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SOME STUDIES ON RABBITS EXPERIMENTALLY INFECTED BY E.COLI O_{157}:H_7 ISOLATED FROM RAW MILK

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ABSTRACT

Escherichia coli O $_{157}$:H_7 causes outbreaks of diarrhea and hemolytic uremic syndrome which can cause acute kidney failure in human. Cattle are identified as the primary source of infection. In this study, the isolation of Escherichia coli O_{157}: H_7 was occurred through out of 100 milk samples from different localities in Egypt. The positive E.coli isolated was 45 from samples then serological identification was done to identify the serotypes of E.coli which revealed that only one sample donated to E.coli O_{157}: H_7.This only positive sample used for infection of rabbit models, which was used to evaluate the pathogenesis of E.coli O_{157}: $_{H7.}$The 10 rabbits were divided into: control group (3 rabbits) and experimental group (7 rabbits). Rabbits were inoculated by feeding tube with 10^9 cfu of E.coliO_{157}: H_7.Fecal samples were collected from animals on days 2, 4, 6 post inoculation to determine the shedding of the pathogen. On day 13 after inoculation, intestine, kidney and liver were collected from slaughtered animals for histopathologic examination. The intestine showed necrosis and desquamation of epithelial lining with hyperplasia and neutrophilic infiltration. Kidneys showed microthrombosis in glomeruli and hyaline casts in the lumen of renal tubules. Liver showed severe congestion with sharp vacuoles in the cytoplasm of hepatocytes giving the cell the signet ring appearance.

***Key words**: Escherichia coli O_{157}:H_7, raw milk, pathology, rabbits.*

INTRODUCTION

Enterohaemorrhagic Escherichia coli (EHEC) has emerged as an important cause of human intestinal diseases in developed countries over the past 20 years (Stuart Naylor *et al.,* 2003). Escherichia coli O_{157}:H_7 causes both outbreaks of diarrhea, hemorrhagic colitis and Hemolytic Uremic Sundrome (HUS) (Diane Baker *et al.*, 2007).

Most patients fully recover from bloody diarrhea, but some develop life-threating diseases of the kidneys and CNS, approximately 15% of the cases in children result in HUS. (Tarr *et al.,* 2005).

E. coli O_{157}: H_7 infections are typically transmitted through consumption of contaminated food or contact with contaminated water, animal feaces or infected animals (Diane Baker *et al.,* 2007).

Raw milk is considered a high risk food as it is highly nutritious and serves as an ideal medium for bacterial growth (Chye *et al.,* 2004).

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Raw milk is known as the main transmission pathway for pathogens resulting in food-borne outbreaks every year (Gillespie *et al.,* 2003 and Rey *et al.,* 2003).

Raw milk exposed to untreated and contaminated water, cattle or human feaces, can easily be contaminated with E.coli.

Unpasteurized milk and dairy products made from raw milk (such as soft cheese) act as vehicles for transmission of E.coli to human (Dweik *et al.,* 2012).

Consumption of raw milk is a high risk behavior leading to morbidity and mortality (Keene, 1999).

Generally, raw milk consumption is a traditional practice among farm families (Jayarao *et al.,* 2006).

Raw milk consumers claim that raw milk is healthier, although equal nutritional value of raw and pasteurized milk has been proved (Bren, 2004).

E.coli O_{157}:H_7 serotypes are identified as enterohemorrhagic E.coli and Categorized in shiga-like toxin- producing Escherichia coli (STEC) (Oksuz *et al.,* 2004).

It Causes haemorrhagic Colitis, hemolytic-ureamicsyndrome (HUS) and thrombocytopenic purpura (TTP) (Zhao *et al.*, 1998).

A vaccine is currently not available to protect humans from infection or disease caused by STEC.

There is a need to define the pathogenic mechanisms by which STEC cause disease and develop strategies for the prevention and treatment of STEC-mediated HUS.

Achieving this goal would benefit from a small animal model that displays gastroenteritis or signs of HUS similar to those accurring in humans.

Naturally infectedrabbits, become infected with EHEC and subsequently exhibit diarrhea and hemorrhagic colitis (Sherman *et al.*, 1983; Ritchie *et al.*, 2003 and Aruna Panda *et al.*, 2010).

The aim of this study was to investigate the prevalence of E.coli O_{157}:H_7 in raw milk and detected its clinical and pathological effect on rabbit model that display signs similar to those occurring in human.

MATERIALS AND METHODS

Isolation of E.coli O_{157}:H_7 from raw milk.

1- Sample Collection

A total of 100 raw milk samples were collected randomly from different farms in Dakahlia province during summer months. The samples were transported to the laboratory in an ice-box and the bacteriological examination was done immediately.

2- Bacterial isolation of E.coli O_{157}:H_7 (USDA, 2002):

25 ml of raw milk sample was added to 225 ml of Trypticase Soya Broth (TSB) and incubated at 37◦c for 24 hrs. The enriched culture were plated on Sorbitol Macconkey agar supplemented with cefixime (0.05 mg/L) and potassium tellurite (2.5mg/L) (CT-SMAC, Merck, Germany) and incubated at 37◦c for 24 hrs.

Five non-sorbitol fermenting colonies (NSF) from each CT-SMAC plate were selected and streaked on to plates containing Eosin methylene blue agar (EMB) and were incubated at 37◦ c for 24 hrs. These isolates, with typical E.colimetalic sheen on EMB, were characterized by biochemical tests including Indole production, methyl red, vogesproskour, citrate utilization and lysine descarboxilase tests and examination with API20 Etest strips (Biomerieux Vitek, Inc, Hazel zood, Mo).

-Serological confirmation included the use of E.coli O157:H7 latex agglutination assay (Unipath, Oxoid, US) and the Bacto E.coli antiserum H7 assay (Difco) according to the manufactures specifications.

The Experimental Portion (Aruna Panda *et al.*, 2010):

1- Animals:

Rabbits (age from 5 to 8 wk) were used. The pathogen status of the rabbits was vertified through health reports from the vendor prior to study intiation.

Rabbits used in this study were free from bacteria including Escherichia coli, Clostridiumdifficile, Pasteurellamultocida, Pasteurellapneumotropica, Salmonella spp and Specific protozoa ectoparasites.

-Fecal samples from all animals used in the study were screened for the presence of Sorbitol-non fermenting strains on sorbitol Macconkey agar. These animals were evaluated for clinical signs such as diarrhea, haemorrhegiccolitis and weight loss. Animals were acclimated for a period of 48 hrs before intiation of the study.

-Animals were cared for and housed individually in cages according to the guide lines of the Guide for the care and use of laboratory Animals (Institute for laboratory Animal Research, 1996).

-Feed and Water wer available adlibiturn to all rabbits through at the study period.

2- Experimental design:

The 10 rabbits used in this study were divided into a control group of 3 animals and experimental group of 7 animals.

-Rabbits were fasted over night to reduce the contents of the intestinal tract and promote bacterial colonization. Water was removed from the rabbit cages 2 hrs before inoculation.

-Each animal (including the controls) then was gavaged with 10ml of 10% sodium bicarbonate solution to increase gastric PH and facilitate colonization by the bacteria in the animals' gastro intestinal tract.

-Then the experimental group were inoculated by intragastric gavage through an infant feeding tube.

-Each animal in the experimental group was gavaged with 10^9 CFU of E.coli O_{157}:H_7 strain suspended in/ ml PBS (phosphate buffered saline).

-Rabbits in the control group were gavaged with PBS only.

-The animals then were returned to housing cages where they were permitted free access to food and water.

3- Clinical assessment:
After inoculation, rabbits were monitored daily for development of clinical signs in the form of diarrhea, hemorrhagic colitis, fever, weight loss or lethargy.

4- Bacterial quantification:
Fecal samples were collected from each animal on day 2,4 and 6 after inoculation to determine shedding and colonization of bacteria in infected rabbits.

-Serial dilution of fecal suspension were spread on Sorbital-Macconkeyagar plates and incubated at 37° c for 24 hrs to determine the number of viable bacteria per gram of feces.

-The Level of colonization of E.coli O_{157}:H_7 was calculated from the total number of Sorbital negative colonies recovered from rabbit feaces.

5- Euthanasia:
On day 15 after inoculation (the end point of the study), rabbits were euthanized and re-isolation of E.coli O_{157}:H_7 from the affected organs (intestine, kidney, liver).

6- Histopathologic examination
Specimens from intestine, both kidneys and liver from each animal were collected and fixed in 10% neutral buffered formalin. Paraffin section of 5u thick were prepared and stained with hematoxylin and Eosin (Bancraft, 1996).

RESULTS

1- Results of bacterial isolation of E.coli O_{157}:H_7 from raw milksamples
From 100 raw milk samples, only one isolate of E.coli O_{157}:H_7 was identified and confirmed by biochemical and serological tests.

2- Recovery of E.coli O_{157}:H_7 from feces of experimently infected rabbits
Fecal Samples were collected on day 2, 4 and 6 after infection from all rabbits, to assess the levels of colonization by E.coli O_{157}:H_7 strain.

-All of the infected rabbits shed the E.coli O_{157}: H_7 strain into the feaces on day 2, 4 and 6 (Table 1).

Table 1: Average of bacterial titressheded in feces of experimentally infected rabbits (CFU/gmfeaces).

Day of Sample Collection	2nd day	4th day	6th day
Infected rabbits (7)	10^3	10^5	10^9
Control rabbits (3)	0	0	0

3- Clinical Findings
All animals were monitored daily for development of clinical signs of disease (diarrhea, fever, lethargy and weight loss).

-Control rabbits had no clinical signs, where as infected rabbits displayed diarrhea and weight loss (Table 2).

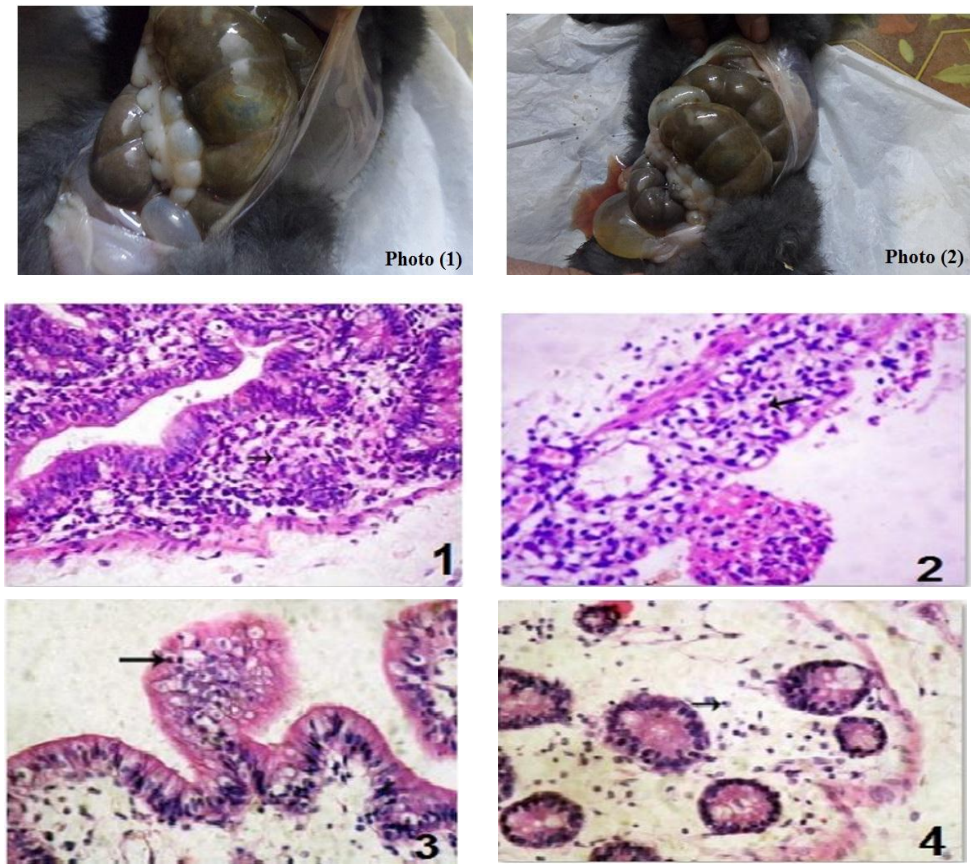
-Diarrhea accurred in 75% of the infected rabbits on 1 or more days after infection 6 of the 7 rabbits (87.5%) infected rabbits exhibited weight loss.

Table 2: clinical signs:

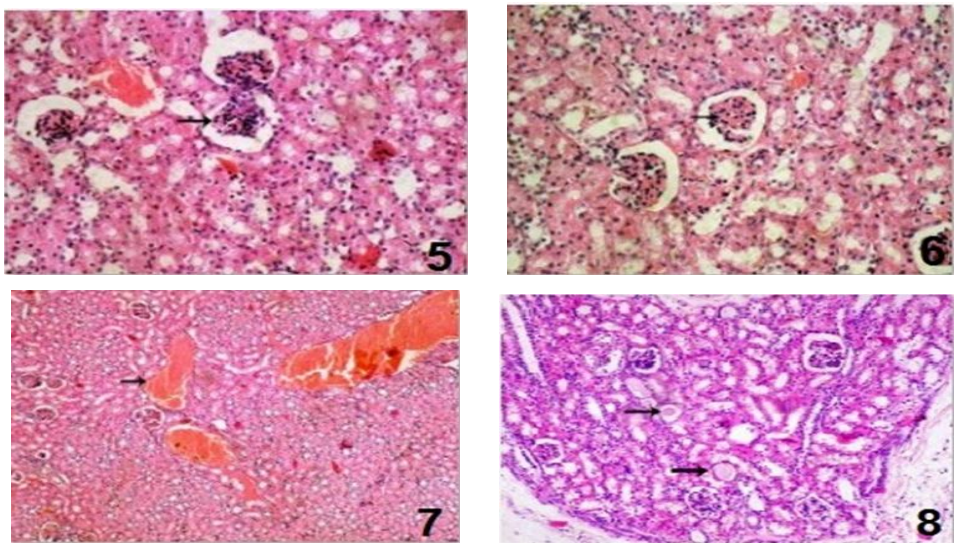
Infection status	No. of rabbits with	
	Weight loss	Diarrhea
Control (3)	0	0
Infected (7)	7	6

4- Macroscopic findings
Multiple pale area on the serosal surface of cecum ranging from 2-3 cm in diameter, in mucosal surface showing multiple foci of haemorrhage and necrosis as shown in photo (1) and (2)

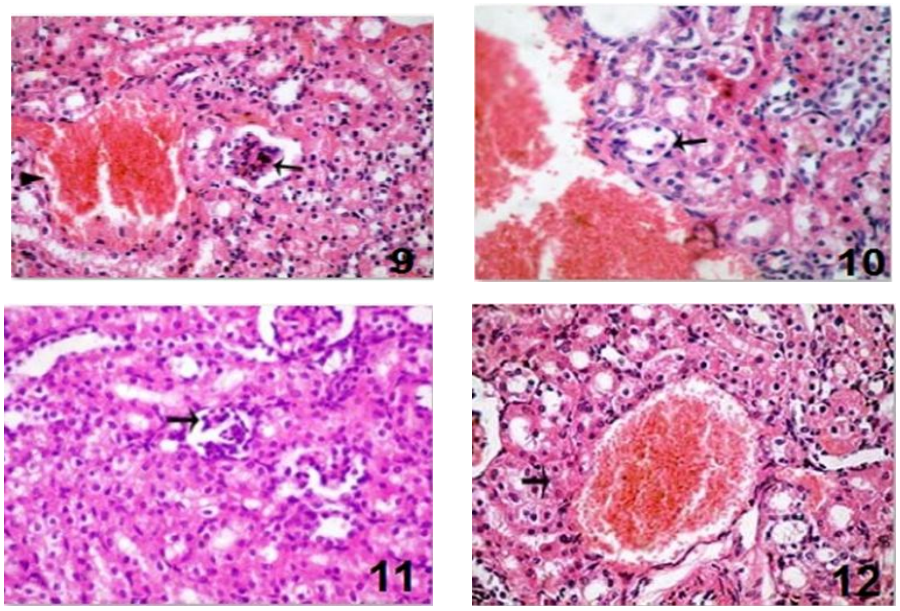
5- Histopathologicalresults



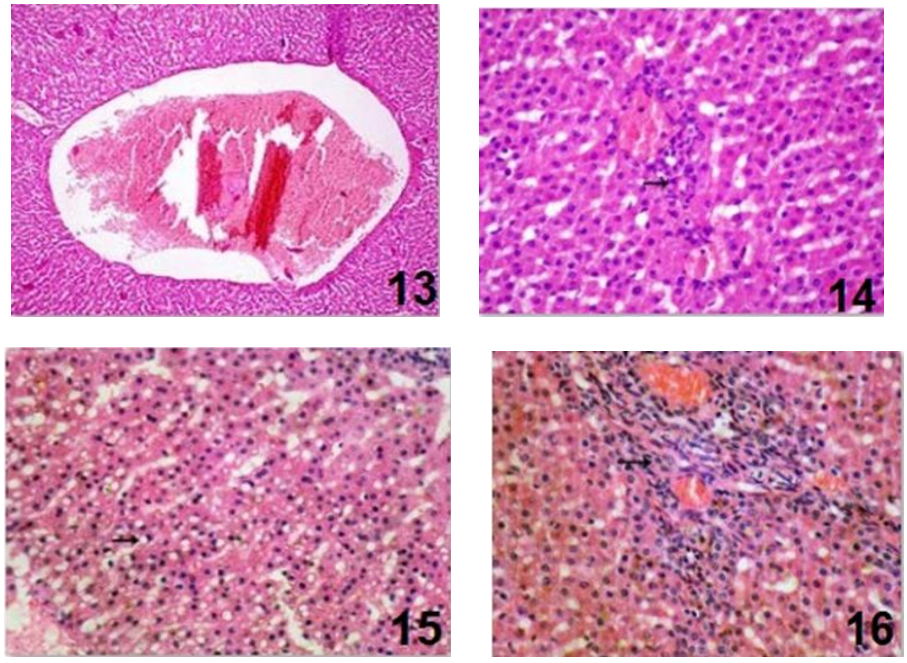
(Fig. 1): Intestine showing neutrophilic infiltrates (arrow) in Lamina propria. (H& E, 400x).
(**Fig 2):** Intestine showing necrosis and desquamation of epithelium with neutrophilic recruitment (arrow). (H &E, 400 x).
(Fig. 3): Intestinal epithelium showing hyperplasia with goblet cells metaplasia and neutrophilic infiltration (arrow). (H&E, 400x).
(**Fig. 4):** Intestine showing edema in Lamina propria and mononuclear cell infiltration (arrow). (H &E, 400x).



(Fig. 5): Kidney showing proliferation of mesangial cells in glomeruli (arrow) with heamorrhage in interstitial tissue (H & E, 400 x).
(**Fig. 6**): Kidney showing microthrombosis in glomeruli (arrow). (H & E, 400x).
(Fig. 7): Kidney showing severe congestion in interstitial blood vessels (arrow) (H & E, 100x).
(Fig. 8): Kidney showing hyaline casts in the lumen of renal tubules (arrow). (H & E, 100x).



(Fig. 9): Kidney showing congestion in interstitial tissue (arrow head) with hyperplasia of mesengial cells and destruction of renal glomeruli (arrow) (H & E, 400x).
(**Fig. 10):** Kidney showing hemorrhage and degeneration of renal tubular epithelium (arrow) (H & E, 400 x).
(Fig. 11): Kidney showing necrosis and destruction of glomeruli (arrow) (H & E, 400x).
(**Fig. 12):** Kidney showing severe congestion in interstitial tissue and necrosis of surrounding renal tissue (arrow) (H & E, 400x).



(Fig. 13): Liver showing severe congestion in central vein with pressure atrophy of surrounding hepatocytes. (H & E, 400 x).
(Fig. 14): Liver showing mild histiocytic and neutrophilic infiltrates. (H &E, 400x).
(Fig. 15): Liver showing sharp, clear vacuoles in the cytoplasm of hepatocytes (arrow) pushing nucleus forming signet-ring appearance (H & E, 400x).
(Fig. 16): Liver showing lympho-histiocytic infiltration and fibroblastic proliferation (arrow), (H & E, 400x).

DISCUSSION

Milk has been associated with out breaks due to E.coli O_{157}:H_7 such as the reported in Canada from non-posteurizedmilk, that affected several children (Jay, 2000) or one reported in Montana from contaminated post-pasteurized milk (Borezyk *et al.,* 1987 and Bielaszewska *et al.,*1997).

In this study, E.coli O_{157}:H_7 was isolated from 1% of raw milk samples by conventional culture, biochemical and serological methods.

The obtained results are nearly similar to that obtained by Reven *et al.,* 2002 who isolated E.coli O_{157}:H_7 in 1.3% (2 isolates out of 150 samples) of non pasteurized milk.

Our findings donot differ greatly from those reported abroad from raw cow milk, it was reported that 6% of raw cow milk samples examined in Egypt and in 3% in Austeria were contaminated with E.coli O_{157}:H_7 (Abdul-Raouf *et al.,* 1996; Allerberger and Dierich, 1997), but (Klie *et al.,* 1997) found that only 0.3% of raw milk samples were contaminated with this serotype in Germany.

Similar studies performed on raw cow small performed in the UK (Scotland) analyzing 500 samples (Coia *et al.,* 2001) and in Netherlands analyzing 1011 samples (Heuvelink *et al.,* 1998a) resulted in E.coli O_{157}:H_7 isolation.

Although according to our finding the incidence of E.coli O_{157}:H_7 in bulk tank is low, the presence of this pathogen in raw milk is important harmful threat in food safety (kaper *et al.,* 2004 and Meng *et al.,* 2007).

-According to Greig (2010), even low dose of E.coli O_{157}:H_7 (10 to 100 cfu) is Sufficient to cause infection and the infection dosage for children is only 1 to 4 cfu (Duncan and Hackney, 1994).

-The prevalence rates of pathogens in raw milk in different studies could be influenced by several factors such as geographical area, farm size, number of animals on the farm, hygiene and farm management practices (Rohr bach *et al.,* 1992 and Jayarao *et al.,* 2006).

The present samples were collected in summer months, and the results agreed with (Cagney *et al.,* 2004) who reported that seasonal distribution of E.coli O_{157}:H_7 with the highest prevalence in summer and the lowest in winter, so it is possible that the contamination rate become even lower than 1% in other seasons.

In the present study, we used E.coli O_{157}:H_7 to infect rabbits. Rabbits are susceptible to O_{157}:H_7 infection and exhibit diarrhea, enteritis, weight loss and the infected rabbits shed bacteria in their feaces. These results were agreed with (Barrett *et al.,* 1989) who investigated the pathogencity of E.coli O_{157}:H_7 in rabbits.

This study was to investigate the pathogenesis of E.coli O_{157}:H_7 in rabbits. Experimentally infected rabbits developed awide range of histopathological changes (Griffin *et al.,* 1990 and Garcia *et al.,* 2008).

The macroscopic finding in this study showed multiple pale area of cecal surface with multiple foci of heamorrhage and necrosis these results agreed with (Aruna Panda *et al.,* 2010).

Our histopathological results declared that the intestine of the infected rabbits showed necrosis and desquamation of epithelial linning with neutrophilic infiltration in lamina propria. The intestinal epithelium showed hyper plasia with goblet cell.

The kidney showed sever congestion in interstitial tissue with necrosis of the surrounding renal tissue, some glomeruli showed micro thrombosis and presence of hyaline casts in the lumen of renal tubulues.

Liver of infected rabbits showed congestion in the central vein with pressure atrophy of the surrounding hepatocytes with neutrophilic infiltration. Liver cells also showed sharp clear vacuoles in the cytoplasm pushing nucleus forming signet ring appearance.

The results agreed with (Aruna Panda *et al.,* 2010) and (Diane Baker *et al.,* 2007).

The microthrombus formed in kidney mainly due to the endothelial damage of glomeruli leading to activation of coagulation factor and adhesion between the platelets and deposition of fibrin. The hyaline casts is formed due to increase protein concentration and desquamation of renal tubular epithelial lining of the tubules and protein urea due to renal glomerular damage.

Toxins of E.coli O_{157}: H_7 leading to severe deleterious effect on hepatic tissue in form of faltty change with signet ring appearance of hepatocytes due to excessive accumulation of fat in hepatocytes and necrosis of hepatocytes with subsequent lymphocytic and histiocytic infiltration.

CONCLUSION

The model of oral infection is likely to more accurately reflect the disease process as it occurs in humans due to oral ingestion of E.coli O157:H7 and to be useful for evaluation of antibodies or vaccines that could intervene in the disease process.

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بعض الدراسات على الارانب المصابة تجريبيا ببكتريا الايشيريشيا القولونية O_{157}:H_7 المعزولة من اللبن الخام

عطيه عبد الله الجداوى ، عبير الرفاعى عبد الغفار ، نسمه رشيد ، رانيا ابراهيم محمد ، ايمان شكرى ، ايمان الخواجه

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تسبب بكتيريا الايشيريشيا القواونية O157:H7 حالات واسعة الانتشار من الاسهال وكذلك حالات البول المدمم الناتج عن فشل وظائف الكلى فى الانسان اما فى الابقار فتعرف على انها المسبب الاول للعدوى البكتيرية. لذلك قمنا فى هذه الدراسة بعزل البكتيريا من الالبان عن طريق تجميع عدد ١٠٠ عينة البان من اماكن مختلفة فى جمهورية مصر العربية وعمل زرع لهذه العينات المختلفة واخذ العينات الايجابية منها والتى تبلغ عددها ٤٥ عينة لعمل الاختبارات التأكيدية والتصنيفية لهذه الميكروبات وتحديد النوع الدقيق لها والتى اظهرت ان عينه واحدة من ٤٥ عينة بعد تصنيفها يوجد بها O157:H7٠تم بعد ذلك اخذ المعزول من هذه العينة وعمل اختبار عدوى للارانب لتقييم الضراوة وذلك فى عدد ١٠ ارانب تم تقسيمها الى مجموعتين: مجموعة ضابطة سالبه وعددهم (٣ ارانب) ومجموعة تم اجراء العدوى لها وعددهم (٧ ارانب). تمت العدوى عن طريق الانبوب المعدى وبتركيز(10^5cfu)ثم تم تجميع عينات البراز عند اليوم ٢و٤ و٦ بعد العدوى لمعرفة وتحديد الخارج من البكتيريا الممرضة وفى اليوم الثالث عشر تم الذبح واخذ الامعاء والكلى والكبد لعمل شرائح الباثولوجيا وقد اظهرت الامعاء وجود نخر وانفصال فى الخلايا الطلائية مع ارتشاح فى خلايا الدم البيضاء. وقد اظهرت الكلى وجود خثرات دموية صغيرة مع وجود ماده هياليئية فى الخلايا المبطنة للانابيب ، اما الكبد فقد اظهر احتقانات شديدة مع وجود دهون فى خلايا الكبد مع اعطاء الخلية شكل حلقى.