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Isolation and characterization of *stx1* and *stx2* toxin-producing *Escherichia coli* in neonatal lambs with diarrhea in Nineveh governorate, Iraq

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ABSTRACT

Escherichia coli causes many health problems in lambs, the most prominent of which is diarrhea, the study aimed to detect the shiga toxins genes in *E.coli* isolated from the suckling lambs affected with diarrhea in Nineveh governorate. 91 fecal samples were collected from suckling lambs from different areas of Nineveh governorate during February 2022.Fecal sampling was performed directly from the rectum and placed in sterile containers, then transferred directly to the research unit of the veterinary medicine faculty at university of Mosul. Standard microbiological methods including culture in EMB, MacConkey and brilliance agars along with biochemical analyzes were performed. Molecular confirmations were done using specific primer sets targeting *uidA* gene for *E.coli* and *Stx1* and *Stx2* for shiga toxin respectively. The result of the present study showed that the prevalence rate of *E.coli* was 58.24%(53/91). Based on the PCR assay, the *uidA*, *Stx1* and *Stx2* were found in all the *E.coli* isolates 100% (53/53). It was concluded that the high isolation rate of *E.coli* from the lambs indicates lack of housing and hygienic procedures both for lactating ewes' health and the environment.

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Keywords: Diarrhea, *E.coli*, PCR, Shiga toxin, Suckling lambs.

INTRODUCTION

Despite the improvement of care and the development of prevention methods, cases of diarrhea still pose a great danger and the most expensive in raising sheep in general and lambs in particular. It is not possible to know the causes of diarrhea from the character and smell of the diarrhea, but rather this is done by microbiological methods, where the causes of diarrhea in lambs are divided into bacterial, viral, parasitic or dietary causes **(Rodostits, 2007).** *E.coli* is one of the main causes of diarrhea in lambs **(Bavaro, 2012).**

E.coli is a commensal microorganism found in the intestines of mammals and is considered one of the most common and important causes of diarrhea in both animals and humans, *E.coli* is able to survive and adapt in the host and its environment by possessing virulence factors (VF) **(Gouali and Weill 2013).** *E.coli*, gram-negative bacilli, is an opportunistic microbe that is active in poor environmental conditions and poor care. The researchers were able to isolate *E.coli* from young lambs at the age of 10 days or less **(Aklilu *et al.*, 2013)**.

It is characterized by rapid infection and spread and it was possible to isolate *E.coli* from lambs of 1-4 day old, and from some symptoms associated with diarrhea accompanied by abundant saliva from the mouth of infected lambs called watery mouth disease **(Kaper *et al.*, 2004; Nadhom, 2016)**.

According to the virulence factors, which includes capsule synthesis, adhesin, toxins production and etc. *E.coli* is divided into intestinal pathogenic *E.coli* (IPEC) and extraintestinal pathogenic *E.coli* (ExPEC) **(Habouria *et al.*, 2019)**. IPEC is classified into Enteropathogenic *E.coli* (EPEC), Enterotoxigenic (ETEC), Enteroaggregative *E.coli* (EAEC), Enteroinvasive *E.coli* (EIEC), and Shiga toxin producing *E.coli* (STEC/VTEC/EHEC) **(Guimarães *et al.*, 2019)**. IPEC infection causes intestinal inflammation and diarrhea, and in some severe cases leads to animals death **(Malberg Tetzschner *et al.*, 2020)**.

The aim of this study was to detect the shiga toxins genes (*Stx*1 and *Stx*2) in suckling lambs affected by *E.coli* with diarrhea in Nineveh governorate.

MATERIALS AND METHODS

Samples

The study was targeted young lambs affected with diarrhea, 91 fecal samples were aseptically collected using sterile swabs from suckling lambs from different sheep flocks in Nineveh governorate during February 2022, samples were collected directly from the rectum and placed in sterile containers, then transferred directly to the research unit of the veterinary medicine faculty at university of Mosul for bacteriological examinations.

Bacterial isolation

Standard microbiological methods including culture in Eosin Methlene Blue (EMB), MacConkey and brilliance agars (Himedia®/India), then the isolates are confirmed using gram stain and biochemical tests (Indole-production, methyl red, Voges-Proskauer, Simmons Citrate, Triple sugar iron, Catalase and Oxidase) were carried out according to **(Quinn *et al.*, 2011).**

Molecular investigation

DNA extraction

The DNA of *E.coli* was isolated depended on the instructions of the Presto™ Mini gDNA Bacteria Kit (Geneaid®, Taiwan). The concentration of DNA of *E.coli* was estimated by using the Biodrop® (UK) and stored at -20°C.

Conventional PCR amplification of DNA

All the primers used in this study were detailed in (Table 1). The molecular confirmation of *E.coli* was performed using species specific primers (*E.coli*: *uidA* gene). While confirmation of the shiga toxin 1 and 2 was done by using 2 set of primers *Stx1* genes (*Stx1*- F gene and *Stx1*-R) and *Stx2* genes (*Stx2*-F gene and *Stx2*-R gene), were amplification using conventional PCR technique by using the thermocycler program which was set according to manufacturer's instructions using special kits supplied by Alpha DNA®, Canada) (table 1).

A total volume of PCR reaction was 25 µL which consist of : (i) 1 µL forward primer, (ii) 1 µL of reverse primer (Table 1), (iii) 12.5 µL of 2×Go Taq Green Mix Master containing (1 unit GoldStar DNA polymerase, 400 µM dNTPs, 3 µM MgCl2, 20 µM (NH4) 2SO4, 75 µM Tris HCl (pH 8.5), yellow and blue dyes which function as loading dye (Promega USA), (iv) 5.5 µL of nuclease-free water (Promega USA), and (v) 5 µL DNA template of *E.coli*. The mixture was placed in PCR reaction tube (Promega USA). The DNA of *E.coli* was amplified by using the thermocycler program which was set as the following: (i) 5 minutes at 95°C for the denaturation, (ii) 35 cycles. Where each cycle consist of denaturation (1 min. at 94ºC); annealing (1 min. at 55 °C for *uidA*, 57 °C for *Stx1* and *Stx2*); and extension (1 min. ant 72ºC) and (iii) 5 min. at 72ºC for the final extension, (Table 1) finally, the PCR products were determined by gel electrophoresis together with DNA marker 623 bp for *uidA*, 347 bp for *Stx1* and 589 bp for *Stx2* ladder in 2% agarose gel (Bio Basic Inc®., Canada).

Table1: Primers used for PCR:

Gene	Primer	Sequence (5' – 3')	Temp °C	Product size (bp)	Reference
uidA	*uidA-1*	CCAAAAGCCAGACAGAGT	55	623 bp	**Moyo *et al.*, 2007**
	uidA-2	GCACAGCACATCCCCAAAGAG			
Stx1	*Stx1-1*	AGTTAATGTGGTGGCGAAGG	57	347 bp	**(Fujioka *et al.*, 2013)**
	Stx1-2	CACCAGACAATGTAACCGC			
Stx2	*Stx2-1*	TTCGGTATCCTATTCCCGG	57	589 bp	**(Fujioka *et al.*, 2013)**
	Stx2-2	CGTCATCGTATACACAGGAG			

RESULTS

The results of the current study of fecal swab samples from suckling lambs suffering from diarrhea showed that out of 91 samples, 53(58,24%) samples were positive for the bacterial isolate of *E.coli* on the medium of EMB, MacConkey and brilliance agars and biochemical tests. *E.coli* growing colonies on EMB was metallic sheen in green color, and pink in MacConkey agar and purple in brilliance agar. The results of biochemical tests were as follows Indole-production (+), methyl red (+), Voges-Proskauer (-), Simmons Citrate (-), Triple sugar iron (+), Catalase (+) and Oxidase (-).

There was a congruence between isolation and biochemical tests results with the result of *uidA* gene PCR product size of 623bp of amplification products on agarose gel (Fig. 1).

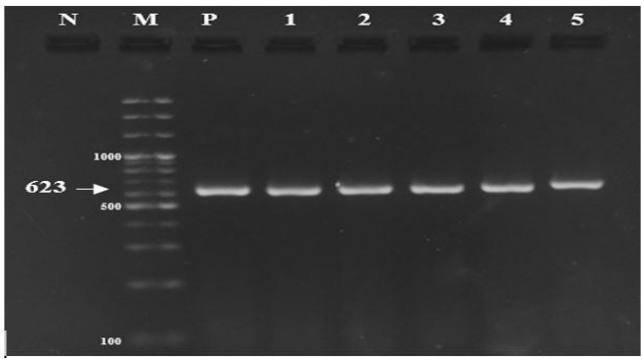

Fig. 1: PCR products of *uidA* gene. Lane M, DNA molecular standard; lane 1-5 positive tested samples giving 623bp product size; lane N, negative control and lane P control positive.

The results also showed that all *E.coli* isolated from different samples possess the genes for shiga toxins 1 and 2 (*Stx1* & *Stx2*) with molecular weights 347bp and 589bp respectively in amplification products on agarose gel as shown in Figures (2,3).

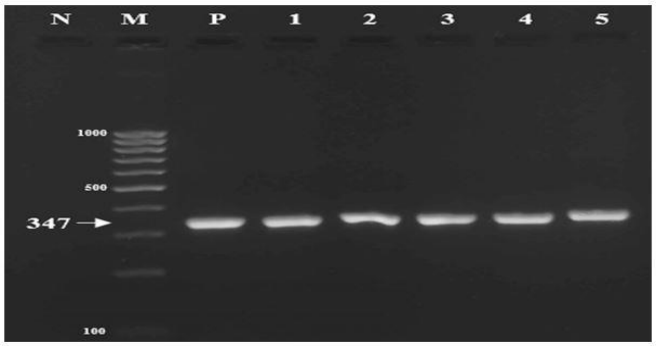

Fig. 2: PCR products of *Stx1* gene. Lane M, DNA molecular standard; lane 1-5 positive tested samples giving 347bp product size; lane N, negative control and lane P control positive.

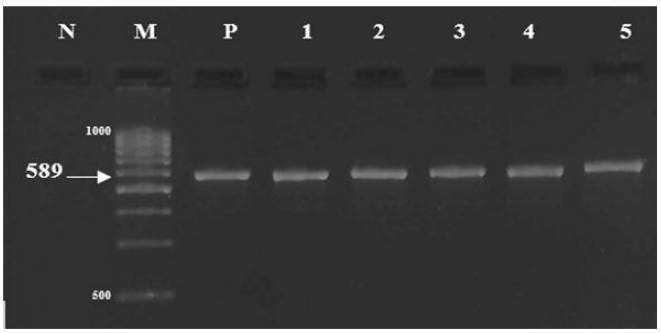

Fig. 3: PCR products of *Stx2* gene. Lane M, DNA molecular standard; lane 1-5 positive tested samples giving 589bp product size; lane N, negative control and lane P control positive.

Discussion

Sheep are regarded the main reservoirs of *E.coli*, which are transmitted to other animals and humans, infection with *E.coli* represents a great challenge for people, especially those who live closely with sheep such as workers, farmers and etc., especially since they do not have any knowledge of the pathogenesis of *E.coli* and its transmission methods **(Shabana *et al.*, 2013; Ferone *et al.*, 2020)**.

E.coli have economic importance, where the death rate in sheep due to infection ranges between 1-5%, and most of them are in the first weeks of 3 to 12 weeks of age, it also leads to a decrease in the quality of wool and a significant decrease in meat production **(Gwyther *et al.*, 1981**).

Although several traditional procedure for *E.coli* detection are avilable, however it pose a problem when accurately diagnosting in different samples, so polymerase chain reaction (PCR) methods have the ability to overcome the problems of conventional methods. In this study, PCR technique used sets of primers derived from the *uidA* gene sequence to detect *E.coli* strains, even those with undetectable b-d-glucuronidase activity by traditional procedure,, can be detected in this way based on the *uidA* gene **(Horakova *et al.*, 2008).**

In our study, out of a total of 91 samples, 53 samples, *i.e* 58,24%, were positive for isolation from diarrheatic lambs, which is less than the results reached by **Tesfaye Sisay, (2013),** which indicated an isolation rate (84%). While it was higher than the percentages reached by **Heuvelink *et al.*, 1995)** in the Netherlands where it reached 4% and in the United Kingdom by 4.8% and 31% in the United States and 68% of the sheep herd in Australia **(Chapman *et al.*, 1997),** Whereas, no sequestration rate was recorded in Norway, Scotland, Ireland, Greece and the United States **(Keenan *et al.*, 1986; Roger *et al.*, 1996).** The reason for this high prevalence in the current study may be attributed to the delay in feeding the first colostrum, clean housing, and failure to implement appropriate preventive measures.

In a study conducted by researchers **(Wani *et al.*, 2009)** on lambs with diarrhea and healthy ones, the prevalence of stx1 and stx2 was 88.5%, and there was a higher prevalence of stx2 than stx1 at a rate of 68.2% and 50%, respectively, and compared with the study conducted by **Brett *et al.*, (2003)** in India and Australia has indicated that stx1 was the most common type in sheep. On the contrary, **Ramachandran *et al.*, (2001)** mentioned in his study in Australia that stx2 is the predominant type in sheep.

The results of the study are linked to many factors, including age, deprivation or delay in feeding colostrum and unclean housing for lambs, which lead

to a high risk of disease **(Olsson *et al.*, 1993)** indicated that the incidence of diseases is associated with a delay in eating colostrum for more than 6 hours. **Alves *et al.*, (2013)** indicated that every hour of delay increases the chance of infection of lambs at a rate of 10%, it was mentioned that 80 mg/ml of IgG is absorbed in the first six hours, and the absorption decreases significantly after this time, the first six hours are critical for maximal absorption of colonic immunoglobulin **(Bond, 2020).**

CONCLUSION

The high isolation rate of *E.coli* from lambs, which clearly indicates the lack of hygiene procedures application with regard to the health of lactating ewes, as well as the sanitary procedures followed in animal housing. Given the overlapping nature of diarrheal cases and the diverse nature of *E.coli*, a deep study should be conducted to understand the microbial distribution of diarrheal cases in lambs. Finally, this study showed that the bacterial isolates of *E.coli* possess *Stx1* and *Stx2* genes in lambs in Nineveh governorate. These results also reveal that the low level of health care and hygiene measures in the sheep-breeding system may increase potential public health risks from the animals.

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Conflict of interest

The authors acknowledge that there is no conflict of interest regarding the research idea and tools, actual, potential and financial, directly or indirectly.

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