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Research Article

Antimicrobial resistance and virulence determinants of *E. coli* in bovine clinical mastitis in dairy farms

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ABSTRACT

Escherichia coli (*E. coli*) is a significant pathogen that causes mastitis in bovine animals and decreases milk quality and production of milk. *E. coli* causes economic losses in the dairy industry all over the world, due to antimicrobial resistance and virulent genes (*stx, eae and cdt)* present in *E. coli*. The present study was conducted to examine the virulence factors, antimicrobial susceptibility, and virulent genes in *E. coli*. Samples were collected from buffaloes from different dairy farms in Faisalabad Punjab, Pakistan. Different techniques were applied for the identification and Isolation of *E. coli*. In the biochemical test Citrate test, Indole test, Gram staining technique, and motility test were used. For virulent genes (*stx, eae and cdt)* and detection PCR technique was used. For the detection of antimicrobial Resistance, the Kirby-Bauer disc diffusion method was used. After the experimentation process, results were statically analyzed by using CRD about the prevalence, virulence, and antimicrobial resistance of *E. coli* in the bovine animals in the Faisalabad Division. After experimentation and statically analyzed results, it was observed that *E. coli* was present in the collected samples in bovine animals. Antimicrobial resistance was high against *E. coli*. For the determination of virulence genes *(stx, eae and cdt)* PCR technique was used. It was observed after the PCR technique that *stx* gene was present in *E. coli*.

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Introduction

Bovine clinical mastitis causes very high economic losses (Camacho et al. 2005). Mastitis is the most complex and costly disease in the dairy industry all over the world. In Pakistan, almost millions of losses occurred every year due to a decrease in milk production and quality of milk (Gomi et al. 2017).

Bovine mastitis can be classified into two groups clinical and subclinical Mastitis based on signs (Burvenich et al. 2003). Various types of pathogens caused Mastitis including viruses, bacteria, Mycoplasma, and other related clinical pathogens (Gao et al. 2017).

Environmental mastitis is caused by *E. coli* which is most toxic and fatal (Camacho et al. (2005).

In bovine mastitis inflammation and infection in the udder is occurred during the parturition and lactation period (Burvenich et al. 2003). If the

pathogen enters through the orifice of the mammary tissue of the bovine animals then mastitis may occur. Morphology of the udder and injuries into the udder play important role in the pathogenesis of mastitis (Wang et al. 2022). Excessive use of antibiotics, farming practices, and milking practices play important role in the development of mastitis (Cao and Cerniglia 1996).

Those strains that cause these diseases are called generally called diarrheigenic *E. coli* strains. Among diarrheagenic *E. coli*, STEC is distinguished by the ability to cause severe illness in humans such as

hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura.

In mastitis, *E. coli* is the most causative agent in dairy animals. But the molecular and development mechanism of mastitis has not yet known. Micro-RNA are recently discover endogenous non-coding RNAs (Wang et al. 2022). The size of *E. coli* is 2.0-6.0 micrometer in length and the width is 1.1-1.5 micrometer. Mostly *E. coli* occurs in soil, water, skin, intestine, air, etc (Dufour et al. 2015).

Bovine clinically mastitis is one of major disease in bovine animals caused by various types of microbes. There is also potential risk to public health because it can be transmit animals to humans and can cause serious other types of infections in humans (Dufour et al. 2015). *E. coli* trigger sporadic mastitis can be vary severe to even fatal forms in bovine animals. Severity of disease of bovine clinical mastitis depend upon the various factors in which presence of pathogenic genes in *E. coli*, host immune system and virulence strains in the host for the infection in bovine animals (Lioui et al. 1999).

E. coli associated with clinical mastitis is high genotypic ally variable and clinical severe (Fahim et al. 2019). Pathogenic variants and typically commensals *E. coli* associated with clinical mastitis. Several studies were conducted to evaluate and determine the pathogenic genes (stx, eae, cdt). Shiga toxin producing *E. coli* are one of pathogens which reported from food borne disease outbreaks. Recurrence or persistence of *E. coli* infection might depend its ability to adhere to, and invasion to mammary epithelium (Elazar and Rosenshine 2008).

E. coli is a genetically heterogeneous group of bacteria whose members are typically nonpathogens that that are part of the normal micro flora of the intestine tract of humans and animals (Abbas et al. 2019). For the intestinal and extraintestinal disease certain subsets of this bacterium have acquired genes that enable them to these diseases. Those strains that cause these diseases are called generally called diarrheigenic *E. coli* strains. Among diarrheagenic *E. coli*, STEC is distinguished by the ability to cause severe illness in humans such as hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura.

In Gram negative bacteria *E. coli* is characterized an early and high expression of cytokines and inflammatory mediators in milk is caused by *E. coli*. Clinical mastitis can cause 10% decrease in milk on quarter level (Bertin et al. 2008). In acute mastitis 10% decrease in milk production in acute mastitis (Adeel and Ullah 2019). Symptoms are not evident in the subclinical mastitis.

In dairy animals especially buffalo's subclinical mastitis is a major problem. Under various framing practices and systems deserves attention in milk due its potential impact in milk production and food security. Two third losses of the total milk production due to affected quarters of animals (Ammar et al. 2013). Contagious pathogens are isolated from mastitis milk. Reported 16.72 and 21.08% clinical mastitis in cows and buffaloes. Staphylococcus and streptococcus microbes isolated in cows and buffaloes were 70-80%. Subclinical and clinical mastitis one of the major infection. In the cows and buffaloes can be diagnosed through somatic cell count (Bag et al. 2021).

Escherichia coli is a Gram-negative organism. *E. coli* is a facultative anaerobic bacterium having a rod shape. The size of *E. coli* is 2.0-6.0 micrometer in length and the width is 1.1-1.5 micrometer. Mostly *E. coli* occurs in soil, water, skin, intestine, air, etc. (Biswas et al. 2020)

Materials and Methods

Sample collection

This research has been conducted in the Institute of Microbiology and pharmacology Lab, Institute of the Microbiology University of Agriculture. A total of 50 samples of mastitic milk were collected from the different Diary farms to evaluate the antimicrobial susceptibility and virulence of *E. coli*. Samples were transported to the laboratory of the University of Agriculture Faisalabad within 24 h at 4°C in the sterile containers. Samples were collected from different dairy farms of bovine mastitic animals. These samples were stored at 4°C in the refrigerator and for the growth, MacConey agar was used. After streaking and incubation at room temperature for 24 hours, it was observed that pink colonies appeared on the Petri plates which showed the growth of *E. coli*. For the further growth of *E. coli,* selective media was used in the laboratory and confirm the growth of *E. coli*. Further typically colonies were picked up and onto nutrient agar for the further biochemical test and identification and morphological characteristics and virulence determinants of *E. coli* in the specific conditions.

Enrichment and isolation of E. coli

Enrichment and isolation of *E. coli* from bovine milk samples were performed according to protocol described by Fahim et al. 2019 with slight modification. One ml sample in each container was collected. For the isolation and enrichment MacConkey agar was used. After streaking the samples in the petriplates and incubated overnight at 374°C in the incubator. Pink color colonies were observed in the petriplates. For the further isolation Luria Bertani media was used in this procedure of isolation.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolated *E. coli* was detected by disc diffusion method as recommended by clinical and Laboratory Standards

institute (CLSI, 2018) and interpreted as susceptible, intermediate and resistant (Balagurunathan et al. 2014). Eight different types of antibiotics were used in the dairy farms and human clinical cases in Faisalabad. Commonly Ciprofloxacin (10µg). Ampicillin (15µg). Clindamycin (10µg), Gentamycin (10µg), Streptomycin (12µg) and Amoxicillin (10µg) Antibiotics were selected in this study. To confirm the reproducibility of the *E. coli* results experiment was performed three times

Molecular detection of E. coli

For the detection of *E. coli* polymerase chain reaction (PCR) was used for identification of eae, stx & cdt genes following protocol described earlier (Barrington et al. 2016). Briefly, for PCR reaction mixture was adjusted to 20 ul with duration time 954°C for 10 min, followed by 30 cycles.

PCR Procedure

With each cycle often consisting of a few distinct temperature increments, PCR entails a succession of 40 repeated temperature changes, or cycles (Blum et al. 2015). The cycle is frequently preceded by one temperature step at an extremely high temperature, followed by one hold at the end for result expansion or temporary stockpiling.

Initialization

In the initialization step DNA is polymerized that require heat by hot-start PCR. 94-964°C temperature is required for the initialization step. Polymerase are also required in the initialization step. Time for this step is 1-10 minutes.

Denaturation

In the denaturation step 94-984°C temperature is required and time is 20-30 seconds. This causes DNA liquefying or denaturation in the denaturation of the denaturation step.

Annealing

In the following stage, temperature is brought about 50-654°C for 20 second. Single-abandoned. In this step DNA layouts are regularly remembered for the response combination. Objective locale containing two single-abandoned supplements. Preliminaries are single-abandoned groupings supplements containing the objective district, supplementing, without doubt, extremely short successions in the finishes of each strand.

Extension /elongation

In this step temperature relies upon the DNA polymerase utilized. 75-804°C however is the ideal temperature for the elongation or extension step in the DNA polymerase step. In this step DNA is extend or elongated by this procedure.

Final elongation

This optional step is carried out for 5–15 minutes after the previous PCR cycle at a temperature of 70–744°C (the range anticipated for the discretionary movement of most polymerases used in PCR) to ensure that any extra single-abandoned DNA is fully extended. In this step DNA polymerase orchestrates another way. The exact time expected for stretching depends on both DNA polymerized.

Final hold

This is the last step cools the chamber to 4-154°C temperature. To check the PCR effectively created the expected DNA.

Visualization of the PCR products on agarose gel

In perception groups, extended on 1.5% agarose gel were electrodes as indicated by of the designated quality. For agarose gel electrophoresis

- 50 ml 1XTAE cushion was taken in a carafe
- 0.75 agarose powder in grams was broken up TAE support 2 3 bubbles in a bubbles until it become a reasonable fluid
- Carafes were refreshed for 5 minutes to diminish the temperature up to 54°C
- 3 ul fluid Ethidium bromide was completely broken down in an agarose blend
- The blend was painstakingly filled in an electrophoresis plate alongside a well-framing brush
- gel was cemented and the brush was eliminated
- Agarose gel plate was set completely dunked in an electrophoresis tank containing TAE cushion in such a place that run from negative to the positive cathode.
- In the principal well, 3ul of 1kb marker was stacked. For each PCR item, 8 ul of item and 3 ul of 0.5x Bromo phenol colors were blended in with the assistance of a pipette and stacked into the gel.
- The gel was run for 35 minutes.
- Gel imagined ID Gel framework under UV light and results were recorded.

PCR detection of antimicrobial resistance genes

E. coli isolated in this study were screened for the presence of antimicrobial resistance genes by PCR based on the phenotypic resistance pattern, genes conferring resistance to β-lactams and tetracycline were screened by PCR by following protocol (Blum et al. 2015). Mixture was adjusted 20 µl and duration time 94°C for 10 min, followed by 30 cycles.

Sequencing and analysis

16s rRNA gene of randomly selected *E. coli* isolates were amplified and sequenced using the primers 8F and 1492 R. Sequencing was performed using

sanger's sequencing technique on an applied biosystems 3500 series analyzer.

Statistical analysis

For the statistical analysis data, p-value less than 0.05 was deemed statistically significant.

Results

Isolation of E. coli in clinical mastitis of bovine animals

A total 50 samples of mastitic milk were collected from bovine animals. After streaking on the MacConey agar growth of *E. coli* was observed (20 %) in 10 samples. Three colonies were selected from each sample. For PCR confirmation specific gene of *E. coli malB* gene was detected. The isolation was further confirmed by targeting Sequencing of 16s rRNA gene randomly selected *E. coli.* After experimentation and detection of *E. coli* it was observed that *E. coli* was present in bovine animals having statistically value was less than (p = 0.05*)* which was significant.

Virulence determinants and phylogenetic group of E. coli

For the virulence of *E. coli* isolates various virulence genes of *E. coli* such as *stx, eae and cdt* genes were further detected by PCR Method. Only *stx* genes was detected in the bovine clinical mastitis that was play important role in the clinical and subclinical mastitis in the bovine animals in the dairy farms in Faisalabad.

Antimicrobial susceptibility

Antimicrobial susceptibility of isolated samples of *E. coli* was determined by Disc diffusion method against the different classes of Antibiotics such as Ciprofloxacin, Ampicillin, Clindamycin, Gentamycin, Streptomycin and Amoxicillin *E. coli*. Resistance was determined 89.5, 80, 50, 20, 10 and 3%, respectively.

Discussion

Bovine clinical mastitis causes very high economic losses. Mastitis is the most complex and costly disease in the dairy industry all over the world. In Pakistan, almost millions of losses occurred every year due to a decrease in milk production and quality of milk. In bovine mastitis inflammation and infection in the udder is occurred during the parturition and lactation period. (Fairbrother et al. 2015).

If the pathogen enters through the orifice of the mammary tissue of the bovine animals then mastitis may occur. Morphology of the udder and injuries into the udder play important role in the pathogenesis of mastitis. Excessive use of antibiotics, farming practices, and milking practices play important role in the development of mastitis (Fazel et al. 2019). Various types of pathogens caused Mastitis including viruses, bacteria, Mycoplasma, and other related Clinical pathogens. In bacterial pathogens, *E. coli* is one of them. *E. coli* causes Endometritis in Bovine animals. Virulence factors of *E. coli* associated with bovine clinical in ppm (Fourichon and Beaudeau 2003).

Different Resistance was observed against different types of antibiotics 94.5, 89.5 and 88.5% resistance was observed against amoxicillin, tetracycline and ampicillin, respectively. Antibiotics which were routinely used in the farms very resistant against *E. coli*. According to a study antibiotic Resistance against clinical bovine mastitis decreased due to developing potential in *E. coli* against antibiotics and routinely associated with clinically bovine mastitis (Bag et al. 2021).

It was identified that a gradual increase in bacterial resistance against a broad spectrum of antibiotics is seriously injurious to humanity in the future. For the control of mortality rate and infection rates in bovine animals, advanced methods should be adopted to control antimicrobial resistance in animals. Applying (ZnoNp), (XRD), and (FE-SEM) resistance can be reduced (Obaidat et al. 2018).

It was identified that *E. coli* is an opportunistic pathogen and is involved in the bovine clinical mastitis of over 200 cows. Different virulence factors were sensed by isolation and identification techniques. There were 26 virulence factors were associated with mastitis in cows. O-serogroups of *E. coli* were assessed by agglutination tests. Cows which contain mastitis cows 24.5% strain of *E. coli* contain (cnf1, cnf2, stx1&stx2) genes. Due to this reason high virulence was determined in the cows by Gao et al. 2017.

A study was about the prevalent and resistance of *E. coli* in the mastitis in cows. After experimentation process it was observed that mastitis caused by *E. coli* acute and chronic. *E. coli* which was isolated from the epithelial layers of cows cause chronic mastitis. These strains which were internalize in epithelial layers. In the acute mastitis *E. coli* was not in the internal layers. It was observed that chronic *E. coli* was treated the (CME), caveolae-mediated endocytosis and receptor mediated endocytosis (RME), double immunofluorescence labeling confocal laser and fluorescence microscopy. Internalization studies demonstrated that chronic strains of *E. coli* persist longer time in interacellularly than acute *E. coli* strains (Fairbrother et al. 2015). Treated bovine clinical mastitis showed low number of intracellular *E. coli* strains in epithelium layers. After experimentation process it was resulted that chronic bovine clinical mastitis used lipid rafts for the movement into epithelial layers and caused bovine clinical mastitis. Due to this mechanism, bacteria hide themselves from the endocytosis, Acidification and other enzymes such as endolysome (Fazel et al. 2019).

Table 1: *E. coli* isolated from bovine animals

Farms	Cattle with clinical mastitis / no of samples	*E. coli* positive samples	*E. coli* positive samples (%)	P value
A	10	3	30	0.118
B	5	*1*	10	0.112
C	5	*1*	10	0.278
D	20	*2*	20	0.233
E	10	*3*	30	0.222
Total	50	*10*		**0.007**

Table 2: Antibiotics used against mastitis in bovine animals

Serial no	**Antibiotics**	**concentration**	**Resistance in %**
1	Ciprofloxacin	10ug	89.5
2	Ampicillin	15ug	80
3	Clindamycin	10ug	50
4	Gentamycin	12ug	20
5	Streptomycin	15ug	10
6	Amoxicillin	10ug	3

Milk samples of cows were used to check the virulence and antimicrobial resistance. After isolation and identification of pathogen *E. coli* by using different procedures and microbiology tests it resulted that *E. coli* was not virulent in the forms of Iran in cows. Antimicrobial susceptibility against antibiotics was high. Antibiotics which are used against mastitis was efficient (Wang et al. 2022).

E. coli is one the pathogens that play role in the bovine clinical mastitis and cause infection. For this purposes mastitic milk samples were collected to check the antimicrobial susceptibility and resistance of *E. coli*. After experimentation and results it was observed that E. coli was present in in the bovine animals in Faisalabad division. Different genes were detected with the PCR confirmation and for antimicrobial resistance. It was resulted that in the virulence genes *STX, CDT and EAE* genes were detected by PCR technique. Just *STX* gene was detected in the bovine animals in Faisalabad division. Antimicrobial resistance various types of antibiotics were used in resistance was observed after experimentation and results process.

Conclusion

This study demonstrated that *E. coli* isolated from clinical bovine mastitis are typical commensal. Major virulence was not caused by the *E. coli*.

Almost all the isolates of *E. coli* are multidrug resistance which are might be associated with the antibiotics.

References

Abbas Q, Han J, Adeel A and Ullah R, 2019. Dairy production under climatic risks: Perception, perceived impacts and adaptations in Punjab, Pakistan. International Journal of Environmental Reseasrch and Public Health 16:45-50.

Adeel A and Ullah R, 2019. Dairy production under climatic risks: Perception, perceived impacts and adaptations in Punjab, Pakistan. International Journal of Environmental Reseasrch and Public Health 16:34-35.

Ammar ER, Zaki A, Khairy N, Moshref BS and Refai MK, 2013.Virulence factors of *Escherichia coli* isolated from recurrent cases of clinical and subclinical mastitis in buffaloes. International Journal of Microbiological Research 4:86-94.

Bag MAS, Khan MSR, Sami MDH, Begum F, Islam MS, Rahman MM, Rahman MT, Hassan J, 2021. Virulence determinants and antimicrobial resistance of *E. coli* isolated from bovine clinical mastitis in some selected dairy farms of Bangladesh. Saudi Journal of Biological Sciences 28:6317-6323.

Balagurunathan Y, Kumar V, Gu Y, Kim J, Wang H, Liu Y, Goldgof DB, Hall LO, Korn R, Zhao B, Schwartz LH, Basu S, Eschrich S, Gatenby RA and Gillies RJ, 2014. Test–Retest reproducibility analysis of lung CT image features. Journal of Digital Imaging 27:805-823.

Barrington GM, Garry FB, Dinsmore RP and Callan RJ, 2016. Use of systemic disease signs to assess disease severity in dairy cows with acute coliform mastitis. Journal of American Veterinary Medical Association 218:567-572.

Bertin Y, Martin C and Livrelli V, 2008. Molecular analysis of Shiga toxinproducing Escherichia coli strains isolated from hemolytic-uremic syndrome patients and dairy samples in France. Applied and Environmental Microbiology 74:2118-2128.

Biswas D, Hanif S, Rana EA and Anower AM, 2020. Study on udder health management practices, reproductive disorders and subclinical mastitis in buffalo herds in coastal region of Bangladesh. Turkish Journal of Agriculture, Food Science and Technology 8:1662-1667.

Blum SE, Heller ED, Sela S, Elad D, Edery N and Leitner G, 2015. Genomic and phenomic study of mammary pathogenic *Escherichia coli*. PLoS One 10:12-20.

Burvenich C, Van Merris V, Mehrzad J, Diez-Fraile A and Duchateau L, 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Veterinary Research 34:521-64.

Camacho AT, Guitian FJ, Pallas E, Gestal JJ, Olmeda S, Goethert H, Telford S and Spielman A, 2005. Serum protein response and renal failure in canine *Babesia annae* infection. Veterinary Research 36:713-22.

Cao WW and Cerniglia CE, 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Applied and Environmental Microbiology 62:1242-1247.

Dufour S, Fairbrother JM, Francoz, D, Nadeau É and Messier S, 2015. Characterization of persistent and transient *Escherichia coli* isolates recovered from clinical mastitis episodes in dairy cows. Veterinary Microbiology 176:126-133.

Elazar S and Rosenshine I, 2008. Mammary pathogenic *Escherichia coli*. Current Opinion in Microbiology 11:60-65.

Fahim KM, Ismael E, Khalefa HS, Farag HS and Hamza DA, 2019. Isolation and characterization of *E. coli* strains causing intramammary infections from dairy animals and wild birds. International Journal of Veterinary Science and Medicine 7:61-70.

Fairbrother JH, Dufour S, Fairbrother JM, Francoz D, Nadeau É and Messier S, 2015. Characterization of persistent and transient *Escherichia coli* isolates recovered from clinical mastitis episodes in dairy cows. Veterinary Microbiology 176:126-133.

Fazel F, Jamshidi A and Khoramian B, 2019. Phenotypic and genotypic study on antimicrobial resistance patterns of *E. coli* isolates from bovine mastitis. Microbial Pathogenesis 132:355-361.

Fourichon C and Beaudeau F, 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. Veterinary Research 34:475-491.

Gao J, Barkema HW, Zhang L, Liu G, Deng Z, Cai L, Shan R, Zhang S, Zou J, Kastelic JP and Han B, 2017. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. Journal of Dairy Science 100:4797-4806.

Gomi R, Matsuda T, Matsumura Y, Yamamoto M, Tanaka M, Ichiyama S and Yoneda M, 2017. Whole genome analysis of antimicrobial resistant and extraintestinal pathogenic *Escherichia coli* in river water. Applied and Environmental Microbiology 83:e02703-16.

Obaidat MM, Bani Salman AE, Davis MA and Roess AA, 2018. Major diseases, extensive misuse, and high antimicrobial resistance of *Escherichia coli* in large- and small-scale dairy cattle farms in Jordan. Journal of Dairy Science 101:2324-2334.

Vivegnis J, Lioui ME, Leclercq A, Lambert B and Decallonne J, 1999. Detection of Shiga-like toxin producing *Escherichia coli* from raw milk cheeses produced in Wallonia. Biotechnology and Agronomical Society of Environment 3:159-164.

Wang W, Li R, Ye T, Zhang X, Chen C, Liang AX and Yang LG, 2022. Preliminary study on gene regulation and its pathways in Chinese Holstein cows with clinical mastitis caused by *Staphylococcus aureus*. Journal of Veterinary Research 66:179-187.