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Research Article

Detection of antibiotic resistance genes in *Lactobacillus* and its role in transferring these genes to *Salmonella*

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Abstract

Antibiotic resistance is a worldwide issue and becoming problematic due to extensive misuse of antibiotics. The present study was aimed to analyze the role of *Lactobacillus* in transmission of antibiotic resistance genes (*tet*M, *erm*B, *sul*2) to *Slamonella* and verification of these genes by quantitative polymerase chain reaction. A total of thirty fecal samples (15 were indigenous and 15 were broilers) were collected and genomic DNA was isolated. Both *Salmonella* and *Lactobacillus* were confirmed by polymerase chain reaction. The expression and quantification of antibiotic resistance genes was done by quantitative polymerase chain reaction. The results indicated that there was higher expression of antibiotic resistance genes in *Lactobacillus* isolated from broiler chicken than the isolates obtained from indigenous birds indicating *Lactobacillus* as a reservoir of antibiotic resistance genes. But the role in transferring these genes to *Salmonella* was found to be non-significant. In conclusion, the excessive use of animal growth promoters in poultry assists in acquisition of antibiotic resistance by *Lactobacillus* and contributes in spread of antibiotic resistant determinants.

Keywords: Antibiotics; Determinants; Quantitative polymerase chain reaction

Introduction

Salmonella enterica is a zoonotic pathogen and transfers from animal to humans with the consumption of animal products, contaminated meat, or other contaminated food products **[1]**. It has been reported that about 1,335 foodborne outbreaks and 36,490 foods related illnesses occur due to *Salmonella enterica* **[2]**. Tetracycline resistance in *Salmonella* is attributed due the efflux pump that expels out tetracycline from the cell **[3]**.Whereas resistance against sulfonamide in *Salmonella* has been recognized due to the presence of *sul* gene which states an insensitive type of dihydrofolatesynthetase**[4]**.

Antibiotic resistance is a global issue and because of this, bacteria are becoming more resistant against antibiotics by developing different mechanisms of antibiotic resistance like: enzymatic degradation, target modification and target substitution **[5]**. This could either be due to mutation or acquirement of a particular antibiotic

resistance (AR) gene by horizontal transfer [6]. The accessibility of antibiotics to be used for treatment of infectious diseases has considerably improved the human health and animal welfare. But massive misuse of antibiotics causes the development of antimicrobial resistance in commensals and pathogenic bacteria [7].

Lactobacillus species isolated from meat and fermented milk products have offered resistance to tetracycline, vancomycin and erythromycin [8]. In pathogenic or commensals strains, AR genes produce an indirect risk for animals and humans and as well as the enlargement of gene pool for different other pathogenic and exogenous bacteria to grab them to advance antibiotic resistance. There are multiple pathways of antibiotic resistance gene transfer including conjugation and transformation as well as transduction. Horizontal gene transfer (HGT) of antimicrobial resistance genes is significantly enhanced by plasmids, transposons and integrons [9]. The AR genes frequently exist on mobile elements like integrons, plasmids and transposons [10].

It has been reported that possible increase of resistance happened because of excessive use of antibiotics [11]. The incidence of AR genes; *erm*(B) and *tet*(M) for erythromycin and tetracycline respectively in *Lactobacilli* represents the most pervasive determinant of resistance. Moreover, in genetic linkage of *Lactobacillus paracasei*, both these genes were frequently stated [12]. The current study is intended for the evaluation of *Lactobacillus* role in transmitting AR genes to *Salmonella*.

Materials and methods

The following research was conducted in Institute of Microbiology University of agriculture, Faisalabad. A total of fifteen samples were taken from three indigenous poultry birds (5 samples from each chicken) and fifteen samples from three broiler birds (5 samples from each chicken). Total thirty fecal samples were taken from the colon region of birds for the isolation of *Lactobacillus* and *Salmonella*. Bead containing collection tubes were used for the collection of all the samples. FavorPrep Stool DNA Isolation Mini Kit was used for genomic DNA isolation following the protocol described [13].

Nano-quantification of extracted DNA

With the help of Nano drop, exact DNA concentration was assessed using 1000 spectrophotometer Thermo scientific®. For this objective, 1µL of freshly extracted DNA was taken and released on thepointed surface of sterilized and specialized Nano spectrophotometer, which provided the accurate concentration of DNA along with the260/280 and 260/230 values [14].

PCR Detection of *Lactobacillus* and *Salmonella*

Lactobacillus and *Salmonellae* were detected by PCR amplification of DNA samples using primers (**LAA;** F:CATCCAGTGCAAACCTAAGAG, R:GATCCGCTTGCCTTCGCA [15] & **InvA;** F:CGGTGGTTTTAAGCGTACTCTT, R:CGAATATGCTCCACAAGGTTA) [16] respectively by using 16 SrRNA micro processed controlled swift Maxi thermal cycler block (Esco technologies Inc. France). In negative control, no sample was loaded.The amplified products were then visualized on 1% agarose gel [17].

Quantitative Polymerase Chain Reaction (qPCR) for detection of *tet*M, *erm*B and *Sul*2 genes

The qPCR also called real time polymerase chain reaction (RT-PCR) analysis was used for the verification of the results that were found from the analysis of Microarray. For this purpose, steps described by [18] were followed. Briefly, genomic DNA up to 20ng/µL was required for each reaction and primers (Table 1) [19] were purchased from Thermo Fisher Scientific and were used by dilution at a ratio of 1:20 from a solution of 5µM. For each reaction, Master Mix of 5µL

Dynamo Flash and ROX up to0.25µLwas added into each valve. After that, 10µL of high performance liquid chromatography (HPLC) water was added and mixed reaction mixer was used for qPCR. The thermal cycler conditions and the reaction mixture's composition is given in (Table 2 & 3).

Expression of qPCR data

Absolute gene copy number was expressed as the no. of copies of resistant genes (reference florescent unit RFU) during each cycle. Absolute abundance was calculated based on the results obtained for the corresponding standard of each resistant gene. Relative abundance was calculated using $E = 10^{-1/slope}$. From this, the ratio of the relative expression of the targeted gene to reference gene (Rpl32) was calculated as follows; ΔCt = Ct target – Ct reference (ΔCt: change of the cycle threshold (Ct) that was calculated during the amplification).

Results and discussion

This study was aimed to detect antibiotic resistance genes in *Lactobacillus* and its role in transmission of these genes to *Salmonella*. The detection of *Lactobacillus* was done by presence of LAA gene consisting of 250 bp (Fig.1) while appearance of 796bp band yielded from Inv-A primer amplification confirmed the presence of *Salmonella* (Fig.2).

The qPCR expression of *tet*M demonstrated that the expression of *tet*M in *Lactobacillus* in case of broiler chicken sample was higher as compared to its expression in *Salmonella* isolated from indigenous chickens representing the role of *Lactobacillus* as a reservoir of *tet*M gene. The qPCR results are also indicating the higher expression of *tet*M gene in broiler as compared to indigenous chickens which may be due to the excessive use of antibiotic growth promoters in broiler chickens (Fig. 3 & Table 4). The results of qPCR for *erm*B gene showed the expression of this gene in *Lactobacillus* and *Salmonella* isolated from indigenous and broiler chicken samples indicating *Lactobacillus* as a reservoir for *erm*B gene. The transfer of these genes to *Salmonella* has been found to be non-significant due to low *erm*B gene expression in *Salmonella* isolated from broiler and indigenous chickens (Fig. 4 & Table 5). The *sul*2results revealed its expression in *Lactobacillus* and *salmonella* in indigenous as well as in broiler chickens. The high expression of *sul*2 gene in *Lactobacillus* of broiler chickens indicates that *Lactobacillus* in broiler chickens serves as a reservoir for *sul2* gene (Fig. 5 & Table 6). The analysis of Ct values for different AR genes in qPCR represents that *tet*M gene is showing the highest expression among the three antibiotic resistance genes. It was found that the most frequent antibiotic resistance gene was *tet*M followed by *erm*B and *sul*2. The qPCR results of present study exhibited that *Lactobacillus* isolated from broiler chickens serves as a reservoir of antibiotic resistance genes due to presence and higher expression level of AR genes (*tet*M, *sul*2 and *erm*B). The transmission of antibiotic resistance genes to *Salmonella* in present study was found to be non-significant because of the very low Ct values and low expression of antibiotic resistance genes in *Lactobacillus*isolated from indigenous chicken samples and in *Salmonella* isolated from both indigenous and broiler chicken specimens. Similar work was performed by **[20]** who detected tetracycline resistant gene (*tet*M) from *Lactobacillus* isolates. Other researchers **[21]** also worked on detection of antibiotic resistance pattern mediated by *tet*M and *tet*L genes in different *Enterococcus* bacteria. Another study **[22]** investigated susceptibility of *Lactobacillus paracasei* to tetracycline and erythromycin and found that this bacterium may act as reservoir for transferring antibiotic resistance genes to other pathogenic bacteria. In another report **[23]**, *erm*B and *tet*M genes were detected from different lactic acid bacterial isolates of

fermented foods. The study done by [24, 25] closely match to our present study who investigated the role of *Lactobacillus* species in transferring *tet*M and *erm*B genes to other *Enterococcus* species. The results of the present research also closely relate to the previous research done by [26] who investigated the role of antibiotic growth promoters in the acquisition of antibiotic resistance genes in commensals and pathogenic bacteria to develop antibiotic resistance.

Table 1. Primers for Erythromycin *erm*(*B*), Sulfonamide (*sul2*), Tetracycline *tet*(M) antibiotic resistance genes

Genes	PrimersSequences (5'-3')
ermB	F: TGGTATTCCAAATGCGTAATG R: CTGTGGTATGGCGGGTAAGT (Zhang *et al.*, 2011)
sul2	F: GCAGGCGCGTAAGCTGA R: GGCTCGTGTGTGCGGATG (Zhang *et al.*, 2011)
***tet* M**	F:CGAACAAGAGGAAAGCATAAG R: CAATACAATAGGAGCAAGC (Zhang *et al.*, 2011)

Table 2. PCR Components and their concentration

S. No.	Component	Quantity	Concentration
1	MgCl2	1.5µL	5mM
2	10x buffer	3µL	…
3	Primer R	1µL	10pmol
4	Primer F	1µL	10pmol
5	RNA-free water	14.3 µL	…
6	dNTPS	1 µL	200 µM
7	Template DNA	3µL	50ng
8	Taq polymerase	0.2 µL	5 U
	Total	25µL	

Table 3. Thermo-cycler conditions for DNA AmplificationCycling 34 repeats

Initial denaturation	94°C	5 min
Denaturation	94°C	60 sec
Annealing	58°C	30 sec
Extension	72°C	2 min 30 sec
Final extension	72°C	5 min

Table 4. Ct values of *tet*M gene in *Lactobacillus, Salmonella*(isolated from both Indigenous and Broiler samples)

	Indigenous	Broiler
Lactobacillus	0.0315	5.16
Salmonella	0.00385	0.0039

Table 5. Ct values of *erm*B gene in *Lactobacillus*, *Salmonella* (isolated from both Indigenous and Broiler samples

	Indigenous	Broiler
Lactobacillus	0.0155	4.18
Salmonella	0.00495	0.0083

Table 6. Ct values of *sul*2 gene in *Lactobacillus*, *Salmonella* (isolated from both Indigenous and Broiler samples)

	Indigenous	Broiler
Lactobacillus	0.0265	3.06
Salmonella	0.00155	0.006

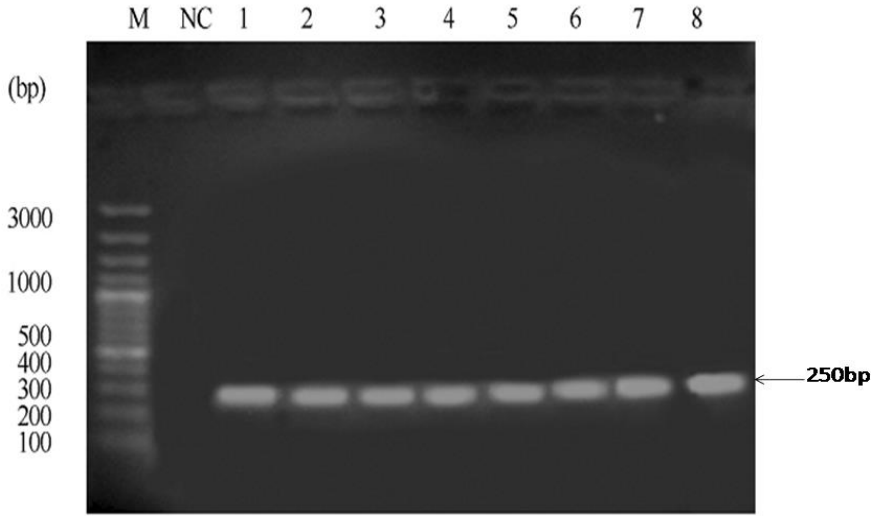

Figure 1. PCR analysis of *Lactobacillus*. Lane M: DNA marker of 3000 bp, Lane NC: negative control, lanes 1-8 indicate the bands for LAA gene (250 bp)

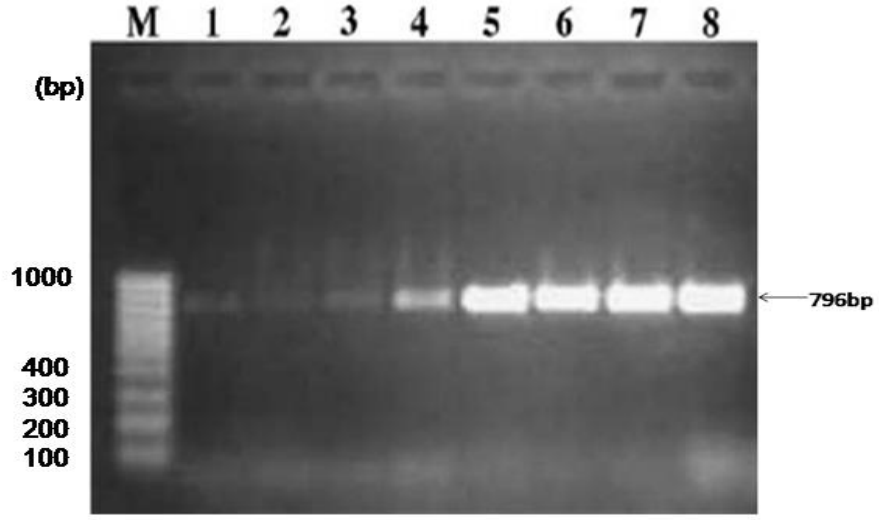

Figure 2. PCR analysis of *Salmonella*. Lane M: marker of 1kb, Lane 1: positive sample, lanes 2: negative control, 3-8 indicate samples showing bands for InvA gene (796 bp) of *Salmonella*

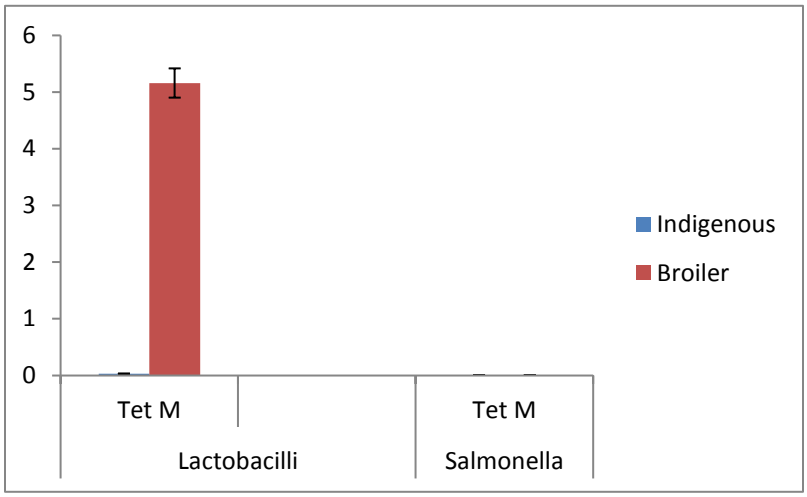

Figure 3.*tet*M gene expression level in indigenous and broiler samples

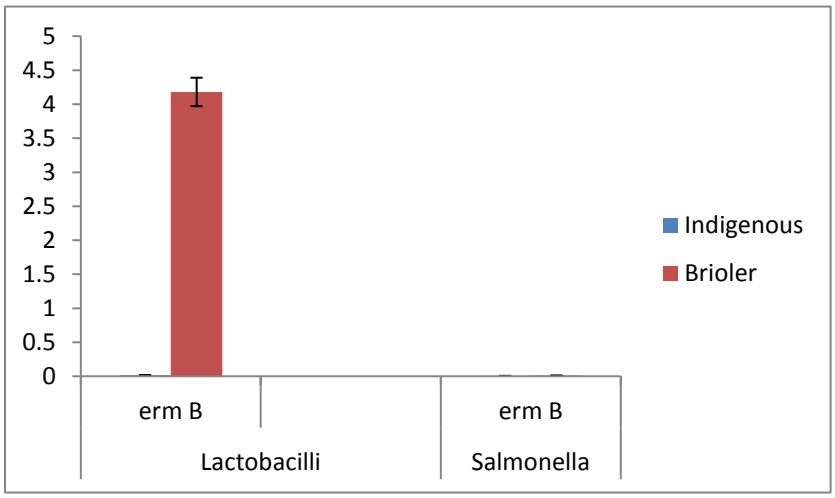

Figure 4.*erm*B gene expression level in indigenous and broiler samples

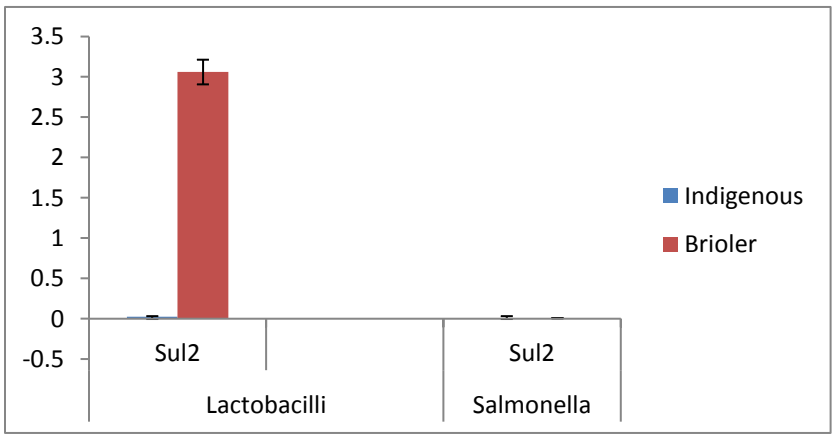

Figure 5. *Sul*2 gene expression level in indigenous and broiler samples

Conclusion

It is concluded that the excessive use of animal growth promoters in poultry assists in acquisition of antibiotic resistance genes by normal micro-biota and hence, development of antibiotic resistance in these strains. The study may assist to offer the future aspects to discover the potential role of horizontal transmission of antibiotic resistance genes from *Lactobacillus* to other pathogenic bacteria.

Authors'contributions

Conceived and designed the experiments: M Ashraf & R Aslam, Performed the experiments: M Ashraf, Analyzed the data: S Ali, Contributed reagents/ materials/ analysis tools: S Ali, Wrote the paper: S Anam & FR Anjum.

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