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MARSLAND PRESS
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Detection of vancomycin resistance in enterococci isolated from poultry

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Abstract: Background: Although enterococci are commensal bacteria of the intestinal tract of animals and humans they are associated with nosocomial infections worldwide. We investigated the occurrence and vancomycin resistance of enterococci in poultry **Methods:** A total of 617 cloacal swabs were collected from diseased poultry diagnosed with gastrointestinal disorders in 6 districts in Egypt. Isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The antibiotic susceptibility testing of all isolates against ticoplanin and vancomycin antibiotics was performed with the MICRONAUT system for Gram-positive bacteria. The presence of 3 resistance-associated genes *van*A, *van*B and *van*C1 was investigated by PCR. **Results:** Four species were identified: *Enterococcus faecium* (n=30), *Enterococcus faecalis* (n=16), *Enterococcus gallinarum* (n=10) and *Enterococcus avaim* (n=8) strains. Antimicrobial resistance profiles of enterococci isolates could be determined (Table 2). Resistance rate to vancomycin were different.*E.feacalis* showed high resistantance rate to both teicoplanin 75.0% and vancomycin 87.5%, followed by *E.faecium* that showed resistance to teicoplanin 40.0% and vancomycin 50.0% and *E.gallinarum resistance to* teicoplanin was 40.0% and vancomycin 30.0% while *E,avaim* showed the lowest rate of resistance to teicoplanin was 25.0% and vancomycin 25.0%. Vancomycin resistance genes were found in 14 isolates. The *van*A were detected in ten isolates of *E.feacalis* only. The *van*B gene was identified in three *E. faecium* isolates and five isolates of *E.feacalis*. The *van*C1 gene was detected in five *E. faecium* isolates. All *E. gallinarum* isolates harboured the *van*C1. *E.avium* did not harboured any of them. **Conclusions:** Vancomycin-resistance was found in several isolates from poultry.

[Wedad Ahmed, Helmut Hotzel, Ashraf Awad Abdeltawab, Mona M. Sobhy, Fatma I. El Hofy. **Detection of vancomycin resistance in enterococci isolated from poultry.** *Nat Sci* 2020;18(3):93-97]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 11. doi:10.7537/marsnsj180320.11.

Keywords: *Enterococcus*, Poultry, vancomycin

1. Introduction:

Vancomycin-resistant *E. faecium* is the second leading pathogen of the priority list of antimicrobial resistance (priority pathogens) published recently by WHO that are a major threat to public health (WHO, 2017).

The genus name *Enterococcus*, originally suggested in 1903 for bacteria previously called *Streptococcus faecalis* and *Streptococcus faecium*, was revived in 1984 when other bacteria were transferred to the genus **(Hardie,1986; Schleifer and Klipper-Balz 1984)**. There are currently 48 members of the genus *Enterococcus* which are published. *Enterococcus faecalis* and *Enterococcus faecium* are the commonest enterococci isolated from human infections **(Naser *et al.* 2005).**

Enterococci were described by The authors as "very hardy and tenacious of life". **(Andrewes and Horder 1906)** studied the biochemical abilities of the Enterococci and manifests that this specific isolate was hemolytic**,** also described organism isolated from fecal samples, that clotted milk and capable to ferment mannitol and lactose they called s faecalis, identical to that observed by MacCallum and Hastings. The scientest used the term faecalis to emphasize its intestinal origin **(Orla-Jensen, 1919).**

Enterococci are Gram-positive facultative anaerobic bacteria that are part of the normal intestinal microbiota, with densities ranging from 10^5 to 10^8 CFU/g of intestinal content **(Yost *et al.* 2011; Dubin and Pamer, 2017).** Members of the genus *Enterococcus*, which includes presently about 40 recognized species, were initially classified as group D streptococci sharing several phenotypic and biochemical similarities, making their identification difficult **(Yost *et al.* 2011).** Enterococci have been proposed as fecal indicator bacteria for microbial source tracking **(Boehm and Sassoubre, 2014)** and

are often used in tracking trends in resistance to antimicrobials for various resistance surveillance systems **(Tyson *et al.* 2018).**

Enterococcus species have emerged as the cause of ~12% of nosocomial infections, with only two species, *Enterococcus faecalis* and *Enterococcus faecium*, causing about 90% of clinical infections **(Torres *et al*., 2018).** Moreover, these two species are considered the third and fourth most prevalent human pathogens worldwide and ranked third in causing bacteremia in Europe and North America, responsible for ~11–13% of all bacteremia cases (**Ammerlaan *et al*. 2013**). *E. faecium* is among the so-called 'ESKAPE' pathogens *(E. faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species),* which cause the majority of the infections in US hospitals and effectively 'escape' the effect of antibacterial drugs **(Rice,2008).**

2. Material and methods

Sample collection and cultivation of Enterococci

A total of 617 cloacal swabs were collected aseptically from diseased poultry with gastrointestinal disorders in small backyards of poultry (layers chicken, broilers, turkeys and ducks) located in 6 districts of Egypt. The swabs were placed into micro-tubes containing sterilized phosphate-buffered saline. They were transported to the laboratory and immediately streaked out onto blood agar containing 5% sheep blood, and incubated at 37.7°C for 48 h (Ulger et al., 2009).

Identification by MALDI-TOF MS

Isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bizzini et al., 2010). Briefly, bacteria from overnight cultures were suspended in 300 µl of bi-distilled water and mixed with 900 µl of ethanol (96% vol/vol; Carl Roth GmbH, Karlsruhe, Germany) for precipitation. After centrifugation for 5 min at 10,000 x g, the supernatant was removed and the pellet was re-suspended in 50 µl of 70% (vol/vol) formic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Fifty microliters of acetonitrile (Carl Roth GmbH) were added, mixed and centrifuged for 5 min at 10,000 x g. One and a half microliter of the supernatant were transferred onto a MTP 384 Target Plate Polished Steel TF (Bruker Daltonik GmbH, Bremen, Germany). After air-drying the material was overlaid with 2 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie GmbH) in a mix of 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich Chemie GmbH). After air-drying spectra were acquired with an Ultraflex instrument (Bruker Daltonik GmbH). The instrument was calibrated with the IVD Bacterial Test Standard (Bruker Daltonik GmbH). Analysis was carried out with the Biotyper 3.1 software (Bruker Daltonik GmbH). Interpretation of results was performed according to the manufacturer's recommendation: score of ≥ 2.3 represented reliable species level identification; score 2.0–2.29, probable species level identification; score 1.7–1.9, probable genus level identification, and score ≤ 1.7 was considered an unreliable identification (Lüthje et al., 2017).

Antibiotic susceptibility testing

The antibiotic susceptibility testing of all isolates was performed with the MICRONAUT system for Gram-positive bacteria using commercial 96-well microtiter plates (Merlin, Bornheim, Germany) according to the manufacturer's recommendations. This system allowed the determination of minimum inhibitory concentrations (MICs) of 22 antimicrobial agents but we considered two glycopeptide antibiotics only teicoplanin and vancomycin in serial dilutions of the antibiotics. Bacteria grown overnight and suspended in NaCl solution (0.9%) to obtain a turbidity corresponding to a McFarland standard of 0.5 (Dr. Lange, CADAS photometer 30, Berlin, Germany). Three hundred microliters of the suspension were added to 11 ml of Mueller–Hinton broth (Oxoid Deutschland GmbH, Wesel, Germany) resulting in a concentration of approximately 10^6–10^7 colony forming units (cfu)/ml. In total, 100 µl of the inoculum were put in each well. After sealing the plates, they were incubated for 18 h to 24 h at 37°C. Reading of plates was done optically. Interpretation was carried out as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2017).

Detection of resistance-associated genes

Genomic DNA was extracted from bacterial cultures using High Pure PCR Template Purification Kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. PCR amplifications of vancomycin resistance genes (*van*A, *van*B and *van*C1) were carried out using primers given in Table 1. PCR products were analyzed by electrophoresis on 2% agarose gel following staining with ethidium bromide and visualizing under UV.

3. Results

Isolation and identification of *Enterococcus* species

Sixty four *Enterococcus* isolates were isolated (Table 2a). Using MALDI-TOF MS three different species were identified representing 30/617 *E. faecium* (4.8%), 16/617 *E. faecalis* (2.5%),10/617*E. gallinarum* (1.6%) and 8/617 *E.aviam* strains (1.2%).

Antimicrobial susceptibility profiles

Antimicrobial resistance profiles of enterococci isolates could be determined (Table 2). resistance rate to vancomycin were different. *E.feacalis* showed high

resistantance rate to both teicoplanin 75.0% and vancomycin 87.5%, followed by *E.faecium* that showed resistance to teicoplanin 40.0% and vancomycin 50.0% and *E.gallinarum resistance to* teicoplanin was 40.0% and vancomycin 30.0% while *E,avaim* showed the lowest rate of resistance to teicoplanin was 25.0% and vancomycin 25.0%.

Detection of antibiotic resistance determinants in enterococci

Vancomycin resistance genes were found in 14 isolates. The *van*A were detected in ten isolates of *E.feacalis* only. The *van*B gene was identified in three *E. faecium* isolates and five isolates of *E.feacalis*. The *van*C1 gene was detected in five *E. faecium* isolates. All *E. gallinarum* isolates harboured the *van*C1. *E.avium* did not harboured any of them.

Table 1. Primer and their sequences used for the detection of vancomycin resistance-associated genes in *Enterococcus* species

Gene	Primer sequences (5´-3´)	Expected amplicon size (bp)	Reference
*van*A	F: ATG AAT AGA ATA AAA GTT GCA ATA R: CCC CTT TAA CGC TAA TAC GAT CAA	1030	Getachew et al., 2012
*van*B	F: AAG CTA TGC AAG AAG CCA TG R:CCG ACA AAA TCA TCC TC	536	Getachew et al., 2012
*van*C1	F:GGA ATC AAG GAA ACC TC R:CTT CCG CCA TCA TAG CT	822	Ünal et al., 2017

Table 2: Antibiotic resistance profile of *Enterococcus faecium* and *Enterococcus gallinarum* isolates from poultry

Antibiotic	*Enterococcus faecium* (n=30)				*Enterococcus Feacalis (n=16)*				*Enterococcus gallinarum* (n=10)				*Enterococcus avaim (n=8)*			
	S	I	R	Resistance rate (%)	S	I	R	Resistance rate (%)	S	I	R	Resistance rate (%)	S	I	R	Resistance rate (%)
Teicoplanin	10	9	11	36.6	2	2	12	75.0	6	0	4	40.0	5	1	2	25.0
Vancomycin	8	7	15	50.0	2	0	14	87.5	6	1	3	30.0	6	0	2	25.0

4. Discussion

Antimicrobial resistance in enterococci is not only of major concern in the clinical setting of hospitals. Bacteria may also affect animal health or may contaminate food of animal origin (Silva et al., 2012). Emergence of the number of infections in humans caused by resistant bacteria that originate from animal reservoirs is of great concern. In fact, results from previous studies showed that transfer of resistance genes from enterococci of animal origin to enterococci in human beings occurred through the food chain (Lester et al., 2006).

In this study, the dominant *Enterococcus* species was *E. faecium* followed by *E. faecalis* and *E. gallinarum* and *E.avaim* which is a similar to the findings of Ünal et al. (2017) who isolated *E. faecium* (60.4%), *E. faecalis* (33.6%) and *E. gallinarum* (2.6%) from broiler samples. No other *Enterococcus* species were detected in this study which could be attributed to different origin and feed contamination (Butaye et al., 1999). *E. faecium* was also the most commonly isolated *Enterococcus* species from poultry cloacal swabs in Turkey (Dilik and İstanbulluoğlu, 2010). In contrast, *E. avium* and *E. gallinarum* were found to be the most predominant *Enterococcus* species in pigeon and duck faeces samples in Egypt (Osman et al., 2019). Pigeon and duck faeces were collected in Cairo city and poor neighborhoods (Osman et al., 2019) while in the presented study samples were collected in farms of six governorates outside the Egyptian metropolis. Thus differences concerning the origin of samples, housing, feeding, breeding but also host specificity may influence study outcome.

E. faecium and *E. faecalis* are the most predominant enterococci species causing human infection worldwide (Billington et al., 2014; Kajihara et al., 2015). They are also a main cause of healthcare-associated infections (Ben Sallem et al., 2016). These two species have also developed resistance to a wide variety of clinically important antibiotics (Bertelloni et al., 2015; Ünal et al., 2017; Kim et al., 2019; Ngbede et al., 2017). In Egypt another often ignored but critical circumstance is the uncontrolled discharge of large amounts of pharmaceutical waste containing active compounds from antibiotic manufacturing plants into rivers and the soil environment in developing countries. This practice contributes to the emergence of antibiotic-resistant organisms resulting in considerable hazard to public health (Grenni et al., 2018). Thus the prominent rates of antibiotic

resistance found in this study may be caused by uncontrolled use of antibiotics for the therapeutic or prophylactic purposes. Antibiotics are still used as growth promoters included in feed for poultry.

In the present a study, *E.feacalis E. faecium* and *E. gallinarum* strains showed resistance to vancomycin which is in accordance with results obtained by Ünal et al. (2017) for broiler cloacal samples in Turkey. Vancomycin resistance was also detected in 10/153 (6.5%) of *Enterococcus* isolates originated from food samples which were collected in different supermarkets and groceries in Egypt (Raafat et al., 2016). The *van*B were detected in both E.feacalis and E.faecium near results were obtained by Osman et al. (2019) found *van*B and *van*C genes in 25.5% and 33.0% in enterococci isolates from poultry in Egypt, respectively. It was comparable to results obtained found in this study. A similar frequency of resistance (23.1%) was found in Egyptian *E. faecium* isolates from hospitals (Moemen et al., 2014).

The *van*A gene could be detected E.feacalis isolates while *van*B and *van*C1 genes were found in *E. faecium* and *E. gallinarum* isolates by PCR. vanC are intrinsic gene in *E.gallinarum*.

Vancomycin resistance in our study reached an alarming rate as it is used for the treatment of enterococcal infections in humans in Egypt in contrast to the situation in the EU (Hao et al., 2016). In contrast to EU where the use of avoparcin which shows chemical similarity to vancomycin is forbitten in livestock feeding avoparcin is widely used in Egypt as growth promoter and for prevention of necrotic enteritis in the poultry production which may led to an increased prevalence of vancomycin resistance in bacteria (Bager et al., 1997).

5. Conclusion

Consumption of antimicrobials is an important risk factor for colonization with multi-drug resistant enterococci because of the suppression of the competitive indigenous microbiota of the gastrointestinal tract.

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