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RESEARCH ARTICLE

Phenotypic and Genotypic Antibiotic Resistance and Virulence Profiling of *Enterococcus faecalis* Isolated from Poultry at Two Major Districts in Bangladesh

Md Shahjalal Sagor [1,2], Muhammad Sazzad Hossain[2], Tarequl Islam[3,4*], Mohammad Asheak Mahmud[1,2], Md. Sujan Miah[1,2], Md Rezaul Karim[2], Md Giasuddin[2] and Mohammed Abdus Samad[2*]

[1]Department of Microbiology, Jagannath University, Dhaka 1100; [2]Animal Health Research Division, Bangladesh Livestock Research Institute, Savar-1341, Bangladesh; [3]Department of Microbiology, Noakhali Science and Technology University, Noakhali-3814, Bangladesh; [4]Department of Biotechnology, Yeungnam University, Gyeongsan 38541, South Korea

*Corresponding author: tarequembg@gmail.com; msamad@blri.gov.bd

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A B S T R A C T

The current study focuses on phenotypic and genotypic antimicrobial tolerance and virulence of *Enterococcus faecalis* (EF) isolated from poultry in Bangladesh. Total 136 cloacal swab samples were collected randomly from meat and egg-producing poultry and analyzed for *E. faecalis*. The overall presence of *E. faecalis* was 21.3% (n=29) where 34.5% commercial broiler (CB) (n=10), 51.7% commercial layer (CL) (n=15) and 13.8% broiler breeder (BB) (n=04) were infected. Among 13 tested antibiotics, the highest resistance was found to penicillin G (100%), followed by streptomycin and tetracycline (97%). However, only imipenem showed high sensitivity (86%) with zero resistance. A significant level of multi-drug resistant (MDR) and possible-extremely drug resistant (XDR) have been observed among 66.52 and 20.69% isolates respectively. The highest MIC values (MIC_{50}/MIC_{90}) were observed for sulfamethoxazole and chloramphenicol (≥1024/≥1024), while only gentamicin showed satisfactory efficiency against *E. faecalis* (≤1/16). Phenotypically vancomycin-resistant isolates were found to carry *van*C_2 and *van*A genes but the *van*B gene was found only among the intermediate isolates. There was a correlation between *van*A, *van*B and *van*C2 genes with virulence genes (*gel*E, *cpd* and *asa*1). Increased level of sequence similarity of multi-drug resistant isolates with Asian and European virulent strains were observed. To our knowledge, this is the first time report on genotypic vancomycin and linezolid resistance in poultry in Bangladesh. This study indicated that multiple antibiotic-resistant *E. faecalis* strains isolated from the poultry of the study areas in Bangladesh could be a possible source for disseminating antibiotic resistance and regarded as a severe threat to public health.

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INTRODUCTION

Enterococci found in diverse food sources, such as meat, milk, cheese, vegetables and water surfaces are essential members of lactic acid bacteria (Jamet *et al.*, 2012). In addition, these bacteria can play a valuable role in many traditional dietary products as they help develop the ripeness and aroma of certain cheeses or fermented sausages (Franz *et al.*, 2011).

Enterococcus sp. is also found in the environment but their elimination is very complicated as they can tolerate harsh and unfavorable conditions (Ayeni *et al.*, 2016). Moreover, internal antibiotic resistance and genes encoding some virulence factors have made enterococci severe infectious agents in clinical microbiology (Gaglio *et al.*, 2016).

E. faecalis is one of the most common members of the Enterococci family. It is usually found in the gut intestine of humans and birds and is considered an opportunistic pathogen, and also associated with nosocomial infections (Fisher and Phillips, 2009). Nowadays, infections related to *E. faecalis* are of significant concern in poultry industries as clinical symptoms of *E. faecalis* infection are associated with despair growth of chicken as well as pulmonary hypertension (Tankson, Thaxton, and Vizzier-Thaxton, 2001), amyloid arthropathy and high mortality within the

first week (Gregersen *et al.*, 2010). Moreover, *E. faecalis* and *E. faecium* are difficult to control by antimicrobials. They pose intrinsic antimicrobial resistance mechanisms and are the third leading cause of hospital-acquired infections in the United States (Report, 2001). Twelves sequence types (STs) of *E. faecalis* were reported in broiler breeder with different clinical conditions (Gregersen *et al.*, 2010) with a specific clone associated with amyloid arthropathy and was named as ST 82 (Petersen *et al.*, 2009). However, the most STs of *E. faecalis* seems likely to stimulate amyloidosis and chronic infections (Gregersen *et al.*, 2010). Unfortunately, data on the epidemiology and pathogenesis of *E. faecalis* infections in poultry farms and industries have still remained scrappy, especially in developing countries like Bangladesh. This has inadequate preventive measures through modern observations signifying that *E. faecalis* represents new zoonosis.

Previous studies have reported the presence of multiple-antibiotic resistant enterococci in chicken meat in Canada, posing a serious threat to the human being (Aslam *et al*., 2012). Moreover, multidrug resistance enterococci was also reported to be associated with bacteremia in children in India (Kapoor and Randhawa, 2005). That is why, it has been suggested that enterococci from meat products should be tested for dynamic antimicrobial resistance (Özmen Toğay *et al.*, 2010). Several studies have focused on *E. faecalis* antibiotic resistance isolated from chicken meat samples. Still inadequate data is available on the spread, detection and antibiotic resistance of isolated *E. faecalis* from chickens in Bangladesh. This study is the first report on the presence of multiple-antibiotics, including necessary antibiotics like vancomycin and linezolid, resistant *E. faecalis* in chicken in Dhaka and Gazipur, the two biggest industrial and farming areas in Bangladesh. It is essential to investigate the presence of multi-drug resistance *E. faecalis* among different poultry groups and their molecular basis of pathogenicity to reveal possible transmission and potential effects of *E. faecalis* among poultry farms.

Present research comprises of four parts: (1) isolation of *E. faecalis* from suspected poultry, (2) determination of their phenotypic & genotypic antimicrobial resistance capability, (3) determination of their virulent properties and (4) partial sequencing of *sod*A gene to characterize the pathogenic group of the isolates to show how different resistant species originated from a series of common precursors. The present study is one of the maiden approaches to investigate pathogenic *E. faecalis* among the poultries in Bangladesh.

MATERIALS AND METHODS

Sample collection and processing: A total of 136 cloacal swab samples were collected randomly from meat and egg-producing poultry, including commercial broiler (CB) (n=31), commercial layer or layer poultry (CL) (n=69) and broiler breeder (n=36) throughout two purposefully selected areas (Mawna, Gazipur; 24°13.16' N 90°24.63' E and Jatrabari, Dhaka; 23°42.54' N 90°26.36' E).

Presumptive identification of *E. faecalis*: Collected samples were pre-enriched with 6.5% NaCl containing brain heart infusion broth (BHI). Primary screening of enterococci was conducted using kanamycin aesculin azide agar. The final isolation was directed using 5% sheep blood agar. Further screening was accomplished by Gram's staining, biochemical tests like catalase test, esculin hydrolysis, sugar (ribose, sucrose, lactose and D-raffinose) fermentation tests and 40ºC temperature tolerance. *E. faecalis* ATCC 29212 was used as a quality control strain.

Polymerase Chain Reaction (PCR): Initially screened isolates were further confirmed by two uniplex (u-PCR I, u-PCR II) polymerase chain reaction (PCR) based on enterococci specific *sod*A gene according to previously published protocol with a slight optimization (Table 1) (Jackson *et al*., 2004).

Antimicrobial susceptibility testing: Antimicrobial susceptibility test was performed by Kirby-Bauer disk diffusion assay according to Clinical and Laboratory Standards Institute (CLSI) standards. Pure culture of *E. faecalis* with turbidity equivalent to 0.5 McFarland solution was tested against antibiotics that were commonly used in veterinary and human medicine.

There are 13 antibiotics' disks (Oxoid, Hampshire, UK) belonging to 10 antibiotic classes were tested against each isolates in antimicrobial susceptibility assay. In this study, isolates resistant to five or more antibiotic classes including two key antibiotic classes were selected as Multi-drug resistant (MDR) and isolates resistant to ≥8 antibiotic classes that are susceptible to only one or two antibiotic classes were selected as possible-Extremely Drug-Resistant (XDR) isolates according to previous guideline (Magiorakos *et al.*, 2012).

Antibiotic resistance gene and virulence factors identification: Genes responsible for vancomycin and gentamicin resistance in enterococci were confirmed in the isolates that were phenotypically resistant to vancomycin and gentamicin respectively. Several single and multiplex PCR (m-PCR II, m-PCR III, u-PCR IV and u-PCR V) were performed to identify the virulence factors. All the genes tested are listed in Table 1.

Determination of Minimum Inhibitory Concentration (MIC) of antibiotics: MIC of eight commonly available antibiotics were determined using microdilution technique according to previously published protocol (Wiegand *et al*., 2008). An array of antibiotic concentration gradients was used ranging from 1024µg/ml to 01 µg/ml using a 2-fold serial dilution method. Unfortunately, the MIC of the remaining antibiotics was not possible due to their unavailability in raw form.

Partial sequencing of *sod*A gene and phylogenetic analysis: Partial sequencing of the *sod*A gene was done for the top five selected isolates based on their phenotypic and genotypic resistance profile data. Cycle sequencing was performed using BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, CA, US). PCR extended product was purified using BigDyeX Terminator purification kit (Applied Biosystems, CA, US). After completing DNA sequencing analysis by 3130 genetic analyzer (Applied Biosystems, CA, US) chromatogram (Table 1), files were used to extract final sequence.

Table 1: Primer used in this study, target genes and relevant amplified product size

Target gene	Primer	Sequence(5'-3')	Amplicon size (bp)	Annealing temp	Ref.
u-PCR I					
*sod*A Enterococcus	d1	CCITAYICITAYGAYGCIYTIGARCC	480	37°C	(Poyart *et al*., 2000)
specific	d2	ARRTARTAIGCRTGYTCCCAIACRTC			
u-PCR II					
*sod*A	FL1	ACTTATGTGACTAACTTAACC	360	55°C	(Jackson *et al*., 2004)
E. faecalis specific	FL2	TAATGGTGAATCTTGGTTTGG			
u-PCR III					
aac (6)Ie-aph(2	acc_F	GAGCAATAAGGGCATACCAAAAATC	505	55°C	(Kao *et al*., 2000)
)Ia	acc_R	CCGTGCATTTGTCTTAAAAAACTGG			
		m-PCR I			
vanA	vanA_F	GCTATTCAG CTGTACTC	783	56°C	(Li *et al*., 2007)
	vanA_R	CAGCGGCCATCATACGG			
vanB	vanB_F	CATCGCCGTCCCCGAATTTCAAA	297		
	vanB_R	GATGCGGAAGA TACCGTGGCT			
vanC1	$vanC_1$_F	GGTATCAAGGAAACCTC	822		
	$vanC_1$_R	CTTCCGCCATCATAGCT			
vanC2/C3	$vanC_2/C_3$_F	CTCC TACGATTCTCTTG	439		
	$vanC_2/C_3$_R	CGAGCAAGACCTTTAAG			
		m-PCR II			
asa1	ASA 11	GCACGCTATTACGAACTATGA	375	56°C	(Vankerckhoven *et al*., 2004)
	ASA 12	TAAGAAAGAACATCACCACGA			
gelE	GEL 11	TATGACAATGCTTTTTGGGAT	213		
	GEL 12	AGATGCACCCGAAATAATATA			
cylA	CYT I	ACTCGGGGATTGATAGGC	688		
	CYT Iib	GCTGCTAAAGCTGCGCTT			
esp	ESP 14F	AGATTTCATCTTTGATTCTTGG	510		
	ESP 12R	AATTGATTCTTTAGCATCTGG			
hyl	HYL n1	ACAGAAGAGCTGCAGGAAATG	276		
	HYL n2	GACTGACGTCCAAGTTTCCAA			
		m-PCR III			
ace	ACE1	AAAGTAGAATTAGATCCACAC	319	56°C	Zoletti *et al*., 2011)
	ACE2	TCTATCACATTCGGTTGCG			
agg	AGG_F	AAGAAAAAGAAGGTAGACCAAC	1553		
	AGG_R	AAACGGCAAGACAAGTAAATA			
cpd	CPD_F	TGGTGGGTTATTTTTCAATTC	782		
	CPD_R	TACGGCCCCTCTGGCTTACTA			
		u-PCR IV			
fsrB	fsrB 1	ATGCTAATCGATTGGATTCTAAAA	710	48°C	(Nakayama *et al*., 2001)
	fsrB 2	TCTTTTTAGGTTTTTCAGTTTGTC			
	efaAfm-2	CTACTAACACGTCACCAATG			

Genome sequences conducted in this study were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under GenBank accession numbers MK947390-MK947394. The evolutionary history was inferred by using the Maximum Likelihood method. Evolutionary analyses were conducted in MEGA X.

Statistics and antimicrobial susceptibility assay data analysis: Survey System 12.0 (Creative Research Systems, California, USA) was used to analyze the prevalence. Kirby-Bauer disk diffusion test were compiled using spreadsheet (MS excel, Microsoft Corporation, Washington, USA). Antibiotic sensitivity data analysis including, % RIS and test measurement were done using WHONET-2019 software (https://doi.org/10.1128/ 978155 5819071.ch48). A heatmap-based dendrogram was calculated on antibiotic resistance values using Morpheus software (Morpheus, 2018). Isolates were typically clustered based on their sensitivity against each antibiotic using categorical coefficient. The antibiotic resistance patterns were visualized through hit-map similarity matrix and hierarchical clustering was done based on one minus Pearson's correlation coefficient and average linkage method.

RESULTS

Isolation of *E. faecalis*: Among 136 collected samples 65 (47.8%; 39.4-56.1) isolates were confirmed as *Enterococcus* and 29 (21.32%; 95% CI: 14-28%) isolates were confirmed as *E. faecalis*. While 34.5% commercial broiler (n=10; 95% CI: 14-42%), 51.71% layer poultry (n=15; 95% CI:12-31%) and 13.79% broiler breeder (n=04; 95% CI:1-24%) have been found to be infected with *E. faecalis* (Table 2).

Antibiotic susceptibility Test: In sensitivity assay, all the isolates or samples were resistant against penicillin-G 100% (n=29; 95% CI: 85-100). After that, maximum resistance were found against streptomycin & tetracycline 96.55% (n=28; 95% CI: 80-100) and erythromycin 82.76% (n=24; 95% CI: 64-94), followed by amoxicillin 65.52% (n=19; 95% CI: 46-81%) and ampicillin 58.62% (n=17; 95% CI: 39-76%). All the other antibiotics were found resistant against less than 50% of isolates including ciprofloxacin 31.03% (n=9; 95% CI: 16-51%), vancomycin 27.59% (n=8; 95% CI: 14-48%), linezolid 24.14% (n=7; 95% CI: 11-44%), chloramphenicol 20.69% (n=6; 95% CI: 9-40%). Lower resistance was observed against nitrofurantoin 13.79% (n=4; 95% CI: 5-33%) and gentamicin 10.34% (n=3; 95% CI: 03-28%). Only imipenem showed promising level of sensitivity (86.21%) (Fig. 1). Around 65.52% isolates (n=19) were multi-drug resistant (MDR) which is alarming for Bangladeshi broiler and poultry industries. Among the MDR isolates, almost 20.69% isolates (n=06) were resistant to ≥8 antibiotic classes out of 10 tested classes and termed as possible-XDR

Table 2: Isolation and prevalence of *E. faecalis* in the study area

Source	Bird Flock Size	Samples (n)	Enterococcus spp (sodA gene) (%; 95%CI)	*E. faecalis* (*sod*A_*FL* gene) (%; 95%CI)
CB	500	36	23 (63.9%; 48.7-79)	10 (27.8%; 13.7-41.8)
CL	10500	69	33 (47.8%; 36-59.5)	15 (21.7%; 12-31.4)
BB	1500	31	09 (29.0%; 13.1-44.8)	4 (12.9%; 1.1-24.6)
Total	12500	136	65 (47.8%; 39.4-56.1)	29 (21.3%; 14.4-28.1)

Note: CB: commercial broiler, CL: commercial layer, BB: broiler breeder, n: numbers

Table 3: Association of phenotypic and genotypic resistance with the presence of virulence genes in the study isolates

Sample ID	Source	N1	N2	MDR	XDR	Resistance gene	Virulence gene
CL-56	Layer poultry	5	3				
BB-01	Broiler breeder	6	4				
BB-04	Broiler breeder	6	4				
CB-09	Commercial broiler	4	4				
CB-16	Commercial broiler	4	4				gelE, cpd
CB-17	Commercial broiler	7	4			vanB, aac*	gelE, cpd
CL-52	Layer poultry	6	4				
CL-53	Layer poultry	6	4				
CL-54	Layer poultry	4	4				
CL-57	Layer poultry	6	4			vanA, vanC2	gelE, cpd
BB-02	Broiler breeder	7	5	MDR		vanB	asa1, gelE, cpd
BB-03	Broiler breeder	7	5	MDR			asa1, cpd
CB-02	Commercial broiler	6	5	MDR		aac*	
CB-03	Commercial broiler	6	5	MDR		aac*	
CB-07	Commercial broiler	7	5	MDR			
CB-28	Commercial broiler	7	5	MDR		vanB	gelE, cpd
CL-38	Layer poultry	7	5	MDR			asa1, gelE, cpd
CL-42	Layer poultry	6	5	MDR		aac*	
CL-43	Layer poultry	8	5	MDR		aac*	
CL-55	Layer poultry	7	5	MDR			
CB-08	Commercial broiler	6	6	MDR			
CB-04	Commercial broiler	7	7	MDR			
CL-39	Layer poultry	9	7	MDR			gelE, cpd
CB-29	Commercial broiler	10	8	MDR	XDR		asa1, gelE, cpd
CL-37	Layer poultry	8	8	MDR	XDR		gelE, cpd
CL-40	Layer poultry	8	8	MDR	XDR	vanC2	gelE, cpd
CL-16	Layer poultry	11	9	MDR	XDR	vanC2	gelE, cpd
CL-18	Layer poultry	12	10	MDR	XDR	vanC2	asa1, gelE, cpd
CL-29	Layer poultry	12	10	MDR	XDR	vanA, vanC2	cpd

Note: aac*: aac(6)le-aph(2)la. N1: Number of antibiotics non-susceptible, N2: Number of classes non-susceptible, MDR: Multi-Drug Resistant, XDR: possible-Extremely Drug Resistant.

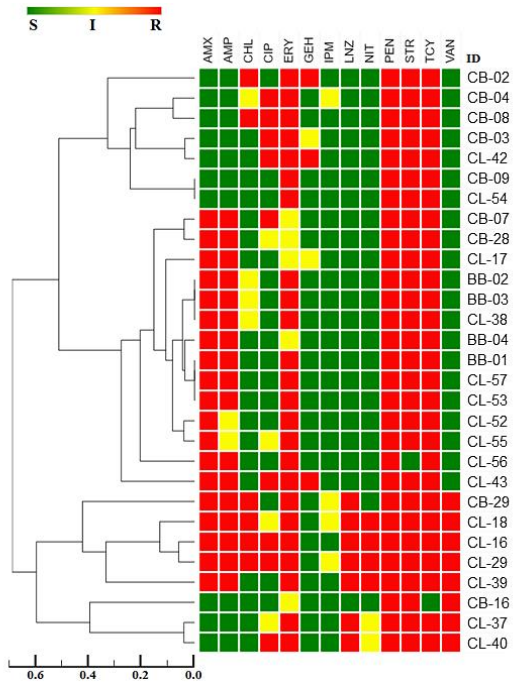

Fig. 1: Dendrogram calculated on antibiotic resistance values using Morpheus software. (Note: R: resistant, I: intermediate, S: sensitive, AMP: ampicillin, AMX: amoxicillin, CHL: chloramphenicol, CIP: ciprofloxacin, ERY: erythromycin, GEH: gentamicin-high, IPM: imipenem, LNZ: linezolid, NIT: nitrofurantoin, PEN: penicillin G, STR: streptomycin, TCY: tetracycline, VAN: vancomycin).

isolate according to the definition provided by the expert panel of ECDC (European Centre for Disease Prevention and Control) and CDC (Centers for Disease Control and Prevention) (Magiorakos *et al.*, 2012).

Antibiotic resistance causing gene: Among the eight vancomycin-resistant isolates, the *van*C2 gene was observed in 62.5% (n=5/8) isolates followed by *van*B 37.5% (n=3/8) and *van*A 2.5% (n=2/8), whereas gentamicin-resistance gene aac(6)le-aph(2)la was observed in 100% (5/5) gentamicin resistant isolates (Table 3).

Minimum Inhibition Concentration (MIC): In broth-microdilution MIC assay, higher value (MIC50/ MIC90) was observed to chloramphenicol and sulfamethoxazole (≥1024/ ≥1024), ciprofloxacin (64/ 512), cefixime (4/ 256) and cefrtiaxone (8/ 128). Comparatively lower MIC values were found against gentamicin (≤1/ 16), oxytetracycline (2/ 64), and oxytetracycline (32/ 64), azithromycin (≤1/ ≥1024) (Table 4).

Virulence of *E. faecalis* for poultry origin: Isolates were tested to find out the presence of virulence factors whereas three virulence factors had been detected including the sex pheromones (*cpd*) in 48.28% (n=14) isolates; gelatinase (*gel*E) in 41.38% (n=12) isolates and aggregation substance (*asa*1) in 17.24% (n=05) isolates (Table 3). Other factors including cytolysin, surface protein, hyaluronidase, collagen-

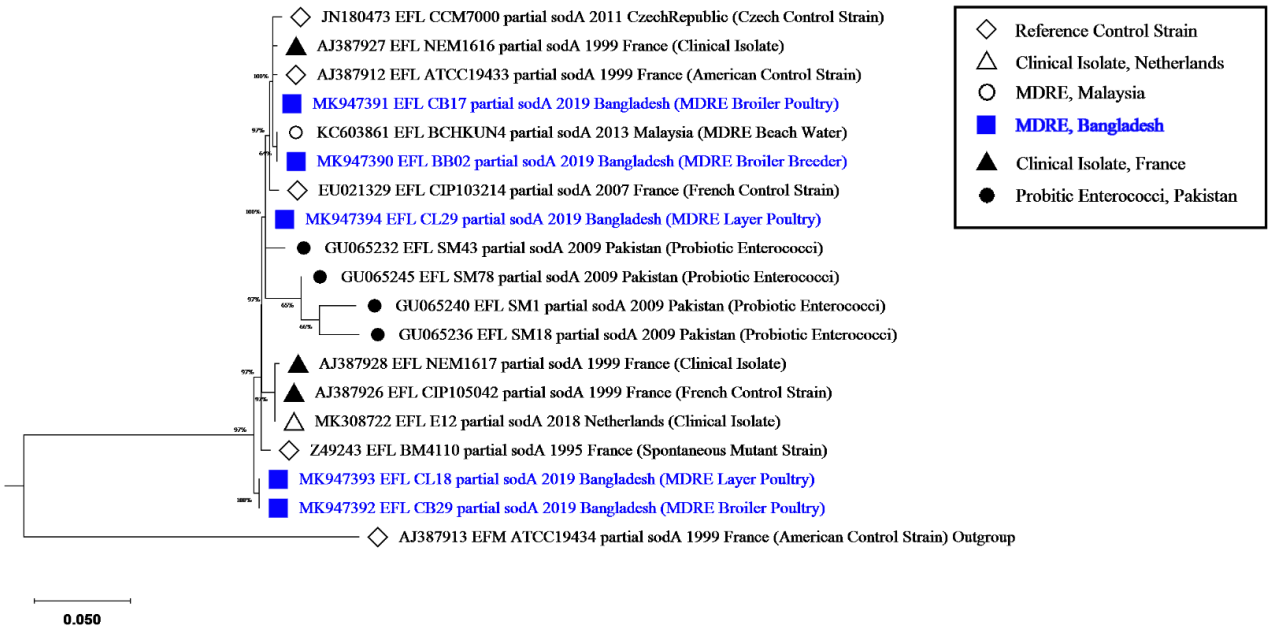

Fig. 2: Phylogenetic tree of *sod*A gene sequence of present study isolates and selected reference (NCBI GenBank) sequence. The tree was constructed based on multiple sequence alignment using MEGA X. Bootstrap value was used as 1000 for tree clustering. Number at the nodes represents the level of bootstrap support (%) based on the neighbor-joining analysis.

Table 4: Distribution of minimum inhibition concentrations (MICs: µg/ml) of eight selected antibiotics obtained by broth micro-dilution

Anti-biotics	Source	Break Points	MIC_{50}/ MIC_{90}	Antibiotic concentration gradients (µg/ml)										
				≤1	2	4	8	16	32	64	128	256	512	≥1024
GEN	CB	≥16 µg/ml	≤1/ 16	5	1		2	2						
	CL		≤1/ 16	10	1	1		3						
	BB		≤1/ ≤1	4										
	Overall		≤1/ 16	19	2	1	2	5	0	0	0	0	0	0
CHL	CB	≥32 µg/ml	≥1024/≥1024									1	1	8
	CL		512/≥1024					1	1	2			5	6
	BB		≥1024/≥1024									1		3
	Overall		≥1024/≥1024	0	0	0	0	1	1	2	0	2	6	17
AZI	CB	≥8* µg/ml	≤1/ 512	5								2	2	1
	CL		≤1/≥1024	8	1								1	5
	BB		≤1/2	3	1									
	Overall		≤1/≥1024	16	2	0	0	0	0	0	0	2	3	6
CIP	CB	≥4 µg/ml	64/≥1024	1			1			3	2	1		2
	CL		64/128	1				1	3	5	4			1
	BB		64/512			1				2			1	
	Overall		64/512	2	0	1	1	1	3	10	6	1	1	3
CFX	CB	≥4* µg/ml	≤1/ 256	6			1			1		1	1	
	CL		8/256	4	2	1	1		1	1		4		1
	BB		2/512	1	1				1				1	
	Overall		4/256	11	3	1	2	0	2	2	0	5	2	1
SUL	CB	≥2* µg/ml	512/ ≥1024	1							2	1	1	5
	CL		≥1024/ ≥1024			1						1	1	12
	BB		512/ ≥1024	1									1	2
	Overall		≥1024/ ≥1024	2	0	1	0	0	0	0	2	2	3	19
CFT	CB	≥4* µg/ml	8/64				5	3		1		1		
	CL		8/128		1	2	7	3			1			1
	BB		8/512			1	1	1					1	
	Overall		8/128	0	1	3	13	7	0	1	1	1	1	1
OXY	CB	≥16 µg/ml	64/64	1	2					6	1			
	CL		2/128	1	8				1	3	2			
	BB		2/2	1	3									
	Overall		2/ 64	3	13	0	0	0	1	9	3	0	0	0

Note: GEN: gentamicin, CHL: chloramphenicol, AZI: azithromycin, CIP: ciprofloxacin, CTX: cefixime, SUL: sulfamethoxazole, CFT: ceftriaxone, OXY: oxytetracycline, CB: commercial broiler, CL: commercial layer, BB: broiler breeder, Breakpoints: isolates showed resistance more or equal of this concentration termed as resistant to that particular antibiotic.MIC_{50}: represents the concentration of each antibiotic inhibiting 50% of the isolates, MIC_{90}: represents the concentration of each antibiotic inhibiting 90% of the isolates. * Breakpoints used for statistical analysis purpose to improvise the epidemiology.

binding protein, a transmembrane protein, endocarditis specific antigen, and aggregation protein were not present among the MDR and XDR isolates.

Phylogenetic analysis: We found a high frequency of similarity of our isolate (CB17, CL29, and BB02) with Malaysian MDRE isolated from beach water and French

control strain CIP103214. On the other hand, a new lineage was also been found in the phylogenetic study, two of our isolates showed quite similar to one French spontaneous mutant strain BM4110 (Pasteur Institute) (Fig. 2).

DISCUSSION

In the last two decades *E. faecalis* has been endangered as an important cause of nosocomial infection (%) due to its acquiring antibiotic resistant traits (Arias and Murray, 2012). The combination of adaptive resistance to novel antibiotics, innate resistance to several major antimicrobials and natural endurance to extreme pH, osmotic pressure and temperature facilitates them to sustain in the environment. *E. faecalis* is an opportunistic pathogen that provokes its ability to cause disease to weakly immune person (Hoelzer *et al*., 2017). Accordingly, *E. faecalis* are usually the most prevalent enterococci species among the isolates recovered from samples such as poultry, cattle, goat, meat and other livestock (Tamang *et al*., 2017). However, very few studies explain the comparative research among those possible reservoirs in a single platform.

The presence of pathogenic enterococci and their similarity in both food animals and human samples indicate that they share the same pathogen and can be a significant source of human infection (Kürekci *et al.*, 2016).

The presence of *E. faecalis* in chickens evolved as a serious threat to human health, a significant public health concern. The ability of tissue adhesion, biofilm formation and antimicrobial resistance readily influence its virulence (Fisher and Phillips, 2009). The present study mainly focuses on the antimicrobial resistance patterns and the correlation between vancomycin resistance and virulence genes in *E. faecalis* isolated from poultry in Bangladesh.

In this study, 21.32% isolates were confirmed as *E. faecalis* where, 34.5% commercial broiler, 51.71% layer poultry and 13.79% broiler breeder isolates were confirmed as *E. faecalis*. This indicates the higher existence of *E. faecalis* in commercial broiler than the commercial layer and broiler breeder. The isolation rate of *E. faecalis* from healthy poultry intestinal content can vary from (13-96%) (Jørgensen *et al*., 2017). So here in this study, the presence of *E. faecalis* in poultry is quite similar to the worldwide case.

In present study, 65.52% (n=19) isolates were multi-drug resistant (MDR) that includes commercial broiler (n=07; 36.84%), layer poultry (n=10; 52.63%), and broiler breeder (n=2; 10.53%) which is dangerously high for broiler and poultry industries in Bangladesh. MDR patterns of the current study are quite similar to the other studies (Kwon *et al.*, 2012). Moreover, 20.69% isolates (n=06) were found as possible-XDR isolate according to ECDC and CDC (Magiorakos *et al.*, 2012). Overall, high levels of resistance were observed to penicillin G (100%), tetracycline (96.55%), followed by amoxicillin (66.52%), ampicillin (58.62%). In contrast, a comparatively low resistance level is observed against nitrofurantoin (13.79%), and only imipenem showed high sensitivity (86.21%) with 13.79% intermediate and zero resistance. None of the isolates was sensitive against all antibiotics. Some previous studies observed higher erythromycin resistant (Ayeni *et al.*, 2016) and medium gentamicin and ciprofloxacin resistant enterococci (Ayeni *et al.*, 2016; Kürekci *et al*., 2016; Sanlibaba *et al*., 2018). Resistance pattern of ampicillin, gentamycin and ciprofloxacin were found higher in another study in India whereas the resistance pattern of amoxicillin and erythromycin were found lower than the current study (Kapoor L, Randhawa VS, 2005). Increased level of penicillin-G and amoxicillin resistance among *E. faecalis* isolates in the current study is a matter to be worried about. Despite low intrinsic resistance to clinical levels of aminoglycosides and macrolides (CLSI 2019), we checked our isolates against streptomycin, erythromycin and gentamicin-high and found high resistance against all of them except gentamicin-high (10%), which reflect their intrinsic condition and susceptibility against a high level of gentamicin antibiotic.

In the broth-microdilution MIC essay, a higher value (MIC_{50}/MIC_{90}) was observed to sulfamethoxazole and ceftriaxone (≥1024/≥1024), chloramphenicol (32/256), and gentamicin (8/128). Comparatively lower MIC values were for ciprofloxacin (32/ 64) and oxytetracycline (32/64). The results may indicate a high consumption of antibiotics for growth promotion in these poultry feeds. Increased frequency of antibiotic resistance in poultry samples is almost similar to the previously published reports in Bangladesh and Turkey (Aslam *et al.*, 2012; Sanlibaba *et al*., 2018). The highest resistance was found against Penicillin G in all three groups, where 100% isolates were resistant to this drug. However, Penicillin G is not generally recommended for enterococcal infections.

Vancomycin has been a plausible alternative to multiple-antibiotic resistant enterococcal infections (Kürekci *et al.*, 2016). In present study, 27.6% EF isolates (BB: 0%, CL: 42.86%, CB: 22.22%) (1.3%, 6.67% and 33.3% CL isolates are *van*A, *van*B and *van*C2 positive respectively whereas 20% CB are vanB positive) were resistant to vancomycin. The prevalence of vancomycin-resistant enterococci in Bangladesh was reported very low (Ahmed *et al*., 2019). Our finding contradicts the recent researches of Bangladesh and worldwide reports (Sanlibaba *et al*., 2018; Ahmed *et al*., 2019).

Vancomycin-resistant genes (*van*A, *van*B and *van*C2) were detected in vancomycin resistance isolates. Five isolates were *van*C_2 positive and two isolates had *van*A gene (Table 3). At the same time, *van*B was found only in three intermediate-resistant isolates. But other intermediate isolates had none of the vancomycin-resistance genes. Therefore, it can be inferred that may be *van*C_2 actively responsible for physical resistance against vancomycin. On the other hand, *van*B may play a vital role in the intermediate stage before becoming fully resistant. Therefore, present data is highly suspicious and vancomycin is no longer reflecting its validity to serve as the solution for treating multiple-antibiotic resistant enterococcal infections in Bangladesh in future.

In Bangladesh, linezolid is available for clinical use and used for vancomycin-resistant enterococcal infections. In present study, 24.14% EF isolates were resistant to linezolid. The rapid increase of linezolid resistance over a short time is alarming and more precautions should be taken to prescribe this antibiotic.

Virulence determinants in *E. faecalis* isolates were similar among the sources where the *gel*E, *cpd* and *asa*1

genes were the most frequent. Similarly, some other studies have reported the same factors in broiler chicken (Rehman *et al.*, 2018).

In the present study, the *asa*1 gene was more common in BB (50%) in comparison with CL (13.33%) and CB (10%). The finding is slightly low from another study (Kwon *et al.*, 2012). Moreover, the higher existence of *asa*1 in BB contradicts recent research (Rehman *et al.*, 2018). The *cpd* gene was the most common virulence factor (48.3%) in *E. faecalis* isolates. Likewise, a high frequency of sex pheromone determinants was found in other research (cad, camE, cCF10 and cOB1) in *E. faecalis* isolated from broiler chicken (Rehman *et al.*, 2018).

The overall incidence of the *gel*E gene was 41.38%, where CL predominates (46.67%). These findings are lower than previous studies (Kwon *et al.*, 2012). In contrast, a high frequency of *gel*E gene among poultry has been reported previously (Rehman *et al.*, 2018). At the same time, a more critical issue is their ability to express the acquired virulence genes, which need more attention and investigation.

Evaluation of correlation between virulence genes and antibiotic resistance among the sources revealed the high frequencies of *cpd*, *gel*E and *asa*1 genes associated with multiple antibiotic resistance (Table 3). CL isolates non-susceptible to seven or more antibiotics pose 2-3 of the virulence gene. The result is also similar for CB and BB. CB isolates non-susceptible to ten antibiotics and BB isolates non-susceptible to seven antibiotics contain all the three virulence genes. The findings are pretty similar to previous research (Aslam *et al.*, 2012).

Correlation between the presence of *van*A, *van*B and *van*C2 genes with virulence genes (*gel*E, *cpd* and *asa*1) was significant. All the *E. faecalis* isolates (n=8) holding any vancomycin resistance genes possess virulence genes (either *gel*E, *cpd* or *asa*1) and 87.5% of them have two or more than two virulence genes. On the other hand, no correlation was found between the presence of gentamycin resistance gene (aac(6)Ie-aph(2)Ia) with virulence genes. Among the five aac(6)Ie-aph(2)Ia positive isolates only one (20%) isolate that also has *van*B, contains two virulence genes. Moreover, only 25% *van* gene non-containing extensively drug-resistant (XDR) *E. faecalis* isolate were virulence gene positive. The findings show a correlation between the presences of *van* genes with virulence genes and possibly these virulence genes facilitate acquiring the *van* genes.

Most of our sequenced MDR *E. fecalis* (MDRE) were highly similar with Asian MDRE and European control strain (Ahmed *et al*., 2019), indicating less possibility of spontaneous mutation but the potential acquisition of drug-resistance genome and virulence gene through horizontal genetic material transfer from another organism (Munita and Arias, 2016). On the other hand, two of our isolates showed similarity with French spontaneous mutant strain BM4110 can be an indication of mutation (Blair *et al.*, 2015). Finally, it is still unambiguous whether the development of MDR and virulence is either due to horizontal gene transfer, spontaneous mutation or a combination of both. An extensive study on this topic is required.

Conclusions: This study focused on phenotypic and genotypic multiple antibiotic resistance profiling of *E. faecalis* from BB, CL and CB in Bangladesh and evaluating their virulence properties. The overall occurrence of MDR and possible-XDR among the *E. faecalis* isolates are highly alarming. Moreover, a correlation between the presence of vancomycin resistance (*van*A, *van*B and *van*C2) genes with virulence genes (*gel*E, *cpd* and *asa*1) was also observed. The presence of Linezolid resistance in poultry is a matter of concern. Our results indicated that *E. faecalis* strains isolated from poultries could be regarded as a potential source for spreading antibiotic resistance. Moreover, the higher existence of multi-drug resistance among *E. faecalis* isolated from each sample group is a serious threat to public health. Therefore, the controlled use of antibiotics in the animal husbandries are highly suggestive in Bangladesh.

Authors contribution: MS Sagor, MS Hossain, T Islam, MA Mahmud, MR Karim, MS Miah, M Giasuddin and MA Samad discussed data and designed the paper. MS Sagor, and T Islam wrote the manuscript. All the authors have read and approved the final manuscript.

REFERENCES

Ahmed I, Rabbi MB and Sultana S, 2019. Antibiotic resistance in Bangladesh: A systematic review. Int J Infect Dis 80:54-61.

Arias CA and Murray BE, 2012. The rise of the Enterococcus: Beyond vancomycin resistance. Nat Rev Microbiol 10:266–78.

Aslam M, Diarra MS, Checkley S, *et al.*, 2012. Characterization of antimicrobial resistance and virulence genes in Enterococcus spp. isolated from retail meats in Alberta, Canada. Int J Food Microbiol 156:222-30.

Ayeni FA, Odumosu BT, Oluseyi AE, *et al.*, 2016. Identification and prevalence of tetracycline resistance in enterococci isolated from poultry in Ilishan, Ogun State, Nigeria. J Pharm Bioallied Sci 8:69-73.

Blair JMA, Webber MA, Baylay AJ, *et al.*, 2015. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42–51.

Fisher K and Phillips C, 2009. The ecology, epidemiology and virulence of Enterococcus. Microbiol 155:1749–57.

Franz CMAP, Huch M, Abriouel H, *et al.*, 2011. Enterococci as probiotics and their implications in food safety. Int J Food Microbiol 151:125-40.

Gaglio R, Couto N, Marques C, *et al.*, 2016. Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. Int J Food Microbiol 236:107-14.

Gregersen RH, Petersen A, Christensen H, *et al.*, 2010. Multilocus sequence typing of Enterococcus faecalis isolates demonstrating different lesion types in broiler breeders. Avian Pathol 39:435-40.

Hoelzer K, Wong N, Thomas J, *et al.*, 2017. Antimicrobial drug use in food-producing animals and associated human health risks: What, and how strong, is the evidence? BMC Vet Res 13:211.

Jackson CR, Fedorka-Cray PJ and Barrett JB, 2004. Use of a genus- and species-specific multiplex PCR for identification of enterococci. J Clin Microbiol 42:3558-65.

Jamet E, Akary E, Poisson MA, *et al.*, 2012. Prevalence and characterization of antibiotic resistant Enterococcus faecalis in French cheeses. Food Microbiol 31:191–8.

Jørgensen SL, Poulsen LL, Thorndal L, *et al.*, 2017. Characterization of Enterococcus faecalis isolated from the cloaca of 'fancy breeds' and confined chickens. J Appl Microbiol 122:1149-58.

Kao SJ, You I, Clewell DB, *et al.*, 2000. Detection of the high-level aminoglycoside resistance gene aph(2")-Ib in Enterococcus faecium. Antimicrob Agents Chemother 44:2876-9.

Kapoor L, Randhawa VS DM, 2005. Antimicrobial resistance of enterococcal blood isolates at a pediatric care hospital in India. Jpn J Infect Dis 58:101–3.

Kürekci C, Önen SP, Yipel M, *et al.*, 2016. Characterisation of phenotypic and genotypic antibiotic resistance profile of enterococci from cheeses in Turkey. Korean J Food Sci Anim Resour 36:352-8.

Kwon KH, Hwang SY, Moon BY, *et al.*, 2012. Occurrence of antimicrobial resistance and virulence genes, and distribution of enterococcal clonal complex 17 from animals and human beings in Korea. J Vet Diagnostic Investig 24:924-31.

Li S, Zhang Z and Mi ZH, 2007. Vancomycin-resistant enterococci in a Chinese hospital. Curr Microbiol 55:125–7.

Magiorakos A-PP, Srinivasan A, Carey RBB, *et al.*, 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18:268–81.

Munita JM and Arias CA, 2016. Mechanisms of Antibiotic Resistance. Microbiol Spect 4:1–32.

Nakayama J, Cao Y, Horii T, *et al.*, 2001. Gelatinase biosynthesis-activating pheromone: A peptide lactone that mediates a quorum sensing in Enterococcus faecalis. Mol Microbiol 41:145-54.

Özmen Toğay S, Çelebi Keskin A, Açik L, *et al.*, 2010. Virulence genes, antibiotic resistance and plasmid profiles of Enterococcus faecalis and Enterococcus faecium from naturally fermented Turkish foods. J Appl Microbiol 109:1084-92.

Petersen A, Christensen H, Philipp HC, *et al.*, 2009. Clonality of Enterococcus faecalis associated with amyloid arthropathy in chickens evaluated by multilocus sequence typing (MLST). Vet Microbiol 134:392–5.

Poyart C, Quesnes G and Trieu-Cuot P, 2000. Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. J Clin Microbiol 38:415-8.

Rehman MA, Yin X, Zaheer R, *et al.*, 2018. Genotypes and Phenotypes of Enterococci Isolated From Broiler Chickens. Front Sustain Food Syst 2:83.

Report N, 2001. National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992-June 2001, Issued August 2001. Am J Infect Control 29:404-21.

Sanlibaba P, Tezel BU and Senturk E, 2018. Antimicrobial resistance of enterococcus species isolated from chicken in Turkey. Korean J Food Sci Anim Resour 38:391-402.

Tamang MD, Moon DC, Kim SR, *et al.*, 2017. Detection of novel oxazolidinone and phenicol resistance gene optrA in enterococcal isolates from food animals and animal carcasses. Vet Microbiol 201:252-6.

Tankson JD, Thaxton JP and Vizzier-Thaxton Y, 2001. Pulmonary hypertension syndrome in broilers caused by Enterococcus faecalis. Infect Immun 69:6318-22.

Vankerckhoven V, Van Autgaerden T, Vael C, *et al.*, 2004. Development of a multiplex PCR for the detection of asal, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among european hospital isolates of Enterococcus faecium. J Clin Microbiol 42:4473-9.

Wiegand I, Hilpert K and Hancock REW, 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163-75.

Zoletti GO, Pereira EM, Schuenck RP, *et al.*, 2011. Characterization of virulence factors and clonal diversity of Enterococcus faecalis isolates from treated dental root canals. Res Microbiol 162:151-8.