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Genetic characterization and clinicopathological features of *Escherichia coli* in neonatal septicemic calves

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Abstract Due to severe diarrhea associated with calf scour and septicemia, colibacillosis is one of the leading causes of neonatal mortality in calves. This study investigated the clinical and hematological manifestations as well as the genetic composition of Shiga toxin-producing *E. coli* (STEC) strains in septicemic neonatal calves. In this study, 47 fecal specimens were collected from diarrheic calves aged between one and three weeks on dairy farms that are unorganized around Basra, Iraq, while twenty age-matched calves were tested as controls. Of the 47 tested samples, 28 fecal samples were tentatively identified as STEC, with a prevalence of 60%. STEC-affected calves had variable rectal temperatures ranging from subnormal to fever, tachycardia, hyperpnea, diarrhea, partial or complete cessation of suckling, mucous membrane congestion, depression, incoordination and rough hair coat conditions. Significant changes were observed in the hemogram and the leukogram during the analysis of blood samples from calves with diarrhea. Molecular analysis of fecal samples demonstrated that local STEC isolates are more closely related to other strains obtained from both human and animal samples worldwide. Based on the results of the current study, we can conclude that the septicemic calves were likely infected by human or environmental resources and could play a major role in further spreading STEC infection.

Keywords: Colibacillosis, neonatal calves, STEC, phenotypic characterization, molecular detection

1. Introduction

A clinical syndrome that is very challenging for veterinarians is diarrhea in farm animals, especially neonatal calves. Cattle industry losses are caused primarily by diarrhea, which is a leading cause of morbidity and mortality during the first few weeks of life for newborn calves (Constable et al., 2016; Shekhar et al., 2017). In addition to deaths, other economic losses are associated with treatment, diagnostics, labor and veterinary intervention, as well as a reduction in the number of herd replacements and the resulting chronic illness (Bazeley, 2003).

Infectious and noninfectious agents, as well as environmental and management factors, all contribute to calf diarrhea (Constable et al., 2016; Izzo et al., 2011); thus, controlling diarrhea effectively is difficult (Al-Alo et al., 2018; Cho & Yoon, 2014). When outbreaks of diarrhea occur, it is essential to identify the causative agent and identify the risk factors and sources of infection so that targeted prevention measures, such as vaccinations, can be taken (Bazeley, 2003; Izzo et al., 2011).

Neonatal diarrhea in calves is mainly caused by a wide range of pathogens—as a single pathogen or in combination—including mainly enterotoxigenic *Escherichia coli* (*E. coli*) (Foster & Smith, 2009). Among young calves, colibacillosis caused by *E. coli* is a major cause of death (Sharma et al., 2006). Infection usually occurs via the oral-fecal route through the ingestion of contaminated food and water. However, transmission via the umbilical vein and nasopharynx is thought to be possible with certain strains of *E. coli* (Radostits et al., 2006).

Although a few serotypes of *E. coli* might induce diseases such as pneumonia, pyelonephritis, and diarrhea (Zhao et al., 2014), there are five diverse types of diarrhea-causing *E. coli*: enterohemorrhagic, enteropathogenic, enteroaggregative, enterotoxigenic, and enteroinvasive (Markey et al., 2013). Phenotypically, *E. coli* is a G-cylindrical, striate, road-shaped, nonspore-forming bacillus. They are aerobic in nature, facultatively anaerobic and made motile by peritrichous flagella (Mishra & Agrawal, 2012; Quinn et al., 2011).

Serious conditions and/or diseases, including colibacillosis, have threatened the livestock industry in Iraq (Saleh et al., 2019) (Naji et al., 2019). There is limited information on these diarrhea-causing *E. coli* strains in newborn calves in Basra, Iraq, due to a lack of documented data. Therefore, the present study aimed to reveal this lack of knowledge about colibacillosis caused by *E. coli* (STEC strain) in newborn Basrah calves through clinical, hematological, phenotypic and genotypic characterization.

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2. Materials and methods

2.1. Animals and Experimental Design

Our experiment was performed between September and December 2018, and neonatal calves from dairy cow farms in different areas of Basra city were examined. Forty-seven neonatal calves (3-21 days of age) with symptomatic colibacillosis and twenty age-matched clinically healthy calves were used as controls in this study. The current study was approved by the general committee of Animal Use and Welfare, University of Basrah, College of Veterinary Medicine, Basra State, Iraq. Swabs and blood specimens were collected from all calves. The general appearance of the body condition, temperature, rate pulse, and rate of respiration were determined according to the methods of (Jackson & Cockcroft, 2008).

2.2. Clinical examination

Calves with diarrhea were examined clinically, and vital signs, such as rectal temperature C, respiratory rate (breaths/minute), and pulse rate (beats/minute), as well as the mucous membranes, were recorded and documented (Naji et al., 2019). However, a careful clinical evaluation was performed, and questionnaires were administered to the owners to evaluate other important signs, such as diarrhea, suckling status, depression, and incoordination. Hair coat quality, as an indicator of animal welfare, was examined under different conditions in all calves throughout the study period as described by (Saleh, 2019). Two main points are taken into consideration when recording hair coat conditions: normal hair conditions (NH) and weak or rough hair conditions (RH).

2.3. Bacterial Isolation and Identification

Fecal samples were collected from both septicemic and clinically healthy calves via sterile dry cotton rectal swabs. Then, the specimens were aerobically cultured at 37°C for 48 hours on EMB agar and on MacConkey agar in the Central Research Unit Laboratory/University of Basrah, College of Veterinary Medicine, Basra, Iraq. According to the gross features of the growing colonies, *Escherichia coli* were identified (Figures 1, 2). Moreover, Gram smears were prepared from each colony type for microscopic examination. Further confirmation was performed based on the biochemical activities of the *E. coli*-positive samples according to the guidelines of Quinn et al. (2011) as well as the Vitek® system. (Table 1). The bacterial isolates suspected to be *E. coli* according to conventional biochemical test results were re-examined by the "bioMerieux VITEK2®" system. An ID-GNB card is a 64-well plastic card containing 41 fluorescent biochemical tests for gram-negative bacteria in the VITEK 2 system (Gavin et al., 2002). According to the manufacturer's instructions, the suspected *E. coli* isolates were analyzed using the "VITEK 2" system.

Table 1 The Results of the Bio-Chemical Properties of *E. coli.*

Test	Gram Stain	Shape	Catalase	Oxidase	O-nitrophenyl-βgalactoside	Methyl red	Urease	Indole	H2S production	Motility	Glucose	Voges Proskauer	Citrate	Lactose
Result	-	Bacilli shape	+	-	+	+	-	+	-	+/	+	-	-	+

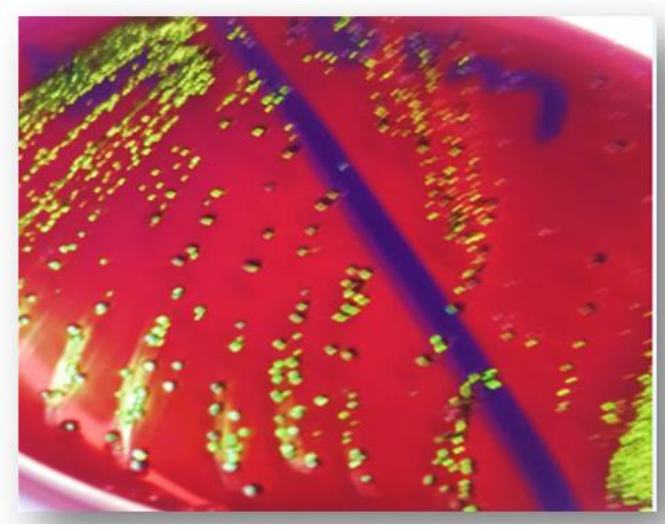

Figure 1 A characteristic metallic green sheen morphology of *E. coli* colonies on EMB media.

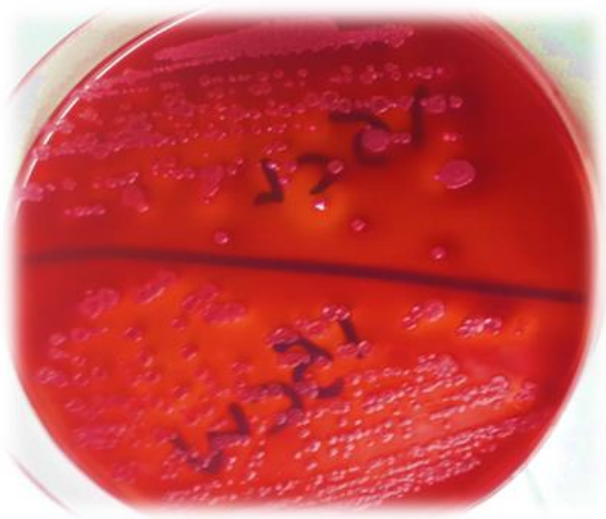

Figure 2 A characteristic circular pink creamy with a smooth central morphology of *E. coli* colonies on Mackonky media.

2.4. Hematology

Blood samples were collected once from the jugular vein of the diseased and control calves for hematological investigation. Blood samples were taken according to the methods of (Jackson, 2013). A complete blood count was performed after five millilitres of blood were collected from the jugular vein aseptically and sent directly to the laboratory in an ice box for analysis. Blood specimens were examined in the Laboratory of Clinical Pathology, College of Veterinary Medicine, University of Basra, using an animal blood counter modified specifically for veterinary use (COUNT 60 *GENEX laboratories,* USA). The following hemogram and leukogram parameters were used: RBC count, Hb, PCV, MCV, MCH, MCHC, and total and differential WBC counts.

2.5. Genomic analysis

A DNA extraction kit was used to extract the DNA of the positive *E. coli* isolates, and sequencing of the gene fragments and standard PCRs were all performed by Apical Scientific Sdn. Bhd. Laboratories, Malaysia (formerly named "1st BASE" laboratories) under the MBS Order ID: 2641 in 2018. *E. coli*-positive colonies were aseptically cut and sent to 1st base laboratories for molecular analysis. The full length 16S rRNA gene (~ 1400 bp) was obtained via "Bacterial Species Barcoding". Includes extraction of gDNA, PCR amplification and purification plus bidirectional PCR product sequencing with data analysis (BLAST to show the top 10 matches from the database)".

2.6. Statistical analysis

The data obtained in the present study were statistically analyzed at P<0.05 using SPSS software. Student's t test was used for comparisons of parametric variables. The chi-square test and/or Microsoft Excel software were also used for nonparametric variables.

3. Results

Clinically, calves suspected to have colibacillosis had variable rectal temperatures ranging from subnormal to fever (36.1 - 41.6 °C), tachycardia, hyperpnea, diarrhea, partial or complete cessation of suckling, mucous membrane congestion, depression, incoordination and rough hair coat conditions (Table 2). The control calves were determined to be healthy based on clinical findings and hematological parameters that were within normal values.

Complete blood count (CBC) parameters were significantly lower in the calves with septicemic colibacillosis than in the healthy controls ($p < 0.05$). However, the CBCs of the septicemic and control calves were within the normal standard values (Table 3).

The overall prevalence of diarrhea caused by *E. coli* in calves in the current study was 60% (28 neonatal calves) (Figure 3). However, *E. coli* was detected in 10% (two neonatal calves) of the clinically normal calves.

Among the 28 septicemic neonatal calf samples identified as *E. coli* by biochemical tests and the VITEK 2 system, 25 were confirmed to be pure *E. coli* isolates via PCR (Figure 4). However, the two isolates obtained from clinically healthy calves were not confirmed to be *E. coli* by PCR. Moreover, whole-genome or complete-genome sequencing of the positive PCR products revealed two main mutant *E. coli* strains, which have the accession numbers LC497324.1 and LC497325.1 in GenBank. These local isolates are more closely related to other strains obtained from both human and animal samples worldwide (Figure 7). The degree of the neighborhood was very high for the "Shiga Toxin-producing *Escherichia coli* (STEC) O145" strain (accession number CP014670.1), which was obtained from human and/or host-associated samples in the USA. Another close correlation was detected with the *E. coli* strain 646 (accession number: CP023200.1), which was isolated from neonatal calves in India. Based on the neighbor-joining phylogenetic tree of the current study (Figure 7), our isolates were considered to be Shiga toxin-producing *Escherichia coli* (STEC).

Table 2 Clinical Examination Results of the Calves with Colibacillosis and Control Calves.

Criteria	Infected Calves (n=47)	Control Calves (n=20)
Temperature (°C; Mean ± SE)	39.51 ± 0.17^{a}	38.77 ± 0.05^{ab}
Pulse Rate (Beat/min; Mean ± SE)	109.13 ± 1.72^{a}	103.65 ± 1.79^{b}
Respiratory Rate (Breath/min; Mean ± SE)	58.13 ± 1.35^{a}	50.3 ± 1.14^{b}
Diarrhea (No.)	47 (100%)	0
Partial Cessation of Suckling (No.)	39 (83%)	0
Cessation of Suckling (No.)	8 (17%)	0
Congested Mucous Membranes (No.)	40 (85%)	0
Depression (No.)	38 (81%)	0
Incoordination (No.)	23 (49%)	0
Rough Hair Coat Condition (No.)	41 (87%)	0

The "a, b" symbols with different superscript values are significantly different (P<0.05).

Table 3 CBC Values for Control and Septicemic Calves (Mean ± SE).

Criteria	Septicemic Calves (n=47)	Control Calves (n=20)
RBC ($x10^{12}$/L)	7.61 ± 0.27^{ab}	7.84 ± 0.28^{a}
Hb (g/L)	9.42 ± 0.35^{ab}	9.75 ± 0.39^{a}
PCV (L/L)	29.67 ± 1.64^{a}	28.17 ± 1.66^{b}
MCV (fL)	39.30 ± 2.19^{a}	36.19 ± 2.02^{b}
MCH (Pg)	12.47 ± 0.49^{a}	12.49 ± 0.38^{a}
MCHC (g/L)	32.54 ± 1.8^{b}	35.62 ± 2.21^{a}
WBC ($x10^{9}$/L)	9.92 ± 0.28^{a}	8.82 ± 0.37^{b}
Neutrophils ($x10^{9}$/L)	4.53 ± 0.19^{a}	4.32 ± 0.18^{b}
Lymphocytes ($x10^{9}$/L)	4.26 ± 0.15^{a}	4.26 ± 0.18^{a}
Monocytes ($x10^{9}$/L)	0.74 ± 0.1^{a}	0.65 ± 0.1^{ab}
Eosinophil ($x10^{9}$/L)	0.29 ± 0.03^{a}	0.26 ± 0.02^{ab}
Basophils ($x10^{9}$/L)	0.08 ± 0.01^{a}	0.09 ± 0.01^{a}

The "a, b" symbols with different superscript values are significantly different (P<0.05).

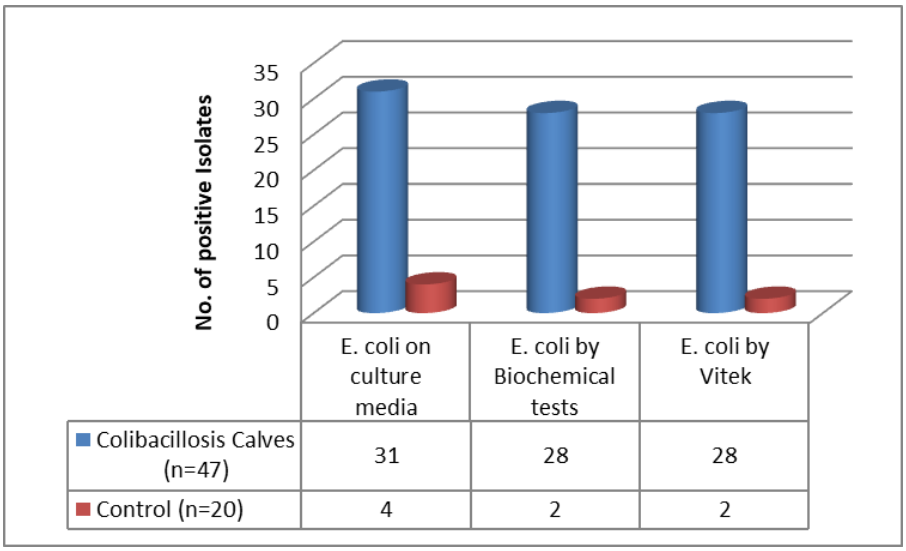

Figure 3 The results of *E. coli* detection depending on the method of identification.

4. Discussion

In the present study, we present a whole-genome sequencing analysis of fecal swab samples obtained from neonatal calves with septicemic colibacillosis combined with a clinicopathological evaluation. Our understanding of the evolution and pathobiology of *Escherichia coli* strains has greatly improved through whole-genome sequencing; this high-adaptability and versatile species can be predicted with a high degree of accuracy from whole-genome sequencing (Robins-Browne et al., 2016). Whole-genome sequencing in the current study will provide additional insight into the strains that are most virulent within a subtype in addition to hematological, clinical and biochemical metadata.

Our findings (those for the LC497324.1 and LC497325.1 isolates) matched those for STEC strains isolated from food poisoning humans and septicemic animals, with a high percentage of compatibility with human-related STEC strains. There was a match with the "Shiga toxin-producing *Escherichia coli* (STEC) O145" strain (CP014670.1) obtained from human and/or host-associated samples in the USA. Another close match was detected with the *E. coli* strain 646 (CP023200.1), which was isolated from neonatal calves in

India, and there was no match with the regional geographic isolates. This finding suggests that the source of infection in neonatal calves could be human sources resulting from poor sanitation conditions in septicemic dairy herds.

Moreover, further spreading of STEC strains to other susceptible people as well as animal infections can have zoonotic impacts on infected calves.

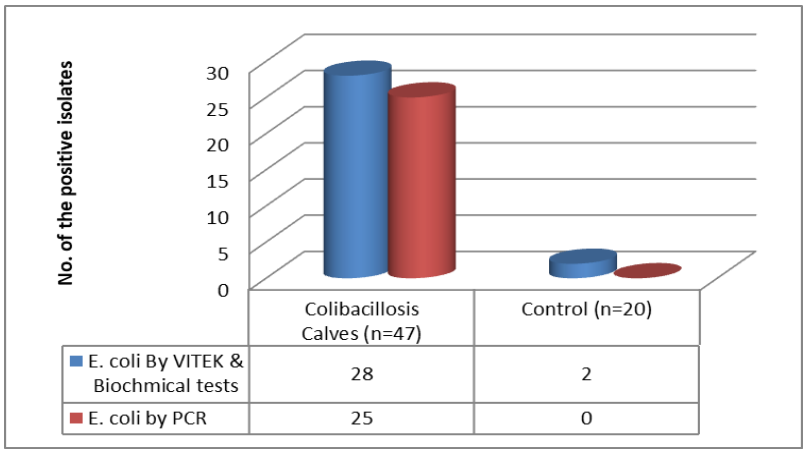

Figure 4 PCR results for both the septic and control calves.

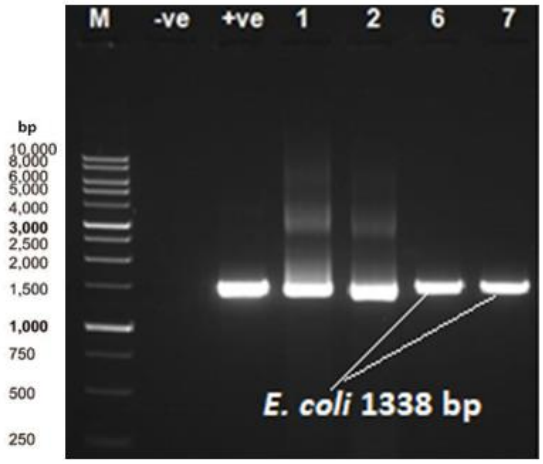

Figure 5 Agarose gel electrophoresis image showing 16S rRNA PCR results, ~ 1.5 kb full length. M: Marker (10000 bp), -ve: PCR no template control, +ve: Positive control (Bacterial gDNA, 10 ng), 6, 7: *E. coli* (1338 bp).

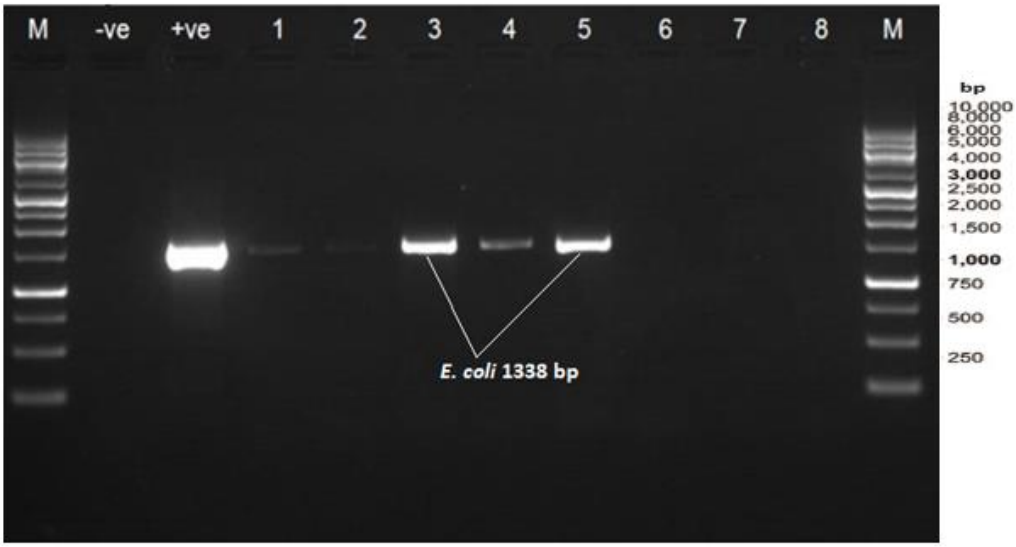

Figure 6 Agarose gel electrophoresis image showing 16S rRNA PCR results, ~1.5 kb full length. M: Marker (10000 bp), -ve: PCR no template control, +ve: Positive control (Bacterial gDNA, 10 ng), 3, 5: *E. coli* (1338 bp).

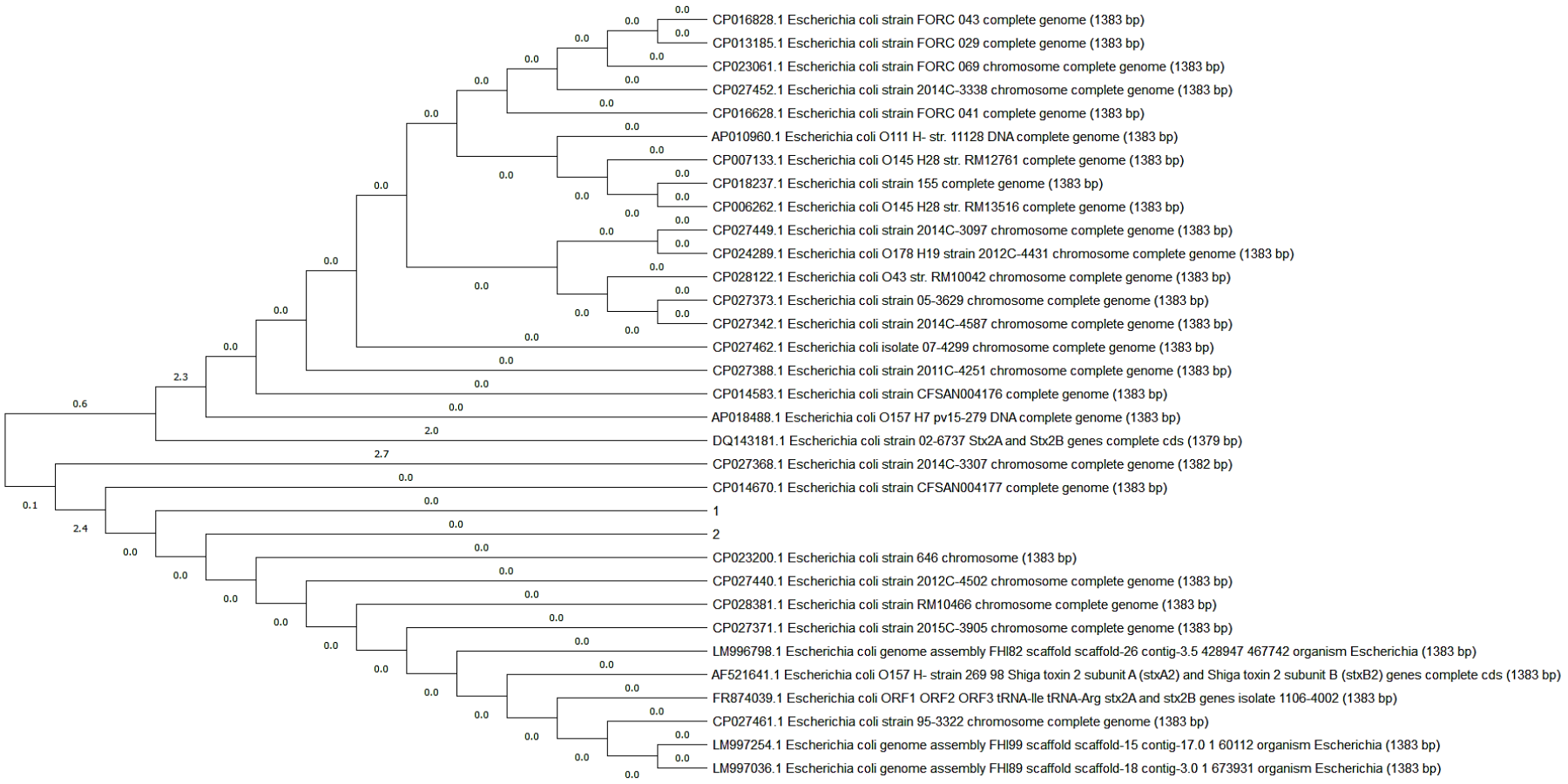

Figure 7 Neonate-associated and reference *Escherichia coli* strains, with whole-genome alignments of 2,272,130 bases each. The maximum likelihood tree (ML) was derived using the RAxML model of GTRGAMMA, with 1000 bootstrap resamplings. The tree was visualized using Dendroscope, and bootstrap values less than 50 were removed. The geographic distribution of the closely related *E. coli* strains to our isolates is described as follows:

CP016828.1: human stool, South Korea; CP013185.1: human stool, South Korea; CP023061.1: human stool, South Korea; CP027452.1: human stool, USA; CP016628.1: food samples, South Korea; AP010960.1: Japan; CP007133.1: Ice cream samples, Belgium; CP018237.1: Human, UK; CP006262.1: feces, Belgium; CP027449.1: c CP024289.1: stool, USA; CP028122.1: bovine fecal samples, USA; CP027373.1: stool, USA; CP027342.1: stool, USA; CP027462.1: USA; CP027388.1: USA; CP014583.1: USA; AP018488.1: hemorrhagic colitis, Japan; DQ143181.1: Canada; CP014670.1: USA; 1 (LC497324.1), 2 (LC497325.1): fecal samples obtained from septicemic calves of the current study; CP023200.1: Neonatal calf feces, Indian isolate; CP027440

Despite the fact that *E. coli* is a versatile bacterial species encompassing both commensal and pathogenic strains, it was confirmed to be the most frequent agent isolated from blood and tissue cultures from septic calves (Fecteau et al., 1997; Lofstedt et al., 1999). Hence, the persistence of STEC in calves with diarrhea in the current study indicates that these animals can serve as reservoirs of the human pathogen *Escherichia coli*. Similarly, recording many pathological signs in calves with septicemia in the current study revealed the extent to which STEC threatens the lives and well-being of these calves. STEC has been extensively studied as a causative agent of diarrheal diseases in calves (Hoey et al., 2002; Pruimboom-Brees et al., 2000). Other effects of STEC, including immunosuppression (Menge et al., 1999) and intestinal colonization (Etcheverria & Padola, 2013), help them survive and reproduce in the host intestine.

The various forms of diarrhea that ranged from bloody to yellowish to watery diarrhea were observed in the calves of the current study. Darrhea is likely caused by STEC strains that colonize and multiply in the small intestine and produce enterotoxins, thereby increasing fluid and electrolyte excretion in the intestinal lumen. Other reports have also demonstrated that pathogenic *E. coli* can colonize and multiply in the epithelium of the small intestine (Fakih et al., 2017; Thiry et al., 2017). According to their reports, the pili of *E. coli* play a major role in the process of attachment and colonization. Upon attachment to specific receptor sites on villous epithelial cells, the bacterial fimbriae proliferate and produce irregular microcolonies that cover the villi.

According to the current study, the increase in body temperature may be a result of the release of exogenous and endogenous pyrogens, including heat-stable enterotoxins and capsular polysaccharides, into the body, as described by (Constable et al., 2016). Thus, an imbalance in body temperature could occur due to the action of the bacterial toxins of the “Thermo-Regulatory Center” of the hypothalamus. Similar to previous reports (Cáceres et al., 2017; Constable et al., 2016), dehydration, weakness, and alterations in respiratory rate, heart rate, and capillary refill time were documented in the present study. The authors found that enterotoxins cause systemic circulation to lose fluid and electrolytes, leading to diarrhea, dehydration, electrolyte imbalance, acidity, circulatory failure, shock, and death.

The present study suggested that newborn calves (one to three weeks of age) are more susceptible to infection as a result of systemic inflammatory syndrome (SIRS), which responds to an effective infection pathway. This suggests that low serum IgG levels in young calves and inadequate intake of colostrum affect the extent of susceptibility to the disease. The results also suggest that the disease is acute, lasting between 1 and 3 days, and has a very low survival rate (Al-Alo et al., 2018; Constable et al., 2016). However, low maternal levels of minerals and vitamins such as vitamin D (Alabada & Saleh, 2020) can suppress neonatal immunity and increase neonatal susceptibility to infection. Overall, the probability of death from colibacillosis in neonatal calves is quite high as a result of the enterohemorrhagic effect of this STIC *E. coli* pathotype.

Hematological changes, as described in our study, are both a consequence of systemic inflammation and evidence of organ dysfunction; our observations are in line with previous data (Biolatti et al., 2012; Irmak et al., 2006).

Finally, progress has been made on zoonotic enterohaemorrhagic *E. coli* (EHEC), which produces "Shiga Toxin", a different problem in cattle because of its minor role as a major pathogen that affects humans. However, approved products have been developed and are now being marketed in several of these areas (Larzábal et al., 2019).

5. Conclusions

In conclusion, to the best of our knowledge, this is the first study investigating the complete genome sequence of STEC in septicemic neonatal calves in Basra, Iraq. The overall prevalence of STEC *E. coli* causing diarrhea in calves in the current study was high (60%). In addition to the ability to accurately predict subtypes and other characteristics of the genome, using complete genome sequencing is also important because of its time and cost. Our results suggest that Colibacillosis in neonatal calves is caused by human and/or environmental infection and that the infection could occur in other countries. This information is important for controlling this disease in both humans and animals.

Acknowledgments

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Ethical considerations

The current study was approved by the general committee of Animal Use and Welfare, University of Basrah, College of Veterinary Medicine, Basra State, Iraq.

Conflict of interest

The authors declare that there are no competing interests in the current study.

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