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DETECTION OF GENE SEQUENCE OF *E. COLI* TOXINS ISOLATED FROM MASTITIC BUFFALO AND COWS MILK.

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SUMMARY

Results obtained indicated that 107 out of 185 milk samples (57.8%) were positive to bacteriological examination. *E. coli* was isolated from 39 out of 107 (36.4%).serological characterization was taken place on *E. coli* isolates and results showed that 19 isolates were serotyped belong to 3 serogroups including O114, O125, O128 with an incidence of 48.7% mean while the rest 20 (51.3%) isolates were serologically untypable with the available antisera. All isolates were tested for their virulence factors, Congo red ability used firstly for the detection of virulence *E. coli*. With reference to toxin production, all isolates were scanned for the presence of enterotoxin (infant mouse suckling assay) and verotoxin (rabbit challenged). 3 isolates out of 39 *E. coli* (7.69%) were enterotoxigenic. It is creditable to mention that 100% concurrence was found between the results of PCR for detection of enterotoxin gene and the other assays used.

INTRODUCTION

Mastitis is an important disease causing economic losses estimated to be 526 millions US dollars annually. Conventional veterinary treatment dependence on costly antibiotics and cure rate is only 60% in field conditions with a problem of milk residues (Varshney and Naresh, 2004).

Mastitis is an important disease affecting animals, causing a lot of economic losses (Lafi and Hailat, 1998). It still remains as one of the most costly diseases to animal agriculture throughout much of the world (National Mastitis Council, 1999).

Coliforms represents one of the most important environmental pathogens causing mastitis , *E. coli* is the predominant coliform species reported as causing intramammary infections in most studies (Todhunter *et al.*,

1991). *E. coli* causes inflammation of the mammary gland in dairy animals around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms. This disease affects mainly high producing animals in dairy herds and may cause several cases of death per year in the most severe cases. It is well known that bacterial, animal and environmental factors are interdependent and influence mastitis susceptibility (Burvenich *et al.*, 2003).

It well known now that *E. coli* produce heat-labile enterotoxins (LT) that are antigenic and similar to cholera enterotoxin and /or a heat stable enterotoxin (ST) that are of small molecular weight and are non antigenic (Frank *et al.*, 1977 & Niazi and Refai 1988). Shiga like toxin producing *E. coli* (STx_1 and STx_2) also known as verotoxin producing *E. coli* (Chanter *et al.*, 1986 and Mainil *et al.*, 1987).

Molecular biology techniques have become integrated into the practice of infectious disease epidemiology (Galane and Le-Roux, 2001). In particular Polymerase chain reaction is an in-vitro amplification technique for enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Erlich *et al.*, 1991).

PCR is used now in large scale as a recent technique for detection of virulence factors of entertoxigenic *Escherichia coli* serotypes (Feng and Monday, 2000 and Makino *et al.*, 2000).

The current work was aimed at chiefly to throw lights on the importance of *E. coli* mastitis with the utilizing of Polymerase Chain Reaction (PCR) as a topical procedure for diagnosis of *E. coli* toxin genes and to substantiate the consequences obtained using conventional methods.

MATERIAL AND METHODS

1- Milk samples:

A total of 185 milk samples were collected from 53 cows and buffaloes from various private farms in Egypt showing clinical signs of mastitis. All samples were taken under aseptic conditions and transferred in ice box to the laboratory as soon as possible.

2- Bacteriological examination of milk samples (Breed *et al.*, 1957)

Milk samples were incubated aerobically at 37°C for 24 h then centrifuged at 3000 r.p.m. for 20 minutes. The cream and supernatant fluid were discarded. A loopfull from the sediment was streaked onto the surface of blood agar, MacConkey's agar and Eosin Methyline Blue agar. The inoculated plates were incubated at 37°C for 24-48 hours and examined for bacterial growth. Suspected colonies, appeared on different media were subcultured, purified, and preserved in semisolid nutrient agar for further identification. Pure colonies were described for their morphological characters, colonial appearance and were biochemically identified according to Quinn *et al.* (2002).

3.Serological identification of *E.coli*:

The isolates were identified serologically by "Seiken" *Escherichia coli* diagnostic antisera (Edward and Ewing, 1972).

4- Virulence factors for *E. coli* isolates:

4.1. Congo red (C.R.) binding test:

Congo red binding is one of the indicators of virulence among *E. coli* isolates. All *E. coli* isolates recovered from milk samples of mastitic cows were tested for its growth status on Congo red medium according to Berkhoff and Vinal (1986).

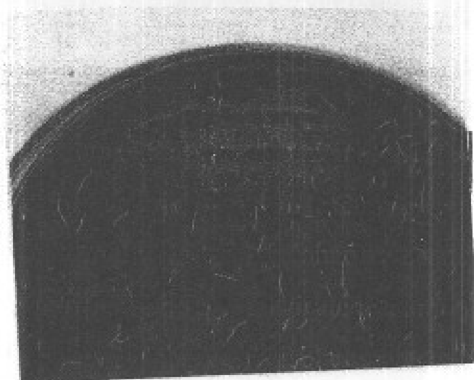


Photo (1) : Showing Congo red Binding activity of *Escherichia coli:*

Cong red positive colonies

Cong red negative colonies

4.2. Verotoxin activity of *E. coli* Serovars:

Detection of cytotoxin of *E. coli* strains isolated from mastitic milk samples using Vero cells was investigated according to Giugliano *et al.* (1982) , toxicity up >50% of the Vero cell was considered toxic.

4.2.1. Assay of verocell cytotoxicity (Kudva *et al.*, 1997):

Volumes (50 µl) of serial twofold dilution of protein content were applied to confluent verocell monolayers. The cell toxin mixtures were incubated at 37°C from 48-72 hours in 7.5% CO_2 atmosphere tension and were examined microscopically for cytopathic effect. Toxicity up >50% of the Vero cells was considered toxin positive.

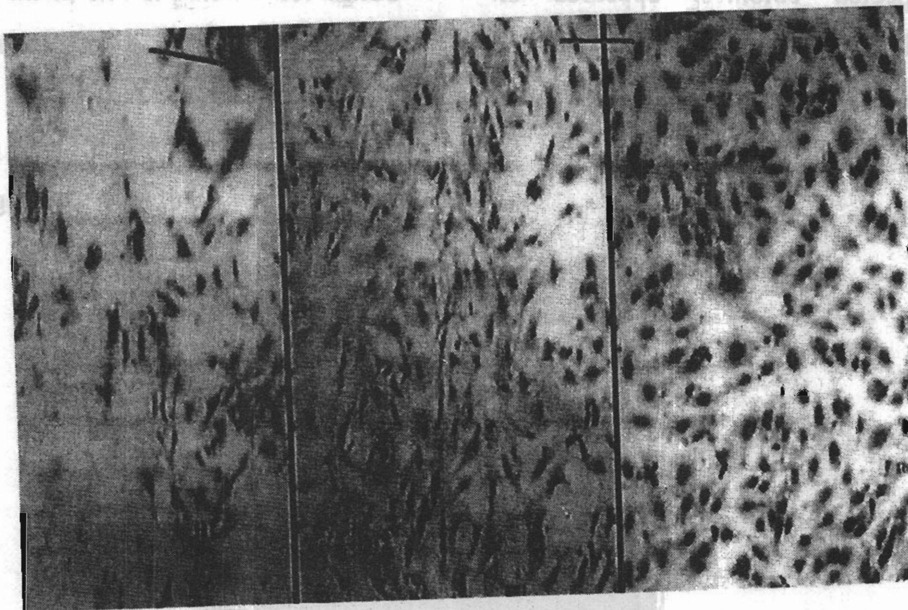


Photo (2) : Showing degree of destruction of Vero cell

4.2.2. Preparation and characterization of rabbit anticytotoxin (O`Brien and Laveck, 1982)

Adult albino rabbits were pre bled and then gave at 7 days intervals, seven sequential intramuscular injections of 1 ml of concentrated culture filtrates emulsified with an equal volume of incomplete adjuvant ($6x10^9$ cfu). ELISA was done to identify the titre of the antitoxic antibodies.

4.2.3. Challenge of infant rabbits with verotoxins:

The verotoxin preparation was diluted in sodium carbonate at final concentration of 10% and was administrated to 3 days old rabbits directly into the stomach through a catheter tube. After inoculation, animals were observed daily for diarrhea. Rabbits were considered to have diarrhea when feces were stocked onto perineum or hind legs. Diarrhea was scored as severe when area was soiled with wet feces. At various times from the day of inoculation, animals were sacrified and portions of the intestine were dissected for VT assay (Pal *et al.*, 1986).

4.3. Identification of enterotoxigenic *Escherichia coli* (ETEC) isolates:

All isolates of *E. coli* were tested for their ability to produce heat stable (ST) enterotoxin by mouse suckling technique and detection of toxin gene by PCR.

4.3.1. Detection of heat stable enterotoxin by mouse suckling technique

The isolated *E. coli* strains were grown over night in brain heart infusion broth and then inoculated in culture medium 10 ml of casamino acids yeast extract medium in a 250 ml Erlenmeyer flask, prepared specifically for production of toxins (Evans *et al.* 1973). After inoculation; the flasks were incubated with shaking on rotatory shaker at 37°C for 24 hours. The culture fluid was centrifuged at 250 x g for 30 min. at 4°C. The supernatant was then taken and subjected to heat treated (80°C for 30 min.) before storing at- 70°C for detection of heat stable enterotoxin (ST).

Heat stable enterotoxin of *E. coli* was detected by suckling mouse assay (Giannella, 1976).

4.3.2. PCR technique for detection of toxigenic *E. coli* enterotoxin (STI) gene and verotoxin STx_1-STx_2 gene.

E .coli plasmid DNA was isolated by the alkaline lysis method (Birnboim and Dolly, 1979). Briefly single colonies from individual isolates were inoculated into 4 ml Luria Bertani (LB) broth (bacto-tryptone 10g. bacto yeast extract 5 g. NaCl 10 g .per 1L H_2O) and grown overnight at 37°C on a shaker. Next day the bacterial culture was transferred to a sterile Eppendorf tube and centrifuged for 15 sec. at 14000 rpm/ 4° C. The supernatant was discarded and this procedure was repeated once.

Then the bacterial pellet was resuspended in 200ul GTE solution (50 mM Glucose, 25mM Tris-HCL pH-8,0 10 mM EDTA; pH-8,0) by vortexing. 300ul cell lysis solution was added (freshly prepared 0.2N NaoH, 1% sodium dodecyl sulfate). Tubes were gently inverted 5 times in order to mix the two solutions. 300ul neutralization solution (5M Sodium Acetate) was added to the tubes and then tubes were centrifuged at 14000 rpm /4° C for 10 min to remove bacterial proteins. Next to the supernatant 800µl isopropanol was added. Tubes were inverted and centrifuged at 14000 rpm/ 4°C for 10 min in order to precipitate plasmid DNA. The pellet was washed once with 70% Ethanol and allowed to dry for 10 min at room temperature with tubes inverted and dissolved in 500ul autoclaved water. To this solution 10µl RNA-ase H (10 mg/ml) was added and the tubes were incubated at 37° C for 30 min. Next the plasmid DNA was extracted with 500µl Phenol / Chloroform. Tubes were vortexed and centrifuged 10 min/4° C. and then the water phase was extracted only with Chloroform with the same centrifugation step. Finally the pure plasmid DNA was precipitated by adding to the supernatant 50µl 3M.

Sodium acetate and 500µl 2-propanol and centrifuged at 14000 rpm / 4° C for 20 min., then washed by 70% Ethanol, allowed to dry for 10 min at room temperature with tubes inverted and dissolved in 50ul autoclaved water. Five micro liters from isolated plasmid DNAs was checked by 0.7% agarose gel electrophoretic analysis with 1 x TAE buffer (0.04M Tris-acetate, 0,001M EDTA) as electrophoretic buffer, and stained with ethidium bromide, visualized under UV light and photographed.

Plasmid DNA was stored at -20° C. ST_1, STx_1, STx_2 toxins genes was detected by PCR using specific primers and

PCR conditions reported by (Tsen and Chi, 1996).

PCR was done in total volume of 20 µl in a thermal cycler.

To 500 ng-1 ng plasmid DNA

2µl 10 x PCR buffer,

2µl DNTPs (25µlM each)

1 µl each primer (pMl0)

0.5 U Taq DNA polymerase

Water to 20 µl

STx_1	Stx_a	STx_2	Stx_a	ST1	Stx_a
	stx_b		stx_b		stx_b

PCR program

Denaturation 94°C / 20 sec.
Annealing 50°C/30 sec.
Extension 72°C / 30 sec.
Final extension 72° / 2 mm.
Total number of cycles 40.
After the amplification the PCR product was resolved on 2% agarose gel and visualized as described above.

Table (1): Primers and PCR to amplify specific gene sequence for different virulence markers

Gene	primer	Oligonucleotide sequence
Stx_1	Stx_a	ACACTGGATGATCTCAGTGG
	Stx_b	CTGAATCCCCCTCCATTAG
Stx_2	Stx_a	CCATGACAACGGACAGCACTT
	Stx_b	CCTGTCAACTGAGCAGCATTTG
St_1	Stx_a	TTAATAGCACGCGGTACAAGCAGG
	Stx_b	CTTGACTCTTCAAAAGAGAAAATTAC

RESULTS

Table (2): Prevalence of bacterial infections among the collected samples with special reference to *E. coli*

Animal species	No. of examined animals	No. of examined quarters	positive samples		*E. coli* positive cases	
			No.	%	No.	%
Cows	32	105	68	64.76	27	39.70
Buffaloes	21	80	39	48.75	12	30.76
	53	185	107	57.8	39	36.44

As show in Table (1) 107 out of 185 quarter's milk samples were positive to bacteriological examination with an incidence of 57.8%. In the same Table from cows milk samples 27 out of 68 samples were positive for *E. coli* with an incidence of 39.70 meanwhile 12 out of 39 samples collected from buffaloes were positive for *E. coli* with an incidence of 30.76 .

Table (3): Serotyping of *E. coli* isolated from mastitic cows and buffaloes milk samples

E. coli serogroups	No.	%
O 128	10	25.6
O125	5	12.8
O114	4	10.3
Untypable	20	51.3
Total	39	

Table (2) showed that *E. coli* isolates were typed serologically and belonged to 3 different serovars viz, 0128 , 0125 0_{114} with an incidence of 25.6 % , 12.8% and 10.25 % respectively.

Table (4): Congo red Binding activity of *Escherichia coli* isolated from mastitic cows and buffaloes in relation to different serotypes

E. coli serogroups	No. of isolates	**Congo red positive**	
		No.	**%**
O 128	10	10	100
O125	5	5	100
O114	4	4	100
Untypable	20	0	0.0
Total	39	19	48.71

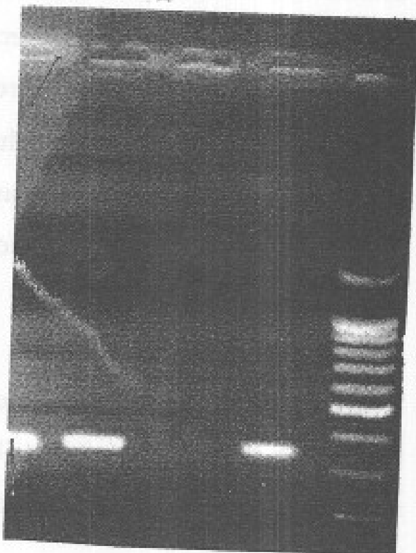
Table (5): Biological properties of verotoxin extracted from *E. coli* serogroups.

Source of isolates	Verocell cytotoxicity titre	Challenged rabbits with verotoxins				
		No. with diarrhoea /No. inoculated	No. with severe diarrhoea /No. inoculated	Vero cell cytotoxicity		
				Proximal	Distal	Mid.
Cows	192	3/3	3/3	+++	+++	++
Buffaloes	192	3/3	2/3	++	++	++
Negative control	-	0/3	0/3			

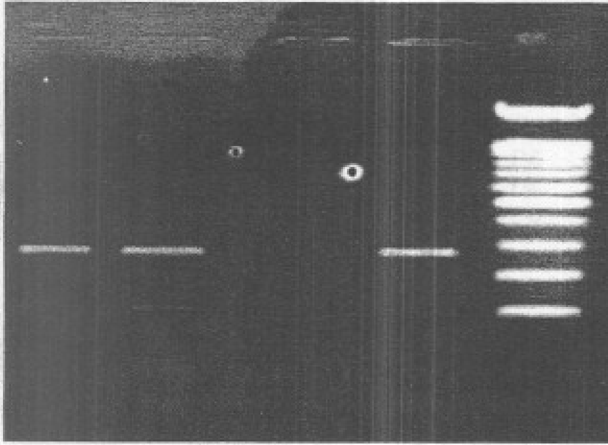
Table (6): Enterotoxigenic activity of *E. coli* serogroups isolated from examined mastitic cows and buffaloes milk samples using infant mouse assay

E. coli serogroups	Clinical mastitis		
	No.	Enterotoxins +ve	
		No.	%
O 128	10	1	10.0
O125	5	1	20.0
O114	4	1	25.0
Non typable	20	0	0
Total	39	3	

Suckling mice assay was used in this study for the detection of enterotoxin production and results showed that 3 isolates of *E. coli* were toxigenic belong to O 128 , O114 and 0.125 (one each).



Lane (0) marker
Lane 1, 3, 4 positive
Lane 2 negative control
Photo (3): positive and negative *E.coli* to ST_1 gene



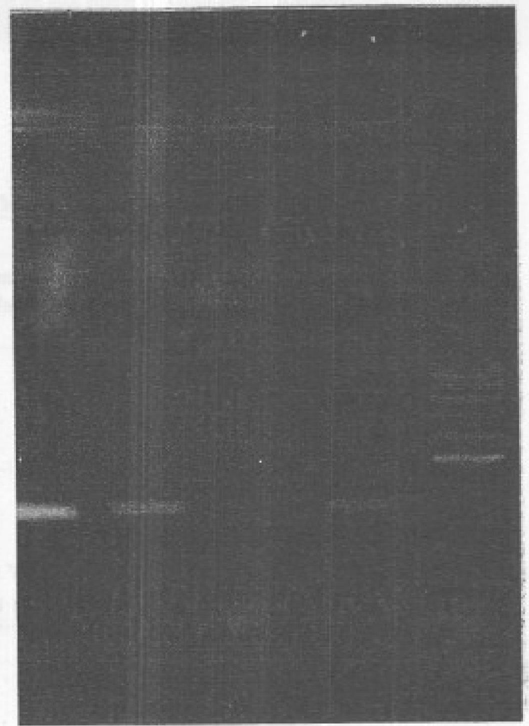


Photo (4): positive and negative *E. coli* to STx_1 gene

Photo (5): positive and negative *E. coli* to STx_2 gene

Lane (0) marker ; Lane 2 negative control ; lane 1, 3, 4 positive

DISCUSSION

Mastitis is a general term which refers to inflammation of the mammary gland, regardless of cause. It is characterized by physical, chemical, and usually bacteriological changes in the milk and by pathological changes in the udder. Early recognition and prompt treatment are important for limiting tissue damage and production losses. However, since treatment is often unrewarding, emphasis should be on mastitis control and prevention (Shearer & Harris, 2003).

Clinical signs of mastitis appeared seven to 14 days with a peak of high fever, more severe anaemia, a second peak of neutrophilia and the complete cessation of milk production from all quarters; extensive and severe pyogranulomatous mastitis developed (Aroch *et al.*, 2003)

Different observations were recorded on this study; first of all; it is not possible to establish an accurate diagnosis of coliform mastitis,

which is based on a specific clinical symptoms mastitis differentiating it from other form of and this observation was in accordance with that mentioned by Schukken *et al.* (1989) as they reported that no single symptom or combination of symptoms is specific for coliform mastitis.

Second observation, was that, most of cases takes place in one quarter and this observation agree with that mentioned by Vaarst and Enevoldsen (1997) who mentioned that Coliform mastitis (6% of the cases) usually occurred in one quarter only and *Escherichia coli* infections were typically (truly) acute cases.

In this study 107 out of 185 quarters milk samples were positive to bacteriological examination with an incidence of 57.8 % and this incidence was in accordance with Jha *et al.*, (1993) and Dhote *et al.*, (1999) as they found that the incidence of mastitis was 18.8% and 20.7% respectively. Table (2) also showed that 39 out of 107 positive quarters were *E. coli* isolated with an incidence of 36.4% and this incidence was in accordance with mutually Ahmed et al.(1988) and Shalaby and Salem (2001).

Serogrouping of the isolates was carried out to bestow an suggestion about the mainly prime serogroupes allied with clinical mastitic cases. Our results showed that *E. coli* isolates were typed serologically and belonged to 3 different serovars viz. 0128 , 0125 ,0 114, with an incidence of 25.6 % , 12.8% and 10.25 % respectively (Table 3) . Additionally, 20 isolates were untypable with a high incidence reach to 51.3 %. It is possible that if there had

been a wider range of diagnostic sera, more of the strains of *E. coli* isolates could have been typed. On the other hand high incidence of untypable give to a high extent that *E. coli* serovars was of a infinitesimal significance for mastitic cases categorization and this observation was recognized by many researchers as Mc- Donald *et al.* (1970) who mentioned . that mastitis had different O serotypes and may not be attributed to epizootic strains as well as Morner *et al.* (1998) who decided that a variety of *E. coli* serovars were detected in mastitic cases .Bacterial virulence factors are required to fight the hosts selection pressure and for the bacteria to colonize, multiply and survive in the udder (Kaipainen et

al., 2002) and from this aspect it was found that virulence factors and in meticulous (particular) enterotoxin production was of importance to be confer.

Congo red dye could be used as a detective of virulence for *E.coli* strains and to distinguish between virulent and a-virulent *E. coli* strains. Our results showed that 48.71% of the isolated *E. coli* could bind actively Congo red dye (Table 4) and this observation coincides with that mentioned by El-Mahrouki *et al.* (2006).

Several virulence factors were detected in pathogenic *E.coli* causing urinary tract infection, diarrhoea , septicaemia and meningitis. These include toxins, adhesins, invasiveness and ability to resist serum complement. Bovine mastitis resembles urinary tract infection as both are ascending caused by bacteria from the environment (Boedeker, 2005).

All *E. coli* serotypes from mastitic cows and buffaloes produced verotoxins and this result consistent with the hypothesis that verotoxin play a major role in the pathogenesis of mastitis caused by *E.coli*. The pathogenicity of this disease probably result from the production of verotoxin or shiga like toxin which efficiency inhibit protein synthesis in mammalian cell free system Genetic profile (STx_1+STx_2) was recorded in our study for serotyped and untyped *E.coli* strains. These results and similar to that detected by Homitzky *et al.* (2005) and EL-Safii *et al.* (2006).

Cytotoxicity production was tested in our study using infant rabbit and results obtained showed that *E. coli* producing cytotoxic activity detected by its capability of inducing clinical signs (diarrhea) in tested rabbit as VT which identified in the luminal content of the mid colon and this results agree with that mentioned by Pal *et al.* (1986) who found that feeding of VT alone to infant rabbit resulted in clinical symptoms and histological changes almost identical to those seen after challenging with live bacteria. Furthermore, bloody diarrhea and signs characteristics of haemorrhagic colitis were seen following intragastric administration of VT.

The expression of colonization factors enables the bacteria to adhere to the intestine, where they produce enterotoxins which elicit physiological changes in intestinal epithelial cells. The toxins at either heat stable toxin (ST) or heat labile (LT).

Bioassays distinguish two types of heat stable toxin ST1 and ST2 (Alam *et al.*, 2006 and Veilleux and Dubreuil, 2006). Suckling mice assay was used in this study for the detection of enterotoxin production and results recorded in table (6) showed that 3 isolates of *E. coli* were toxigenic belong to two serovars , 0128 , 0 114, and one untypable and this remark were in accordance with that mentioned by El-Mahrouki *et al.* (2006).

Conventionally, microbiological testing of mastitic milk has involved isolation of microorganisms from milk samples and performing specific biochemical, and in some cases serological, tests to confirm the presence or absence of suspected pathogens. Given the public attention milk have received as sources of food-borne disease, there has been considerable interest in the application of rapid detection techniques that require hours rather than days for completion. Hypothetically, rapid detection methods could reduce the time from the initial sampling to confirmation so that conclusive results would be available by the time to process the milk (Pillai and Ricke, 1995).

Many researchers recommended the modern molecular diagnostic techniques. They concluded that in comparison to culture method, PCR was found to be more sensitive rapid and accurate method for diagnosis. In addition, PCR technique minimizes labor needed for isolation, (Pinnow *et al.*, 2001). So, it becomes expedient and also considered necessary for a technique to be applicable for detection of toxigenic isolates and PCR is one of the promising techniques, results obtained here using sucking mouse techniques and rabbit challenge which revealed that 3 isolates were positive for both assay were in concurrence with that of PCR with an incidence of 100% (photo 3,4 and 5)

Unfortunately with no unambiguous conclusion, virulence factors studied didn't give a clear idea about its mode of action as act alone or in combination to help in establishment at the infection site and subsequently causing a disease condition.

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تحديد سلسة الجينات المفرزة للسموم للميكروب القولونى المعزول من البان الجاموس والابقار المصابة بالتهاب الضرع.

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اظهرت النتائج ان 107 من 185 عينة البان مصابة بالتهاب الضرع الظاهرى كانت ايجابية للفحص البكتريولوجى بنسبة عزل 57.8% ونم عزل الميكروب القولونى من 39 عينة بنسبة مئوية 33.0% .

تم اجراء الاختبارلت السيرولوجية على المعزولات حيث تبين ان 19 عترة تم تصنيفها سيرولوجيا وكانت تتبع ثلاث انواع سيرولوجية تشمل

O114, O125, O128 اما باقى المعزولات فكانت غير مصنفة بأى من الانواع السيرولوجية المستخدمة.

تم اختبار ضراوة المعزولات حيث تم اختبارها اولا باستخدام اختبار الارتباط بصبغة الكونغو الحمراء وذلك للتميز بين العترات الممرضة والاخرى الغير ممرضة .

تم اختبار المعزولات للقدرة على افراز السموم وذلك للسموم المقاومة للحرارة باستخدام اختبار الفئران الرضيعة وايضا للسموم القابلة للحرراة باستخدام الارانب واظهرت النتائج ان ثلاث عترات ايجابية لكلا الاحتبارين بنسبة 7.69%.

تم اجراء اختبار سلسلة البلمرة التفاعلية على العترات المعزولة حيث اكدت النتائج عن وجود نفس العترات مفرزة للسموم بنسبة تاكيدية تصل 100% .