Diversity of vanA-type vancomycin-resistant Enterococcus faecium isolated from broilers, poultry slaughterers and hospitalized humans in Greece

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Objectives: This study investigated the prevalence of vancomycin-resistant enterococci (VRE) in the broiler production environment after the avoparcin ban and their epidemiological relationship with human clinical VRE from the same geographical regions in Greece.

Methods: Caecal contents from broilers (n = 500) from eight livestock farms and faecal samples from poultry slaughterers (n = 50), all collected in two slaughterhouses during 2005–08, were analysed for species and vancomycin resistance gene identification using multiplex PCR. Sixty-three human clinical vancomycin-resistant Enterococcus faecium (VREF) isolates, obtained during 2006–09, were also examined. Discriminant analysis (DA) was used to establish the relationship of antimicrobial resistance profiles (ARPs) among broiler, poultry slaughterer and human clinical VRE. PFGE was conducted to study the genetic relatedness among VREF from the different sources.

Results: A total of 120 VRE were recovered from 113 (22.6%) broiler samples. VREF carrying the vanA gene were predominant, being recovered from 72 (14.4%) samples from five (62.5%) broiler farms. Concerning poultry slaughterers, VREF were recovered from 10 (20%) samples. Susceptibility testing revealed that broiler VREF were consistently resistant to tetracycline, whereas 93.7% of clinical VREF were resistant to ampicillin. Furthermore, 92.1% of clinical VREF compared with 54.4% of broiler VREF were multiresistant (resistant to at least five antimicrobial classes). DA classified broiler and human clinical VREF into their corresponding source with high classification rates (100% and 85.7%, respectively), while the classification rate of poultry slaughterer VREF was relatively low (50%), with 40% of them classified closely to broiler VREF. PFGE patterns were clearly related to the source of the VREF, with broiler isolates being clustered distinctly from all human isolates.

Conclusions: A remarkable persistence of VREF was observed in the broiler production environment even >10 years after the avoparcin ban. Human and broiler VREF belonged to clearly unrelated populations, strongly indicating no clonal spread of VREF among the different sources, even between broilers and poultry slaughterers, despite them sharing common ARPs, as also supported by DA.

Keywords: VRE, antimicrobial resistance, discriminant analysis, PFGE

Introduction

During the last two decades, vancomycin-resistant enterococci (VRE) have emerged as important nosocomial pathogens, due to the widespread use of glycopeptides, mainly vancomycin, in hospitals.¹ Meanwhile, the use of the glycopeptide avoparcin as a growth promoter in food-producing animals also resulted in the emergence of VRE in livestock worldwide,²–⁴ indicating that these animals might be a potential reservoir for vancomycin resistance determinants. The use of avoparcin has been banned...
since 1997 in the European Union. To date, nine types of vancomycin resistance have been identified: VanA, VanB, VanC, VanD, VanE, VanG, VanL, VanM and VanN, with VanA, which results in high-level vancomycin and teicoplanin resistance and is related mainly to Enterococcus faecium isolates (vancomycin-resistant E. faecium (VREF)), being the most common in humans and animals in Europe.7,8

According to updated epidemiological data, Greek reports showed higher rates for clinical VREF (22.5%) compared with the majority (23 out of 28) of European countries (reporting rates <10%),9 for which VanA was the predominant type.10–14 In addition, the isolation of VRE and a considerably high prevalence of VREF in pigs as well as in urban and hospital wastewater has also been reported in Greece.15,16 However, no genetic association has been established between isolates from pigs and those from hospitalized patients.15 Although VRE from poultry have previously been reported in Greece,17 there is no knowledge on their prevalence in broilers after the avaparcin ban, as well as on their genetic relatedness to VRE from broiler production workers and hospitalized humans in the same geographical regions. In other countries, individual cases have raised questions regarding the clonal relatedness of VREF from poultry and the humans in close contact with them.8,18,19 We aimed at investigating the prevalence of VRE among broilers and poultry slaughterers as well as their possible epidemiological relationships with clinical VRE from the same geographical regions in Greece, on the basis of their antimicrobial resistance profile (ARP) and clonality.

Materials and methods

Sample collection and VRE isolation and identification

The caecal contents of 500 randomly selected healthy broilers from eight conventional livestock farms (named B1–B8) in northern and central Greece were obtained in two poultry slaughterhouses, between May 2005 and June 2008. During the same time period, 50 faecal samples were also collected from poultry slaughterers working at the two poultry slaughterhouses. For enrichment, 1 mL of a 10-fold-diluted faecal sample in 0.9% NaCl was added to 9 mL of Enterococcus broth (Becton Dickinson) supplemented with 6 mg/L vancomycin (Sigma Chemical Co.). After incubation for 48 h at 37°C, 100 μL was subcultured onto Enterococcus agar (Becton Dickinson), also supplemented with 6 mg/L vancomycin.20 A maximum of four to five colonies were selected according to their colony morphology and esculin hydrolysis. Identification of the presumptive VRE was performed by conventional biochemical tests.21 All isolates were subcultured and stored at –80°C.

In addition, 63 human clinical VREF were included in this study, of which 48 originated from adult (n = 13) and neonate (n = 35) samples from Hippokration University Hospital, and 15 were from adult samples from Larissa University Hospital. The isolates tested corresponded to all clinical VREF, in contrast to none of the poultry slaughterer samples. VREF carrying the vanA gene were predominant, being recovered from 72 (14.4%) samples from five (62.5%) broiler farms (B1, B3, B5, B6 and B7), while Enterococcus gallinarum harbouring the vanC gene were recovered from 41 (8.2%) samples also obtained from five farms (B1, B2, B3, B4 and B5). Only one farm (B8) was negative for VRE. Concerning poultry slaughterers, VRE were recovered from 14 (28%) samples, of which 10 (20%) were positive for VREF and 4 (8%) for E. gallinarum. The MICs of vancomycin and teicoplanin (Sigma Chemical Co.) were determined by the broth microdilution method according to CLSI criteria.26

PCR amplification of van genes

Multiplex PCR analyses were performed with whole genomic DNA (NucleoSpin; Macherey-Nagel) from all presumptive VRE exhibiting vancomycin MICs of ≥2 mg/L and by amplification with primers specific for the vanA, vanB, vanC1–C2, vanD, vanE, vanG, ddl-E. faecium and ddl-Enterococcus faecalis genes, and by using reference strains (Institute Pasteur, France), as previously described.25

Discriminant analysis (DA) of ARPs

The relationship of ARPs among broilers, poultry slaughterers and clinical VREF was determined using DA, based on all 14 antimicrobials tested. DA has previously been carried out as a multiple ARP analysis to classify isolates into groups of different origin, such as animal and human isolates.26–28 Briefly, this multivariate statistical analysis separates a dataset of variables, e.g. ARPs, into a number of predefined groups using linear combinations.26 ARPs with similar variances yield similar discriminant scores; therefore, they are grouped together when plotted. According to DA, in this study, ARP data were converted into binary code on the basis of susceptibility or resistance26,27 and analysed with StatistiXL software (version 1.8; http://www.statistiXL.com), thus calculating the number of isolates of each source (broilers, poultry slaughterers and hospitalized patients) that were classified into the corresponding source. The average rate of correct classification (ARCC) was determined by averaging the percentages of isolates classified into their corresponding source, as previously described.28

PFGE typing

PFGE of SmaI-digested genomic DNA29 was performed in a CHEF-DR III system (Bio-Rad Laboratories) and the banding patterns were interpreted according to the criteria established by Tenover et al.30 Fingerprinting II Software 3.0 (Bio-Rad Laboratories) was used to calculate Dice correlation coefficients and to generate a dendrogram by the unweighted pair group with arithmetic averages clustering method.

Results and discussion

Prevalence and antimicrobial susceptibility of VREF isolates

A total of 120 VREF were recovered from 113 (22.6%) broiler samples. VREF carrying the vanA gene were predominant, being recovered from 72 (14.4%) samples from five (62.5%) broiler farms (B1, B3, B5, B6 and B7), while Enterococcus gallinarum harbouring the vanC gene were recovered from 41 (8.2%) samples also obtained from five farms (B1, B2, B3, B4 and B5). Only one farm (B8) was negative for VRE. Concerning poultry slaughterers, VRE were recovered from 14 (28%) samples, of which 10 (20%) were positive for VREF and 4 (8%) for E. gallinarum. The MICs of vancomycin and teicoplanin were ≥128 mg/L for 97.5% and ≥32 mg/L for 72.2% of broiler VREF, respectively. Interestingly, all clinical VREF, in contrast to none of the poultry slaughterer
VREF, exhibited vancomycin and teicoplanin MICs of >128 and >32 mg/L, respectively.

The 14.4% prevalence of broiler VREF indicates their remarkable persistence even more than a decade after the avoparcin ban. This prevalence rate is consistent with updated epidemiological data from other European countries showing rates among broilers varying up to 23%. Notably, older studies from European countries reported rates up to 80%. Concerning VREF-positive broiler farms, a rate of 81.8% was reported 5 years after the avoparcin ban in the UK. The relatively high rate of VREF-positive broiler farms found in our study more than a decade later further supports the remarkable persistence of VREF. The data available on VREF prevalence among humans working in broiler and food production, with rates varying from 5% to 27.8%, are consistent with our results. Of note is the occurrence of VREF-positive broiler farms found in our study more than a decade later further supports the remarkable persistence of VREF. The data available on VREF prevalence among humans working in broiler and food production, with rates varying from 5% to 27.8%, are consistent with our results. Of note is that European studies on healthy volunteers in the community have reported VREF prevalence of up to 7%. The aforementioned VREF persistence in broilers observed in this study occurred without any apparent glycopeptide selection, as also previously reported. The extensive use in agriculture and veterinary practice of other antimicrobials that exhibit genetic resistance coselection with glycopeptides could explain the occurrence of VREF among broilers. A genetic linkage between macrolides and vancomycin resistance has been reported previously. Indeed, in our study, a significant rate of resistance to erythromycin (54.4%) was observed among broiler VREF. Similarly, the high occurrence of resistance to erythromycin (57.4%) among clinical VREF indicates a possible association with the use of this antimicrobial in hospitals.

In line with previous reports, broiler VREF were consistently resistant to tetracycline (100%), a compound used extensively in veterinary medicine. On the other hand, the overwhelming majority (93.7%) of clinical VREF were resistant to ampicillin, which could be explained by the widespread use of this compound in hospitals. In contrast, low rates of resistance to tetracycline (9.5%) and ampicillin (16.5%) were observed among clinical and broiler VREF, respectively. The high rates of resistance to ampicillin among clinical VREF, combined with the high rates of resistance to ciprofloxacin (87.3%) could also possibly be associated with the spread of the so-called hospital-adapted, epidemic clonal types of VREF among hospitalized patients. Concerning poultry slaughterers, VREF exhibited low and moderate resistance to ampicillin (10%) and ciprofloxacin (30%), respectively, similar to the rates observed in broilers (16.5% and 30.4%, respectively). Additionally, they exhibited moderate resistance to tetracycline (40%), whereas almost all of them were susceptible to high-level aminoglycosides, as these antimicrobials are rarely used in the community. In contrast, the rate of resistance to high-level aminoglycosides was high among clinical (66.7% to high-level gentamicin and 50.8% to high-level streptomycin) and moderate among broiler (21.5% to high-level gentamicin and 41.8% to high-level streptomycin) VREF, in agreement with previously reported studies in Greece and other countries, possibly reflecting the continuous usage of these compounds in hospital and broiler production environments. However, the possibility that the observed co-resistances to antimicrobials might be a stable characteristic of VREF isolates in our region, especially in these environments, cannot be excluded. Finally, all VREF were susceptible to the last-resort agent, linezolid.

**Analysis of ARPs of VREF isolates**

Fifty-two distinct ARPs were revealed, with broiler, poultry slaughterer and human clinical VREF exhibiting 31, 6 and 20 types, respectively (Table 1). Remarkably, broiler VREF shared only one type (11i) with clinical VREF and three types (3i, 4iii and 5v) with poultry slaughterer VREF. The latter exhibited one type (9viii) in common with clinical VREF. The existence of variant ARPs in combination with the different resistance rates among isolates from the different sources (Table 1) might reflect the broad spectrum of the selection pressure of the antimicrobial agents used in each environment.

Furthermore, the existence of variant ARPs types within the same source environment (Table 1) reflects the varying selection pressure even within each different niche. Indeed, no apparent predominant ARP type was observed among broiler VREF, with three types (3i, 4i and 7vii) shared by 39.2% (31 out of 79) of them. Two predominant ARP types (7vii and 9viii; Table 1) were observed among clinical VREF, with type 7vii being, however, restricted to outbreak-related isolates. Meanwhile, 43 out of 79 (54.4%) broiler and 58 out of 63 (92.1%) clinical VREF were multi-resistant (resistant to at least five antimicrobial classes), whereas 9 out of 10 (90%) poultry slaughterer VREF were resistant to four or fewer classes (Table 1). The ARCC inferred by DA of the ARPs was 78.6%. Specifically, broiler and human clinical VREF were correctly classified at a rate of 100% and 85.7%, respectively, showing a clear discrimination and association with their source (Figure 1). In contrast, the classification rate of poultry slaughterer VREF was just 50%, indicating their low source specificity, with 40% of them classified closely to broiler source as they shared common ARPs (Table 1 and Figure 1).

**PFGE analysis of VREF isolates**

PFGE analysis of all 152 VREF isolates from the three sources revealed 118 distinct PFGE restriction patterns (Figure 2). On a similarity level of >75%, corresponding to six or fewer different DNA fragments according to the criteria established previously, a percentage of 71.7% of VREF were grouped into eight clusters. An isolate’s PFGE pattern was clearly related to its source and broiler VREF were clustered distinctly from human VREF (Figure 2), which strongly indicated no clonal spread among the different sources. Meanwhile, a high genetic diversity within each source, as demonstrated by the existence of more than one cluster, was observed (Figure 2). Specifically, 71 out of 79 broiler VREF were grouped into four distinct clusters, designated C1–C4. The existence of genetically indistinguishable PFGE patterns of broiler VREF from farms of different geographical regions (Figure 2; C2 cluster) as well as the distribution of PFGE patterns of VREF of one farm (B1) in all four C clusters (Figure 2) suggest that besides clonal spread, horizontal transmission of vancomycin resistance might also have occurred. The clonal dissemination could be due to the spread of VREF-colonized broilers from a common hatchery. The latter scenario as well as the intensive and widespread use of antimicrobials in veterinary practices may have contributed to the maintenance of VREF among broiler farms. Indeed, farms sharing multiresistant ARP types were observed (Figure 2; B3 and B5 farms). Concerning clinical VREF, 37 out of 63 appeared to belong to four distinct clusters, designated H1–H4, exhibiting <60% similarity.
Table 1. Distribution of ARPs of VREF isolates from broilers, poultry slaughterers and hospitalized patients

<table>
<thead>
<tr>
<th>ARP(\alpha)</th>
<th>ARP type</th>
<th>No. (%) of isolates</th>
<th>broilers ((n = 79))</th>
<th>poultry slaughterers ((n = 10))</th>
<th>hospitalized patients ((n = 63))</th>
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<td>1 (10)</td>
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<tr>
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<td>1 (10)</td>
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<td>10v</td>
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Continued
Clonal and/or horizontal transmission might also have occurred among clinical VREF. The H1 and H2 clusters shared patterns of isolates with common ARP types (9viii and 10v), from different hospitals, possibly through patient transfer, whereas most of the outbreak-related VREF sharing the same ARP type (7vii), were separated into H3 and H4 clusters. Interestingly, poultry slaughterer isolates exhibited distinct patterns unrelated to the C or H clusters.

**Table 1. Continued**

<table>
<thead>
<tr>
<th>ARP type</th>
<th>broilers (n = 79)</th>
<th>poultry slaughterers (n = 10)</th>
<th>hospitalized patients (n = 63)</th>
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<td>VAN TEC TET AMP SXT ERY PEN CIP HLS HLG NEM</td>
<td>11i 1 (1.2)</td>
<td>1 (1.6)</td>
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<tr>
<td>VAN TEC SXT AMP ERY PEN CIP HLS HLG NEM BAC</td>
<td>11ii 1 (1.6)</td>
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<tr>
<td>VAN TEC TET AMP SXT ERY PEN CIP HLS HLG NEM BAC</td>
<td>12i 2 (3.2)</td>
<td></td>
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</tbody>
</table>

Abbreviations of antimicrobials (disc content): VAN, vancomycin (30 μg); TEC, teicoplanin (30 μg); BAC, bacitracin (10 IU); AMP, ampicillin (10 μg); PEN, penicillin (10 IU); HLS, high-level streptomycin (300 μg); NEM, neomycin (30 μg); ERY, erythromycin (15 μg); TET, tetracycline (30 μg); CIP, ciprofloxacin (5 μg); CHL, chloramphenicol (30 μg); SXT, sulfamethoxazole/trimethoprim (23.75/1.25 μg). Grey shading indicates ARP types shared among isolates from two out of three sources. Bold AMP and TET represent ampicillin and tetracycline resistance of broiler and human clinical VREF, respectively.

**Figure 1.** DA plot of ARPs of VREF isolates originating from the three sources. All broiler ARPs were correctly classified into the broiler source (classification rate 100%). The classification rate of human clinical VREF into the human clinical source was 85.7% (54 isolates) corresponding to 12 ARPs, whereas 3 (3 isolates (4.8%)) and 5 (6 isolates (9.5%)) ARPs were classified closely to poultry slaughterer and broiler sources, respectively; the arrow indicates the common ARP shared by broiler and human clinical VREF. The classification rate of poultry slaughterer VREF was 50% corresponding to two ARPs, whereas three ARPs (40% of isolates) and one ARP (10% of isolates) were classified closely to broiler and human clinical sources, respectively.

to the C clusters (Figure 2).
Figure 2. Clustering of the VREF isolates by their PFGE patterns in comparison with ARP types. The dendrogram is based on analysis by the unweighted pair group with arithmetic averages clustering method; percentage similarities are shown above the dendrogram. PFGE patterns of VREF were delineated with a 75% similarity cut-off value corresponding to six or fewer different DNA fragments, according to the criteria established by Tenover et al. and indicated by the broken vertical line. The ARP type and the source of VREF are shown on the right-hand side of the PFGE patterns. The dots indicate the source, with the ARP type of each isolate indicated near each dot. P.SL., poultry slaughterers; CL.H., hospitalized adult patients in Hippokration Hospital; CL.N.H., hospitalized neonates in Hippokration Hospital; CL.L., hospitalized patients in Larissa Hospital. C1, C2, C3 and C4 represent broiler (chicken) clusters, while H1, H2, H3 and H4 represent human clusters.
Conclusions
In the current study, broiler and clinical VREF isolates belonged to clearly unrelated populations, as supported by PFGE patterns as well as the DA of the ARPs. The clonal non-relatedness between these two sources was in line with previous reports.\textsuperscript{45,46} It is worth noting that clonal relatedness has also not been observed among hospitalized patients and pigs concerning Greek VREF isolates,\textsuperscript{15} whereas, in contrast, common clones among these two populations were found in other countries.\textsuperscript{6,17} Furthermore, individual cases with common clones among poultry and the humans in close contact with them have been reported previously.\textsuperscript{8,18,19} However, PFGE analysis in this study did not reveal any sharing of clones between broilers and poultry slaughterers, despite them sharing common ARPs, as supported by DA. The information gained may serve as a basis to further clarify (i) whether ‘broiler-specific’ vanA elements circulate both in the broiler production environment and among humans, and (ii) the mechanisms of VREF long-term persistence in the broiler production environment in Greece.

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Transparency declarations
None to declare.

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