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Basant
MVSc. Animal Biotechnology, Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Sciences, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Parma Ram Gorachiya
Ph.D., Livestock Products Technology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Narendra Singh
Ph.D., Scholar, Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Corresponding Author
Basant
MVSc. Animal Biotechnology, Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Sciences, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Phylotyping of *Escherichia coli* Isolates from calf diarrhea

Basant, Parma Ram Gorachiya and Narendra Singh

Abstract
Calf diarrhea is one of the major health challenges in cattle herds. *Escherichia coli* is the most common cause of calf diarrhea. In the current study, we isolated a total of 27 isolates from diarrheic calves. The preliminary identification was carried out by the development of a green metallic sheen on the Eosin Methylene Blue agar plate. After that molecular level confirmation by amplification of 16S rRNA gene. The positive isolates were subjected to phylogroups. The B1 and A were most prevalent while phylogroup C, D, and E were less likely to be associated with calf diarrhea.

Keywords: Calf diarrhea, *E. coli*, Quadruplex PCR

Introduction
Newborn calves play an important role in animal production for meat production as well as breeding purposes worldwide. Calf diarrhea is a disease of neonatal and young animals and is well-known for productivity and economic loss to livestock owners throughout the world. The main causative agent for diarrhea includes enterotoxigenic *Escherichia coli* (*E. coli*), which causes noninflammatory secretory diarrhea to 3-5 days old calves mainly, Salmonella sp. causes foul-smelling feces containing blood and mucus which further leads to septicemiamainly to 2-12 weeks of calves, Clostridium perfringens which causes fatal hemorrhagic enteritis up to 8 weeks of age and another known causative agents are Bovine Rotavirus, Bovine Coronavirus and Cryptosporidium parvum (Cho *et al.*, 2014) [1].
E. coli consists of commensal and pathogenic strains of great diversity, therefore the classification of those microorganisms in phylogenetic groups through the combination of gene clusters is vital to understanding *E. coli* pathogenesis and interaction with its hosts (Croxen *et al*., 2013) [2]. An understanding of the *E. coli* genomic structure showed that the strains belonging to the various phylogroups are also related to the source of isolation (Clermont *et al*., 2013) [3]. Since its introduction in 2000 (Clermont *et al*., 2000) [4], phylogenetic typing using PCR has become widely used because of its simplicity and speed, allowing the differentiation of virulent strains (B2 or D) and commensal lineages (A and B1). This method was improved in 2013 (Clermont *et al*., 2013) [3] and a quadruplex PCR can identify seven phylogroups (A, B1, B2, C, D, E, and F). This study aimed to find an association of different phylogroups with calf diarrhea in the Bikaner district of Rajasthan.

Material and Methods
Sample collection and bacterial isolation: A total of 100 fecal samples were collected from diarrheic calves from private dairy farms nearBikaner, Rajasthan in the year 2018 and 2019. 1 gram of fecal sample was diluted in PBS followed by overnight incubated in with the nutrient broth. the enriched sample was streaked on MacConkey Agar Plate. The pink colonies (lactose fermentors) obtained from MacConkey Agar were subjected to further streaked on selective media EMB Agar. The characteristic Growth of colonies on EMB agar was a metallic sheen of colonies. The molecular levels of conformation of EMB positive isolates were carried out by performing 16sRNA-gene-specific PCR as described previously (Khalad *et al.*, 2010) [5] using forward primer 5'-GCTTGACACTGAACATT-3' and reverse primer 5'-GCACTTATCTCTTCCGCATT-3'.

Amplification of genes encoding for phylogenetic groups: After isolation of genomic DNA by using the phenol-chloroform method, the phylogroups of *E. Coli* isolates were carried out by performing Quadruplex-PCR as per described previously (Clermont *et al.* 2013) [3].

The PCR product was analyzed by agarose gel electrophoresis to examine the amplicon sizes and the phylogenetic group to which an isolate belonged was done as per the method of Clermont *et al.* (2013) [3].

Results and Discussion

Isolation and confirmation of *E. coli* isolate: Preliminary isolation and identification of E coli isolates were carried out by culture characteristic (Differential media, Selective media) and gram staining. A total of 27 isolates were recovered in the study which showed pink colonies on MacConkey lactose Agar and also produces green metallic seen on EMB agar which is characteristic of E. coli. Besides this, all 27 isolates were found positive for species-specific 16srRNA ribotyping (all isolates showed an amplicon of 662bp). Thefindings were similar to Meena *et al*., 2015 [6] where 40 isolates of *E. coli* from healthy poultry were phenotypically confirmed by metallic sheen on EMB agar and all the isolates were genotypically confirmed by 16SrRNA gene.

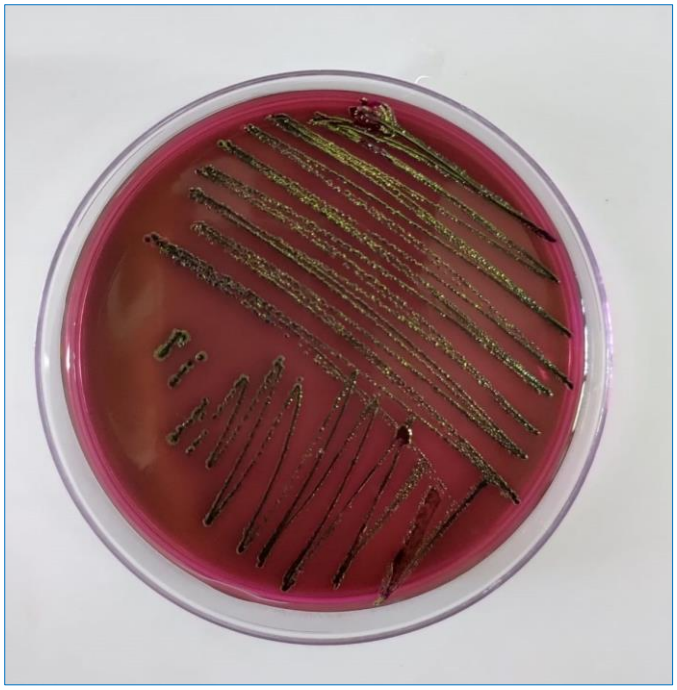

Fig 1: Metallic sheen colony of E. *coli* on EMB agar plate

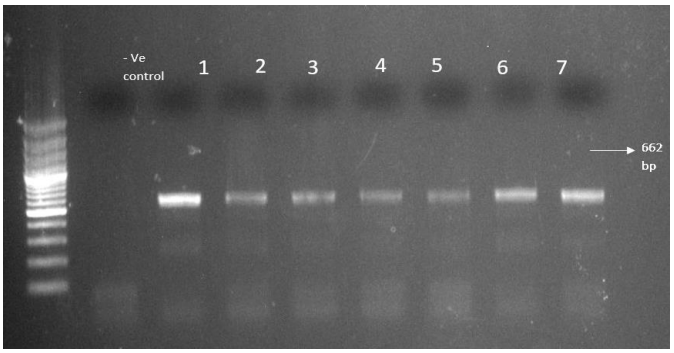

Fig 2: PCR based species-specific 16 S r RNA ribotyping of *E. coli* isolates

Quadruplex PCR-based Phylogenetic Grouping of E. Coli: The phylogrouping of *E. Coli* isolates was carried out by performing Quadruplex-PCR as per described previously described by Clermont *et al*, 2013. The phylogenetic group analysis revealed that the most prevalent group B1 had 11 (40.75%) isolates followed by group A having 7 (25.92%) isolates. Only one isolate belongs to Phylogroup C, D, and E each. The 5 (18.51%) isolates were found to not be associated with any phylogenetic groups (Table-1). The findings were almost similar to Müştak *et al.*, 2015 [7]. in which groups A1 and B2 were found the most prevalent by triplex and quadruplex PCR assays in *E. coli* isolates from mastitic samples and also most of the urinary tract isolates belonged to A1 by triplex and B2 by quadruplex PCR assays.

Table 1: Quadruplex PCR-based Phylogenetic Grouping of *E. Coli*

Phylogrouop	Number of *E. Coli* isolates
Phylogroup-A	7
Phylogroup-B1	11
Phylogroup-C	2
Phylogroup-D	1
Phylogroup-E	1
Phylogroup-U	5

Conclusion

E. coli is the most common causative agent of calf diarrhea. Easy and early identification of pathogens plays an important role in the treatment of such diseases. In the present study, all the isolates from calf diarrhea samples showed a specific metallic sheen colony on EMB agar which is characteristic of *E. coli* isolates, and showed 16s r RNA specific gene amplification. In phylogenetic grouping by quadruplex highest isolates belonged to B1 followed by A, U, C, D, and, E.

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