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VIRULENCE GENES AND ANTIMICROBIAL RESISTANCE PROFILE OF *Escherichia coli* ISOLATES FROM DIARRHOEIC NEONATAL DROMEDARY CAMELS

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ABSTRACT

In the present study, *Escherichia coli* were isolated from rectal swabs of total 48 (20.68%) diarrhoeic neonatal dromedary camels during the five years study period in an organised farm. The PCR for amplification of virulence genes revealed that 31 (64.58%) isolates harboured at least one virulence gene. The detection rates of *stx1, stx2, eae, F41, K99* and *sta* virulence genes were 4.16%, 2.08%, 35.41%, 14.58%, 18.75% and 16.66%, respectively. Based on occurrence of these virulence genes the isolates were pathotyped into shigatoxigenic *E. coli* (STEC) (6.25%), enteropathogenic *E. coli* (EPEC) (20.83%) and enterotoxigenic *E. coli* (ETEC) (29.16%). Atypical combinations of EPEC+ETEC (8.33%) were also detected. The *E. coli* isolates from all three neonatal camels having acute haemorrhagic enteritis and mortality were found to be of STEC type. In antibiotic sensitivity test, most prevalent resistance was observed against amoxicillin, cloxacillin, erythromycin and lincomycin whereas lowest resistance was observed against gentamicin and amikacin. Findings of this study indicate that neonatal camels are the probable reservoir of multidrug resistant and zoonotic STEC. Young age (below 7 days), housing system with loose sandy ground and winter season were identified as important risk factors for high incidence of neonatal camel calf diarrhoea in the present study.

Key words: Antimicrobial resistance, camel, *Escherichia coli*, neonatal diarrhoea, PCR, virulence genes

The occurrence of neonatal diarrhoea or colibacillosis caused by *Escherichia coli* in neonatal camels is one of the important infectious diseases as it incurs significant economic losses due to high morbidity and mortality rate resulting from severe diarrhoea and septicemia (Mohammed *et al*, 2003). It was believed that strains of *E. coli* colonise the host's intestine with different virulence factors and induce diarrhoea by escaping the immune system (Cho and Yoon, 2014; Desvaux *et al,* 2020). *E. coli* causing neonatal calf diarrhoea were mainly pathotyped on the basis of presence of virulence factors as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and shigatoxigenic *E. coli* (STEC) which include subgroup enterohaemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and enteroadherent *E. coli* (EAdEC) (Andrade *et al,* 2012). These pathotypes have mostly been related to mild to severe diarrhoea causing high rates of morbidity and mortality particularly during the early neonatal period in calves (Andrade *et al,* 2012). Each diarrhoeagenic *E. coli* pathotype represents a collection of strains that possess similar virulence factors and cause similar diseases with similar pathology (Robins-Browne *et al,* 2016). The STEC is considered as zoonotic pathogen causing haemorrhagic colitis and haemorrhagic uraemic syndrome in human beings (Nataro and Kaper, 1998). The pathogenicity of STEC is mediated mainly through Shiga toxins 1 and 2 encoded by stx1 and stx2 genes, respectively (Paton and Paton, 1998). The pathogenicity of ETEC is attributed to the expression of fimbrial antigens F41, F5 and F17, and the elaboration of one or more enterotoxins like heat-stable enterotoxins (sta) and heat-labile enterotoxins (LT) (Ryu *et al,* 2020). The EPEC pathotype involved in young calf diarrhoea and dysentery induce attaching and effacing (AE) lesions on intestinal cells due to the production of the protein intimin (eae) (Mainil and Fairbrother, 2014).

During the past decade, drug resistance in enterobacteriaceae has increased worldwide which is considered as potential threat for public health (Prestinaci *et al,* 2015). Since *E. coli* is present as gut commensal in humans and animals, it has become one of the microorganisms that are commonly resistant to

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antimicrobials due to the selective pressure imposed by the antimicrobial drugs used in the treatment of food animals and humans (Zhao *et al,* 2012). In present study, characterisation of *E. coli* pathotypes isolated from diarrhoeic neonatal dromedary camels and their antimicrobial resistance pattern was investigated.

Materials and Methods

Animals, sample collection and pathological studies

This study was conducted during the period from 2016 to 2020 in an organised dromedary camel farm situated at Bikaner, India which is an arid region within Thar desert. The neonatal camels of the farm were kept along with their dam in a corral having loose sandy soil with free access to colostrum since birth. The faecal swabs were directly collected from the rectum of the diarrhoeic neonatal camels using sterile cotton swabs for bacterial culture. These calves were not given any antimicrobials before sampling. The faecal samples were also collected in sterile containers (sterile clinicol™, Himedia, India) and investigated for presence of oocysts of coccidia and cryptosporidia and eggs of helminths using direct wet mount examination method. The necropsy was performed on 3 diarrhoeic neonatal camels having mortality and tissue samples from internal organs were collected in 10% formal saline for histopathology. The formalin fixed tissue samples were embedded in paraffin, cut into 4–5 micron sections using a semi automatic microtome (Wewox®, India) and stained with haematoxylin and eosin (HE) stain.

Bacterial culture and antimicrobial susceptibility testing

The rectal swab samples collected from diarrhoeic neonatal camels were inoculated on MacConkey and Eosin Methylene Blue (EMB) agar media and incubated at 37 °C for 18-24 hours. These *E. coli* colonies were further cultured on Mueller Hinton agar for determination of antimicrobial susceptibility using the agar disc diffusion method (Humphries *et al,* 2018). The antibiotic discs were chosen keeping in mind their common use in livestock farming in India. The antibiotic discs (HiMedia, India) included in the present study were: amoxicillin (AMX) (10µg), cloxacillin (COX) (5µg), cefotaxime (CTX) (30µg), tetracycline (TE) (30µg), doxycycline hydrochloride (DO) (30µg), erythromycin (E) (15µg), lincomycin (10µg), gentamicin (GEN) (10µg), amikacin (AK) (30µg), streptomycin (S) (10µg), amoxicillin/ sulbactum (AMS) (30/15µg), trimethoprim (TR) (5µg), ciprofloxacin (CIP) (5µg), enrofloxacin (EX) (10µg), ceftriaxone (CTR) (30µg) and chloramphenicol (C) (30µg). The diameter of the zones of inhibition was measured by antibiotic zone scale™ (HiMedia, India) and the zones were graded as sensitive and resistant to the drugs tested by referring to Zone Size Interpretative Chart (HiMedia, India) in accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, Clinical and Laboratory Standards Institute (CLSI) (Humphries *et al,* 2018). Multiple-antibiotic resistance was defined as resistance to 2 or more antibiotic classes.

DNA extraction and PCR for detection of virulence genes

Pure and characteristic lactose fermenting pink coloured colonies from MacConkey agar were selected for DNA extraction using Mericon™ DNA bacteria kit (Qiagen, Germany). The PCR for molecular identification of *E. coli* isolates was carried out using the primer sequence targeting uidA gene encoding β- glucuronidase of *E. coli* to amplify a 486-bp fragment (Heininger *et al*, 1999). For detection of different virulence genes (*stx1, stx2, eae, K99, F41* and *sta*), multiple PCR was performed using different primer sets (Table 1) and PCR cycling conditions described previously (Franck *et al,* 1998).

Results

Incidence, clinical and pathological findings

In the present study, *E. coli* was isolated from rectal swab samples of 48 (20.68%) diarrhoeic neonatal camels over the period of 4 years with case fatality rate of 6.25%. No significant difference was found in occurrence of diarrhoea in male and female neonatal camels. No evidence of any parasitic infection was found in any of the faecal samples collected from these diarrhoeic neonatal camels. An association between age of neonatal camels and occurrence of diarrhoea was observed. The incidence of diarrhoea was appreciably more in camels of age group below 7 days (n=41) compared to camels of age group 7 days and above (n=7) (Table 2). The important clinical signs in diarrhoeic neonatal camels were profuse, foul-smelling, yellow to pale yellow or greenish, watery to pasty diarrhoea soiling the tail and hindquarters. In 3 neonatal camels having mortality, the severity of the symptoms were more pronounced and characterised by anorexia, weakness and mucous and blood mixed faeces.

The necropsy of these neonatal camels showed gross lesions of dark red diffusely congested small and large intestinal mucosa with presence of moderate amount of mucous and blood mixed contents in the lumen (Fig 1). The abomasum also showed dark red congested mucosa with blood mixed contents. The liver was enlarged and showed multifocal pale areas throughout its surface. Kidneys were enlarged with moderate to severe congestion. The other organs were not showing any significant gross changes. Histopathology of small intestine revealed areas of desquamation with presence of free epithelial cells in lumen, mucosal epithelial necrosis, hyperemia of the villi, villus stunting and fusion, and mild to moderate infiltration of eosinophils in the lamina propria and crypt region (Fig 2). The submucosa showed mild to moderate thickening, oedema and dilatation and congestion of submucosal capillaries. Histopathology of liver showed prominent vacuolar degenerative changes in hepatocytes with congestion of central vein and sinusoidal capillaries. Histopathology of kidney showed congestion of glomerular capillaries and occasionally atrophied and distorted glomeruli.

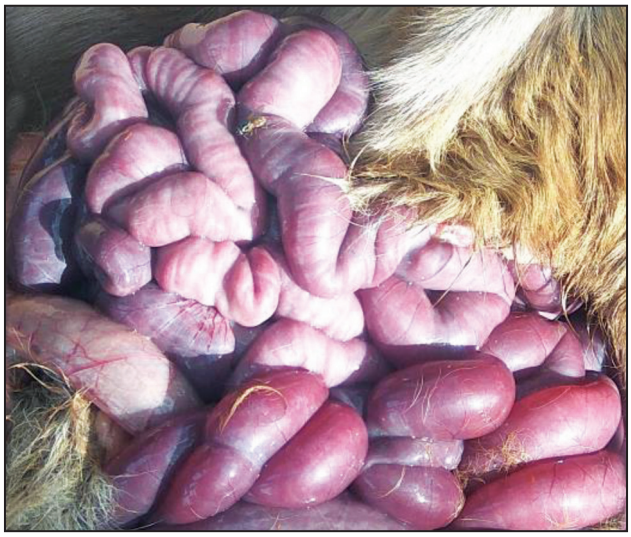

Fig 1. Moderate to severely congested small intestinal loops.

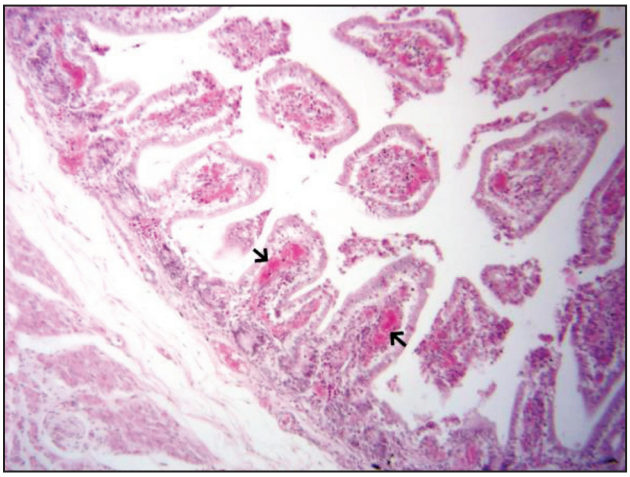

Fig 2. Histopathology of small intestine showing desquamation of villous epithelium and atrophied villi with hyperemic blood vessels (arrow). HE X 100.

Table 1. Details of primers used for amplification of different virulence genes.

Pathotype	Target virulence gene	Oligonucleotide sequences (5′-3′)	Product size (bp)	References
STEC	*stx1*	F: TTCGCTCTGCAATAGGTA R: TTCCCCAGTTCAATGTAAGAT	555	Paton *et al*, 1995
	stx2	F: GTGCCTGTTACTGGGTTTTTCTTC R: AGGGGTCGATATCTCTGTCC	118	Paton *et al*, 1993
EPEC	*eae*	F: ATATCCGTTTTAATGGCTATCT R: AATCTTCTGCGTACTGTGTTCA	425	Yu and Kaper, 1992
ETEC	*F41*	F: GCATCAGCGGCAGTATCT R: GTCCCTAGCTCAGTATTATCACCT	380	Fidock *et al*, 1989
	K99	F: TATTATCTTAGGTGGTATGG R: GGTATCCTTTAGCAGCAGTATTTC	314	Roosendaal *et al*, 1984
	sta	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	190	Sekizaki *et al*, 1985

Table 2. Summary of age, sex and result of virulence gene PCR in diarrhoeic neonatal camels.

Total camels	Sex		Age (days)		Result of virulence gene PCR					
	Male	Female	<7 days	≥7 days	*stx1*	*stx 2*	*eae*	*F41*	*K99*	*sta*
48	21	27	41	7	2 (4.16%)	1 (2.08%)	17 (35.41%)	7 (14.58%)	9 (18.75%)	8 (16.66%)

Table 3. Summary of virulence gene profile and pathotypes of *E. coli* isolates from diarrhoeic neonatal camels.

Pathotype	Virulence genes	No. of isolates (%)	Total No. of isolates (%)
STEC	*stx1+eae*	2 (4.16%)	3 (6.25%)
	stx2+eae	1 (2.08%)	
EPEC	*eae*	10 (20.83%)	10 (20.83%)
ETEC	*F41*	5 (10.41%)	14 (29.16%)
	K99	1 (2.08%)	
	K99+ sta	6 (12.5%)	
	sta	2 (4.16%)	
Mixed (EPEC+ETEC)	*eae+ F41*	2 (4.16%)	4 (8.33%)
	eae+ K99	2 (4.76%)	
None	None	17 (35.41%)	17 (35.41%)

Distribution of virulence genes and pathotypes

The screening for presence of virulence genes in *E. coli* isolates from diarrhoeic neonatal camels revealed 31 (64.58%) isolates possess one or more virulence genes, whereas in 17 (35.41%) of the isolates no virulence genes could be detected and considered non-pathogenic (Table 3). The individual incidence of virulence genes *viz.*, stx1, stx2, eae, F41, K99 and sta in *E. coli* isolates were 2 (4.16%), 1 (2.08%), 17 (35.41%), 7 (14.58%), 9 (18.75%) and 8 (16.66%), respectively (Table 2). Based on the distribution of virulence genes, the *E. coli* isolates were pathotyped as STEC (6.25%), EPEC (20.83%), ETEC (29.16%) and mixed type (8.33%) (Table 3). The *E. coli* isolates from all the 3 neonatal camels having acute haemorrhagic enteritis and mortality were found to be of STEC type.

Antimicrobial resistance

The antimicrobial resistance profiles of all *E. coli* isolates against the tested antibiotics are summarised in Table 4.

Discussion

In this study, a high incidence of neonatal camel calf diarrhoea was observed which was mainly attributed to unhygienic living conditions, overcrowding and damp floors which support and shelter infectious agents (Cho and Yoon, 2014). Since camels are seasonal breeders, maximum calving in India usually took place in winter season particularly in the months of January and February. Moreover, the calving shed of the present study had loose sandy soil in which wet conditions prevailed for longer time due to winter months. Highest prevalence rate of *E. coli* infection during winter season was also recorded in cattle and buffalo calves in earlier studies (Awad *et al,* 2020). The neonatal calves below 7 days showed maximum incidence of calf diarrhoea which was mainly attributed to the non appearance of humoural immunity and pessimistic response to cell mediated immunity in calves (Mohan *et al,* 1990). The clinical signs of yellowish or greenish diarrhoea with occasional presence of mucous and blood in *E. coli* infected camels of the present study were in agreement with camel calf diarrhoea cases reported earlier (Yeshiwas and Fentahun, 2017). Similarly, the pathological lesions of acute enteritis with desquamation of mucosal epithelium and atrophy of villi were frequently reported in intestine of diarrhoeic calves due to *E. coli* infection (Awad *et al,* 2020).

Table 4. Summary of antimicrobial resistance profile of *E. coli* isolates.

Antimicrobial group	Antimicrobial agents	No. of resistant *E. coli* isolates (%)
β- lactams	Amoxicillin	48 (100%)
	Cloxacillin	48 (100%)
	Cefotaxime	6 (12.5%)
	Cephoxitin	12 (25%)
Tetracyclines	Tetracycline	27 (56.25%)
	Doxycycline	24 (50%)
Macrolides	Erythromycin	48 (100%)
	Lincomycin	48 (100%)
Aminoglycosides	Gentamicin	5 (10.41%)
	Amikacin	5 (10.41%)
	Streptomycin	22 (45.83%)
β- lactamase inhibitor	Amoxicillin/ sulbactum	21 (43.75%)
Folate inhibitor	Trimethoprim	40 (83.33%)
Quinolones	Ciprofloxacin	20 (41.66%)
	Enrofloxacin	21 (43.75%)
Cephalosporins	Ceftriaxone	11 (22.91%)
Chloramphenicol	Chloramphenicol	33 (70.83%)

The antimicrobials used for susceptibility testing in the present study were routinely used in veterinary practice as therapeutics or as growth promoters in India. The antibiotic resistance pattern observed in *E. coli* isolates of the present study was comparable with those observed in *E. coli* isolates from camel and cattle calves from Tunisia and India (Bessalah *et al,* 2016; Sharma *et al,* 2017). The high prevalence of antimicrobial resistance against commonly used drugs in human medicine in *E. coli* isolates of the present study pointed to the judicious use of antimicrobials in livestock farms. Although, there was difference in occurrence of virulence factors in *E. coli* isolates, however, the antimicrobial susceptibility pattern was more or less similar among them. This may be due to the fact that all diarrhoeic neonatal camels of the present study were from a farm where continuous use of these antibiotics for various types of ailments was practicsed since long time.

Characterisation and identification of genes encoding virulence factors and subdivision of diarrhoeic *E. coli* into pathotypes were necessary for understanding the disease epidemiology and pathogenesis (Garcia *et al,* 2020). The majority of diarrhoeic neonatal camels of the present study were found infected with ETEC infection during first 6 days after birth was in agreement with earlier studies (Guler *et al,* 2008; Foster and Smith, 2009). The 2nd most prevalent pathotype in diarrhoeic neonatal camels was EPEC with detection rate of 20.83% which was comparable with previous studies in diarrhoeic cattle and buffalo calves (Guler *et al,* 2008; Awad *et al,* 2020). However, Foster and Smith (2009) claimed that the significance of EPEC as a calf pathogen was questionable as it can be isolated from both healthy and diarrhoeic calves. The STEC pathotypes were detected in 6.25% of *E. coli* isolates which was lower than those reported in previous studies in diarrhoeic cattle and buffalo calves (Awad *et al,* 2020). In the present study, the *E. coli* isolated from neonatal camels having acute haemorrhagic enteritis and mortality were found to be of STEC type. Globally, STEC was an important cause of life threatening diarrhoeal disease in both animals and humans and association of STEC pathotype in causing haemorrhagic dysentery in young calves had been frequently reported (Robins-Browne *et al,* 2016). The shiga toxins destroyed intestinal microvilli resulting into haemorrhagic diarrhoea in calves (Nataro and Kaper, 1998). Mixed pathotypes with combinations of EPEC with ETEC were frequently reported in earlier studies (Awad *et al,* 2020; Ryu *et al,* 2020). It was speculated that these atypical combinations may result in the emergence of new pathotypes which may be more pathogenic and cause severe diarrhoea in calves (Awad *et al,* 2020).

Among diarrhoeic neonatal camels of the present study, ETEC were the most common pathotype, whereas STEC were found responsible for acute haemorrhagic enteritis and mortality. Young age (less than 7 days), calving pen with loose sandy ground and peak winter season were found associated with high incidence of neonatal camel calf diarrhoea in the present study. The studies on virulence genes and antimicrobial resistance pattern in *E. coli* isolates from diarrhoeic neonatal camels can be crucial for camel farmers and veterinarians in selection of antimicrobials for effective management and prevention of the disease and to minimise the emergence of multidrug resistant *E. coli* which may pose health risks to both animals and humans.

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