

Serotyping, virulence gene expression and phenotypic characterization of *E. coli* O157:H7 in colibacillosis affecting buffalo calves in Basra governorate

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Abstract

The objective of the current study was to detect the clinical signs of colibacillosis in buffalo calves, isolate *E. coli* O157:H7, detect its virulence gene *eaeA* using PCR and estimate its prevalence. The current study sampled 120 buffalo calves aged 1 day to 5 months from the Al- Basra veterinary Hospital and Private veterinary clinic within the Basra province between October 2017 and July 2018. A total of 100 calves were naturally diarrheic and the other 20 calves served as controls. The clinical signs in the diarrheic subjects included a significant increase in body temperature, heart rates, respiration rates and capillary refill time as compared to control group. Other clinical signs included whitish to yellowish watery diarrhea with tincture of blood, anorexia, weakness, depression, weak suck reflex, dry oral mucous membranes, cold extremities, weak peripheral pulse, dehydration and death. Using phenotypic characterization tools like MacConky agar, EMB agar, biochemical tests and Viteck, 83 out of 100 diarrhea samples confirmed *E. coli*. Using CT- SMACT agar, 31 out of 83 *E. coli* isolates were *E. coli* O157:H7 positive. The PCR result indicated that 47 out of the 83 isolated *E. coli* samples were positive for *eaeA* virulence gene. In conclusion, this study is a debut in the report of *E. coli* and *E. coli* O157:H7 isolation and genes identification in buffalo calves in Iraq. Therefore, proper prevention and control measures are requisite to curtail the mortality and morbidity rate caused by Colibacillosis.

Keywords: Colibacillosis, *E. coli* O157:H7, Buffalo calves, Basra, Iraq

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التصنيف المصلي، التعبير الجيني للضراوة والتوصيف المظهري للإشريكية القولونية O157: H7 المعزولة من عجول الجاموس في محافظة البصرة

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الخلاصة

كان الهدف من الدراسة الحالية تحديد العلامات السريرية التي تسببها الإصابة بالإشريكية القولونية في عجول الجاموس وعزل عترة *E. coli* O157:H7 وتحديد جين الضراوة *eaeA* باستخدام تقنية تفاعل سلسلة البلمرة. شملت الدراسة الحالية ١٢٠ عينة من عجول الجاموس تراوحت أعمارها من يوم واحد إلى ٥ أشهر الواردة للمستشفى البيطري والعيادات البيطرية الخاصة في محافظة البصرة خلال الفترة من شهر تشرين الأول ٢٠١٧ إلى تموز ٢٠١٨. شملت الدراسة ١٠٠ من العجول التي تعاني من الإصابة بالإسهال و ٢٠ عجل كانت سليمة سريريا اعتبرت كمجموعة سيطرة. كانت العلامات السريرية في العجول المصابة تتميز بزيادة معنوية في ارتفاع في درجة الحرارة، معدل ضربات القلب، ومعدل التنفس وزيادة معدلات زمن رجوع الدم في الأوعية الدموية في العجول المصابة بالإسهال بالمقارنة مع مجموعة السيطرة. وشملت العلامات السريرية الأخرى إسهال اصفر إلى ابيض وفي بعض الحالات كان مصحوبا بدم، فقدان الشهية،

ضعف عام، خمول، ضعف أو عدم القدرة على الرضاعة، جفاف وبرودة في الأغشية المخاطية والأطراف، جفاف والنقور. من خلال استخدام أدوات التصنيف النمطي مثل وسط الماكونكي، وايوسين مثيلين بلو واختبارات البايوكيميائية واستخدام تقنية الفايترك تم تشخيص ٨٣ عينة موجبة بالاشريشكية القولونية من أصل ١٠٠ عينة كانت مصابة بالإسهال في عجول الجاموس. كذلك تم تشخيص ٣١ عينة كانت مصابة *E. coli* O157:H7 باستخدام وسط SMAC-CT. أظهرت نتائج تفاعل سلسلة البلمرة أن ٤٧ عينة من أصل ٨٣ كانت موجبة لجين الفوعة *eaeA*. تعتبر الدراسة الحالية هي الأولى التي تم من خلالها عزل وتشخيص الاشريشكية القولونية *E. coli* O157:H7 وعامل الضراوة من عجول الجاموس في العراق، لذلك نوصي بأخذ التدابير الوقائية ووضع طرق السيطرة المناسبة للحد من نسب الإصابة والهلاكات نتيجة للإصابة بهذا المرض.

Introduction

Colibacillosis is a challenging and debilitating disease affecting the cattle industry (1). It is caused by a multiple range of aetiological agents, out of which *Escherichia coli* was recognized as the most important bacterial cause (2). Colibacillosis occurs in all age groups of animals with morbidity up to 50% (1). *Escherichia coli* belong to a large group of bacteria of the family Enterobacteriaceae. These commensal bacteria are harmless and mostly found in the large intestine of birds and mammals. Although few strains of *E. coli* can cause disease conditions like pyelonephritis, diarrhea and pneumonia (3,4). Five different classes of *E. coli* that causes diarrhea are enteroinvasive, enterotoxigenic, enteropathogenic, enteroaggregative and enterohemorrhagic (5). The species *E. coli* is a gram-negative straight cylindrical rod like, non-spore-forming bacilli, measuring 1.1-1.5 * 2.0-6.0 µm, they are aerobic and facultative anaerobic in nature, rendered motile by peritrichous flagella (6,7).

Escherichia coli typing is based on the capsular (K) polysaccharide, somatic (O), flagellar (H) antigens is used in epidemiology (8). *Escherichia coli* (*E. coli* O157:H7) is a toxin producing bacteria that causes intestinal diseases in humans and animals resulting into bloody diarrhea. Severe cases can as well lead to life threatening kidney problems especially in children and neonates. Domestic and wild animals serve as carriers of this bacteria as it has been isolated and identified in cattle, sheep, pigs, deer, dogs and poultry (9,10). But cattle have been pinned as the main carrier (11). Infected animals, especially the young ones shed the bacteria in their faeces, the livestock get infected by *E. coli* O157:H7 via ingestion of the bacteria in contaminated feeds, fomites and feces of infected animals (12).

This important group of diarrheagenic *E. coli* is characterized by the ability to induce attaching and effacing A/E lesions on intestinal epithelium, and absence of Shiga toxin production (13,14). A chromosomal pathogenicity island designated locus of enterocyte effacement (LEE) contains the genes responsible for the A/E phenotype, among them *eae* and *tir*, which encode for an outer membrane protein called intimin and its translocated receptor Tir, respectively (15). The diarrheagenic *E. coli*

causes high morbidity and mortality rate in calves resulting into a huge economic losses (16).

There is paucity of information on this diarrheagenic *E. coli* in buffalo in Basra- Iraq, as no documented data avails. Therefore, the present study aimed at unveiling this dearth of knowledge on Colibacillosis caused by *E. coli* O157:H7 in buffalo in Basra using phenotypic and molecular techniques.

Materials and methods

Animal and study design

The present study was conducted on 120 buffalo calves aged 1day to 5 months old, a total of 100 calves were naturally suffering from diarrhea and 20 clinically normal calves served as negative control. The study period was between October 2017 to July 2018 in the College of Veterinary Medicine, Al-Basra Veterinary Hospital and another private veterinary clinic with Basra province.

Clinical examination

Diarrheic calves were examined and scores of vital signs were properly recorded and documented.

Samples and bacterial isolate

Rectal swab samples were collected from the calves using sterile swab sticks. Samples were then cultured on MacConkey agar and Eosin Methylene blue agar (Lab M, UK) and incubated aerobically at 37 °C for 48 hours. Thereafter, growing colonies were phenotypically characterized for and *E. coli* samples were identified. *E. coli* positive cultured samples were further grown on Cefixime Tellurite - Sorbitol MacConkey agar (CT-SMAC) to detect the *E. coli* O157:H7. Further confirmation was done using biochemical tests and Veitick. The virulence gene *eaeA* was finally detected using PCR.

DNA extraction and Polymerase chain reaction (PCR)

DNA of the *E. coli* isolate was extracted using bacteria DNA extraction kit (Promega / USA) following the manufacturer's instructions. The PCR technique was used to detect the polymorphisms of the virulence gene (*eaeA*) using forward and reverse primers (GACCCGGCACAAGCATAAGC and

CCACCTGCAGCAACAAGAGG, respectively) (17). The primers were designed by Alpha DNA in Canada. Amplification reaction was performed using a DNA thermo-cycler, 1.5% agarose gel electrophoresis was stained with ethidium bromide under UV light (Table 1).

Table 1: The reaction mix (25 µl) of multiplex PCR

Chemicals	Volume
PCR green master mix	12 µl
Primer forward	1 µl
Primer reverse	1 µl
DNA	3 µl
Deionized distilled water	8 µl

Statistical analysis

All data were analyzed statistically using IBM SPSS software “version 11.5, copyright © 2019”. Data was considered statistically significant at (P < 0.05).

Results

The results from 100 fecal swabs taken from diarrheic buffalo calves had 83 samples confirmed as *E. coli* based on phenotypic characterization. Out of the 83 *E. coli* isolates, 72 isolates were confirmed based on vetick test, about 31 isolates were detected as *E. coli* O157:H7 based on SMAC-CT Agar (Fig. 1). The suspected colonies of *E. coli* O157:H7 appeared small, circular colorless with smoky center 1-2 mm in diameter on Sorbitol MacConky agar plus cefixime - potassium tellurite (SMAC-CT). Table 2 shows results of biochemical test on the bacterial isolate.

Table 2: Biochemical tests results

Genus	Simmons Citrate	Urease	Indol test
<i>E. coli</i>	Negative	Negative	Positive

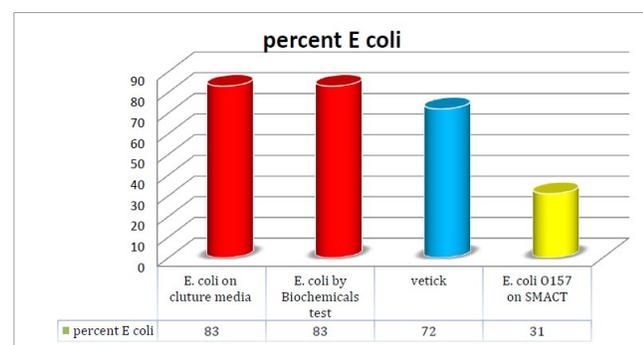


Figure 1: The percentage of *E. coli* depended on method isolation.

Clinical signs

The results of present study showed that the calves during the first 4 weeks of life are more susceptible for infection (Fig. 2).

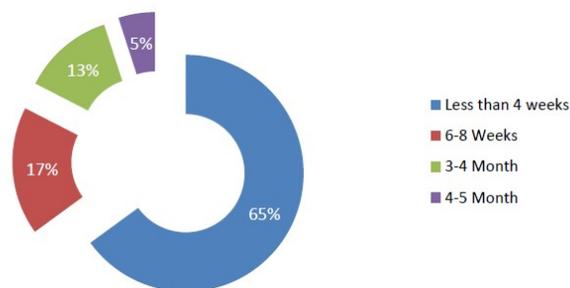


Figure 2: Relation between age and infection.

The clinical sings in diarrheic calves which confirmed infection with *E. coli* were a significant increase in body temperature, heart rates, respiration rates and Capillary refill time as compared to control group (Table 3).

Table 3: physiological parameters in infected animals and control groups

Parameter	Control group ± SE	Infected group ± SE
Body temperature °C	39.25 ± 0.51	41.7 ± 0.46**
Heart rate /mint	93.73 ± 7.44	109.3 ± 4.41**
Respiratory rate/ mint	37 ± 3.05	52 ± 5.22**
Capillary refill time	1.8 ± 0.12	5.2 ± 1.3**

** P<0.01 values are mean ± stander error of mean.

Other clinical signs were white to yellow watery diarrhea with tincture of blood, anorexia, weakness, depression, weak sucking reflex, dry mucous membranes, cold extremities, weak peripheral pulse, dehydration and death (Table 4).

Table 4: Clinical sings in infected animals

Clinical signs	Numbers (%)
Yellow diarrhea	56 (67.4%)
Bloody diarrhea	17 (20.48%)
White diarrhea	10 (12.04%)
Anorexia	71 (85.54%)
Weakness	62 (74.69%)
Depression	59 (71.08%)
Dehydration	53 (63.85%)
Oral mucous membranes are dry and cool	48 (57.83%)
Weak peripheral pulse	44 (53.0%)
Cold extremities	31 (37.34%)
Death	23 (27.71%)

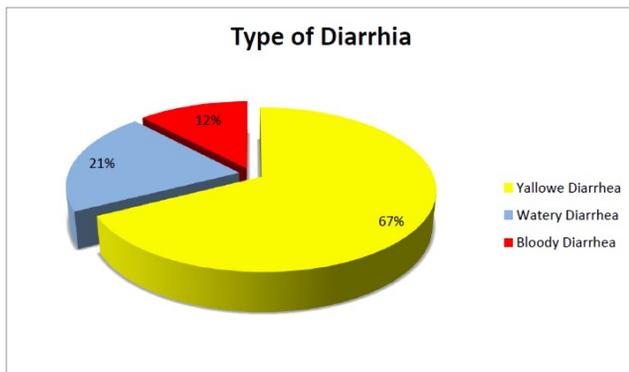


Figure 3: Types of diarrhea in infected animals.

The results of PCR showed that out of 83 isolates that were positive to *E. coli* via cultural and biochemical methods, only 47 isolates were positive for virulence gene *eaeA* using PCR (Fig. 4).

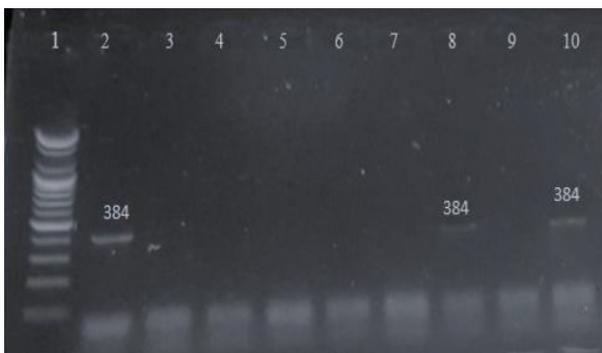


Figure 4: PCR detection of *E. coli* virulence gene *eaeA* at amplification of 384 bp as visualized using agarose gel electrophoresis. Lane 1,8 and 10 were positive for *eaeA* virulence other lanes were negative.

Discussion

The results of the present study indicated that gram stain and other biochemical tests used in the study revealed that *E. coli* was responsible for disease condition observed in the sampled calves. This present finding is in resonance with those documented by 15. The *E. coli* O157:H7 positive result obtained from the CT-SMAC test is in agreement with the reports of Garcia *et al*; Alani (18,19). The authors found out that a typical *E. coli* O157:H7 appeared as colorless colonies and did not ferment sorbitol on SMAC agar while most non-O157 strains fermented sorbitol appeared as pink color colonies on SMAC agar.

The current study also indicated that calves within the first 4 weeks of life are more susceptible to the infection as this might be due to the systemic inflammatory response

syndrome to an active infectious process. This finding correlates with the reports of Constable *et al*; Alani; Biolatti *et al* (9,19,20). These authors have associated the quantum of susceptibility of this infection to young calves majorly due to low levels of serum IgG in calves and inadequate colostrum intake. They also submitted that the illness is per acute with a course varying from 24 to 96 hours having a survival rate less than 12%.

Findings in the present study also revealed that the observed diarrhea in this infection varies from watery yellow to bloody diarrhea in infected calves. We can opine that the diarrhea occurs due to Enterotoxigenic effects of *E. coli* (ETEC) strains that colonize and proliferate in the upper small intestine and produce enterotoxins, thereby, causing an increase in net secretion of fluid and electrolytes into the gut lumen and hence the resultant diarrhea. This result is in total agreement with the findings of Alani; Fakhri *et al* (19,21). Other reports have also supported the fact the bacteria can colonize and hence proliferate in the intestinal epithelial (16). Thirty in their report have referred the adhesion of *E. coli* to intestinal epithelial cells has been mediated by bacterial pili. The bacterial fimbriae attach to specific receptor sites on villous epithelial cells, following which the bacteria multiply and forms microcolonies that cover the surface of the villi.

The increase in body temperature observed in the current study might indicate the liberation of endogenous and exogenous pyrogens such as a heat-stable enterotoxin and capsular polysaccharide. This is quite in harmony with the findings of Constable *et al* (9). Constable had reported that the bacteria toxins exerts effect on the thermoregulatory center of the hypothalamus thereby altering the thermostatic level. The other clinical signs such as heart rate, respiratory rate, capillary refilling time, weakness, dehydration is similar to those observed and reported by Constable *et al*; Cundon *et al*; Caceres *et al* (9,22,23). These authors reported that the production of the enterotoxin results in net secretion of fluid and electrolytes from the systemic circulation into the lumen of the intestine, resulting in varying degrees of diarrhea, dehydration, electrolyte imbalances, acidemia, circulatory failure, shock, and death.

The results of present study indicated that the prevalence of *eaeA* gene was 56.62% as only 47 out of 83 isolates of *E. coli* were positive. This is typical of the findings of Alani, 2016. Alani had earlier reported that the percentage of *eaeA* gene was 53.12%. However, the result of the current study is lower than that reported by Syngde *et al*; Schouten *et al*; Alam and Zurek (24-26). These authors have reported that all tested isolates of *Escherichia coli* O157:H7 in cattle were positive for *eaeA* in their various studies. These discrepancies in the prevalence report by different authors might be associated to due to different models and numbers of experimental animals, different

diagnostic technique and geographical locations where such studies were carried out.

The public health outcry is that the *eaeA* positive isolates can cause intimate adherence, attaching and effacing lesions in intestinal epithelial cells of both human and animal. Therefore, this suggests a potential threat to the public health sector. The *eaeA* gene had been found in *E. coli* strains which do not belong to the EPEC serotypes and which are negative for shiga-toxins, and these strains have also been referred to attaching and effacing *E. coli* (27).

In conclusion therefore, this present study is a debut and the first to document the prevalence of *E. coli* and *E. coli* O157:H7 as it relates to colibacillosis in buffalo calves in Basra – Iraq.

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