High Prevalence of Vancomycin Non-Susceptible and Multi-Drug Resistant Enterococci in Farmed Animals and Fresh Retail Meats in Bangladesh

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Abstract

The emergence of antimicrobial resistant *Enterococcus* spp., a leading cause of untreatable nosocomial infection, in food animals and dissemination to humans is a public health concern. The study was conducted to determine the prevalence and antimicrobial resistance, and virulence characteristics of *Enterococcus faecalis* and *Enterococcus faecium* in food animals and meats in Bangladesh. *Enterococcus* spp., were confirmed using *sod*A gene specific PCR, and antimicrobial resistance and virulence properties were characterized by PCR. *Enterococcus* spp. were recovered from 57% of the collected samples (n=201/352). Farm samples yielded significantly higher ($p \leq 0.05$) prevalence (62%) compared to retail meat samples (41%) and *E. faecalis* (52%) was most frequently isolated species. High proportions of isolates exhibited resistance to tetracycline (74%), erythromycin (65%) and ciprofloxacin (34%). Fifty-one isolates were vancomycin non-susceptible enterococci (VNSE), of which forty-seven were MDR and 20 were linezolid resistant, a last line drug for VNSE. Virulence factors such as gelatinase (*gel*E), aggregation factor (*asa*1) and sex pheromone (*cpd*) were detected along with vancomycin resistance gene (*van*A, *van*B and *van*C2/C3) in VNSE isolates. The high prevalence of MDR enterococci in food animals and retail meats may lead to infection in consumers with concomitant reduced therapeutic options available for treatment.

Introduction

Enterococci are Gram-positive bacteria belonging to the lactic acid bacterial group which consists of over 50 diverse species, and inhabit the gastrointestinal flora of humans and a wide variety of warm-blooded animals as well as insects (Santestevan et al. 2015). Over the past few decades, *Enterococcus* spp., particularly *Enterococcus faecium* and *Enterococcus faecalis*, have emerged as one of the most challenging healthcare associated pathogens, and is one of the three most common causes of nosocomial infections worldwide (Wang et al. 2015). The spectrum of enterococci-caused infections in humans includes urinary tract infections, peritonitis, endocarditis, neonatal sepsis or even life-threatening infections such as septicemia and meningitis, and with about 80-90% of all enterococcal clinical infections being caused by *E. faecalis* and *E. faecium* (Arias and Murray 2012). Additionally, enterococci are often used as indicators of fecal contamination in food and water, and are able to compete and survive in harsh environments (Lebreton et al. 2014).

Enterococci have low susceptibility to beta-lactams and intrinsic resistance to several antimicrobial classes including sulphonamides, cephalosporins, and low concentrations of aminoglycosides. Indeed, enterococci are able to acquire and exchange genetic elements, including AMR genes, and thereby serve as reservoirs of transferable resistance circulating in Gram positive bacteria (Hollenbeck and Rice 2012; Wang et al. 2015). Acquisition of resistance to antimicrobials such as vancomycin and linezolid in enterococci is of considerable clinical concern, and is the development of multi-drug resistance (MDR) which could restrict treatment options. Vancomycin resistance is conferred by *van* genes such as *vanA*, *vanB*, *vanC1*, *vanC2/C3*, and *vanD* (Kolář 2018). Epidemiological studies have described the prevalence of MDR enterococci in different sources including human, animal, food of animal origin and even in environmental sources such as soil and surface water (Bennani et al. 2012; Cassenego et al. 2011; López-Salas et al. 2013; Torres et al. 2018). The emergence and rapid spread of MDR poses a serious therapeutic challenge for effective antimicrobial therapy in human infections, due to scarcity of newer antimicrobial agents as well as fewer remaining therapeutic options caused by Gram positive organisms (Aslam et al. 2012; Wang et al. 2015). An additional concern is the presence of virulence genes and diverse virulence factors have been reported in enterococci that increase their ability to colonize hospitalized patients and contribute to infections in humans (Mannu et al. 2003).

Antimicrobial resistant enterococci have been observed in food animals and food of animal origin, such as poultry, duck, swine and cattle (Vignaroli et al. 2011), which is an indication of fecal contamination; meat products are therefore at risk of becoming contaminated during the slaughtering process (Boehm and Sassoubre 2014; Tyson et al. 2018; Wang et al. 2015). The literature suggests food animals as reservoir of antimicrobial resistant enterococci, and contaminated products could enter the food chain (Aslam et al. 2012; Hammerum et al. 2010; Hoelzer et al. 2017; Ogier and Serror 2008) in the absence or lack of strict food safety measures in place. Moreover, enterococci from farm animals and food of animal origin can transfer their resistance genes, located on mobile genetic elements, to human endogenous flora as well as transient bacteria, including pathogens (Vignaroli et al. 2011).

The irrational use of antimicrobials with growth promotion properties in livestock and poultry has been identified as a risk factor for the emergence of antimicrobial resistant *Enterococcus* spp (Hoelzer et al. 2017). In Bangladesh, there are over 250 antibiotics registered for human and 100 antibiotics registered for veterinary medicine use according to the Directorate General of Drug Administration (DGDA), Bangladesh database (DGDB 2018). However, vast majority of the veterinary medicinal products registered are categorized by the World Health Organization as critically important antibiotics (CIAs) in human medicine (World Health Organisation 2019)

In Bangladesh, the prevalence and diversity of enterococci such as *E. faecalis* and *E. faecium* in livestock, poultry and in animal origin food products is poorly defined. Furthermore, little is known about the antibiotic resistances and virulence genes harboured in these bacteria. To address this evidence gap, we have undertaken this study to determine the prevalence of enterococci in farm animals and retail meat, and explored their AMR patterns. *E. faecalis* and *E. faecium* were isolated and tested for susceptibility using a panel of eight antimicrobials important in human and veterinary medicine, and vancomycin resistant isolates were further screened for the presence of virulence genes.

Materials And Methods

Sample collection and isolation of *Enterococcus* spp.

A total of 352 samples were collected from selected farms and at retail markets of Savar, Gazipur, and Dhaka from 2016 to 2017. The sampling frame for the farm component constituted poultry (n=136), cattle (n=35), goat (n=29) and camel (n=30). Cloacal swabs were collected from poultry using sterile swab, and for cattle, goat, and camel samples approximately 5 g of freshly excreted fecal contents was collected into 25 ml PBS. For meat products, approximately 5 g of chicken (n=60), beef (n=32) and mutton (n=30) were collected aseptically into 10 ml PBS, and stored on ice for transport to the laboratory within 3-5 hours. All samples were processed using routine bacterial culture for enterococci as per standard protocols (Kuiken et al. 2005) and as outlined below. In brief,

samples (cloacal swab or 1ml of homogenized meat) were cultured in selective pre-enrichment broth (brain heart infusion broth [BHI]; BD Difco™, USA) supplemented with 5% NaCl at 37°C for 24-48 hours. Primary isolation of enterococci was done using kanamycin aesculin azide (KAA) agar (Oxoid, UK) at 37°C for 18-24 hours and further screening was done by transferring 3-5 characteristic brown colonies from KAA agar to 5% sheep blood agar (Oxoid, UK) for another 24 hours at 37°C, followed by biochemical and temperature tolerance test (i.e. 10°C then to 40°C) (Guerrero-Ramos et al. 2016). Final confirmation of species identification was done for one or two selected pure isolates for each primary positive sample, using u-PCR I and m-PCR I based on enterococci specific *sod*A gene. Genus specific primer for *Enterococcus* spp., and species-specific primer for *E. faecalis* and *E. faecium* were used for molecular confirmation of enterococci isolates according to previously published protocol (Jackson et al. 2004; Poyart et al. 2000) with a slight modification. DNA extraction was performed using conventional boiling method (Huq et al. 2012). List of primer and amplified target DNA size used in this study are provided in Supplementary material 1.

Antimicrobial susceptibility assay

Kirby-Bauer disk diffusion assay was performed according to Clinical and Laboratory Standards Institute standards M02-A12 and M07-A10 (Tuohy et al. 2000). Quality control strain *E. faecalis* ATCC 29212 was used as control for antimicrobial susceptibility testing. *E. faecalis* and *E. faecium* isolates were susceptibility tested using a panel of eight antimicrobials commonly used in veterinary and human medicine (Oxoid, Hampshire, UK) belong to phenicols (chloramphenicol: 30µg), quinolones (ciprofloxacin: 5µg), macrolides (erythromycin: 15µg), oxazolidinones (linezolid: 30µg), glycopeptides (vancomycin: 30µg), penicillins (penicillin G: 10µg), nitrofurans (nitrofurantoin: 300µg), and tetracyclines (tetracycline: 30µg). Among the all isolated enterococci a subset of pure *E. faecalis* and *E. faecium* culture (n=117) representing individual positive samples were selected for antibiogram study. Isolates non-susceptible to three or more antibiotic classes were defined as MDR. Isolates non-susceptible to 7 or 8 of the antibiotics were termed as possible-Extremely Drug Resistant (XDR) isolates according to published criteria (Magiorakos et al. 2012). Isolates non-susceptible to vancomycin were termed vancomycin non-susceptible enterococci (VNSE).

Statistics and data analysis

Descriptive statistics and univariate analysis were performed using SPSS Statistics 20 software (IBM corp, USA) and The Survey System 12.0 (Creative Research Systems, USA). Inhibition zone data in antibiotic susceptibility assay were analyzed using BacLink and WHONET-2019 software (WHO Collaborating Centre for Surveillance of Antimicrobial Resistance, USA) (Stelling et al. 2007). Comparative analysis of antibiotic resistance was done using online data analysis tool 'Morpheus' created on R Studio interface to identify isolates that exhibited dual antibiotic resistance patterns (Morpheus 2018). For this analysis the cattle, goat, and camel samples were combined to form a group termed 'Livestock' as farming system for these species are largely similar in Bangladesh. Moreover, same butcher shop sells all these meat species sharing the same slaughtering facilities and equipment and similar processing techniques. A p value of ≤ 0.05 was considered significant.

Antibiotic resistance gene and virulence factors identification

The presence of the vancomycin resistance genes *van*A, *van*B, *van*C_1 and *van*C_2/C_3 were determined using a multiplex PCR (m-PCR II) according to previously published protocol. Published PCR primers were also used to assess the presence of ten virulence factors: aggregation substance (*asa*1), gelatinase (*gel*E), cytolysin (*cyl*A), enterococcal surface protein (*esp*), hyaluronidase (*hyl*), collagen-binding protein (*ace*), transmembrane protein (*fsr*B), endocarditis specific antigen (*efa*), aggregation protein (*agg*), and sex pheromones (*cpd*) (Strateva et al. 2016; Vankerckhoven et al. 2004; Zoletti et al. 2011). All PCR primers used are given in (Supplementary material 1)

Results

Isolation of Enterococcus spp.

A total of 211 *Enterococcus* spp. comprising 115 (105 single and 10 co-isolation) *E. faecalis*, 36 (26 single and 10 co-isolation) *E. faecium* and 60 *enterococci* not assigned to a species by PCR were isolated from 352 collected samples, summarized in Table 1 and detailed in Supplementary material 2. Overall prevalence of *Enterococcus* spp. was 57% (95%, CI 52-62). In poultry, farm samples yielded significantly higher recovery of enterococci ($p < 0.05$) compared to poultry meat samples of retail markets. Conversely, for livestock a greater proportion of meat samples were positive for enterococci compared to fecal samples (Table 1). In case of samples collected on farm, a significantly greater proportion of poultry isolates were culture positive for *E. faecalis* compared to livestock, however, retail livestock meat carried the greater proportion of *E. faecalis* than poultry meat (Table 1).

Table 1
Prevalence of enterococci in corresponding farm and fresh meat samples. Significant p- value are marked as bo

Isolates	Poultry (n=196)							Livestock (n=156)							p-value	Risk Esti
	Farm (n=136)		Meat (n=60)		p-value	Risk Estimate		Farm (n=94)		Meat (n=62)		p-value	Risk Estimate			OR
n (%)	95% CI	n (%)	95% CI	OR	95% CI	n (%)	95% CI	n (%)	95% CI	OR	95% CI			OR	95% CI	
E. faecalis	50 (36.8%)	28.7 - 44.9%	12 (20.0%)	10.0 - 31.6%	**.021**	2.3	1.1-4.8	18 (19.1%)	11.7 - 27.7%	25 (40.3%)	29.0 - 51.6%	**.006**	0.4	0.2-0.7	.415	1.2
E. faecium	10 (7.4%)	3.7 - 12.5%	6 (10.0%)	3.3 - 18.3%	.575	0.7	0.2-2.1	08 (8.5%)	3.2 - 14.9%	2 (3.2%)	0 - 8.1%	.317	2.7	0.6-13.6	.682	1.3
E. faecalis & *E. faecium*)	5 (3.7%)	0.7 - 7.4%	0 (0.0%)	0 - 0%	.326	-	-	4 (4.3%)	1.1 - 8.5%	1 (1.6%)	0 - 4.8%	.649	2.7	0.3-24.8	.755	0.8
Other Enterococci	19 (14.0%)	9.7 – 19.5%	3 (5.0%)	1.1 - 11.3%	.055	1.9	0.8-3.7	28 (29.8%)	23.7 - 36.6%	10 (16.1%)	29.0 - 51.6%	.125	1.8	0.7-3.9	.335	1.3
Overall	84 (61.8%)	53.7 - 68.5%	21 (35.0%)	18.9 - 41%	**.028**	2.1	1.1-4.1	58 (61.7%)	52.8 - 69.3%	38 (61.3%)	52.0 - 70.1%	.087	0.5	0.2-1.1	.381	1.1

Antimicrobial Susceptibility

High prevalence of VNSE (44%) was observed in both farm (41%) and meat (52%) isolates, in addition, resistance level was also noteworthy for tetracycline (74%) and erythromycin (65%). Moreover, twenty isolates (17%) were resistant to linezolid which includes 14 and 6 isolates from farm and meat respectively. MDR was observed in 80% of the isolates with a similar proportion for both *E. faecalis* (79%) and *E. faecium* (81%). One *E. faecium* isolate and seven *E. faecalis* isolates were possible-XDR, having resistance to ≥7 antibiotic classes. Almost all the *E. faecalis* and *E. faecium* from poultry, including poultry farms (n=57/60) and poultry meat (n=9/9) were MDR. The full antibiotic resistance patterns and profiles towards the eight antimicrobials tested is presented in Table 2 and Figure 1

Table 2
Antibiotic resistance of enterococci among tested poultry, poultry meat, livestock and livestock meat isolates

Enterococci Sources	Isolates	Antimicrobials								
		CHL	CIP	ERY	NIT	LID	PEN	TET	VAN	MDR
Poultry	*E. faecalis* (n=46)	9 (20%)	17 (37%)	38 (83%)	6 (13%)	8 (17%)	21 (46%)	44 (96%)	9 (20%)	43 (93%)
	E. faecium (n=14)	1 (7%)	6 (43%)	11 (79%)	2 (14%)	0 (0%)	3 (21%)	13 (93%)	1 (7%)	14 (100%)
	Overall (n=60)	10 (17%)	23 (38%)	49 (82%)	8 (13%)	8 (13%)	24 (40%)	57 (95%)	10 (17%)	57 (95%)
Poultry Meat	*E. faecalis* (n=5)	2 (40%)	2 (40%)	5 (100%)	2 (40%)	3 (60%)	2 (40%)	4 (80%)	3 (60%)	5 (100%)
	E. faecium (n=4)	1 (25%)	2 (50%)	4 (100%)	1 (25%)	1 (25%)	2 (50%)	4 (100%)	1 (25%)	4 (100%)
	Overall (n=9)	3 (33%)	4 (44%)	9 (100%)	3 (33%)	4 (44%)	4 (44%)	8 (89%)	4 (44%)	9 (100%)
Livestock	*E. faecalis* (n=15)	0 (0%)	2 (13%)	7 (47%)	0 (0%)	3 (20%)	0 (0%)	7 (47%)	2 (13%)	9 (60%)
	E. faecium (n=11)	0 (0%)	0 (0%)	6 (55%)	0 (0%)	3 (27%)	1 (9%)	5 (45%)	2 (18%)	6 (55%)
	Overall (n=26)	0 (0%)	2 (8%)	13 (50%)	0 (0%)	6 (23%)	1 (4%)	12 (46%)	4 (15%)	15 (58%)
Livestock's Meat	*E. faecalis* (n=20)	0 (0%)	7 (35%)	4 (20%)	1 (5%)	2 (10%)	2 (10%)	9 (45%)	0 (0%)	11 (55%)
	E. faecium (n=2)	0 (0%)	1 (50%)	1 (50%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	0 (0%)	1 (50%)
	Overall (n=22)	0 (0%)	8 (36%)	5 (23%)	1 (5%)	2 (9%)	2 (9%)	10 (46%)	0 (0%)	12 (55%)
Overall	*E. faecalis* (n=86)	11 (13%)	28 (33%)	54 (63%)	9 (10%)	16 (19%)	25 (29%)	64 (74%)	14 (16%)	68 (79%)
	E. faecium (n=31)	2 (6%)	9 (29%)	22 (71%)	3 (10%)	4 (13%)	6 (19%)	23 (74%)	4 (13%)	25 (81%)
Overall Resistance (n=117)		13 (11%)	37 (32%)	76 (65%)	12 (10%)	20 (17%)	31 (27%)	87 (74%)	18 (15%)	93 (80%)

Note: CHL: Chloramphenicol, CIP: Ciprofloxacin, ERY: Erythromycin, NIT: Nitrofurantoin, LID: Linezolid, PEN: Penicillin G, TET: Tetracycline, VAN: Vancomycin, MDR: Multi-drug Resistance.

Detection Of Antibiotic Resistance Genes

A total of 51 VNSE isolates were further characterized to determine the presence of vancomycin resistance genes (Table 3). Twenty isolates were PCR positive for at least one vancomycin resistance gene, of which nine isolates were phenotypically resistance to vancomycin. Nonetheless, eleven isolates harbored at least one vancomycin resistance gene without being phenotypically resistant, though these were vancomycin non sensitive. The most commonly detected vancomycin resistance genes were *van*A *and van*$C_{2/3}$.

Table 3
Resistance profile, vancomycin resistance gene and virulence factors observed in multidrug resistant isolates from different sources

Sample ID	Source	Isolate	Resistance Profile	N. Res	VAN	Van resistance gene	Virulence gene
BCS 016	Pountry Cloaca	*E. faecalis*	ERY VAN	2	R		*gel*E, *cpd*
LBM 002	Beef	*E. faecalis*	ERY VAN	2	I		*asa*1
LBM 011	Beef	*E. faecalis*	ERY VAN	2	I		*asa*1, *gel*E, *cpd*
L-En 017	Camel Fecal	*E. faecium*	ERY VAN	2	I		
LBM 014	Beef	*E. faecalis*	CIP ERY VAN	3	I		*asa*1, *gel*E, *cpd*
LBM 023	Beef	*E. faecalis*	CIP ERY VAN	3	I		*asa*1, *gel*E
LBM 028	Beef	*E. faecalis*	CIP ERY VAN	3	I		*asa*1, *cpd*
L-En 028	Camel Fecal	*E. faecium*	ERY TET VAN	3	I		
BBS 009	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I		*gel*E, *cpd*
BBS 012	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I		*cpd*
BCS 031	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I	*van*C1, *van*C2/3	*asa*1, *gel*E, *cpd*
BCS 032	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I	*van*C1, *van*C2/3	*gel*E, *cpd*
BCS 033	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I		*gel*E
LBM 001	Beef	*E. faecalis*	CIP ERY TET VAN	4	I		*gel*E, *cpd*
LBM 016	Beef	*E. faecalis*	CIP ERY LNZ VAN	4	I		*asa*1, *gel*E, *cpd*
LCM 047	Chicken Meat	*E. faecalis*	CIP ERY TET VAN	4	I		*gel*E, *cpd*
LCS 006	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I		*gel*E
LCS 008	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I	*van*A	*asa*1, *gel*E
LCS 008	Pountry Cloaca	*E. faecium*	CIP ERY TET VAN	4	I	*van*A	*asa*1, *gel*E
LCS 011	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I		*gel*E, *cpd*
LCS 013	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I		*asa*1, *gel*E, *cpd*
LCS 032	Pountry Cloaca	*E. faecium*	CIP ERY NIT VAN	4	R		*asa*1, *gel*E, *cpd*
LCS 048	Pountry Cloaca	*E. faecalis*	CIP ERY TET VAN	4	I		
L-En 009	Camel Fecal	*E. faecalis*	CIP ERY LNZ VAN	4	I	*van*C2/3	*gel*E, *cpd*
L-En 014	Camel Fecal	*E. faecalis*	ERY TET LNZ VAN	4	R	*van*A	*asa*1, *gel*E
L-En 015	Camel Fecal	*E. faecium*	ERY TET LNZ VAN	4	R	*van*A	
L-En 033	Goat Fecal	*E. faecalis*	ERY TET LNZ VAN	4	I	*van*C1	*asa*1, *gel*E
L-En 033	Goat Fecal	*E. faecium*	ERY TET LNZ VAN	4	I	*van*C1	*asa*1, *gel*E
LMM 015	Mutton	*E. faecalis*	CIP ERY TET VAN	4	I		
BBS 007	Pountry Cloaca	*E. faecalis*	CHL CIP ERY TET VAN	5	I		*gel*E, *cpd*
BCS 013	Pountry Cloaca	*E. faecalis*	CHL CIP ERY TET VAN	5	I		*gel*E, *cpd*
LCM 037	Chicken Meat	*E. faecium*	CHL CIP ERY TET VAN	5	I		
L-En 027	Camel Fecal	*E. faecium*	ERY PEN TET LNZ VAN	5	R	*van*B	*asa*1, *gel*E, *cpd*
BCS 029	Pountry Cloaca	*E. faecalis*	CHL ERY PEN TET LNZ VAN	6	R		*asa*1, *gel*E, *cpd*
BCS 035	Pountry Cloaca	*E. faecalis*	CHL CIP ERY NIT TET VAN	6	I	*van*C1,	*asa*1, *gel*E
BCS 035	Pountry Cloaca	*E. faecium*	CHL CIP ERY NIT TET VAN	6	I	*van*C1	*asa*1, *gel*E
LBM 022	Beef	*E. faecalis*	CHL CIP ERY PEN TET VAN	6	I		*asa*1, *gel*E
LCM 054	Chicken Meat	*E. faecalis*	CIP ERY PEN TET LNZ VAN	6	R		*gel*E, *cpd*
LCM 057	Chicken Meat	*E. faecalis*	CIP ERY NIT TET LNZ VAN	6	R		*gel*E, *cpd*

Note: N. Res: Number of antibiotic classes to which the corresponding isolate was non-susceptible. CHL: Chloramphenicol, CIP: Ciprofloxacin, ERY: Erythromycin, NIT: Nitrofurantoin, PEN: Penicillin G, TET: Tetracycline, LID: Linezolid, VAN: Vancomycin. R: Resistant, I: Intermediate.

Sample ID	Source	Isolate	Resistance Profile	N. Res	VAN	Van resistance gene	Virulence gene
LCS 003	Pountry Cloaca	*E. faecalis*	CIP ERY PEN TET LNZ VAN	6	R		*gel*E, *cpd*
LCS 037	Pountry Cloaca	*E. faecalis*	CIP ERY NIT TET LNZ VAN	6	R		*gel*E, *cpd*
LCS 039	Pountry Cloaca	*E. faecalis*	ERY NIT PEN TET LNZ VAN	6	R		*gel*E, *cpd*
LCS 040	Pountry Cloaca	*E. faecalis*	CIP ERY NIT TET LNZ VAN	6	R	*van*C2/3	*gel*E, *cpd*
BCS 030	Pountry Cloaca	*E. faecalis*	CHL CIP ERY NIT PEN TET VAN	7	I	*van*C1	*gel*E
LCM 040	Chicken Meat	*E. faecium*	CIP ERY NIT PEN TET LNZ VAN	7	R		*gel*E
L-En 003	Camel Fecal	*E. faecalis*	CHL CIP ERY NIT TET LNZ VAN	7	R	*van*A, *van*C2/3	*gel*E, *cpd*
LBM 025	Beef	*E. faecalis*	CHL CIP ERY NIT PEN TET LNZ VAN	8	I	*van*A	*asa*1, *gel*E
LCM 036	Chicken Meat	*E. faecalis*	CHL CIP ERY NIT PEN TET LNZ VAN	8	R	*van*A	*gel*E, *cpd*
LCS 016	Pountry Cloaca	*E. faecalis*	CHL CIP ERY NIT PEN TET LNZ VAN	8	R	*van*C2/3	*gel*E, *cpd*
LCS 018	Pountry Cloaca	*E. faecalis*	CHL CIP ERY NIT PEN TET LNZ VAN	8	R	*van*C2/3	*asa*1, *gel*E, *cpd*
LCS 029	Pountry Cloaca	*E. faecalis*	CHL CIP ERY NIT PEN TET LNZ VAN	8	R	*van*A, *van*C2/3	*cpd*

Note: N. Res: Number of antibiotic classes to which the corresponding isolate was non-susceptible. CHL: Chloramphenicol, CIP: Ciprofloxacin, ERY: Erythromycin, NIT: Nitrofurantoin, PEN: Penicillin G, TET: Tetracycline, LID: Linezolid, VAN: Vancomycin. R: Resistant, I: Intermediate.

Detection Of Virulence Genes

The 51 VNSE isolates were also tested for the presence of ten virulence factors. The gelatinase gene (*gel*E) was observed in 79% isolates (42), aggregation factor (*asa*1) in 38% isolates (20), and the sex pheromones (*cpd*) in 66% isolates (35) (Table 3). Other virulence factors, including cytolysin, surface protein, hyaluronidase, collagen-binding protein, transmembrane protein, endocarditis specific antigen, and aggregation protein were not observed among these isolates.

Discussion

To assess the prevalence of AMR enterococci in healthy finisher livestock and poultry, and their products in Bangladesh, we screened 230 samples from livestock and poultry at farms and 122 meat samples at retail markets. Our findings indicate that *Enterococcus* spp. are relatively common in poultry and livestock animals in Bangladesh, though the overall prevalence of *E. faecium* was lower than *E. faecalis*. Nonetheless, poultry has been demonstrated as the leading source of *Enterococcus* spp. in several studies conducted elsewhere (Maasjost et al. 2015; St□pień-Pyśniak et al. 2016). The prevalence of *E. faecalis* was significantly higher in poultry farms than at other livestock farms, but retail livestock meat yielded a higher prevalence of *E. faecalis* than poultry meat. This is likely to be indicative of post-slaughter contamination as well as the absence of, or lack of enforcement of, food safety regulations during the slaughtering process and meat product handling in Bangladesh.

Poultry and livestock are essential to food security in Bangladesh, the potential dissemination of resistant *Enterococcus* spp. in the food production continuum and strengthening of food safety regulations needs to be addressed. In the context of One Health and to further inform food safety interventions in Bangladesh, it is also important to identify the major reservoirs and their dissemination downstream of the production continuum, which was the intent of our study. Our data indicates that more than half of the samples were contaminated with *Enterococcus* spp. and the most predominant species was *E. faecalis*, the third most commonly identified pathogen in hospitals associated with increased mortality (Coque 2008). These findings are consistent with several preceding studies (Ngbede et al. 2017; Poeta et al. 2006; Yoshimura et al. 2000). There is no extensive data available on AMR amongst enterococci in livestock and poultry in Bangladesh because a national AMR surveillance program has not yet been established. However, one study showed high prevalence of enterococci among chickens (Banik et al. 2018) consistent with our study. Two relevant studies in involving human cases have implicated enterococci (Akram Hossain 2016; Suchi et al. 2018).

High prevalence of VNSE (44%; n=51) has been observed in this study which is higher than some other studies (Cosentino et al. 2010; Maasjost et al. 2015; St□pień-Pyśniak et al. 2016). Nonetheless, most alarming finding was the co-occurrence of linezolid resistance and VNSE, where most linezolid (90%) resistant isolates were VNSE, which could drive a therapeutic crisis for treating infection caused by VNSE (Bialvaei et al. 2017). In addition, this study detected low susceptibility of isolates to antimicrobials that are concurrently being used for both human infection and animal production in Bangladesh. In our study, the frequency of resistance to tetracyclines and macrolides were relatively high whereas comparatively low resistance were observed to DNA synthesis inhibitors including nitrofurantoin (10%) and ciprofloxacin (31%), which are consistent with similar studies conducted elsewhere (Cosentino et al. 2010; Maasjost et al. 2015; St□pień-Pyśniak et al. 2016).

MDR prevalence was high in farm and in fresh meat sold at retail markets (i.e. products originating from the same geographical locations where they were raised) and most of the VNSE isolates were also MDR (92%, 47/51). Remarkably, isolates that were possible-XDR were mostly recovered from poultry farms, this also raises the potential public health concern of exposure to and consumption of products from this species. These findings may be reflective of the

indiscriminate use of antimicrobials in poultry in Bangladesh, (Ahmed et al. 2019) notably, the high use of CIAs (Imam et al. 2020) and thus support the need for surveillance of AMR and monitoring of AMU to inform changes in usage policy and to better understand AMR and AMU relationships. The comparative pairwise antibiotic resistance matrix reveals that the combination between chloramphenicol or ciprofloxacin with nitrofuratoin, linezolid or vancomycin may produce better efficiency against multidrug resistant isolates as combined resistance level to those antibiotics is very low compared to the other antibiotics (Figure 2). These findings warrant further research.

The presence of *van*A gene in our study, primarily responsible for vancomycin resistance (Torres et al. 2018) further highlight the widespread dissemination of these resistant strains in animal populations. However, there was a discrepancy in detection of vancomycin resistance gene in our study; it was detected in both resistance and intermediate phenotypes, which might be showing that resistance is not dependent on gene presence alone rather depends on the gene expression level. We also found the presence of *van*C1 and *van*C2/3 gene among *E. faecalis* which was previously thought to be species specific to *E. gallinarum* and *E. casseliflavus*, respectively (Clark et al. 1998). The *vanC* gene cluster can be located on plasmids (as well as the chromosome), and can be transferred to other enterococci such as *E. faecium* and *E. faecalis*, (Moura et al. 2013; Sun et al. 2014) however the location of the *vanC* gene in the isolates from this study was not determined and may warrant further study in the future. We observed that most genotypic vancomycin-resistant enterococci (VRE) isolates were phenotypically non-resistant to vancomycin indicating vancomycin-variable enterococci (VVE), as described previously (Downing et al. 2015; Thaker et al. 2015). This could be a result of the mutation in *van* gene cassette including *van*SR or *van*HAXY gene cluster or any other novel mutations.(Hong et al. 2008) This particular finding has impact in clinical settings, where the misidentification in enterococcal infections may result to challenges in the development of an efficacious treatment regimen. The preceding studies found the relevance of VVE with only *van*A gene, but in this study, we also found the association of *van*B, *van*C1 and *van*C2/3 with VVE which would require further investigation. We also found some vancomycin resistant isolates without having any major VRE gene which may be due to the other type of resistance mechanism and beyond the scope of our current study. However, this discrepancy in vancomycin resistance pattern may evolves as a new therapeutic challenge for clinical setting. An immediate improvisation in diagnostic technique for VVE and continuous surveillance for VVE is utmost necessary as some other studies suggested the same (Downing et al. 2015; Szakacs et al. 2014).

Moreover, these VRE gene have been associated with vancomycin resistance and can easily be transferred to another susceptible isolates since there is a probability to be clustered in a mobile genetic elements like plasmid or transposons (Torres et al. 2018) and investigation of this in the future would be informative. This transfer could occur within livestock farms and their environment or throughout the food production chain from farm to fork as evidenced by the detection of MDR *E. faecalis* and *E. faecium* from diverse food animal species such as poultry, cattle, and goat and meat products/meat-derived products (Hammerum et al. 2010; Hoelzer et al. 2017).

The high prevalence of virulence indicators like gelatinase, sex pheromones, and aggregation factors were found among the MDR isolates. The presence of these factors in MDR enterococci can contribute to the colonization or formation of bacterial biofilm-like vegetations among immuno-compromised patients through urinary tract or blood and subsequently turned into untreatable urinary tract infection (UTI) or endocarditis respectively (Sharifi et al. 2013).

Conclusion

Our study indicated that the livestock and poultry fresh meat are frequently contaminated with *Enterococcus* spp., possibly due to insufficient food safety practices in the slaughtering and meat processing systems in Bangladesh. Mostly poultry meat samples were contaminated with *E. faecalis* indicative that poultry is potentially an important source of resistant enterococci that can infect people via the food chain. The presence of enterococci resistant to WHO's CIA in food animals can pose an unprecedented threat to public health. The most alarming finding was the co-occurrence of vancomycin and linezolid resistance in enterococci in livestock and poultry. Moreover, the prevalence of gelatinase, pheromones and aggregation factor in MDR enterococci, in the face of poorly enforced (or lack of) food safety regulations in livestock and poultry in Bangladesh could plausibly lead to their widespread dissemination and persistence in nature. This study calls for an urgent need to reduce the use of WHO's CIA in livestock and poultry and enhancement of food safety practices at the farms and slaughter plants in Bangladesh to reduce the public health implications of *Enterococcu*s spp., particularly VRE.

Declarations

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Authors' contributions

Conceptualization: Mohammed A. Samad; Methodology, Formal Analysis and investigation: Md Shahjalal Sagor, Muhammad Sazzad Hossain, Mohammad Asheak Mahmud, Md Samun Sarker; Writing – original draft preparation: Mohammed A. Samad, Md Shahjalal Sagor; Writing – review and editing: Fahria A. Shownaw, Md Rezaul Karim, Zakaria Mia, Md Samun Sarker, Roderick M. Card, Agnes Agunos, Lindahl Johanna; Funding acquisition: Mohammed A. Samad; Resources: Mohammed A. Samad; Supervision: Mohammed A. Samad, Lindahl Johanna.

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Code or data availability: On Request

Competing Interests: None

Ethical Approval: This study was conducted under the ethical regulation constructed by Bangladesh Livestock Research Institute (ARAC: 01/10/2016:01).

Consent to participate: Verbal consent from the authority of each farm was taken before collecting the samples from the farm.

Consent for publication: We give our consent for the publication of the submitted manuscript.

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Figures

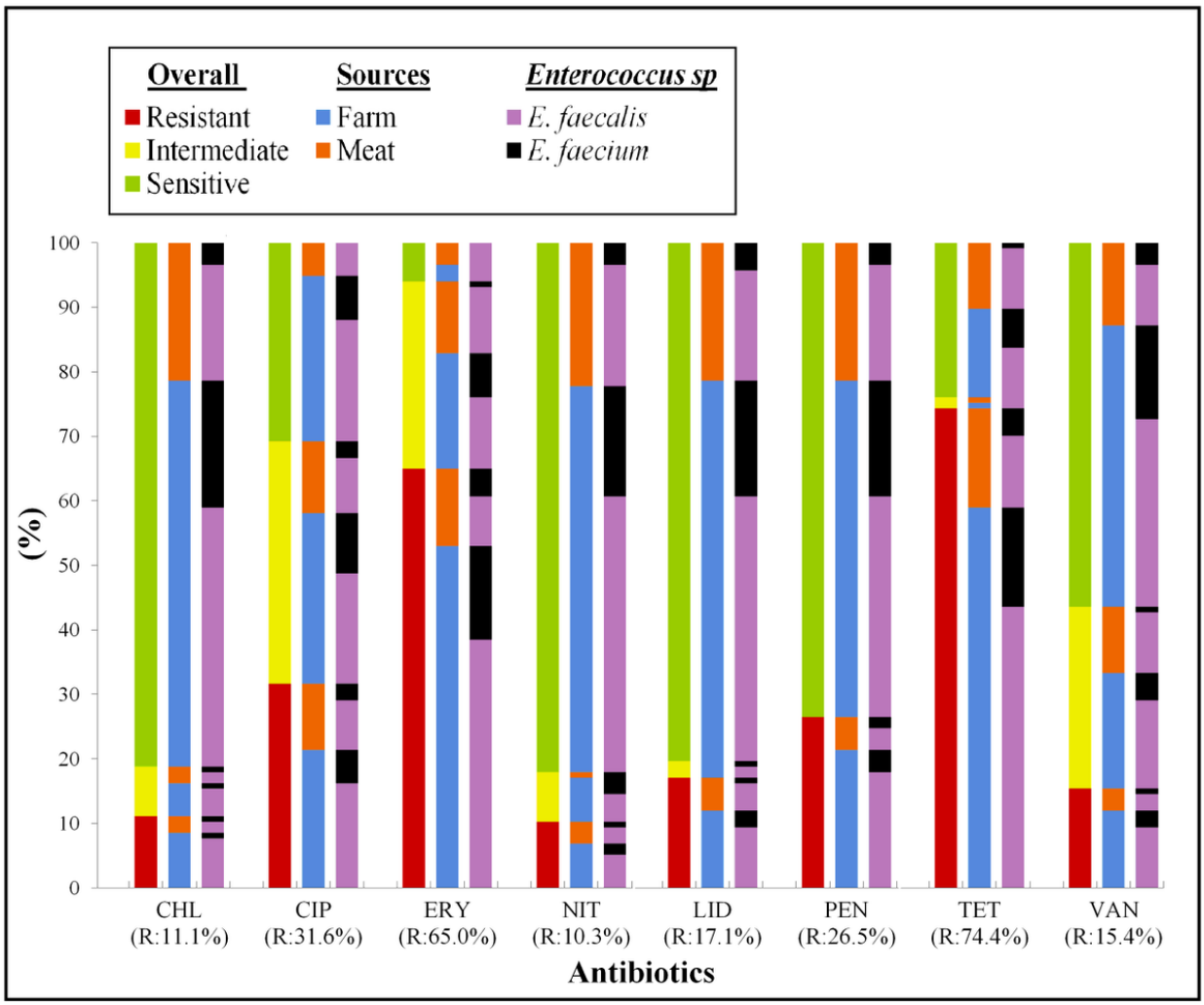

Figure 1

Overall antimicrobial resistance of Enterococci isolates against each antibiotic: 117 isolates comprising 86 E. faecalis and 31 E. faecium originated from farm (n=86) and meat (n=31), were tested against 8 antibiotics. Of three vertically related adjacent bars for each antibiotic, the left bar representing the overall antimicrobial sensitivity of corresponding antibiotic against all isolates; middle bar shows the origin of resistant, intermediate or sensitive isolates; and the right bar express the species of isolates (E. faecalis or E. faecium) themselves related to the source and antimicrobial criteria. Note: R: Resistance, CHL: Chloramphenicol, CIP: Ciprofloxacin, ERY: Erythromycin, NIT: Nitrofurantoin, LID: Linezolid, PEN: Penicillin G, TET: Tetracycline, VAN: Vancomycin.

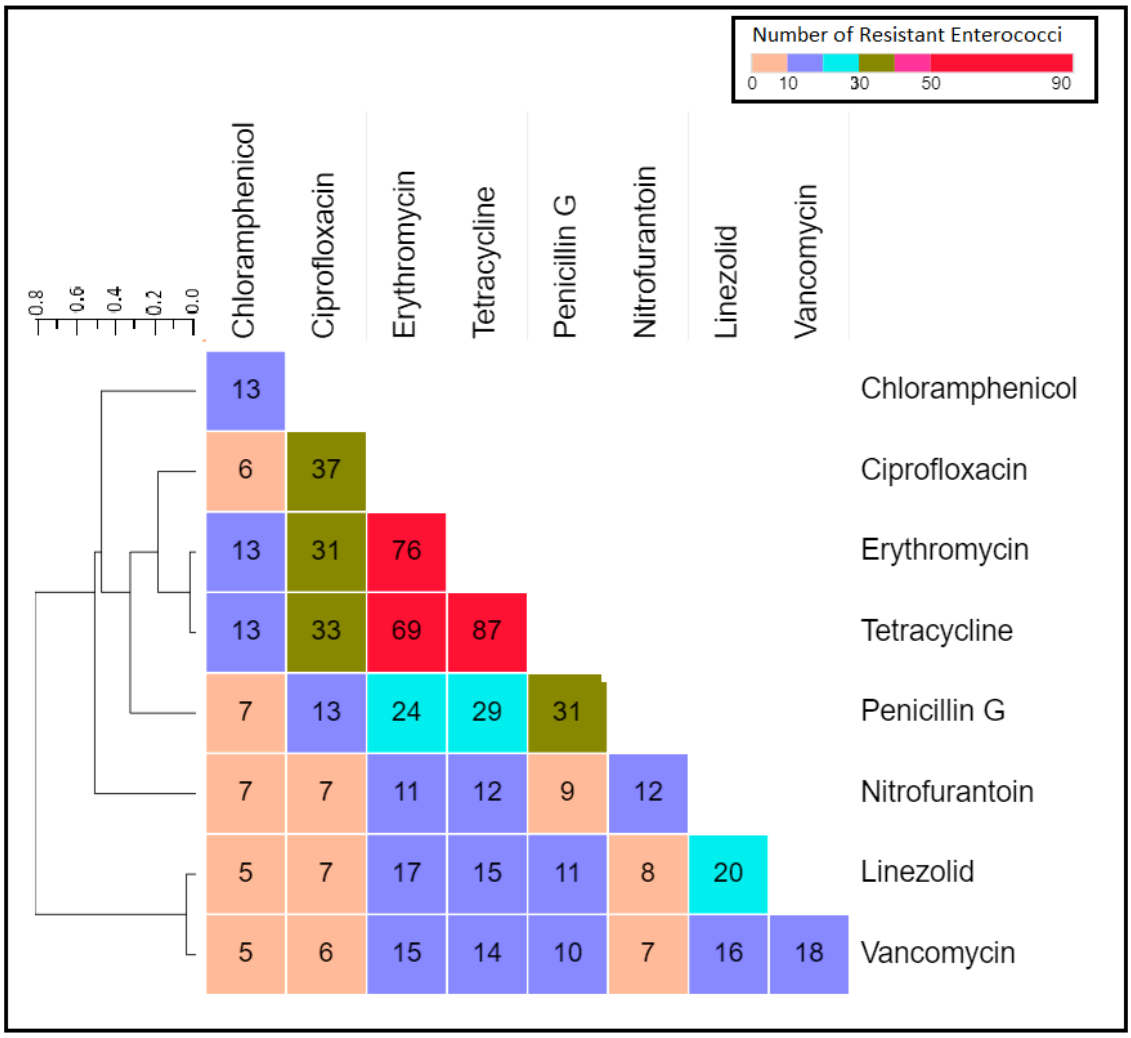

Figure 2

Comparison and dendrogram cluster of pairwise antimicrobial resistance pattern against the all-tested antibiotics: The dual antibiotic resistance pattern was visualized through hit-map similarity matrix and hierarchical clustering was done based on one minus Pearson correlation coefficient and average linkage method. In above matrix the value of each square representing the number of resistant enterococci against both corresponding vertical and horizontal antimicrobials.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SamadSupplementary02PrevalenceOfEnterococciInFarmAndMeat.docx
- SamadSupplementary03AMRpatternHostSppeciesGroups.xlsx
- SamadSupplementary04ASTzoneData.xlsx
- SamadSupplementary01ListOfPrimers.docx