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Presence and mechanism of macrolide-lincosamide resistance in Enterococcus columbae strains belonging to the intestinal flora of pigeons

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Faecal samples from 50 pigeons all originating from different lofts were screened for the presence of macrolide and lincosamide (ML)-resistant isolates of Streptococcus gallolyticus and Enterococcus columbae by plating the samples onto selective media. Sixty-eight ML-resistant E. columbae strains were recovered from the faecal samples of 29 animals. Two of these samples also harboured ML-resistant S. gallolyticus strains. The \textit{erm}(B) gene was detected in 58 E. columbae and in five \textit{S. gallolyticus} isolates. Four of these \textit{E. columbae} isolates also carried the \textit{mef}(A) gene. Five \textit{E. columbae} strains possessed the \textit{mef}(A) gene in the absence of \textit{erm}(B). On the basis of the sequence of the complete \textit{erm}(B) gene, 10 \textit{E. columbae} isolates clustered together in six groups. In two of these isolates, the \textit{erm}(B) gene sequence was identical to that of \textit{S. gallolyticus} strains, indicating that exchange of resistance genes might occur between pathogenic and non-pathogenic bacterial species belonging to the pigeon's intestinal flora.

Introduction

The specific taxonomy of the intestinal flora of pigeons has until recently been underexploited territory. Baele \textit{et al.} (2002) described \textit{Enterococcus columbae} as the major Gram-positive component of the pigeon gut microflora. In contrast to the intestinal flora of other animals, other enterococcal species such as \textit{Enterococcus faecium} and \textit{Enterococcus faecalis} are rarely found in these birds (Devriese \textit{et al.}, 1987; Baele \textit{et al.}, 2002). De Herdt \textit{et al.} (1994a) also considered \textit{Streptococcus galloyticus}, previously known as \textit{Streptococcus bovis}, as a putative inhabitant of the pigeon intestinal tract, although to a lesser extent than \textit{E. columbae} (Baele \textit{et al.}, 2002). \textit{S. galloyticus}, a facultatively pathogenic bacterium, is a well-known cause of sepsis, endocarditis, meningitis and colorectal malignancies (Klein \textit{et al.}, 1977; Ballet \textit{et al.}, 1995; Grant \textit{et al.}, 2000). In streptococci and enterococci, ML resistance often finds its origin in the presence of \textit{erm}(B) genes (Jensen \textit{et al.}, 1999). These genes encode for erythromycin-resistant methylases (Erm enzymes), which alter the target site of the macrolide antibiotics in the bacterium and thereby convey ML and streptogramin B compound cross-resistance. The high prevalence of ML resistance among \textit{S. galloyticus} was recently reinforced by Kimpe \textit{et al.} (2003), who found 42% of the tested strains harbouring the \textit{erm}(B) gene. Identical sequences for this gene were detected in human and pigeon
**Materials and Methods**

### Samples

Faecal samples from 50 pigeons, all originating from different lofts, were plated on Slanetz and Bartley (Oxoid, Basingstoke, UK) agar and on Columbia (Oxoid) agar supplemented with 5% sheep blood and aztreonam. In order to select resistant strains, three plates of both agars were supplemented with 1 mg/l erythromycin, 8 mg/l erythromycin or 10 mg/l lincomycin. The plates were incubated for 24 h (Columbia plates) and 48 h (Slanetz and Bartley plates) in a 5% CO2-enriched environment at 37°C. From each plate, all phenotypically different colonies were purified and stored at -70°C.

### Identification

All isolates were identified using the tDNA-polymerase chain reaction (PCR) technique (Baele et al., 2000, 2001). Briefly, genomic DNA was extracted by suspending one colony of a bacterial culture in 20 μl lysis buffer (0.25% sodium dodecyl sulphate, 0.05 M NaOH). After heating at 95°C for 5 min, 180 μl sterile distilled water was added and centrifuged at 16 000 × g for 5 min. The spacers in between the tRNA genes were amplified using the primer T5A (5'-TTATTTCCTCCCGTTAAA-3') and the fluorescently labeled primer T3B (5'-AGATAGATGTCAGACGCACG-3'). The PCR running conditions were as follows: denaturation at 93°C for 1 min, 35 cycles of denaturation at 93°C for 1 min, annealing at 57°C for 1 min, 72°C for 1 min, followed by elongation at 72°C for 5 min. Gel electrophoresis on 1.5% agarose was used to visualize the amplicons in the presence of 1 μg ethidium bromide/ml.

### Sequencing

The sequence of the complete erm(B) gene, which is 738 bp in length, was determined for 10 E. columbae strains and two S. gallolyticus strains. All 10 sequenced enteroococcal strains were isolated from different pigeons, while the S. gallolyticus strains were isolated from samples that also harboured erm(B)-positive E. columbae strains.

For this purpose, the PCR amplicons of 738 bp, which were obtained using the primers 5'-ATGACAAAAATAAATTTTT-3' and 5'-TTATTTCCTCCCGTTAAA-3', were sequenced using the BigDye Terminator Cycle Sequencing kit (PE Biosystems) adopting the outwardly directed primers, 5'-CCATACCACAGATGTTCCAG-3' and 5'-AGATAGATGTCAGACGCACG-3', as well as the earlier mentioned primers for the erm(B) screening. This was performed on an ABI Prism™ 310 Genetic Analyzer. The electropherograms were exported and converted to GeneBase (Applied Maths, Kortrijk, Belgium) using Abicon (Applied Maths).

The sequencing procedure, including PCR amplification, was performed three times. All sequences were compared with one another and with the erm(B) gene sequences of 10 S. gallolyticus strains isolated from pigeons as published previously (Kimpe et al., 2003). The resulting sequences were also compared with those available at GenBank.

### Results

#### Samples and identification

From 47 animals, at least one resistant strain per animal was isolated. Out of a total of 294 strains isolated, 68 isolates were designated as E. columbae and were recovered from 29 different animals. Five S. gallolyticus strains were detected in two faecal samples that also harboured ML-resistant E. columbae. The remaining ML resistant isolates mainly belonged to the genera Lactobacillus and Enterococcus.

#### Resistance genotype

The erm(B) gene was detected in 58 E. columbae isolates. Four of them also carried the mef(A) gene. Five E. columbae strains possessed the mef(A) gene in the absence of erm(B). The remaining five phenotypically resistant E. columbae strains tested negative for both the erm(B) and mef(A) gene. Four of these strains were isolated from plates containing 10 mg/l lincomycin and one strain from a plate containing 1 mg/l erythromycin. The strains identified as S. gallolyticus all carried the erm(B) gene.

#### Sequencing

Sequences of all 10 E. columbae and 12 S. gallolyticus strains showed a similarity of at least 99.7%. On the basis of their erm(B) sequence, the 10 E. columbae isolates clustered together in six groups with identical sequences. Three of these

S. gallolyticus strains, indicating that exchange of resistance genes or strains might occur between humans and pigeons (Kimpe et al., 2003).

Next to erm(B) genes, mef(A) genes may also enforce resistance to macrolides evoking efflux of the antibiotic compound out of the bacterium (Roberts et al., 1999). These mef(A) genes have been recognized in several streptococcal species (Arpin et al., 1999). In S. gallolyticus, however, its presence was limited to a mere 2% (Kimpe et al., 2003).

In several animal species, intestine-inhabiting E. faecium and E. faecalis have been frequently reported to carry acquired resistance to antimicrobial agents (Butaye et al., 2001). This has raised concerns that their resistance genes might be transferred to other pathogenic micro-organisms in the gut, resulting in therapeutic failures should infections due to these pathogens occur. This disquieting possibility led us to screen the intestinal microflora of pigeons for ML-resistant E. columbae, the major enteroococcal floral constituent of the pigeon gut. To obtain better insights into possible exchanges of resistance genes between non-pathogenic E. columbae and facultatively pathogenic S. gallolyticus strains, the erm(B) genes present in these bacteria were compared.

Following positive identification, all isolates were supplemented with 1 mg/l erythromycin, 8 mg/l erythromycin or 10 mg/l lincomycin. The strains were plated on Slanetz and Bartley plates in a 5% CO2-enriched environment at 37°C. From each plate, all phenotypically different colonies were purified and stored at -70°C.

Resistance genotype determination

Following positive identification, all E. columbae and S. gallolyticus strains were screened for possession of the erm(B) and mef(A) genes. Published sequences (Sutcliffe et al., 1996) were used for the derivation of the primers, amplifying specific PCR products of 640 base pairs (bp) for the erm(B) gene and 400 bp for the mef(A) gene. PCR mixtures of 50 μl each contained 1.5 mM MgCl2, 2.5 u Taq DNA polymerase, 200 μM each DNTP, 100 pmol both primers and 2.5 μl DNA sample. The PCR running conditions were as follows: denaturation at 93°C for 3 min, 35 cycles of denaturation at 93°C for 1 min, annealing at 57°C for 1 min, 72°C for 1 min, followed by elongation at 72°C for 5 min. Gel electrophoresis on 1.5% agarose was used to visualize the amplicons in the presence of 1 μg ethidium bromide/ml.

Sequencing

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groups contained one strain, two contained two strains and one contained three strains. Two of these clusters also harboured *S. gallolyticus* strains.

Upon inclusion of the sequences of *erm*(B) genes from other genera and species from Genbank, other enterococcal species, such as *Enterococcus hirae* (AF406971) and *E. faecalis* (Y00116), could also be located in one of the clusters with both *S. gallolyticus* and *E. columbae*. The remaining deposited *erm*(B) sequences all differed in at least one nucleotide from those found in the other clusters.

The *erm*(B) sequence of the *S. gallolyticus* strains isolated from the faeces of two pigeons differed from that of *E. columbae* strains isolated from the same samples in one or two nucleotides.

**Discussion**

In this study, 58% of the tested animals harboured ML-resistant *E. columbae* bacteria as part of their intestinal microflora and up to 94% may be considered as ML resistance carriers in the gut. This high level of ML resistance undoubtedly raises questions regarding the transferability of resistance genes to bacteria with a pathogenic potential.

The broad distribution of the *erm*(B) gene in numerous bacterial genera, including *Enterococcus* and *Streptococcus* (Leclerq & Courvalin, 1991), indicates that this gene may be readily transferred between different genera. The finding that divergent genera and species harbour *erm*(B) genes, when consulting Genbank, likewise testifies to this. In order to assess the risk of actual transfer of *erm*(B)-mediated resistance from *E. columbae* to *S. gallolyticus*, the *erm*(B) sequences of both species isolated from the same animals were determined. They proved to be different in one or two base pairs. Considering that *S. gallolyticus* was very rarely isolated in this study, previous sequencing studies on the *erm*(B) gene from 10 pigeon *S. gallolyticus* strains (Kimpe et al., 2003) were included for comparison. Two *erm*(B) sequences captured from *E. columbae* strains were identical to those obtained from *S. gallolyticus* isolates. Comparison of all *erm*(B) sequences generated in this study with those found in Genbank resulted in the detection of identical *erm*(B) genes from other enterococci and other genera and species for only one of the six *E. columbae* clusters. This strengthens the theory that *erm*(B) genes of *E. columbae* and *S. gallolyticus* are more related to one another than to those harboured by other bacterial genera and species. In the light of these findings, the hypothesis of the transfer of *erm*(B) genes within the intestinal niche between *E. columbae* and *S. gallolyticus* is worth considering and needs to be further investigated. Future studies, including those regarding localization of the identical *erm*(B) genes on plasmids and/or transposons, may shed more light onto the possible exchange of resistance genes between both species.

The very sporadic isolation of *S. gallolyticus* from the faecal samples is in accordance with the findings of Baele et al. (2002) but is in marked contrast to the data obtained by De Herdt et al. (1994a), who catalogued *S. gallolyticus* as part of the intestinal flora. This discrepancy may be due to the use of a selective medium by De Herdt et al. In our study only ML-resistant *S. gallolyticus* strains were selected, and this may have resulted in underestimation of the number of *S. gallolyticus* carriers. However, ML resistance rates in this species have been reported in up to 48% of the tested strains (Kimpe et al., 2002), so higher detection rates would have been expected should *S. gallolyticus* be part of the normal intestinal flora.

Five phenotypically resistant *E. columbae* strains carried neither the *erm*(B) gene nor the *mef*(A) gene. The genetic background for the ML resistance in these isolates could be due to the presence of less ubiquitous genes like *erm*(A), which has been described in enterococci (Schmitz et al., 2000; Jensen et al., 2002). Other genetic mechanisms such as ribosomal mutation of the 23S rRNA have also been described as causing macrolide resistance in other bacterial genera, most specifically in *Streptococcus pneumoniae* (Pihlajamäki et al., 2002). Hitherto, the occurrence of similar mutations in the genus *Enterococcus* has not been reported. Additionally, since these mutational variations are generally known to cause low-level resistance to lincosamides (Jalava et al., 2001) and four out of these five strains were isolated from high-level lincosamide plates, ribosomal mutation seems less probable.

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**References**


Macrolide resistance in *E. coli* 53


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