Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Serotypes, Genotypes, Virulence Factors and Antimicrobial Resistance Genes of *Escherichia coli* Isolated in Bovine Clinical Mastitis from Eastern China

Javed Memon[1], Jam Kashif[2], Nazir Hussain[2], Muhammad Yaqoob[2], Asghar Ali[2], Rehana Buriro[2], Jamila Soomro[2], Mohammad Farooque Hassan[3], Benazir Sahito[2] and Fan Hongjie[1]*

[1]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, P.R. China; [2]Sindh Agricultural University, Tandojam, Pakistan; [3]Shaheed Benazir Bhutto University of Veterinary and Animal Sciences, Sakrand, Sindh, Pakistan
*Corresponding author: fhj@njau.edu.cn

ARTICLE HISTORY (15-490)

Received: November 28, 2015
Revised: June 22, 2016
Accepted: June 26, 2016
Published online: August 23, 2016

Key words:
E. coli
Genotypes
Mastitis
Resistance genes
Serotypes
Virulence

ABSTRACT

Coliform mastitis is still significantly problematic disease to treat and control in practice, which is testament to the intricacy and mutability of the condition. We examined 103 mastitis *E. coli* isolates collected from 22 dairy farms in eastern China, for their serotypes and prevalence of virulence and antimicrobial resistance genes. Of the sixteen serotypes characterized through serum agglutination test, O39 followed by O92 and O123 were the most predominant serovars. The genotyping of the isolates was also determined by using ERIC-PCR. Phylogenetic analysis of DNA fingerprints was performed by using SPSS data editor, which yielded ten distinct genotypes (A-J). 14 virulence genes and 10 antimicrobial resistance genes were checked in all 103 isolates by PCR assay. Most prevalent virulence genes were, *TraT*, *FimH*, *papC*, *iucD*, *F4 (K88*) and *sfa*; but *F17A*, *F41*, *stx1*, *intimin*, *CNF1*, *CNF2*, *LT* and *ST* were not present in any isolate. Among all investigated resistance genes, 48% isolates carried *CTX-M* and *qnrS*. In addition, *tetA*, *tetB*, *sul1*, *sul2* were also found in high frequency. Statistical analysis revealed an unconditional association between virulence and resistance genes as the $P<0.05$. To our knowledge, this is most updated report on serotypes, genotypes, prevalence of resistance and virulence genes, and their significant association with each other in mastitis *E. coli* isolates from eastern China.

To Cite This Article: Memon J, Kashif J, Hussain N, Yaqoob M, Ali A, Buriro R, Soomro J, Hassan MF, Sahito B and Hongjie F, 2016. Serotypes, genotypes, virulence factors and antimicrobial resistance genes of *Escherichia coli* isolated in bovine clinical mastitis from Eastern China. Pak Vet J, 36(4): 493-498.

INTRODUCTION

Mastitis *E. coli* is environment-originated opportunistic pathogen and considered as a common cause of bovine intra-mammary infection (IMI) subsequent of *Staphylococcus aureus* (Khan *et al*., 2013; Kempf *et al*., 2015). Pathogenesis of *E. coli* infection depends on some specific virulence factors that enhance their capability to colonize under some favorable condition, so far no specific virulence factor has been shown to cause coliform mastitis (Wenz *et al.*, 2006; Blum and Leitner, 2013), however many studies have been carried out to detect the virulence genes in mastitis *E. coli* isolates (Suojala *et al.*, 2011; Liu *et al*., 2014). The frequency of coliform mastitis is increasing among the countries, but very little is known about the gentic distribution and serotypes of *E. coli* isolates involved in bovine mastitis infection particularly in China. Serotyping and genotyping have substaintial importance in distinguishing the pathogenic and nonpathogenic bacteria, because particular serogroups are constantly linked to certain clinical disorders (Kaper *et al.*, 2004), so far link between *E. coli* serotypes and mastitis has not been well established, however pathogenic *E. coli* has been frequently isolated in clinical mastitis cases (Wenz *et al.*, 2006). While genotyping helps in better understanding of clonal differences among the bacterial isolates from different regions on the basis of their genetic determinants mainly virulence and resistance genes (Kaper *et al.*, 2004; Bandyopadhyay *et al.*, 2012). *E. coli* has ability to develop the resistance against frequently used antimicrobials, which is one of the main reasons for treatment failure of coliform mastitis (Suojala *et al.*, 2011). Accurate information about antimicrobial resistance and presence of resistance and virulence determinants is necessary for effective treatment, though studies have shown unconditional association between resistance and virulence genes of *E. coli* isolates (Aslam *et al.*, 2012), which is likely due to their dissemination within strains through mobile genetic elements, especially plasmid (Yaqoob *et al.*, 2013; Javed *et al*., 2015).

In China, particularly in eastern region, no recent epidemiological study focused on the prevalence of bovine coliform mastitis despite of increasing incidences worldwide. Therefore, the present study was carried out with objects: 1) to investigate the serotypes of *E. coli* strains characterized in mastitic milk, their genotypes to understand the biological relationship among isolates and 2) And probe for presence of virulence and antimicrobial resistance determinants for prognosis in effective treatment of coliform mastitis particularly in this region.

MATERIALS AND METHODS

***E. coli* Isolates:** *E. coli* isolates (n=103) probed in this study, which were identified from 299 bovine clinical mastitis milk samples through a chain of laboratory techniques (Memon *et al.*, 2013). Briefly, bacterial culture of isolated pathogens was subjected to Gram stain, biochemical tests and specific media for *E. coli* identification (Chrom agar). The identified *E. coli* isolates were further verified through (16S rDNA) gene amplification assay and sequenced (Memon *et al.*, 2013).

Serotyping: *E. coli* isolates were retrieved from microorganism storage system and cultured overnight in Luria-Bertani broth at 37°C. The isolates were characterized for their serotypes through slide agglutination assay, for which 181"O" antigens and H7 antisera were purchased from China Institute of Veterinary Drugs Control, Beijing. The flagella antigen H7 was tested only in isolates belonging to O157 serovar. The serotyping was performed according to the previously described procedure (Schroeder *et al.*, 2002).

Detection of Virulence genes (VGs): Simple PCR was carried out to amplify the virulence and resistance genes in 103 isolates. Fourteen virulence genes were selected, which were previously reported for *E. coli* strains identified in mastitic milk including *F41*, *F17A*, *ST*, *LT* and *F4 (ETEC)*, *sfa*, *Trat* and *FimH* (ExPEC), *CNF1*, *CNF2*, *papC* and *iucD* (UPEC), *stx* and intimin (*eaeA;* STEC) (Wenz *et al.*, 2006; Suojala *et al.*, 2011). The examined virulence genes their fragment sizes and annealing temperatures are listed in Table1.

Table 1: Oligonucleotide sequences, amplicons size, annealing temperature, accession numbers and references of VGs and ARGs examined in mastitis *E. coli* (103) isolates.

Virulence and resistance genes	Oligonucleotide sequences	Size in bp	Annealing Temp	Accession numbers	Reference
F41 F	GCATCAGCGGCAGTATCT	380	52		Bandyopadhyay *et al.* (2012)
F41 R	GTC CCTAGCTCAGTATTATCACCT				
FimH F	GATCTTTCGACGCAAATC	389	52	JN408573.1	Moulin-Schouleur *et al.* (2006)
FimH R	CGAGCAGAAACATCGCAG				
Intimin F	ATATCCGTTTTAATGGCTATCT	425	55		Paton and Paton (1998)
Intimin R	AATCTTCTGCGTACTGTGTTCA				
stx1 F	ATAAATCGCCATTCGTTGACTAC	180	55		Paton and Paton (1998)
stx1 R	AGAACGCCCACTGAGATCATC				
sfa1 F	CTCCGGAGAACGGGTGCATCTTAC	410	52		Van Bost *et al.* (2003)
sfa2 R	CGGAGGAGTAATTACAAACCTGGCA				
f17A F	GCAGAAAATTCAATTTATCCTTGG	537	52		Van Bost *et al.* (2003)
f17A R	CTGATAAGCGATGGTGTAATTAAC				
CNF1 F	GGCGACAAATGCAGTATTGCTTGG	552	52		Pass *et al.* (2000)
CNF1 R	GACGTTGGTTGCGGTAATTTTGGG				
CNF2 F	ACTGAAGAAGAAGCGTGGAATA	654	52		Kaipainen*et al.* (2002)
CNF2 R	ATAAGTTGAGCCGAGCGAGG				
TraT F	GATGGCTGAACCGTGGTTATG	307	55	X14566.1	Kaipainen*et al.*(2002)
TraT R	CACACGGGTCTGGTATTTATGC				
*iucD*F	AAGTGTCGATTTTATTGGTGTA	778	60	AY230263.1	Ewers *et al.* (2005)
iucD R	CCATCCGATGTCAGTTTTCTG				
papC F	GACGGCTGTACTGCAGGGTGTGGCG	328	60	DQ010312.1	Ewers *et al.* (2005)
papC R	ATATCCTTTCTGCAGGGATGCAAT A				
LT F	TTACGGCGTTACTATCCTCTCTA	275	52		Bandyopadhyay *et al.* (2012)
LT R	GGTCTCGGTCAGATATGTGATTC				
ST F	TCCCCTCTTTTAGTCAGTCAACTG	163	52		Bandyopadhyay *et al.* (2012)
ST R	GCACAGGCAGGATTACAACAAAGT				
F4(K88) F	ATCGGTGGTAGTATCACTGC	601	52		Ojeniyi *et al.*(1994)
F4(K88) R	AACCTGCGACGTCAACAAGA				
blaSHV F	TT ATCTCCCTGTTAGCCACC	795	55		Yaqoob*et al.* (2012)
blaSHV R	GATTTGCTGATTTCGCTCGG				
tetA F	GGCGGTCTTCTTCATCATGC	502	64	FJ794040.1	Perreten and Boerlin (2003)
tetA R	CGGCAGGCAGAGCAAGTAGA				
tetB F	CATTAATAGGCGCATCGCTG	930	64	FJ917423.1	Perreten and Boerlin (2003)
tetB R	TGAAGGTCATCGATAGCAGG				
qnrA F	ATTTCTCACGCCAGGATTTG	516	53		Park *et al.* (2006)
qnrA R	GATCGGCAAAGGTTAGGTCA				
qnrB F	GATCGTGAAAGCCAGAAAGG	469	53		Park *et al.* (2006)
qnrB R	ACGATGCCTGGTAGTTGTCC				
qnrC F	GGGTTGTACATTTATTGAATC	447	50		Wang *et al.* (2009)
qnrC R	TCCACTTTACGAGGTTCT				
qnrS F	ACGACATTCGTCAACTGCAA	417	53	FR873842.1	Park *et al.* (2006)
qnrS R	TAAATTGGCACCCTGTAGGC				
Sul1 F	GTGACGGTGTTCGGCATTCT	779	68	JN596280.1	Perreten and Boerlin (2003)
Sul1 R	TCCGAGAAGGTGATTGCGCT				
Sul2 F	CGGCATCGTCAACATAACCT	721	66	JN012467.1	Perreten and Boerlin (2003)
Sul2 R	TGTGCGGATGAAGTCAGCTC				
CTX-M F	CGCTTTGCGATGTGCAG	550	55	JN794060.1	Yaqoob*et al.*(2000)
CTX-M R	ACCGCGATATCGTTGGT				

Detection of antimicrobial resistance genes (ARGs): Selection of resistance genes was based on the antimicrobial sensitivity profiles of *E. coli* isolates. Minimal inhibitory concentration (MIC) results showed the percent isolates resistant against betalactam (93 to 99%), cephalosporin (54 to 66%), fluoroquinolones (40 to 74%), oxytetracycline (91%) and 88% against sulfadiazine-trimethoprim (Memon *et al.,* 2013). The selected resistance genes, along with their amplicon sizes and annealing temperatures are listed in table-1. PCR products of representative genes were sent for sequencing to Invitrogen Corp. (Shanghai, China). The obtained gene sequences were blast with National Center for Biotechnology Information (NCBI, http://www. ncbi.nlm. nih.gov) and their accession numbers are listed in Table1.

DNA fingerprinting: All the *E. coli* strains were genotyped by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR. Genomic DNA extracted through commercially available DNA extraction kit (Geneaid Biotech, Taiwan). DNA template used to amplify ERIC sequences as per procedure described previously (Bandyopadhyay *et al.,* 2012).

ERIC-PCR generated fingerprints of *E. coli* strains were evaluated by Quantity-1 software and SPSS data editor was used for statistical analysis and plotting a dendrogram using Hierarchical Cluster Analysis method (average linkage between groups) to understand the genetic relationship between isolates.

Statistical analysis: The association between VGs and ARGs was assessed by Fisher's exact tests (Analytical Software, Tallahassee, FL, USA). The relationship between VGs and ARGs was deemed as significant at $P<0.05$.

RESULTS

Serotyping: Sixteen serotypes were characterized in 103 *E. coli* isolates and the most prevalent "O" antigen in this region was O39 (20 strains), followed by O92 (17 strains) and O123 (15 strains) as shown in Table 2.

Virulence genes (VGs): Of all the investigated virulence genes the most predominant was *TraT* gene followed by *FimH*, *papC*, *iucD*, *F4* and *sfa* (Fig. 3 & 4). All the isolates contained either one or maximum four virulence genes, totally nineteen different combinations of VGs found in our isolates (Fig. 1).

Antimicrobial resistance genes (ARGs): Of the ten examined antimicrobial resistance genes, six were present. Most prevalent resistance gene was *sul2* followed by *qnrS*, *CTX-M*, *tetA*, *sul1*, and *tetB* (Fig. 3 & 4). Totally, 26 combinations of ARGs were found in all 103 isolates, majority of isolates carried 3, 4 and 5 resistance genes in group (Fig.1).

Association between VGs and ARGs: The statistical analysis revealed the significant association between some virulence and resistance genes. Accordingly, *papC* showed a potential association with *sul2* and *qnrS* resistance genes and *FimH* was significantly associated with *qnrS*. However, *iucD* was found associated with *CTX-M*, *sul2* and *tetA* genes ($P<0.05$) (Table 3). Other virulence genes including *TraT*, *F4* and *sfa* were not found associated with any antimicrobial resistance genes.

DNA fingerprinting: DNA fingerprints based phylogenetic dendrogram yielded ten genotypes (A-J). ERIC-PCR based genotyping combined the genetically similar isolates in same genotype group which can be observed in dendrogram (Fig.1). VGs and ARGs profile are placed in front isolates for better understanding the similarity and/or difference between all genotypes (Fig.1).

DISCUSSION

E. coli is most common cause of bovine mastitis even in well-managed herds, thus causing considerable economic loss to dairy industry, worldwide. So far, very little is known about the *E. coli* serotypes involved in mastitis. In our study, we confirmed that in divergent geographical regions, various *E. coli* serotypes were involved in bovine mastitis. As we found sixteen serotypes in 103 isolates and the most common serovars were O39, O92 and O123 that were widely disseminated on investigated cattle farms of eastern China. Whereas, O146, O8 and O150K, O8K were reported as the most prevalent serovars in mastitis-infected cattle in USA and Netherlands, respectively (Wenz *et al.,* 2006).

Gram negative pathogens especially *E. coli* is prone to lateral gene transfer, therefore its virulence factors were well established and acquisition of virulence determinants offers evolutionary track to pathogenicity. In this study, the presence of virulence genes in almost all mastitis *E. coli* isolates was much higher than previously reported 40% in Finland and 37% in Iran (Ghanbarpour and Oswald, 2010; Suojala *et al.,* 2011). Several innate resistant VGs play role in IMI have been characterized, of which *TraT* gene has been reported in 31-40% of mastitis *E. coli* isolates in Finland (Kaipainen *et al.,*2002), which is much lower than 95% of our studied isolates. High frequency of fimbria *FimH* in 77% of isolates was similar with the findings of Dogan *et al.* (2006), whereasanotherstudy has shown its 100% prevalence in mastitis *E. coli* isolates (Fernandes *et al.,* 2011).

Table 2: Frequency and distribution of "O" antigen type identified in mastitis *E. coli* (103) isolates

Farms (Numbers)	O antigen type	Frequency	Percentage (%)	Prevalence (Farms No.)
Nanjing (I)	63, 112, 124, 158	2	1.9 each	O63 (VII), O112 (I), O124 (I), O158 (I)
Taizhou (II)	5, 157, 186	3	2.9 each	O5 (I), O186 (V), O157 on 2 farms (I & VII)
Xuzhou (III)	12, 111	4	3.9 each	O12 (VIII), O111 on 2 farms (X & XI)
Huainan (IV)	131	5	4.9 each	On 2 farms (II & III)
Lianyungang (V)	162	6	5.8	On 3farms (VII, VIII & X)
Weifang (VI)	50	7	6.8	On 3 farms (I, III & VI)
Zaozhuang (VII)	180	8	7.7	On 4 farms (I, IV, VI & XI)
Hangzhou (VIII)	123	15	14.6	On 4 farms (I, II, IV & IX)
Nanping (IX)	92	17	16.5	On 7 farms (I, VI & X)
Fengyang (X)	39	20	19.4	On 7 farms (I & VI)
Nanchang (XI)				

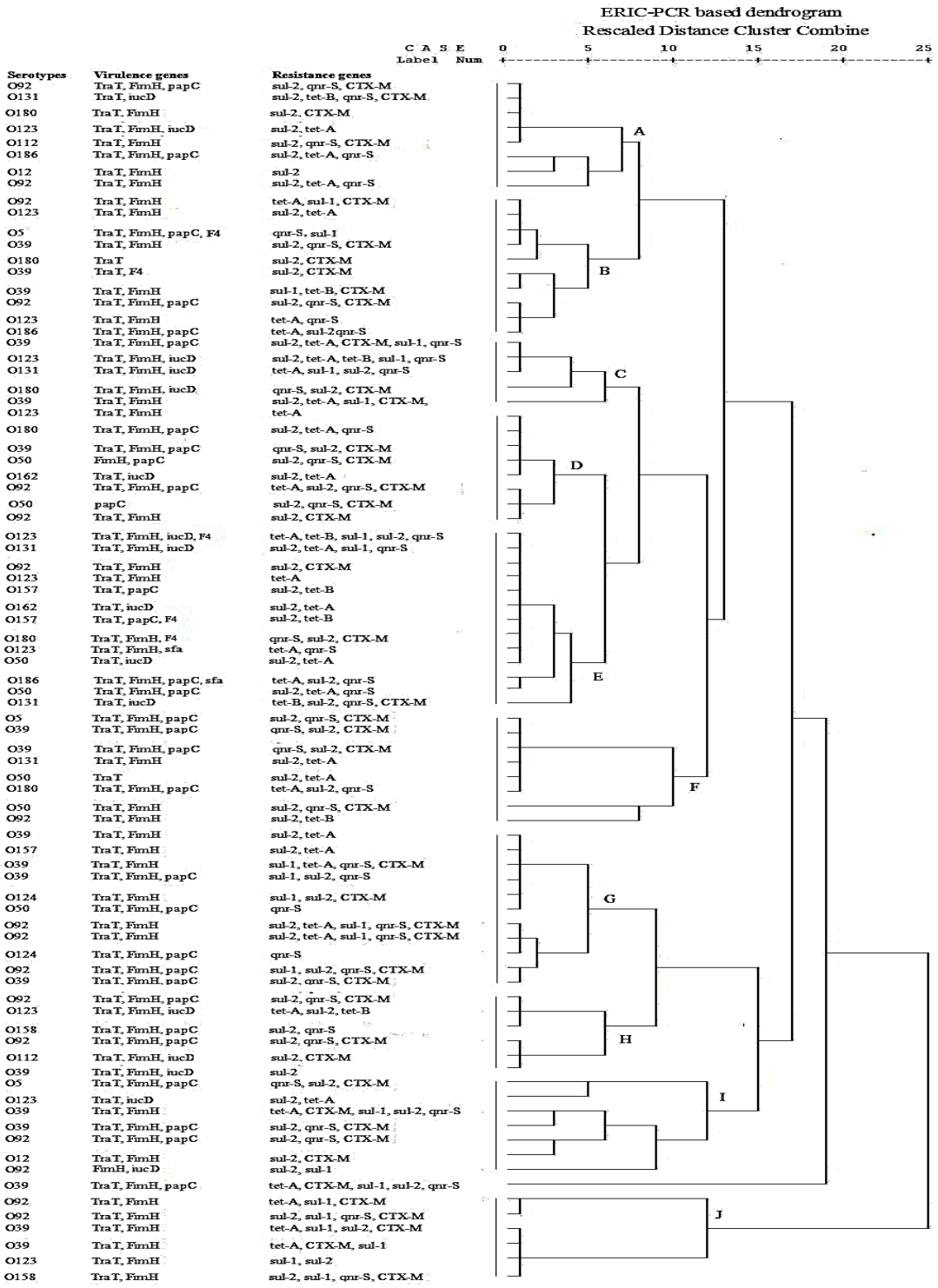


Fig. 1: ERIC-PCR based dendrogram: Percentages of similarity between profiles calculated using SPSS Data Editor and dendrogram generated using Hierarchical Cluster Analysis with cluster method (average linkage between groups). Serotypes, virulence and resistance genes are placed in front of their strain number.

Table 3: Association of virulence and resistance genes in mastitis *E. coli* strains

Virulence	*CTX-M*			*Sul1*			*Sul2*			*tetA*			*tetB*			*qnrS*		
Genes (present in strains)	+49	-54	P	+29	-74	P	+83	-20	P	+48	-55	P	+14	-89	P	+50	- 53	P
TraT (98)	46(94)	52(96)	0.111	29(100)	69(93)	0.120	79(95)	19(95)	0.141	48(100)	50(90)	0.106	12(86)	86(97)	0.159	47(94)	51(96)	0.111
FimH (79)	42(86)	37(68)	0.090	29(100)	50(68)	0.060	64(77)	15(75)	0.150	40(83)	39(71)	0.102	7(50)	72(81)	0.116	47(94)	32(60)	0.041*
papC (34)	20(41)	14(26)	0.083	7(24)	27(36)	0.127	32(38)	2(10)	0.038*	12(25)	22(40)	0.083	2(14)	32(36)	0.130	30(60)	4(7)	0.000*
iucD (18)	4(8)	14(26)	0.038*	5(17)	13(18)	0.223	18(22)	0(0)	0.041*	13(27)	5(9)	0.028*	5(36)	13(15)	0.085	6(12)	12(23)	0.102
F4(K88) (6)	4(8)	2(4)	0.222	0(0)	6(8)	NS	4(5)	2(10)	0.253	2(4)	4(7)	0.275	2(14)	4(4)	0.173	0(0)	6(11)	NS
sfa (3)	2(4)	1(2)	0.363	0(0)	3(4)	NS	2(2)	1(5)	0.388	0(0)	3(5)	NS	0(0)	3(3)	NS	3(6)	0(0)	0.245

The association between VGs and ARGs was considered as significant when *P* values were less than 0.05 (*P<0.05). The positive (+) numbers showed the presence of ARGs in number of strains and negavtive (-) numbers showed the absence of ARGs in mastitis *E. coli* strains. Number of virulence genes positive for isolates were placed in boxes of VGs row and their percentages (%) in positive and negative ARGs isolates were given in relevent brackets.

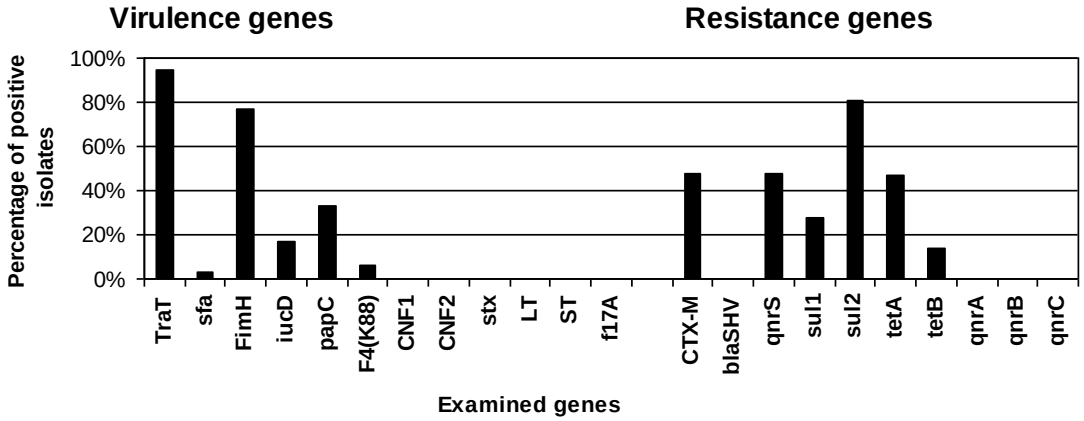
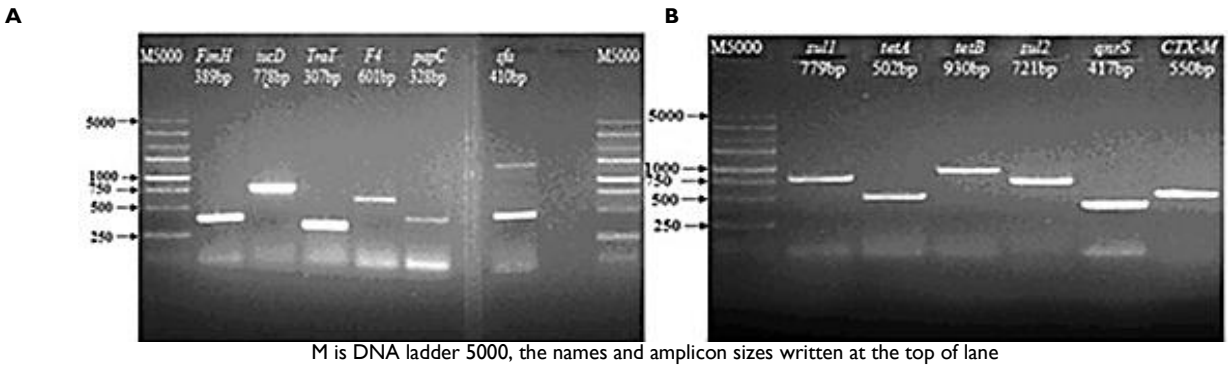


Fig.2: Detected virulence and resistance genes (percentage) in mastitis *E. coli* (103) isolates



M is DNA ladder 5000, the names and amplicon sizes written at the top of lane

Fig.3: Detected Virulence genes (A) and Antimicrobial resistance genes (B) in mastitis *E. coli* isolates

In this study, existence of *papC* and *sfa* genes in mastitis *E. coli* isolates was in agreeement with previous reports of Kaipainen *et al.* (2002) and Suojala *et al.* (2011). The fimbria F4 was also detected in six isolates, which has not yet reported in mastitis *E. coli* isolates. These fimbrial adhesins play role in pathogenesis of bacterial infection through binding to host cell receptors (Bertin *et al.,* 1996). Most commonly reported virulence gene in mastitis *E. coli* isolates was *iucD*, and its presence was reported in all isolates (Lin *et al.,* 1998), in other studies it was reported as 11-16% in mastitis *E. coli* (Ghanbarpour and Oswald, 2010; Suojala *et al.,* 2011), similarly we also found *iucD* in 17% isolates. Virulence genes *TraT, FimH* and *papC* were present alone as well as in combinations in our isolates and this support the findings of Kaipainen *et al.* (2002), that virulence gene were present in combinations in mastitis *E. coli* isolates. However, our findings disagreed with US study showing no combinations of VGs in mastitis *E. coli* isolates (Wenz *et al.,* 2006). Our results showed that all the studied mastitis *E. coli* isolates were pathogenic, as all isolates were carrying a variety of virulence genes. It was assumed that high incidence of *Trat* (95%) and *FimH (*77%) in our isolates could be one of the reasons for bovine coliform mastitis in this region, since their role in mastitis pathogenesis needs further molecular investigation.

It is well reported that majority of mastitis *E. coli* are lacking *ST, LT, stx1, stx2, CNF1, CNF2* and *intimin (eae)* virulence genes (Dogan*et al.,* 2006), our results are also same, no any isolate carried *F17A, F41, stx1, CNF1, CNF2, LT, intimin* and *ST genes*.

Majority of mastitis *E. coli* isolates are resistant (Liu *et al*., 2014), high prevalence of resistance genes i.e. *CTX-M, sul1, sul2, tetA, tetB and qnrS* is in harmony with our MIC results (Memon *et al.,* 2013). Presence of *CTX-M* and *qnrS* gene (48%) in mastitis *E. coli* isolates is firstly reported particularly in China. Furthermore, the unconditional association between resistance and virulence genes in our isolates was prominent. Another association between *CTX-M* and *iucD* gene was pragmatic, which is in accordance with a previous report on association of β lactam genes with VGs in mastitis *E. coli* and in pigs *E. coli* isolates (Wang *et al.,* 2010; Suojala *et al.,* 2011). Whereas, the association of sulfonamide and tetracycline resistance genes (*sul2* and *tetA*) with *iucD* and *papC* was evident, similar findings were also observed in our previous study on *E. coli* isolates from chicken (Yaqoob *et al.,* 2013). High prevalence of *qnrS*and its unconditional association with *FimH* and *papC is* first time reported in mastitis *E. coli* from China. It is in contrast with previous report claiming no association between *qnrS* and *papC* in *E. coli* isolates (Da Silva and Mendonca 2012), however, it is in agreement with Zhao *et al.* (2009) reported association between *papC* and *CTX-M* genes. Increased prevalence of a various resistance genes in mastitis *E. coli* isolates conferring resistance against commonly used antimicrobials in veterinary practice, especially for the treatment of mastitis infection, thus intimating the proper selection and careful use of antibiotics.

ERIC-PCR based DNA polymorphism patterns generated dendrogram showed ten distinct genotypes expressing 80-90% similarity with each other. Similarity between isolates within same cluster was 95-99% irrespective to combinations of VGs and ARGs suggested the presence of some other uninvestigated genes presence

in the isolates of same cluster. The highly virulent isolates represented the genotypes (B, C, E, G and H) and most resistant isolates were in genotypes (A, B, C, G, and J). High genotypic similarity in isolates may be due to similar management, treatment patterns and/or climate conditions of study area.

Conclusions: Our results showed variety of *E. coli* serotypes were involved in bovine mastitis infection which were closely related at gene level. Thus, isolates with different serotypes may have similar genes. Mastitis *E. coli* isolates were highly pathogenic and genetically resistant which is alarming and could be the main reason for the clinical mastitis treatment failure. High virulence potential in isolates indicating the indiscriminate use of commonly used antibiotics which provide opportunity to pathogen to become more virulent. Furthermore, genetically related mastitis *E. coli* isolates were prevailing at different locations which may be the consequence of selection pressure of antibiotics, animal transition from one place to another, similar husbandry practices and management. More research is necessary to appraise the role of *E. coli* in bovine mastitis infection to develop proper approach for treatment and control of coliform mastitis.

Acknowledgements: This study supported by the National Natural Science Foundation of China (No. 31172319), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and Department of Livestock and Fisheries Government of Sind, Pakistan under the "ASPL-II" project.

Author's contribution: FH and JM designed the study. JK, NH, MY and AA executed and helped in experiments. NH, RB, MFH, JS and BS supported in analyzing the data and interpretation. All authors reviewed critically the interpreted data and consented on final version.

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