SHORT COMMUNICATION

Phylogenetic Analysis and Antimicrobial Resistance of *Escherichia coli* Isolated from Diarrheic Piglets

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Abstract

Escherichia coli is one of the major causative agents of diarrhea in neonatal piglets worldwide. The present study was conducted to examine the occurrence of *E. coli* carrying virulence genes and antibiotic resistance associated with piglet diarrhea in and around Shirwal, District Satara. 30 fecal samples were randomly collected from the piglets with history of diarrhea for the isolation of *E. coli* followed by screening of virulence genes among the isolates by specific PCR. Further *E. coli* isolates were subjected to study phenotypic antibiotic resistance by disc diffusion method. The overall occurrence of *E. coli* was 40% (12/30). On molecular characterization, all the *E. coli* isolates were found positive for 16S ribosomal RNA gene. The highest antibiotic resistance was found for Amikacin (100%) followed by Cloxacillin/Clavulate, Chloramphenicol, Enrofloxacin, Gentamicin and Metronidazole.

Keywords: Antibiotic drug resistance, Phylogenetic analysis, Piglets Diarrhea.

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Introduction

Neonatal diarrhea is a multifactorial condition commonly present in pig farms and leads to economic losses due to increased morbidity and mortality of piglets. The microorganisms associated with enteritis and diarrhea in suckling piglets are, rotavirus A, Enterotoxigenic *Escherichia coli* (ETEC) and *Clostridium perfringens* type C. *E. coli* is the most common bacterium grouped as commensal *E. coli*, intestinal pathogenic *E. coli* and extra-intestinal pathogenic *E. coli*. The distinction between pathogenic and non-pathogenic strains of *E. coli* is made based on the different adhesion factors (Chaudhari *et al*., 2017) and virulence factors (Muglikar *et al*., 2019). The pathogenic strains of enteric *E. coli* are recognized as enterotoxigenic (ETEC), enteropathogenic (EPEC) and verotoxigenic (VTEC) based on their virulence properties. These pathogenic strains are associated with intestinal infections causing diarrhea as well as extra-intestinal infections (Hammermueller *et al.,* 1995; Sancak *et al.,* 2004). The infection spreads through direct contact via contaminated food and water (Zweifel *et al.,* 2010). The strains of ETEC associated with intestinal colonization in piglets express F4, F5, F6, F17, F18 and F41 fimbriae (Dubreuil, 2008). The piglets neonatal diarrhea leads to economic threat to both small scale and commercial pig producers. Therefore, the study was undertaken to isolate and characterize *E. coli* based on *16S r*RNA from clinical diarrheic cases of piglets.

Materials and Methods

A total 30 fecal samples from diarrheic piglets were randomly collected aseptically and brought to the laboratory during December 2019 to December 2020. The samples were inoculated in nutrient broth and incubated at 37°C for 12 hours and streaked on MacConkey agar for primary isolation. The lactose fermenting pink colonies were streaked on Eosin Methylene Blue (EMB) agar for selective isolation of *E. coli*. The colonies having greenish metallic sheen were confirmed as *E. coli* and were subjected for morphological, cultural and biochemical characterization following the method of Cruickshank *et al*. (1975).

The *E. coli* isolates were screened for virulence potential by hemolysin production on 5% sheep blood agar. The *E. coli* isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method as described by Cruickshank *et al.* (1975) using 30 µg each of Amikacin, Cloxacillin/Clavulanic acid, Chloramphenicol, Amoxicillin/Clavulanic acid, Enrofloxacin, Ciprofloxacin, and 10 µg each of Gentamicin, Metronidazole, Sulpha trimethoprim. Hi Media discs were used for sensitivity

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test. The diameter of zones of inhibition was measured to nearest millimeter and interpretation was made as described by the manufacturer.

Molecular Detection of *E. coli*

DNA of all *E. coli* isolates was extracted using DNA Mini Kit (Hi Media Pvt Ltd) according to the manufacturer's protocol. For molecular identification of *E. coli*, primers for *16S rRNA* gene of *E. coli* were selected (Tonu *et al.,* 2011). The reaction was carried out in a total volume of 25 µL consisting 3 µL of DNA as a template, 1 µL (5 pmol) of each forward & reverse primer and 12.5 µL of Hichrome PCR master mix (Hi Media Pvt Ltd), 7.5 µL of nuclease free water. The PCR mixture was then subjected to the following cycling conditions: 50°C (1 cycle for 2 min); 95°C (1 cycle for 5 min); 40 cycles at 95°C (45 s), 50°C (1 min), and 72°C (1 min); and 72°C (1 cycle for 7 min) in a thermal cycler (Eppendorf).

The 16S rRNA gene fragment of *E. coli* was detected by using ECO-F CAGTCGTCTCCGAAGTTAACAA a forward primer and ECO-R CTCTACGCATTTCACCGCTAC as a reverse primer (Tonu *et al.,* 2011). The amplified DNA was checked for presence of 704 bp product specific for *E. coli* by electrophoresis in 1.5% agarose gel, 100 bp ladder was used for marker and ethidium bromide as a tracing dye under UV illuminator.

Sequencing and Phylogenetic Analysis

The amplified *16S rRNA* fragments of these isolates were purified using the QIAquick gel extraction kit (Qiagen,) following the manufacturer's instructions and sequenced at gene Ombio Technologies Pvt. Ltd. The sequences of the *16S rRNA* gene have been deposited in the GenBank database under the accession number MW11053. Genes sequenced in this study were compared with the sequences available in public domain using NCBI BLAST (Basic Local Alignment Search Tool) server. The sequences were analyzed using BLAST and the Clustal-W (CLUSTAL 2.1 multiple sequence alignment) to generate sequence alignment reports. Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 was used for construction of phylogenetic tree. The bootstrapped phylogenetic tree was constructed using Neighbor-joining method.

Results and Discussion

Out of 30 diarrheic piglet fecal samples screened, 12/30 (40%) samples were found positive for *E. coli* isolates, which were gut acting, as reported by Zweifel *et al.* (2010). Our observations are in concordant with a previous study on diarrheic piglets, where 29.93% ETEC and 2.54% EPEC prevalence were recorded (Rajkhowa and Sarma, 2014).

The *in-vitro* antibiotic sensitivity pattern of all 12 *E. coli* isolates indicated maximum (100%) sensitivity towards sulpha/trimethoprim, while 50% and 16.66% isolates were found sensitive to amoxicillin/clavulanic acid and

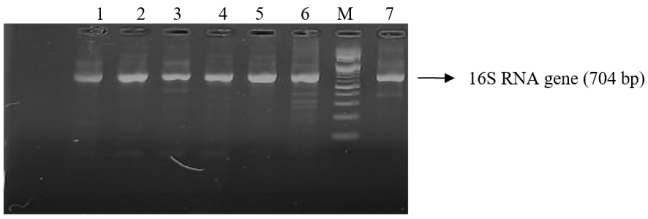

Fig.1: Amplification of *E. coli* (704 bp) of piglet faecal samples. Lane M-Molecular weight marker (100bp ladder) Lane-1,2,3,4,5,6 = Positive *E. coli* samples, Lane-7=Positive control

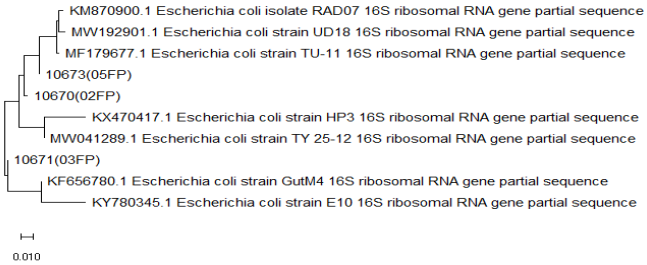

Fig. 2: Sequence homology analysis of *E. coli* strains detected from diarrheic piglet

ciprofloxacin, respectively. The isolates showed high resistance towards amikacin (100%), followed by cloxacillin/clavulanic acid, chloramphenicol, enrofloxacin, gentamicin, and metronidazole. These findings agree with the reports of Bashar *et al.* (2011) and Parul *et al.* (2014).

The *E. coli* isolates were subjected to molecular characterization for the detection of *16S* rRNA gene. All 12 *E. coli* isolates were found positive for *16S* rRNA (Fig. 1). Shakuntala *et al.* (2017) reported that out of 152 *E. coli* isolates, 18 (11.8 %), 2 (1.3%) and 23 (15.1%) isolates were Shiga-toxin-producing *E. coli* (STEC), atypical enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. Coli* (ETEC), respectively, by PCR. Rajkhowa and Sarma (2014) found 29.93% ETEC and 2.54% EPEC prevalence of diarrheic piglets by PCR. Rhouma *et al.* (2021) explained that genomic DNA could be used to assess the fecal microbiota diversity and composition using the V4 region of the 16S rRNA gene in piglets.

Sequence homology analysis of *E. coli* strains was detected from diarrheic piglet specimen by PCR and was confirmed by nucleotide sequencing and phylogenetic analysis. On phylogenetic analysis of the sequence of PCR products (Accession Nos: 10671, 03FP), 98% and 100% homology were seen with the genome sequences (Ac. No: KX470417 *E. coli* 16 S rRNA) and (Ac. No: MW041289) of *E. coli* (Fig. 2). The 16S rRNA *E. coli* partial sequences generated in present study revealed close homology as well as with the earlier published 16 S *E. coli* sequences available on NCBI.

Conclusion

The study revealed a high incidence of *E. coli* (40%) in diarrheic piglets in Western Maharashtra. The highest antibiotics tolerance was observed in piglet isolates showing

indiscriminate use of these antibiotics. *E. coli* antibiotic resistance in piglets is alarming, and this study has raised awareness to overcome antibiotic abuse to treat and prevent *E. coli* colibacillosis infection in piglets.

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ANNOUNCEMENT: SVSBT-NS-2022

IX Annual Convention and National Seminar of SVSBT

The ***IX Annual Convention*** and ***National Seminar*** of The Society for Veterinary Science & Biotechnology ***(SVSBT) on "Recent Biotechnological Advances in Health and Management to Augment Productivity of Livestock and Poultry"*** will be **organized at Ramayanpatti, Tirunelveli - 627 358, Tamil Nadu, during September 22-24, 2022** (Thursday, Friday & Saturday) by Veterinary College & Research Institute, Tirunelveli - 627 358, TANUVAS, (TN). The detailed Brochure cum Invitation showing Theme Areas/ Sessions, Registration Fee, Bank Details for online payment and deadlines, etc. has been floated on the Whats Apps and e-mails. Accordingly, the organizing committee of ***SVSBT NS-2022 invites abstracts*** of original and quality research work on theme areas of seminar limited to 250 words by e-mail on svsbttnns2022@gmail.com or mopandian69@gmail.com latest by 30th August, 2022 for inclusion in the Souvenir cum Compendium to be published on the occasion.

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