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Antibiogram, Haemolysis and Biofilm Properties of *Escherichia coli* from Bovine Mastitis

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ABSTRACT

Background: *Escherichia coli* cause mammary gland inflammation in dairy cows with striking local and sometimes severe systemic clinical symptoms. This disease affects many high producing cows in dairy herds and may cause several cases of death per year in the most severe cases. The present study was planned to evaluate the antibiogram and virulence factors of the isolated bacteria responsible for mastitis.

Methods: In this study, 300 milk samples were collected and subjected to identify clinical mastitis (based on clinical symptoms) and sub clinical mastitis (using California mastitis test). *E. coli* was isolated and identified from mastitis positive samples and further tested for antibiogram and virulence factors such as haemolysis, biofilm formation and *fimH* and *pap* genes presence.

Result: Out of 300 milk samples collected, 121 (40.33%) were positive for mastitis. *E. coli* was isolated from only 30 (24.79%) out of 121 mastitis positive samples. On Serotyping for somatic antigen, 13 *E. coli* isolates belonged to somatic serogroup O83, 2 to O157 and one each to O8, O20, O49, O119, O128 and O145. The highest sensitivity of *E. coli* isolates was recorded for ciprofloxacin, followed by gentamicin, tetracycline, nitrofurantoin, chloramphenicol, amikacin, cefixime, trimethoprim, ampicillin/sulbactam, ceftriaxone, cefoperazone, cefotaxime, kanamycin, cefotaxime/clavulanic acid and amoxycillin in the decreasing order. Multidrug resistance (MDR) was recorded in 96.66% of isolates. Out of 30 *E. coli* isolates, 46.66% were haemolytic and 40.0% positive for Congo red dye binding. Biofilm production was shown by 76.66% isolates. Molecular characterization revealed presence of *fimH* gene in 9 isolates, but *pap* gene was not detected in any of the strain. It may be concluded that *E. coli* is an emerging environmental mastitogen in cows and isolation of MDR strains with virulence factors is of serious concern.

Key words: Antibiogram, *Escherichia coli,* Mastitis, Virulence factors.

INTRODUCTION

Mastitis is the most economically significant diseases of dairy animals. It is widespread in dairy herds and is associated with a significant reduction in milk yield, increased costs of production and deteriorated milk quality. Mastitis triggered by *E. coli* is usually sporadic and clinical signs vary from very severe or even fatal forms to mild mastitis in which cows have only local signs in the udder (Sikrodia *et al*., 2020). While the severity of the disease depends on host immune response and genetic makeup, virulence of the bacterial strains involved may also play a role (Fernandes *et al*., 2011). The point sources of coliform bacteria that cause infections include bedding materials, soil, manure and other organic matter in the environment of cows. The portal of entry into the mammary gland for Gram-negative bacteria is the teat canal (Hogan and Larry, 2003). Incidence of coliform mastitis increases during climatic periods that maximize populations in the environment.

A high incidence of clinical and sub-clinical mastitis in cows due to Gram positive cocci was reported from the Malwa region of India (Ghose *et al.,* 2001, 2003). However no studies had been done to study coliform mastitis in this area. Hence, the present study was undertaken.

MATERIALS AND METHODS

A total of 300 milk samples were collected aseptically from cows from various organised and unorganised herds. All samples were processed and tested in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandary, Mhow. The pooled milk samples were subjected to california mastitis test (CMT) (Schalm *et al*., 1971) for the diagnosis of subclinical mastitis (SCM), whereas clinical mastitis was detected by the examination of udder. The samples found positive for clinical and subclinical mastitis were included for further studies.

Isolation and identification of *E. coli*

Milk samples from clinical cases and those showing +2 or +3

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reactions in CMT, were cultured bacteriologically to isolate and identify *E. coli* bacteria. Each sample was inoculated in Brain heart infusion (BHI) broth (Hi Media) and incubated aerobically at 37°C for 18-24 hrs. Subsequently a loopful of bacterial growth from BHI broth was on Eosin Methylene Blue (EMB) agar (Hi Media) and incubated aerobically at 37°C for 24 hrs. A single, well isolated colony with metallic sheen on EMB agar after confirming as Gram negative rods was transferred on nutrient agar (NA) slant. The NA slants were incubated aerobically at 37°C for 24 hrs and thereafter stored at 4°C for the preservation of isolates.

The presumptive identification of bacterial isolates as *E. coli* was accomplished by colonial and bacterial morphology and confirmed by a battery of biochemical tests, *viz*- ONPG, catalase, oxidase, MR, VP, urease, nitrate reduction, indole, citrate utilization, carbohydrate fermentation and growth on triple sugar iron agar as per the procedure described by Barrow and Feltham (1993).

The bacterial isolates identified as *E. coli* on the basis of cultural, morphological and biochemical characteristics were sent to National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli (H.P.) for serotyping of somatic (O) antigen.

Antibiotic sensitivity test of *E. coli*

In vitro antibiotic sensitivity test (AST) of *E. coli* isolates was conducted as per the method of Bauer *et al*. (1966) using antibiotic discs (Hi Media) of amikacin, amoxicillin, ampicillin, cefixime, cefoperazone, ceftriaxone, cefotaxime/ clavulanic acid, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nitrofurantoin, tetracycline and trimethoprim. Diameters of the clear zone of inhibition around antibiotic discs were measured in mm. The interpretation of the result was made in accordance with the instructions of the manufacturer.

Virulence factors of *E. coli*

Haemolysis

The haemolytic activity of *E. coli* was studied on blood agar plate as per Agarwal *et al*. (2003). Zone of complete haemolysis (β hemolysis) around the bacterial colonies after 24 hrs incubation was noted as strongly positive and the zone which appeared after incubation of two days was recorded as weakly positive. Alpha haemolysis was characterized by a hazy zone of partial haemolysis (green discoloration), containing a proportion of unlysed cells and less clearly demarcated from the surrounding medium than β haemolysin activity.

Biofilm formation

A qualitative assessment of biofilm formation by tube method was determined as per the method described by Mathur *et al.* (2006). The culture was inoculated in 5 ml Tryptone soya broth (TSB) (Hi Media) and incubated for 12 hrs at 37°C after which 50% of spent media from each tube was gently aspirated and replaced by equal quantity of fresh TSB, with sucrose (0.25%). The tubes were reincubated at 37°C for 12 hrs. Subsequently, the broth was decanted and tubes were gently washed twice with PBS, kept inverted for drying and stained with 0.1% safranin solution. Excess stain was removed and the tube was gently washed with deionised water. Tubes were then dried in an inverted position and observed for biofilm formation.

Congo red dye binding assay

The test was performed as an indicative of invasiveness following the method of Ishiguro *et al*. (1985). The isolates were streaked on tryptone soya agar media (Hi Media) containing 0.03% Congo red dye and incubated for 48 hrs at both 37°C and 25°C. A positive reaction was indicated by the appearance of intense orange or brick red colonies. A negative result was evidenced by pale or white colonies.

Molecular characterization of *fimH* and *pap* genes

The isolates were cultivated by inoculating in BHI broth (Hi Media) and incubating at 37°C for 12-18 hours (overnight grown culture). DNA was extracted using a kit (HipurA Bacterial genomic DNA purification kit, HiMedia) as per the manufacturer's instructions. The PCR was performed for the amplification of *fimH* and *pap* of *E. coli* using suitable primers (Fernandes *et al.,* 2011) (Table 1). It was carried out in final reaction volume of 12.6 µl using master-mix (Sigma) in 0.2 ml thin wall PCR tube (Table 2).

The DNA amplification reaction was performed in Thermocycler (Eppendorf Research, Germany) with a pre-heated lid. The cycling conditions for PCR included the steps shown in Table 3. PCR products were kept at -20°C until further analysis by agarose gel electrophoresis. The amplified PCR products (10 µl) were electrophoresed in a 1.5% agarose gel (in TBE buffer), stained with ethidium-bromide solution.

RESULTS AND DISCUSSION

In the present study 121(40.33%) out of 300 milk samples from cows were found positive for mastitis. The incidence of mastitis was higher in unorganized farms (42.70%) as compared to organized farms (36.11%) (Fig 1). The overall prevalence reported in the present study is in close agreement with the results of Birhanu *et al*. (2017), but is lower than the findings of Zeryehun and Abera (2017), who reported a prevalence of more than 50%. The difference in the prevalence of mastitis could probably be due to differences in farm management practices, breed and age of animals, production status, stage of lactation, season *etc*.

Out of 121 mastitis positive samples, *E.coli* was isolated from only 30 (24.79%) samples (Fig 2). Sangeetha *et al.* (2020) also reported 25.7% incidence of *E. coli* from cases of mastitis, but Sharma *et al.* (2015) and Sudheer *et al.* (2019) reported a higher rate of isolation.

Amongst 30 *E.coli* isolates, 21 were typed into 8 different 'O' serogroups, while the remaining 9 were untypable. The most common serogroup was O83 (43.33%) followed by O157 (6.66%). The other 'O' serogroups isolated in the present study were O8, O20, O49, O119, O128 and O145

Table 1: Details of primers used for PCR reaction.

Target gene	Primer sequence	Product size
fimH	Forward 5′- TGCAGAACGGATAAGCCGTGG	508 bp
	Reverse 5′- GCAGTCACCTGCCCTCCGGTA	
Pap	Forward 5′- GCAACAGCAACGCTGCATCAT	336 bp
	Reverse 5′-AGAGAGAGCCACTCTTATACGGACA	

Table 2: Components used in PCR mixture.

Component	Volume (in µl)
Nuclease free water	2.3
Template	2.0
Forward primers	1.0
Reverse primers	1.0
PCR master mix	6.3
Total quantity	12.6

Table 3: Steps and conditions of thermo cycling for *fimH* and *pap* gene by PCR.

Steps		Temp./ Time (*fimH* primers)	Temp./ Time (pap primers)
Initial denaturation	Temp.	94°C	94°C
	Time	5 min	2 min
Denaturation	Temp.	94°C	94°C
	Time	1 min	60 sec
Annealing	Temp.	57°C	63°C
	Time	1 min	30 sec
Extension	Temp.	72°C	72°C
	Time	1 min	90 sec
Final extension	Temp.	72°C	72°C
	Time	7 min	5 min

Steps 2 to 4, 35 cycles for *fimH* gene and 30 cycles for *pap* gene.

(3.33% each). The serotypes O8, O20, O49, O83, O119, O128 and O157 have been isolated from earlier also from bovine mastitis in different frequencies (Iguchi *et al*., 2015; Sharif *et al.*, 2017). O157 serotype recovered in the present study is one of the most important STEC that causes severe diseases in humans. However, until its 'H' antigen is also characterized, it will be too early to implicate pathogenicity of present *E. coli* isolates of O157 type in human diseases.

The results of antibiogram studies (Fig 3) revealed that ciprofloxacin (93.33%) was most effective drug against *E. coli,* followed by gentamicin (80%), tetracycline and nitrofurantoin (76.66%) and chloramphenicol (63.33%), amikacin and cefixime (46.66%) and trimethoprim (43.33%). On contrary, they were resistant to amoxicillin (96.66%), kanamycin (93.33%), cefotaxime/clavulanic acid (73.33%), ampicillin/sulbactam (66.64%), cefixime and cefotaxime (53.33% each) and ceftriaxone (43.33%). Our results are in corroboration with reports of Perez *et al.* (2017) and Sikrodia *et al*. (2020) who also reported ciprofloxacin to be most effective against *E. coli* isolates from bovine mastitis. However, Puvarajan *et al.* (2020) recorded low (38.0%) sensitivity for ciprofloxacin and a high sensitivity to ceftriaxone and cefotaxime in *E. coli* causing bovine mastitis, which is contradictory to the present report. Sudheer *et al.* (2019) observed high resistance to cephalosporins in *E. coli* causing bovine mastitis. Tetracycline resistance was found in 23.64% of isolates, which is similar to report of Marashifard *et al*. (2019), but contradictory to those of Chandrasekaran *et al.* (2015). The antimicrobial resistance pattern of the bacterial population in the cow's environment can vary between herds, reflecting the quantitative and qualitative aspects of antimicrobial treatments. The use of antimicrobials may also select bacteria with virulence factors linked to antimicrobial resistance (Lehtolainen *et al.,* 2003).

Multiple drug resistance (MDR) was observed in 96.67% of the isolated *E. coli* strains. The percentage of MDR isolates recovered in the present study is in close association with the studies done by Srinivasan *et al*. (2007) and Jena *et al.* (2014) who reported 90.7 and 100 per cent in MDR strains of *E. coli*, respectively.

Antibiotic-resistant bacteria pose a severe challenge to both clinicians and dairy animal producers because they have a negative impact on therapy. Development of resistance has been attributed to the extensive therapeutic use of antimicrobials (Abo-Shama, 2014) as exemplified by high resistance towards beta-lactam antibiotics and low towards tetracyclines, in our study. The usage of antibiotics correlates with the emergence and maintenance of antibiotic resistant traits within pathogenic strains. These traits are coded by genes that may be carried on the bacterial chromosome, plasmids, transposons or on gene cassettes that are incorporated into integrons (Daka *et al.,* 2012) and thus are easily transferred among isolates.

E. coli species is very diverse comprising of commensal as well as pathogenic strains clustered in different pathovars based on clinical data and specific virulence properties (Kempf *et al*., 2016). Pathogenicity of strains is conditioned by a specific repertoire of virulence factors located on the mobile genetic elements and transmitted by horizontal gene transfer (Baidy-Chudzik *et al.,* 2015).

Among 30 isolates, 14 were found to be haemolytic. Haemolysins are identified as important virulence factors of enterohaemorrhagic *E. coli* (EHEC), which also produce verotoxin, verocytotoxin or shiga toxins affecting the cell membrane.

Biofilm production was demonstrated by 23.33% *E. coli* isolates. Biofilms are highly organised communities of microorganisms structured within an array of exopolysaccharides (EPS) and adhering to a living or inert surface with the function of protecting the microorganisms in stress

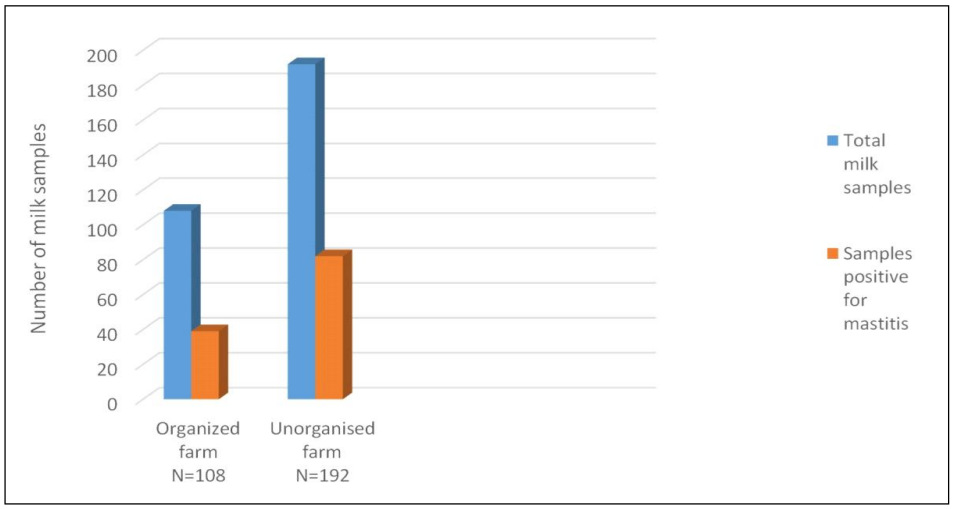


Fig 1: Incidence of mastitis in dairy farms.

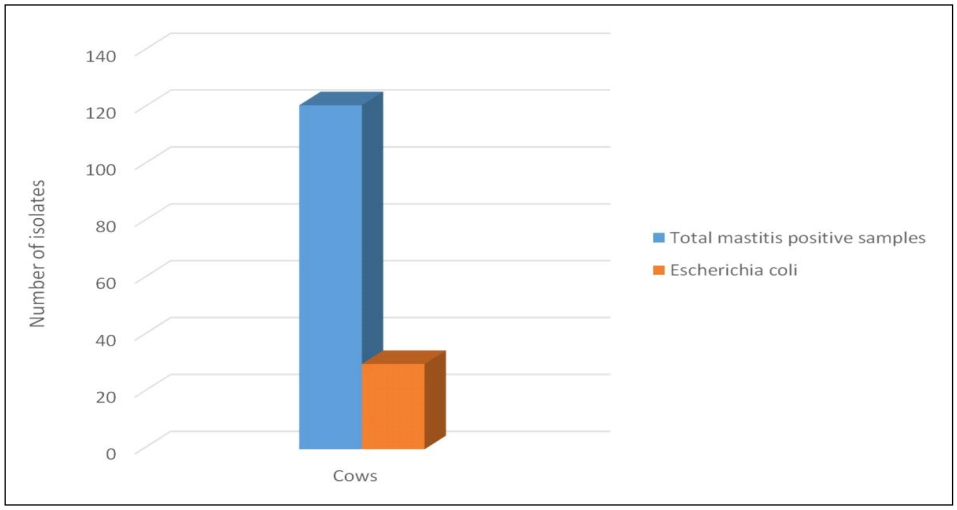


Fig 2: Incidence of *Escherichia coli* in dairy cows.

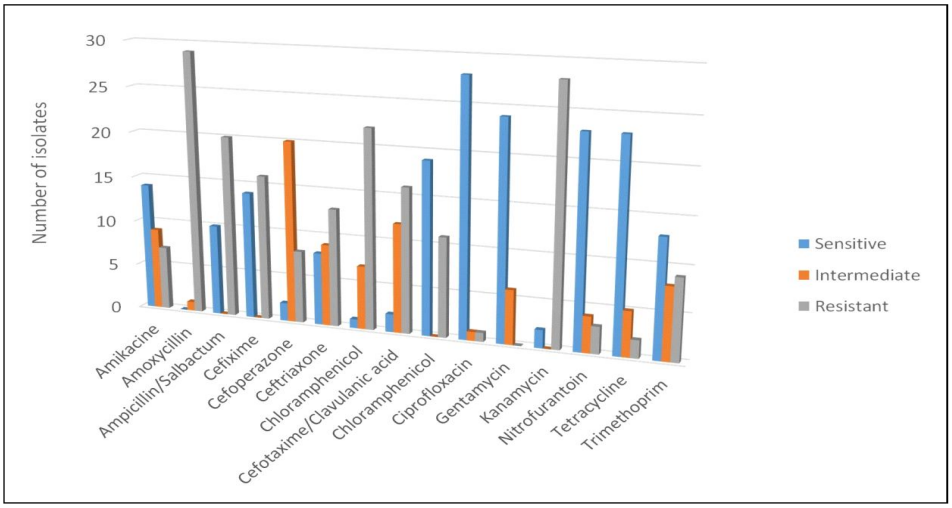
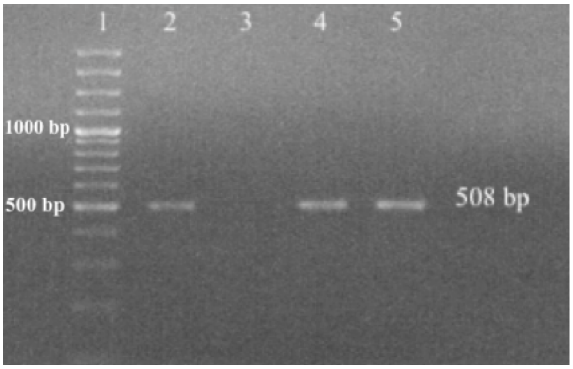


Fig 3: Per cent sensitivity of *E.coli* to different antimicrobial agents.



Lane 1: 100 bp DNA ladder (Fermantas).
Lane 2: Positive control.
Lane 3: Negative control.
Lane 4 and 5: Positive samples.

Fig 4: PCR Amplification of *Fim H* gene (508 bp).

environments. The biofilm potentially plays an important role in the development of persistent infections as well as recurrent clinical symptoms after antibiotic therapy despite quite good *in vitro* antimicrobial susceptibility of the agent. They are associated with antimicrobial treatment failure (Melchior *et al*., 2006).

During the present investigation, total of 12 (40.0%) isolates were found positive for the Congo red binding test. The results are in accordance with Lamey *et al*. (2013) who reported 38.1% *E. coli* isolates from bovine mastitis positive for Congo red binding activity. The ability to bind CR dye has been proposed as a marker for the invasive property of *E. coli* (Sharma *et al*., 2006).

The severity of mastitis in bovine and the pathogenicity of *E. coli* are greatly affected by the presence of genes coding virulence factors. The organism produces a large number of potential virulence factors, such as capsule, biofilm production and pili, which have important roles in the pathogenesis and colonization in mammary gland; Type 1 fimbriae are the most common adhesive organelles of *E. coli,* which mediate the adhesion of the organism to the host's mannose-containing glycoproteins (Dubravka *et al*., 2015). In the present study *fimH* gene was demonstrated in nine isolates (Fig 4). The *fimH* is an important virulence associated gene associated with the expression of curli fimbriae that has an influence on biofilm formation (Dubravka *et al*., 2015). Similar to this study the detection of *fimH* gene was also reported by Dogan *et al*. (2006) and Fernandes *et al*. (2011). However, *pap* gene was not found in any of the isolates as also reported earlier (Fernandes *et al*., 2011), but contrary to those of Kaipainen *et al*. (2002). A low prevalence of virulence genes in *E. coli* associated with bovine mastitis was also recorded by Marashifard *et al*. (2019). Thus the present results indicate that the pathogenicity of *E. coli* in bovine mastitis is not a consequence of specific virulence factors. Only isolates with successful combinations of virulence factors will be capable of causing disease.

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Conflict of interest

All authors declare that they have no conflict of interest.

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