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Research Article

MOLECULAR CHARACTERIZATION AND ANTIBIOGRAM OF ESCHERICHIA COLI ISOLATES IMPLICATED IN CALF MORTALITY

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ABSTRACT

Blood and tissues from 52 carcasses of buffalo calves and cow calves were collected during post-mortem examination to study the incidence of *Escherichia coli* infection from June, 2007 to March, 2008. A total of 23 *E. coli* isolates were obtained from liver, lung and blood. Various serotypes of *E. coli* identified were O60, O20, O114, O164, O4 and O12. *In-vitro* drug sensitivity pattern revealed that the isolates were most sensitive to a combination of ceftriaxone and sulbactum and were most resistant to oxytetracycline and streptomycin. The isolates were confirmed as *E. coli* by polymerase chain reaction assay as a band of 232 bp was observed in all 23 isolates. Of the 19 *E. coli* isolates tested for the presence of plasmid, 18 isolates possessed plasmids of molecular weights ranging from 4-20 kb. The serotypes resistant to one or more than one antimicrobials were found to possess plasmids of high molecular weight (20 Kb).

Key words: *Escherichia coli*, serotype, plasmid profiling, polymerase chain reaction assay

Diseases of bovine calves are known to cause heavy financial losses to dairy industry not only because of mortality but also from decreased productivity of recovered calves, their limited scope for their selection as breeding animals, delay in rebuilding of herds, loss of lactation etc. Thus to attain maximum gains from livestock industry, it is important that young calves should be reared into healthy and productive animals. Neonatal calf mortality has been reported to be very high in cattle and buffalo (Khan and Khan, 1991). Various causes of bovine calf mortality are bacterial diseases (colibacillosis, salmonellosis, pasteurellosis etc.), viral diseases (rotavirus infection), nutritional disorders and pathological conditions (hepatitis, pneumonia). *Escherichia coli* (*E. coli*) infection is one of the major diseases involved in the calf mortality (Merchant and Packer, 2002; Bhardwaj and Chugh, 1988). The present work was undertaken to study molecular characterization and antibiogram of *E. coli* isolates from bovine calves.

MATERIALS AND METHODS

The study was conducted on 52 bovine calves (35 buffalo calves and 17 cow calves) carcasses during June, 2007 to March, 2008. During post-mortem examination, blood sample from each carcass was collected from heart for bacteriological examination. Pieces of liver and lungs were also collected in sterile petridish for bacteriological examination.

Isolation of *E. coli*: Blood and tissue samples were inoculated on to blood agar and Mc-Conkey's lactose agar (MLA) plates (Cruickshank *et al.*, 1965). The inoculated plates were incubated at 37°C for 24-48 hours and examined for the presence of bacterial growth, if any. Blood agar plates were also observed for the presence of haemolysis. Primary identification of the bacterial growth was done by colony morphology and Gram's staining. The *E. coli* isolates were subjected to different biochemical tests such as IMViC (Indole, Methyl Red, Voges Pro-Skaur and citrate utilization test), nitrate reduction test, urease test and hydrogen sulphide production test on triple sugar iron medium for further characterization (Cruickshank *et al.*, 1965).

Serotyping: The *E. coli* isolates were got serotyped from the Central Research Institute, Kasauli.

***In-vitro* Chemotherapeutic Sensitivity:** Chemotherapeutic sensitivity was performed as per the disc-diffusion method (Bauer *et al.*, 1966).

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Concentration of various drugs (mcg) used in *in-vitro* chemotherapeutics sensitivity test were: streptomycin, enrofloxacin and gentamicin (10 each); oxytetracycline, neomycin, kanamycin, amikacin, cefotaxime and ceftazidime (30 each); and cefatriaxone+sulbactum (30+15) and furoxone (100). The plates were then incubated at 37°C for 18-24 hours and observed for sensitivity by measuring the zones of inhibition. Results were noted as sensitive (S) and resistant (R) on the basis of the table provided by the manufacturer for zone size interpretation.

Plasmid Profiling of *E. coli*: Plasmid DNA from 19 *E. coli* isolates was extracted using alkaline lysis method (Sambrook and Russel, 2001). Plasmid DNA (10 µl) was then electrophoresed on 0.7% agarose gel in 0.5 X Tris borate EDTA (TBE) buffer using 1Kb DNA ladder (Fermentas) as a marker.

DNA Extraction: DNA was extracted by rapid boil and chill method (Medici *et al.*, 2003). For this, a single colony from overnight grown culture of *E. coli* isolates was inoculated in 25 µl of Tris EDTA buffer, boiled at 99°C for 15 minutes and then cooled immediately by putting on ice, the resultant template DNA was stored at –20°C till further use.

PCR Assay: Forward and reverse oligonucleotide primers were 5'ATCAACCGAGATTCCCCCAGT3' (Eco 223) and 5'TCACTATCGGTCAGTCAGGAG3' (Eco 455) (Riffon *et al.*, 2001). The PCR reaction was performed in a thermalcycler (Biorad cycler, USA). PCR reaction mixture contained 200 M dNTPs mix, 1X PCR buffer, 1.5 mM MgCl2, 20 pmol of each primers, 2.5 U Taq DNA polymerase (Fermentas), 5 µl DNA to make total volume 25 µl. Nuclease free water was taken as a negative control. PCR amplification was done with initial denaturation at 94°C for 45 seconds, annealing at 64°C for one minute and extension at 72°C for 2 minutes (35 cycles) followed by a step of final extension at 72°C for 10 minutes.

Analysis of PCR Products: After amplification, 10 µl of the amplified PCR products were subjected to electrophoresis in 1.5% agarose gel in 0.5 X TBE (Amresco) containing ethidium bromide (0.2g/ml). A 100 bp gene ruler (Fermentas) was used as a marker.

RESULTS AND DISCUSSION

Isolation of *E. coli*: *E. coli* organisms were isolated from 11 bovine calves (nine buffalo calves and two cow calves). A total of 23 *E. coli* isolates were obtained from liver (11), lung (4) and blood (8) indicating that they might have caused bacteraemia/septicaemia. It is worth to mention that in these cases, lesions were seen in a number of organs indicating systemic infection (Lehreena, 2008). Various workers (Joon and Kaura, 1993; Verma *et al.*, 2001) have also reported isolation of *E. coli* from the carcasses of bovine calves.

Serotypes Detected: Various serotypes of *E. coli* were O60 (4), O20 (3), O114 (2), O164 (1), O4 (1) and O12 (1) (Table 1). Remaining six isolates were untyped. In most cases, the serotype was same in the isolates of the same animal except one case in which isolate from lung was serotyped as O12 whereas isolate from liver was untyped. Major serotypes isolated were O60 and O20 followed by O114. In a cow-calf there was only one serogroup i.e. *E. coli* O60 was isolated from liver and blood. Most of these serotypes have been reported to be pathogenic for domestic animals and poultry including Haryana state (Jindal *et al.*, 1999; Naveda *et al.*, 2000; Kanwar *et al.*, 2005). Nevertheless, pathological lesions noticed in various organs of bovine calves associated with *E. coli* infection in this study also support pathogenic nature of these serotypes. Calf mortality at the farms which were the source of the present study, too revealed a number of serotypes of *E. coli* associated with enteritis and hepatitis but only two serotypes namely O12 and O20 were common with the present study (Singh, 2005) indicating the introduction of new serotypes in the herd. The serotype O114 has not been reported from diseased bovine calves. However, it has been reported in healthy chickens in the eastern province of Saudi Arabia (Ghamdi *et al.*, 1999). Furthermore, *E. coli* belonging to serotypes O114 and O164 are considered to be enteropathogenic (EPEC) and enteroinvasive (EIEC) in human beings, respectively (Scotland *et al.*, 1981; Todorova *et al.*, 1990). Thus, these two serotypes of *E. coli* isolated from buffalo calves are important from public health point of view.

***In-vitro* Drug Sensitivity:** The *E. coli* isolates were

Table 1
Serotypes and plasmid profile of *E. coli* isolates from bovine calves

Specimen	Serotype	No. of plasmids possessed	Molecular size (kb)
Liver	O60	4	20, 10, 7, 5
Blood	O60	4	20, 10, 7, 5
Liver	O60	3	20, 10, 5
Lung	O60	3	20, 10, 5
Liver	UT	5	20, 10, 7, 5, 4
Blood	O12	1	10
Liver	O164	3	20, 10, 7
Liver	O4	3	20, 10, 7, 5
Lung	O12	3	20, 10, 7, 5
Liver	UT	Nil	Nil
Liver	UT	2	20,10
Lung	UT	1	20
Liver	UT	1	20
Blood	UT	1	20
Lung	O114	1	20
Liver	O114	1	20
Liver	O20	1	20
Lung	O20	1	20
Liver	O20	1	20

UT=Untypable

found most sensitive to ceftriaxone+sulbactum (95.6%) followed by gentamicin (91.3%), amikacin (91.3%), kanamycin (91.3%), neomycin (86.95%), cefotaxime (82.9%), ceftazidime (82.9%) and furoxone (65.2%). The isolates were found most resistant to oxytetracycline (90.5%) and streptomycin (71.5%). The resistance of *E. coli* to various chemotherapeutic drugs ranged from 4.8 to 90.5%. The results of the present study are similar to those reported by other workers in calves and sheep (Singh *et al.*, 2006; Sharma *et al.*, 2007). A significant increase in the resistance of *E. coli* isolates to tetracycline, ampicillin,

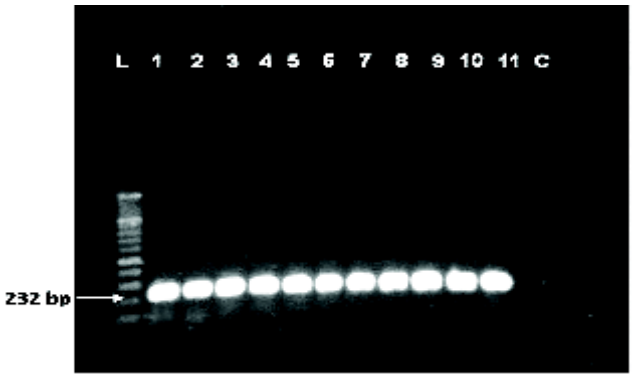

Fig 1. PCR analysis of *E. coli* isolates obtained from liver, lung and blood samples in bovine calves. L=100bp ladder; Lanes=1-11 *E. coli* isolates from clinical samples; C=Negative control

oxytetracycline, doxycycline and streptomycin in both humans and animals including chicken had earlier been reported (Ghamdi *et al.*, 1999, Olewa *et al.*, 2008). Higher resistance of these antibiotics might be attributed to their widespread use. Furthermore, plasmid mediated drug resistance in *E. coli* isolates in human beings has been reported (Wang *et al.*, 2003; Olewa *et al.*, 2008). Plasmid profiling revealed that *E. coli* isolates showing multiantibiotic resistance possessed medium to large sized plasmids indicating increase in plasmid associated resistance.

Plasmid Profiling: Out of 19 *E. coli* isolates, 18 isolates possessed plasmids (Table 1). Molecular weights of the plasmids isolated ranged 4 to 20 Kb. Some isolates possessed single sized plasmids while the other had multiple plasmids of different sizes. Number of plasmids in isolates from liver ranged from 1 to 5 with size of 20, 10, 7, 5, 4 Kb. Whereas in the lung, the number varied from1 to 3 with size of 20, 10, 5 Kb and in blood, it was 1 to 4 with size 20, 10, 7, 5 Kb. The antibiogram of all the *E. coli* isolates was compared with their plasmid profile (Table 2). Plasmid size of 20 Kb was found in all isolates except one. The serotype which was resistant to one or more than one antibiotics were found to possess plasmids of high molecular weight i.e. 20 Kb. Antibiotic resistance patterns revealed that over 94.7% of the isolates showing multidrug resistance possessed medium to large sized plasmids (7, 10, 20kb). These results coincide with the findings of Smith *et al.* (2003). This indicated that animals could be a source of dissemination of plasmid resistant *E. coli* in the environment. Furthermore, 60.7% of *E. coli* isolates from different human clinical samples were found to harbour single sized to multiple sized plasmids with size ranging from 6.557 to 23.222 kb. Nevertheless, Todorova *et al.* (1990) reported that 92% of *E. coli* serotype O164 strains possessed two small plasmids of molecular weights 9.06 and 7.248 Kb.

PCR Assay: Results of PCR assay revealed that 20 samples were confirmed as *E. coli* yielding an amplified product of 232bp, whereas one of the samples was negative for *E. coli* (Fig. 1). PCR would be the better method for identification of bacterial organisms like *E. coli* in subclinical cases. Furthermore, the major advantage of PCR lies in the possibility of the elimination

Table 2

Comparison of antibiogram of *E. coli* isolates with their plasmid profiles

Resistance pattern	Frequency of occurrence	No. of plasmids	Plasmid size (kb)
S,O,G,K,Ak,Cl,Ce	1	2	20,10
S,O,N,K,Ak,Fx	1	3	20,10,7
S,O,Ex,Fx	1	3	20,10,5
S,O,N,Fx	1	1	10
S,O,Ex	3	1	20
S,O	2	1	20
S,Ex,G,Fx	1	4	20,10,7,5
O,N,Ex,Ca,Fx	1	5	20,10,7,5,4
O,Ex,Ce,Ca	3	1	20
O,Ex	2	3	20,10,7
O,Fx	2	4	20,10,7,5
Fx	1	3	20,10,7

S=Streptomycin; O=Oxytetracycline; G=Gentamicin; K=Kanamycin; Ak=Amikacin; N=Neomycin; Ex=Enrofloxacin; Fx=Furoxone; Ce=Cefotaxime; Ca= Ceftazidime; Cl=Ceftriaxone+Sulbactum

of culture, rapidity and easy analysis.

The present study thus revealed that *E. coli* infection was the major infection involved in the calf mortality. Ceftriaxone+sulbactum, gentamicin and kanamycin were found to be the most effective drug against *E. coli.* Plasmid profiling of *E. coli* revealed that the isolates showing multiantibiotic resistance possessed medium to large sized plasmids indicating increase in plasmid associated resistance genes.

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