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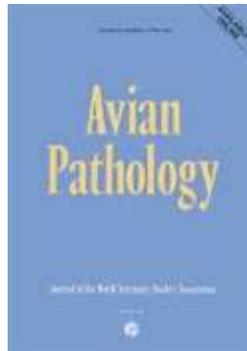
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High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the *erm(B)* gene (revision of manuscript CAVP-2006-0103 which got timed-out)

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High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the *erm*(B) gene

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High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the *erm(B)* gene

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Abstract

A total of 73 isolates of *Enterococcus* spp. carrying the *erm(B)* gene were obtained from cloacal swabs of broiler chickens derived from 13 different farms in Belgium. The *erm(B)* gene encodes resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS_B). The isolates belonged to eight different species: *Enterococcus avium* (8), *Enterococcus casseliflavus* (11), *Enterococcus cecorum* (8), *Enterococcus durans* (7), *Enterococcus faecalis* (10), *Enterococcus faecium* (17), *Enterococcus gallinarum* (7) and *Enterococcus hirae* (5). Acquired resistance to tetracycline was detected in 68 of the isolates and in 62 of these it was associated with the presence of the resistance genes *tet(L)*, *tet(M)*, *tet(O)* or *tet(S)*. In three *E. faecium* isolates that were phenotypically susceptible to tetracycline, *tet(L)* or *tet(M)* was present. The transposon integrase gene (*int* gene) of the Tn916/Tn1545 transposon family, was detected in 18 of the 54 isolates that contained the *tet(M)* gene. It was concluded that acquired resistance to tetracycline antibiotics is often present in enterococci from poultry carrying the *erm(B)* gene. The use of tetracyclines in poultry may therefore co-select for resistance to MLS_B antibiotics which may be important as alternative therapy for enterococcal infections in humans.

Introduction

Enterococci are part of the normal microbiota belonging to the gastrointestinal and genitourinary tract of humans and animals (Murray, 1990; Reiber *et al.*, 1995; Facklam *et al.*, 1999). Although they are generally considered as commensal bacteria, they have the potential to cause a number of infections. Over the past two decades enterococci, especially *Enterococcus faecium* and *Enterococcus faecalis*, have been increasingly identified as causative agents of nosocomial infections in humans, paralleled with increasing antimicrobial resistance (Mundy *et al.*, 2000). They have been associated with urinary tract infections and endocarditis in immunocompetent patients, bacteraemia or sepsis in immunocompromised patients and may lead to procedure-related infections including meningitis, urinary tract infections and intra-abdominal infections (Koch *et al.*, 2004).

In poultry, clinical infections with enterococci are relatively rare. Nevertheless, *E. faecalis*, *E. faecium*, *Enterococcus durans*, *Enterococcus cecorum* and *Enterococcus hirae* have occasionally been associated with a variety of pathologies including endocarditis, septicaemia, central nervous disorders and lameness (Devriese *et al.*, 1991; Cardona *et al.*, 1993; Goodwin *et al.*, 1996; McNamee & King, 1996; Wood *et al.*, 2002). Chadfield *et al.* (2004) associated *E. faecalis*, in combination with *Streptococcus gallinaceus*, with septicemia in an avian leucosis virus group J (ALV-J)-positive broiler parent flock with a high prevalence of endocarditis. In contrast to observations in man, this seems to be the only report in poultry where enterococci caused disease in immunocompromised animals.

The presence of antimicrobial resistance genes in *Enterococcus* species, especially those on mobile elements, is regarded as a hazard for animal and human health. Poultry enterococci carrying antimicrobial resistance genes, may not only transfer these genes to other, possibly

pathogenic bacteria in the chicken gut but upon transfer to zoonotic bacteria, they also may pose a human health hazard. Furthermore, these enterococci may be transferred, directly or indirectly to man where they might be able to cause disease or further disperse their antimicrobial resistance genes among the gastro-intestinal bacterial community.

Macrolide, lincosamide and streptogramin B (MLS_B) antibiotics are used in both human and veterinary medicine and may be important as alternative therapy for treatment of enterococcal infections in man (Portillo *et al.*, 2000; Molander *et al.*, 2003; De Leener *et al.*, 2005). Different mechanisms of acquired resistance against these antibiotics have been described with target modification, active efflux and enzymatic inactivation as the major modes of action. Target modification results in macrolide-lincosamide-streptogramin B (MLS_B) resistance and finds its origin in a mutation in the 23S rRNA gene or is encoded by *erm* (erythromycin-ribosome-methylase) genes (Roberts *et al.*, 1999). Acquired resistance to these antibiotics in enterococci is often associated with the presence of the *erm*(B) gene (Jensen *et al.*, 1999; Roberts *et al.*, 1999; Aarestrup *et al.*, 2000; Portillo *et al.*, 2000; Aarestrup *et al.*, 2002; Martel *et al.*, 2003b; De Leener *et al.*, 2005).

Tetracyclines are frequently used in poultry, being relatively cheap and effective against a wide variety of microorganisms (Chopra & Roberts, 2001). They inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome. Different mechanisms of acquired resistance to tetracyclines have been described with ribosomal protection, efflux of the antibiotic from the bacterial cell and enzymatic inactivation of the antibiotic as major modes of action (Chopra & Roberts, 2001; Roberts, 2005). There are currently 38 different tetracycline resistance (*tet*) and oxytetracycline resistance (*otr*) genes described (Roberts, 2005). Thus far, *tet*(L), *tet*(M), *tet*(O) and *tet*(U) genes have been identified in poultry enterococci. The *tet*(S) gene has only been described in enterococci from beef, pork and human

origin (Aarestrup *et al.*, 2000; Wilcks *et al.*, 2005) and the *tet(K)* gene was demonstrated in an *E. faecalis* strain of human origin (Roberts & Hillier, 1990). The *tet(M)* gene is most commonly located in the bacterial chromosome. It can be carried by conjugative transposons of the Tn916/Tn1545 family, which is the most promiscuous of the conjugative transposons described, with a large host range that includes both Gram-positive and Gram-negative genera (Chopra & Roberts, 2001; Roberts, 2005). While Tn916-like transposons only carry the *tet(M)* gene, carriage of *tet(M)* is associated with the *erm(B)* gene in Tn1545-like transposons (Rice, 1998). The transposon integrase gene (*int* gene) of this family of Tn916/Tn1545 transposons, has been identified in enterococci of human and porcine origin (Weaver, 2000; De Leener *et al.*, 2004).

The aim of this study was to determine the presence of tetracycline resistance genes and the *int* gene in *Enterococcus* isolates from broilers carrying the *erm(B)* gene, as an aid in assessing the potential risk of poultry enterococci acting as a pool of resistance genes. Use of tetracyclines in broilers will not only favour spread of resistance to these antibiotics, but there is also the possibility for co-selecting resistance to MLS antibiotics in enterococci which are simultaneously resistant to both groups of antimicrobials.

Material and Methods

Bacterial strains. Cloacal swabs were collected in 2002 on 13 Belgian farms from 26 four-week-old broilers (two animals/farm). These swabs were inoculated on Slanetz and Bartley (SL) agar (LAB M, Bury, UK) supplemented with either 1µg/ml erythromycin or 10µg/ml lincomycin, allowing isolation of enterococci with decreased susceptibility to macrolides and lincosamides (Martel *et al.*, 2003a). Media were incubated in a 5% CO₂ atmosphere at 37°C for 48 h.

Up to ten morphologically different colony types were selected per plate and were further identified using tRNA intergenic length polymorphism PCR (tDNA-PCR) (Baele *et al.*, 2001). They were tested for the presence of the *erm(B)* gene using PCR, as previously described (De Leener *et al.*, 2004). Isolates derived from the same chicken that belonged to the same *Enterococcus* species were included only once in the final results.

Susceptibility testing. All *Enterococcus* isolates carrying the *erm(B)* gene were tested for susceptibility to tetracycline and for 40 of these isolates erythromycin susceptibility was also studied. Minimal inhibitory concentration (MIC) values were determined on Mueller Hinton II agar plates (LAB M) containing two-fold serial antibiotic dilutions ranging from 128 µg/ml to 0.06 µg/ml. The antibiotics were dissolved in an appropriate solvent and then further diluted in distilled water as outlined by the Clinical and Laboratory Standards Institute (CLSI (formerly NCCLS) M31-A2, 2002). The reference strain used for determination of MIC values was *E. faecalis* ATCC 29212.

The bacteria under test were first grown on Mueller Hinton II agar plates (35°C, overnight) and then diluted in phosphate buffered saline (PBS) to a turbidity of 0.5 on the McFarland scale. These suspensions were diluted 1/10 in PBS and approximately 1×10^4 colony forming units of the strains were inoculated on to the plates with a multipoint inoculator (Denley Mast, Sussex, England). Plates were incubated anaerobically (Gaspakplus, BBL) at 35°C for 20h. The MIC value was defined as the lowest concentration producing no visible growth. Antimicrobial-free agar plates were included as a control for normal growth. Resistance or susceptibility was interpreted using the breakpoints by the CLSI (M100-S17, 2007).

Detection of tetracycline resistance genes and the *int* gene. All *Enterococcus* isolates carrying the *erm*(B) gene were tested for the presence of *tet*P(B), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(U), *tet*(W) and *int* genes. DNA preparation and visualization were performed as previously described (Martel *et al.*, 2001). The primers, amplification protocols and amplicon sizes for the above genes were derived from published references and the described positive controls were likewise included (Sutcliffe *et al.*, 1996; Descheemaeker *et al.*, 2000; Aminov *et al.*, 2001; Ng *et al.*, 2001; Nishimoto *et al.*, 2005). The positive control for *tet*(U) was obtained from Dr. M. Roberts (University of Washington, Seattle, USA).

Results

Bacterial strains. From 26 broilers 93 *Enterococcus* isolates were obtained. In 73 of these isolates, belonging to eight different species, the *erm*(B) gene was detected (Table 1).

Susceptibility testing. The MICs of erythromycin and tetracycline for reference strain *E. faecalis* ATCC 29212 fell within the acceptable quality ranges of MICs for reference strains outlined by the CLSI (M31-A2, 2002). The MIC of erythromycin was > 128 µg/ml for all broiler isolates tested, demonstrating resistance (CLSI, M100-S17, 2007). The critical concentration differentiating between susceptibility and resistance against tetracycline for *Enterococcus* spp. is 16 µg/ml, as given in CLSI guidelines (M100-S17, 2007). A bimodal frequency distribution range of MICs of tetracycline was seen only for *E. faecium*: for five isolates MICs varied between 0.25 and 8 µg/ml, indicating susceptibility, while for the other isolates MICs varied between 32 and >128 µg/ml, indicating resistance. All isolates belonging to the other

Enterococcus species were considered to be resistant to tetracycline antibiotics as the MICs of tetracycline all fell in the high ranges (16 to >128µg/ml).

Detection of tetracycline resistance genes and the *int* gene. The presence of *tet* and *int* genes in the 73 enterococci carrying the *erm*(B) gene is summarized in Table 1. Four different *tet* genes were detected: *tet*(L), *tet*(M), *tet*(O) and *tet*(S). In three of the five *E. faecium* isolates that were phenotypically susceptible to tetracycline, *tet* genes were detected: two isolates carried *tet*(M) and one carried *tet*(L). None of them carried the *int* gene. Two *E. avium*, two *E. casseliflavus*, one *E. durans* and one *E. faecalis* strain that were phenotypically resistant to tetracycline were negative for the *tet* and *int* genes tested. In 65 of the 73 (89%) *erm*(B) positive enterococci, *tet* genes were detected. The *int* gene was demonstrated in 18 of the 54 (33%) isolates which contained both the *tet*(M) and *erm*(B) genes.

Discussion

The results of this study demonstrate that the gastrointestinal tract of broilers constitutes a reservoir of enterococci with acquired resistance to both tetracycline and MLS antibiotics. As enterococci can survive in the environment for prolonged periods and as they are among the most thermotolerant of the non-sporulating bacteria (Franz *et al.*, 1999), contamination of transport containers with antibiotic-resistant enterococci poses a risk for transfer of these bacteria to other flocks and indirectly to the final meat product. All of the *Enterococcus* species isolated in the current study, except for *E. cecorum*, have previously been recovered from poultry litter and

transport containers (Hayes *et al.*, 2004) and all except for *E. cecorum* and *E. avium* have been recovered from poultry meat (Hayes *et al.*, 2003).

Several enterococcal strains are resistant to low pH and bile salts (Lewenstein *et al.*, 1979; Hancock & Gilmore, 2000) and therefore those strains ingested with food or through direct contact with animals, may survive in the human gastrointestinal tract and the presence of antimicrobial resistance genes in these enterococci may thus pose a risk. Indeed, Lancaster *et al.* (2003) suggested that the high prevalence of macrolide- and tetracycline-resistance in oral bacteria from non-antibiotic treated children might be due to acquisition of resistance genes from food. Furthermore, van den Bogaard *et al.* (2002) found a high correlation between the prevalence of antimicrobial resistance of faecal enterococci in broilers, broiler farmers, and poultry slaughterers. This indicates that intense contact with these animals is a risk factor for colonization of humans with resistant enterococci.

Deleted: and

Deleted: laying hens and laying hen farmers as well as in broilers

E. faecalis and *E. faecium* are by far the most important enterococci isolated from healthy and diseased human adults. However, *E. gallinarum*, *E. avium*, *E. hirae*, *E. casseliflavus*, *E. durans* and *E. cecorum* have also been isolated from patients with a variety of infections (Murray, 1990; Greub *et al.*, 1997; d'Azevedo *et al.*, 2004). The finding of *E. cecorum* strains in human patients further strengthens the possibility of transfer of enterococci from poultry to humans (De Baere *et al.*, 2000; Greub *et al.*, 1997; Hsueh *et al.*, 2000).

In 89 % of the *erm*(B) positive enterococci, *tet* genes were also present. The frequent use of tetracyclines in poultry may therefore co-select for resistance to MLS_B antibiotics which may be important as alternative therapy for enterococcal infections in man (De Leener *et al.*, 2005; Portillo *et al.*, 2000; Molander *et al.*, 2003).

Our study confirms that tetracycline resistance genes *tet*(L), *tet*(M), *tet*(O) and *tet*(S) may be present in enterococci (Wilcks *et al.*, 2005) but could not demonstrate the presence of *tet*(K) or

tet(U) (Chopra & Roberts, 2001) in *erm(B)*-positive enterococci from poultry. Aarestrup *et al.* (2000) did not find *tet(K)* in enterococci isolated from humans, broilers and pigs. Unlike these authors, who reported that *tet(S)* only occurs in enterococci of human origin, we found *tet(S)* in poultry isolates of *E. avium*, *E. casseliflavus* and *E. gallinarum*. *Tet(S)* was first described in *Listeria monocytogenes* (Charpentier *et al.*, 1993) and later in *E. faecalis* (Charpentier *et al.*, 1994), including isolates from beef and pork but not from poultry meat (Huys *et al.*, 2004; Wilcks *et al.*, 2005). Carriage of *tet(S)* on a Tn916-like transposon in a *Streptococcus intermedius* isolate has been described recently by Lancaster *et al.* (2004), increasing the likelihood of dissemination of this gene among different bacteria.

In this study, *tet(M)* was the most frequently encountered tetracycline-resistance gene. This gene is most commonly located in the bacterial chromosome and can be carried by conjugative transposons of the Tn916/Tn1545 family. In Tn1545-like transposons, *tet(M)* is associated with *erm(B)* (Clewell *et al.*, 1995; Rice, 1998). However, while 74% of the *erm(B)* positive strains also carried *tet(M)*, the *int* gene of the Tn916/Tn1545 family, was identified in only 33% of these strains, indicating a relatively low occurrence of Tn1545-like transposons in poultry *Enterococcus* isolates. These findings are in contrast with a much higher occurrence of Tn916/Tn1545 transposons in enterococci isolated from pigs and humans in Belgium (De Leener *et al.*, 2004). Indeed, De Leener *et al.* (2004) found that 77% of porcine and 70% of human *erm(B)* and *tet(M)* positive enterococci also carried the *int* gene. This might indicate that pigs may constitute a more important reservoir of transferable MLS_B and tetracycline resistance for humans than poultry. In order to obtain better insights in this possible exchange of resistance genes between different farm animals and humans, the molecular diversity of the Tn1545-like transposons from porcine, poultry and human isolates should be studied. Determination of the allelic variation of the *int* gene on Tn1545-like elements might be useful for this purpose.

In our study, three *E. gallinarum* isolates and one *E. avium* isolate carried *tet(O)*. *Tet(O)*-mediated tetracycline resistance in intestinal enterococci isolated from broilers has been described earlier by Aarestrup *et al.* (2000) and, when investigating tetracycline resistance determinants in raw food, Wilcks *et al.* (2005) found this gene to occur only in enterococci isolated from poultry meat. The presence of this gene has been described in human *E. faecalis*, but is rare (Aarestrup *et al.*, 2000).

Three of the investigated strains were shown to carry *tet* genes without displaying phenotypic tetracycline resistance. This lack of correlation between resistance phenotype and genotype may be due to defective expression of the resistance gene and has been described earlier (Martineau *et al.*, 2000; Martel *et al.*, 2003b).

In conclusion, our study shows high levels of phenotypic and genotypic tetracycline resistance in *Enterococcus* isolates from broilers carrying the *erm(B)* gene, encouraging us to speculate that the frequent use of tetracyclines in poultry may co-select for resistance to MLS antibiotics which may be important as alternative therapy for enterococcal infections in man.

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Table 1. Distribution of transposon integrase (*int*) and tetracycline resistance (*tet*) genes among enterococci from broilers containing the *erm(B)* gene

<i>Enterococcus</i> species (number of isolates)	<i>int</i>	<i>tet</i> (L)	<i>tet</i> (M)	<i>tet</i> (S)	<i>tet</i> (L) + <i>tet</i> (M)	<i>tet</i> (L) + <i>tet</i> (O)	<i>tet</i> (L) + <i>tet</i> (S)	<i>tet</i> (M) + <i>tet</i> (O)	<i>tet</i> (L) + <i>tet</i> (M) + <i>tet</i> (S)	no <i>tet</i> genes
<i>E. avium</i> (8)	0	1	0	0	2	1	1	0	1	2
<i>E. casseliflavus</i> (11)	7	0	8	1	0	0	0	0	0	2
<i>E. cecorum</i> (8)	1	0	2	0	6	0	0	0	0	0
<i>E. durans</i> (7)	4	0	5	0	1	0	0	0	0	1
<i>E. faecalis</i> (10)	1	0	3	0	6	0	0	0	0	1
<i>E. faecium</i> (17)*	2	3	7	0	5	0	0	0	0	2
<i>E. gallinarum</i> (7)	3	0	1	3	0	0	0	3	0	0
<i>E. hirae</i> (5)	0	1	2	0	2	0	0	0	0	0
Total (73)	18	5	28	4	22	1	1	3	1	8

* Five *E. faecium* isolates were phenotypically susceptible to tetracyclines. All other *Enterococcus* isolates were resistant.