Pitfalls of antimicrobial susceptibility testing of enterococci isolated from farming broilers by the disk diffusion method

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Accurate antimicrobial susceptibility testing is essential for bacterial control. Although minimum inhibitory concentration (MIC) determination is the reference method, disk diffusion (DD) testing is often performed.

Antimicrobial susceptibility of broilers enterococci to enrofloxacin (ENR), gentamicin (GEN), oxytetracyclin (OXT), streptomycin (S) and vancomycin (VAN) was evaluated by both methods. Three types of discrepancies were considered: minor error (one test is intermediate, the other is positive or negative); major error (false-resistant DD result) and very major error (false-susceptible DD result).

Levels of agreement between methods to ENR, GEN, OXT, S and VAN were, respectively, 32.4%, 52.9%, 70.6%, 79.4% and 100%. Minor errors were the most frequent. Major errors were found for ENR, S and OXT while very major errors were found for S and GEN.

False susceptibility and resistance levels in DD testing may lead to the antimicrobial drug inadequate choice. Specific breakpoints for poultry pathogens and their correspondence in DD testing should be established.

Keywords antimicrobial susceptibility; broilers; disk diffusion; *Enterococcus*; minimum inibitory concentration

1. Introduction

Enterococci are gram-positive, facultative anaerobic bacteria bacteria that belong to the commensal microbiota that colonises the intestinal tract of many animal species, including mammals and birds. They are usually considered non pathogenic, but in the last decade they have been frequently associated with several infections in humans, such as nosocomial infections [1, 2, 3], endocarditis, urinary and genital tract infections, meningitis and septicaemia [2].

As members of the normal intestinal microbiota, they are exposed to the vast majority of the antimicrobial compounds administered to their host, which can lead to a high probability of acquiring and transferring antimicrobial resistance genes [2, 4, 5], and also of transferring those genes to other bacteria present in the faecal environment [5]. These features render enterococci ideal bacteria for controlling parameters related to antimicrobial susceptibility studies.

Antimicrobial susceptibility testing is essential for the adequate control of antimicrobial resistance transfer from enterococci to other bacteria genera. Although the minimum inhibitory concentration (MIC) determination is the reference method for antimicrobial susceptibility testing, the disk diffusion method (DD) is often chosen, as it provides rapid results at reduced costs, being easy to perform and interpret, allowing for the evaluation of the bacterial culture purity, and presenting a good inter-laboratory reproducibility [6]. Nevertheless, some studies refer to the occurrence of discrepancies between the susceptibility results obtained by DD and MIC testing [2, 7, 8], and between *in vitro* susceptibility testing methods and drug performance *in vivo* [8]. To address these shortcomings, the Clinical Laboratory Standards Institute (CLSI) frequently evaluates and modifies the interpretive criteria for both methods, including adaptation of MIC breakpoints, revision of the categories, determination of species' breakpoints, establishing new screening test concentrations and methods [8].

Antibiotics are widely used in veterinary medicine, as therapeutic agents or growth promoters [5], rendering veterinary field isolates a useful tool for studies concerning the comparison of susceptibility testing methods. This study aimed at comparing DD susceptibility results of enterococci isolated from slaughtered broilers with MIC values of four antimicrobials commonly used in veterinary medicine, and also of vancomycin, in order to evaluate its adequacy for enterococci field isolates testing.

2. Material and Methods

2.1. Bacterial strains

Thirty-four enterococci isolates were used, belonging to a collection of intestinal commensal microbiota (*E. coli* and *Enterococcus* sp.) from poultry slaughtered for human consumption in two Portuguese slaughterhouses. Bacteria were

isolated and identified according to Martins da Costa et al. [9] and kept frozen at -80ºC until further processing. Isolates were identified as *Enterococcus faecalis* (n=11; 32.35%), *E. faecium* (n=21; 61.76%), *E. durans* (n=1; 2.94%), and *E. gallinarum* (n=1; 2.94%).

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) values of vancomycin (VAN; Sigma, V861987), enrofloxacin (ENR; Sigma, E3369), oxytetracyclin (OXT; Sigma, 04636), streptomycin (S; Sigma, S6501) and gentamicin (CN; Sigma, G1264) were determined by broth microdilution, following CLSI guidelines for veterinary susceptibility testing [10].
All antimicrobials were purchased from Sigma. Stock solutions (1.28 mg/mL) were prepared and stored at -80º C for a maximum of 15 days until testing. Briefly, ten two-fold dilutions of each drug were prepared in Mueller-Hinton Broth (MHB, Oxoid, CM0405) and distributed in 96-well microtiter plates (Nunc®). Prior to testing, isolates were grown in Columbia agar plates supplemented with 5% sheep blood (bioMérieux) and incubated at 37ºC for 24 h. Isolated colonies were suspended in MHB, adjusted by optical density, distributed into the wells to a final concentration of approximately $5x10^5$ CFU/mL and incubated at 37ºC for 22±2 h under normal atmosphere. All isolates were at least tested twice in separate occasions. Susceptibility breakpoints used, based on CLSI document M31-A3, are described in Table 1. MIC was considered the lowest dilution inhibiting visible bacterial growth. Reference strain *Enterococcus faecalis* ATCC 29212 was used as control for MIC determination [10].
Antimicrobial susceptibility testing was also performed by DD on Mueller-Hinton agar plates (Biokar Diagnostics) [10]. The disks, purchased from Oxoid, contained the following antimicrobial compounds: vancomycin (VAN, 30 µg, Oxoid, CT0058B), enrofloxacin (ENR, 5 µg, Oxoid, CT0639B), oxytetracyclin (OXT, 30 µg, Oxoid, CT0041B), streptomycin (S, 10 µg, Oxoid, CT0047B) and gentamicin (CN, 10 µg, Oxoid, CT0024B). Classification of isolates' susceptibility was made according to the inhibition zone diameter, based on CLSI document M31-A3 (Table 1).

Table 1 MIC susceptibility breakpoints (µg/mL) and DD susceptibility zone diameter (mm) of vancomycin (VAN), enrofloxacin (ENR), oxytetracyclin (OXT), streptomycin (S) and gentamicin (CN). Oxytetracycline (OXT) breakpoints were extrapolated from the class representative tetracycline, (CLSI document M31-A3).

	MIC Susceptibility breakpoints (µg/mL)			Zone Diameter (mm)		
	Susceptible (µg/mL)	Intermediate (µg/mL)	Resistant (µg/mL)	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
VAN	≤ 4	8-16	≥ 32	≥ 17	15-16	≤ 14
ENR	≤ 0.5	-	≥ 4	≥ 23	17-22	≤ 16
OXT	≤ 4	8	≥ 16	≥ 19	15-18	≤ 14
S	≤ 32	-	≥ 64	≥ 15	13-14	≤ 12
CN	≤ 4	8	≥ 16	≥ 15	13-14	≤ 12

2.3. Comparative analysis

Susceptibility results for each isolate were compared (Table 2) and three types of discrepancies were considered: minor error, when one test result is intermediate and the other are either susceptible or resistant; major error, a false-resistant DD testing result; very major error, a false-susceptible DD testing result (Table 3).

3. Results

MIC and DD distribution values of the five antimicrobials tested to enterococci field isolates are summarised in Table 2. MIC values for the quality control organism were within recommended ranges for all the antimicrobial agents tested [10].

Table 2 Antimicrobial susceptibility distribution of 34 enterococci isolated from Portuguese broilers to five antimicrobial compounds as determined by the two methods.

	Antimicrobial compounds									
	VAN[a]		ENR[b]		S[c]		OXT[d]		CN[e]	
Results	MIC[f]	DD[g]	MIC	DD	MIC	DD	MIC	DD	MIC	DD
Sensitive	34	34	8	2	2	4	11	2	4	13
Intermediate	-	-	22	9	-	2	-	1	2	5
Resistant	-	-	4	23	32	28	23	31	28	16
Total	34	34	34	34	34	34	34	34	34	34

[a]vancomycin, [b]enrofloxacin, [c]streptomycin, [d]oxitetracycline, [e]gentamicin, [f]minimum inhibitory concentrations, [g]disk diffusion

MIC and DD results were compared (Table 3). The overall level of agreement between methods for VAN, ENR, S, OXT and CN was, respectively, 100%, 44.1%, 85.3%, 73.5% and 67.6%. Minor errors were observed for ENR and CN, major errors were detected for ENR, S and OXT, and very major errors were found for S and CN.

Table 3 Comparison between antimicrobial susceptibility results of 34 enterococci isolated from Portuguese broilers to five antimicrobial compounds as determined by the two methods: susceptibility results discrepancies and agreement level.

	Antimicrobial compounds					
MIC[a] vs DD[b] Results	VAN[c]	ENR[d]	S[e]	OXT[f]	CN[g]	Susceptibility results discrepancies
S[h] vs S	34	2	-	2	2	-
S vs I[i]	-	4	1	-	2	-
S vs R[j]	-	2	1	9	-	major errors (n=12)
I vs S	-	-	-	-	2	minor errors (n=19)
I vs R	-	17	-	-	-	
I vs I	-	5	-	-	-	-
R vs S	-	-	4	-	9	very major errors (n=13)
R vs I	-	-	1	1	3	-
R vs R	-	4	27	22	16	-
Agreement	34 (100%)	15 (44.1%)	29 (85.3%)	25 (73.5%)	23 (67.6%)	

[a]minimum inhibitory concentrations, [b]disk diffusion, [c]vancomycin, [d]enrofloxacin, [e]streptomycin, [f]oxitetracycline, [g]gentamicin, [h]susceptible result, [i]intermediate result, [j]resistant result

4. Discussion

Enterococci are comprised in the normal intestinal microbiota of most animals, being involved in the acquisition and transference of antimicrobial resistance genes [2, 4, 5]. These features render enterococci ideal bacteria for controlling parameters related to antimicrobial susceptibility studies.

In order to evaluate the accuracy of DD testing for enterococci field isolates, we compared DD and MIC susceptibility results of enterococci isolated from faecal samples of slaughtered broilers for four antimicrobials commonly used in veterinary medicine and also for vancomycin.

Our study shows the occurrence of discrepancies between the susceptibility results obtained by the two methods used. A high level of agreement was found for VAN, S and OXT. A medium level of agreement was obtained for CN, and a lower level of agreement was detected for ENR. A relatively high percentage of minor errors was observed (55.9%), whereas the percentages of major and very majors errors was lower (35.3% and 38.2%, respectively). It is important to refer that all very major errors observed occurred with aminoglycosides (S and CN), which is in agreement with the results by Kronvall [6]. The fact that no errors were observed in relation to VAN differs from previous studies that attributed errors in glycopeptides DD to difficulties in the antibiotic diffusion through the agar medium [8], and recommended the use of highly accurate DD or MIC susceptibility tests to determine VAN resistance, in order to not underestimate the prevalence of vancomycin-resistant enterococci [2, 8].

Karmarkar et al. [8] also described divergences between MIC and DD susceptibility patterns of *E. faecalis* and *E. faecium* clinical isolates regarding several antimicrobial compounds. These authors observed the occurrence of major errors for CN, and of very major, major and minor errors for VAN. Divergent results were also obtained by Jorgensen et al. [7] when determining enterococci susceptibility by MIC, E-test strips and DD.

The errors observed point out the need for a critical evaluation of DD results of enterococci broiler isolates. DD testing can be affected by many factors, including the standard method chosen, the composition, electrolyte concentration, pH and depth of agar medium, the medium batch, the interaction between the drug impregnated discs and the agar, the density of the inoculum used, and the interaction between the bacteria and the antibiotic [6, 7, 11, 12, 13]. Variability in the inhibition zones diameter is also observed among laboratories [6].

DD false-resistant isolates (major errors) may be responsible for an inadequate choice of the antimicrobial drug selected for control and treatment of broilers infection. This may lead to the use of second-choice drugs, usually more recent and expensive, and thus contribute to economic losses and the selection of resistant strains. Therefore, antimicrobial susceptibility testing should ideally be performed by determining MIC values for each antimicrobial drug. Recently, this has become a viable option due to the availability of commercial systems for MIC determination by broth microdilution.

Even if DD and MIC testing results showed full agreement, the issue of lack of data on specific breakpoints remains. Only VAN susceptibility breakpoints are specific for enterococci. ENR susceptibility breakpoints are defined according to the animal species (birds), and the breakpoints for S, OXT and CN are not specific for enterococci or poultry isolates. They are derived from human data [10] and do not necessarily represent accurate susceptibility breakpoints for enterococci isolated from slaughtered broilers.. Enterococci from slaughtered broilers may present specific resistance characteristics, remaining at risk for clinical treatment failure, regardless of appropriate dosing or susceptibility level determined by the antimicrobial susceptibility test applied [8].

Further studies are required to establish the specific breakpoints for enterococci field isolates and their correspondent in DD testing, and also to evaluate discrepancies between *in vitro* susceptibility testing methods and drug performance *in vivo*.

Acknowledgements The support by CIISA ("Centro de Investigação Interdisciplinar em Sanidade Animal") from the Faculty of Veterinary Medicine, Lisbon, is gratefully acknowledged.

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