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Role of Bacterial and Parasitic Pathogens in Occurrence of Neonatal Diarrhoea in Goat-Kids

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ABSTRACT

Infectious diarrhoea in neonates of animals is one of the most common and economically important conditions encountered in the livestock industry. Faecal samples (n=210) from diarrhoeic neonatal goat-kids of different livestock sheds of ICAR-CIRG, Makhdoom, Mathura (U.P.), were aseptically collected, and immediately processed for isolation of bacterial pathogens and parasitic evaluation. A total of 178 isolates of *E. coli* from 210 samples were identified on the basis of cultural, morphological, biochemical and molecular characteristics. Out of 178 *E. coli* isolates, 3.93 % (7/178) isolates were identified as STEC by PCR amplification of *stx*-1 and *stx*-2 gene. A total of 64 isolates of *E. coli* were sent to National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli for the serotyping. The common serogroups of *E. coli* responsible for neonatal diarrhoea in goat-kids were identified as O36, O26, O59, O29, O43, O91, O82, O9 and O171, out of which, 46.15% were O36, O26 and O59. *Cryptosporidium* spp. infection was detected in 46 samples out of 148 faecal samples by ZN staining and nested PCR.Based on cultural, morphological, biochemical and molecular characteristics,16 isolates of *Salmonella* spp. and 5 of *Klebsiella* spp. were identified from 210 fecal samples. The present study concluded that *E. coli* followed by *Cryptosporidium* spp. and *Salmonella* spp. were the prevalent infectious agents associated with neonatal diarrhoea in goat-kids.

Keywords: *E. coli, Salmonella*, *Cryptosporidium*, *Klebsiella*, Goat-Kids, Neonatal-Diarrhoea

The major problem encountered in goat farming is higher morbidity/mortality among neonatal kids, which leads to severe economic loss to goat farmers/entrepreneurs. Many factors contribute to the mortality in neonatal goat-kids. Diarrhoea is the single major cause of mortality in neonatal goat-kids (Singh *et al.,* 2018). Infectious diarrhoea in neonates of farm animals is one of the most common and economically devastating conditions encountered in the livestock industry (Draksler *et al*., 2002). The infectious causes of diarrhoea have been well studied in cattle, pigs and lambs; however, not much work has been done in India/abroad to determine the enteric pathogens that cause diarrhoea in goat-kids (Smith and Sherman, 2009). Diarrhoea during the first month after birth is important cause of mortality in neonates of the farm animals (Radostits *et al*., 2006). The mortality due to diarrhoea may be as high as 60% (Kritas, 2002). In goat-kids, *Escherichia coli, Salmonella* spp. and *Cryptosporidium* spp. are considered as the most prevalent organisms associated with diarrhoea (Ozmen *et al*., 2006; Paul *et al*., 2014). Among the diarrhoegenic bacterial pathogens in neonatal animals, *E. coli* and *Salmonella* spp. are the most common and economically most important (Coura *et al.,* 2015). *Cryptosporidium* spp. may cause mortality up to 40% leading to severe economic loss to farmers

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(Johnson *et al*., 1999). *Klebsiella*s spp, a Gram-negative, bacterium may cause diarrhoea in farm animals (Ryan and Ray, 2004; Herrera-Luna *et al.,* 2009). Considering the importance of infectious diarrhoea in neonatal goat-kids, the present study was planned to isolate, and to identify different bacterial and parasitic pathogens associated with diarrhoea in neonatal goat-kids, so that control and preventive strategies against the pathogens may be devised.

MATERIALS AND METHODS

Sample collection

During Dec 1, 2012–March 31, 2014; 210 faecal samples were collected from diarrhoeic neonatal goat-kids (n=210) of Jamunapari, Jakhrana and Barbari breeds of goat from different livestock sheds of the Institute (ICAR-CIRG, Makhdoom, Mathura) using sterile swabs (HiMedia). The swabs were kept on the ice, and immediately brought to the Laboratory, Division of Animal Health, ICAR-Central Institute for Research on Goats, Makhdoom, Farah, Mathura (Uttar Pradesh), India.

Isolation and identification of *E. coli*

All faecal samples collected were initially inoculated on sterile blood agar (5% de-fibrinated sheep blood), and incubated at 37º C for 18-24 hr. Thereafter, colonies from the blood agar were inoculated on MacConkey agar, and incubated overnight at 37 ºC. The pink coloured colonies grown on MacConkey agar, were further re-inoculated on Eosin Methylene Blue (EMB) agar, and incubated at 37ºC for 18-24 hr.

Biochemical tests

For biochemical identification of *E. coli*, *Salmonella* spp. and *Klebsiella* spp., the biochemical tests *viz.* Catalase, Oxidase and IMViC were performed as per the standard bacteriological protocols (Cowan and Steel, 1975).

Detection of pathogenicity of *E. coli*

Congo red dye agar test was carried out to determine the invasiveness (pathogenicity) of the *E. coli* isolates as per the technique recommended by Berkhoff and Vinal (1986). The test was performed using soyabean casein digest medium containing 0.03% Congo red dye. Appearance of red colonies within 72 hr was recorded as a positive reaction (invasive *E. coli*). The colonies which did not bind the dye, and remained white or grey even after 72 hr, were declared negative for Congo red dye binding activity.

Serotyping of isolates

A total of 64 isolates of *E. coli* were sent to National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, India for the serotyping.

Molecular detection of *E. coli*

The genomic DNAs from all *E. coli* isolates (n=178) was extracted by hot-chill method as described by Yang *et al.* (2008). The molecular identification of *E. coli* was done by PCR amplification of the universal stress protein A (*usp*A) gene using species specific primers, (F-5'-CCGATACGCTGCCAATCAGT-3' & R-5-ACGCAGACCGTAGGCCAGAT-3') as used by Mishra *et al.* (2017). The annealing temperature was kept at 55°C for 0.5 min. Thereafter, the identification of shiga toxin producing *E. coli* (STEC) or verotoxin producing *E. coli* (VTEC) was done by PCR amplification of its *stx*-1 and *stx*-2 genes as per the protocols of Islam *et al.* (2007) and Rahman *et al.* (2008), respectively. The sequences of the primers used for the PCR amplification of *stx*-1 gene were F-5'-CACAATCAGGCGTCGCCAG CGCACTTGCT-3' and R-5'-TGTTGCAGGGATCAG TCGTACGGGGATGC-3', whereas for *stx*-2 gene, were F-5'-ATCCTATTCCCGGGAGTTTACG-3' and R-5'-GCGTCATCGTATACACAGGAGC-3'. The annealing temperature for the above PCRs was kept at 58 °C for 0.5 minute.

Isolation and identification of *Salmonella* spp. and *Klebsiella* spp.

The diarrhoeic samples (n=210) intended for *Salmonella* spp. isolation were initially inoculated into Rappaport Vassiliadis broth (10-15 ml), and incubated overnight at 37 ºC. The overnight growth was inoculated on blood agar. After overnight incubation, the colonies grown on blood agar were inoculated on Bismuth Sulphite agar (BSA), and the colonies on BSA were further inoculated on

Deoxycholate Citrate agar (DCA). The molecular detection of *Salmonella* spp. was done by PCR amplification using the published genus specific primers (Cohen *et al*., 1993) (F-5'-ACTGGCGTTATCCCTTTCTCTGGTG-3' & R-5'-ATGTTGTCCTGCCCCTGGTAAGAGA-3'). The annealing temperature was kept at 60 °C for 0.5 min. Likewise, the samples, intended for *Klebsiella* spp. isolation, were immediately inoculated on the sterile blood agar plates, and incubated at 37 ºC for 24 hr. Thereafter, the growth on blood agar showing catalase positive, oxidase negative and Gram-negative reactions were inoculated on MacConkey and EMB agars, and then incubated at 37 ºC for 24 hr. The growth from EMB agar was then inoculated on *Klebsiella* selective agar, and incubated at 37 ºC for 24 hr.

Detection of *Cryptosporidium* spp.

A total of 148 faecal samples from diarrhoeic neonatal goat-kids were targeted for presence of *Cryptosporidium* spp. For detection of the oocysts of *Cryptosporidium* spp., modified ZN staining method was used. The nested PCR targeting the SSUrRNA gene of *Cryptosporidium* was performed for its confirmatory diagnosis using the published oligonucleotide primers (Rieux*et al.,* 2013). The sequences of the primers for primary PCR were F-5'-TTCTAGAGCTAATACATGCG-3' and R-5'CCCATTTCCTTCGAAACAGGA-3', whereas for secondary/nested PCR, were F-5'-GGAA GGGTT GTATTTATTAGATAAAG-3' and R-5'-AAGG AGTAAGGAACAACCTCCA-3'. The annealing temperatures for primary and secondary PCR were kept at 56°C for 1 min and 57 °C for 1 min, respectively.

RESULTS AND DISCUSSION

The growth on blood agar showing catalase positive, oxidase negative and Gram negative reactions (Gram negative rods, Fig. 1A) was then inoculated on MacConkey agar. Pink colored colonies (lactose fermentation) on MacConkey agar (Fig. 1B) were further inoculated on EMB. Colonies with a characteristic metallic green sheen on EMB agar (Fig. 1C) were indicative of E. *coli.* The results of IMViC tests were found as +ve, +ve, –ve, -ve. In PCR amplification of the *usp*A, the size of the amplified product was found 884 bp (Fig. 1D).

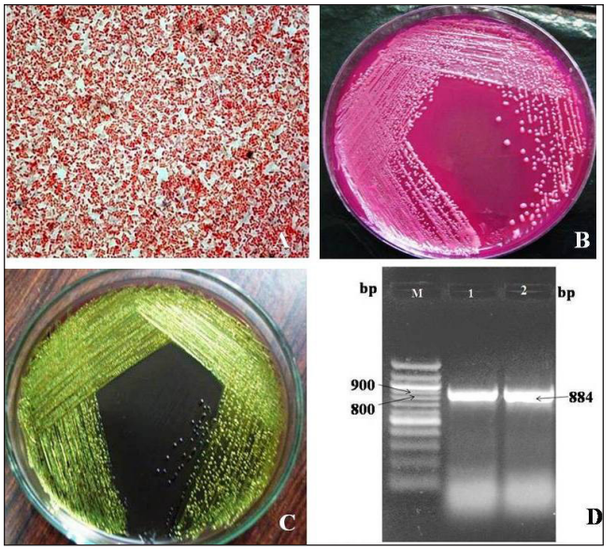

Fig. 1: Culture and isolation of *E. coli* from faecal samples of goat kids. **(A)** Gram's staining: *E. coli* as Gram negative rods. **(B)** Growth characteristics on MacConkey agar showing lactose fermentation. **(C)** Growth characteristic on Eosin Methylene blue agar showing characteristic green metallic sheen. **(D)** PCR amplification of *uspA* gene; Lane M; Marker; Lane 1, 2: Amplified products

From 210 faecal samples, 178 isolates of *E. coli* were identified on the basis of cultural, morphological, biochemical and molecular characteristics (Table 1). All of the 178 isolates showed 100% Congo red binding activity (Fig. 2), where as Kalorey *et al.* (2002) reported 89.09% Congo red binding in his study.

Fig. 2: *E. coli* showing Congo red binding activity

Out of 178 isolates of *E. coli* from the diarrheic neonatal kids, 3.93 % (7/178) were identified as STEC by PCR amplification of *stx*-1 and *stx*-2 gene (Fig. 3A, B). Contrary to this finding, Wani *et al*. (2003), Bhat *et al*. (2008) and Bandyopadhyay *et al*. (2011) reported higher percent positivity, that is,6%, 17.8% and 32%, respectively.

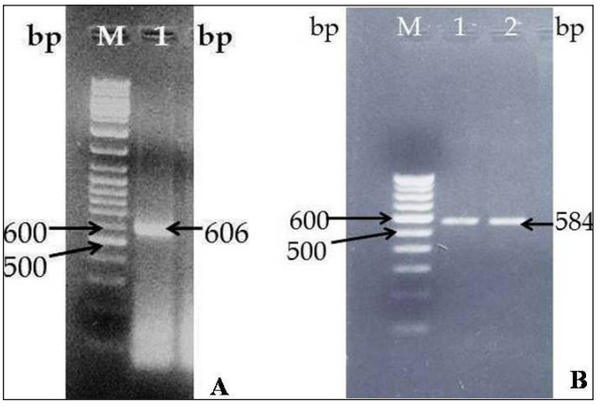

Fig. 3: PCR Amplification of *stx-1* and *stx-2* gene of STEC. **(A)** Lane-M: Marker; Lane-1: Amplified product; **(B)** Lane-M: Marker; Lane-1,2: Amplified product

A total of 64 isolates of *E. coli* were sent to National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli for the serotyping. The common serogroups of *E. coli* responsible for neonatal diarrhoea in goat-kids were identified as O36, O26, O59, O29, O43, O91, O82, O9 and O171, out of which, 46.15% were O36, O26 and O59. In another study, Mishra *et al.* (2019), O88, O22, O11 and O83 were found as the most frequently isolated serogroups from diarrheic neonatal goat-kids, whereas Pachaury and Kataria (2012) found O55 as the most frequently serogroup associated with diarrhoea in neonatal goat-kids.

The growth on blood agar showing catalase positive, oxidase negative and Gram negative reactions (Fig. 4A), black colored colonies on BSA (Fig. 4B) and colonies with a black center surrounded by a wide light-colored rim on DCA (Fig. 4C) were indicative of *Salmonella* spp. The results of IMViC tests were obtained as -ve, +ve, -ve, +ve. Cultural, morphological and biochemical characteristics indicated the confirmatory diagnosis of *Salmonella* spp. The above findings were in accordance with the earlier findings of Saha *et al*. (2014). The molecular confirmation of *Salmonella* spp. was done by the PCR and the size of the amplified product was obtained as 496 bp (Fig. 4D). In present study, 7.62% (16/210) samples were positive for *Salmonella* spp. (Table 1). Zaki *et al*. (2010) found 27% of diarrhoeic faecal samples positive for *Salmonella* spp. in his study, whereas Munoz *et al*. (1996) reported *Salmonella* spp. presence in 2.7% samples. In five cases of the neonatal diarrhoea, *Salmonella* was found as single causative agent of the diarrhoea indicating its ability to cause disease alone.

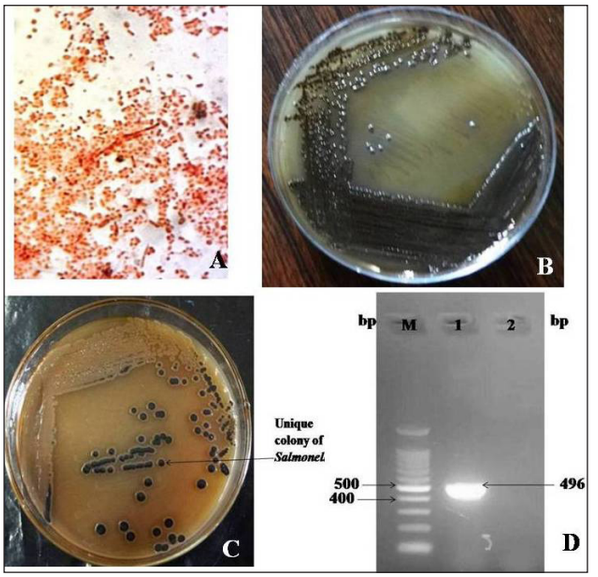

Fig. 4: Culture and isolation of *Salmonella* from faecal samples of goat kids. **(A)** Gram's staining: *Salmonella* as Gram negative rods. **(B)** Growth characteristics on Bismuth Sulphite agar showing black coloured colonies. **(C)** Growth characteristic on Deoxycholate citrate agar. **(D)** PCR for identification of Salmonella; Lane M; Marker; Lane 1: Amplified product; Lane-2: Control

For *Klebsiella* spp. isolation, the colonies showing catalase positive, oxidase negative, IMViC reactions as -ve, -ve, +ve, +ve and Gram negative reactions (Fig. 5A), were selected. Mucoid pink colored colonies (lactose fermentation, Fig. 5B) on MacConkey agar and Fish-eye type colonies (a dark center surrounded by a wide, light-colored, mucoid rim) on EMB agar (Fig. 5C) were indicative of *Klebsiella* spp. Presence of the purple coloured colonies on the *Klebsiella* selective agar (Fig. 5D) indicated the confirmatory diagnosis of *Klebsiella* spp. Presence of *Klebsiella* spp. was found in 2.38% of diarrheic samples (5/210) of the neonatal goat-kids

as presented in Table 1. In our study, diarrhoegenic *E. coli* was also isolated from the five *Klebsiella* positive samples. Hence, *Klebsiella* spp. alone was not found to cause diarrhoea in the goat-kids. This finding is contrary to the findings of Ryan and Ray, (2004) and Herrera-Luna *et al.* (2009), in which *Klebsiella* spp. alone was found capable to cause diarrhoea.

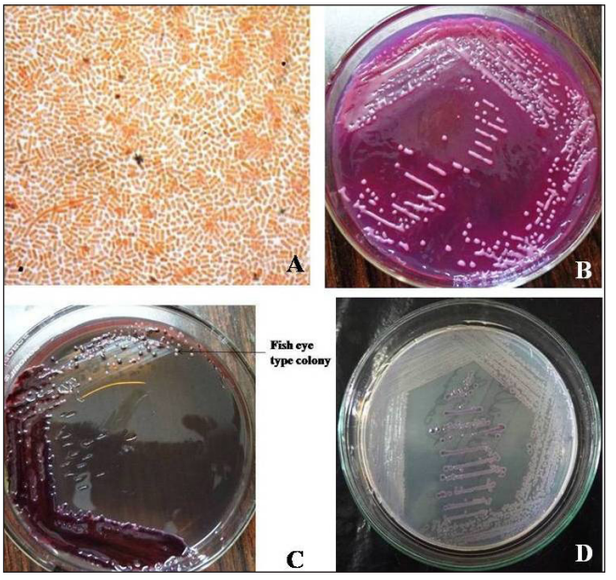

Fig. 5: Culture and isolation of *Klesiella* from faecal samples of goat kids. **(A)** Gram's staining: *Klebsiella* as Gram negative rods. **(B)** Mucoid colonies on MacConkey agar. **(C)** Fish eye type colonies on EMB. **(D)** Klebsiella on selective media

Table 1: The breed wise distribution of the identified bacterial pathogens from the neonatal diarrhoea in goat-kids

Breed of goat	Number of Faecal Samples Collected	Percent (%) of *E. coli* Positive Samples	Percent (%) of *Salmonella* Positive Samples	Percent (%) of *Klebsiella* positive Samples
Jamunapari	99	83.83	8.08	2.02
Barbari	100	86.00	6.00	3.00
Jakhrana	11	81.82	18.81	—
Total	**210**	**84.76**	**7.62**	**2.38**

Out of 148 samples, the presence of *Cryptosporidium spp*. was found in 46 samples by modified ZN staining method (Table 2; Fig. 6A) and the nested PCR targeting the SSU rRNA gene of *Cryptosporidium spp.* It resulted in to the amplicon with size of 834 bp (Fig. 6B). From 16 samples positive for *Cryptosporidium* spp., no bacterial agent targeted in the present study was isolated indicating its capability to cause neonatal diarrhoea as sole pathogen. Munoz *et al*. (1996) detected *Cryptosporidium parvum* in diarrhoeic goat-kids with 42% positivity but not in non-diarrhoeic animals, which supports our finding.

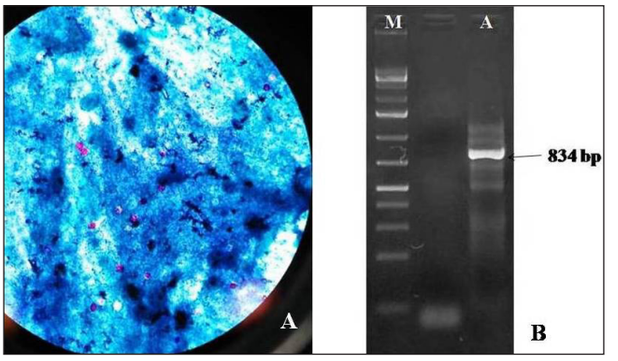

Fig. 6: Showing *Cryptosporidium* spp., **(A)** By modified ZN staining, **(B)** Amplification of 18s sRNA gene of *Cryptosporidium* spp., Lane-M: 100bp marker; Lane-a: Nested product

Table 2: Breed wise presence of *Cryptosporidium* in diarrhoeic feces of the neonatal kids

Breed of goat	Total Number of Diarrheic Samples	Total Positive Samples for *Cryptosporidium*	Percent (%) of *Cryptosporidium* Positive Samples
Barbari	61	19	31.2
Jamunapari	55	18	32.8
Jakhrana	32	9	28.1
Total	**148**	**46**	**30.7**

The results of current study suggest that *E coli* were found to be the main causative agents associated with the neonatal diarrhoea in the goat kids. *Salmonella* spp. and *Cryptosporidium* spp. were also found capable to cause the diarrhoea in the kids. Other researchers (Fassi-Fehri *et al*., 1988; Ozmen *et al*., 2006; Paul *et al*., 2014) also indicated same findings in their studies. The results from the study would serve as basic data for designing the diagnostics, prophylaxis and the package of practices to control/prevent the diarrhoea in the neonatal goat-kids. The data would help in understanding the pattern of

distribution of the bacterial and parasitic agents associated with diarrhoea in the kids.

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