *Enterococcus cecorum* and on ISO agar (Oxoid) for enterococci. Tablets containing the following antimicrobial agents were used: erythromycin (78 µg), tylosin (150 µg), lincomycin (19 µg) and clindamycin (25 µg) (Rosco Diagnostics, Taastrup, Denmark). After overnight incubation at 37°C in a 5% atmosphere, the zone diameters were measured.

2.3. Detection of erm*(B)*

Part of the *erm*(B) gene (640bp) was amplified with a PCR reaction using primers 5'-GAAAAGA/GTACTCAACCAAATA-3' and 5'-AGTAACGGTACTTAAATTGTTTAC-3' (8,13).

3. RESULTS

3.1. Isolation and identification

Out of the 26 cloacal swabs, 279 resistant strains were isolated. One hundred and forty-one of the strains belonged to known enterococcal or streptococcal species. From each swab, one representative per species and per antibiotic was retained to determine the resistance phenotype and genotype. In this way, 110 strains were further analysed: 13 *Streptococcus alactolyticus*, 11 *Enterococcus cecorum*, 18 *E. gallinarum*, 20 *E. faecium*, 11 *E. faecalis*, 11 *E. avium*, 5 *E. hirae*, 8 *E. durans* and 13 *E. casseliflavus* strains.

3.2. Resistance phenotype and genotype

Ninety-seven out of 110 enterococci and streptococci (88%) were resistant to all the antimicrobials tested. Ten strains (9%) showed resistance to lincosamides only. One strain (*E. gallinarum*) was resistant to erythromycin and both lincosamides and two strains (*S. alactolyticus* and *E. cecorum*) were resistant to lincomycin only.

Ninety out of the 97 strains resistant to all of the four tested antimicrobials carried the *erm*(B) gene. One *E. hirae*, exhibiting phenotypical resistance against lincomycin and clindamycin only, contained the *erm*(B) gene. The *erm(*B) gene was detected in 83% of the isolates studied. From each of the 26 broilers, at least one strain carrying the *erm*(B) gene was isolated.

4. DISCUSSION

In this study, eight enterococcal and one streptococcal species were found in the intestinal flora of three to five week old broilers with *E. faecium* being the predominant enterococcal species. This is in agreement with results of Devriese et al (5). In contrast to our results, *E. avium*, *E. gallinarum* and *E. casseliflavus* were hardly or not at all detected in the study conducted by Devriese et al. (5).

According to other studies (7,8,12), resistance against macrolide and lincosamide antibiotics (ML phenotype) was the predominant resistance phenotype among the studied isolates. Furthermore, this study shows that the *erm*(B) gene coding for resistance against macrolide, lincosamide and streptogramin antibiotics is widespread among the intestinal flora of broilers.

Seven enterococcal strains showed the ML phenotype and did not test positive for the *erm*(B) gene. Five out of these 7 strains originated from the same farm, indicating that *erm* genes different from *erm*(B) or other genes coding for resistance to the macrolides and lincosamides were present on this particular farm (11).

One *E. hirae* strain showed the L phenotype although it harboured the *erm*(B) gene. This apparent disagreement between resistance phenotype and genotype may be explained in several ways. The gene expression indeed depends on several factors like the structure of the promoter and the mRNA and the sequence associated with the AUG start signal (6). These factors determine the gene expression and might be responsible for the failure of expression in the disk susceptibility tests. Martineau et al. (9) described a 98.5% correlation between the resistance phenotype and genotype for erythromycin. The species involved were *Staphylococcus aureus* and *Staphylococcus epidermidis*. For certain other antibiotics there was a 100% correlation.

In conclusion, the *erm*(B) gene was found to be widespread among enterococci and streptococci from poultry.

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GENETICALLY DIVERGENT *ENTEROCOCCUS FAECIUM* ISOLATES CONTAINING IDENTICAL *ERM*(B) GENES

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This study was conducted in order to obtain better insights into the possible exchange of antibiotic resistance between man and pigs. Twenty macrolide- and lincosamide-resistant, *erm*(B)-positive *Enterococcus faecium* strains isolated from pigs (7 strains), pork carcasses (4 strains) and humans (9 strains) were typed using multilocus sequence typing (MLST). The coding sequence of the *erm*(B) gene was determined for all isolates and these results were combined with the MLST data. Molecular analysis revealed 17 different sequence types and 7 different *erm*(B*)* sequences. Identical *erm*(B) gene sequences were detected in porcine and human isolates with very different sequence types. One pig strain possessed a sequence type identical to that of human hospital isolates but had a different *erm*(B) type.
Our findings indicate that horizontal transfer of *erm*(B) genes between porcine and human strains occurs. Furthermore, there are indications that exchange of strains occasionally occurs between pigs and humans.

Deze studie werd uitgevoerd om een beter inzicht te krijgen in de mogelijke overdracht van antibiotica resistentie van dier naar mens. Twintig macrolide en lincosamide resistente, *erm*(B)-positieve *Enterococcus faecium* stammen die geïsoleerd werden bij varkens (7 stammen), varkenskarkassen (4 stammen) en mensen (9 stammen), werden getypeerd met multilocus sequence typing (MLST). De coderende sequentie van het *erm*(B) gen werd bepaald voor alle isolaten en deze resultaten werden gecombineerd met de MLST data.
Er werden 17 verschillende sequentietypes en 7 verschillende *erm*(B) sequenties teruggevonden bij de 20 onderzochte isolaten. Identieke *erm*(B) gensequenties werden gedetecteerd bij varkens- en humane isolaten met verschillende sequentietypes. Slechts één varkensisolaat had een sequentietype dat identiek was aan dat van een humaan isolaat maar had een verschillend *erm*(B) type.
Onze resultaten laten vermoeden dat de horizontale transfer van *erm*(B) genen tussen porciene en humane stammen relatief frequent voorkomt. Daarnaast zijn er indicaties dat er occasioneel ook uitwisseling van stammen tussen varkens en mensen plaatsgrijpt.

1. INTRODUCTION

Enterococci are normal intestinal bacteria of humans and animals (19). Although no pathology is seen in pigs, these bacteria are responsible for a wide variety of infections in humans (endocarditis; urinary tract infections; intra-abdominal, pelvic and wound infections; bacteremias) (18). Selective antibiotic pressure and the capacity of enterococci to acquire antibiotic resistance genes has resulted in antibiotic-resistant strains and limitations to treatment. The number of nosocomial infections caused by multidrug resistant *Enterococcus faecium* has increased in the past decade (4,5,9,12).

Macrolide, lincosamide and streptogramin (MLS) antibiotics are important as alternative therapy for treatment of infections in man (20,21). Acquired resistance against these antibiotics has frequently been described in enterococci originating from humans as well as from animals (6,17,21). The *erm*(B) gene conferring cross resistance against MLS antibiotics is very common among pig-derived enterococci (1,13,17,22).

Two types of antimicrobial drug resistance transfer from animal to humans are of importance. The direct way of resistance transfer occurs when resistant zoonotic bacteria infect humans. In the indirect way, resistance genes are transferred horizontally from animal to human strains. Pork carcasses can be contaminated by faecal or

tonsillar pig flora during the slaughter process. In this way, resistances present in these bacteria may spread through the food chain and can be considered as an indirect zoonotic problem (2,3,23).
This study was conducted in order to obtain better insights into the possible exchange of antibiotic resistance between man and pigs. For this purpose macrolide and lincosamide resistant, *erm*(B)-positive *E. faecium* strains isolated from pigs and humans were typed using multilocus sequence typing (MLST) and the coding sequence of their *erm*(B) gene was determined.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

A total of 20 *erm*(B)-positive *E. faecium* strains isolated from humans (9 strains), pigs (7 strains) and pork carcasses (4 strains) were used in this study. The human isolates were derived from hospitalized and non-hospitalized patients. The porcine isolates originated from different farms and slaughterhouses. The isolates were identified as *E. faecium* using a PCR described by Kariyama et al. (14). The detection of the *erm*(B) gene was performed with a PCR described by Martel et al. (16).

2.2. MLST

MLST was performed as described by Homan et al. (10,11). Internal fragments of 7 housekeeping genes (*adk, atpA, ddl, gyd, gdh, purK* and *pstS*) were amplified by PCR with the following sets of primers: *adk*1: 5'-TAT GAA CCT CAT TTT AAT GGG-3' and *adk*2: 5'-GTT GAC TGC CAA ACG ATT TT-3', *atpA*1: 5'-CGG TTC ATA CGG AAT GGC ACA-3' and *atpA*2: 5'-AAG TTC ACG ATA AGC CAC GG-3', *ddl*1: 5'-GAG ACA TTG AAT ATG CCT TAT G-3' and *ddl*2: 5'- AAA AAG AAA TCG CAC CG-3', *gdh*1: 5'-GGC GCA CTA AAA GAT ATG GT-3' and *gdh*2: 5'-CCA AGA TTG GGC AAC TTC GTC CCA-3', *gyd*1: 5'-CAA ACT GCT TAG CTC CAA TGG C-3' and *gyd*2: 5'-CAT TTC GTT GTC ATA CCA AGC-3', *purK*1: 5'-GCA GAT TGG CAC ATT GAA AGT-3' and *purK*2: 5'-TAC ATA AAT CCC GCC TGT TTY-3', *pstS*1: 5'-TTG AGC CAA GTC GAA GCT GGA G-3' and *pstS*2: 5'-CGT GAT CAC GTT CTA CTT CC-3'. All these genes were amplified using the same PCR running conditions which consisted of an initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 5 min. Each 50 µl PCR mixture contained buffers and *Taq* polymerase from SphaeroQ (Leiden, The Netherlands).
PCR products were purified with a PCR purification kit (Qiagen) and sequenced with PCR forward and reverse primers, an ABI PRISM Big Dye Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems, Foster City, Calif.) and an ABI 3700 DNA sequencer (Perkin Elmer). Different sequences of a given locus were given allele numbers, and each unique combination of alleles (the allelic profile) was assigned a sequence type (ST).
Computer analysis of the MLST data was performed with Bionumerics software (Applied Maths, Kortrijk, Belgium) and all results were compared with the database of the Research Laboratory for Infectious Diseases (RIVM). This database contains MLST data from epidemiologically unlinked *E. faecium* isolates from humans and livestock in The Netherlands, Australia, United Kingdom, United States and France, and is available on the Internet (http://efaecium.mlst.net).

2.3. Sequencing of the erm*(B) gene*

The coding sequence of the *erm*(B) gene was amplified using primers ORR1: 5'-ATGAACAAAAATATAAAATATT-3' and ORR2: 5'-TTATTTCCTCCCGTTAAA-3' and the following conditions: initial denaturation at 94°C; 35 cycles at 94°C for 1 min, 41°C for 1 min, 72°C for 1 min; and final extension at 72°C for 5 min. Reactions were performed in 50 µl volumes using *Taq* PCR Master Mix Kit (Qiagen) and the amplification products were purified with QIAquick PCR Purification Kit (Qiagen). The *erm*(B) genes were sequenced using the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer) and the following primers E1: 5'GAAAAGA/GTACTCAACCAAATA3'; E2: 5'AGTAACGG TACTTAAATTGTTTAC3'; E3: 5'CCATACCACAGATGTTCCAG3'; E4: 5'AGATAGATGTCA GACGCACG3' on an ABI Prism 3100 genetic analyzer. The electropherograms were exported and converted to Genebase (Applied Maths) using Abiconv (Applied Maths).

3. RESULTS

3.1. MLST

Application of MLST revealed 17 different STs among the 20 isolates studied which can be grouped in 5 genogroups. The results are summarized in Table 1. In the RIVM database (10) genogroup A mainly contains isolates from non-hospitalized humans and pigs. Seven isolates of which 2 are human isolates, 3 are pig isolates and 2 isolates from pork carcasses belong to this cluster. Isolates related to hospital outbreaks are clustered in genogroup C1. This complex contains 4 isolates from hospitalized patients and one isolate derived from a pig.

Five faecal isolates from humans and pigs belong to genogroup C, a cluster that contains isolates from hospitalized patients and a variety of sources, such as dogs and cats. One pig isolate clustered in genogroup B and one isolate recovered from a pig carcass clustered in genogroup D. Genogroup B and D predominantly contain isolated from poultry and veal calves respectively. Finally one isolate from a pig carcass belonged to a group of highly deviant STs not related to the major genogroups A, B, C, C1, and D.

Table 1

strain	**origin**	**source**	***adk***	***atpA***	***ddl***	***gyd***	***gdh***	***purK***	***pstS***	**ST**	**Geno-group**	***erm*(B)-type**
HE2	human	urine	1	7	1	5	1	1	1	18	C1	3
HE3	human	faeces	5	4	2	1	1	9	1	6	A	2
HE10	human	abdominal fluid	1	1	1	1	1	1	1	17	C1	6
HE15	human	faeces	1	2	3	1	1	26	1	136	C	1
HE22	human	wound fluid	1	7	1	1	1	1	11	19	C1	7
HE28	human	faeces	5	5	3	1	1	9	1	137	A	2
HE29	human	faeces	1	2	3	1	1	26	1	136	C	1
HE35	human	faeces	1	9	3	1	1	6	1	25	C	3
HE40	human	?	1	1	1	1	1	1	1	17	C1	6
AME136	pig	faeces	5	3	5	1	1	3	14	138	A	1
AME129	pig	faeces	5	5	2	1	1	9	1	5	A	4
AME81&	pig	faeces	1	7	1	5	1	1	1	18	C1	6
AME4	pig	faeces	1	15	1	1	1	2	1	121	C	3
AME61	pig	faeces	1	29	2	1	12	6	6	143	B	4
AME31	pig	faeces	5	3	5	1	1	3	1	139	A	2
AME46	pig	faeces	1	4	5	1	1	3	1	29	C	1
VZ200	pig	carcass	5	3	2	1	1	9	1	140	A	2
SH48	pig	carcass	5	28	2	2	1	12	34	144	NA*	4
LO122'	pig	carcass	5	5	5	1	1	9	1	141	A	5
WV235	pig	carcass	8	5	4	1	12	6	1	142	D	4

*NA: Not Assigned

3.2. Sequencing of the erm*(B) gene*

Sequence alignment and UPGMA-clustering of the 20 sequenced *erm*(B) genes resulted in 7 different sequences, *erm*(B)-types 1 to 7, which are more than 99% identical (Figure 1). *Erm*(B)-types 1, 2, 3 and 6 were found in human isolates as well as in isolates originating from pigs. *Erm*(B)-types 4 and 5 were only detected in porcine strains, whereas *erm*(B)-type 7 belonged to a human isolate. *Erm*(B)-types 1 and 2 are coding for an identical amino acid sequence.

4. DISCUSSION

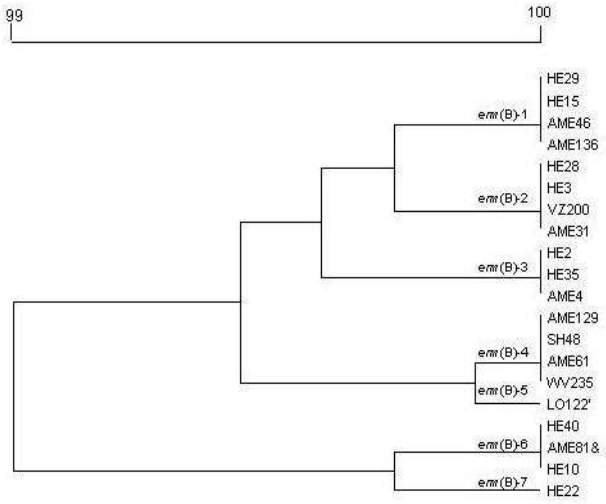
Two main routes of exchange of antibiotic resistance genes between humans and animals are considered. Clonal spread occurs when zoonotic bacteria infect humans (8,15,24). Since porcine and human strains with identical STs and *erm*(B)-sequences were not detected in the present studies, clonal spread from *E. faecium* strains from pigs to humans or from humans to pigs was not demonstrated.

On the other hand, resistance genes can be transferred horizontally from animal to human strains (7,25). Identical *erm*(B) gene sequences were detected 5 separate times in porcine and human isolates with unrelated STs. For example, the human isolate HE15 and the porcine isolate AME46 belong to the genetically unrelated ST 136 and ST 29 differing in three of the seven loci and both carry *erm*(B)-type 1. The presence of identical *erm*(B) gene sequence in these two different STs is a strong indication for horizontal transfer between porcine and human strains.

The porcine isolate AME81& has ST 18 and is the only animal isolate that was genetically indistinguishable from a human clinical isolate. Furthermore, this isolate also clustered among isolates related to hospital outbreaks in genogroup C1. This may indicate spread of a human strain to pigs.

In conclusion, our findings strongly indicate that transfer of *erm*(B) genes between porcine and human strains occurs. Furthermore, there are indications that exchange of strains occasionally occurs between pigs and humans. More isolates will be analyzed to clarify *erm*(B) gene transfer from pigs to humans.

Figure 1: Dendrogram showing 7 different *erm*(B) gene sequences among the 20 studied isolates (UPGMA clustering)



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STREPTOCOCCUS BOVIS AS INDICATOR BACTERIUM FOR CATTLE

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The purpose of the study was to demonstrate *Streptococcus bovis* to be a more appropriate Gram-positive indicator bacterium compared to *Enterococcus faecalis* and *Enterococcus faecium*. Faecal samples were taken from 927 animals in three different age categories located on ten dairy farms and ten beef cattle farms. *Streptococcus bovis* was isolated on all twenty farms, whereas *E. faecalis* and *E. faecium* were isolated on ten and three of the twenty farms, respectively. In total, *S. bovis* was detected in 30.6% of all samples. Susceptibility testing was performed on 102 *S. bovis* isolates from ten dairy farms by means of a disk agar diffusion test. Only 21 isolates (20.6%) were susceptible to all antimicrobials tested. It was concluded that *S. bovis* is suitable as a Gram-positive indicator bacterium for cattle because of the high prevalence and because of their ability to harbour resistance determinants.

Het doel van deze studie was aan te tonen dat *Streptococcus bovis* meer geschikt is als indicator bacterie dan *Enterococcus faecalis* en *Enterococcus faecium*. Daartoe werden op tien melkveebedrijven en tien zoogkoeienbedrijven meststalen genomen van 927 dieren verdeeld over drie leeftijdscategorieën. *Streptococcus bovis* werd op alle twintig bedrijven geïsoleerd (100%). *Enterococcus faecalis* werd slechts op tien van de twintig bedrijven geïsoleerd en *E. faecium* op drie van de twintig bedrijven. In totaal werd in 30.6% van de stalen *S. bovis* gedetecteerd. Op 102 isolaten afkomstig van tien melkveebedrijven werden gevoeligheidsbepalingen verricht door middel van een disk agar diffusie test. Slechts 21 isolaten (20.6%) waren gevoelig voor alle geteste antibiotica.
Uit deze resultaten kunnen we besluiten dat *S. bovis* zich beter leent tot het gebruik als indicatorbacterie dan *E. faecalis* en *E. faecium.* Dit blijkt enerzijds uit de prevalentie, anderzijds uit de waargenomen resistentie.

1. INTRODUCTION

National antibiotic resistance monitoring programmes consist of consumption data of antimicrobials and susceptibility data of pathogenic, zoonotic, and indicator bacteria [2, 12]. Indicator bacteria are isolated in the faeces of healthy animals in order to estimate the reservoir of potential transferable resistance genes in the bacterial population related to food animals. *Escherichia coli* and enterococci (*Enterococcus) faecalis* and *E. faecium)* have been chosen as Gram-negative and Gram-positive indicator bacteria, respectively, because of their high prevalence in the faeces of healthy livestock and because of their ability to harbour several resistance determinants [5]. In addition, indicator bacteria have a zoonotic aspect.
In cattle however, enterococci are only frequently isolated in pre-ruminating calves [8], an age group which is relatively unrelated to slaughtered cattle. Enterococci were rare in older cows, in contrast with *Streptococcus bovis* that predominated, especially in dairy cows [8]. According to some investigators, streptococci are little receptive to antimicrobial resistance genes [3]. However, in human medicine there is a recent unexpected increasing resistance to penicillin in *Streptococcus pneumoniae* [9]. In veterinary medicine, a high prevalence of resistance was observed in *Streptococcus suis* and ß-haemolytic streptococci for penicillin (G) [4], and in *S. bovis* for macrolides [11] Concerning the zoonotic aspect of *S. bovis*, although bovine aspecific serotypes were involved, the organism is recognized as a cause of endocarditis in elderly people and a uncommon cause of septicaemia and meningitis in newborn infants [11].

The purpose of this study was to demonstrate that *S. bovis* is more suitable as a Gram-positive indicator bacterium compared to *E. faecalis* and *E. faecium.*

2. MATERIALS AND METHODS

2.1 Sample collection

From 927 animals on ten dairy farms and ten beef farms faecal samples were aseptically obtained aseptically. Samples were taken from 50% of all the animals present, divided over three different age categories: younger than six months, between six and twelve months and older than twelve months. On one beef farm only 20% of the animals were sampled. The samples were transported in cool conditions to the laboratory and investigated within four hours after sampling.

2.2 Bacterial identification

From each faecal sample, one loop of undiluted material was directly inoculated on Slanetz and Bartley agar (Oxoid) that had been cooked until the medium turned pink (approximately three min) [6, 7] and incubated for 48h at 37°C in 5% CO_2 . Preliminary identification of *S. bovis* colonies was based upon morphology (smooth, pink colonies without a red centre). Further identification consisted of a negative culture of the organisms on Brain Heart Infusion (Oxoid) containing 6.5% NaCl and a positive amylase test. Amylase activity was demonstrated by spot-inoculation on Colombia agar (Oxoid) supplemented with 1% starch. Clear zones surrounding growth spots after the plates were flooded with Gram's iodine solution were evidence for amylase activity [8].

2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the isolates was performed by a disk agar diffusion test according to the guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS) [1]. The medium used was Mueller-Hinton II agar supplemented with 5% sheep blood (Oxoid). Following antibiotics were tested: benzylpenicillin, tetracycline, erythromycin, lincomycin, neomycin, gentamicin, enrofloxacin and oxacillin (Rosco). The agar plates with oxacillin neo-sensitabs were incubated at 30°C. *Enterococcus faecalis* ATCC 29212 was used as a reference strain.

Measurement of the inhibition zones was done semi-quantitatively (mm) and registered using a standardised automatic reading system (SIR-SCAN 2000®). Interpretation of these results as sensitive, intermediate or resistant was done as stipulated in the neosensitabs users guide according to NCCLS guidelines (available at www.rosco.dk).

3. RESULTS

Streptococcus bovis was isolated on all twenty farms. *Enterococcus faecalis* was isolated on ten of the twenty farms and *E. faecium* only on three of the twenty farms. On the dairy farms, 117 of 453 samples (25.8%) were positive for *S. bovis.* On the beef farms, 167 of 474 samples (35.2%) were positive for *S. bovis*. In total, *S. bovis* was isolated from 30.6% of all samples. The detection rates of *S. bovis* in relation to the three investigated age categories are presented in Table 1.

Table 1: Prevalence of *Streptococcus bovis*

	< 6 months	**6-12 months**	**> 12 months**	**Total**
Dairy farms	17/51 (33.3%)	10/64 (15.6%)	90/338 (26.6%)	**117/453 (25.8%)**
Beef cattle farms	29/76 (38.2%)	17/94 (18.1%)	121/304 (39.8)	**167/474 (35.2%)**
Total	**46/127 (36.2)**	**27/158 (17.1%)**	**211 (32.9%)**	**284/927 (30.6%)**

Susceptibility testing was performed on 102 *S. bovis* isolates from ten dairy farms. Only 21 isolates (20.6%) were susceptible to all antimicrobials tested, 15 (14.7%) were resistant to at least one antimicrobial and 66 isolates (64.7%) were intermediate resistant to at least one antimicrobial. Table 2 gives an overview of the percentages of sensitive *S. bovis* isolates in the different age categories for different antibiotics. All of the investigated isolates were sensitive to penicillin and oxacillin. However, of the 102 isolates tested 37 (62.7%) were resistant or intermediate resistant to neomycin and 42 (41.2%) to enrofloxacin. The rate of tetracycline resistance was very low (1%). In calves, 20% of the isolates were resistant or intermediate resistant to erythromycin and lincomycin.

Table 2. Percentage of sensitive *S. bovis* isolates in the different age categories

Age	N	peni	oxa	tet	neo	gen	enr	ery	linc
<6	15	100	100	100	66.7	100	80	80	80
6-12	9	100	100	88.9	22.2	77.8	22.2	100	100
>12	78	100	100	100	32.1	96.2	35.9	98.7	100
TOTALE	**102**	**100**	**100**	**99**	**36.3**	**95.1**	**41.2**	**96.1**	**97.1**

peni: penicillin; oxa: oxacillin; tet: tetracycline; neo: neomycin; gen: gentamicin; enr: enrofloxacin; ery: erythromycin; linc: lincomycin

4. DISCUSSION

It was concluded that *S. bovis* is suitable as a Gram-positive indicator bacterium for cattle because of the higher prevalence of *S. bovis* in faeces compared to the prevalence of *Enterococcus* spp. and because of their ability to harbour resistance determinants. *Streptococcus bovis* predominated in all age categories. The present findings largely agree with earlier reports. Also in a study from Devriese et al. [8] *S. bovis* predominated in young cattle and dairy cows but in pre-ruminating calves *E. faecalis* was isolated most frequently. However, in that study the prevalence of *S. bovis* in young cattle and dairy cows was higher than in our study. *Streptococcus bovis* was isolated from 53% of the young cattle and from 91% of the dairy cows.

According to some investigators, streptococci are not very receptive to antimicrobial resistance genes [3] but only 21 isolates (20.6%) investigated in this study were susceptible to all antimicrobials tested. Also other investigators observed a high percentage of resistance in streptococci, both in human and in veterinary medicine [4, 9, 11].

Concerning the zoonotic aspect of *S. bovis*, even though different subtypes of a bacterial species may affect man and animals, the ability of bacterial strains to transfer genes between each other means that either population may act as a reservoir of resistance genes [10].

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DANMAP:
MONITORING OF ANTIMICROBIAL RESISTANCE IN BACTERIA FROM ANIMALS FOODS AND HUMANS IN DENMARK

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DANMAP – PROVIDING DATA ON ANTIMICROBIAL RESISTANCE

In 1995, The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) was established by the Danish Ministery of Food Agriculture and Fisheries and the Danish Ministery of Health. Since 1996, DANMAP reported annually on the occurrence of antimicrobial resistance is pathogenic and indicator bacteria from animals, food and humans in Denmark. Furthermore, DANMAP reports data on antimicrobial usage in animals and humans.
The objectives of the programme are to provide data on trends in the occurrence of antimicrobial resistance, to monitor consumption of antimicrobial agents, and to investigate associations between use of antimicrobials and occurrence of resistance in animals and humans.

ISOLATES FROM ANIMALS

Bacterial isolates included in the monitoring programme are collected from animals at slaughter (E. coli, enterococci and Campylobacter), as well as from diagnostic submissions (Staphylococcus hyicus from pigs and Staphylococcus aureus (mastitis) from cattle and E. coli from diarrhoea in cattle and pigs and septicaemia in poultry). Salmonella isolates from subclinical infections as well as from cases of clinical salmonellosis are included.
The samples from animals at slaughter are collected by meat inspection staff or company personnel and sent to the Danish Veterinary Institute (DVI) for examination. The number of samples for each plant has been determined in proportion to the number of animals slaughtered per year. Each sample represents one herd or flock. They are collected once a month (weekly for broilers). The broiler, cattle and pig slaughter plants included in the surveillance programme account for 98%, 80% and 95%, respectively, of the total production of these animal species in Denmark. Accordingly, the bacterial isolates may be regarded as representing a stratified random sample of the respective populations, so that the occurrence of resistance provides an estimate of the true occurrence in the populations.
Among all Salmonella isolates serotyped at DVI only one isolate of each serotype per farm is selected for the DANMAP report. The DVI is the national reference laboratory for Salmonella in animals, feeding stuffs and food, and receives isolates for typing.
Bacterial isolates from diagnostic submissions are selected by systematic random sampling among isolates from submissions to the DVI, the Cattle Health Laboratory in Ladelund and the laboratory of the Federation of Danish Pig Producers and Slaughterhouses in Kjellerup. Accordingly, the programme achieves nationwide coverage for these pathogens.

ISOLATES FROM FOOD

All food samples are collected at wholesale and retail outlets by the Regional Veterinary and Food Control Authorities during routine inspection, or on request for the DANMAP surveillance programme. The collection of food samples for analyses of indicator bacteria (enterococci and *E. coli*) is planned and coordinated by the Danish Veterinary and Food Administration. Samples are collected from Danish and imported foods.

ISOLATES FROM HUMANS

Salmonella spp. and *Campylobacter* spp. from humans are selected by random sampling of isolates grown from faeces samples submitted to the Statens Serum Institut for diagnostic purpose. For *Salmonella* Typhimurium all isolates are subjected to susceptibility testing.
To monitor the level of resistance among healthy individuals a running surveillance comprising approximately 200 stool samples per year were initiated in 2002. Subjects selected for participation in the surveillance were found through the Danish Civil Register system (CPR), which is a continuously updated register of all residents in Denmark. Indicator bacteria (enterococci and *E. coli*) from the samples are subjected to susceptibility testing.
All *Staphylococcus aureus* blood isolates and all *Streptococcus pneumoniae* blood and spinal fluid isolates, nationwide, are sent to the Statens Serum Institut and are available to DANMAP. Furthermore *Escherichia coli* and coagulase-negative staphylococci obtained from either blood or urine samples submitted for microbiological diagnostic are available.

SUSCEPTIBILITY TESTING

Susceptibility testing of bacterial isolates from animals and food is carried out using broth micro dilution (Trek Diagnostic Systems Ltd.). Testing of bacterial isolates from humans is carried out using either broth micro dilution or tablet/disk diffusion. All *Campylobacter* isolates are tested using plate dilution. The antimicrobial panels used include antimicrobial substances representing the main groups of therapeutic agents as well as antimicrobial growth promoters.

VETSTAT – PROVIDING DATA ON ANTIMICROBIAL CONSUMPTION

Since 1996, statistics on the total sales of antimicrobials for veterinary use in Denmark have been performed, based on reports from the pharmaceutical companies to the Danish Medicines Agency. These data did not included information on usage in individual target animal species.
From 2001, the Danish register of veterinary medicines, VetStat, has replaced the returns on annual sales from the pharmaceutical industry. The VetStat data are collected close to the point of use, ie. the pharmacies, the veterinary practises, and the feed mills.
VetStat comprise detailed information on all prescription medicine on herd level, including information on farm identity, animal species, age group, disease class, drug identity, amounts of drug and prescribing veterinarian.
In Denmark, all therapeutic antimicrobial drugs are prescription only. Approximately 80% of the total amounts of antimicrobials are sold directly to the animal owners from the pharmacies on prescription. Due to stringent legislation, antimicrobial drugs for use in veterinary practise can only be obtained at the pharmacies. The veterinary practitioners report all usage of prescription medicines in animals to VetStat. The feed mills report sales of medicated feed sold to farms and aquaculture.
The detailed information in VetStat enables standardisation of drug usage, taking into account the potency of the drugs and the animals in which they are used. Standardised animal daily dosages (ADD) have been defined for each species and every therapeutic formulation. The general principles for standardisation of dosage for animals is parallel to the defined daily dosage in humans, however, ADD are calculated for each age group in VetStat. The introduction of ADDs facilitates comparison of antimicrobial usage between different animal species and compounds.
The VetStat programme monitors veterinary use of drugs, hereby providing data on which control measures and legislations regarding veterinary drug administration can be based. Furthermore, in combination with the DANMAP programme, the VetStat programme provides valuable data for pharmaco-epidemiological research.

MACROLIDE AND LINCOSAMIDE RESISTANCE IN HUMAN AND PIGEON *STREPTOCOCCUS GALLOLYTICUS* STRAINS

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Sixty-five pigeon and 30 human *S. gallolyticus* strains were checked phenotypically for their resistance to macrolide and lincosamide (ML) antibiotics. By means of the disk diffusion method, 13 human and 28 pigeon strains were found to be constitutively resistant to the tested antibiotics. Simultaneous screening for the presence of *erm*(B) and *mef*(A) genes using PCR, revealed that the *erm*(B) gene was present in 40 out of these 41 phenotypically resistant *S. gallolyticus* strains while the *mef*(A) gene was detected in only one resistant and one susceptible human-derived strain. The *erm*(B) genes of ten human and ten pigeon *S. gallolyticus* strains were sequenced and compared. The sequences showed to be identical in four human and seven pigeon strains, while the *erm*(B) sequence of the remaining strains differed in one to five nucleotides. These findings could indicate a possible exchange of resistance genes between human and pigeon strains.

Door middel van de agardiffusietest werden 65 duiven en 30 humane *Streptococcus gallolyticus* stammen onderzocht op hun fenotypische gevoeligheid voor macroliden en lincosamiden antibiotica. Tegenover deze antibiotica werd constitutieve resistentie gezien bij 13 humane en 28 duivenstammen. Deze stammen werden onderzocht op de aanwezigheid van het *erm*(B) en het *mef*(A) gen met behulp van PCR. Het *erm*(B) gen werd gedetecteerd bij 40 van deze 41 fenotypisch resistente isolaten, terwijl het *mef*(A) gen enkel aangetoond werd bij één fenotypisch resistente en één fenotypisch gevoelige humane stam. Bij de vergelijking van 10 humane en 10 duiven *S. gallolyticus* stammen op gebied van *erm*(B) sequentie, werden vier humane en zeven duiven isolaten identiek bevonden. De *erm*(B) sequentie van de resterende stammen verschilde in één tot vijf baseparen. Deze bevindingen kunnen eventueel wijzen op de uitwisseling van dit resistentiegen tussen duiven en humane stammen.

1. INTRODUCTION

In veterinary as well as in human medicine, macrolide, lincosamide and streptogramin B antibiotics constitute important classes of antimicrobial agents (MLS_B antibiotics). Over the last years, however, along with the expanded use of MLS antibiotics, an increasing number of resistant strains among different bacterial species, both human and animal related, has emerged (8).

Acquired streptococcal resistance to macrolide antibiotics can largely be put down to two mechanisms, as described by Cornaglia (2). The first mechanism, target site modification, is mediated by adenine-N^6-methyltransferases which are encoded by *erm* (erythromycin-resistant-methylase) genes. As these enzymes induce the methylation of the 50S ribosomal subunit, which is the target site of the MLS_B antibiotics, the binding of the latter is hindered, causing the MLS_B-resistance phenotype to surface.
Next to *erm*(B) genes, *mef*(A) genes may also enforce resistance to 14- and 15-membered macrolides (M-phenotype) by evoking active efflux of the antibiotic compound out of the bacterium (11).

The facultatively pathogenic *S. gallolyticus* bacterium is a well-known cause of septicemia in pigeons (4) and has been the subject of a previous antimicrobial drug resistance study in which resistance to macrolide and lincosamide (ML) antibiotics was a frequently noted phenomenon, with nearly half of the tested strains being phenotypically resistant (7). These fairly high levels of MLS resistance jeopardize the efficiency of antibiotic treatment in this animal species. Additionally these disturbing data might pose a threat to human health. In human beings, *S.*

gallolyticus, formerly known as *S. bovis* (5), might be associated with human endocarditis (1) and gastrointestinal lesions, especially colorectal malignancies (10).

2. MATERIALS AND METHODS

2.1. Bacterial strains

A total of 95 *S. gallolyticus* strains were included in this study, made up by 65 pigeon and 30 human strains. All pigeon strains were isolated in Belgium between 1999 and 2001. Forty-nine of the pigeon isolates originated from internal organs of diseased pigeons and the remaining sixteen strains were obtained from faecal samples, all from different lofts. The human *S. gallolyticus* strains all originated from clinical samples, mainly from endocarditis and blood cultures.
S. pyogenes strains STP 016 and STP 023 (from human origin) were used as positive control strains in the *erm*(B) and *mef*(A) detecting PCR tests, respectively (3).

2.2. Disk susceptibility testing

Antimicrobial susceptibility phenotypes were determined by disk diffusion on Columbia blood agar (Oxoid, Basingstoke, UK), using the following antimicrobial agents: clindamycin, erythromycin, lincomycin and tylosin, all obtained from Rosco diagnostics, Taastrup, Denmark.

2.3. PCR

DNA was extracted from bacterial cells that were grown overnight on Columbia agar (Gibco) with 5% bovine blood for 24 h at 37°C in a 5% CO_2 enriched environment. Primers for *erm*(B) and *mef*(A) screening of the strains were derived as published previously (13). DNA amplification was performed in a thermal cycler (model 9600 GeneAmp PCR system, Perkin-Elmer, Zaventem, Belgium) with the following conditions: 93°C for 3 min, 35 cycles of 93°C for 1 min, 57°C for 1 min, 72°C for 1 min, followed by 72°C for 5 min. Subsequently, a mixture of 5 µl amplicon with 2 µl sample buffer (50% glycerol, 1 mM cresol red) was electrophoresed in a 1.5% agarose gel for 50 min at 175V in 0.5 TBE buffer (Amresco, Ohio, U.S.A.).

2.4. Sequencing

The sequences of the *erm*(B) gene of ten human and ten pigeon *S. gallolyticus* strains that screened positively in the *erm*(B) PCR test, were determined. The entire *erm*(B) gene was sequenced using the BigDye Terminator Cycle Sequencing kit (PE Biosystems) and the following outwardly directed primers, 5'CCATACCACAGATGTTCCAG3' and 5'AGATAGATGTCAGACGCACG3' (unpublished results), as well as the earlier mentioned primers for the *erm(*B*)* screening on a ABI Prism™310 Genetic Analyzer. The electropherograms were exported and converted to GeneBase (Applied Maths, Kortrijk, Belgium) using Abiconv (Applied Maths). Sequences were compared mutually.

3. RESULTS

3.1. Disk susceptibility testing

The ML phenotype was displayed by 13 out of the 30 human *S. gallolyticus* strains. Twenty-one out of the 49 and seven out of the 16 pigeons strains isolated from the internal organs and faeces, respectively, likewise displayed the same phenotype. The remaining strains were fully susceptible. The M phenotype was not seen.

3.2. PCR

Neither the *erm*(B) gene nor the *mef*(A) gene were encountered in 53 out of the 54 susceptible *S. gallolyticus* strains. The remaining phenotypically susceptible human-derived strain was found to carry the *mef*(A) gene.

All 28 pigeon resistant *S. gallolyticus* strains were shown to be *erm*(B) positive in the PCR assay. None of these strains carried the *mef*(A) gene. The *erm*(B) gene was detected in 12 out of the 13 resistant human *S. gallolyticus* strains while the *mef*(A) gene was present in the one remaining resistant human strain.

3.3. Sequencing

Four out of ten human and seven out of ten pigeon strains possessed exactly the same sequence for the *erm*(B) gene. The seven pigeon strains in question consisted of four faecal and three organ derived strains. The sequence is deposited in GenBank under accession number AY183117. The sequences of the remaining strains differed in one to five nucleotides.

4. DISCUSSION

Phenotypic resistance to ML antibiotics was encountered in 43% of all strains tested. This is in agreement with our previous study which reported 45 and 48% of the pigeon *S. gallolyticus* strains being resistant to macrolides and lincosamides, respectively (7).

In their survey on the presence of *erm* genes in gram-positive bacteria from humans and animals, Jensen *et al.* (6) stated that the *erm*(B) gene occurred in 90% of the resistant streptococci tested. Our findings are no exception to this. The resistant *S. gallolyticus* strain lacking the *erm*(B) gene tested positive for the *mef*(A) gene normally entailing the M phenotype. The discrepancy between its genotype and phenotype most likely is associated with the involvement of other resistance genes such as *lnu*, *lnu* like or *ermTR* genes, which have, though to a lesser extent, been identified in streptococci, or a hitherto uncharacterised gene. The *ermTR* gene has fairly recently been apprenticed in *Streptococcus* species by Seppälä *et al.* (12), mediating a MLS_B phenotype simultaneously masking the M phenotype which normally results from the *mef*(A) gene.

Another discrepancy between genotype and phenotype was found in a phenotypically susceptible strain that carried the *mef*(A) gene. Martineau *et al*. (9) suggest several factors that could explain the sensitive phenotype in genotypically resistant staphylococci. The hypothesis that the gene is present in the bacterium but is being deprived of its expression due to regulatory factors, is a plausible explanation.

5. ACKNOWLEDGEMENTS

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MACROLIDE RESISTANCE IN PORCINE STREPTOCOCCI: A HUMAN HEALTH HAZARD?

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In order to obtain better insights into the possible exchange of resistance genes between human and porcine streptococci we performed three studies. First, the sequences of the *erm*(B) genes of streptococcal isolates from humans, pigs and pork carcasses were compared. Secondly, *in vitro* filter matings between human and porcine streptococci were carried out. Finally, the presence of different tetracycline resistance genes was determined in streptococci isolated from pigs and pork carcasses. Identical *erm*(B) gene sequences were present in strains from humans, pigs and pork carcasses. This gene was exchanged between human and porcine strains *in vitro*. Apposed to human strains, the *tet*(O) gene was the predominant tetracycline resistance gene in the porcine and pork carcass streptococcal strains. Our results suggest that, although transfer of the *erm*(B) gene from human to porcine strains and vice versa is possible, it might be a rather rare phenomenon.

Om een beter inzicht te krijgen over de mogelijke uitwisseling van antibiotica resistentiegenen tussen humane en varkens streptokokken, werden drie experimenten uitgevoerd. Eerst werden de sequenties van de *erm*(B) genen van streptokokken geïsoleerd van mensen, varkens en varkenskarkassen vergeleken. Ten tweede werd een *in vitro* overdracht studie uitgevoerd tussen humane en porciene streptokokken. Ten derde, werd het voorkomen van verschillende tetracycline resistentie genen bepaald bij streptokokken afkomstig van varkens en varkenskarkassen. Identieke *erm*(B) gen sequenties waren aanwezig bij streptokokken van mensen, varkens en varkenskarkassen. Het *erm*(B) gen kon ook *in vitro* uitgewisseld worden tussen humane en porciene streptokokken. In tegenstelling tot bij humane streptokokken is het *tet*(O) gen het meest voorkomende tetracycline resistentie gen bij porciene streptokokken. Uit onze resultaten blijkt dat overdracht van het *erm*(B) gen tussen humane en porciene streptokokken mogelijk is, maar dat het waarschijnlijk niet frequent gebeurt.

1. INTRODUCTION

Transfer of antibiotic resistance from production animals to humans is a major public health issue. Two types of resistance transfer from animals to humans are of importance. The direct way of resistance transfer occurs when resistant zoonotic bacteria such as *Salmonella* serotypes, *Campylobacter* spp. or *Yersinia* spp. infect humans, either by direct contact or through the food chain. In the indirect pathway of resistance transfer, resistance genes are transferred horizontally from animal to human strains. If this transfer occurs to human pathogenic bacteria, treatment failure most likely is an important consequence.
One of the most important antibiotic groups affected by resistance in humans and in farm animals is the macrolide-lincosamide-streptogramin B (MLS_B) antibiotic family (1, 4, 5). In a previous study, we found 71% of the *Streptococcus suis* isolates from swine to be resistant to macrolides and lincosamides (7). This high incidence of resistance seriously complicates the therapy of many bacterial diseases in animals but above all has become a controversial issue, as there may be a risk of resistance transfer to humans.
Cross-resistance against macrolides, lincosamides and streptogramin B antibiotics (MLS_B) is mostly encoded by *erm* (erythromycin ribosome methylation) genes. These genes encode a methyltransferase, which (di)methylates the 23S rRNA. Different classes of *erm* genes have been described (10). In pigs as well as in humans, the MLS_B phenotype in streptococci is mostly encoded by the *erm*(B) gene (5, 6). This finding underscores the possible exchange of this

resistance gene between human and porcine streptococci. A first step to be undertaken in the confirmation of this hypothesis, is the comparison of the genes in different strains at the DNA sequence level. Therefore, in this study, the sequences of the *erm*(B) genes from streptococci from humans, pigs and pork carcasses were compared. *In vitro* mating experiments were performed in order to detect if the *erm*(B) gene could be transferred between human and porcine streptococci. In human streptococci the presence of the *erm*(B) gene is frequently linked to the *tet*(M) gene. Therefore, finally, the presence of different tetracycline resistance genes in porcine streptococci was determined.

2. MATERIALS EN METHODS

2.1 Bacterial isolates

All strains used in this study were isolated between 1999 and 2002 and showed acquired resistance against erythromycin, tylosin, lincomycin and clindamycin as determined in disk diffusion tests (6, 7). This strain collection consisted of twenty-one human streptococci (two *S. oralis-mitis* sp., eight *S. pneumoniae*, five *S. pyogenes*, one *S. salivarius*, three *S. sanguinus* and two *S. thermophilus* strains), 160 porcine streptococci (nine *S. alactolyticus*, four *S. bovis*, one *S. corrodens*, one *S. dysgalactiae*, eight *S. gallolyticus*, four *S. hyointestinalis* and 133 *S. suis* strains) and 118 streptococci originating from pork carcasses (one *S. agalactiae*, 39 *S. alactolyticus*, seven *S. bovis*, 19 *S. dysgalactiae*, 10 *S. hyointestinalis*, 39 *S. suis* strains and three *S. porcinus* strains). The human *S. pyogenes* and *S. pneumoniae* strains were isolated from patients with tonsillopharyngitis and bacteremia, respectively. The other human streptococci were isolated from throat swabs of eight healthy volunteers. Ninety-two *S. suis* isolates, belonging to different serotypes, were isolated from lesions of diseased pigs. The other porcine streptococcal strains originated from tonsillar and colon swabs of healthy pigs. The strains from pork carcasses were cultured from swab samples taken from carcasses in four slaughterhouses. The pigs originated from different farms. All the strains were identified using tDNA-PCR (3).

2.2 Sequencing

The PCR products of *erm*(B) genes of 21 human, 22 porcine and 15 streptococci originating from pork carcasses, derived with primers 5'ATGAACAAAAATATAAAATATT3'and 5'TTATTTCCTCCCGTTA AA3', were cloned with the TOPO TA Cloning kit (Invitrogen, Paisly, UK). The sequences of the *erm*(B) genes were determined using the BigDye Terminator Cycle Sequencing kit (PE Biosystems) and the following primers: 5'GAAAAGA/GTACTCAACCAAATA3', 5'AGTAACGGTACTTAAATTGTTTAC 3', 5'CCATACCACAGATGTTCCAG3' and 5'AGATAGATGTCAGACGCACG3' on an ABI Prism^{TM}3100 Genetic Analyzer. The electropherograms were exported and converted to GeneBase (Applied Maths, Kortrijk, Belgium) using Abiconv (Applied Maths).

2.3 Mating procedure

Transferability of the *erm*(B) gene between porcine *S. suis* (one serotype 9 and one serotype 7) strains and human *S. pyogenes*, *S. pneumoniae* and *S. oralis* streptococci was examined by filter mating. The *S. suis* serotype 9, *S. pyogenes*, *S. pneumoniae* and *S. oralis* donor strains carried, beside the *erm*(B) gene, also the *tet*(M) tetracycline resistance gene. Acceptor strains were susceptible to macrolides but showed acquired resistance to enrofloxacine. The donor and recipient strains were grown in BHI broth at 37°C and 5% CO_2 atmosphere for four hours. Cultures of donor and recipient were mixed (donor/acceptor ratio 1/10) in 5 ml BHI broth and the mixture was filtrated through a sterile nitrocellulose 0.45 µm pore-size filter. Filters were incubated for 18 h on Columbia blood agar plates at 37°C in 5% CO_2 environment. The bacteria were washed from the filters with 2ml PBS by vortexing for 30 s. Dilutions of mating mixtures were spread onto Columbia blood agar plates supplemented with 8 µg/ml erythromycin (isolation donor strains), 8 µg/ml erythromycin and 2 µg/ml enrofloxacine (isolation conjugants), or 2 µg/ml enrofloxacine (isolation acceptor strains) and incubated for 24-48 h (37°C, 5% CO_2).

2.5 PCR detection of tet *genes*

A PCR assay was performed to detect the presence of *tet*P(B), tet(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T) and *tet*(W) gene in all the strains isolated from pigs and pork carcasses. The primers were derived from published sequences (2, 9, 13). The PCR mix (total volume 50µl) contained 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase, 200 µM of each dNTP, 100 pmol of both primers and 2.5 µl DNA sample. The PCR programme consisted of an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, annealing temperature (*tet*(K), *tet*(L), *tet*(S) 50°C; *tet*(M) 55°C; *tet*(O) 58°C; *tet*P(B); *tet*(Q) 63°C; *tet*(T) 44°C; *tet*(W) 64°C) for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. PCR products were separated by electrophoreses on a 1.5 % agaraose gel and visualized by ethidium bromide staining.

3. RESULTS

The comparison of the encoding sequences of the *erm*(B) gene (738 bp) is shown in Figure 1. The sequences showed a similarity between 98.7% to 100%. Identical *erm*(B) genes were present in streptococcal strains isolated from humans, pigs and pork carcasses.

The data shown in Table 1 represent the results of successful matings. The *S. suis* serotype 9, *S. pyogenes*, *S. pneumoniae* and *S. oralis* strain carried, besides the *erm*(B) gene, also the *tet*(M) resistance gene. This gene was transferred to the recipient strains together with the *erm*(B) gene.

The results of the PCR's to detect the presence of the different tetracycline resistance genes are shown in Table 2.

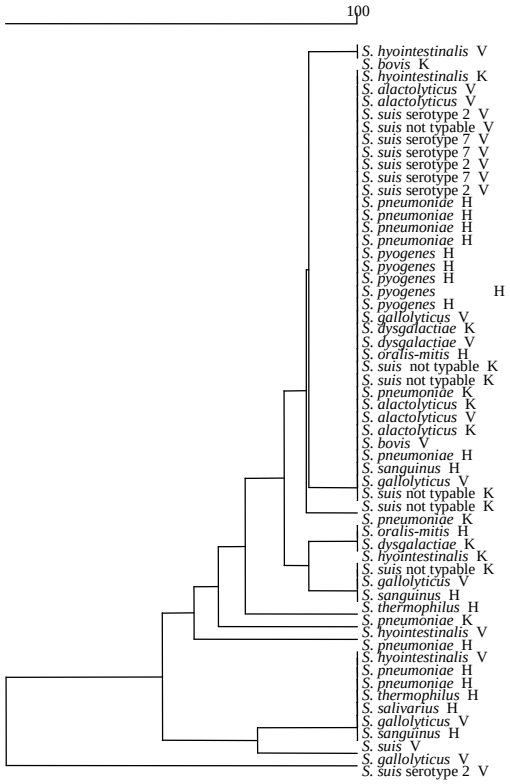

Figure 1. Comparison of the *erm*(B) sequences of 21 human, 22 porcine and 15 streptococci originating from pork carcasses. V streptococci isolated from pigs; K streptococci isolated from pork carcasses; H streptococci isolated from humans

Table 1. Conjugal transfer of the erythromycin resistance gene *erm*(B) from *erm*(B)-positive *S. suis*, *S. pyogenes*, *S. pneumoniae* and *S. oralis* donors to erythromycin-susceptible *S. suis*, *S. pyogenes* and *S. pneumoniae* recipients.

Donor strain	Recipient strain	Transfer frequency
S. suis serotype 9	*S. suis* serotype 2	6.3×10^{-7}
	S. pyogenes	4.9×10^{-6}
S. suis serotype 7	*S. suis* serotype 2	5.2×10^{-6}
	S. pneumoniae	1.6×10^{-9}
S. pneumoniae	*S. suis* serotype 2	4.0×10^{-6}
	S. pyogenes	5.7×10^{-4}
S. pyogenes	*S. suis* serotype 2	1.6×10^{-7}
	S. pneumoniae	1.1×10^{-6}
S. oralis	*S. suis* serotype 2	4.8×10^{-7}
	S. pyogenes	8.3×10^{-8}
	S. pneumoniae	2.3×10^{-7}

Table 2. Presence of tetracycline resistance genes in streptococci from pigs and pork carcasses.

	number	*erm*(B)	*tet*(K)	*tet*(L)	*tet*(M)	*tet*(O)	*tet*(Q)	*tet*(S)	*tet*(T)	*tet*(W)	*tet*P(B)
S. agalactiae	1	1	0	0	0	1	0	0	0	0	0
S. alactolyticus	48	39	0	35	6	36	0	2	0	0	0
S. bovis	11	9	0	9	2	10	0	0	0	0	0
S. dysgalactiae	21	12	5	2	16	11	0	0	0	0	0
S. hyointestinalis	14	11	1	6	13	4	0	0	0	0	0
S. porcinus	3	2	1	6	1	2	0	0	0	0	0
S. suis	172	141	43	40	21	105	0	1	1	11	10
S. corrodens	1	1	0	1	1	1	0	0	0	0	0
S. gallolyticus	8	8	1	1	5	5	0	0	0	0	0
S. pluranimalium	1	1	0	0	0	0	0	0	0	0	0

4. DISCUSSION

Identical *erm*(B) gene sequences were present in strains belonging to different streptococcal species isolated from pigs, pork carcasses and humans. From these results it is not possible to discriminate if these strains have obtained the resistance gene from a common reservoir or that horizontal gene transfer between the human and porcine streptococci has occurred.

The filter mating experiments described in this study demonstrate that transfer of the *erm*(B) gene can occur at high frequencies under laboratory conditions of intimate cell-to-cell contact, from human to porcine streptococci and from porcine to human streptococci. Combined with the results from the *erm*(B) sequencing, this finding suggests that the high prevalence of the *erm*(B) both in humans and pigs might be due to horizontal genetic transfer within and between host species.

In *S. pyogenes* and *S. pneumoniae* the presence of the *erm*(B) gene is frequently linked to the *tet*(M) gene, both located on the same conjugative transposon (8,12). *Erm*(B) positive porcine or pork carcass strains that also carried the *tet*(M) gene were detected in only 51 of 225 strains, whereas 153 *erm*(B) positive strains also carried the *tet*(O) gene. The latter gene is rare in human streptococci (11). These results suggest that, although transfer of the *erm*(B) gene from human to porcine strains and vice versa is possible, it might be a rather rare phenomenon.

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ANTIBIOTICS AND RESISTANCE: MECHANISM, GENETICS, SPREADING, RESISTANCE, SELECTION AND EPIDEMIOLOGY

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1. Definition of Antibiotics

(Sande and Mandell, 1985; Bywater, 1991)

1.1. Natural Antibiotics

Natural antibiotics are chemical substances produced by various species of microorganisms (bacteria and fungi) that suppress or kill the growth of bacteria. Hundreds of natural antibiotics have been identified, and nearly 100 have been developed to the stage where they are of value in the therapy of infectious diseases. The first natural antibiotic was benzylpenicillin. Other examples are streptomycin, chloramphenicol, tetracyclines and macrolides.

1.2. Semi-synthetic antibiotics

Semi-synthetic antibiotics are derivatives of natural antibiotics. These derivatives are obtained by small alterations in structural formulas of natural antibiotics. For instance, soon after the introduction of benzylpenicillin, one side chain of its structure was altered by one oxygen atom by a small variation in the growth medium for the *Penicillium*, resulting in phenoxymethylpenicillin. This derivative has the virtue of being acid-stable and is suitable for oral administration. After chemical identification of natural antibiotics many derivatives have been, or are still produced and tested for their antibacterial activity. Other examples of semi-synthetic antibiotics are the penicillinase resistant semi-synthetic penicillins such as nafcillin, cloxacillin and flucloxacillin.

1.3. Synthetic antibiotics

Synthetic antibiotics formerly called chemotherapeutics are chemically synthesised. The first compound with chemotherapeutic activity that was used therapeutically was Prontosil, an azo dye structurally related to sulphanilamide. Soon afterwards the sulphonamides were developed, and they still play an important role in therapy of infectious diseases. More recent examples of synthetic antibiotics are the nitrofurans and the quinolones.

1.4. Mechanisms of action

Their mechanisms of action fall into four categories:

- inhibition of cell wall synthesis (ß-lactam antibiotics, vancomycin, bacitracin);
- damage to cell membrane function (polymyxins, polyenes);
- inhibition of nucleic acid function (nitroimidazoles, nitrofurans, quinolones, rifampin) or intermediate metabolism (sulphonamides, trimethoprim);
- inhibition of protein synthesis (aminoglycosides, fenicols, lincosamides, macrolides, streptogramins, pleuromutilins, tetracyclines).

2. GENETICS OF RESISTANCE

Antibiotics have been used for more than 60 years. During this period a tremendous selection pressure has been exerted on bacterial eco-systems in humans and animals and has led to the emergence of resistant bacteria. Looking at the history of antibiotic agents, development of bacterial resistance has been an expected but rather an unpredictable phenomenon (Huovinen et al., 1997). Bacteria isolated from patients 60 years ago had virtually no resistance genes (Hughes and Datta, 1983). Similarly, strains resistant to new antibiotic agents have not been

seen among several species of bacteria until these agents had been used for years or decades. The most reliable information on the characteristics of bacteria from the pre-antibiotic era comes from studies of the "Murray-collection", microbial pathogens that were collected between 1914 and 1950. These organisms are completely sensitive to the common antibiotic agents. Even though sulphonamides were introduced into clinical practice in the mid-1930s, the "Murray collection" is susceptible to this class of drugs. Nevertheless, many of the "Murray strains" carry plasmids and are capable of promoting conjugative transfer (Davies, 1997).

When antibiotics were first introduced for treatment of common bacterial infections, development of antibiotic resistance during therapy was not expected, because the frequency of mutation to resistance in bacteria was thought to be too low. It was at that time unknown that even in nature, bacteria can collect and exchange genetic information with extraordinary ease and lack of species specificity (Davies, 1994). However, antibiotic resistance exemplifies „par excellence“ Darwinism. Resistance has developed rapidly, as has been observed after the introduction of most of the ”new” antibiotics.

Table 1: Antibiotic discovery and resistance development
(C. von Eiff et al, 1997; Davies, 1997; Kliebe et al., 1985; O’Brien, 1997)

Antibiotic	Discovered	Introduced into clinical use	Resistance identified
Penicillin	1940	1943	1940 (Methicillin 1961/5)
Streptomycin	1944	1947	1947, 1956
Tetracycline	1948	1952	1956
Erythromycin	1952	1955	1956
Vancomycin	1956	1972	1987
Nalidixic acid	1960	NA	NA
Gentamicin	1963	1967	1970
Third generation cephalosporins	NA	1980	1985
Fluoroquinolones	NA	1983	1985

All resistance has a genetic basis, which might either be a fixed part of the bacterial genome or be transferable between bacteria. Whenever antibiotics are used, bacteria will inevitably develop resistance, either by mutation, gene acquisition, or a combination of both. Surviving resistant strains have emerged under protection and selection by the antibiotic.

Bacterial resistance to antibiotics may be intrinsic (natural) or acquired. Intrinsic resistance (non-susceptibility) is a characteristic of bacterial species that are homogeneously resistant to a particular antibiotic, either because they lack the cellular mechanisms by which that particular antibiotic exerts its action or because the bacterial wall is impermeable to the antibiotic. The latter is commonly encountered in Gram-negative species. Acquired resistance can originate from chromosomal mutation or from the acquisition of transferable genetic material already present within related or unrelated bacterial populations. Acquired resistance can be found in every pathogenic bacterial species as well as in the commensal flora of man and animals, but the prevalence varies considerably between bacterial species and even between subspecies. For example, Gram-positive bacteria except staphylococci and enterococci, often lack the ability to acquire R-plasmids.

2.1. Chromosomal resistance

This type of resistance develops from mutations in the nucleotide sequences of the bacterial chromosome resulting in the synthesis of proteins or other macromolecules that differ sufficiently from the original chemical entities to interfere with the antibiotic activity. Mutations can occur continuously and irrespective of the presence of antibiotics, but are generally lost or “repaired” by cellular mechanisms. Transfer of mutations takes place during multiplication (vertical transfer). The mutation frequency is low, usually in the range of 10^{-6} to 10^{-10} per generation. However, mutants with increased antibiotic resistance are advantageous to the bacterium only when antibiotics are used. Sensitive bacterial sub-populations will be eliminated while the antibiotic remains in the environment in concentrations above the MIC. Development of resistance resulting from mutations is usually specific to the selecting antibiotic agent or closely related antibiotics.

Chromosomal resistance is inherited clonally. Development of chromosomal resistance within a population exposed to an antibiotic is usually a gradual, step-wise process effected by several successive mutations, but for some antibiotics a single mutation may produce a dramatic increase in the MIC. Resistant mutants emerge less frequently *in vivo* than *in vitro*, probably because mutations leading to resistance are often associated with other cell changes, which may possess disadvantages for the bacterium. In general the number of the resistant mutants will decrease after cessation of exposure. Therefore, the development of resistance in many bacterial species caused by chromosomal mutations is regarded as a smaller problem than transferable resistance by some

scientists. Nevertheless, this depends mainly on factors like survival capacity of the mutants, cross-resistance and co-resistance to other antibiotics or substances and their use.

2.2. Transferable resistance

Bacteria have extremely efficient genetic transfer systems capable of exchanging and accumulating resistance genes. Certain bacterial genes, including genes encoding for resistance, can move between chromosomal and extra-chromosomal DNA elements in bacteria. They may move between bacteria belonging to the same or different species or to bacteria of different genera (horizontal transfer). Antibiotic resistance genes on plasmids and transposons flow to and from Gram-positive and Gram-negative bacteria, and among bacteria which inhabit vastly different ecological niches (Levy, 1997). Inter-species transfer implies that once transferable resistance genes have developed, bacteria carrying these genes will remain potential gene donors for other bacteria.

Resistance genes commonly occur in the natural bacterial flora and not all transferable resistance has been induced by use of man-made antibiotics. The first report in the mid-1950s of transferable antibiotic resistance genes was in Japan (Davies, 1997). The transfer of antibiotic resistance genes in natural environments has a very broad host range and can happen even between phylogenetically distinct bacterial genera, such as between Gram-positive and Gram-negative bacteria („trans-Gram-conjugation/promiscuity“, Courvalin, 1994).

The most important vehicles for transfer of resistance genes in bacteria are plasmids, transposons and integrons. Because of their mobility, transferable resistance elements are more likely to persist at a low level in an eco-system even in the absence of antibiotic selection pressure than chromosomal resistance.

2.2.1. Plasmids

Plasmids are extrachromosomal, replicable circular DNA molecules that may contain resistance genes. They replicate independently of bacterial chromosomal DNA. Plasmids are important in bacterial evolution, because they affect replication, metabolism, bacterial fertility as well as resistance to bacterial toxins (bacteriocins), antibiotics and bacteriophages, thus providing a better chance of survival and propagation. Nevertheless, in general plasmids are not necessarily required by the bacterium for its survival. They have been identified in most bacterial species and can be either conjugative or non-conjugative. They may have the capacity to be transferred (conjugative plasmids) or co-transferred (non-conjugative plasmids) from one bacterium to another, thus resulting in wide spread dissemination of plasmid-encoded characteristics within a bacterial ecosystem. Genes encoded by plasmids are intrinsically more mobile than chromosomal genes because plasmids can be transferred within and as well as between different species. The frequency of plasmid transfer (i.e. conjugation) may be as high as $1{:}10^5$, so the acquisition of new resistance determinants can occur much more readily than by genetic mutation. R-plasmids are plasmids containing resistance genes. A single R-plasmid may code for resistance to up to 10 different antibiotics simultaneously. Many different R-plasmids have been identified. Plasmids from human and animal isolates seem to be very similar.

Dissemination of plasmids may occur by clonal distribution and by intra-species and inter-species transfer resulting in a gradual increase of the proportion of microorganisms within a bacterial community carrying one or more R-factors. Although some resistance plasmids are non-conjugative, they may often be transferred (mobilised) to a recipient if they co-inhabit a cell with a conjugative plasmid. In contrast to mutation-based chromosomal resistance, acquisition of an R-plasmid generally confers resistance to clinically achievable levels of an antibiotic in a single step. In Gram-negative bacteria the transfer/acquisition of further plasmid-mediated characteristics, such as virulence and enterotoxin production, is in some instances facilitated by the presence of R-plasmids. Furthermore, a single bacterial cell can contain many different plasmids and each plasmid can carry more than one resistance gene, thus forming so-called gene cassettes. More than 40 gene cassettes have been identified (Amábile-Cuevas et al., 1995; Davies, 1996; Hall, 1997)

2.2.2. Transposons

Transposons (jumping genes) are short sequences of DNA that can move between plasmids, between a plasmid and the bacterial chromosome or between a plasmid and a bacteriophage (bacterial virus). Unlike plasmids, transposons are not able to replicate independently and must be maintained within a functional replicon (e.g. plasmid or chromosome). Transposons in Gram-negative bacteria are non-conjugative, in Gram-positive bacteria and *Bacteroides* spp. they can either be conjugative or non-conjugative. However, if a transposon in Gram-negative bacteria is part of the DNA of a conjugative plasmid, horizontal transfer is possible. Transposons, including those carrying resistance genes, are easily acquired by plasmids and then incorporated into bacterial DNA. Often several transposons are clustered on the same plasmid, resulting in the transfer of multiple resistance determinants with a single conjugation (Burns, 1995). Plasmids of different origin may also carry several sets of identical resistance genes. The intracellular transfer of transposons between plasmids, between

bacterial chromosomes and plasmids as well as an inter-bacterial transfer of plasmids and conjugative transposons can result in rapid development of resistance within several bacterial populations. The major impact of transposons on the emergence of antibiotic resistance is that they can expand the host range of bacteria species to which resistance can be spread. Expression of resistance genes located on transposons, e.g. production of specific enzymes, may require the presence of the antibiotic(s) in question. Furthermore, the presence of the antibiotic will promote transfer of resistance. Antibiotics create an environment in which possession of resistance determinants is advantageous and, in addition, the rate of transfer of resistance genes will increase.

2.2.3. Integrons and gene cassettes

Integrons are DNA structures containing a recombination site, integration machinery and a promoter region that actively integrate resistance genes (Hall, 1997). They can be located in the chormosomal DNA, but are often located on transposons and can therefore be mobile. They contain gene cassettes that encode for resistance to different antibiotics and heavy metals such as mercury. An example of an integron is the one present in genes of *Salmonella* Typhimurium DT104. Gene cassettes are a family of mobile elements that include a single gene and a recombination site. There are more than 40 cassettes identified and, of them, all but five contain resistance genes (Hall, 1997).

2.3. Mechanisms for inter-bacterial transfer of resistance

Several mechanisms have been identified for transfer of genetic material, including resistance genes, between bacteria. An important mechanism is bacterial conjugation, whereby a plasmid or other genetic material is transferred from the donor bacterium to the recipient via a cytoplasmatic bridge. Conjugation may occur between bacteria of the same species, within species of the same genera or between species of different families. Other ways of inter-bacterial transfer are transduction (transmission by bacteriophages) and transformation (direct transfer of free DNA originating, for example from lysed bacteria).

3. MULTIPLE-DRUG RESISTANCE (MULTIRESISTANCE)

Within the past few years, several divergent organisms have emerged as significant causes of morbidity and mortality, including infections caused by bacteria that are refractory to therapy because of resistance to many antibiotic agents (Levy, 1998). Examples are *Salmonella* typhi, penicillin resistant pneumococci, methicillin resistant *Staphylococcus aureus* (MRSA) and multiresistant mycobaceria. Simultaneous resistance in one bacterium against three or more classes of antibiotics by various resistance mechanisms generally encoded by different genes is defined as **multiresistance**. Exceptions are for instance multi-drug-resistance (MDR)-genes, which encode for different resistance phenotypes by using the same mechanism (e.g. efflux). **Cross-resistance** is defined as resistance to different antibiotics by the same resistance mechanism. In general these antibiotics belong to the same class. Multiresistance in bacteria is generally attributed to the acquisition of transposons, integrons and/or plasmids bearing genetic determinants for different mechanisms of resistance. If a bacterium is multiresistant with genetically linked resistance determinants, it will not easily lose its resistance to a particular antibiotic, even when this drug is not used for a long period of time. One explanation would be that the gene, which encodes for resistance to that antibiotic can remain present as a result of the use of other antibiotics to which the determinant is genetically linked (co-selection). Another explanation would be that the plasmid encoding the gene is not counter-selected in the absence of chloramphenicol. Because of the intensive use of antibiotics in hospitals and animal husbandry, hospital strains of bacteria and bacteria in animals might “collect” resistance genes. As a result, in these environments in general a larger number of multiresistant bacteria can be detected than in environments with less selection pressure.

3.1. Gram-positive bacteria

3.1.1. General

In some bacteria large numbers of transposable elements have been discovered carrying virtually all possible combinations of known resistance genes . In hospitals, as a result of selection by antibiotic use, nosocomial infections caused by multi-/methicillin-resistant *Staphylococcus aureus* (MRSA), coagulase negative staphylococci and glycopeptide-resistant *Enterococcus faecium (*McDonald et al. 1997) as new Gram-positive challenges (Mouthon and Mainardi, 1996). Outbreaks of multiresistant *Mycobacterium tuberculosis* in HIV-infected patients in the USA and Europe have focused international attention (Anonymous, 1998). Community-acquired infections with multiresistant *Streptococcus pneumoniae* and *Shigella sonnei* also cause treatment problems both, in the developed and the developing world .

Methicillin-resistant *Staphylococcus aureus* (MRSA) MRSA-isolates, which are also resistant to all penicillins including ß-lactamase-resistant penicillins, cephalosporins and carbapenems are frequently also resistant to other

antibiotics, especially macrolides, quinolones, aminoglycosides, lincosamines and trimethoprim/sulfamethoxazole. These multiresistant strains cause serious therapeutical problems (Eiff et al., 1997; Voss et al., 1994). The glycopeptides, vancomycin and teicoplanin presently remain the cornerstone of treatment ('last resort' reserve antibiotics) for all MRSA infections in human medicine. Therefore, clinical acquisition of vancomycin resistance by *S. aureus* (MRSA) would be catastrophic (Segal-Maurer et al., 1996). However, recently infections with vancomycin-intermediate resistant *Staph. aureus* strains (VISA) in Japan and the USA have been reported .

3.1.2 Glycopeptide-resistant enterococci

Multiresistant / glycopeptide -resistant enterococci are currently emerging nosocomial pathogens. They have already become the second most common bacterium recovered from nosocomial infections, and the third most common cause of nosocomial bacteriaemia in the USA. One of the major reasons why these organisms have thrived in the hospital environment in this country is their intrinsic resistance to several commonly used antibiotics and their ability to acquire resistance to all currently available antibiotics as shown in table 2 (Moellering, 1998).

Table 2: Multiple drug resistance in *Enterococcus faecium*:

Intrinsic resistance	Acquired resistance
ß-lactams (particularly cephalosporins, penicillinase-resistant penicillins)	ß-lactams (high concentrations, by PBB or ß-lactamases)
Aminoglycosides (low concentrations)	Aminoglycosides (high concentrations)
Fluoroquinolones	Fluoroquinolones
Trimethoprim-sulphonamides	Glycopeptides (vancomycin, teicoplanin)
Clindamycin	Tetracyclines
	Erythromycin
	Chloramphenicol
	Fusidic acid
	Nitrofurantoin

The emergence of vancomycin resistant enterococci (VRE) mainly results from the intensive use of glycopeptides, especially the parenteral and oral use of vancomycin in hospitals. The acquisition of or colonisation with VRE by hospitalised patients has also been associated with a number of other factors like the length of hospital stay, underlying disease, intensity of antibiotic exposure, additional use of broad-spectrum drugs (like cephalosporins etc.). A recent survey demonstrated greater mortality in patients associated with VRE bacteriaemia compared to patients with vancomycin-susceptible enterococcal bacteriaemia (36,6 % versus 16,4 %) (Segal-Maurer et al., 1996). VRE with the *vanA*-gene have been isolated extensively in animals and food products in Europe. The glycopeptide avoparcin, which can induce cross-resistance to vancomycin and teicoplanin has been used as an antibiotic feed additive in food producing animals the EU for nearly 20 years. However, in Europe only a few clinical outbreaks of infections with VRE have occurred in humans in comparison to the USA, where infections occurred despite the fact that avoparcin has not been authorised for use in animals in the USA (McDonald et al., 1997). Moreover, European strains of VRE are generally not multiresistant.

The streptogramin-combination of quinupristin and dalfopristin is a promising new reserve-antibiotic for treatment of infections with MRSA and VRE. However, the use of virginiamycin as feed additive has already selected for resistance in animals in Europe as shown in enterococci in poultry (Werner and Witte, 1998).

3.2. Gram-negative bacteria

Gram-negative bacteria are very important pathogens of humans and animals. The first well-documented bacterial outbreak involving multiresistant bacteria was an epidemic of *S.* typhi (typhoid fever, which is solely a human pathogen) in Mexico in the early 1970s with more than 10 000 confirmed cases in 1972. The *S.* typhi strain involved carried genes encoding for chloramphenicol- (the former drug of choice for treatment of this infection), ampicillin-, streptomycin- and sulphonamide-resistance (Amábile-Cuevas et al., 1995). Recently multiresistant (including fluoroquinolone-resistant) enteropathogenic bacteria responsible for community-acquired infections, such as zoonotic and pathogenic *Salmonella* and *Campylobacter* (Acar and Goldstein, 1997; Gold and Moellering, 1996) and *E. coli* have been described (Kim et al., 1994; Murphy and Echevarria , 1998).
Zoonotic infections like salmonellosis are examples of the development and spread of multiresistant bacteria from animals to man via the food chain. Recent data from several European countries show a decreasing prevalence of susceptible *S.* typhimurium strains. This is caused by the clonal spread of multiresistant *Salmonella* typhimurium DT104, which is present in all species of farm animals, especially in poultry, cattle and

pigs. This phage type of *S.* typhimurium causes severe illness, particularly in cattle (Angulo, 1997; Liesegang et al., 1997; Van Pelt and Leeuwen; 1998; Wall, 1997; Wray, WHO, 1997). The use of antibiotics in food animal production may be one of the factors contributing to the rapid dissemination of multiresistant *S.* typhimurium DT104 (Wegener, 1998). Nevertheless, other factors might be important as well, which can be illustrated by the example of *S.* Enteritidis PT4, where a global clonal spread of a bacterium occurred that is in general very susceptible to all antibiotics.

Resistance genes causing so-called ACSSuT type resistance (resistance against **a**mpicillin, **c**hloramphenicol, **s**treptomycin, sulphonamides, **t**etracyclines) occur as a group of chromosomally integrated resistance determinants with a tendency to acquire further resistance determinants (i.e. trimethoprim and fluoroquinolones). Resistance to fluoroquinolones can also be acquired by mutation. This latter resistance type is quite often found in Salmonella spp. and causes marked concern because of the chance of therapy failure. In many countries fluoroquinolones are considered the first choice drug in humans for treatment of acute gastrointestinal infections. In 1996 more than 95 % of *S.* typhimurium DT104 strains isolated from humans and sent to the Public Health Laboratory Service (PHLS) Laboratory for Enteric Pathogens in the UK (Threlfall, 1997) and about 90 % of the *S.* typhimurium DT104 registered in the National Salmonella Reference Centre in Germany (Liesegang et al., 1997) were multiresistant to at least four antibiotics. In these countries *S. typhimurium DT104* is the 2nd most important *Salmonella* causing infections in humans.

4. Evolution of Resistance

Whenever antibiotics are used bacteria will inevitably develop resistance, either by mutation, gene acquisition, or a combination of both. Resistant strains have survived under the protection and selection by the antibiotic. Little is known how those resistance genes first emerged or why it took quite a long period for many of them to emerge. Some of these genes may have awaited mutations necessary to evolve from ancestral genes within the species. Other genes may have already existed in environmental bacteria and have been transferred to species that are of more (veterinary) medical interest (O'Brien, 1997).

Extra chromosomal resistance plasmids were already present in bacteria isolated in the pre-antibiotic era. Most resistance genes in bacteria are identical or homologous to those found in antibiotic-producing microorganisms in the soil (see table 3). Antibiotic resistance must be as ancient as antibiotic synthesis and resistance genes were already present in natural environments in soil and water long before the therapeutic use of antibiotics commenced 50 years ago with the advent of penicillin. Their presence is believed to be related to the production of antibacterial agents by saprophytic organisms such as actinomycetes to protect these antibiotic producing species against self-destruction (Burns, 1995; Bergogne-Bérézin, 1997). For instance, plasmids in *Streptomyces* spp. often include resistance genes to the same antibiotics these organisms produce. Therefore, soil and the natural environment might constitute a huge reservoir for antibiotic resistance genes.

Table 3: Resistance mechanisms found in bacteria with biochemical homologues in antibiotic producing organisms (in soil)

Antibiotic	**Resistance mechanisms**
Penicillins, Cephalosporins	ß-lactamases, Penicillin-binding proteins
Aminoglycosides	Acetyltransferases Phosphotransferases Nucleotidyltransferases
Chloramphenicol	Acetyltransferases
Tetracyclines	Efflux system Ribosomal protection
Macrolides Streptogramins Lincosamines	Ribosomal RNA methylation Esterases Phosphotransferases Acetyltransferases
Glycopeptides	vanA-Ligase

However, widespread emergence of genes expressing resistance to drugs with antibiotic properties occurred only after the agent became widely used. Within the European Union antibacterial drugs available for clinical use have increased within 36 years from 5 antibiotics (plus sulphonamides) in 1959 to 102 different molecules (Bergogne-Bérézin, 1997) in 1995. Since the first introduction of antibiotics in the late 1940s an inexorable propagation of antibiotic resistance genes in bacterial pathogens, zoonotics and commensals has occurred. In

particular, the production and use of large quantities of antibiotics have undoubtedly contributed to the selection of bacterial clones possessing resistance genes. Moreover, recently DNA sequences containing antibiotic-resistance genes were even found in commercial antibiotic preparations (Davies, 1997). The resistance gene pool in the environment is readily accessible to bacteria, which are exposed to strong selective pressures by antibiotic usage in hospitals, for veterinary and agricultural purposes or as growth promoters in animal husbandry (Davies, 1994).

4.1. Resistance mechanisms

Over the years bacteria have developed numerous, and often elegant, ways to escape the action of antibiotic agents. The most common antibiotic resistance mechanism is drug inactivation. Concerning evolution of antibiotic resistance bacteria utilise three main **resistance strategies** (Moreillon, 1995):

1. Modification of their permeability: Bacteria modify their permeability either by becoming impermeable to antibiotics or by actively excreting the drug accumulated in the cell.
2. Modification of the antibiotic: Bacteria produce enzymes capable of modifying and directly inactivating antibiotics.
3. Modification of target: bacteria modify the structure of the antibiotic's target molecule, usually an essential metabolic enzyme of the bacterium - and thus escape the drug's toxic effect.
4. Overproduction of the target.

To understand resistance mechanisms the knowledge of targets of commonly used classes of antibiotics is essential as summarised in table 4.

Table 4: Main bacterial resistance strategies against classes of antibiotics

Class of antibiotics	**Cellular target**	**Modification of**				**Over-production**
		bacterial cell permeability		**antibiotic Inactivation**	**target**	
		Decreased influx	**Increased efflux**			
ß-Lactams	Penicillin-binding proteins	+	-	+	+	-
Aminoglycosides	30S ribosomal subunit	+	-	+	+	-
Sulphonamides **Trimethoprim**	Dihydropteroate synthetase Dihydrofolate reductase	+	-	-	+	+
Macrolides and Lincosamides	50S ribosomal subunit	+	+	+	+	-
Streptogramins	50S ribosomal subunit	+	+	+	+	-
Tetracyclines	30S ribosomal subunit	-	+	+	+	-
Chloramphenicol	50S ribosomal subunit	+	-	+	-	-
Quinolones (Fluoroquinolones)	DNA gyrase Topoisomerase IV	+	+	-	+	-
Glycopeptides	Pepdidoglycan precursor	-	-	-	+	-

4.2. Persistence of antibiotic resistance

To evaluate the persistence of antibiotic resistance is difficult since the genetic ecology of antibiotic resistance is complex and analysis is usually retrospective. Although antibiotics achieve selection for a new type of resistance quite rapidly, a removal of the antibiotic reverses this trend only slowly. As the selected resistant bacteria (for instance by tetracyclines) are just as „fit" as the susceptible flora; they continue to propagate and persist (Levy, 1997). Furthermore, in cases where a bacterium is multiresistant with genetically linked resistance determinants,

resistance will remain present as long as other antibiotics are still used to which the determinant is genetically linked (co-selection).

Nevertheless, in some areas in the world it was seen that stopping the use of tetracycline, streptomycin (Morell, 1997) or chloramphenicol caused the resistance to decrease very slowly (over the years).

4.3. Factors that influence antibiotic resistance of bacteria

Subtherapeutic use

Pharmacokinetic characteristics of different classes of antibiotics may favour the development of resistance as well as dose regimen (e.g. insufficient, too short duration of treatment or long-term use), active concentration or route of excretion of the drug (Davies, 1994). Especially long term use of sub-MIC concentrations (subtherapeutic doses) is regarded as one of the major factors responsible for development of resistance, exerting a potent selective pressure for the emergence of resistant clones that already pre-existed in the bacterial population (Bergogne-Bérézin, 1997; Corpet et al., 1989). The progressive emergence of insensitive bacteria and of acquired resistance in human clinical settings and the veterinary fields reflects the "tuning of these microorganisms to antibiotic polluted" eco-systems (Courvalin, 1996).

The **amount of antibiotic consumption** is also a selective force (Prescott, 1997; Swartz, 1997). Some studies have shown that antibiotic usage has directly contributed to an increased prevalence of resistance (Pradier et al., 1997; DANMAP, 1998). The association of local antibiotic consumption and development of resistance has been shown in many hospital studies (Alos et Carnicero, 1997; Huovinen et al., 1997) and in some studies in veterinary medicine (Rassow and Schaper, 1996; Klarmann, 1997). Using antibiotics for treatment, therapy, metaphylaxis or prophylaxis in human and veterinary medicine and agriculture has exerted an enormous global selective pressure (Acar and Goldstein, 1997; Cohen, 1997; Levy, 1997). The evolution and dissemination of resistance is clearly associated with use, over- and misuse of antibiotics (Courvalin, 1996).

4.4. Evolution of resistance in special antibiotic classes

ß-lactamases are enzymes found in most bacterial species, which are able to hydrolyse the ß-lactam ring of ß-lactam antibiotics. In Gram-negative bacteria and all *Enterobacteriaceae* chromosomally encoded ß-lactamases confer resistance (Davies, 1997). Although these enzymes have protected bacteria against naturally occurring ß-lactams long before the introduction of synthetic antibiotic agents, the numbers and varieties of ß-lactamases have increased dramatically since the introduction of modern penicillins and cephalosporins. The development of ß-lactam antibiotics responds to the evolution of bacterial resistance to this class of antibiotics. A single base change in the gene for a ß-lactamase can change the substrate specificity of the enzyme. Such changes occur frequently, especially in the *Enterobacteriaceae*. Stepwise selection of variants within the extended ß-lactamases classes has been documented, Most bacterial species can synthesise at least one of the more than 200 described ß-lactamases (Bush, 1997; Medeiros, 1997). In addition, genes encoding the enzymes have been hopping out of the chromosome onto plasmids and then back onto the chromosome, resulting in virtually unrestrained transferability of many enzymes. Also, outbreaks of infections with ß-lactam-resistant bacteria can be traced to the introduction of specific classes of ß-lactams or the introduction of a specific agent.

Aminoglycosides resistance mechanisms in all types of bacteria have become more complex with the increased usage of aminoglycosides in time. Combinations of mechanisms occurred widening the spectrum of aminoglycoside resistance in all genera (Miller et al., 1997), for example 53 different inactivating enzymes have been observed in *Enterobacteriaceae* in Europe.

Sulphonamides and **trimethoprim**, both synthetic antibacterial agents, were firstly used in 1932 and 1962, respectively. A dramatic increase in resistance to trimethoprim along with the high-level resistance to sulphonamides has been observed during the past two decades. The number of genes encoding plasmids for resistance against dihydrofolate reductase is already 17, 16 in Gram-negative bacteria and one in *S. aureus*.
Resistance mechanisms show a remarkable evolutionary adaptation. Most of these genes are transferred by efficient gene cassettes. The genetic linkage of genes for both, trimethoprim and sulphonamide resistance largely invalidates the argument for using the combination of trimethoprim and a sulphonamide to prevent the development of resistance (Huovinen, 1997).

Regarding resistance in **macrolides, lincosamides and streptogramins** amongst others three methylases (ERMA, ERMB and ERMC) have been identified. The corresponding genes are located either on transposons (*erm*A, *erm*B) or on small plasmids (*erm*C). Among these *erm* genes, *ermC* has been detected most frequently in Staphylococci from humans and animals (Werckenthin et al., 1997). Other transferable mechanisms of resistance

to streptogramins involve inactivating enzymes such as streptogramin-A acetyltransferase (*sat*-gene) and streptogramin-B hydrolase (*vgb*-gene) and have been described in staphylococci and enterococci (Zervos, 1997).

Primary target of **fluoroquinolone** action is DNA gyrase, a type II bacterial topoisomerase composed of two A subunits and two B subunits, encoded by *gyrA* and *gy B* genes, respectively. Topoisomerase IV is another important target, especially in Gram-positive organisms. As quinolones did not exist in nature they had no analogues among microbial biosynthetic products before their development and introduction into human and veterinary medicine. A single mutation in the "quinolone resistance determining region" of the bacterial genome provides an increment for survival, a subsequent „hot spot" mutation produces even higher levels of resistance (Davies, 1997). The emergence of resistance to fluoroquinolones in all species of bacteria was recognised soon after the introduction of these compounds for clinical use more than 10 years ago and evolved extremely rapidly.

Resistance genes (*tet*-genes) are known to be the basis for **tetracycline resistance** in Gram-positive bacteria. These resistance determinants promote efflux of the drug out of the bacterial cell, inactivation of tetracycline or target protection and are transposon-associated. Some of them are found in Gram-positive and as well in Gram-negative bacteria; e.g. *Pasteurella* spp. can contain *tet*-genes from Gram-positive and Gram-negative origin or both. The conjugative transfer, mobilisation and transposition of conjugative transposons that encode tetracycline resistance in some bacteria might also be regulated by the presence of tetracyclines. At least a 100-fold increase in gene transfer was observed in bacteria harbouring the transposon when exposed to low concentrations of tetracyclines. The implications of these findings are alarming. Not only the expression of the antibiotic resistance gene is dependent on the antibiotic, but the antibiotic also provokes the transfer of its own resistance genes. Subinhibitory concentrations of antibiotics (such as tetracyclines) may also stimulate cell-to-cell contact, and thus gene transfer, by causing subtle changes in bacterial outer membrane structure. The tetracycline group best exemplifies widespread dissemination of resistance genes within the bacterial population. This class of antibiotics has been widely used for all imaginable purposes and the ecology of the *tet* determinants is a model case for resistance-gene dissemination (Roberts, 1997).

Literature:

References can be obtained from the author

LEVEL OF TETRACYCLINE AND AMPICILLIN RESISTANT LACTOSE-POSITIVE ENTERIC COLIFORMS IN DIFFERENT AGE CATEGORIES OF FATTENING PIGS

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To evaluate the importance of the porcine intestinal flora as antimicrobial resistance reservoir, a cross-sectional field trial was conducted, in which the level of tetracycline and ampicillin resistant lactose-positive enteric coliforms (LPEC) was determined in fattening pigs of three age categories. Faecal samples were collected on thirty-two randomly selected pig herds and analysed using an agar dilution technique. A large number of LPEC were resistant to tetracycline (57.8 ± 21.7%) and ampicillin (36.8 ± 23.4%). For both antibiotics, treated animals had a significant higher level of resistance than non-treated animals (tetracycline $p=0.02$; ampicillin $p<0.01$). The level of resistance to tetracycline was not significantly different between age categories ($p=0.07$), whereas the level of resistance to ampicillin was significantly different ($p<0.01$). The results of this study reflect the effect of the selection pressure exerted by the use of antimicrobial agents in Flemish pig herds. These results also indicate that the effect of age on the level of antimicrobial resistance differs for two frequently used antibiotics in pigs.

Om het belang van de normale darmflora van varkens als bron van antibioticum resistentie te evalueren, werd een dwars-doorsnede studie uitgevoerd, waarbij het niveau van tetracycline en ampicilline resistente faecale lactose-positieve coliformen (LPEC) werd bepaald bij vleesvarkens van drie verschillende leeftijden. Faecesstalen werden verzameld op 32 at random geselecteerde varkensbedrijven en de stalen werden onderzocht met behulp van een agar dilutie techniek. Tetracycline (57.8 ± 21.7%) en ampicilline (36.8 ± 23.4%) resistentie werd frequent gedetecteerd bij de LPEC. Zowel voor tetracycline ($p=0.02$) als ampicilline ($p<0.01$) was de resistentie significant hoger bij behandelde dieren. Het niveau van tetracycline resistentie bleek niet significant verschillend tussen de leeftijdscategorieën ($p=0.07$). Voor ampicilline resistentie daarentegen, was dit wel significant verschillend ($p<0.01$). Deze resultaten tonen het effect van de selectiedruk uitgeoefend door het gebruik van antibiotica in Vlaamse varkensbedrijven. Bovendien tonen deze resultaten dat de invloed van leeftijd op de antibioticum resistentie verschillend is voor twee veelgebruikte antibiotica bij varkens.

1. INTRODUCTION

Antibiotics are used in livestock production for the treatment of diseases and to enhance growth of animals [6]. The selection pressure exerted by these antimicrobials has caused the emergence of antimicrobial resistance, not only in pathogenic bacteria, but also in non-pathogenic bacteria of the commensal flora [1,12]. The commensal flora represents a reservoir of resistant bacteria and resistance genes. Especially the intestinal flora constitutes a large reservoir of resistance genes [12], which may be the cause of resistance in humans. Contamination of carcasses with faecal flora occurs during slaughtering, causing food of animal origin as a possible vehicle to transport resistant bacteria and resistance genes between animals and humans [3,9,12].
In order to evaluate the importance of the porcine intestinal flora as antimicrobial resistance reservoir, a cross-sectional field trial was conducted in West and East Flanders, in which the level of tetracycline and ampicillin resistant lactose-positive enteric coliforms (LPEC) was determined in fattening pigs of different age categories. The antibiotics were selected because they, or related antibiotics, are regularly used in growing and fattening pigs.

2. MATERIALS AND METHODS

2.1 Selection of farms

The herds were selected from the Sanitel-database. The selection was based on three criteria: (1) closed or semi-closed, (2) located in West-or East-Flanders and (3) at least 150 sows and 600 fattening pigs. A total of 821 farms fulfilled these criteria. Fifty farms were randomly selected out of this list and were contacted by letter to inform them about the investigation. Shortly hereafter, the farms were contacted by phone. Cooperation was on a voluntary basis. Thirty-two farmers were willing to cooperate, four farmers had stopped their activities or were intending to do so and 14 farmers didn't want to cooperate (response rate of 64%). Between March and October 2003, all thirty-two farms were visited on a single occasion. During the visit, a questionnaire was filled in and faecal samples were collected.

2.2 Sampling

Samples were taken from fattening pigs of three different age categories: 10 ± 2 weeks, 18 ± 2 weeks, 26 ± 2 weeks. All animals in every age category were raised under the same housing conditions and were given the same treatment regimen, with the exception of some individual treatments. In this study 'treated' means that the animals received, at least once during their life, a treatment with tetracycline or ampicillin, or related antibiotics.

In each age category, 16 pigs (4 pens, 4 animals per pen) were randomly selected for the collection of faecal samples. Samples were taken via rectal stimulation and were put in a 30 ml sterile recipient. Immediately after sampling, the samples were transported to the laboratory where they were processed within a few hours after sampling.

2.3 Bacteriological analysis and determination of resistance

The faecal samples of each pen were pooled to a volume of one gram to minimize the influence of an individual treatment. The pooled samples were suspended in 9 mL of phosphate buffered saline. After vortexing, 10-fold dilutions were made. The quantification of sensitive and resistant LPEC was done by means of an agar dilution procedure. Therefore, two series of six tenfold dilutions were plated on three media: (1) MacConkey agar N° 3 (Oxoid) without antibiotic, (2) MacConkey agar N° 3 (Oxoid) with 16 µg/mL oxytetracycline (Sigma) and (3) MacConkey agar N° 3 (Oxoid) with 4 µg/mL ampicillin (Sigma). The plates were incubated aerobically at 37°C for 24 hours and lactose-positive colonies were counted [4]. The breakpoint for oxytetracycline was based on the NCCLS guidelines [8]. The breakpoint for ampicillin is in accordance with the CRG guidelines [2], which indicates that *Enterobacteriaceae* with MIC ≤2µg/mL are sensitive.

The level (%) of antibiotic resistance in each faecal sample was calculated using the following formula: the average number of colony forming units (CFU) growing on the two plates containing antibiotics divided by the average number of CFU on the two antibiotic-free control plates x 100 [4,12].

2.5 Statistical analysis

The results were analysed using multivariate linear mixed models with herd as random factor (S-Plus, 2000). Results are presented as mean ± standard deviation.

3. RESULTS

The faeces obtained from the pigs in this study contained an average LPEC count of 6.36 ± 0.92 $\log_{10}$ CFU/g faeces for the 10 ± 2 weeks group, 6.38 ± 0.84 $\log_{10}$ CFU/g faeces for the 18 ± 2 weeks group and 6.55 ± 0.63 $\log_{10}$ CFU/g faeces for the 26 ± 2 weeks group.

A large number of these LPEC (57.8 ± 21.7%) appeared to be resistant to tetracycline. Also for ampicillin, a relatively high level of resistant LPEC (36.8 ± 23.4%) was observed. For both antibiotics, treated animals had a significant higher level of resistance than non-treated animals. This difference was more pronounced for ampicillin (45.5 ± 21.4% vs. 20.7 ± 17.1 %) ($p<0.01$) than for tetracycline (66.4 ± 18.3% vs. 53.0 ± 21.2%) ($p=0.02$).

In Table 1, the level of tetracycline and ampicillin resistant LPEC according to age category and treatment regimen are presented. The level of resistance to tetracycline was not significantly different between age categories ($p=0.07$). Ampicillin resistance did significantly differ between the age categories ($p<0.01$).

Table 1: Level of tetracycline and ampicillin resistant lactose-positive enteric coliforms (LPEC) according to age and treatment regimen

	Tetracycline resistant LPEC (%)		Ampicillin resistant LPEC (%)	
Age category (weeks)	Treated[a]	Non-treated[b]	Treated[c]	Non-treated[d]
10 ± 2	65.5 ± 19.3 (7)	56.4 ± 25.8 (25)	58.1 ± 26.5 (20)	27.5 ± 26.6 (12)
18 ± 2	66.4 ± 23.8 (9)	53.6 ± 20.2 (23)	37.2 ± 15.1 (21)	20.1 ± 11.4 (11)
26 ± 2	66.9 ± 13.2 (11)	48.4 ± 17.2 (21)	41.2 ± 17.0 (21)	14.6 ± 8.1 (11)
Total resistance per treatment regimen (%)	66.4 ± 18.3%[e]	53.0 ± 21.2%[f]	45.5 ± 21.4%[g]	20.7 ± 17.1 %[h]

[a,c]Resistance in animals who received tetracyclines or ampicillin/amoxycillin, respectively. The number of farms, which used these antibiotics in the respective age category, is stated between brackets.
[b,d]Resistance in animals who did not receive tetracyclines or ampicillin/amoxycillin, respectively.
[e,f] Values with different superscript are significantly different ($p<0.01$)
[g,h] Values with different superscript are significantly different ($p=0.02$)

4. DISCUSSION

Although the division of the samples in treated and non-treated was based on what the farmer could remember, we are confident that all farmers were able to give reliable information on whether the animals were treated or not, since most treatments were done on a regular basis.

Our overall results show that resistance to tetracycline is relatively high in Flemish pig herds. This is in accordance with what has been observed by Moro et al. (1998). Although treated animals have a significant higher level of resistance, the level of resistance in the non-treated group is also high. The former is probably due to the intensive use of tetracyclines (oxytetracycline, tetracycline, chlortetracycline and doxycycline). The latter is probably due to the long persistence of tetracycline resistance [1,5]. According to other authors [6,7], younger animals have higher resistance levels than older animals. In this study no significant decrease in resistance with age was observed for tetracycline, in either treated animals or non-treated animals. In contrast, a small increase could be seen in the treated animals. This is probably due to the higher use of tetracyclines in older animals.

The overall results for ampicillin also show a relatively high level of resistance, which is higher than found by Moro et al. (1998). This can be explained by the inclusion of treated animals to calculate the overall result and by the use of a low breakpoint of 4 µg/mL, which may also include intermediate bacteria. The high level of resistance was mainly pronounced in the treated group. The significant difference between the treated group and the non-treated group is probably a result of the intensive prophylactic and therapeutic use of ampicillin and amoxycillin in nursery pigs. Looking at the differences between age categories, both groups show a decrease in resistance, as animals grow older. For the treated group, the higher level of resistance in younger animals can be explained by the frequent use of ampicillin and amoxycillin in nursery pigs, but less in fattening pigs. The results in the non-treated group are in accordance with the reduction of resistance with age as generally described in literature [6,7].

This study shows that the intestinal flora of pigs contains a large reservoir of resistance, which is easily influenced by treatment. These results also indicate that the effect of age on the level of antimicrobial resistance differs for two frequently used antibiotics in pigs. As antimicrobial resistance develops easily, it is hard to get rid of [10]. Therefore, a low level of antibiotic resistance in the intestinal flora of food animals should be considered as a distinguishing quality and safety mark [11].

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STAPHYLOCOCCUS AUREUS STRAINS FROM RABBITS ARE RARELY RESISTANT TO ANTIBIOTICS

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Fifty-six *Staphylococcus aureus* isolates recovered between 1998 and 2003 from 31 rabbit farms with and without problems of chronic staphylococcosis, were screened for antibiotic resistance. Four isolates showed resistance to erythromycin and lincomycin. These isolates were positive for the *erm*(C) gene in the PCR. Eleven strains were resistant to tetracyclines. All these isolates were positive for the *tet*(K) gene. In the agar dilution test, five isolates showed resistance to penicillin. However, in the disk diffusion test 12 isolates showed resistance due to the production of penicillinase. None of these 12 resistant isolates carried the *mec*(A) gene determining resistance to penicillinase-stable penicillins and cefalosporins. Only one strain showed resistance to gentamicin, and all strains were susceptible to enrofloxacin and neomycin. This study demonstrates that antibiotic resistance in *S. aureus* isolates originating from rabbits is rare compared to resistance in *S. aureus* isolates originating from other animals and from humans.

Zesenvijftig *Staphylococcus aureus* stammen, verzameld tussen 1998 en 2003 op 31 konijnenbedrijven met en zonder problemen van chronische staphylococcose, werden onderzocht op antibioticumresistentie. Vier isolaten waren resistent aan erythromycine en lincomycine. Deze isolaten waren positief voor het *erm*(C) gen in de PCR. Elf stammen waren resistent aan tetracyclines. Al deze stammen waren positief voor het *tet*(K) gen. In de agardilutie test waren vijf isolaten resistent aan penicilline. In de schijfdiffusietest echter bleken 12 isolaten penicillinase te produceren. Geen enkele van deze 12 resistente isolaten bezat het *mec*(A) gen dat codeert voor resistentie tegenover de penicillinase-stabiele penicillines en cefalosporines. Slechts één stam was resistent aan gentamicine. Alle stammen waren gevoelig aan enrofloxacine en neomycine. Deze studie toont aan dat antibioticumresistentie bij *S. aureus* isolaten afkomstig van konijnen zeldzaam is vergeleken met resistentie bij *S. aureus* isolaten afkomstig van andere dieren en van mensen.

1. INTRODUCTION

In rabbits, *Staphylococcus aureus* bacteria infect small dermal lesions and invade subcutaneous tissue (20). In individual rabbits, all *S. aureus* infections have a similar clinical appearance, with lesions of pododermatitis, subcutaneous abscesses and mastitis (11, 20, 3, 14, 21, 7). Sporadically, internal organ abscesses are observed as well, predominantly in lungs, liver and uterus. This leads to poor production results, infertility and death. Suckling young may die as a result of agalactia in the doe. At rabbit flock level, two types of *S. aureus* infections can be distinguished. In the first type, the infection remains limited to a small number of animals; this type only has a minor economical importance. In the second type of infection, caused by the so-called high virulence strains, *S. aureus* causes an epidemic spread of disease in the rabbitry. This leads to chronic problems.
It is generally accepted that infections with high virulence *S. aureus* strains in rabbits cannot be eradicated with antibiotics (3, 14, 21). The reason for this remains unknown. Not much information is available on the antimicrobial resistance of *S. aureus* strains from rabbits. The purpose of this study was to determine the prevalence of resistance

phenotypes for a number of antibiotics among rabbit *S. aureus* isolates and to screen for the genes responsible for their resistance.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

In the present studies, 56 rabbit *S. aureus* strains were tested. Nineteen of these strains were high virulence strains, all isolated from commercial rabbitries with chronic problems of staphylococcosis. Eighteen of these strains belonged to the biotype – phagetype combination "mixed CV-C" – 3A/3C/55/71 and RAPD type a. One strain belonged to the biotype – phagetype combination "mixed CV-C" – 29/79/42E/92/D11/HK2 and RAPD type m (7, 13). The other strains were low virulence strains, belonging to other biotypes, phagetypes and RAPD types.

2.2 Dilution susceptibility testing

Minimal Inhibitory Concentrations (MICs) of the following antimicrobial agents were determined: tetracyclines, lincomycin, gentamicin, erythromycin, neomycin, penicillin, and enrofloxacin. The MICs were determined using the NCCLS (National Committee for Clinical Laboratory Standards) agar dilution method (19). Bacteria were inoculated in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, England) and, after overnight incubation at 37°C, suspended in 0.9% NaCl solution to a 0.5 McFarland standard. By means of a Steers inoculum applicator, a 1/10 dilution of these suspensions was inoculated on Mueller-Hinton II agar (Beckton Dickinson, Le Pont de Claix, France) containing tetracycline, lincomycin, gentamicin, erythromycin and neomycin concentrations ranging from 0.12 µg/ml to 64 µg/ml and enrofloxacin and penicillin concentrations ranging from 0.03 µg/ml to 8 µg/ml. The plates were incubated at 37°C and observed after 24h. The MIC was defined as the lowest concentration producing no visible growth. Criteria for resistance were those formulated in the NCCLS Approved Standard (19).

2.3 Disk susceptibility testing

In order to detect ß-lactamase (penicillinase) activity, penicillin G susceptibility tests were additionally determined by disk diffusion on Isosensitest agar (Oxoid, Basingstoke, England) using Penicillin Low Neo-Sensitabs (Rosco Diagnostics, Taastrup, Denmark). The plates were inoculated with the above described 0.5 McFarland standard suspension and after overnight incubation at 37°C, zone edges were examined. ß-lactamase positive strains show heaped-up borders, while inhibition zones of susceptible strains have diffuse margins.

2.4 PCR assay on antibiotic resistance genes

A PCR assay was performed to detect the presence of *tet*P(B), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(T), *tet*(W), *erm*(A), *erm*(B) and *erm*(C) in all isolates. The isolates that proved to be resistant in the penicillin disk diffusion test were screened for the presence of the *mec*(A) gene.

To prepare DNA, one colony of bacterial cells was suspended in 20 µl lysis buffer (0.25% SDS, 0.05N NaOH) and heated at 95°C for 5 minutes. The cell lysate was spinned down by short centrifugation at 16000 g, and then diluted by adding 180 µl distilled water. Another centrifugation for 5 minutes at 16000 g was performed to remove the cell debris. Supernatants were frozen at -20°C until further use.

The primers, annealing temperatures and positive controls are shown in Table 1.

After amplification, 4 µl amplicon was mixed with 2 µl sample buffer (50% glycerol, 1 mM cresolred). Electrophoresis was performed using a gel containing 1.5% Agarose I (Amresco, Solon, USA) and 50 µg/l ethidium bromide in 1 x TBE buffer (0.9 M TrisHCl, 0.9 M boric acid, 0.02 M EDTA). Gels were run for 70 min at 175 V in a tank containing 0.5 x TBE buffer (0.45 M TrisHCl, 0.45 M boric acid, 0.01 M EDTA). After electrophoresis, gels were visualized under U.V. light and photographed. The Gene Ruler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot, Germany) was used as a DNA size marker.

3. RESULTS

3.1 Susceptibility testing

Four strains were resistant to erythromycin and lincomycin. There was only one other strain that showed multiple resistance, to gentamicin and penicillin. All the other strains were either susceptible to all antibiotics or resistant to only one antibiotic. All strains were susceptible to neomycin and enrofloxacin. The highest levels of resistance in the agar dilution test were found to tetracyclines (11 strains), followed by penicillin (5 strains). In the disk diffusion test for penicillin however, twelve of the 56 strains were found to produce ß-lactamase. No differences were found in

antibiotic resistance between high virulence strains and low virulence strains. Overall, fourteen (25%) of the 56 strains were resistant to at least one antibacterial drug tested.

Table 1.

Class targeted	**Primer sequence**	**PCR annealing temp (°C)**	**Amplicon size (bp)**	**Positive control**	**Reference**
Erm(A)	5'TCTAAAAAGCATGTAAAAGAA3' 5'CTTCGATAGTTTATTAATATTAGT3'	52°C	640	Staphylococcus aureus RN/389	(23)
Erm(B)	5'GAAAAGA/GTACTCAACCAAATA3' 5'AGTAACGGTACTTAAATTGTTTAC3'	57°C	640	Streptococcus pyogenes SYP016	(23)
Erm(C)	5'TCAAAACATAATATAGATAAA3' 5'GCTAATATTGTTTAAATCGTCAAT3'	49°C	640	Staphylococcus aureus R/V4220/PE/94	(23)
Tet(K)	5'TTATGGTGGTTGTAGCTAGAAA3' 5'AAAGGGTTAGAAACTCTTGAAA3'	50°C	1159	Escherichia coli EC7	(24)
Tet(L)	5'ATAAATTGTTTCGGGTCGGTAAT3' 5'AACCAGCCAACTAATGACAAGT3'	50°C	1077	E. coli C65	(24)
Tet(M)	5'ACAGAAAGCTTATTATATAAC3' 5'TGGCGTGCTAGAGTCAC3'	55°C	171	E. coli EC6	(1)
Tet(O)	5'ACGGA/AGTTTATTGTATACC3' 5'TGGCGTATCTATAATGTTGAC3'	58°C	171	E. coli tet(O)	(1)
*Tet*P(B)	5'AAAACTTATTATATATAGTG3' 5'TGGAGTATCAATAAATTCAC3'	46°C	169	E. coli EC70	(1)
Tet(T)	5'AAGGTTTATTATATAAAAGTG3' 5'AGGTGTATCTATGATATTTAC3'	44°C	169	Streptococcus pyogenes tet(T)	(1)
Tet(W)	5'GAGAGCCTGCTATATGCCAGC3' 5'GGGCGTATCCACAATGTTAAC3'	64°C	168	Escherichia coli tet(W)	(1)
Mec(A)	5' AAA ATC GAT GGT AAA GGT TGG C3' 5' AGT TCT GCA GTA CCG GAT TTG C3'	55°C	533	Staphylococcus aureus ULB1	(2)

3.2 PCR assay on antibiotic resistance genes

The four strains resistant to erythromycin and lincomycin were shown to be *erm*(C) positive in the PCR assay. None of the strains carried the *erm*(A) or the *erm*(B) gene. All of the strains resistant (MIC of ≥16 µg/ml) to tetracyclines were *tet*(K) positive in the PCR assay. None of the strains were positive in the PCR assay for *tet*P(B), *tet*(L), *tet*(M), *tet*(O), *tet*(T), and *tet*(W).

The strains resistant to penicillin in the disk diffusion test all were negative in the PCR assay for the *mec*(A) gene.

4. DISCUSSION

The relatively low percentage (25%) of isolates resistant to one or more antibiotics contrasts with the values found in bovine (49.7%) isolates (18). Penicillin resistance (21%) is low compared to levels described for human (over 90%) as well as bovine (around 50%) isolates (12, 6, 26). The levels of resistance to erythromycin, lincomycin and neomycin are comparable to those found in bovine isolates (6, 26). There is a striking difference between the level of resistance of the rabbit isolates to tetracyclines (20%) and the level of resistance (>90%) formerly described in poultry isolates (10). In human medicine, methicillin-resistant *S. aureus* strains are a major problem, with resistance levels up to 36% (26, 5). In veterinary medicine, however, methicillin-resistant *S. aureus* are reported only occasionally (8, 15, 25, 4). This is in agreement with the results of this study, as the *mec*(A) gene encoding for methicillin resistance was not detected in the PCR assay. Only ß-lactamase positive strains were tested here, because *mec* genes are typically found in such strains. No resistance was detected against enrofloxacin, and only 1 rabbit *S. aureus* strain out of 56 was resistant to gentamicin. This is in agreement with the situation for bovine *S. aureus* isolates in most countries, except for Germany, where resistance for bovine *S. aureus* isolates was reported to be 10.1% for enrofloxacin and 51.2% for gentamicin (26).

Four out of 56 rabbit *S. aureus* isolates were resistant to erythromycin and lincomycin, although these antibiotics are of no use in rabbits, because of their toxicity (9, 22, 17). The fact that these strains are resistant to erythromycin as well as lincomycin is in agreement with their carriage of the *erm*(C) gene which encodes resistance to lincosamides, macrolides and streptogramine B antibiotics (16). A possible explanation for the occurrence of the *erm*(C) gene among rabbit *S. aureus* isolates is the use of spiramycine in rabbits.

With penicillin susceptible to staphylococcal ß-lactamases, interpretations of MIC-tests may be difficult. The very small bacterial inocula used in the NCCLS procedure do not allow sufficient production of this enzyme to become easily detectable.

In view of the fact that antibiotic resistance percentages are low in rabbit *S. aureus* strains, we can conclude that there have to be other reasons why antibiotic therapy is failing in eradicating high virulence *S. aureus* strains at rabbit flock level. Hypotheses about the reason of persistence include the existence of carrier-rabbits and the survival of the bacterium in the environment. However, the exact cause remains to be elucidated.

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DESCRIPTIVE EPIDEMIOLOGY OF THE RESISTANCE OBSERVED IN *ESCHERICHIA COLI* ISOLATED FROM HEALTHY CATTLE, PIGS AND BROILERS, THEIR MEAT AND MEAT PRODUCTS

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From October 2002 to 2003, a pilot surveillance study on resistance was conducted co-ordinated by the Veterinary working party of the Belgian Antibiotic Policy Co-ordination Committee (BAPCOC). Non-pathogenic *E. coli* were isolated from cattle, beef, pigs, pork, broilers and chicken meat. Fecal samples were taken from healthy animals, swabs were taken from carcasses at the slaughterhouse and from meat at cutting plants and meat processing plants.
Susceptibility was tested using the disk diffusion method according to NCCLS against ampicillin/amoxicillin, florfenicol, apramycin, nalidixic acid, chloramphenicol, tetracycline, trimethoprim, enrofloxacin, gentamicin, sulphonamides and ceftiofur. Resistance in *E. coli* from living animals was highest in broilers and pigs. *E. coli* isolated from meat and meat products were more resistant when originating from pork and broiler meat. When comparing resistance of isolates originating from the living animal with resistance of isolates originating from its corresponding meat, resistance percentages were generally higher in cattle and pigs than in beef and pork but for *E. coli* originating from broilers and chicken meat the resistance percentages were similar.

Dit zijn deels de resultaten afkomstig van een pilootstudie uitgevoerd tijdens 2002 en 2003 door de werkgroep diergeneeskunde van de Belgische Commissie ter Coordinatie van het Antibioticabeleid (BAPCOC).
Niet pathogene *E. coli* werd geisoleerd uit runderen, varkens, braadkippen en hun respectievelijke vlees en vleesproducten. Bij levende gezonde dieren werden in bedrijven meststalen afgenomen. Bij karkassen in slachthuizen of verwerkt vlees in de uitsnijderijen en de vleeswerwerkings bedrijven werden swabs genomen.
Alle isolaten werden getest met de “disk diffusie methode” (NCCLS) voor resistentie tegenover ampicilline/amoxilline, florfenicol, apramycine, naladixinezuur, chloramphenicol, tetracycline, trimethoprim, enrofloxacine, gentamicine, sulfonamiden en ceftiofur. Bij het levende dier werden de hoogste resistentiepercentages geobserveerd bij de braadkip en het varken en dit was ook het geval voor kippenvlees en varkensvlees waar de resistentie hoger was dan in rundsvlees. Voor het rund en het varken kan er gesteld worden dat bij het levende dier de resistentiepercentages hoger liggen dan voor respectievelijk rundsvlees en varkensvlees terwijl er geen verschillen vastgesteld werden voor de kiemen afkomstig van de levende braadkip en het vlees afkomstig van braadkippen.

1. INTRODUCTION

Antimicrobial resistance in animal bacteria may be a risk for public health because the gastro-intestinal flora of healthy animals constitutes a reservoir of resistance genes and resistant bacteria. Resistant bacteria can influence directly or indirectly (through the spread of mobile genetic determinants) resistance among human bacteria. However, these hazards are still very badly characterized. A first step is the execution of surveillance studies in which basic resistance levels are determined throughout the different ecosystems. Therefore, we conducted a study to determine the resistance of non-pathogenic *E. coli* isolated from cattle, beef, pigs, pork, broilers and chicken meat.

2. MATERIALS & METHODS

2.1. Sampling

The peripheral veterinary laboratories sampled healthy cattle, pigs and poultry. One sample per randomly selected herd was taken, and only non-diarrheic and non-septicaemic animals were taken into consideration, to avoid the inclusion of pathogenic *E. coli*. For cattle it was chosen to sample from animals below four months of age in order to maximize the probability of isolating *Enterococcus spp*. (data not presented here). Pigs were sampled at the weight of around 70 kg. Samples from chickens (broilers) were taken during the fattening period.
McConkey agar plates inoculated with bovine faecal material were provided by the "*Association Régionale de Santé et Identification Animales"* and plates inoculated with the pig and chicken faecal samples respectively by "*Dierengezondheidszorg Vlaanderen*", Torhout and Drongen.
A total of 401 *E. coli* were isolated from the three animal species (128 from broilers, 127 from cattle and 146 from pigs). Selection of colonies, identification of the isolate, and susceptibility testing was performed at the Veterinary and Agrochemical Research centre.
From meat and meat products a total of 549 *E. coli* were isolated originating from beef (212), from broiler meat (151) and from pork (186). Samples included swabs from carcasses, meat cuts and minced meat (table 1). Selection of colonies, identification of the isolate, and susceptibility testing was performed at the Scientific Institute of Public Health.

Table 1. Number of *E. coli* isolates over the different matrices

Species	Carcass	Filet	Mince	Piece	Total
Beef	175		17	20	212
Chicken	73	54	24		151
Pork	99		48	39	186

2.2. Antimicrobial susceptibility testing

Susceptibility for ampicillin/amoxicillin, florfenicol, apramycin, nalidixic acid, chloramphenicol, tetracycline, trimethoprim, enrofloxacin, gentamicin, sulphonamides and ceftiofur was tested with the disk diffusion method according to NCCLS guidelines using Rosco Tablets (Taastrup, Denmark) except for florfenicol (Mast diagnostics) and ceftiofur (Biorad-Sanofi).
Breakpoints used are shown in table 2.

Table 2. Breakpoints for *E. coli* antimicrobial susceptibility testing, upper and lower limits for intermediate results

	Amox	Ceftio	Genta	Apra	Nal	Enro	Trim	Sulf	Chlor	Flor	Tetra
Upper	19	20	22	19	24	22	19	22	24	18	22
Lower	17	18	20	17	21	17	17	20	21	16	20

2.3. Statistical analysis

The percentage of isolates resistant against a given antibiotic in a given matrix is defined as the number of resistant isolates divided by the total number of isolates tested (being the number of resistant plus intermediate plus susceptible isolates). To compare the antimicrobial resistance percentages between isolates from the different matrices we simulated the uncertainty distribution around each proportion point estimate through Bayesian inference (binomial assumption with non-informative beta priors) [2]. For the multi-resistance we used the parametric (multinomial) bootstrap [1]. The uncertainty is visualized by graphing the 2.5% and 97.5% percentiles of the obtained (posterior) distributions (red lines in the graphs) while the height of the bar in these graphs represent the median (50^{th} percentile) of that distribution.
Comparison between the matrices is done by looking at the overlap of the two-sided 95% intervals. If there is no overlap we assume the difference significant, realizing that this is not entirely correct, as there is no hypothesis tested. A good way to interpret it is that there is a very high probability that difference exists between the compared true population parameters. For the results we only state a parameter as higher or lower if there is no overlap of the 95% intervals.

3. RESULTS

All antimicrobial resistance percentages for the different matrices are graphed in figures 1-3.
E. coli from cattle were shown to be less resistant against ampicillin (42%) and trimethoprim (17%) than those from broilers (ampicillin 67%, trimethoprim 52%) and pigs (ampicillin 62%, trimethoprim 68%). They were also less resistant against nalidixic acid (15%) compared to chicken (41%) and less resistant against tetracycline (45%) and sulphonamides (52%) compared to pigs (tetracycline 80%, sulphonamides 73%). The proportion of isolates susceptible for all tested antibiotics was highest in *E. coli* isolated from cattle (38%) while this was 14% in pigs and 12% in chickens.
For *E. coli* originating from meat and meat products similar trends were observed in the way that bacteria originating from beef had significant lower resistance against amoxicillin (9%), nalidixic acid (4%), trimethoprim (5%), tetracycline (10%) and sulphonamides (17%) compared to chicken meat (amoxicillin 59%, nalidixic acid 39%, trimethoprim 41%, tetracycline 52%, sulphonamides 57%) and pork (amoxicillin 22%, trimethoprim 30%, tetracycline 48%, sulphonamides 44%). Isolates from beef were also more susceptible for all antibiotics tested (47%) than those from chicken meat (11%) and pork (21%).
When comparing resistance of isolates originating from the living animal with resistance of isolates originating from its corresponding meat, resistance percentages are higher in cattle and pigs than in beef and pork, respectively. In *E. coli* originating from broilers and chicken meat the resistance percentages were similar.

Figure 1. Median and 95% credible intervals of resistance observed in *E. coli* from beef and bovines

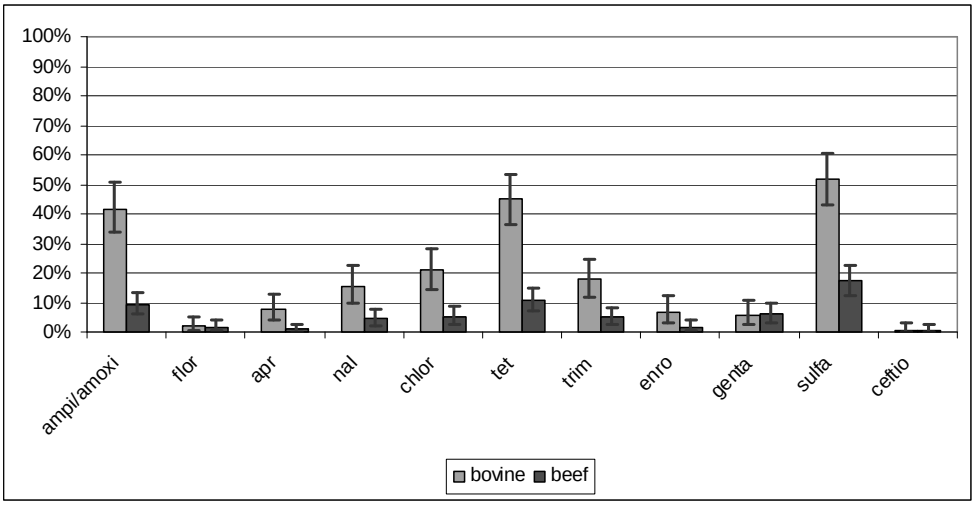


Figure 2. Median and 95% credible intervals of resistance observed in *E. coli* from pigs and pork

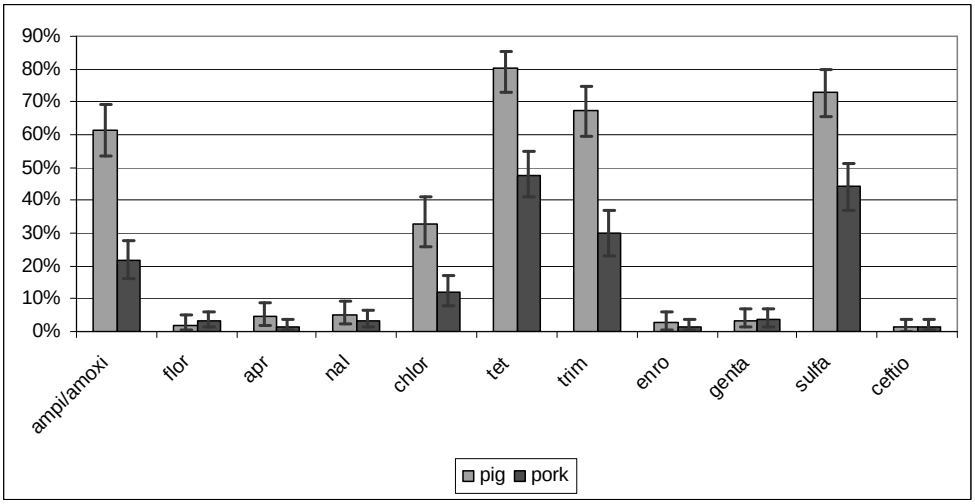
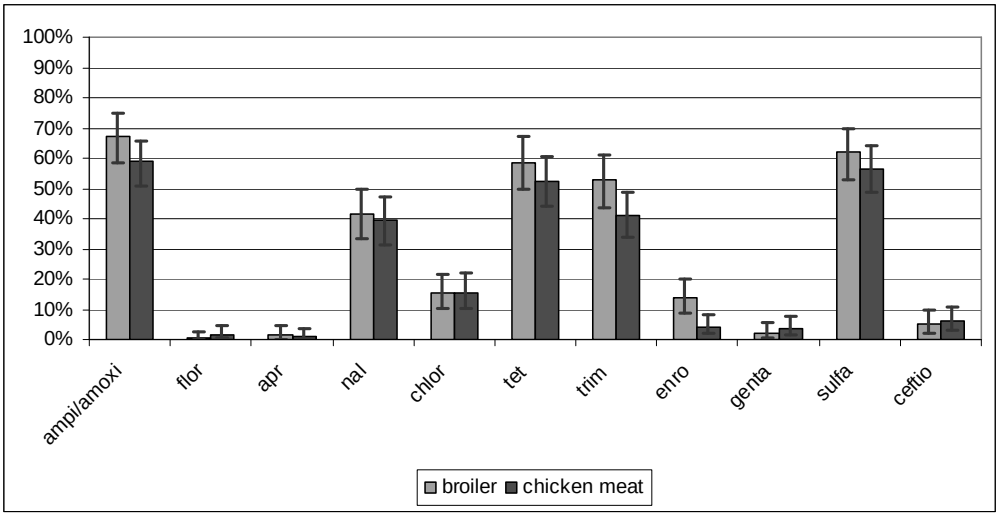


Figure 3. Median and 95% credible intervals of resistance observed in *E. coli* from broilers and chicken meat



4. DISCUSSION

Although in Belgium no concrete consumption data for veterinary antibiotics are available, it is accepted that ampicillin/amoxicillin, nalidixic acid, tetracycline, trimethoprim, sulphonamides are most widely used. The results presented here show that highest resistance percentages in *E. coli* from food animals were observed against these antibiotics.

In general, resistance that we observed in *E. coli* from cattle was lower than resistance observed in pigs and broilers. Similar, resistance observed in strains from beef was lower than those isolated from pork and chicken meat. This is not only visible in lower observed resistance percentages but also in a higher percentage of isolates that were susceptible to all tested antibiotics.

The resistance percentages of isolates from cattle are in general higher than those from beef and resistance observed in *E. coli* from pigs is higher than for pork. In contrast, resistance percentages in *E. coli* originating from broilers and chicken meat were similar. Those differences may be due to differences in slaughter procedures or sampling area's on the carcass (e.g. the neck skin of broilers). In addition, it should be noted that samples from cattle were from calves of less than 4 months of age, while beef usually originates from older animals. Further research is needed to gain more insight.

Though the difference is not significant the resistance against ceftiofur is highest among strains from broilers and chicken meat. There is no obvious explanation for these relative high levels of resistance certainly because in this animal species, the use of ceftiofur is not allowed in Belgium.

Regarding the statistical analysis we like to emphasize that no hypothesis was tested, and in consequence a null hypothesis was not rejected at a certain significance level. The reason for this is that the sampling set-up did not allow to check for over-dispersion, since this can lead to an increase of type I errors (e.g. incorrect conclusion of a significant difference). This problem has been looked at in another part of the pilot project and is presented in these proceedings by Catry et al.

ACKNOWLEDGEMENTS

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