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RESEARCH ARTICLE
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Virulence associated factors and antibiotic sensitivity pattern of *Escherichia coli* isolated from cattle and soil

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Abstract

Aim: The present study was conducted to isolate pathogenic *Escherichia coli* from the faeces of apparently healthy cattle and soil of the farms to determine their susceptibility to commonly used antibiotics.

Materials and Methods: A total of 90 samples (70 faecal and 20 soil samples) were collected aseptically and processed under required conditions for the isolation of *E. coli*. To confirm the isolates as *E. coli*, various biochemical tests like IMViC were performed. To assess the virulence of isolates, they were subjected to Congo red dye assay and hemolysis assay. Antibiotic sensitivity pattern of pathogenic isolates was studied by Disc diffusion method.

Results: The prevalence of *E. coli* was observed to be 85.71% and 20% from the faecal and soil samples, respectively. Based on the phenotypic characteristics on CT SMAC and MUG Sorbitol, none of the isolates were found to be *E. coli* O157. The percent positivity on Congo red dye assay was 44.28% for faeces and 5% for soil while only faecal *E. coli* (4.28%) were found to be positive for hemolysis assay. The antibiogram of all 35 pathogenic isolates against 8 antibiotics showed that majority of pathogenic strains exhibited high level of sensitivity to Ceftriaxone (95%), Ciprofloxacin (93%), Amikacin (90%), Gentamycin (89%) and low level of sensitivity against Ampicillin (8%) and Streptomycin (5%). All isolates were 100% resistant to Amoxicillin and Tetracycline.

Conclusion: Cattle act as main reservoirs of pathogenic *E. coli* that may enter the food chain by faecal contamination and pose potential public health hazards.

Keywords: alpha hemolysis, antibiogram, congo red dye assay, *E. coli,* virulence factor.

Introduction

Various studies have been conducted worldwide to isolate pathogenic bacteria that may be a cause of concern for human or animal health. *Escherichia coli* is such a commensal microbe which is the major part of normal aerobic microbial population of the intestine of humans and warm blooded animals. Its presence is considered as major indicator of faecal contamination in food and water [1]. Many of the strains of *E. coli* could emerge as pathogens due to the presence of certain pathogenic features and virulence genes which are located on transmissible genetic elements and this distinguishes them from ordinary commensal strains [2].

Pathogenic *E. coli* have become a significant health concern, especially *E. coli* O157:H7, which is associated with human diseases like hemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and thrombocytic thrombocytopaenic purpura (TPP). Cattle is considered to be the major reservoir of many pathogenic *E. coli* especially serotype O157:H7. The contamination of raw milk, meat and their products by cattle faeces have been the cause of major foodborne outbreaks all around the world. Antimicrobial drug resistance in bacterial isolates that have potential to enter our food supply is a growing public health concern. Antibiotics are being frequently incorporated as sub therapeutic and animal food supplements to cure and prevent disease in animals. This non judicious use of antibiotics generate a selective pressure that has led to the emergence of antibiotic resistance in the microbes including *E. coli* [3].

Considering the impact of pathogenic *E. coli* on humans, animals and its considerable public health significance, the present study was conducted to discern the virulence factors and antibiotic susceptibility profile of *E. coli* isolated from faeces of apparently healthy cattle as well as environment (soil) from dairy farms of Uttar Pradesh.

Materials and Methods

A total 90 samples comprising of 70 rectal swab samples of healthy adult crossbred dairy cows, and 20 soil samples were collected from two organised dairy farms located at Mathura and Bareilly districts of Uttar Pradesh in a period of one year from December 2012-November 2013. For the soil sample collection, 1 cm deep in to the farm soil was dug and approximately 20 gm of soil collected where animals were housed. Isolation of *E. coli* was done as per the method described by

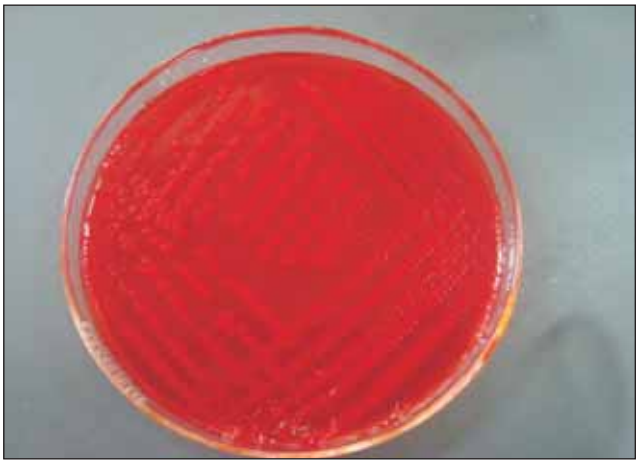

Figure-1: *E. coli* showing brick red colonies on Congo red medium

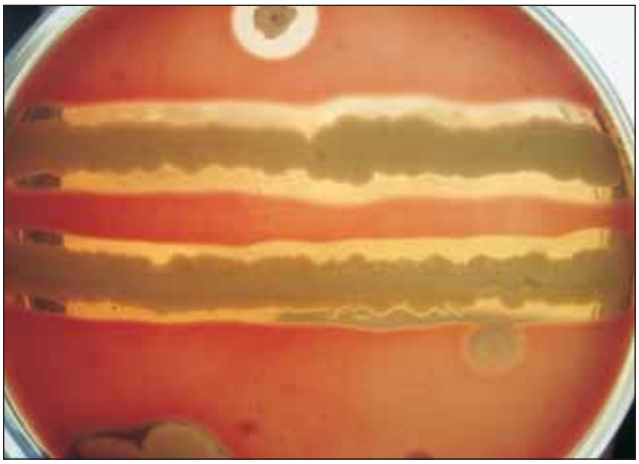

Figure-2: *E. coli* showing alpha hemolysis on washed sheep blood agar

Merchant and Backer [4] with slight modification. One gm of faecal samples and 20 gm of soil sample were collected aseptically and enriched with 10 ml and 90 ml trypticase soya broth (TSB) (containing 1mg/100 ml of acriflavin) respecti-vely for 18 hrs at 37 °C. Sub culturing was done using Mac Conkey's Agar (MLA) plates. Lactose fermenting, small, pink colonies were transferred to Eosin Methylene Blue (EMB) Agar plates for demonstration of characteristic "green metallic sheen" following incubation for 24 hrs at 37°C. Different biochemical tests like indole, methyl red, voges- proskauer, citrate, nitrate reduction, oxidase and sugar fermentation were performed by kit (KB010 Hi *E. Coli* identification kit, Himedia, India).

Phenotypic characterisation of isolates: Biochemically confirmed *E. coli* isolates were streaked over CT- SMAC (Cefixime tellurite sorbitol MacConkey Agar), and kept for incubation at 37°C for 24 hrs. *E. coli* O157:H7 produced sorbitol-non fermenting colorless colonies while non O157 fermented the sorbitol and appeared pink on this medium [5]. The isolates were also streaked on MUG sorbitol agar which differentiates *E. coli* O157 from non O157 [6]. *E. coli* O157:H7 which is glucuronidase negative did not show fluorescence while other *E. coli* fluoresced under UV light due to hydrolysis of 4-methyl-umbelliferyl-β-D glucuronidase (MUG) by the enzyme glucuronidase.

In- vitro* pathogenicity test of *E. coli

Congo red (CR) dye binding assay: Congo red (CR) dye binding assay is used as a phenotypic marker to distinguish between virulent and avirulent strains of *E. coli.* All isolates were streaked on Congo red agar medium comprising trypticase soya agar enriched with 0.05% Congo red dye and 0.15% bile salts and incubated at 37°C for 24 hrs. The plates were further incubated at room temperature for an additional 48 hrs. The CR positive *E. coli* isolates produced brick red colonies (Figure-1) which became wrinkled after 48 hrs at room temperature while CR negative *E. coli* did not bind the dye and produced white colonies [7]. Verotoxic *E. coli* serotype O26 and strain ATCC 25992 were used as positive and negative controls respectively, for this assay.

Hemolysis assay: The ability of certain *E. coli* strains to lyse erythrocytes of mammalian species is termed as hemolysis [8]. Four different types of hemolysins viz alpha, beta, gamma and enterohemolysin (E-hly) are produced by different pathogroups of *E. coli.* Alpha hemolysin (Hly), considered as a prototype of a large family of pore-forming toxins, named repeat in toxin (RTX), is a cell free factor which causes clear zone around colonies on blood agar plates within 4-8 hrs of incubation [9]. To assess the haemolytic properties of *E. coli* isolates, all were streaked on 5% washed sheep blood agar supplemented with 10 mM $CaCl_2$. *E. coli* serotype O34 and strain ATCC 25992 were used as positive and negative controls respectively, for this assay. Positive isolates produced clear zone of hemolysis (Figure-2).

Antimicrobial sensitivity of *E. coli:* *In-vitro* antibiogram of all the pathogenic isolates was performed by Disc diffusion method [10] on Muller Hinton agar. Isolates were tested against 8 commonly used antibiotics *viz.* Amikacin 10µg, Ampicillin 10µg, Amoxicillin 10µg, Cefotaxime 5µg, Ciprofloxacin 10µg, Gentamycin 10 µg Streptomycin 10 µg and Tetracycline10µg. Zones of complete inhibition were measured according to standards of Clinical Laboratory Standard Institute (CLSI). *E. coli* Strain ATCC 25992 was used as a negative control.

Results

Out of 70 faecal and 20 soil samples which were screened for isolation of *E. coli,* a total of 64 isolates (60 from faeces and 4 from soil) were obtained which were biochemically confirmed. The prevalence of *E. coli* was found to be 85.71% and 20% from the faeces and soil, respectively. For the isolation of pathogenic *E. coli* serotype O157, all 64 isolates were streaked over CT SMAC and resulting colorless colonies were further transferred to MUG sorbitol. None of the isolates

Table-1. Antimicrobial susceptibility pattern of *E. coli* isolate from cattle faeces and soil

Antibiotic	No. of isolates tested	Sensitivity %	Resistance %
Amikacin (AK)	35	90	10
Ampicillin(AMP)	35	8	92
Amoxycillin(AMX)	35	0.0	100
Ceftriaxone(CTR)	35	95	5
Ciprofloxacin(CIP)	35	93	7
Gentamycin(GEN)	35	89	11
Streptomycin(S)	35	5	95
Tetracycline(TE)	35	0.0	100

were found to be non-fluorescent on MUG sorbitol showing a zero prevalence of O157. To assess the *in-vitro* pathogenicity, all 64 isolates were subjected to Congo red dye binding (CR) and hemolysis assay. A total of 35 isolates (31 faecal and 4 soil isolates) were positive on CR assay while on sheep blood agar only 3 faecal isolates and none from soil produced the desired clear zone of alpha hemolysis. The percent positivity of CR assay was 44.28% for faeces and 5% for soil while only faecal *E. coli* (4.28%) were found to be positive for hemolysis assay. The result of antimicrobial sensitivity pattern of all 35 pathogenic isolates against 8 commonly used antibiotics are presented in Table-1. The majority of pathogenic strains exhibited a high level of sensitivity to Ceftriaxone, Ciprofloxacin, Amikacin and Gentamycin and low level of sensitivity against Ampicillin and Streptomycin. All the isolates showed 100% resistance to Amoxicillin and Tetracycline.

Discussion

In the present study prevalence of *E. coli* in cattle faeces was reported to be 85.71% while it was found to be 20% in soil. Similar results were obtained by Fluckey *et al.* [11], while lower prevalence of *E. coli* was found by Elseisy *et al.* [12] and Ahmed *et al.* [13]. The variation in the prevalence of *E. coli* in various studies might be due to the influence of different factors like age, sex, breed, place and probably seasons. A wide range of prevalence (0.2 to 48.9%) of O157 from cattle faeces has been reported worldwide [14]. In the present study, prevalence of O157 was found to be zero which is almost similar to the lower limit of wide range of prevalence (0.2 to 48.9%) of O157 reported from cattle faeces worldwide. Thran *et al.* [15] and Scott *et al.* [16] also reported the zero prevalence of O157 in cattle faeces.

Congo red dye binding (CR) assay could be used as phenotypic marker to distinguish invasive and non invasive strains of *E. coli*. CR assay had been studied by various workers [7, 17,18] and all of them found 50% isolates from poultry and environmental sources to be positive for this assay. In the present study, 44.28% and 5% isolates from cattle faeces and soil, respectively, were found to be CR positive. There are only few studies on CR assay of *E. coli* of bovine origin. Sharma *et al.* [19] found 47.42% positive value which was in accordance to our study while a much higher value (89%) was obtained by Elseisy *et al.* [12] and a much lower positivity (20.4%) was obtained by Kalorey *et al.* [20].

Alpha-hemolysin (Hly) is a common exotoxin produced by *E. coli* that enhances virulence in a number of clinical infections of animals [21]. It is a cell free haemolytic factor which produces large clear zones of haemolysis often apparent after 4 hrs of incubation on washed or unwashed sheep blood agar [22]. The alpha-haemolysin has activity against human lymphocytes [23]. Enterohemolysin (E-hly) generally produced by VTEC group of *E. coli* are pathogenic to humans. There is approximately 60% relatedness between E-hly and (Hly) and both haemolysins belong to the RTX family of toxins [23]. In this study, alpha hemolysis was produced by 4% isolates of faecal origin and none of the soil isolates showed this characteristic. The findings are in agreement with various worker Lorenz *et al.* [24] and Shekh *et al.* [25] showed 2.8% haemolytic activity while high haemolytic activity observed in study of Elseisy *et al.* [12] as 34%.

All 35 pathogenic *E. coli* isolates from faeces and soil samples subjected to antimicrobial sensitivity profile, showed high resistance to Ampicillin, Amoxicillin, Streptomycin and Tetracycline. The findings of present study are in agreement with earlier reports [3, 26, 27]. High level of sensitivity was reported to Amikacin, Ciprofloxacin, Ceftriaxone and Gentamycin this was in accordance to the finding of Romanus *et al.* [28] and Zinnah *et al.* [29], while many workers have found contrasting results as in the studies of Fluckey *et al* [11] and Joshi *et al.* [30]. This increasing resistance pattern of *E. coli* may be attributed to the over use and non judicious use of various antimicrobials. Commensal *E. coli* face various selective pressures in the environments of intestine which further favours the development, persistence and dissemination of robust strains that may be resistant to antimicrobial agents.

Conclusion

Pathogenic *E. coli* isolated from cattle faeces and soil may contaminate the food chain and may be a source of major public health hazards. Antibiotic resistant organisms that are constantly shed into the environment through the faeces of apparently healthy cattle may cause serious antibiotic resistant infections to human beings. So decisive efforts must be made to maintain hygienic conditions at dairy farms and at food processing plants. The high prevalence of antimicrobial resistance among strains of *E. coli* depicts the immense need for strict measures to regulate the use of antimicrobials within the food chain.

Author's contributions

Parul, BB, BS and UJ executed the study design and analysed the data. Parul performed the entire study under the guidance of BB. Parul drafted and revised the manuscript with the help of BS and UJ. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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