MOLECULAR CHARACTERIZATION, DETECTION AND QUANTIFICATION OF *ESCHERICHIA COLI* STRAINS ISOLATED FROM DIARRHOEIC LAMBS USING BOTH GRADIENT PCR AND SYBR GREEN REAL TIME PCR

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ABSTRACT

There are different types of pathogenic *Escherichia coli* (*E. coli*) carrying various types of genes. *E. coli* has universal stress protein (*usp*) genes which are species specific and confirm its presence. The *uspA* gene is important for survival of *E. coli* during cellular growth, adhesion and motility. Likewise, enteropathogenic *Escherichia coli* (EPEC) are an important cause of infantile diarrhoea which colonizes the small intestinal epithelial lining and causes lesions in the microvilli. There are several different well-characterized and putative adhesive factors involved in this process including bundle forming pilin (BFP) protein, and well defined *bfpA* gene. In the present study, *E. coli* were isolated from a total of 100 diarrhoeic lambs of Udaipur region located in southern part of Rajasthan. These isolates were screened by a series of biochemical tests and then subjected to amplification of the genomic DNA using universal stress protein A (*uspA*) gene for *E. coli* resulting into a single amplicon of 884 bp. These isolates were then subjected to characterization of enteropathogenic *E. coli* (EPEC) using bundle forming pilin protein A (*bfpA*) gene. The amplification of *bfpA* gene resulted into a single amplicon of 326 bp. A normal polymerase chain reaction (PCR) was performed to amplify genes and quantification of amplified products of both the genes were obtained by using fluorescent probes or fluorescent DNA-binding dyes and real-time PCR instruments that measure fluorescence while performing the thermal cycling for PCR.

***Key words*:** Isolates, characterization, *E. coli*, *usp*A gene, *bfpA* gene, RT-PCR

Introduction

Pathogenic *Escherichia coli* are considered as a major cause of large scale epidemics of gastrointestinal illness in man and animals (Deshmukh and Karpe, 2006). Domestic and wild animals are sources of *E. coli* but ruminants primarily sheep, goats and cattle have been identified as major reservoirs and source for human infection (Kiranmayi *et al*., 2010 and Rahimi *et al*., 2012). Bacterial culture examination is a time consuming procedure thus, there was a need to evolve a faster as well as sensitive and specific test to identify an etiological agent.

Diagnostics using DNA based tools, such as polymerase chain reaction (PCR) have higher specificity and speed, compared to culture based methods (Louie, 2000). *E. coli* strains causing diarrhoea were among the first pathogens for which molecular diagnostic methods were developed. Virulence gene probes (DNA hybridization) are specific and sensitive diagnostic and epidemiologic tools for the detection and typing of *E. coli* virulence genes (Chizhikov *et al*., 2001). Over the past several years, real-time PCR has become the leading tool for the detection and quantification of DNA or RNA.

Real-time PCR is a variation of the standard PCR technique that is commonly used to quantify DNA or RNA in a sample. Using sequence specific primers, the number of copies of a particular DNA or RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA-binding dyes and real-time PCR instruments that measure fluorescence while performing the thermal cycling for PCR. Among the several chemistries available for real-time PCR assays, SYBR green based assays are the most widely used. Previously, real-time PCR based methods have been reported for detection of many pathogens of veterinary importance (Aguero *et al*., 2007). SYBR green based real time PCR assay was used earlier to differentiate enteropathogenic *E. coli* (EPEC), Shigatoxic *E. coli* (STEC) and other enteroaggregative *E. coli* (EAEC), and it was concluded in that study that it may be used as an effective diagnostic tool compared to conventional PCR, for detection of *E. coli* strains (Bischoff *et al*., 2005; Guion *et al*., 2008).

Escherichia coli have six *usp* genes namely A, C, D, E, F and G. But for the survival of *E. coli* during cellular growth, adhesion and motility (Nachin *et al*., 2005) the *usp*A gene is required. Various stresses such as heat shock, nutritional starvation, osmotic pressure and the presence of toxic agents etc can induce the synthesis of *usp* protein (Nystrom and Neidhardt, 1992). The primer *uspA* can be used as a genetic marker to detect the *E. coli* from other species of microflora present in the gut of kids (Rajput *et al*., 2014).

Enteropathogenic *Escherichia coli* (EPEC) are an important cause of infantile diarrhoea in developing countries (Nataro and Kaper, 1998; Bugarel *et al*., 2011). EPEC colonizes the small intestinal epithelial lining, leading to 'attaching and effacing' (A/E) lesions in the microvilli, characterized by intimate aggregation and attachment of bacteria (Knutton *et al*., 1989; Allen-Vercoe *et al*., 2006). *E. coli* which produces AE lesions but not Shiga toxin, are called enteropathogenic *E. coli* (EPEC) (Kaper 1996; Bhat *et al.,* 2008). There are several different well-characterized and

putative adhesive factors involved in this process including Bundle forming pilin (BFP) protein and there is a set of defined *bfpA* mutants and transformants supporting the role of BFP (Cleary *et al*., 2004).

Materials and Methods

Sample collection on farms

A total of 78 *E. coli* isolates obtained from 100 diarrhoeic lambs belonging to organized farm and private flocks located at different places around Udaipur situated in the southern part of Rajasthan state (India) were investigated. Samples were mainly collected for a period of one year between 2017 and 2018. Diarrhoeic lambs of 0-3 months were selected for sample collection. Swabs were taken from the rectum of lambs and placed immediately in 1 ml of 0.1 M sterile phosphate buffered saline (PBS) and diluted 1:10 in PBS and then transported over ice to laboratory for isolation and identification of bacterial cultures.

Isolation and identification

Faecal samples were collected directly from rectum of diarrhoeic lambs with the help of sterile cotton swabs. Rectal swabs were collected in sterile test tubes and transported over ice to laboratory for isolation and identification. Faecal samples were inoculated on MacConkey agar medium (Fig. 6) for preliminary characterization aerobically at 37 °C for 24-48 hours. Colonies were medium size and bright pink to red with flat or elevated surface and complete white edges on MacConkey agar. For confirmation these colonies were picked up and inoculated on Eosin Methylene Blue (EMB) agar (Fig. 7) and colonies showing characteristic metallic sheen on EMB agar were considered as *E. coli.* The purified cultures of *E. coli* were stored in nutrient broth for further identification by biochemical tests. All the isolates were stained by Gram's Method (Fig.5) to determine the purity of the isolates. Primary identification of *E. coli* isolates was done by biochemical tests *viz.* Indole, Methyl red reduction, Voges proskaeur and citrate utilization (Quin *et al*., 2002). The secondary identification of *E. coli* isolates were done by the following tests *viz*. carbohydrate fermentation, urea hydrolysis, production of H_2S on TSI as per Edwards and Ewing (1972)

Molecular characterization of *E. coli*

Isolation of genomic DNA

Genomic DNA was isolated following the method of Wilson (1987) with slight modifications. About 100 µl of 18 hr broth culture (single colony inoculated in nutrient broth) was inoculated in 5 ml of nutrient broth and subjected to incubation at 37 ºC in an orbital shaker at 200 rpm overnight. Bacterial pellet was obtained by centrifugation at 10,000 rpm for 5 minute at 4 ºC. The pellet was resuspended in 567 µl of TE buffer by repeated pipetting. Then 30 µl of 10 per cent SDS and 3 µl of 20 mg per ml proteinase K were added to give a final concentration of 100 µg/ml protinase K in 0.5 per cent SDS. Mixed thoroughly and incubated at 37 ºC for 1 hr. The lysate was treated with 100 µl of 5 M NaCl and 80 µl cetyl trimethyl ammonium Bromide (CTAB) (10 per cent w/v in 0.7 M NaCl w/v) with further incubation at 65 ºC in a water bath for 10 min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 w/v) were added in each tube, mixed gently and centrifuged at 13,000 rpm for 5 min. The supernatant was collected in a separate tube and DNA was precipitated by incubating the tubes at -20 ºC overnight after adding 1/10 volume of ammonium acetate (7.5 M) and double the volume of chilled absolute ethanol. Finally the DNA pellet was obtained by centrifugation at 13,000 rpm for 20 min at 4 ºC. The DNA pellet was washed with 70 per cent ethanol dried and resuspended in 100 µl sterile nuclease free water. DNA was finally stored at -80 ºC in small aliquots.

Primers

The primers for PCR amplification of *Escherichia coli* specific sequences, *uspA* and *bfpA* used in the present study were procured from Bio-innovations Pvt. Ltd. Mumbai shown in the Table 1.

Amplification of *uspA* and *bfpA* sequence of *E. coli* by gradient PCR

A gradient PCR (Cole Parmer Techne prime) was used for amplification of genes. Genomic DNA of *E. coli* was used as the template for PCR reactions. 'Universal stress protein A' (*uspA*) sequence (884 bp) which is species specific for *E. coli* was amplified by standard PCR protocol using the primers used by Osek (2001) and Mishra *et al.* (2017). Further genomic DNA were used as the template for PCR reactions for confirming enteropathogenicity of *E. coli* by using 'Bundle forming pillin' (*bfpA*) gene sequence (326 bp) which is specific for Enteropathogenic *E. coli* (EPEC) was amplified by standard PCR protocol using the primers as used by Gunzburg *et al.* (1995) and Rajput *et al.* (2014).

For both *uspA* and *bfpA* genes the following reagents were taken in a 25 µl reaction mix, in a 0.2 ml, thin walled PCR tube for PCR reactions. The following cycling conditions were used in the gradient PCR as shown in Table 3 for both the genes. The amplified products were confirmed for the expected size (884bp) and (326 bp) in 1.5 per cent agarose gel in 1X Tris Acetate EDTA (TAE) buffer as per the method of Sambrook and Russel (2001).

Amplification of *uspA* and *bfpA* sequences of *E. coli* by Real time PCR

Amplification of genes was carried out in Real time PCR (AriaMx, Agilent Technologies).The genomic DNA of *E. coli* was used as the template for Real time PCR reactions, *uspA* sequence (884 bp) and *bfpA* sequence (326 bp) of *E. coli* was amplified by standard Real time PCR protocol using primers of genes as above.

PCR was performed in thin walled tubes using the Genesure™ Sybr Green/ROX q PCR master mix-200 x 25 µL (Puregene Genetix) as described by the manufacturer. All runs included a negative DNA control without template and optimized reaction mixtures for *uspA* specific SYBR Green assays included 1x master mix, a 150 nmole concentration of each primer, and 2 µl of template DNA in a 25 µl PCR mixture.

Nuclease-free water	: 9 µl
RT-PCR Master mix	: 12.5 µl
uspA Primer (forward) (50 pmol)	: 0.75 µl
uspA Primer (reverse) (50 pmol)	: 0.75 µl
Genomic DNA	: 2 µl

After PCR amplification, T_m curve analysis was performed. The PCR products were cooled to 65 ºC and then slowly heated

to 95 ºC at a rate of 0.1 ºC/s. Fluorescence signals obtained were continuously monitored to confirm amplification specificity. T_m peaks of the products were calculated based on initial fluorescence curves (*F*/*T*) by plotting the negative derivative of fluorescence over temperature versus temperature (*dF*/*dT* versus *T*).

Results

Out of 100 faecal swabs collected from diarrhoeic lambs, only 78 (78 per cent) were found to possess *E. coli*.The *uspA* genes from *E. coli* strains were successfully amplified using species specific primers. PCR amplification resulted into a single amplicon of 884 bp (Osek, 2001; Rajput *et al.,* 2014 and Mishra *et al.,* 2017) as illustrated Fig. 1. The primer *uspA* was used as a genetic marker to detect the *E.coli* from other species of microflora present in the gut of kids (Rajput *et al.,* 2014). These *E.coli* isolates for *uspA* gene were also screened by SYBR green real time PCR, based on melt curve analysis (Fig. 3). The T_m product 1 {-R'(T)} of approximately 83.5 ˚C, a melting peak above threshold 100 and cycle quantification Cq (ÄR) values were between 10 and 20 were considered as positive amplicons as illustrated in Table 1 and Fig. 3, whereas the other noisy peaks less than 80 ºC and threshold line were primer dimers.

In present study, *E.coli* isolates from diarrhoeic lambs were used to identify enteropathogenic *E. coli* (EPEC) using the same primers of *bfpA* gene as used by Gunzberg *et al.* (1995) and Rajput *et al.* (2014)**.** *E. coli* isolates obtained from diarrhoeic lambs were positive for *bfpA*, giving a 326 bp

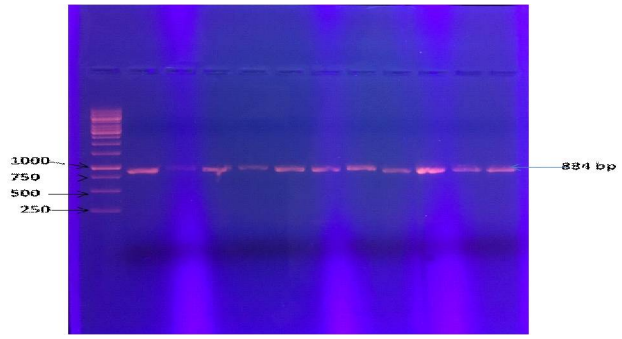


Fig. 1: Representing polymerase reaction (PCR) results of universal stress protein (*uspA*) gene of *Escherichia coli* isolated from lambs using 1kb DNA ladder

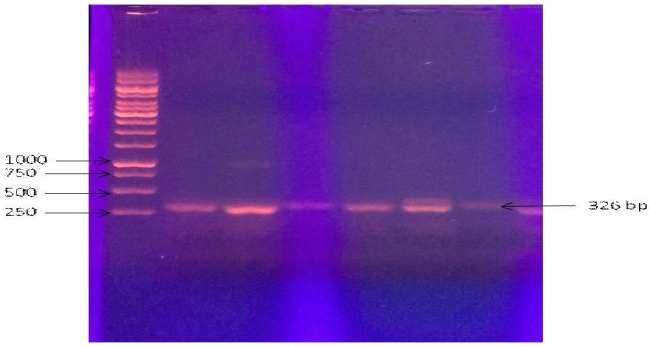
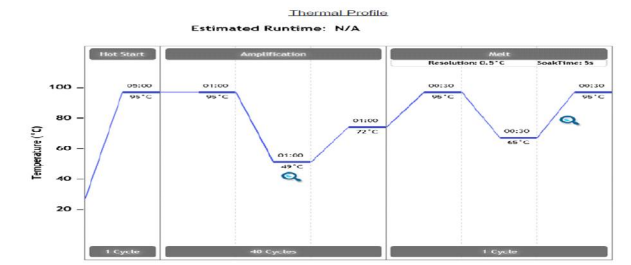
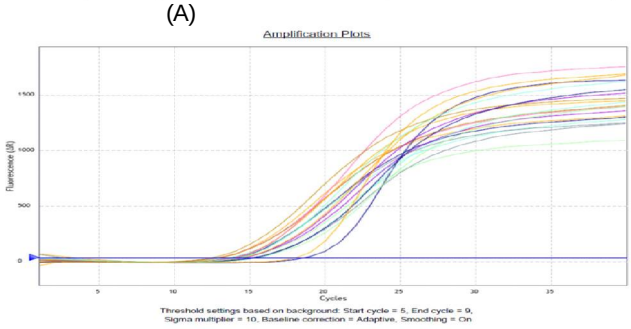


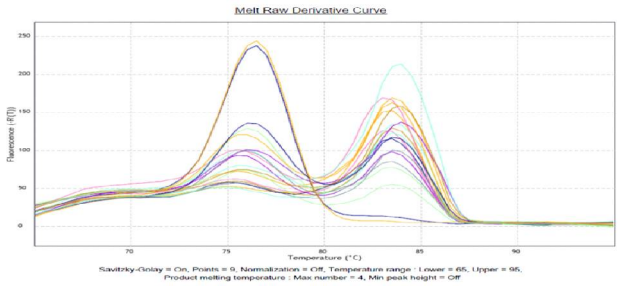
Fig. 2: Representing polymerase reaction (PCR) results bundle forming pilin protein (*bfpA*) gene of *Escherichia coli* isolated from lambs using 1kb DNA ladder



(A)



(B)

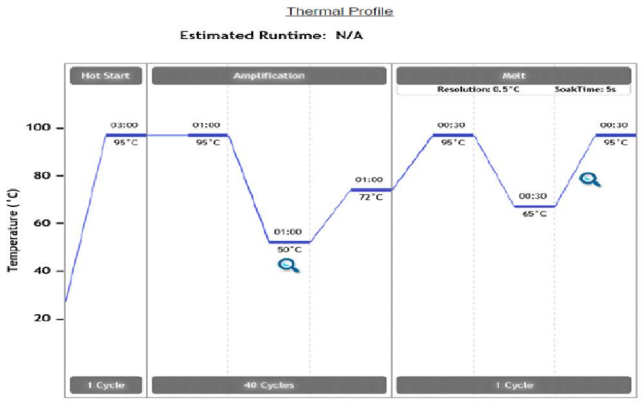


(C)

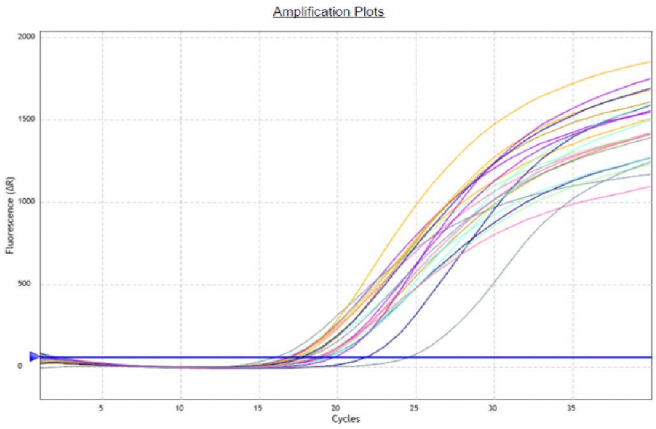
Fig. 3: SYBR green chemistry based Real time PCR assay of us*p*A gene.
(A) Thermal Profile; (B) Cq (cycle quantification) or cycle threshold (Ct) of Positive control; (C) Melt Raw Derivative Curve
Melting peak of approximately 83.5 °C the threshold 100 are positive amplicons. Whereas, the other noisy peaks less than 83.5 °C and threshold line are primer dimmers.

amplicons indicating it as EPEC as illustrated (Fig. 2). These *E. coli* isolates for *bfpA* gene were also screened by SYBR green real time PCR, based on melt curve analysis and cycle quantification (Fig. 4). The SYBR green real time assay for *bfpA* gene based screening of EPEC has a specificity of 99 per cent when compared with non EPEC strains and other gram negative bacteria (Singh *et al*., 2018). The T_m product 1 {-R'(T)} of approximately 83.5 ºC, a melting peak above threshold 100 and cycle quantification Cq (ÄR) values were between 10 and 30 were considered as positive amplicons as illustrated in Fig. 4. Whereas the other noisy peaks less than 80 ºC and threshold line were primer dimers.

In the present study, the overall prevalence of EPEC (*bfpA*) was 67.94 per cent in neonatal diarrhoeic lambs depicted in Table 4. EPEC is reported to be the major cause of diarrhoea in infants of almost all the species including lambs. This is true with the current study, where *E. coli* isolates from diarrhoeic lambs were used to identify enteropathogenic *E. coli* (EPEC) using the same *bfp*A gene as used by the Rajput *et al.* (2014). The overall prevalence of EPEC (*bfpA*) in diarrhoeic

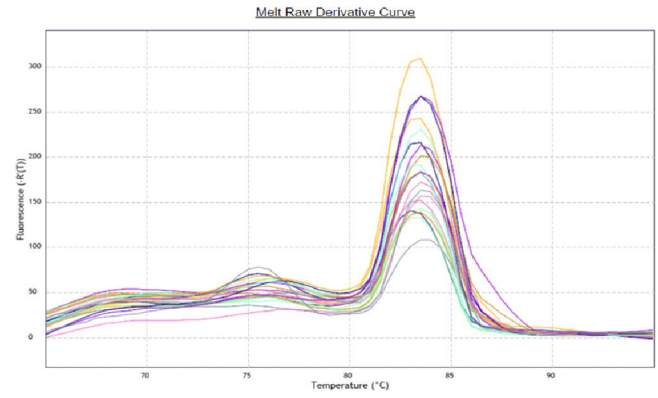


(A)



Threshold settings based on background:start cycle = 5, end cycle = 9, Sigma multiplier = 10, Baseline correction = Adaptive, Smoothing = On

(B)



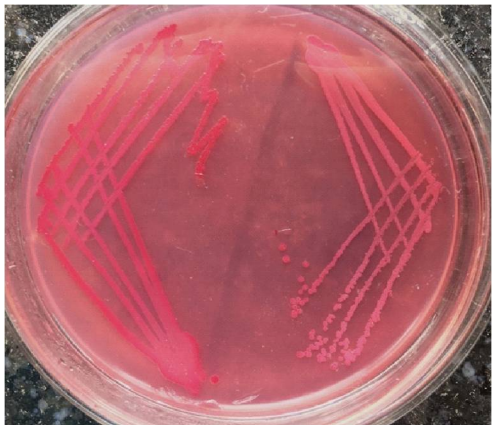
Savitzky-Golay = On, Points = 9, Normalization = Off, temperature range: Lower = 65, Upper = 95, Production melting temperature : Max number = 4, Min peak height = Off

(C)

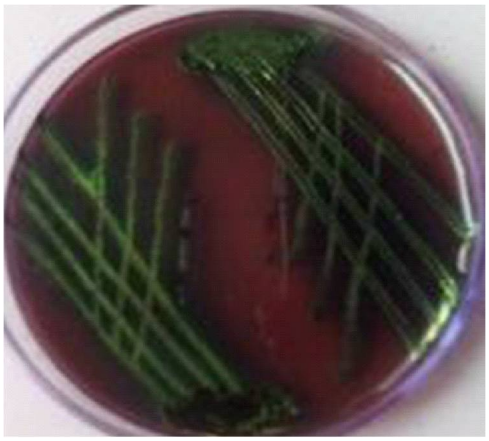
Fig. 4: SYBR green chemistry based Real time PCR assay of *bfp*A gene for screening EPEC.
(A)Thermal Profile; (B) Cq (cycle quantification) or cycle threshold (Ct) of Positive control; (C) Melt Raw Derivative Curve
Melting peak of approximately 83.5 °C the threshold 100 are positive amplicons. Whereas the other noisy peaks less than 83.5 °C and threshold line are primer dimers

lambs at the age of 0-30 days, 31-60 days and 61-90 days was 38.46 per cent, 17.94 per cent and 11.53 per cent,

(Fig. 5). Gram negative rods of *E. coli*



(Fig. 6). Lactose fermenting *E. coli* colonies on Mac conkey agar



(Fig. 7). *E. coli* showing metallic sheen on EMB agar

respectively. There was highly significant difference in the overall prevalence of EPEC in diarrhoeic lambs at different age groups (P<0.01). The present finding reveals that prevalence of colibacillosis due to EPEC (*bfpA*) decreases with the advancement of age. The present findings were similar to findings of Singh *et al*. (2018) who reported 68.08 per cent incidence in neonatal diarrhoeic kids which is higher than the earlier findings of Rajput *et al.* (2014) who reported 48 per cent in diarrhoeic kids, findings of Wani *et al.* (2004) who reported 26.6 per cent incidence of EPEC, where as the findings

Table1: Details of primers used in the present study

S. No.	Oligo name	Sequence 5'-3'	Base pairs	gene	Tmp. (°C)	Amplicon size (bp)
1	*uspA* (forward)	5'CCGATACGCTGCCAATCAGT3'	20 bp	*uspA*	54	884
2	*uspA* (reverse)	5'ACGCAGACCGTAAGGGCCAGAT3'	22 bp		59	
3	*bfpA* (forward)	5'AATGGTGCTTGCGCTTGCTGC3'	21 bp	*bfpA*	56	326
4	*bfpA* (reverse)	5'GCCGCTTTATCCAACCTGGTA3'	21 bp		54	

Table 2: Reagents taken in a 25 µl reaction for both the genes

S.No.	*uspA*		*bfpA*	
1.	Nuclease-free water	9 µl	Nuclease-free water	9 µl
2.	2x Master mix	12.5 µl	2x Master mix	12.5 µl
3.	*uspA* Primer (forward) (50pmol)	0.75 µl	*uspA* Primer (forward) (50pmol)	0.75 µl
4.	*uspA* Primer (reverse) (50pmol)	0.75 µl	*uspA* Primer (reverse) (50pmol)	0.75 µl
5.	Genomic DNA	2 µl	Genomic DNA	2 µl

Table 3: Cyclic conditions used in the real time PCR

S. No.	**Cyclic conditions**	***uspA***	***bfpA***
1.	Initial denaturation	94°C for 5 min.	94°C for 5 min.
2.	Denaturation (30 cycles	95°C for 1 min	95°C for 1 min
3.	Annealing	49°C for 1min	50°C for 1min
4.	Extension	72°C for 1 min	72°C for 1 min
5.	Final extension	72°C for 10 min.	72°C for 10 min.

Table 4: Prevalence (per cent) of *bfpA* gene in different age groups of positive samples of colibacillosis affected lambs with real time PCR

Type	Virulent gene	0-30days			31-60 days			61-90 days			Overall total
		Male	Female	Total	Male	Female	Total	Male	Female	Total	
EPEC	*bfpA*	20.51 (16)	17.94 (14)	38.46 (30)	10.25 (8)	7.69 (6)	17.94 (14)	6.41 (5)	5.12 (4)	11.53 (9)	67.94 (53)

of Bhat *et al.* (2008) reported much lower prevalence of 12.5 per cent in diarrhoeic lambs. The variations in the prevalence of EPEC may be due to host species, geological, climatic, seasonal determinants etc.

Conclusion

From present study we can conclude that biochemical characterization screened *E. coli* isolates from diarrhoeic faeces, while molecular characterization helped in identifying up to species level by using *usp*A gene. The pathogenicity of *E. coli* was confirmed by using pathogenic marker *i.e* bundle forming pilus gene (*bfpA*). The of percentage of EPEC in the 0-1month age group was found significantly higher than the lambs of 1-3 months age group, thus indicating considerable influence of age. The molecular characterization was done by using molecular tools such as gradient PCR and real time PCR and found that real time PCR was more fast, sensitive and precise in detecting genes, thus helping in faster diagnosis.

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