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RESEARCH ARTICLE

Phenotypic and Molecular Analysis of Antibiotic Resistance in Lactobacilli of Poultry Origin from Lahore, Pakistan

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ABSTRACT

Acquired antibiotic resistance in lactobacilli poses a significant threat to public health. This study presents phenotypic and molecular analysis of antibiotic resistance in lactobacilli of poultry gut origin. A total of 105 *Lactobacillus spp.* isolates including 59 from indigenous and 46 from commercial poultry were recovered. Lactobacilli were identified by genus specific polymerase chain reaction (PCR) targeting 16SrDNA-23S rDNA inter-spacer region. Minimum inhibitory concentrations of different antibiotics were determined by broth micro dilution method. Antibiotic resistance genes for erythromycin [*erm*(B)] and tetracycline [*tet*(M)] were amplified by PCR and sequenced for homology analysis. Antibiotic resistance against cephradine (67.39% vs 55.93%), cefuroxime (100% vs 77.96%), erythromycin (86.95% vs 38.98%), tetracycline (41.30% vs 32.2%), ciprofloxacin (91.30% vs 84.74%), levofloxacin (97.82% vs 81.35%) and ofloxacin (95.65% vs 88.13%) were comparatively higher in lactobacilli of commercial poultry origin as compared to indigenous poultry. Resistance against ampicillin was slightly higher in back yard (49.15%) as compared to commercial poultry (43.47%). Overall, a high level resistance against cefuroxime (87.61%), levofloxacin (88.57%), ofloxacin (91.42%), ciprofloxacin (87.61%), and moderate level resistance against cephradine (60.95%), ampicillin (46.66%), tetracycline (36.19%) and erythromycin (60%) was observed. Erythromycin and tetracycline resistant genes [*erm* (B), and *tet*(M), respectively] were successfully amplified from phenotypically resistant lactobacilli. Sequencing analysis revealed that *erm*(B) gene had >99% similarity with *erm*(B) gene of *Enterococcus faecium* while *tet*(M) had >99% similarity with *tet*(M) of *E. coli*. It is concluded that lactobacilli of poultry gut origin contain acquired antibiotic resistance and its transmission to other bacterial strains is a significant threat to public health.

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INTRODUCTION

Vaccines and antibiotics are generally used to control infectious diseases (Park *et al.*, 2016). Prophylactic use of antibiotics is quite common in many countries including Pakistan (Asghar *et al.*, 2016; Carrique-Mas *et al.*, 2015; Saeed *et al.*, 2017). It leads to the emergence of antibiotic resistant microorganisms including pathogens and commensals (Magouras *et al.*, 2017). The antibiotic resistance is becoming an alarming issue across the world (Saeed *et al.*, 2017).

Normal microbiota of poultry gut includes Lactic Acid Bacteria, *E. coli*, Clostridia and Campylobacter. Lactobacilli are generally one of dominant group of microbes in poultry gut (Lu *et al.*, 2003). Lactobacilli are gram positive, catalase negative bacilli comprising of more than 150 species. Dominant lactobacilli in poultry gut include *L. salivarius, L. reuteri, L. gallinarum, L. crispatus and L. acidophilus* etc. (Hilmi *et al.*, 2007). Many strains of lactobacilli are also used as poultry probiotics which provide various benefits i.e increased feed conversion ratio, better immune response and

mycotoxins binding (Park *et al.*, 2016). Being a permanent microbiota of poultry gut, lactobacilli are highly exposed to antibiotics. Exposure to antibiotics may lead to development of antibiotic resistance in lactobacilli. Lactobacilli harboring acquired antibiotic resistance can transfer this resistance not only to other commensals in gut but also to pathogenic bacteria (Gueimonde *et al.*, 2013; Nawaz *et al.*, 2011). This problem is further compounded by the fact that lactobacilli may enter in human food chain and ultimately human gut and become a reservoir of antibiotic resistance genes (Gueimonde *et al.*, 2013). Transfer of antibiotic resistant determinant genes between commensal microbiota of gut and pathogens is a widely occurring phenomenon (Huddleston, 2014; van Schaik, 2015).

Development of antibiotic resistance can be controlled by rationale use of antibiotics in clinical and veterinary setups. Many countries have already taken initiatives and prohibited the prophylactic use of antibiotics especially those which are also used in human clinical setup (Magouras *et al.*, 2017; Prestinaci *et al.*, 2015). Antibiotic resistance in commensals especially lactobacilli of poultry origin has rarely been described in Pakistan. Therefore, present study was designed to analyse the antibiogram of lactobacilli of poultry origin, to determine responsible antibiotic resistance genes and to explore the role of lactobacilli in antibiotic resistance transmission.

MATERIALS AND METHODS

Isolation of lactic acid bacteria: A total of 60 samples of poultry droppings, caeca and cloaca were collected from commercial broiler (n=30) and indigenous poultry birds (n=30) from Lahore, Pakistan. Lactic acid bacteria were isolated by plating 10 fold serially diluted samples on deMan Rogosa and Sharp**e** (MRS) media (pH, 5.5). MRS plates were incubated at 35±2.5ºC for 48 hours in anaerobic conditions. After incubation, distinguished colonies, presumptive of lactobacilli, were selected from MRS plates, purified and stored in MRS broth supplemented with 20%(v/v) glycerol at -20ºC until further analysis.

Identification of lactobacilli: Lactobacilli were presumptively identified to genus level by their morphology on MRS media, Gram's staining and catalase test. DNAs of lactobacilli were extracted by TIANGEN bacterial DNA extraction kit (Tiangen Biotech, China) following the instructions of manufacturer. Lactobacilli were identified by polymerase chain reaction(PCR) targeting 16S rDNA-16S/23S rDNA interspace region by primers (XB5 and LbLMA1-R, Table 1), specific for Lactobacillus *spp.*, as described previously (Asghar *et al.*, 2016). Amplicons (~250 bp) were separated on Agarose gel electrophoresis (1.5%), stained by ethidium bromide and visualized in UV trans-illuminater.

Antibiotic susceptibility testing: Minimum inhibitory concentrations (MICs) of ampicillin, cephradine, cefuroxime, erythromycin, tetracycline, ofloxacin, ciprofloxacin and levofloxacin against lactobacilli (n=105) were determined by broth micro dilution method in 96 well microtiter plates as described previously (Nawaz *et al.*, 2011). Two-fold dilutions of antibiotics (0.25µg/ml-128 µg/ml) were prepared in MRS broth using microtiter plate wells. Doubly diluted antibiotics (50 µl) were inoculated with lactobacilli (100 µl, ~3 ×10^4 CFU). Inoculums of lactobacilli (~1 McFarland) were prepared by suspending their fresh growth in normal saline followed by dilution (1/10^3) in MRS broth. Microtitre plates were incubated at 37ºC for 24 hours in anaerobic conditions followed by observation of visible growth or turbidity. Minimum concentrations of antibiotic inhibiting the visible growth of isolates were considered as MICs. Isolates were designated as resistant, intermediate and sensitive to ampicillin, tetracycline and erythromycin following the breakpoints given by European food safety authority (EFSA, 2012). Breakpoints for cephradine, cefuroxime, ciprofloxacin, levofloxacin, and ofloxacin were adopted from Clinical and Laboratory Standards Institute. MIC50 and MIC90 were also determined as concentration of antibiotics showing total inhibition of 50% and 90% of isolates, respectively.

Molecular characterization of antibiotic resistance in lactobacilli: Antibiotic resistance genes for erythromycin *erm*(B) and tetracycline *tet*(M) were amplified by PCR using specific primers (Table 1) as described previously (Nawaz *et al.*, 2011). Briefly, 25 µl PCR reaction mixtures contained 50 ng DNA, 10 pmol of each primer and 12.5µl of Accu primeTM super Mix II. Amplifications were carried out using following program: initial denaturation at 95ºC for 5 min; followed by 40 cycles of final denaturation at 95ºC for 1 min, annealing at 50 ºC for *erm*(B) and 55 ºC for *tet*(M) for 1 min, initial extension at 72ºC for 1 min, and a final extension at 72ºC for 7 min. Amplicons were resolved by 1.5% (w/v) agarose gel electrophoresis, stained by ethidium bromide (0.5 µg/ml) and visualized under UV trans-illumination. Amplicons were purified and sequenced by 1st Base DNA Sequencing Service. Sequences were analysed by Chrome lite (free version) and their homology with other sequenced *erm*(B) and *tet*(M) genes was determined by NCBI (BLAST). Sequences were submitted to NCBI GenBank to obtain accession numbers.

RESULTS

Isolation and identification of lactobacilli: Samples of poultry gastrointestinal origin (n=60) were processed for the isolation of lactic acid bacteria on MRS agar. Lactic acid bacteria produced cream colored colonies of about 1-2 mm in diameter. Staining revealed that isolates were Gram positive bacilli or coccobacilli in different arrangements. Gram positive, catalase negative rods were considered *Lactobacillus.* All other isolates were removed from further analysis. Total number of lactic acid bacteria and *Lactobacillus spp*. Isolated from indigenous and commercial poultry are given in Table 2. Out of 240 isolates, 105 were identified as lactobacilli and confirmed by PCR using genus specific primers (XB5-F and LbLMA1-R) as mentioned in Table 1. All lactobacilli (n=105) revealed ~250 bp *Lactobacillus* genus specific amplicon. Out of 105 lactobacilli, 59 were from indigenous poultry and 46 from commercial poultry.

Table 1: Primers used in polymerase chain reaction

Target genes	Primer sequence (5′ → 3′)	T_a°C	Size (bp)
16S/23S spacer	XB5-F :GCCTTGTACACACCGCCCGT LbLMA1R:CTCAAAACTAAACAAAGTTTC	55	250
erm(B)	F:GAAAAGRTACTCAACCAAATA R:AGTAACGGTACTTAAATTGTTTAC	50	642
tet(M)	F:GTTAAATAGTGTTCTTGGAG R:CTAAGATATGGCTCTAACAA	55	576

bp: Base pairs; T_a: Annealing temperature of primers.

Table 2: Lactic acid bacteria isolated from poultry

Sample	Isolates from Indigenous poultry		Isolates from commercial poultry	
	LAB	*Lactobacillus spp.*	LAB	*Lactobacillus spp.*
Droppings	63	28	34	13
Caeca	40	17	53	23
cloaca	26	14	24	10
Total	129	59	111	46

LAB: lactic acid bacteria.

Antibiotic susceptibility pattern of *Lactobacillus spp*.: Minimum inhibitory concentrations (MICs) of different antibiotics against *Lactobacillus spp.* are presented in Table 3. Antibiotic resistance against cephradine (67.39% vs 55.93%), cefuroxime (100% vs 77.96%), erythromycin (86.95% vs 38.98%), tetracycline (41.30% vs 32.2%), ciprofloxacin (91.30% vs 84.74%), levofloxacin (97.82% vs 81.35%) and ofloxacin (95.65% vs 88.13%) were comparatively (statistically non-significant, P>0.05) higher in commercial poultry. Resistance against ampicillin was slightly higher in indigenous poultry (49.15%) as compared to commercial poultry (43.47%). Overall, a high level of resistance against cefuroxime (87.61%), levofloxacin (88.57%), ofloxacin (91.42%), ciprofloxacin (87.61%), and moderate level resistance against cephradine (60.95%), ampicillin (46.66%), tetracycline (36.19%) and erythromycin (60%) was observed. MIC 50 and MIC 90 of all antibiotics against lactobacilli are presented in Table 4 and Table 5 respectively. MIC50s of ampicillin, cefuroxime, erythromycin, tetracycline, ciprofloxacin, levofloxacin, ofloxacin of indigenous poultry were 2-4 fold higher (2, 64, 16, 16, 32, 32, 128 µg/ml, respectively) as compared to lactobacilli isolated from commercial poultry (4, 128, 16, 32, 64, 128, >128 µg/ml, respectively). MIC90 of lactobacilli isolated from indigenous and commercial poultry were similar. Overall, MIC90 of ampicillin, cephradine, cefuroxime, erythromycin, tetracycline, ciprofloxacin, levofloxacin and ofloxacin against lactobacilli of poultry origin were 8, 32, >128, 32, 64, 128, >128, >128 µg/ml, respectively.

Molecular characterization of antibiotic resistance in lactobacilli: Selected isolates, highly resistant to erythromycin (MIC >32) and tetracycline (MIC >64) were subjected to amplification of *erm*(B) and *tet*(M) genes, respectively. All selected erythromycin resistant lactobacilli (n=08) had *erm*(B) while tetracycline resistant lactobacilli (n=08) had *tet*(M) gene. *Erm*(B) gene of PDL40 and *tet*(M) gene of PDL28 were sequenced and their sequences were deposited in NCBI GenBank. GenBank Accession numbers of *erm*(B) of PDL40 and *tet*(M) of PDL28 are MF983811 and MF983812, respectively. Sequence similarity analysis through BLAST revealed that *erm*(B) gene of PDL40 had >99% similarity with *erm*(B) gene of *Enterococcus faecium* while *tet*(M) gene of PDL28 had >99% similarity with *tet*(M) gene of *E. coli*.

DISCUSSION

Antibiotic resistance is a great public health concern from last few decades (Prestinaci *et al.*, 2015). It is estimated that by 2050, antibiotic resistance may cause 10 million deaths annually. It is dire need of time to adopt multipronged strategy to secure the future of human race (Jasovský *et al.*, 2016). Development of new vaccines, antimicrobials, antibiotic resistance reversal, rationale use of antibiotics and broader understanding of emergence of antibiotic resistance may provide hope (Allen *et al.*, 2014). Therefore, identification of new microbial niches and antibiotic resistance reservoirs is absolutely necessary. In present study, a total of 240 lactic acid bacteria of poultry origin were isolated. Out of total 240 lactic acid bacteria, 105 isolates were identified as lactobacilli which confirmed the dominance of lactobacilli in poultry gut microbiota (Lu *et al.*, 2003). Many previous studies have also isolated lactobacilli of poultry origin (Asghar *et al.*, 2016; Rocha *et al.*, 2014). In present study, lactobacilli were isolated from commercial as well as indigenous back-yard poultry. A slightly higher number of lactobacilli were recovered from indigenous poultry (n=59) as compared to commercial poultry (n=46). It has been demonstrated that organically reared backyard poultry contain higher gut microbial diversity (Park *et al.*, 2013).

Antibiotic resistance is generally species and strain specific in lactobacilli. Breakpoints provided by EFSA or CLSI were adopted for interpretation of antibiotic susceptibility testing of lactobacilli in this study (EFSA, 2012). Antibiotic resistance can be acquired or intrinsic. Acquired antibiotic resistance is transferable and intrinsic resistance is not transferable to other bacteria. Resistance against penicillins, cephalosporins, macrolides and tetracycline is generally acquired in lactobacilli (EFSA, 2008; Nawaz *et al.*, 2011). In present study, lactobacilli were moderately resistant to ampicillin (49/105, 46.66%). Lower level of ampicillin or penicillin resistance has been detected in lactobacilli of different origins i.e fermented foods (Nawaz *et al.*, 2011), human gut (Klare *et al.*, 2007), chicken meat (Shazali *et al.*, 2014) and poultry gut (Dec *et al.*, 2017; Shazali *et al.*, 2014). Our results are in agreement with a previous study (Dec *et al.*, 2017), which declared moderately higher level of ampicillin resistance in lactobacilli. Resistance to ampicillin is mainly encoded by beta-lactamases which breaks the beta-lactam ring of ampicillin (Tenover, 2006). Cephradine and cefuroxime resistance in lactobacilli is also considered as acquired. Most of the isolates in this study were resistant to cephradine (64/105, 60.95%) and cefuroxime (92/105, 87.61). Furthermore, higher resistance to cephalosporins in isolates of commercial poultry origin indicates higher selective pressure of these antibiotics in commercial poultry gut. Similar results regarding resistance to cephalosporins have been reported previously as well (Nawaz *et al.*, 2011). Resistance of lactobacilli to cephalosporins can also be of intrinsic nature with unknown mechanisms (Sharma *et al*., 2018).

Table 3: Antibiotic resistance pattern of *Lactobacillus spp.* against selected antibiotics

Origin of lactobacilli		N	Antibiotic resistance pattern of *Lactobacillus spp.*							
			AMP n (%)	CED n (%)	CXM n (%)	ERY n (%)	TET n (%)	CIP n (%)	LVX n (%)	OFL n (%)
Indigenous poultry	Droppings	28	14(50)	21(75)	28(100)	13(46.42)	9(32.14)	27(96.42)	25(89.28)	27(96.42)
	Caeca	17	6(35.29)	5(29.41)	16(94.1)	1(5.88)	5(29.41)	11(64.70)	12(70.58)	13(76.47)
	Cloaca	14	9(64.28)	7(50)	2(14.28)	9(64.28)	5(35.71)	12(85.71)	11(78.57)	12(85.71)
	Total	59	29(49.15)	33(55.93)	46(77.96)	23(38.98)	19(32.2)	50(84.74)	48(81.35)	52(88.13)
Commercial poultry	Droppings	13	6(46.15)	6(46.15)	13(100)	13(100)	4(30.76)	12(92.30)	12(92.30)	13(100)
	Caeca	23	7(30.43)	17(73.91)	23(100)	18(78.26)	12(52.17)	20(86.95)	23(100)	21(91.30)
	Cloaca	10	7(70)	8(80)	10(100)	9(90)	3(30)	10(100)	10(100)	10(100)
	Total	46	20(43.47)	31(67.39)	46(100)	40(86.95)	19(41.30)	42(91.30)	45(97.82)	44(95.65)
Grand Total		105	49(46.66)	64(60.95)	92(87.61)	63(60)	38(36.19)	92(87.61)	93(88.57)	96(91.42)
MIC breakpoint (µg/ml)			≥4[b]	≥64[b]	≥64[b]	≥1[b]	≥16[a]	≥4[a]	≥8[a]	≥8[a]

[a]Breakpoints adopted from Clinical and Laboratory Institute to designate an isolate as resistant; [b]Breakpoints (reference values) adopted from European Food Safety Authority to designate an isolate as resistant; N: Number of *Lactobacillus spp.* tested by micro dilution broth method, n: number of resistant isolates, AMP: Ampicillin, CED: Cephradine, CXM: Cefuroxime, ERY: Erythromycin, TET: Tetracycline, CIP: Ciprofloxacin, LVX: Levofloxacin, OFL: Ofloxacin.

Table 4: MIC50 of different antibiotics against *Lactobacillus spp.* isolated from poultry

Origin of lactobacilli		N	MIC50 (µg/mL) of different antibiotics against *Lactobacillus spp.*							
			AMP	CED	CXM	ERY	TET	CIP	LVX	OFL
Indigenous poultry	Droppings	28	2	16	>128	128	32	32	64	128
	Caeca	17	2	8	64	16	8	64	32	64
	Cloaca	14	4	16	64	16	32	128	64	128
	Total	59	2	16	64	16	16	32	32	128
Commercial poultry	Droppings	13	4	16	>128	32	32	128	128	>128
	Caeca	23	2	32	128	32	32	128	>128	>128
	Cloaca	10	8	32	128	32	32	64	128	>128
	Total	46	4	16	128	32	32	64	128	>128
Overall		105	4	16	128	16	32	64	128	>128

MIC 50= Minimum inhibitory concentration required to inhibit the growth of 50% of tested organisms. N = Number of *Lactobacillus spp.* tested by micro dilution broth method, AMP: Ampicillin, CED: Cephradine, CXM: Cefuroxime, ERY: Erythromycin, TET: Tetracycline, CIP: Ciprofloxacin, LVX: Levofloxacin, OFL: Ofloxacin.

Table 5: MIC90 of different antibiotics against *Lactobacillus spp.* isolated from poultry

Origin of lactobacilli		N	MIC90 (µg/mL) of different antibiotics against *Lactobacillus spp.*							
			AMP	CED	CXM	ERY	TET	CIP	LVX	OFL
Indigenous Poultry	Droppings	28	8	128	>128	>128	>128	>128	>128	>128
	Caeca	17	8	64	>128	32	64	>128	128	>128
	Cloaca	14	8	32	>128	32	64	>128	>128	>128
	Total	59	8	32	>128	32	64	128	128	>128
Commercial Poultry	Droppings	13	8	32	>128	32	64	>128	>128	>128
	Caeca	23	8	32	>128	32	64	128	>128	>128
	Cloaca	10	8	32	>128	32	128	>128	>128	>128
	Total	46	8	32	>128	32	64	128	>128	>128
Overall		105	8	32	>128	32	64	128	>128	>128

MIC 90= Minimum inhibitory concentration required to inhibit the growth of 90% of organisms. N = Number of *Lactobacillus spp.* tested by micro dilution broth method, AMP: Ampicillin, CED: Cephradine, CXM: Cefuroxime, ERY: Erythromycin, TET: Tetracycline, CIP: Ciprofloxacin, LVX: Levofloxacin, OFL: Ofloxacin.

Erythromycin and tetracycline are commonly used in poultry to treat and control mycoplasmosis, chlamydiosis and *Clostridium perfringens* (Dec *et al.*, 2017). Moderate level of erythromycin (63/105, 60%) and tetracycline resistance (38/105, 36.19%) reported in present study is in agreement with many of the previous studies (Dec *et al.*, 2017; Dec *et al.*, 2015; Egervärn *et al.*, 2009; Ishihara *et al.*, 2013; Karapetkov *et al.*, 2011; Lonkar *et al.*, 2005). Erythromycin resistance is generally encoded by *erm*(A), *erm*(B) and *erm*(C) while resistance to tetracycline by *tet*(M), *tet*(K), *tet*(S), *tet*(L) and *tet*(O) (Dec *et al.*, 2017; Dec *et al.*, 2015; Egervärn *et al.*, 2009). Resistance genes including *erm*(A), *erm*(B), *erm*(C), *tet*(M), *tet*(K), *tet*(S) and *tet*(O) have been detected from lactobacilli isolated from poultry gut, fermented foods and human gut in previous studies from different parts of the world (Carrique-Mas *et al.*, 2015; Dec *et al.*, 2017; Dec *et al.*, 2015; Ishihara *et al.*, 2013; Karapetkov *et al.*, 2011; Nawaz *et al.*, 2011; van Schaik, 2015). Although, *erm*(B) or *tet*(M) have also been successfully amplified from pathogenic bacteria (Farid *et al.*, 2015). To the best of author's knowledge, it is the first report of presence of transferable *erm*(B) and *tet*(M) genes in lactobacilli of poultry origin in Pakistan. Tetracycline resistance gene *tet*(M) can confer cross resistance to oxytetracycline, chlortetracycline, doxycycline and minocycline while erythromycin resistant gene *erm*(B) to macrolides, lincosamides and streptogramins (Dec *et al.*, 2017). Similarity (>99%) of *erm*(B) and *tet*(M) genes, sequenced in this study, with *erm*(B) gene of *Enterococcus faecium* and *tet*(M) of *E. coli* respectively, indicate their potential transferable nature. Transfer of *erm* (B) and *tet*(M) genes of lactobacilli to other genera including pathogens have also been reported previously (Nawaz *et al.*, 2011).

Conclusions: It is concluded that acquired antibiotic resistance is present in lactobacilli of indigenous as well as commercial poultry origin which may pose a threat to public safety. Presence of *tet*(M) and *erm*(B) genes insinuate for implementation of strict regulatory policies regarding prophylactic use of antibiotics in poultry.

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Authors contribution: MN, AG, and AJ conceived and designed study. NS, and IK executed the experiments. NS and MN 5 analyzed the data. MN, AM and RY prepared the Manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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