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Research Article

FIRST REPORT OF ISOLATION OF ENTEROPATHOGENIC *ESCHERICHIA FERGUSONII* FROM THE FAECES OF ELEPHANTCALF *(ELEPHAS MAXIMUS)*

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ABSTRACT: A wild elephant calf of 6-8 months of age was brought to the Centre for Wildlife Rehabilitation and Conservation (CWRC), Bokakhat, Assam, India, having symptoms of diarrhea and debility. The fecal sample was collected and studied for isolation and identification of bacteria. Eosin Methylene Blue (EMB) agar media showed moderate growth of bacterial colonies with a characteristic green metallic sheen. The isolates were subjected to Gram stain and biochemical tests (lactose, sucrose, and sorbitol). Polymerase chain reaction (PCR) amplification with universal primers showed an amplicon of 300 bp. The PCR amplified product after nucleotide BLAST showed 98.95 % similarity with the sequence of enteropathogenic *Escherichia fergusonii*.

Keywords: Coli bacillosis, Elephant, *E. fergusonii*, Enteropathogenic, Primers.

INTRODUCTION

Neonatal diarrhea is a common phenomenon caused by infectious pathogens which include bacterial, viral, and parasitic organisms apart from contaminated food, water, or some other ingested milk products [1, 2]. The development of resistance among bacteria against the anti-bacterial agents and lack of development of newer effective anti-bacterial at regular intervals limits the role of using them to get rid of these problems easily [3, 4, 5]. The presence of infective organisms in the surrounding environment leads to the development of food, water, and milk-borne infections [6, 7]. It is also true that when the elephant calves when raised with skimmed milk, insufficient consumption of colostrum is a frequent cause of the development of collibacillosis in newborn calves. Colostrum and its components are effective against a wide range of common pathogens including rotavirus, streptococcus species, and *E. coli. Escherichia coli* is a very common pathogen in diarrhoeic calves and companion animals [8]. *Escherichia fergusonii* is a closely related organism to *E. coli* apart from its relation to non-human primates [9]. *Escherichia fergusonii* could be obtained from fecal samples of goats, sheep, horses, turkeys, ostrich, chicken, cattle, pigs, dogs [10], newborn foals [11], warm-blooded animals [12], and from beef samples [13]. The pathogen is associated with abortion, diarrhea, and mastitis in cattle and sheep [14]. It has also been isolated from human clinical samples like blood, urine, abdominal wounds, and feces [15].In the present study, *E. fergusonii* was isolated from the fecal sample of a diarrhoeic calf. It is an emerging multi-drug resistant zoonotic pathogen [1] and hence a detailed study is required to know the status of the disease in elephants due to its scanty information.

MATERIALS AND METHODS

Case history

A wild elephant calf of 6-8 months of age, rescued by the villagers was brought to the Centre for Wildlife Rehabilitation and Conservation (CWRC), Bokakhat, Assam with the complaint of debility and diarrhea. The calf was treated with a combination of ciprofloxacin (3000 mg) and tinidazole (3600 mg) which showed no clinical improvement. Instead, the condition of the calf deteriorated further exhibiting

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symptoms of lethargy, weakness, profuse watery diarrhea followed by ataxia, tremor, muscular twitching, bloat, swelling of eyelids, dilatation of pupil, recumbency, comatose, and ultimately death. The fecal sample was collected within two hours of death and brought for bacteriological examination.

Bacterial isolation

The rectal and fecal swab samples were collected in a sterile container containing normal saline solution and were kept under refrigeration temperature (2ºC) until processed. The sample was a liquid, off-white color with a foul odor. The fecal samples were inoculated into MacConkey agar (HIMEDIA; Catalogue No.SM081; 100 ML) and incubated at 37°C for 24 h. The colonies appeared to be dry, partial pink color lactose fermenting colonies on MacConkey plates similar to other strains of the *Escherichia* family, however, a little skepticism drove us to go for the lactose fermentation test for reliability on the phenotypic outcome. Further, it was inoculated on Eosin Methylene Blue (EMB) agar (HIMEDIA; Catalogue No.MV022; 500G) Colonies (*Escherichia* spp.) showed a green metallic sheen which was subsequently purified on nutrient agar plates (Fig. 1). Isolates were phenotypically identified as Gram-negative small cocco-bacilli using conventional Gram's staining technique and were subjected to further biochemical (lactose, sucrose, and sorbitol) and finally confirmation molecular technique.

DNA isolation

DNA was extracted using a DNA Extraction kit (Nucleo-pore, Genetix; Catalogue No. NP-1003T; 20 reaction pack size) according to the manufacturer's specifications. The concentration of the extracted DNA was determined by the Nanodrop (Thermo scientific).

Polymerase chain reaction

Universal primers of 27F and 1492R with sequence 5' AGAGTTTGATCMTGGCTCAG 3' and 5' TACGGYTACCTTGTTACGACTT 3 were used. The PCR reactions were performed with a 25 µL total reaction volume resulting from 12.5 µl of master mix (Thermo Scientific), 0.5 µl of each primer, 11 µl nuclease-free water and 1 µl of DNA templates. Thermal cycling conditions followed were: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, final extension at 72°C for 7 min with 36 cycles [15]. The PCR products were subjected to gel electrophoresis in 1.5% 1X Tris-EDTA (TAE) buffer agarose gel with 3 µl ethidium bromide for 1 hat 90 Volts and visualized under trans illuminator as per standard procedures. For size comparison of the amplicons, a 100 bp and DNA ladder marker (Thermo Scientific) was used.

Nucleotide sequence analysis

The amplified PCR product was purified and sequenced by 1st BASE DNA sequencing; in Malaysia. The nucleotide sequence of the amplified PCR product was subjected to Phylogenetic analysis and standard Nucleotide BLAST analysis to compare with the reference sequences available in the NCBI GenBank. The sequences were aligned using ClustalW (https://www.genome.jp/tools-bin/clustalw) and the phylogenetic tree was made using the MEGA 7 software (Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets [16].

Antibiotic sensitivity test

To determine the antibiotic susceptibilities of *E. fungusonii* isolates, the disk diffusion method was applied using Mueller-Hinton Agar (HIMEDIA; Catalogue No. MV022M173; 500G). The antimicrobial agents and their concentration per disc used in the present study were ceftriaxone and tazobactam (30/10 µg), enrofloxacin (5 µg), amoxicillin and sulbactam (10/10 µg), amoxicillin and clavulanic acid (30 µg), ciprofloxacin (5 µg) and gentamicin (10 µg). Interpretation of the test was done based on the zone sizes around the antibiotic discs as described [17]. The most sensitive antibiotic was identified and was recorded for further *in-vitro* studies in the event of future reporting of similar diseases for rendering therapeutic management at the field level.

RESULTS AND DISCUSSION

In the present study, the fecal sample and rectal swab from the affected calf were collected aseptically and inoculated on MacConkey and Eosin Methylene Blue (EMB) agar plates. The suspected isolates showed a green metallic sheen (Fig. 1) on EMB plates and were subjected to Gram's stain. Small Gram-negative, non-capsulated, non-motile, and non-spore-forming cocco-bacilli ranging from 0.5 to 1 µm in size under oil immersion (100X objective) were detected in Gram staining. The isolate was purified on nutrient agar plates and subjected to further biochemical tests using the API 20E identification kit (API 20 E Analytical Profile Index (Ref. 20 190). The isolate was unable to ferment the lactose, sucrose, and sorbitol. *Escherichia fergusonii*, although typically unable to ferment lactose,

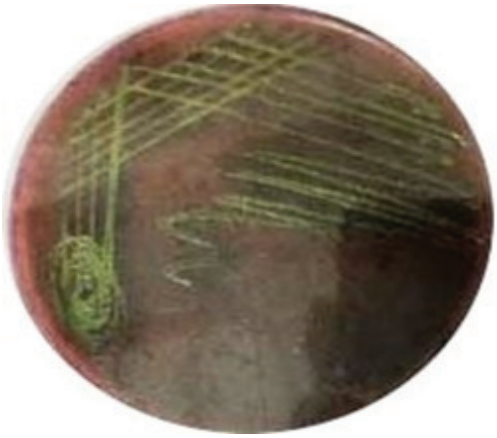

Fig. 1. Green metallic sheen on EMB agar plate.

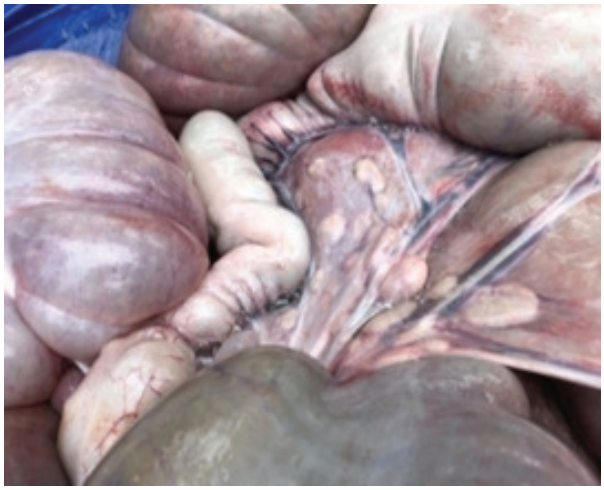

Fig. 2. Enlargement of mesenteric lymph nodes.

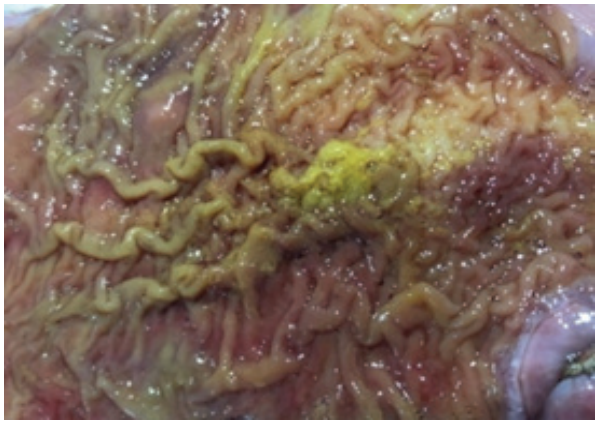

Fig. 3. Haemorrhagic gastritis.

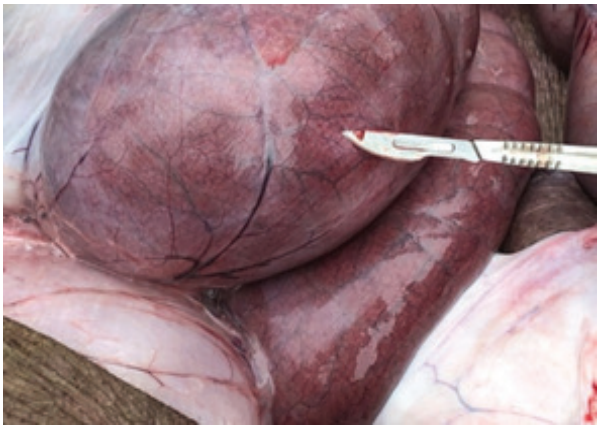

Fig. 4. Congestion in the serosal surface of stomach and intestine.

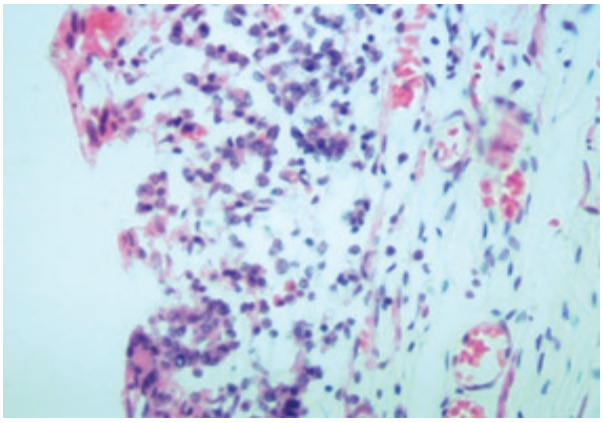

Fig. 5. Sloughing of necrosed epithelial cells of intestinal villi. (At scale bar = 40 µm, 40X magnification).

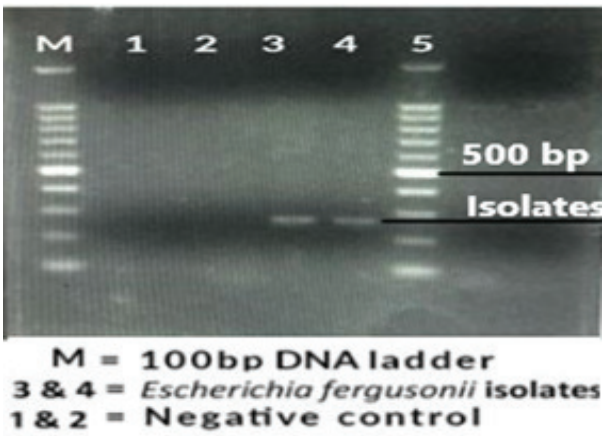

Fig. 6. PCR results of *E. fergusonii.*

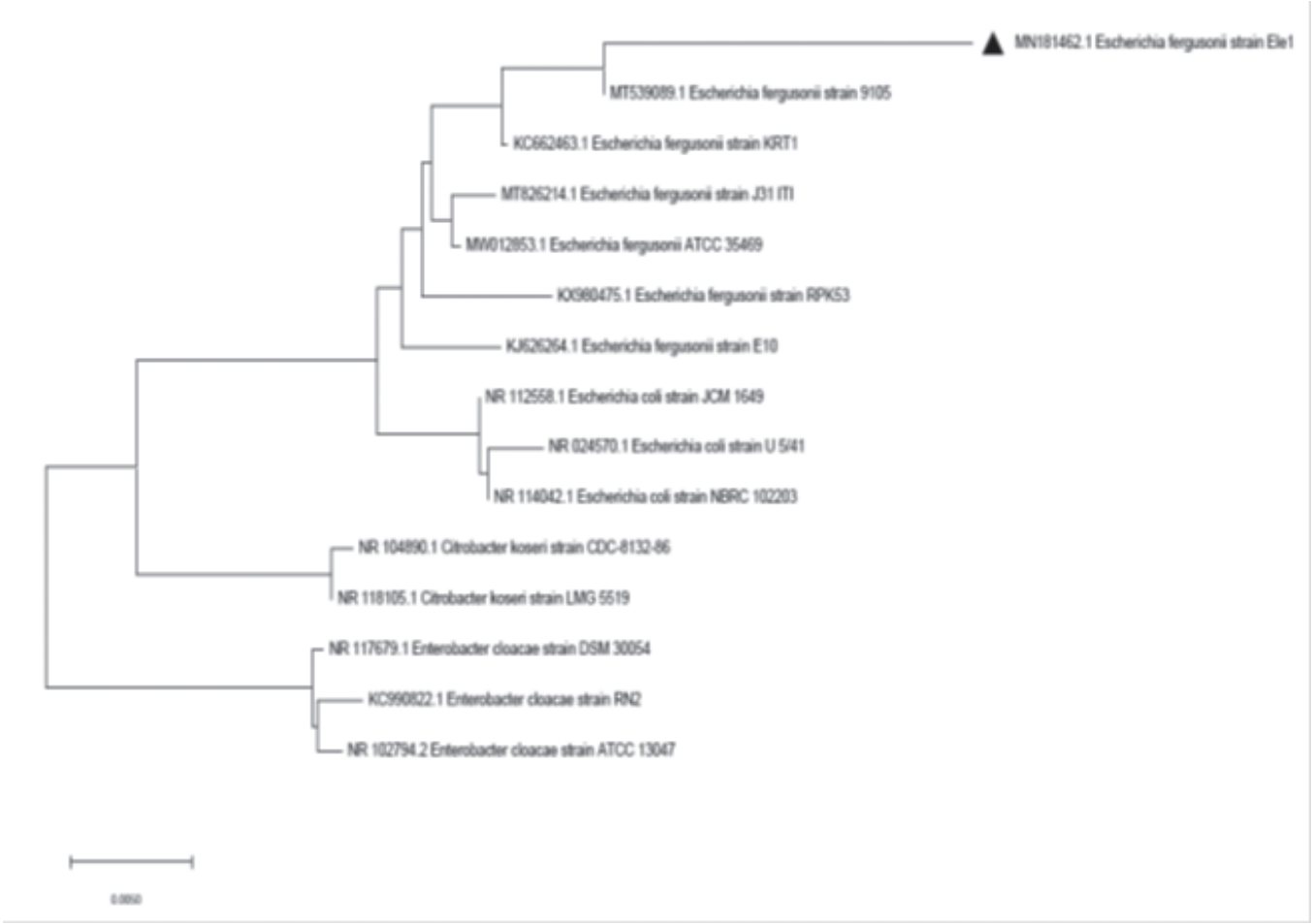

Fig. 7. Phylogenetic tree based on partial 16S rRNA gene sequences showing relationship between *E fergusonii* and other closely related members of Enterobacteriaceae. [The *E fergusonii* isolated in this study has been marked as "Δ". The sequences were aligned with Clustal W and the tree was constructed by the Neighbour-joining method using MEGA 7 software].

can occasionally exhibit pink-colored colonies on lactose-fermenting media like MacConkey agar. This can happen due to the presence of other factors or enzymes that may lead to a weak or partial fermentation of lactose, resulting in a subtle color change. However, it is important to note that the pink coloration in *E. fergusonii* colonies on lactose-fermenting media is not as reliable or intense as in lactose-fermenting strains of *Escherichia coli*. Therefore, when working with *E. fergusonii*, confirming the identification using additional tests or molecular methods is crucial to avoid misinterpretation or misidentification. Therefore, the presence of *E. fergusonii* was also confirmed in the fecal sample of the elephant calf by polymerase chain reaction (PCR) amplifying the 16s rRNA gene using the universal primers (27F and 1492R) (Fig. 6). The amplified PCR product showed a fragment size of 300 bp and was outsourced for sequencing. The nucleotide sequence was submitted to the NCBI GenBank and the accession number MN181462.1 was obtained. The percent identity observed with other *E. fergusonii* sequences are as follows- 99.14% with *E. fergusonii* (MT539089.1), 99.05% with *E. fergusonii* (MT826214.1) and *E. fergusonii* ATCC 35469 (MW012853.1), 98.86% with *E. fergusonii* (KC662463.1), 98.67% with *E. fergusonii* (KX980475.1) and 98.48% with *E. fergusonii* (KJ626264.1). Furthermore, the phylogenetic analysis revealed that the *E. fergusonii* isolated in this study (MN181462.1) clustered with the other representative *E. fergusonii* sequences available in the GenBank while sequences of the other members of the Enterobacteriaceae selected for this study formed distinctly separate clusters (Fig. 7).

Gross lesions of the affected calf showed enlargement of mesenteric lymph nodes (Fig. 2),

marked hemorrhagic gastritis (Fig. 3), and congestion in the serosal surface of the stomach and intestine (Fig. 4). Histopathological examination of the affected elephant calf showed severe congestion in the submucosa and muscularis of the intestine. The intestinal villi were flattened with sloughing of the epithelium and necrosis (Fig. 5).

Following its first isolation in 1985 from various human samples *E. fergusonii* has also been recovered from faecal samples of goat, sheep, horse, turkey, ostrich, chicken, cattle, pig and recently from fish. The presence of *E. fergusonii* in faecal sample of elephant calf was confirmed in the present study on the basis of morphological, cultural, and biochemical characteristics. It was further confirmed by polymerized chain reaction (PCR) using the universal primers (27F and 1492R) and Standard Nucleotide BLAST. Isolation and confirmation of *E. fergusonii* from elephant calf is the first document of its kind in Assam, India. It has also been detected from Bangladeshi children [18] as well as from diarrhoeic patient of Odisha, India [15].The rescued elephant calf under the present study being raised with skimmed milk under close custody of caretakers of wildlife rehabilitation and conservation centre, have every possibility of acquiring Escherichia infection with *E. fergusonii* from the caretaker and other attendants during the period of stay at rehabilitation center. The rescued elephant calf usually came in contact with the staff/caretaker during feeding (three times/day). Majority of reported *E. fergusonii* isolations in other parts of the world are case reports but data on host species dynamic, reservoirs and transmission are currently unknown.

Molecular detection of multiple antimicrobial résistance factors in all *E. fergusonii* isolated from juvenile monkeys was attributed to regular human contact for feeding, bathing and cudding and thereby assumed to be the possible route of transmission into nonhuman primate population.

CONCLUSION

Isolation and identification of *E. fergusonii* is important because it may emerge as antibiotic-resistant gut bacteria in the near future. In order to prevent future outbreaks, it is necessary to conduct more investigations on its zoonotic implications, host reservoir, and multidrug-resistant isolates, especially at fringe areas where the occurrence of spillover infection due to wildlife-domestic interface spillover episodes are very common. Molecular identification tests described in the present study has clearly established that enteropathogenic *E. fergusonii* was responsible for causing colibacillosis in elephant calf.

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