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Characterization of ESBL producing mammary pathogenic *Escherichia coli* isolated from mastitis affected cattle in India

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Abstract
Bovine mastitis is a globally prevalent disease affecting dairy cattle. It impacts the quantity and quality of milk produced and can lead to the loss of production animal as a whole coliform bacteria are the most common pathogens that have been isolated frombovine mastitis. *Escherichia coli* have been identified in a greater number of bovine mastitis in comparison to other coliforms. Mammary pathogenic *Escherichia coli* (MPEC) strains have virulence attributes to resist the host innatedefenses and thrive in the mammary gland environment. Mammary pathogenic *E. coli* (MPEC) has been identified as a newly recognized pathotype responsible for instigating mastitis in dairy animals. MPEC have acquired specific virulence factors (VFs) that might help the bacteria toinvade the mammary gland, survive, and multiply in milk turning the mammary gland into an amenableopportunistic habitat for these bacteria. As a part of a larger study we have isolated various bacterial pathogens from mastitis affected cattle. We were able to identify four multi drug resistant *E. coli* among these bacterial pathogens which were also shown to be biofilm forming. This paper describes the characterization of these biofilm forming ESBL MPEC isolated from mastitis affected cattle in India.

Keywords: Coliform mastitis, *Escherichia coli*, extended spectrum betalactamase resistant

1. Introduction
Bovine mastitis is a globally prevalent disease affecting dairy cattle. It impacts the quantity and quality of milk produced and can lead to the loss of production animal as a whole [1]. More than 140 species of microorganisms have been identified as contributors to bovine mastitis [2]. Coliform bacteria are the most common pathogens that have been isolated from bovine mastitis [3]. *Escherichia coli* has been identified in a greater number of bovine mastitis in comparison to other coliforms. Mammary pathogenic *Escherichia coli* (MPEC) strains have virulence attributes to resist the host in nate defenses and thrive in the mammary gland environment.

Escherichia coli constitute a highly diverse group of commensal inhabitants within the gastrointestinal tract. However, due to the remarkable genomic adaptability of this organism [4, 5, 6] it has given rise to pathogenic strains capable of inducing various diseases. Among these, bovine mastitis can occur when the teat skin becomes contaminated with fecal matter. Mammary pathogenic *E. coli* (MPEC) has been identified as a newly recognized pathotype responsible for instigating mastitis in dairy animals [7, 8].

As a part of a larger study we have isolated various bacterial pathogens from mastitis affected cattle. We were able to identify four multi drug resistant *E. coli* among these bacterial pathogens which were also shown to be biofilm forming. This paper describes the characterization of these biofilm forming ESBL MPEC isolates.

2. Materials and Methods
2.1 Sample Collection
The samples described in this study were obtained as a part of a larger study of bovine mastitis. Briefly, Animals with clinical mastitis showing inflammation of udder, abnormal physical character of milk such as clot formation, discoloration; alterations in viscosity, abnormal smell or presence of blood were sampled for milk. The udder was washed with sterile water and dried with sterile towel before collection of milk in a sterile container.

The sample is stored at 4 °C and processed within 4 hours of collection.

2.2 Isolation of *E. coli*

A loopful of milk sample was streaked on nutrient agar (Himedia, India) andthen sub-cultured on selective media: HiCrome™ *E. coli* Agar (Himedia, India) All plates were incubated aerobically at 37 for 24 h. The plates were examined for colony morphology and pigmentation after 24 and 48 h.

2.3 Selection of ESBL *E. coli*

The organism cultured on *E. coli* selective media was then screend for the presence of Extended Spectrum β-Lactamase-production characteristic by subculturing on HiCrome™ ESBL Agar Base with AC3F Selective Supplement containing Ceftazidime, Cefotaxime, Ceftriazone, Aztreonam and Fluconazole

2.4 Antibiotic Susceptibility Testing

E. coli isolates suspected as ESBL producers were subsequently confirmed using anautomated microdilution system (VITEK 2, bioMérieux, Germany) according to the manufacturer's instructions. For this study, the test card ASTN289 (bioMérieux Deutschland GmbH) was used, which included the following antibiotics: piperacillin, piperacillin / tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxacin, tigecycline, fosfomycin, colistin and trimethoprim/sulfamethoxazole.

2.5 Molecular characterization

E. coli genomic DNA was extracted from bacterial cultures using the NucleoSpin® Microbial DNA (TAKARA, Japan) according to the instructions of the manufacturer. The 16s rRNA gene was amplified using universal 16S rRNA bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-GGTTACCTTGTTACGACTT-3'). PCR products were visualized on a 1% agarose gel stained with ethidium bromide under UV light to confirm the presence of a 1,350 bp band. PCR products were purified using QIAquick PCR Purification Kit prior to bi-directional sequencing using primers 27F and 1392R using an ABI PRISM 3730xl DNA Analyzer available at GenoseqDx, Chennai, India. The sequences were compared with global 16s rRNA sequences using NCBI BLAST.

2.6 Assay for biofilm formation

E. coli isolate was inoculated in Luria-Bertani (LB) liquid medium (Himedia, India) so that the colony formation unit (CFU) per milli liter (mL) was around 1×10^9 after a shaking incubation at 37 °C for 24 hours. This was diluted 1:100 in fresh LB medium and 200ul was added into 96 wells flat bottomed cell culture plates and incubated at 37 °C for 24 hours. After 24 h, the exhaust medium isaspirated, and the biofilms are washed three times in a gentle stream of tap water. The excess water was removed and then the plate was inverted on a filter paper and tapped gently to remove any remaining liquid. Crystal Violet (CV) staining solution (0.1%) is added to the sample well (100ul per well) and incubated for 20 min at room temperature on a bench rocker oscillating at 20 rpm. Then 125µL of 30% acetic acid in water is added to each well of the microtiter plate and incubated for 15 minutes to solubilize the CV. The solubilized CV is transferred to a new flat bottomed 96 well ELISA plate (Nunc, USA) and the absorbance is quantified in a plate reader at 550 nm using 30% acetic acid in water as the blank.

3. Results and Discussion

Resistance of bacteria to beta-lactam antibiotics is due to the production of beta-lactamases that hydrolytically cleave the beta-lactam ring, leading to inactivation of the antibiotic. The emergence of antibiotic-resistant bacteria in animals is often attributed to the indiscriminate use of antibiotics. Presence of antibiotic-resistant bacteria in milk and dairy products is a serious concern due to the immediate threat to human health and due to the larger public health concerns *E. coli* is a leading cause of acute clinical mastitis in dairy cattle worldwide [5, 7, 9]. *E. coli* has been identified to harbor and transmit several antimicrobial resistance genes to other pathogenic bacteria, making it a pathogen of concern in bovine masitits [10, 11].

In the present study we aimed to identify the *E. coli* mastitis in cattle and characterization of the isolates. A total of 30 milk samples were collected and processed for bacterial isolation. All of the 30 milk samples showed bacterial growth on nutrient agar. The cultures are then sub-cultured on the HiCrome™ *E. coli* Agar. Four isolates that produced blue-green coloured colonies are picked and used for further processing.

Although, HiCrome™ *E. coli* Agar is selective medium, considering the possibility of false positives, DNA was extracted from all the four blue green colony producing isolate and 16s rRNA gene of 1350 bp was amplified by PCR (Fig.1). The PCR products were purified and sequenced. The sequence data showed all the isolates to be of *E. coli* by a NCBI nucleotide BLAST analysis.

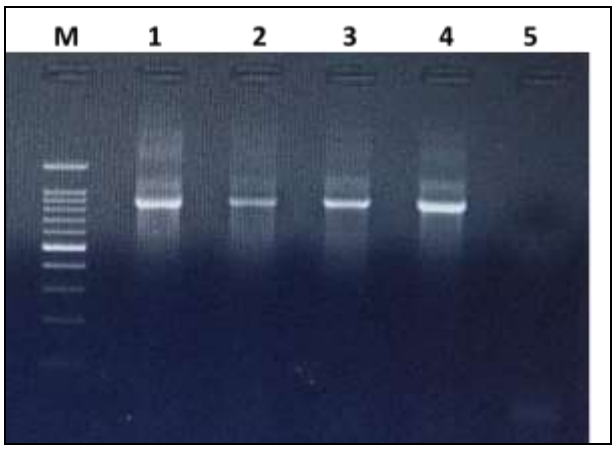

Fig 1: Polymerase chain reaction amplification of 1350bp of 16s rRNA gene of the *E. coli* isolates. Lanes 1 to 4: *E. coli* isolates, Lane 5: negative control, M: DNA Marker.

The *E. coli* isolates were characterized for their antimicrobial sensitivity in VITEK system and their sensitivity and resistance pattern are shown in Table 1. All the four isolates are characterized as ESBL producing due to their resistance to betalactam antibiotics. All the four MPEC isolates were sensitive to Imepenam, Meropenam and Amikacin. It is interesting to note that one of the isolate was also resistant to Colistin and Tigecycline demonstrating the extended spectrum of antimicrobial resistance.

The biofilm production assay showed that one out of the four *E. coli* isolates was able to produce a strong biofilm (absorbance @ λ550 nm > 0.7) while the other three were able to produce biofilms on much lower scale (absorbance @ λ550 nm 0.2 to 0.3). Biofilms are formed on tissue/ organ surface by bacterial populations encapsulated by a protective matrix of secreted carbohydrates, proteins, and DNA. Such formation in vivo helps in protection against the host immune system and also enables enhanced resistance to antibiotics. Biofilm formation within the udder will result in treatment failure and can cause recurrent infections.

Table 1: Antimicrobial sensitivity/ resistance characteristics of the *E. coli* isolates

Isolate ID	Piperaziline	Piperazilline tazobactum	Cefotaxime	Ceftazidime	Cefepime	Aztreonam	Imipenem	Meropenem	Amikacin	Gentamicin	Tobramycin	Ciprofloxacin	Moxifloxacin	Tigecycline	Fosfomycin	Colistin	Trimethoprim/ sulfamethoxazole
MPEC-1	R	I	R	R	R	R	S	S	S	R	R	R	R	S	S	S	R
MPEC-2	R	I	R	R	R	R	S	S	S	R	S	S	S	S	S	S	R
MPEC-3	R	I	R	R	R	R	S	S	S	R	R	S	R	S	R	S	R
MPEC-4	R	I	R	R	R	R	S	S	S	R	R	R	R	R	R	R	R

4. Conclusion

This study shows a 13% overall prevalence of *E. coli* in all bovine mastitis cases with bacterial etiology. *E. coli* is often related to severe clinical mastitis and some cases it has led to death of the affected animal [12]. The development of multidrug resistant bacterial strains in dairy animals has widespread public health consequences. Our results demonstrate the need for a large scale investigation into antimicrobial resistance pattern in dairy animals. Carefull and restricted use of antibiotics in animal health care is crucial to prevent such emergence of resistant organisms.

5. Conflict of Interest

Not available

6. Financial Support

Not available

7. References

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