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Full Length Research Paper

Detection of tetracycline resistant *E. coli* and *Salmonella* spp. in sewage, river, pond and swimming pool in Mymensingh, Bangladesh

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Antibiotic resistant bacteria and resistance genes in the environment are major health problem globally. The present study was undertaken to detect antibiotic resistant *Escherichia coli* and *Salmonella* spp. in sewage, river, pond and swimming pool. Emphasis was given on tetracycline resistant phenotype and genotype, since, tetracycline is a widely used antibiotics. Isolation and identification of antibiotic resistant *E. coli* and *Salmonella* spp. were based on morphology, staining, cultural, and biochemical properties, disk diffusion test and PCR. A total of 47 samples were collected from Mymensingh, Bangladesh. Among the 47 samples, 36 (76.59%) were found positive for *E. coli* and 42 (89.36%) for *Salmonella* spp. Phenotypically, all isolates were found resistant to tetracycline as revealed by disk diffusion test. Isolated *E. coli* were resistant to chloramphenicol (5.5%), streptomycin (16.6%) and ampicillin (97.2%) while *Salmonella* spp. to chloramphenicol (07.1%), ciprofloxacin (07.1%), streptomycin (19.1%) and ampicillin (100%). All bacterial isolates were sensitive to gentamycin. PCR result showed that 77.77 and 80.95% phenotypically tetracycline resistant *E. coli* and *Salmonella* spp. were positive for *tetA* gene. From this study it is concluded that tetracycline resistant *E. coli* and *Salmonella* spp. widely present in sewage, river, pond and swimming pool water are of great public health concern.

Key words: Environment, sewage, antibiotic resistance, *tetA*, *E. coli, Salmonella* spp., polymerase chain reaction, public health.

INTRODUCTION

Concern over the threat posed by antibiotic resistant bacteria and resistance genes to human health has turned greater attention also to the environmental dimensions of the problem. Only fairly recently, acknowledge has been made on the role of the environment as a source and dissemination route for antibiotic resistance (Karkman et al., 2019). Antibiotics are used as prescribed medications to control clinical infections. They are also included in feeds for livestock and poultry as growth promoters. From human and animal, these pharmaceuticals are excreted

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from the body in our environment including water bodies and sewage through urine or feces (Lood et al., 2017).

Antibiotics in the environment act as a selective pressure to induce bacterial antimicrobial resistance (AMR). *E. coli* and *Salmonella* spp. are Gram negative enteric bacterium of the family Enterobacteriaceae and ubiquitous in the environment (Scott et al., 2002). Environment harboring antibiotic resistant bacteria (ARB) act as a source or reservoir for the spread of antibiotic resistance genes vertically into the other bacteria, thus making the situation more aggravated. In addition, from environment people can directly get exposed to this ARB or indirectly through food chain (George, 2019).

Tetracycline is one of the widely used antibiotics in veterinary and human medicine. They are also used as growth promoters for livestock and aquaculture (Li et al., 2010). Tetracycline is a broad-spectrum agents having effect against a range of Gram positive and Gram negative bacteria through inhibition of bacterial protein synthesis due to the activity of tetracycline resistance genes including *tetA-E* (Levy et al., 1999; Guillaume et al., 2000). In many cases, *tetA* is found more commonly in clinical *E. coli* than other *tet* gene family (Sengeløv et al., 2003).

ARB and their resistance genes represent a serious threat for human health since diseases caused by these resistant bacteria cannot be treated by standard therapies. Both the ARB and ARGs have been detected extensively in waste water samples globally (Bouki et al., 2013). In Bangladesh, sewage and water treatment system is not well developed. Various types of clinics and hospitals are often established near the water body in Bangladesh could be the major source of antibiotics in aquatic environments (Siddiqui et al., 2015). Even biological waste material from diagnostic laboratory and hospital are directly disposed in drain water without any treatment that are loaded with pathogenic microbes. In addition waste materials from municipal, agricultural, livestock and poultry farms are also dumped in water bodies could be contaminated with antibiotics resistant bacteria.

Recently in Bangladesh antibiotic resistant *Salmonella* spp. and *E. coli* were detected from pond water and sewage samples respectively by Mahmud et al. (2019) and Sobur et al. (2019). Previously Zahid et al. (2009) reported the occurrence of multidrug resistance (MDR) *E. coli* in surface water in Bangladesh. However, not molecular based adequate surveillance data are available in Bangladesh on the occurrence of tetracycline resistant *E, coli* and *Salmonella* spp. in sewage, river, pond and swimming pool water. These surveillance data on AMR are crucial to support the National Action Plan of AMR of Bangladesh Government to take necessary steps to tackle the AMR related hazards. Therefore, the present study aimed to explore the presence and rate of the public health important tetracycline resistant *E. coli* and *Salmonella* spp. in untreated sewage, river, pond and swimming pool water samples.

MATERIAL AND METHODS

Collection of samples

Aseptically, sampling was carried out over the period of June to July, 2018 on random basis. A total of 47 water samples were collected from different areas of Mymensingh, Bangladesh including 20, 4, 20 and 3 from sewage, river, pond and swimming pool water, respectively. From each case 250 ml water samples were collected aseptically in sterile glass bottle labeled properly and transported to the laboratory maintaining cool chain for immediate processing.

Isolation and identification of *E. coli* and *Salmonella* spp.

Isolation and identification of *E. coli* and *Salmonella* spp. were carried out based on initial culture in nutrient broth (6 h at 37°C aerobically) followed platting on Eosine Methylene Blue (EMB) agar and Xylose Lysine Dextrose (XLD) agar plates (Hi Media, India) respectively. Culture plates were aerobically incubated at 37°C for 24 h followed by observing the cultural characteristics, morphology, staining, and biochemical test as described by Bergey et al. (1974). Isolation of *E. coli* and *Salmonella* spp. were confirmed by PCR targeting 16S rRNA gene and *imvA* genes, respectively as described subsequently.

Extraction of genomic DNA

Genomic DNA for the PCR was extracted by boiling method as described previously by Mahmud et al. (2018). In brief, initially 100 µl of deionized water was taken into an Eppendorf tube. A pure bacterial colony of *E. coli* or *Salmonella* spp. from overnight culture on EMB or XLD agar plate at 37°C was gently mixed with deionized water. The tube was then transferred into boiling water and boiled for 10 min, then immediately transferred into ice for cold shock for about 10 min, and finally centrifuged at 10,000 rpm for 10 min. Supernatant from each tube was collected and used as template DNA for PCR. The extracted DNA was stored at -20°C until use.

E. coli and *Salmonella* spp. specific PCR

Primers and protocol used for the detection of *E. coli* and *Salmonella* spp. is listed in Table 1. All the PCR were done in a final 25 µl reaction with 12.5 µl master mixture 2X (Promega, USA), 2 µl genomic DNA (30 ng), 1 µl each primer (10 picomol) and 8.5 µl nuclease free water.

Thermal profile for PCR

Thermal condition was consisted of initial denaturation at 95°C for 5 min followed by 30 cycles each of denaturation at 94°C for 30 s, optimal annealing temperature for each primer set (Table 1), extension at 72°C for 1 min and final extension at 72°C for 10 min.

Visualization of amplified products

Amplified PCR products were analyzed by electrophoresis in 1.5% agarose gel. Ethidium bromide was used to stain product which were visualized under ultraviolet trans-illuminator (Biometra,

Table 1. Primers used for the detection of *E. coli, Salmonella* spp. and *tetA* gene.

Target gene	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)	References
invA	F: ATCAGTACCAGTCGTCTTATCTTGAT R:TCTGTTTACCGGGCATACCAT	211		Shanmugasundaram et al. (2009)
EC 16*S* rRNA	F: GACCTCGGTTTAGTTCACAGA R:CACACGCTGACGCTGACCA	585		Candrian et al. (1991)
tetA	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	577		Woodford and Livermore (2009)

Table 2. Isolation of *E. coli* in sewage, river, pond and swimming pool.

Source of sample	Number of sample	*E. coli*		*Salmonella* spp.	
		Number of positive samples	Occurrence (percentage)	Number of positive samples	Occurrence (percentage)
Sewage	20	17	85	19	95
River	04	03	75	04	100
Pond	20	15	75	18	90
Swimming pool	03	01	33.33	01	33.33
Total	47	36	76.59	42	89.36

Germany). 100 bp DNA ladder (Promega, USA) was used as molecular weight marker.

In vitro antibiotic sensitivity test

Six commonly prescribed antibiotics (HiMedia, India) namely chloramphenicol (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), tetracycline (30 µg), streptomycin (10 µg) and ampicillin (2 µg) were selected for the sensitivity test. Antibiogram were done by disk diffusion method using Mueller Hinton (HiMedia, India) agar media as described by Mamun et al. (2017). A McFarland 0.5 standard was maintained for each culture suspension of bacterial isolates. The results of the test were recorded as sensitive, intermediately sensitive, or resistant by the recommendations of CLSI (2016).

Molecular detection of the *tet*A gene

Isolated *E. coli* and *Salmonella* spp. that were found phenotypically resistant to tetracycline were further screened to detect tetracycline resistance gene, *tetA* by PCR using the primers and protocol as presented in Table 1. All the PCR were done as stated previously. Thermal condition consisted initial denaturation at 95°C for 5 min followed by 30 cycles each of 95°C for 60 s, 57°C for 60 s, 72°C for 1 min and final extension at 72°C for 10 min. Agarose gel 1.5% was used to analyzed and amplified the PCR product by electrophoresis.

RESULTS

Isolation of *E. coli* and *Salmonella* spp.

Among the 47 samples collected, 36 (76.59%) were found positive for *E. coli*. On sample basis, the highest occurrence was in sewage (85%) and lowest in swimming pool (33.33%; Table 2). On the other hand among the 47 samples, 42 (89.36%) were found positive for *Salmonella* spp. Occurrence of *Salmonella* spp. was highest in river and lowest in swimming pool (Table 2).

Antibiogram profile

All the isolates were subjected to antibiogram study. Phenotypically among 36 *E. coli* isolates, two were found resistant to chloramphenicol, six to streptomycin, 35 to ampicillin and all to tetracycline (Table 3). On the other hand phenotypically among the 42 *Salmonella* spp. isolates, three were found resistant to chloramphenicol, three to ciprofloxacin, eight to streptomycin, and all to tetracycline and ampicillin. Notable finding is that all the isolated *E. coli* and *Salmonella* spp. were found resistant to tetracycline, while all were sensitive to gentamicin.

Detection of *tetA* gene

Tetracycline resistant phenotypes were screened for the detection of *tetA* gene by PCR (Figure 1). Among the 36 *E. coli* 28 (77.80%) isolates were found positive for *tetA* gene (Table 4). In case of *Salmonella* spp. among the 42 isolates, 34 (80.90%) were found positive for *tetA* gene.

DISCUSSION

Because of the rapid emergence and spread of antibiotic

Table 3. *In vitro* antibiotic sensitivity test of the isolated *E. coli* and *Salmonella* spp.

Name of antibiotic	Isolated bacteria	Sensitive	Intermediate	Resistant
Gentamicin	E (36)	33 (91.6%)	03 (08.3%)	0 (0.00%)
	S (42)	34 (80.9%)	08 (19.1%)	0 (0.00%)
Chloramphenicol	E (36)	26 (72.2%)	08 (22.2%)	02 (05.5%)
	S (42)	30 (71.4%)	09 (21.45%)	03 (07.1%)
Ciprofloxacin	E (36)	24 (66.6%)	12 (33.3%)	0 (0.00%)
	S (42)	31 (73.8%)	08 (19.1%)	03 (07.1%)
Streptomycin	E (36)	23 (63.8%)	07 (19.4%)	06 (16.6%)
	S (42)	16 (38.1%)	18 (42.8%)	08 (19.1%)
Ampicillin	E (36)	0 (0.00%)	01 (02.7%)	35 (97.2%)
	S (42)	0 (0.00%)	0 (0.00%)	42 (100%)
Tetracycline	E (36)	0 (0.00%)	0 (0.00%)	36 (100%)
	S (42)	06 (14.2%)	0 (0.00%)	42 (100%)

*E = E. coli, S= Salmonell*a spp.

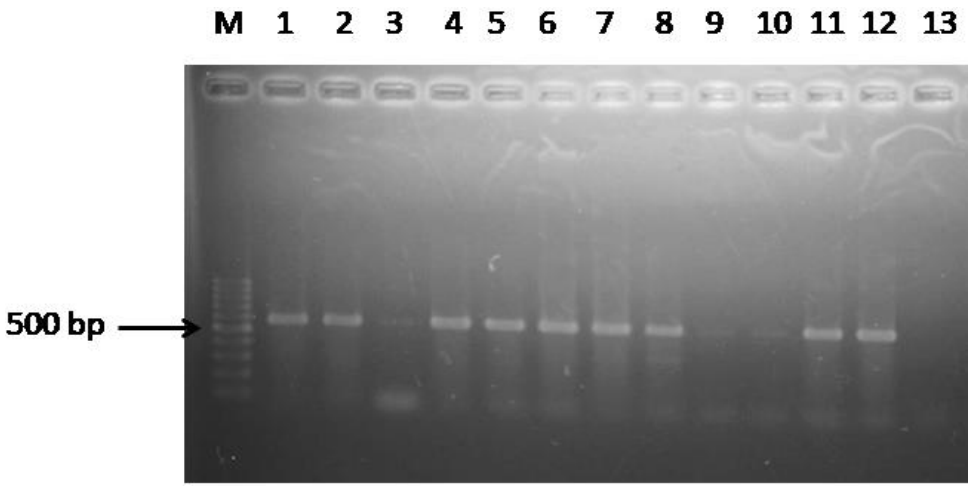

Figure 1. PCR amplification of 577 bp amplicon of *tetA* gene. Lanes 1-6: *E. coli*, lanes 7-11: *Salmonella* spp. M 100 bp. Lane 12: positive control, lane 13: negative control.

Table 4. Distribution of *tetA* gene among the isolated *E. coli* and *Salmonella* spp.

Source of sample	Tetracycline resistant *E. coli*		Tetracycline resistant *Salmonella* spp.	
	No. of phenotype tested	*tetA* positive	No. of phenotype tested	*tetA* positive
Sewage	17	14/17 (82.3%)	19	17/19 (89.4%)
River	03	2/3 (66.6%)	04	1/4 (25%)
Pond	15	11/15 (73.3%)	18	15/18 (83.33%)
Swimming pool	01	1/1 (100%)	01	1/1 (100%)
Total	36	28 (77.77%)	42	34 (80.95%)

resistant bacteria and their resistance genes among humans, animals and the environment at global scale, antibiotic resistance is now considered as a one health challenge. Environment is a major source for antibiotic resistant bacteria that are of great public health concern. Most of the researches on AMR focused on human and animal, and there is a lack in AMR situation in the environment in LMICs such as in Bangladesh. In this

study, the occurrence of antibiotic resistant *E. coli* and *Salmonella* spp. in sewage, river, pond and swimming pool in Mymensingh, Bangladesh with molecular level was investigated.

In this study *E. coli* and *Salmonella* spp. were found to be widely distributed in the environmental samples analyzed. The overall occurrence of *E. coli* and *Salmonella* spp. were 76.59 and 89.36%, respectively. One of the most striking finding of this study is that all the isolates of *E. coli* and *Salmonella* spp. were found resistant to tetracycline phenotypically. This observation is further supported by the detection of *tetA* gene. Resistance against tetracycline is usually associated with *tet* gene family. Tetracycline is a widely used antibiotic (Hassan et al., 2015). Long time wide spread use of tetracycline in veterinary and human medicine could be lined with these observed resistant against tetracycline. Peak et al. (2007) and Huang et al. (2019) also found tetracycline resistance genes in various types of waste water and sewage. It is also important to note that few isolated *E. coli* and *Salmonella* spp. in this study were found to be MDR in nature for example, resistant against tetracycline, ampicillin and streptomycin. Rashid et al. (2015) earlier reported the presence of MDR *E. coli* in various aquatic sources in Bangladesh.

It is not uncommon to detect these ARB in environmental samples as evident from the recent work of Divya and Hatha (2019), Proia et al. (2019) and Liu et al. (2018), who also detected antibiotic resistant *E. coli* and *Salmonella* spp. in various environmental samples including tropical estuarine water, waste water, sewage etc. In this study, tetracycline resistant *E. coli* and *Salmonella* spp. in water collected from sewage, river, pond and swimming pool were detected. Wei et al. (2018) detected several member of Enterobacteriaceae in swimming pool water in Guangzhou, China and in Imo river water in Nigeria (Ihejirika et al., 2011). Contamination of surface water with biological waste including fecal materials could be associated with the occurrence of these resistance bacteria in these environmental samples.

In Bangladesh, Zahid et al. (2009) carried out an investigation on the prevalence of multiple ARB and their chromosomal determinants in surface water. From 147 samples, they isolated 103 bacterial species of which 65% were *E. coli* including isolates resistant to tetracycline. While Siddiqui et al. (2015) showed presence of antibiotic resistant *Salmonella* spp. in hospital waste and many of which eventually ended up in the sewage in Bangladesh, the present study findings support both of these earlier observations.

E. coli and *Salmonella* spp. are enteric bacteria. Although not all the strains of *E. coli* and *Salmonella* spp. are pathogenic in nature, some strains are capable of causing serious illness in animal and human including enteritis. Both of them are also zoonotic in nature (Vasco et al., 2016). Detection of antibiotic resistant *E. coli* and *Salmonella* spp. in sewage, river, pond and swimming pool as evident in this study are very alarming from the public health point of view. Antibiotic resistance is a global health problem. Disease caused by ARB are very difficult to treat, needs special attention and expensive to treat. Human can easily get exposed to these resistant strains from sewage, river, pond and swimming pool. Moreover, occurrence of antibiotic resistant bacteria in the environmental samples observed in this study is an indication of serious environmental pollution and hazard. Many of the resistant genes are mobile in nature. Environment contaminated with resistant bacteria and resistance genes act as source or reservoir for AMR that can easily transmit to other bacterial species.

There are a number of limitations associated with this study. Although tetracycline resistant *E. coli* and *Salmonella* spp. in various environmental samples have been detected here, not much samples were analyzed. In addition, the virulence properties of these isolates were also not investigated. Molecular basis of other resistant phenotypes were not focused. More detail study focusing on these limitations will provide a better understanding of AMR in environmental samples.

Conclusion

Environmental contamination is a global health challenge of the 21st century. In this study a wide spread occurrence of antibiotic resistant *E. coli* and *Salmonella* spp. in sewage, river, pond and swimming pool including *tetA* gene responsible for resistance against tetracycline were detected. The presence of these resistant bacteria in this environment is of great public health concern. Many strains of *E. coli* and *Salmonella* spp. are pathogenic in nature and there is potentiality for transmission of these pathogens to human from the contaminated environment. Disease caused by resistant isolates is difficult to treat. Food chain and animal are also at the risk of contamination. It is suggested that establishing active surveillance system across the nation for detection of ARB and ARGs in various environmental samples will assist in reducing hazards associated with AMR on animals and humans.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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Full Length Research Paper

Detection of mutations in *ampC* promoter/attenuator gene in *Escherichia coli* from dairy cows in Rio de Janeiro and Mato Grosso, Brazil

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***Escherichia coli* present the ampC naturally, and the observation of phenotypical resistance to cefoxitin is related to this gene deregulation. Mutations in the regulatory region in *ampC* cause exaggerated expression. The most frequent alterations in the *E. coli* AmpC promoter/attenuator leading to this overexpression is described at the positions: -88, -82, -42, -18, -1 and +58. Mastitis studies were carried in Rio de Janeiro and Mato Grosso, Brazil. Two cefoxitin and amoxicillin-clavulanic acid-resistant *E. coli* from farms animals were unusually detected once these characteristics are not observed together in this species. The objective of this work was to determine if these isolates had a chromosomal gene mutation, determining AmpC hyperproduction. After DNA sequencing, mutations were observed at -88, -82, -73, -18, -1 and +58 positions, confirming the initially suspected AmpC hyperexpression. In Brazil, this is the first work to report *E. coli* hyperproducing this enzyme.**

Key words: *Ampc* attenuator, AmpC hyperproduction, *ampC* promoter, bovine feces, mastitic milk.

INTRODUCTION

AmpC is a serine-β-lactamase that belongs to group 1 of Bush-Jacoby-Medeiros and class C of Ambler classifications. This enzyme is codified by the chromosomal gene, *amp*C, which is a natural gene in *Escherichia coli* species. The *amp*C is a non-inducible gene in this species because it has lost its regulator gene. So, the resistance to cephamycins is not phenotypically observed in this species (Ambler, 1980; Bush and Jacoby, 2010). The hyperproduction of AmpC in *E. coli* caused by spontaneous mutations that produce

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deregulation of *ampC* has been reported and is responsible to resistance to first, second and third-generation cephalosporins and to Extended-Spectrum Beta-Lactamase (ESBL) inhibitors (Siu et al., 2003; Jorgensen et al., 2010; Kohlmann et al., 2018). This hyperproduction does not cause resistance to cefepime that is related to ESBL co-production or Extended-Spectrum AmpC (ESAC) production in the isolates. AmpC may occur in bacteria producing another β-lactamase as ESBL, and it decreases the therapeutic options in the treatment of bacterial infectious diseases (Kojima et al., 2005; Mammeri et al., 2006; Rodríguez-Martínez et al., 2012).

Two regions are associated with controlling the enzyme production. The first is the promoter region that contains two important boxes, -35 box and -10 box, located between -42 and -18 positions. Another critical region is the attenuator of the *ampC* that is located between +17 and +37 locations (Olsson et al., 1983; Caroff et al., 1999; Corvec et al., 2002; Siu et al., 2003; Jorgensen et al., 2010). The mutations in the *ampC* gene may occur alone or in combination, although a single mutation in a specific position is sufficient to cause high enzyme production (Olsson et al., 1983).

The most frequent insertions or deletions in hyperproducers *ampC E. coli* occurs in -88, -82, -42, -18, -1 and +58 positions, but mutations at -32, -11, +6, +24 and +31 (Olsson et al., 1983; Caroff et al., 1999; Caroff et al., 2000; Haenni et al., 2014). There are data in the literature about these alterations in human clinical strain, but in *E. coli* from animal samples, it is not frequently demonstrated. Considering *E. coli* isolated from animals, these mutations had been described in Spain, Denmark and Japan (Briñas et al., 2002; Olesen et al., 2004; Kojima et al., 2005; Hiroi et al., 2011). However, there are no data about AmpC-hyperproducing *E. coli* in Brazil. The aim of this work was to detect the mechanism responsible for AmpC phenotypic characteristics observed in two *E. coli* from feces and milk in dairy cows during an antimicrobial resistance study.

MATERIALS AND METHODS

E. coli were isolated from milk and feces of cows on dairy farms in Rio de Janeiro (RJ) and Mato Grosso (MT), Brazil, within six years (2009-2015) (protocol no. CEUA-3664040915, Federal Rural University of Rio de Janeiro, Brazil). Routine biochemical tests identified 238 *E. coli* isolates, which was further confirmed by matrix-assisted laser desorption/ionization time-of-flight MS assay (Rodrigues et al., 2017; Santiago, 2017). Antimicrobial resistance of the *E. coli* isolates was obtained by the disk diffusion method, and two strains (G27 and S10) was suspected to AmpC hyperproduction. The cefoxitin-resistance was confirmed by MIC, according to CLSI (2017). To evaluate *ampC* promoter/attenuator was used the primers AB1 (5′-GATCGTTCTGCCGCTGTG-3′) and AmpC2 (5′-GGGCAGCAAATGTGGAGCAA-3′), yielding a 271-bp amplification product (Corvec *et al*., 2002). They were sequenced (ABI 3130xl, Applied Biosystems, São Paulo, Brazil) and analyzed by DNA Sequence Assembler version 4 (HeracleBioSoft, Arges, Romania) and Mega software version 7 (Caspermeyer, 2016). The sequences were deposited in a GenBank database (Genbank accession numbers: MK559376, MK559377 and MK559378). *E. coli* ATCC 25922 obtained from FIOCRUZ (Rio de Janeiro, Brazil) were used as a control for phenotypic and genotypic tests (CLSI, 2017).

RESULTS AND DISCUSSION

The isolates were sequestered from Agar MacConkey and thereafter subjected to Gram test to confirm the morphological and tinctorial characteristics. The isolates were identified as *E. coli* by phenotypic laboratory tests in accordance with Koneman et al. (2010). All *E. coli* were confirmed by matrix-assisted laser desorption/ionization time-of-flight MS assay (Rodrigues *et al*., 2017).

After specie identification, these isolates were submitted for antibiotics tests for detection of resistance to β-lactams. So, two *E. coli* isolates, G27 and S10, presented resistance to cefoxitin, amoxicillin and amoxicillin-clavulanic acid and susceptibility to cefepime, suspected to AmpC hyperproduction. G27 and S10 presented MIC < 4 (CLSI 2017). The *E. coli* G27 was isolated from dairy cows' milk samples in suspected mastitis cases in Rio de Janeiro (2010), and the *E. coli* G27 was isolated from dairy cows' milk samples in suspected mastitis cases in Rio de Janeiro during evaluations in 2010, and the E. coli S10 was isolated in cow feces from Mato Grosso, in 2014.

These isolates were submitted to PCR and the primers used include the -35 box, the -10 box, and the attenuator segment. The sequencing of the regulatory region of G27 and S10 were analyzed using DNA Sequence Assembler version 4 (HeracleBioSoft, Arges, Romania) and Mega software version 7. Some alterations were observed in important positions. G27 and S10 *ampC* regulatory region revealed the mutations previously described in the literature responsible for causing the AmpC hyperproduction. Both isolates presented the most common substitutions for -88, -82, -18, -1 and +58 positions, although they have also shown a replacement at -73 position (Table 1).

Many authors described alterations in important regions in *E. coli ampC* regulator from human and animals samples. Naturally, *E. coli* produces AmpC enzyme in a low quantity because it is responsible for wall maintenance as a biological function (Johnson et al., 2013; Santiago et al., 2016).

The *ampC* promoter studies demonstrated -1 and +58 mutations are associated with increased strength of promoter taking higher gene transcription in *E. coli* (Olsson et al., 1983; Caroff et al., 1999; Corvec et al., 2002; Jorgensen et al., 2010; Haenni et al., 2014). Siu et al. (2003) and Yu et al. (2009) found replaced C (cytosine) by T (timine) at -58 position as in this study.

In other studies involving *E. coli* from animal was detected this species expressing resistance to cefoxitin with mutations at -88, -82 -42, -32, -18, -1, +37 +58 and +70 positions in promoter region of *ampC* gene (Briñas et

Table 1. Nucleotide mutation in the *ampC* promoter/attenuator of *Escherichia coli* G27 and S10 compared with *Escherichia coli* ATCC 25922.

Isolate	Origin	Nucleotide mutation					
		- 88	- 82	- 73	- 18	- 1	+ 58
E. coli ATCC 25922	-	C	A	T	G	C	C
G27	Milk	T	G	C	A	T	T
S10	Feces	T	G	C	A	T	T

C, cytosine; A, adenosine; T, thymine; G, guanine.

al., 2002; Olesen et al., 2004; Kojima et al., 2005; Hiroi et al., 2011; Haenni et al., 2014). The mutation in -73 position was only observed in human isolates studied by Yu et al. (2009). They described one *E. coli* containing mutation at -73 position and it was associated with other mutations, among them at 80, -28, -1, +58 and +82 position.

The AmpC-hyperproducing *E. coli* has not been reported in dairy cattle. However, many positions of mutation observed in *E. coli* beef cattle, broiler, and meat were described in human samples (Briñas et al., 2002; Hiroi et al., 2011). These changes demonstrate that there is a relationship between the transmission of these bacteria in the food chain and dissemination through the environment.

Another important fact is that some mutations were observed in human isolates before been reported in animal samples. That way, we believe the dissemination of these bacteria occurred before 1999, but only years later; the studies involving animals were published. This may have happened due to the new paradigm implemented by the One Health concept in 2007.

Interestingly, the occurrence of AmpC-hyperproducing *E. coli* was low during the period evaluated. den Drijver *et al*. (2018) studied the prevalence of AmpC-producing *E. coli* from a Dutch teaching hospital and affirmed these characteristics had been declined. In Brazil, AmpC-hyperproducing *E. coli* had not been reported until now, so it is challenging to state about the epidemiology of these isolates.

These mutations demonstrate that AmpC enzyme has been hyperproduced by these isolates. This study indicates that AmpC-hyperproducing *E. coli* also exist in Brazil, specifically in dairy herds. This is the first Brazilian report to consider hyperproduction of AmpC enzyme in *E. coli* isolated from dairy cows. Future studies can be conducted with the aim of identifying other animals and animal products containing *E. coli* with these characteristics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Methicillin-resistant *Staphylococcus* spp. in the nasal cavity of dental surgeon's professors

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The aim of the study is to investigate the methicillin-resistant *Staphylococcus* spp. nasal colonization among dental surgeon professors. Dental surgeon professors of a Higher Education Institution (HEI) responded to a questionnaire covering sociodemographic, employment and behavioral data, and were subjected to clinical specimen collection by nasal swab. Identification and susceptibility testing of bacteria were performed by automated method (Vitek 2 compact™). Susceptibility to mupirocin was tested by disk-diffusion method. The detection of *mec*A and *lukS*-F genes was performed by PCR. The genetic similarity among the isolates was determined by Pulsed Field Gel Electrophoresis. Four (9.7%) dental surgeon professors were colonized by methicillin-resistant *Staphylococcus* spp. and claim have provided care to patients without wearing surgical masks (1/4) and/or gloves (4/4), and had the habit of keeping surgical masks on the chin (1/4). Two *S. aureus* and one *S. epidermidis* isolates were *mec*A gene positives. MLS_B complex (inducible), mupirocin and sulfamethoxazole/trimethoprim resistance were also detected. The *lukS-F* gene was not detected in any *S. aureus* and no genetic similarity was found among the isolates. Dental surgeon professors were found to be colonized with methicillin-resistant *Staphylococcus* spp. and declared noncompliance to infection control practices, posing risk of infection to themselves, patients, students and their families.

Key words: Occupational dentistry, antimicrobials/antimicrobial resistance, dental education, infection control, bacteria, infectious disease(s).

INTRODUCTION

The dental surgeon (DS) provides treatment to patients with various medical problems by peculiar procedures including the continuous use of instruments that generate droplets and aerosols, which enhances his exposure to a

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wide variety of microorganisms, including pathogenic bacteria, favoring the colonization (Centers for Disease Control and Prevention 2016; Harrel and Molinari, 2004; Centers for Disease Control and Prevention 2013). Additional factors that make healthcare workers (HCW) vulnerable to colonization are the non-adherence to biosecurity measures (Centers for Disease Control and Prevention 2016; Siegel et al., 2007; Centers for Disease Control and Prevention, 2003). Additionally, unlike hospital settings, the DS works in clinics where, sometimes, the clinical care is conducted in the same area where the reprocessing of dental reusable devices/ instruments is performed, due to the absence of a specific area for this purpose (Alvarenga et al., 2010).

Colonization status poses risks to the HCW since, in an episode of imbalance of the microbiota and immune system, an endogenous infection may be developed (Kim et al., 2018; Zervou et al., 2014; Albrich and Harbarth 2008). In addition, it poses risks to the patient, once the colonized HCW becomes a reservoir and a potential source of bacteria in the epidemiological chain of Healthcare-Associated Infections (HAI) (Ugolotti et al., 2018; Kim et al., 2018; Zervou et al., 2014; Costa et al., 2014; Albrich and Harbarth 2008). However, studies on the colonization of dental HCW with multidrug-resistant bacteria are scarce (Khairalla et al., 2017), particularly, in clinical practice in higher educational institutions, which reflects the reality of clinical care treatments in outpatient dental clinics.

Among the more relevant multidrug-resistant bacteria in the context of HAI, methicillin-resistant *Staphylococcus aureus* (MRSA) stands out. This infectious agent is associated with high morbi-mortality rates worldwide (Grundmann et al., 2006) and belongs to the ESKAPE group (*Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *Enterobacter* spp.), composed of bacteria that are often multidrug-resistant (Rice, 2008). Coagulase-negative *Staphylococcus,* especially, methicillin-resistant *Staphylococcus epidermidis* (MRSE), previously reported as contaminant, also represent important pathogens in the context of the HAI (Soumya et al., 2017; Becker et al., 2014). Thus, the aim of this paper was to investigate the nasal colonization of DS professors with methicillin-resistant *Staphylococcus* spp. These HCW were chosen because they practice in various medical specialties and dedicate themselves to the academic teaching and guidance for students in dental clinical practice.

MATERIALS AND METHODS

This study was conducted in a Higher Education Institution (HEI) in the Central-West region of Brazil. The institution has a total of 106 dental offices organized in polyclinics, providing an average of 4.500 consultations per month in several dental specialties. All DS involved in academic teaching and guidance activities of the institution were invited to participate. The faculty team was composed of 53 DS, and 43 of them were in clinical practice. The inclusion criteria were: To be a dental surgeon, to be an employee of the HEI, and to have a role in providing guidance in academic clinical activities during the period of the samples collection. The exclusion criteria were: Suspicion of upper respiratory tract infection at the time of the samples collection, and who were using or had used any antimicrobial in the last 30 days prior to the samples collection. The project was approved by the Ethics and Research Committee (protocol number 509.774) and the Informed Consent was read and signed by the participants.

Data and sample collection

The eligible DS who agreed to participate in the study responded to a questionnaire related to socio-demographic, employment and behavioral aspects. Nasal specimens were obtained by sterile swab moisturized with sterile saline (0.9%) (Askarian et al., 2009; Scarnato et al., 2003), and were stored in tubes containing *Stuart* transport medium (Copan®, Brescia, Italy). The tubes were transported to the laboratory of bacteriology at room temperature and processed within 12 h.

The nasal swab was immersed in Brain Heart Infusion (BHI) broth and mixed on vortex for 1 min and incubated for 18/24 h at 35°C. Following incubation, the broth culture was inoculated onto mannitol salt agar and tryptic soy agar (TSA) supplemented with 4.0% NaCl and 6 µg/mL of oxacillin (primary culture), followed by incubation at 35°C for up to 72 h. The colonies suggestive of *Staphylococcus* sp. were initially identified by their macroscopic and microscopic characteristics, by Gram stain, and streaked onto mannitol salt agar and incubated at 35°C for 24 h to isolate pure cultures. Colonies were subcultured onto nutrient agar and incubated for 24 h at 35°C, to perform the test of catalase production and storage into microtubes containing tryptic soy broth with 20% of glycerol, at -20°C.

The biochemical identification (Vitek™ 2 GP card) and evaluation of antimicrobial susceptibility, the detection of methicillin resistance and the induced resistance to the Macrolide-lincosamide-streptogramin B group (MLS_B) (Vitek™ 2 - AST-GP-P585) were performed by automated method using the Vitek 2 Compact™ system, according to the manufacturer instructions for use. Susceptibility to mupirocin (20 µg) was analyzed by disk-diffusion method (Clinical and Laboratory Standards Institute, 2015) and the interpretation of the test was done following the recommendations of the British Society for Antimicrobial Chemotherapy (British Society for Antimicrobial Chemotherapy, 2015). The standard strain (ATCC 25923) was used as a quality control.

mecA and *lukS-F* genes detection

All *Staphylococcus* sp. identified by the Vitek 2 Compact™ system were submitted to genomic DNA extraction (Aires de Sousa et al., 2007) and subjected to PCR for detection of *mec*A gene (Murakami et al., 1991), which is responsible for the alternative pathway for the synthesis of a modified PBP (PBP2a or PBP2'), using the primers: F 5'-TCCAGATTACAACTTCACCAGG-3' and R 5'-CCACTTCATATCTTGTAACG-3'. Cycle condition: 4 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 53°C, 1 min at 72°C, and an additional extension of 4 min at 72°C. The detection of *lukS*-F gene, which encodes the Panton-Valentine leukocidin (PVL), was performed in all *S. aureus* identified, using the primers: PVL1: 5' - ATCATTAGGTAAAATGTCTGGACATGATCCA- 3' and PVL2: 5'-GCATCAASTGTATTGGATAGCAAAAGC – 3' (Lina et al., 1999), under the following cycle condition: 5 min at 94°C, 25 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C and an additional extension of 7 min at 72°C.

Table 1. Sociodemographic, employment and behavioral characteristics of dental surgeon professors (N = 41).

Variable	N	%
Age (years)		
<50	17	41.5
≥50	24	58.5
Experience (years)		
01 - 15	12	29.3
16 - 30	18	43.9
31 - 45	11	26.8
Clinical practice area*		
Esthetic/prosthetic dentistry	18	43.9
Periodontics/implantology/buccomaxilofacial surgery	11	26.8
Endodontics	07	17.0
Pediatric dentistry	08	19.5
Clinical activity (working hours per week)		
01 - 10	18	43.9
11 - 20	12	29.3
21 - 40	07	17.1
< 40	04	9.8
Currently working in hospital settings		
Yes	05	12.2
No	36	87.8
Ever worked in hospital settings		
Yes	12	29.3
No	29	70.7

* Possibility of more than one alternative.

Pulsed Field Gel Electrophoresis

The chromosomal DNA macrorestriction profile of the isolates was determined by Pulsed Field Gel Electrophoresis (PFGE), after bacterial chromosome digestion with *Sma*I (Chung et al., 2000). The PFGE was performed with 1% agarose gel in Tris-Borate-EDTA 0.5X buffer solution using the CHEF DRII system (Bio-Rad Laboratories). Images were captured with the Molecular Imager Gel Doc XR (Bio-Rad™) and analyzed by BioNumerics program (version 5.0; Applied Maths, Ghent, Belgium). The construction of the dendrogram was established by using the similarity coefficient of Dice (Dice, 1945), based on the position and presence of the bands and the phylogenetic analysis algorithm UPGMA (Unweighted Pair-Groups Method), using unweighted average clustering (Sneath and Sokal, 1975). The tolerance and optimization parameters were set to 0.7 and 1.0%. Each cluster of isolates will be defined as a grouping of profiles (n ≥ 2), presenting a similarity coefficient above 80% (Carriço et al., 2005).

RESULTS

Forty-one (77.3%) of the 53 DS professors actively involved in teaching participated in this study. Table 1 presents the socio-demographic and employment characteristics of participants. Of the 41 DS professors, 31.7% (13/41) were colonized in the nasal cavity with *Staphylococcus* spp. and 9.7% (4/41) were colonized with methicillin-resistant *Staphylococcus*. Table 2 presents the socio-demographics, employment and behavioral risk characteristics of the four DS professors colonized with methicillin-resistant *Staphylococcus*, who are identified as A, B, C and D. Cases of upper respiratory tract infections (tonsillitis), before sample collection, and use of antimicrobials (clavulanic acid and amoxicillin combined with clavulanic acid), not within the 30 days prior sample collection, were confirmed by 2/4 DS professors.

Three MRSA were isolated, denominated MRSA 1 (from DS professor A), MRSA 2 (from DS professor B), MRSA 3 (from DS professor C) and 1 MRSE (from DS professor D). Isolates MRSA 1 and MRSA 3 were susceptible to cefoxitin screen test (disk-diffusion), but *mec*A gene positive (Figure 1), thus considered MRSA. MRSA 2 was resistant to oxacillin and to cefoxitin screen test, although *mec*A gene negative. Inducible resistance to MLS_B complex was observed in two (50.0%) of the isolates (MRSA 1 and MRSE) (Table 3).

MRSA 1 was mupirocin-resistant and MRSE was trimethoprim/sulfamethoxazole-resistant. MRSE also presented intermediate resistance to quinolones, ciprofloxacin and norfloxacin (Table 4). All methicillin-resistant *Staphylococcus* spp. were susceptible to

Table 2. Sociodemographic, employment and behavioral characteristics of dental surgeon professors colonized with methicillin-resistant *Staphylococcus* spp. (N = 4).

Socio-demographic and employment characteristics	Dental surgeons*			
	A	B	C	D
Age (years)	≥50	≥50	<50	≥50
Experience (years)	25	37	14	31
Clinical practice area*	Esthetic/ prosthetic dentistry	Esthetic/ prosthetic dentistry	Pediatric dentistry	Endodontics
Clinical activity (working hours per week)	11 and 20	1 and 10	1 and 10	11 and 20
Behavioral risk characteristics				
Hand hygiene not performed upon changing torn gloves	X	X	X	
Gloves not used during some patient care			X	X
Dental care has been provided to patients without using gloves	X	X	X	X
Dental care has been provided to patients without using surgical masks		X		
Surgical cloth mask has been used by dentist	X	X		X
Habit of wearing masks on the chin	X			
Not changing wet masks			X	

*Dental surgeons colonized with methicillin-resistant *Staphylococcus* spp. identified by the letters A, B, C and D.

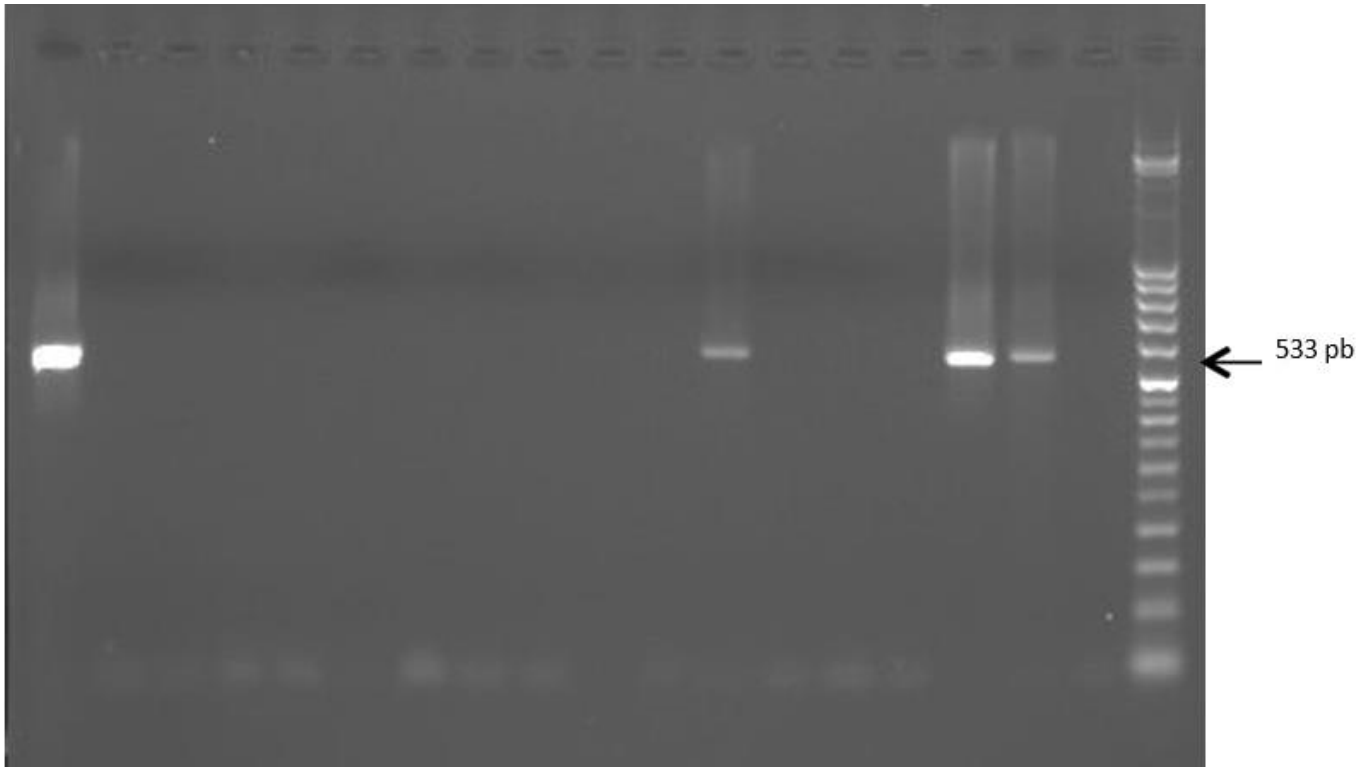

Figure 1. Electrophoresis for detection of *mec*A gene in *Staphylococcus* spp. isolated from the nasal cavity of dental surgeons. Columns 1 to 16: Strains of *Staphylococcus* spp.; column 17: Positive control (USA 300); column 18: Negative control; column 19: 50 bp molecular weight marker. Column 1: Methicillin-resistant *Staphylococcus aureus* (MRSA) 1; Column 2: MRSA 2; Column 12: MRSA 3; Column 16: Methicillin-resistant *Staphylococcus epidermidis* (MRSE).

Table 3. Phenotypic and genotypic characterization of resistance to methicillin and phenotypic resistance to MLS_B complex of methicillin-resistant *Staphylococcus* spp. (N = 4) isolated from the nasal cavity of dental surgeon professors.

Markers for methicillin resistance	MRSA 1	MRSA 2	MRSA 3	MRSE
Oxacillin	S	R	S	R
Minimum inhibitory concentration for oxacillin (mcg/mL)	≤0.25	≤4	0.5	≤4
Cefoxitin (screening test)	-	+	-	+
mecA gene	+	-	+	+
Markers for inducible resistance to MLS_B complex				
Clindamycin	R	I	S	R
Inducible Clindamycin resistance test	+	-	-	+
Phenotypic resistance to MLS_B complex	MLS_B (i)	-	-	MLS_B (i)
Erythromycin	R	R	S	R

S= susceptible; I=intermediate; R=resistant; (+) = positive; (-) = negative; Inducible resistance to Macrolide, Lincosamide and Streptogramin B - MLS_B (i).

Table 4. Susceptibility profile of methicillin-resistant *Staphylococcus* spp. (N = 4) isolated from the nasal cavity of dental surgeon professors.

Antimicrobials	MRSA 1	MRSA 2	MRSA 3	MRSE
Mupirocin	R	I	S	S
Benzylpenicillin	R	R	R	R
Ciprofloxacin	S	S	S	I
Norfloxacin	S	S	S	I
Trimethoprim/Sulfamethoxazole	S	S	S	R

*S= susceptible; R=resistant; I=intermediate; P =positive; N =negative.

moxifloxacin, vancomycin, teicoplanin, gentamicin, tigecycline, linezolid, rifampicin and fusidic acid. All (100%) isolates were *luk-F* gene negative. There was no genetic similarity among the MRSA isolates.

DISCUSSION

Biohazard exposure is widely addressed in guidelines for HCW and there has been a wide discussion about blood borne pathogens (Kuhar et al., 2013; Schillie et al., 2013), however little discussion about multidrug-resistant bacteria has taken place (Centers for Disease Control and Prevention, 2016; Centers for Disease Control and Prevention, 2003). Most studies about multidrug-resistant bacteria colonization in HCW address those who work in hospital settings (Albrich and Harbarth, 2008). Thus, it highlights the importance of analyzing the nasal colonization with methicillin-resistant *Staphylococcus* spp. among DS professors (9.7% - 4/41) working in clinical practice orientation in HEI. These professionals, as well as those who work in hospitals, provide direct patient care and are exposed to biohazards and are at risk of acquiring HAI. A similar prevalence of nasal colonization with MRSA was reported among DS from a university in Egypt (9.7% - 3/31) (Khairalla et al., 2017).

The four professionals colonized with methicillin-resistant *Staphylococcus* spp. worked in specialty clinics in endodontics, pediatric dentistry or esthetic/prosthetic dentistry, areas where the use of rotational instruments is frequent. It is well known that medical devices/ instruments that generate droplets and aerosols increase the dispersion of particles in the air containing water, saliva, pathogenic microorganisms and even blood, factors which contribute to the colonization of HCW (Harrel and Molinari, 2004; Centers for Disease Control and Prevention, 2003). Additionally, colonized DS professors presented risk behaviors such as low compliance with standard and transmission-based precautions. Improper use and/or no use of gloves were reported (Table 2), which exposes the hands of these HCW to contamination with infectious agents and may be transferred to the nasal cavity. Removing gloves to facilitate the dental procedure was reported by about 50% of dentists participating in a study in Poland (Garus-Pakowska et al., 2017). The colonization of the gloves, in turn, leads to contamination of hands with direct contact. Colonization of nasal cavity and hands among DS with MRSA was also confirmed in dental clinics at a university in Egypt (Khairalla et al., 2017).

Improper use and/or no use of surgical masks were also reported by DS professors colonized with methicillin-

resistant *Staphylococcus* spp. (Table 2). In Poland, 6.5% of dentists reported never use protective equipment, including procedure masks, which is the main protective barrier against nasal cavity colonization (Garus-Pakowska et al., 2017). A study that assessed the contamination of different areas of DSs' faces during dental procedures identified the presence of spatters throughout the face being more concentrated around the nose, probably due to close proximity of the HCW to the oral cavity to obtain a better view of the area (Nejatidanesh et al., 2013).

Cases of upper respiratory tract infections were reported by HCW colonized with methicillin-resistant *Staphylococcus* spp. Albrich and Harbarth (2008) showed that the prevalence of HCW colonized with MRSA who had subsequent infections was 5.1%. The most frequent diseases were cutaneous and soft tissue, followed by upper respiratory tract infections. Among dentists, prolonged exposure to procedures in which there is production of aerosols was associated with the presence of symptoms such as persistent or productive cough, nasal congestion, runny nose, sneezes, eye irritation, cutaneous eruptions, pruritus or dry skin (Allsopp et al., 1997). These findings indicate bacterial colonization as an adjuvant in the occurrence of adverse effects in occupational health (Costa et al., 2014; Albrich and Harbarth, 2008).

There was no genetic similarity among the MRSA isolates, implying an absence of clones and transmission among DS professors. However, the colonized status increases the possibility to spread these bacteria from symptomatic professionals with upper tract infections to patients, their family members, community setting as well as the occurrence of outbreaks (Lis et al., 2009; Lu et al., 2008). Furthermore, it is worth highlighting the potential of direct transmission from professor to students, since they are in continuous contact during clinical orientation and practice at HEIs. In addition, the risks those noncompliant DS professors pose on students, with regards to the preventive measures, since the professor is considered to be a role model and can influence students' behavior and skills (Morais et al., 2017; Betancourt et al., 2011).

In this study, a genotypic resistance pattern (presence of *mecA* gene) with a phenotypic methicillin-susceptible profile was detected in two isolates (MRSA 1 and MRSA 3). It can be explained by a phenomenon called heteroresistance, when two subpopulations coexist in a culture, where all cells can carry the genetic information for resistance, however only a small number expresses it, therefore, in the absence of genotypic characterization of isolates, these could be wrongly identified as methicillin-susceptible (Andrade-Figueiredo and Leal-Balbino, 2016). The opposite, isolate with phenotypic methicillin-resistant profile and genotypic susceptibility profile (absence of *mecA* gene) (MRSA 2), was also identified. Two possibilities may explain these findings. Firstly, it is the hyperproduction of β-lactamase, which results in partial hydrolysis of the beta-lactam ring, or the modification of other Penicillin-Binding Proteins, known as Borderline resistance, and the treatment of infections caused by this microorganism could be inefficient even with the use of high doses of oxacillin (Hryniewicz and Garbacz, 2017). Secondly, it is the presence of a *mecA* gene homologue, the $mecA_{LGA251}$ gene, known as *mecC* gene (Ito et al., 2012). Bacteria that carry this gene can colonize and cause disease in humans and in a wide range of other host species and it was able to adapt rapidly in high concentrations of oxacillin *in vitro* (Milheiriço et al., 2017).

MRSE was the microorganism that showed to accumulate the highest number of drug resistance mechanisms, being intermediate to ciprofloxacin and norfloxacin, and resistant to trimethoprim/sulfamethoxazole. Similar results were reported for MRSE isolated from HCW in a cancer hospital centre (Costa et al., 2014), pointing to the need for follow-up cultures of these microorganisms given the multidrug-resistance and the difficulty for the infection treatment (Soumya et al., 2017). Inducible MLS_B complex resistance was observed in MRSE and MRSA 1 isolates and resistance to mupirocin in MRSA 1. Total resistance rate has been shown to be higher in MRSA isolates of dental staffs than in isolates from environmental surfaces in dental service (Khairalla et al., 2017). It should be noted that the topical use of mupirocin is the most widely used treatment option for decolonization and its high rate of resistance has been related to mistakes in how bacterial decolonization is conducted (McConeghy et al., 2009).

In conclusion, DS professors were colonized in the nasal cavity with methicillin-resistant *Staphylococcus* spp. with different resistance mechanisms and reported noncompliance with preventive measures, such as the use of gloves and surgical masks. These findings highlight that DS professors are reservoirs of these infectious agents which pose a threat to their own health and place them as potential disseminators. Educational and strategic activities to increase adherence to standard and transmission-based precautions are required not only for the HCW's own safety but also for the patient, students, other dental staff and community/family contact safety, and to ensuring quality of academic education, since students mirror professors' behavior.

It should be noted that the results of this study were reported individually to the DS professors, with a letter explaining the implications of being colonized and the preventive measures to be taken. In addition, a newsletter containing the results of the research was delivered to the HEI directors in order to clarify the importance of sending the results to the Dental Infection Control Committee of the HEI for implementation of appropriate precautions.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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Extended spectrum beta-lactamase production and plasmid mediated quinolone resistance among lactose non-fermenting *Enterobacteriaceae* isolated from poultry sources in Calabar, Nigeria

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This study investigated the co-carriage of plasmid mediated quinolone resistance (PMQR) and extended spectrum beta-lactamase (ESBL) producing lactose non-fermenting (LNF) *Enterobacteriaceae* isolated from poultry birds. This was a descriptive cross-sectional study carried out between September, 2016 and March, 2017. The Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility patterns. ESBL screening disc kit was used to detect ESBL activities. Detection of ESBL and PMQR genes was carried out by means of polymerase chain reaction. In total, 207 LNF *Enterobacteriaeae* isolates were recovered from the cloacal swabs of poultry birds within the Calabar Metropolis. ESBL-producing isolates were 162 (78.3%) while fluroquinolone resistant isolates were 194 (93.7%). Among the ESBL-producing isolates, resistance to Ciprofloxacin, Norfloxacin, Levofloxacin, Ofloxacin and Nalidixic acid was 55 (34.2%), 26 (16.1%), 35 (21.7%), 50 (31.1%), and 162 (100%), respectively. About 65% of the quinolone resistant isolates were positive for at least one of the PMQR and ESBL genes in this study. Strict antimicrobial screening, surveillance of resistant isolates as well as the judicious practice of antimicrobial administration in the poultry setting with special emphasis on fluoroquinolones is advised given the high prevalence of co-existent ESBL and PMQR genes.

Key words: LNF enterobacteriaeae, Extended spectrum beta-lactamases, quinolone resistance.

INTRODUCTION

A serious concern is arising on the coexistence of Extended beta lactamase (ESBL) and Plasmid-mediated quinolone resistance (PMQR) producing non-lactose fermenting *Enterobactericeae* in animal husbandry which could be dangerous to humans especially in strains that may not be routinely screened for antibiotic resistance

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(Oghenevo et al., 2016; Yangkam and Bassey, 2015).

The past decade has witnessed a rise in the use antimicrobial agents in both the clinical and the veterinary setting with the concomitant increase in the development of antimicrobial resistance (Orji et al., 2005; Schwarz et al., 2001; Xiong et al., 2018). Resistance is common to the most frequently used antibiotics. The frequently recommended antimicrobial agents for common infections caused by *Enterobacteriaceae* are beta-lactams and quinolones/fluoroquinolones (in severe cases) (Bajaj et al. 2016).

Resistance to quinolone is chromosome-mediated via a mutation of the DNA gyrase encoding genes (*gyrA* and *gyrB*) and the topoisomerase IV encoding genes (*par*C and *parE*) (Strahilevitz et al., 2009). However, there seems to be a linkage between resistance to quinolone and beta lactam antibiotics. Production of ESBL is plasmid mediated. Resistant plasmids carrying genes encoding for ESBL usually carry genes encoding for quinolone resistance and this has given rise to PMQR in the *Enterobacteriaceae* family (Ni et al., 2016; Sun et al., 2014).

Three mechanisms for PMQR have been detected since 1988 (Tran and Jacoby 2002). They include, plasmid genes *qnr*A, *qnr*B, *qnr*C, *qnr*D and *qnr*S which codes for the quinolone resistance proteins (Qnr) of the pentapeptide repeat family that protects DNA gyrase and topoisomerase from quinolone inhibition, acetylation of quinolones with an appropriate amino nitrogen target by a variant of the common aminoglycoside acetyltransferase AAC(6')-Ib, thus reducing their activity and the plasmid mediated *oqx*AB and *qep*A genes used for efflux pump mechanisms (Tran and Jacoby 2002; Jacoby et al., 2014).

These plasmid-mediated mechanisms only provide low-level resistance that by itself does not exceed clinical breakpoint for susceptibility but nonetheless facilitates selection for higher-level resistance and makes infections by pathogens much more difficult to treat (Pourahmad Jaktaji and Mohiti, 2010). Currently, there is a dearth of data regarding the co-carriage of ESBL and PMQR genes in *Enterobacteriaceae* from poultry sources in Nigeria. Therefore, this study aimed at investigating the prevalence of PMQR and ESBL determinants in lactose non-fermenting *Enterobacteriaceae* from poultry sources in Calabar, Nigeria.

MATERIALS AND METHOD

This study was carried out within the Calabar metropolis in Cross River State, Nigeria. Calabar is the capital city of Cross River State in the South-South geopolitical zone of Nigeria. Cross River State shares boundaries with Benue State to the North, Ebonyi and Abia States to the west and to the east by the Republic of Cameroun. The city is administratively divided into Calabar municipal and Calabar South Local Government Areas (LGAs). Calabar covers a surface area of about 406 km^2 (157 $mile^2$) and a population of 371,022 at the 2006 census.

Study design

This was a descriptive cross-sectional study designed to investigate the prevalence of PMQR and ESBL determinants in lactose non-fermenting *Enterobacteriaceae* from poultry sources. This study was carried out in the Department of Microbiology, University of Calabar teaching Hospital, Calabar, Nigeria from September 2016 to March 2017.

Ethical consideration

The Ethical Committees of the selected hospital approved the protocol for this study. Approval was also obtained from the Cross River State Ministry of Health, conveyed via CRS/MH/ CGSE-H/018/Vol/123 and the Health Research Ethical Committee of University of Calabar.

Isolation and identification of species

The samples included cloacal swabs obtained from healthy birds in major poultry farms and markets within the Calabar metropolis. Sample collection method was by random sampling. Sterile cotton gauze moisturized with 70% alcohol was used to clean the surrounding of the cloaca of the birds, and a sterile cotton swab was inserted about two inches into the cloaca and whirled for about two seconds. The used swab sticks were stored in 10% buffered peptone water prior to transportation to the laboratory within four hours.

The samples inoculated on MacConkey agar and Xylose lysine deoxycholate agar. Lactose non-fermenting *Enterobacteriaceae* were identified using standard culture methods and conventional biochemical tests. The DNA of each lactose non-fermenting isolate was extracted and used for the amplification of the 16s rRNA region of the DNA of the isolates using a specific primer set (27F: 5'-AGAGTTTGATCMTGGCTCAG-3', 1492R: 5'-CGGTTACCTTGTTACGACTT-3'), by means of the ABI 9700 Applied Biosystem thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mix included: X2 Dream Taq Master Mix supplied by Inqaba biotec, South Africa (Taq polymerase, DNTPs, $MgCl_2$), the primer sets at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation at 95ºC for 5 min, denaturation at 95ºC for 30 s, annealing at 52ºC for 30 s, extension at 72ºC for 30 s for 35 cycles and final extension at 72ºC for 5 min. The product was resolved on a 1% agarose gel at 120V for 20 min and viewed by means of a UV trans-illuminator (Inqaba biotec, Pretoria - South Africa).

DNA sequence analysis was performed using direct sequencing of both strands by means of the BigDye Terminator kit on a 3510 ABI sequencer (Inqaba biotecl, Pretoria - South Africa). The obtained DNA sequences were edited using TraceEdit. Highly similar sequences were downloaded from GenBank in the National Center for Biotechnology Information using BLASTn (http://www.ncbi.nlm.nih.gov/blast). The Lactose non-fermenting isolates were confirmed by a 100% match with the 16s rDNA of the downloaded sequences. One hundred and seventy-two (172), lactose, non-fermenting *Enterobacteriaceae* were identified following 16S rRNA sequence analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out using commercially available antimicrobial discs types (LiofilChem Diagnostic ID USA) which included; Ciprofloxacin (CIP-5 µg),

Norfloxacin (NOR-10 µg), Levofloxacin (LEV-5 µg), Ofloxacin (O-5 µg), Nalidixic acid (NA-30 µg), Clotrimoxazole (CLO-50 µg), Amikacin (AK-30 µg), Imipenem (IMI-10 µg), Chloramphenicol (C-30 µg), Cefpodoxime (PX-10 µg), Ceftazidime (CAZ-30 µg), Cefotaxime (CTX-30 µg), Ceftriaxone (CRO-30 µg), Cefepime (FEP-30 µg), Aztreonam (ATM-30 µg). The quality control strain used was *E. coli* ATTC 25922. The susceptibility of the isolates to the antimicrobial agents was determined by means of the Kirby-Bauer disk diffusion method as described by the Clinical and Laboratory Standard Institute (Ferreira et al., 2011)

Transferring 4 to 5 confirmed *Salmonella* colonies in a tube containing 2.5 ml sterile normal saline by means of a sterile inoculating loop to prepare a bacterial lawn. The suspension was vortexed and its turbidity compared with barium chloride (0.5 McFarland Turbidity Standard; 1.0×10^8 CFU/µL). The optical density of the standard was regularly monitored with a spectrophotometer at λ=625nm and 1cm light path (ODλ=0.08 - 0.1) (Cheesbrough, n.d.). One hundred micro liters (100 µL) of the inoculum was spread on Iso-sensitest agar plates. The excess inoculum was siphoned with sterile Pasteur pipettes. Plates were allowed to dry at room temperature in a laminar flow hood. The discs containing predetermined amounts of the antimicrobial agents were then dispensed onto the bacterial lawn using a pair of sterile forceps and gentle pressure applied to ensure complete contact with the agar. The disks were placed 15 mm away from the edge of the plate and 25 mm apart from each other. The plates were inverted within 15 min after the discs were dispensed, and incubated at 37°C for 16 to 18 h. After incubation, they were examined by reading the diameters of the inhibition zones and interpreted in accordance with the description of the United States Clinical and Laboratory Standard Institute (Pallecchi et al. 2012).

Phenotypic ESBL detection

The screening test for the detection of ESBL activity was carried out by means of the ESBL screening disc kit (Cefotaxime, Cefotaxime + Clavilanic acid, Cefotaxime + Clxacillin and Cefotaxime+Clavilanic acid + Cloxacillin) by LiofilChem Diagnostic, ID. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 was used as positive and negative control strains respectively for ESBLs production. (Mathai et al., 2002).

Molecular detection of ESBLs

All the isolates that were phenotypically resistant to the beta-lactam antimicrobial agents used in this study were screened for some relevant ESBL encoding genes (bla_{SHV}, bla_{OXA} and bla_{CTX-M}) by means of polymerase chain reaction (Abrar et al., 2019).

The bla_{CTX-M}, bla_{SHV}, bla_{OXA} genes were amplified using the following primer sets respectively: CTX-M/F: 5'-CGCTTTGCGATGTGCAG-3' and CTX-M/R: 5'-ACCGCGATATCGTTGGT-3', SHV/F: 5'-CGCCTGTGTATTATCTCCCT-3' and SHV: 5'-CGAGTAGTCCACCAGATCCT-3', OXA/F: 5'-AGCCGTTAAAATTAAGCCC-3' and OXA/R: 5'-CTTGATTGAAGGGTTGGGCG-3' on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 µL for 35 cycles. The PCR mix included: X2 Dream Taq Master Mix supplied by Inqaba biotec, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 min; denaturation, 95ºC for 30 s; annealing, 52ºC for 30 s; extension, 72ºC for 30 s for 35 cycles and final extension, 72ºC for 5 min. The PCR product was resolved on a 1% agarose gel at 120 V for 20 min and visualized on a UV trans-illuminator (Inqaba biotech, South Africa).

Molecular detection of PMQR genes

All the isolates that were phenotypically resistant to the fluoroquinolone antimicrobial agents used in this study were screened by means of polymerase chain reaction for the detection of *qnr*A, *qnr*B, *qnr*S and *qep*A (Chen et al. 2012; Kao et al. 2016).

Statistical analysis

The relationships between the lactose non-fermenting *Enterobacteriaceae* isolates and fluoroquinolone resistance and PMQR determinants were evaluated using the Chi-square or Fisher's test where necessary. The data generated in this study was analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA, version 22.0.). P-values of less than 0.05 (< 0.05), was considered statistically significant.

RESULTS

Antibiotics resistance patterns are shown in Table 1. A total of 207 Lactose Non-Fermenting (LNF) *Enterobacteriaeae* isolates were recovered from the cloacal swabs of poultry birds within the Calabar Metropolis. *Proteus mirabilis* was the most common bacterial isolate 118 (57.0%), followed by *Salmonella enterica* 39 (18.8%) while *Providencia rettgeri* 8 (3.9%) was the least common species.

All the isolates showed varied resistance to the 11 antibiotics and were most resistant to Nalidixic acid (93.7%), ceftazidime 179 (86.5%), cefotaxime 182 (87.9%) and cefpodoxime 179 (86.5%). Among the fluoroquinolones used in this study, resistance to nalidixic acid was the most prominent while resistance to Norfloxacin was the least. Among the beta lactam antibiotics, resistance to cefotaxime was the highest.

ESBL was phenotypically detected in 162(78.3%) of the 207 isolates. In Table 2 are shown the frequency of ESBL genes among the *Enterobacteriaceae* isolates under study. The genes bla_{CTX-M} and bla_{SHV} were the highest occurring 119(57.5%), while bla_{OXA} was the least ESBL gene detected 27(13%).

Among the ESBL-producing isolates, the rate of resistance to ciprofloxacin, norfloxacin, levofloxacin, ofloxacin and nalidixic acid was respectively 55 (34.2%), 26 (16.1%), 35 (21.7%), 50 (31.1%) and 162 (100%). The resistance to these fluroquinolone anitbiotics was significantly higher when compared to the non-ESBL-producing isolates ($P<0.05$). However, among the ESBL-producing isolates, there was no significant difference in the rate of fluoroquinolone resistance when compared with beta-lactam resistance ($P>0.05$).

Of the 207 isolates, 194 were resistant to quinolone antibiotics. About 65.5% (127) of the quinolone resistant

Table 1. Antimicrobial resistance pattern of LNF *Enterobacteriaceae* species isolated from poultry sources.

Antibiotics	Percentage (%) resistance					Total (n=207)
	Salmonella enterica (n=39)	*Proteus mirabilis* (n=118)	*Proteus vulgaris* (n=22)	*Providencia stuartii* (n=20)	*Providencia rettgeri* (n=8)	
Ciprofloxacin	48.7	22.9	36.4	5.0	0.0	26.6
Norfloxacin	20.5	13.6	13.6	0.0	0.0	13.0
Levofloxacin	51.3	6.8	36.4	0.0	12.5	17.9
Ofloxacin	84.6	7.6	31.8	0.0	12.5	24.2
Nalidixic acid	100	94.1	90.9	80.0	100	93.7
Ceftazidime	97.4	84.7	77.3	80.0	100	86.5
Ceftriaxone	87.2	62.7	54.5	100	0.0	58.9
Cefotaxime	87.2	87.3	77.3	100	100	87.9
Cefpodoxime	87.2	84.7	77.3	100	100	86.5
Cefepime	87.2	90.7	36.4	0.0	0.0	72.0
Aztreonam	94.9	82.2	86.4	35.0	0.0	77.3
Imipenem	38.5	16.9	4.5	0.0	0.0	17.4
Amikacin	41.0	44.9	9.1	0.0	0.0	34.3
Clotrimoxazol	89.7	70.3	68.2	30.0	50.0	69.1
Chloramphenicol	41.0	52.5	0.0	20.0	12.5	40.1

Table 2. Prevalence of ESBL-producing genes in LNF Enterobacteriaceae isolated from poultry sources.

Gene	No.(%) detection					Total (n=207)
	Salmonella enterica (n=39)	*Proteus mirabilis* (n=118)	*Proteus vulgaris* (n=22)	*Providencia stuartii* (n=20)	*Providencia rettgeri* (n=8)	
*bla*OXA	13 (33.3)	14 (11.9)	0	0	0	27 (13.0)
*bla*SHV	24 (61.5)	82 (69.5)	12 (54.5)	1 (5.0)	0	119 (57.5)
*bla*CTX-$_M$	33 (84.6)	70 (59.3)	16 (72.7)	0	0	119 (57.5)

Table 3. Prevalence of PMQR genes in LNF Enterobacteriaceae isolated from poultry sources.

Gene	No.(%) detection					Total (n=194)
	Salmonella enterica (n=39)	*Proteus mirabilis* (n=111)	*Proteus vulgaris* (n=20)	*Providencia stuartii* (n=16)	*Providencia rettgeri* (n=8)	
*qnr*A	3(7.7)	0	0	0	0	3(1.5)
*qnr*B	36(92.3)	61(55.0)	20(100)	1(6.3)	3(37.5)	121(62.4)
*qnr*S	20(51.3)	7(6.3)	0	0	0	27(13.9)
*qep*A	14(35.9)	9(8.1)	1(4.5)	2(10.0)	8(100)	34(17.5)

isolates were positive for at least one of the PMQR genes and ESBL genes used in this study. In Table 3 are shown the distribution of PMQR genes in the 194 quinolone resistant isolates. The *qnr*B gene was the most common PMQR gene detected 121 (62.4%), followed by *qep*A 34 (17.5%), *qnr*S 27 (13.9%) and *qnr*A 3 (1.5%).

Using the *S. enterica* isolates as an example, in table 4 is illustrated the co-existence of the ESBL and PMQR genes in LNF Enterobacteriaceae. All 39 *S. enterica* isolates demonstrated resistance to fluoroquinolones while 34 isolates were found to produce ESBL. Among the 34 ESBL-producing isolates, 41% (14) carried at least one PMQR gene, that is, one PMQR gene was co-carried with ESBL gene in the plasmid.

DISCUSSION

PMQR genes have been reported to be carried on mobile gene elements and can be easily transferred among

Table 4. Coexistence of ESBL and PMQR genes in *Salmonella enterica* (n=39) isolated from poultry sources

PMQR gene	ESBL genes	No. of isolates
*qnr*A	CTX-$_M$ + SHV	2
	CTX-$_M$ + OXA	1
*qnr*B	CTX-$_M$	7
	SHV	3
	CTX-$_M$ + SHV	10
	CTX-$_M$ + OXA	2
	CTX-$_M$ + SHV + OXA	11
*qnr*S	CTX-$_M$	4
	SHV	1
	CTX-$_M$ + SHV	8
	CTX-$_M$ + SHV + OXA	7
*qep*A	CTX-$_M$	1
	SHV	1
	CTX-$_M$ + SHV	4
	CTX-$_M$ + SHV + OXA	7

different bacterial strains and species (Osińska et al., 2016; Redgrave et al., 2014). This potential exacerbates the development of multi drug resistance because PMQR reportedly reduces microbial susceptibility to antibiotics and supports the occurrence of resistance-associated mutations on bacterial chromosomes, thus making *Enterebacteriaceae* infections much more difficult to treat. In this study, we look into the co-carriage of PMQR and ESBL genes in LNF *Enterebacteriaceae* isolates in Calabar, Nigeria.

All 207 LNF *Enterobacteriaceae* demonstrated varied degrees of resistance to the 11 antibiotics used in this study. However, the isolates were most resistant to Nalixidic acid and were least resistant to Imipenem, Levofloxacin and Norfloxacin. This partially agrees with a similar study carried out in Azerbaijan and Iran on ESBL-PMQR co-carriage where resistance to Nalixidic acid was highest (68.5%) closely followed by resistance to Levofloxacin (55%) and Norfloxacin (65%) (Azargun et al., 2018). The injudicious and common use of Nalixidic acid in comparison to other fluroquinolones, Levofloxacin and Norfloxacin could be the reason for such high resistance to Nalixidic acid in this region. Hence we recommend strict selection and rotation of antimicrobial agents coupled with the continuous monitoring of susceptibility profiles of antimicrobial agents to determine best treatment options

Our results further revealed that 78% (162) of the isolated produced ESBL and the most prominent genes of the 3 ESBL-producing genes tested were the *bla*$_{SHV}$ and the *bla*$_{CTX-M}$. This correlates with several studies that have reported both genes as the most prevalent ESBL-producers (Giske et al. 2008) There was also high prevalence of PMQR genes (65%) among the 194 isolates that were resistant to fluroquinolones with *qnr*B being the most prevalent. This agrees with several studies that have suggested of the added advantage given by PMQR genes to fluoroquinolone resistance.

Upon further analysis, we discovered that resistance to fluoroquinolones was significantly higher among ESBL-producing isolates than non-ESBL-producing isolates. This implies that co-carriage of ESBL and PMQR genes is associated with ESBL-producing isolates. This result agrees with several studies in Asia and Africa but disagrees with a few other studies, as specific effects of PMQR co-carriage on treatment outcomes has been difficult to document (Jacoby et al., 2014; Jiang et al., 2012; Shakya et al., 2013) Nonetheless, such high prevalence of multidrug resistance among ESBL-producing isolates poses a serious challenge to antimicrobial therapy.

About 65.5% (127) of the quinolone resistant isolates were positive for at least one of the PMQR genes and ESBL genes used in this study. To further buttress on the issue of co-carriage, our study using *S. enterica* isolates demonstrated that among the 34 ESBL-producing isolates, 41% (14) carried at least one PMQR gene, that is, at least one PMQR gene was co-carried with ESBL gene in its plasmid. This was in consonance with a previous study in which a high proportion of PMQR genes was observed among isolates possessing the ESBL genes (17).

The association of ESBL genes and PMQR genes are of importance for public health concerns. However, this

study did not include all the known ESBL genes and PMQR genes and molecular epidemiology was not performed, which could have further buttressed the evidences of the study. This study demonstrates high prevalence of LNF Enterobacteriaceae in a poultry setting. This agrees with several other studies done in USA and China (Projahn et al., 2018; Schwaiger et al., 2012). Due to the high prevalence of co-resistance to beta-lactam and fluoroquinolone antibiotics; we recommend the judicious practice of antimicrobial administration in the poultry setting with special emphasis on fluoroquinolones. We also recommend the continuous surveillance and monitoring of multidrug resistant isolates which should aid in proper antimicrobial administration in both humans and animals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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