B-4882
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RESEARCH ARTICLE Indian Journal of Animal Research

Virulence Determinants of *Escherichia coli* Isolated from Buffalo Subclinical Mastitis

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ABSTRACT

Background: *E. coli* associated buffalo mastitis is comparatively least studied area in microbes in India. Mastitis is an inflammatory reaction of the parenchyma of the mammary gland that can be of an infectious, traumatic or toxic nature.

Methods: Total 300 buffalo milk samples were collected. *E. coli* were isolated from the mastitis samples and characterized for virulence factors haemolysis, biofilm formatipon, invasiveness and molecular characterization of *fimH* and *Pap* Gene.

Result: Out of total 300 buffalo milk samples 52.33% were found positive for sub clinical mastitis. *E. coli* was recovered only from 17.19% samples. Hemolytic activity was exhibited in 23 (85.18%) out of 27 examined *E. coli* strains. Invasive property also has been determined in 12 (44.44%) isolates. A higher percentage of isolates were found positive for biofilm production by CRA (70.37%) than by tube (33.33%) method. In present study, *fim*H gene was detected in 8 isolates (29.62%). However, none of the isolate was found positive for *pap* gene.

Key words: Buffalo, *E. coli,* Subclinical mastitis.

INTRODUCTION

Buffaloes are the second largest source of milk but mastitis is a major impediment for milk production. The multiplicity of the cause and emergence of resistance due to indiscriminate and prolonged use of antibiotics in absence of antibiogram is a major hurdle in the control of mastitis (Khan *et al*., 2004).

The bovine udder is an ideal environment for microbial growth. The point sources of coliform bacteria include contaminated environment, bedding materials, soil, manure and other organic matter in the environment or the animal itself (Waseem *et al*., 2020). Necrosis of the mammary epithelium occurs during severe, naturally occurring clinical *E. coli* mastitis (Bradley and Green, 2001). The most common mastitis causing bacterial pathogens are *Staphylococcus aureus*, *Escherichia coli*, streptococci, *etc.* (Gao *et al.,* 2017; Yang *et al*., 2020).

Mastitis-causing *E. coli* strains may also have virulence factors, to support their colonization, adherence and survival in the udder, like toxins, adhesins, proteins secreted into host cells, polysaccharide capsules and O-antigens and other mechanisms to resist killing by complement or to scavenge iron. The genes for virulence factors may be present in the bacterial genome or may reside extra chromosomally on plasmids (Harel and Martin, 1999). Considering above factors, the study has been planned to isolate *E. coli* from buffalo mastitic milk samples and determination of its associated virulence factors.

MATERIALS AND METHODS

The study was carried out in the Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Mhow (M.P.) in the year 2019.

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How to cite this article: Singh, A., Chhabra, D., Gangil, R., Sikrodia, R., Shukla, S. and Sharda, R. (2022). Virulence Determinants of *Escherichia coli* Isolated from Buffalo Subclinical Mastitis. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4882.

Submitted: 09-02-2022 **Accepted:** 09-08-2022 **Online:**

Sample collection

Total 300 milk samples were collected from buffaloes that were found positive for clinical mastitis and subclinical mastitis, belonging to various organized and unorganized dairy farms situated in and around Mhow and Indore cities.

California mastitis test (CMT)

The test was performed as per method described by Schalm *et al*. (1971). Formation of gel was recorded as a positive reaction and grades were scored as trace +, ++, +++, depending upon the degree of gel formation.

Isolation and identification of *E. coli*

Samples found positive in CMT were enriched in brain heart infusion (BHI) broth with incubation at 37°C for 24 hrs followed by subculture on nutrient agar, MacConkey agar and selective media eosin methylene blue (EMB) agar. The organisms were confirmed on the basis of bacterial morphology, cultural characteristics and biochemical tests

(Barrow and Feltham, 1993). All the *E. coli* strains isolates producing purple-black colour colonies with black centre and greenish metallic sheen on EMB agar considered as positive (Fig 1). All the *E. coli* isolates were sent to Central Research Institute, Kasauli (H.P.) for serotyping of somatic (O) antigen.

Haemolysis

Haemolytic activity of *E. coli* was demonstrated on blood agar plate as per the method of Agarwal *et al.* (2003). The plates were exhibiting "greening" or clearing of the agar around areas of bacterial growth as an indication of alpha or beta hemolytic activity.

Biofilm formation

A qualitative assessment of biofilm formation by tube and Congo red agar methods was determined as per method described by Mathur *et al.* (2006), Ludarkar (2011) and Nachammai *et al.* (2016). In tube method, all the samples were inoculated in 5 ml tryptose soya broth (TSB) and after 12 hrs incubation 50% media were replaced with the TSB containing 0.25% sucrose followed by staining with 0.1% safranin solution. A visible film lined the inner wall and bottom of the tube was considered positive for biofilm formation. Congo red agar was prepared by using blood agar, glucose and congo-red dye as described by Nachammai *et al.* (2016). Black colonies with a dry crystalline consistency indicated biofilm production; non-biofilm producers usually remained pink.

Congo red dye binding assay

The test was performed to determine the congo red binding ability of the *E. coli* isolates as an indicative of invasiveness following the method of Ishiguro *et al.* (1985). The isolates were streaked on tryptone soya agar 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 appearance of intense orange or brick red colonies (Fig 2). A negative result was evidenced by pale or white colonies.

Molecular detection of Virulence gene *(fimH and Pap* gene*)* DNA extraction

DNA was extracted from all 27 *E. coli* isolates. Bacterial pellet was formed from 1.5 ml of an overnight bacterial culture of each isolate and DNA was extracted from each pellet as per manufacturer instructions supplied with the kit (HipurA Bacterial genomic DNA purification kit, Hi Media). DNA was stored at -20°C till further use. The PCR amplification was carried out for detection of two genes *viz*. *fimH* and *pap* gene of *E. coli* using specific published primers (Fernandes *et al.,* 2011). The nucleotide sequences of the forward and reverse primers for amplification of 508 bp product of *fimH* gene were as-forward (5′- GCAACAGCAACGCTGCATCAT-3′) and reverse (5′- GCAGTCACCTGCCCTCCGGTA-3′) . The nucleotide sequences of the forward and reverse primers were *pap* F (5′-CAGTTAATGTGGTGGCGAAG-3′) and *pap* R (5'-AGAGAGAGCCACTCTTATACGGACA -3') for amplification of 336 bp product of *pap* gene. The PCR reaction was optimized for both amplification by adding 12.5 µl of 2X master mix, 1.0 µl of forward and reverse primer (20 pm/µl) each, 5 µl of extracted DNA (150-200ng) and the reaction was made up to 25 µl using nuclease free water. The amplification cycles for *fimH* gene were initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. Annealing temperature for *pap* gene was 63°C for 30 sec and rest of the cycles were same as *fimH* gene The negative control consisted of sterile water instead of DNA template. The amplified products were electrophoresed in 1.5% agarose gel and visualized in gel documentation system.

RESULTS AND DISCUSSION

Worldwide, reports have documented increasing incidence of bovine subclinical and clinical mastitis including reports from India (Sharma *et al.,* 2012, Nalband *et al*., 2020, Waseem *et al*., 2020). In present study total 300 milk samples of buffaloes were screened for mastitis and 157 samples (52.33%) were found positive for mastitis. Similarly, Bhanot *et al*., 2012 who reported high (78.1%) prevalence of buffalo mastitis but very low prevalence was recorded by Farooq *et al*., 2008. In the current study, the prevalence of

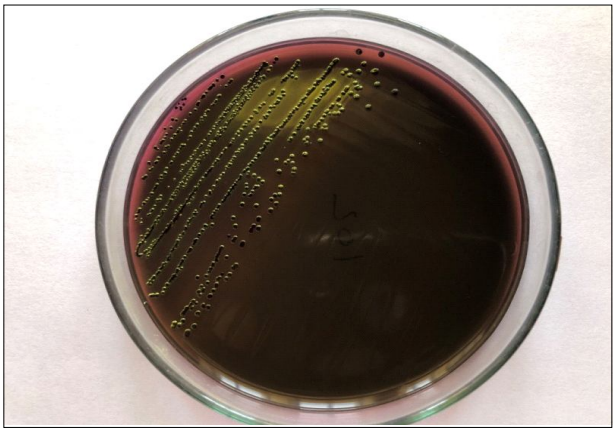

Fig 1: Colonies of *E. coli* on EMB agar showing metallic sheen.

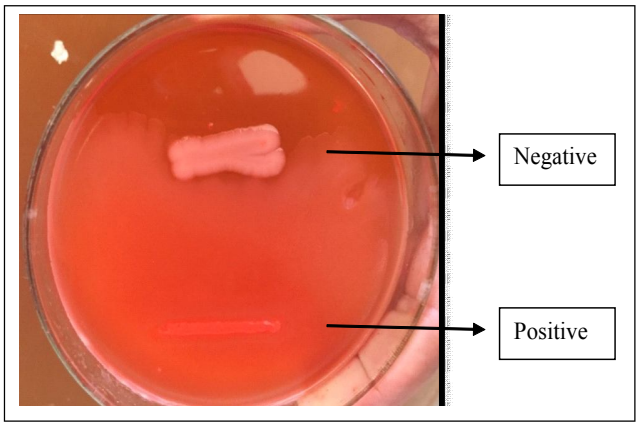

Fig 2: Congo red agar plate showing brick red colonies.

Table 1: Frequency of 'O' serotypes of *E. coli* isolates of buffalo subclinical mastitis.

Sr. no.	Serotypes	Isolate no.	No. of isolates (N=27)	Percentage of serotype
1.	O8	4, 7, 19, 22, 24,25	6	22.22%
2.	O11	2, 21	2	7.4%
3.	O83	11, 17, 20	3	11.11%
4.	O86	27	1	3.7%
5.	O88	8, 15, 18	3	11.11%
6.	O120	5, 6, 14	3	11.11%
7.	O128	1	1	3.7%
8.	O157	3, 10, 16, 23	4	14.81%
9.	Untypable	9, 12, 13, 26	4	14.81%
Total	Eight	27	27	

mastitis in buffaloes was found 42.14% and 59.21% on organized farm and unorganized farms, respectively. Ali *et al*. (2011) also reported the higher prevalence at individual holding buffalo farms (52%) as compared with small holdings (48%) and organized (32%) farms. Variations may be due to differences in farm management practices and hygienic conditions breed and age of animals, geographic location, production status and stage of lactation, season, etc. Predisposing factors for subclinical mastitis in buffaloes include, level of milk production, body weight, calving period, udder type and hygiene conditions for milking (Hussain *et al*., 2013).

E. coli is the major pathogen causing environmental mastitis and exhibit a wide range of systemic disease severity. The incidence of *E. coli* in buffalo mastitic milk samples observed in present study was 17.19% being in close agreement with Ali *et al.,* 2011; *Bhanot et al., 2012;* El-Sayed Lamey *et al.,* 2013; Waseem *et al.,* 2020 who observed 16.18%, 16.3%, 18.47%, 14.4% frequency of *E. coli,* respectively in buffalo milk samples. However, Kumar, 2009, Shawky *et al.,* 2013, Charaya *et al.,* 2014 reported high frequency of *E. coli i.e*. 50%, 25.92%, 20.10%, respectively in buffalo milk samples Beyond that various factors such as bactericidal action of bovine sera, opsonisation by IgM with subsequent phagocytosis and killing by neutrophils, non availability of iron due to its binding by lactoferrin, *etc*. Prevent multiplication or establishment of *E. coli*. These inherent properties of udder defense against *E. coli* infection might be responsible for low incidences of *E. coli* mastitis (Gyles and Thoen, 1993).

Six isolates of *E. coli* were typed as O8 and 4 as O157. Three isolates of each were O83, O88, O120 and two isolates of O11. Two isolates one each belonged to serogroup O86 and O128, while four isolates were untypable for 'O' antigen (Table 1). Total eight types of serotypes were found in buffalo milk. Out of which serotype O8 (22.22%) was predominant.

Hemolytic activity could be used a phenotypic marker of virulence factor of *E. coli*. Hemolytic activity was detected in 23 (85.18%) out of 27 examined *E. coli* strains recovered from buffalo mastitis milk samples. This is in agreement with the findings (81.25%) of Gad El-Said, 2005. Although records of low haemolytic activity i.e. 5.6%, 12.5% and 13.5% by Sayed (2014), Zaki *et al.* (2004) and El-Sayed Lamey *et al.* (2013), respectively are also available. Out of total 23 serotyped *E. coli* only 3 isolates (13.04%) belonging each to O8, O83 and O157 serotypes and out of 4 only 1 (0.25%) untypable *E. coli* were nonhemolytic.

Congo red agar dye binding assay (CRBA) was performed to differentiate invasive and noninvasive *E. coli* isolates. The ability of CR dye has been proposed as a marker for the invasive property of several enteropathogens (Payne and Finkelstien, 1977). Studies have shown that CR binding ability is due to presence of an outer membrane protein, which is absolutely required for virulence (Ishiguro *et al*., 1985). Out of the total 27 *E. coli* isolates, 12 (44.44%) were positive for congo red dye binding (CR^+). El-Sayed Lamey *et al.,* 2013 reported 24 (38.1%) *E. coli* isolates had Congo red binding activity, 20 (31.75%) was invasive which is in close agreement with present findings. Out of total 23 serotyped *E. coli* 10 isolates belonging to 6 serotypes and out of 4 two untypable isolates were positive for CR+. Two serotypes O86 and O120 were found negative for CR. Sharma *et al.* (2006) reported that congo red dye binding test was 100% specific and this test can be used for primary screening of noninvasive *E. coli* from potentially invasive *E. coli.* On the other side, some authors found that congo red binding activity did not correlate well with pathogenicity (Zaki *et al.,* 2004).

The biofilm potentially place an important role in the development of persistent infections and are associated with antimicrobial treatment failure (Melchior *et al*., 2009). In present study, higher percentage of isolates were found positive for biofilm production by CRA (70.37%) than by tube (33.33%) method. Contrary to our findings, Deka (2014) reported higher percentage in tube method (57%) than by CRA (20%) method. Lower per cent in CRA (48.03%) was reported by Dhanawade *et al*. (2010). Our finding is contradictory to Deka, 2014 who recommended tube method is better than CRA and Knobloch *et al*., 2002 who also not recommended the CRA method for biofilm detection. Asfour and Darwish, 2014 reported lower sensitivity and specificity of CRA biofilm detection

Out of total 23 serotyped *E. coli* 10 isolates (43.47%) and 2 (50%) untypable isolates out of 4 were positive for CRA. Only one serotype O120 was found negative for CRA. Out of total 23 serotyped *E. coli,* 6 and 16 isolates were positive for tube method and Modified CRA methods, respectively.

The organism produces a large number of potential virulence factors which have important roles in the pathogenesis and colonization in mammary gland these include capsule, flagella and fimbriae. Type 1 fimbriae of *E. coli* mediates the adhesion of the organism to the host's mannose containing glycoprotein. Presence of virulence genes affects the severity of mastitis. In present study *fim*H gene (508bp) was detected in 8 isolates (29.62%) (Fig 3), which indicate the adhesion properties of bacteria (Memon *et al.,* 2016). The *fimH* gene is an important virulence

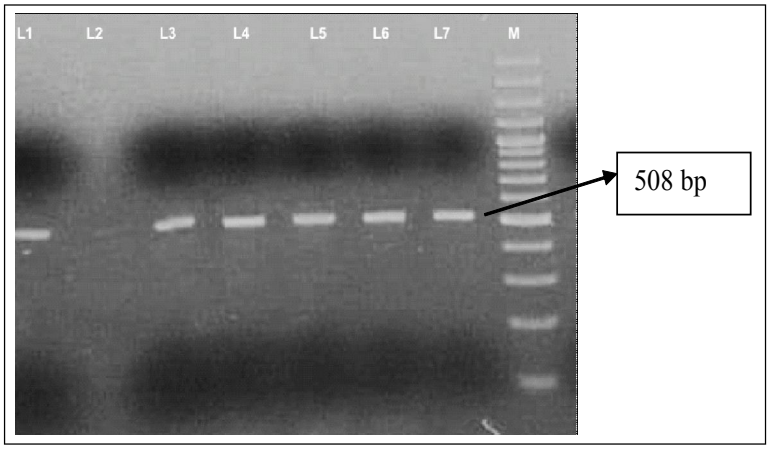

Fig 3: PCR amplification of *fimH* gene of *E.coli.*
L1: Positive control.
L2: Negative control.
L3-L7: Positive samples.
M: 100 bp DNA ladder.

associated gene associated with the expression of curli fimbriae and cellulose and has influence on biofilm formation (Dubravka *et al*., 2015). In isolates *pap* gene was not detected, similar to the result reported by Fernandes *et al*. (2011).

CONCLUSION

Thus, the present result indicates that the pathogenicity of *E. coli* in buffalo mastitis is not a consequence of specific virulence factors. Only isolates with successful combinations of virulence factors will be capable of causing disease.

ACKNOWLEDGEMENT

The authors are thankful to College and NDVSU University officials for providing necessary facilities for research.

Conflict of Interest: None.

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