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GENOTYPING OF E.COLI O157:H7 ISOLATED FROM BUFFALOES

BY

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ABSTRACT

The present study was concerned with E.coli O157:H7 isolated from buffalo-calves. From the total examined samples (215), which were collected from buffaloe farms, the total prevalence of E.coli isolated from buffaloe- calves was 47.91%, while in apparently healthy buffalo-calves and diarrheic buffalo-calves were 23% and 69.57% respectively. Further more, the incidences of sorbitol and non sorbitol fermenting E.coli were 44.65% and 3.26% respectively. Serological identification of E.coli O157:H7 from the total E.coli isolates revealed 93.2% negative (sorbitol fermenting E.coli) and 6.79% positive (non sorbitol fermenting E.coli). Hemagglutination (HA) activity of E.coli O157:H7 isolates showed that 100% positive HA [mannose resistant hemagglutination) (MRHA) and mannose sensitive hemagglutination (MSHA)] for human, guinea pig and chicken RBCs. While the test was 42.9% positive MRHA, 28.6% positive MSHA and 28.6% negative HA with bovine RBCs. The prevalence of enterotoxigenic E.coli O157:H7 isolates was 71.43%. Detection of α hemolytic E.coli O157:H7 was 29% while β-hemolytic E.coli O157:H7 was 43%. There were differences in isolates susceptibilities to different antimicrobial agents. Amoxicillin, colistin sulphate, flumequine, imipenem, had 100% susceptibilities on E.coli O157:H7 isolates. PCR assay for E.coli O157:H7 isolates revealed positive amplification of 255 bp fragment of shiga toxin 2 gene was observed in 80% of examined E.coli O157:H7 isolates. Moreover, positive amplification of 384 bp fragment of intimin gene was determined in 60% of examined E.coli O157:H7 isolates.

INTRODUCTION

E.coli is the most frequently encountered Gram negative organism causing diarrhea which is a problem in young calves, especially in suckling beef calves **(China *et al.,* 1998).** E.coli O157:H7 is considered the predominant pathogen in the enterohemorrhagic E.coli (EHEC) group and it was first identified in 1982 in the United States as a human pathogen **(Riley *et al.,* 1983; Sidjabat Tambunan and Bensink, 1997).** Epidemiological investigation has linked several outbreaks of disease in human caused by E.coli O157:H7 with consumption of bovine products (meat and raw milk) **(Wells *et al.,* 1991; Upton and Coia 1994).**

Multiplex PCR was developed for detection of virulence genes of E.coli **(Kimata *et al.,* 2005).** A novel multiplex PCR assay used 5 sets of primers that specifically amplify segments of the eae A, Stx-1, Stx-2, fliC and rfbE genes, this PCR system successfully distinguished serotype O157:H7 from other serotypes of E.coli, provided accurate profiling of the shiga-like toxins and the intimin adhesion in individual strain and did not cross-react with the background bacterial flora in bovine feces **(Hu *et al.,* 1999).**

The aim of this investigation was directed to the following:

1- Bacteriological examination of fecal samples collected from apparently healthy and diarrheic buffalo-calves.

2- Serological identification of E.coli O157 isolates.

3- Determination the antibiogram of the E.coli O157:H7 isolates.

4- Investigation of some virulence factors associated with O157:H7 isolates including:

   - Hemagglutination activity.
   - Enterotoxin assay using infant mice.
   - Hemolysin detection using sheep erythrocytes.

5- Detection of some virulence genes of the O157:H7 isolates (Stx and eae) using PCR.

MATERIALS AND METHODS

Sampling:

215 feacal sample were collected from buffaloe farms including 100 sample from apparently healthy buffalo-calves and 115 sample from diarrheic buffalo-calves. Samples were collected in sterile polyethylene bags, and they were sent to laboratory in an ice box with a minimum of delay.

Isolation and identification of E.coli O157:H7 strains:

According to Koneman *et al.* (1992) and Quinn *et al.*, (1994). Samples were cultured onto MacConkey agar medium for 24h at 37°C, then a part of single typical lactose fermenting colony was tested for sorbitol fermentation by culturing on sorbitol macConkey agar and incubated at 37°C overnight. A part of single isolated colony was picked up from sorbitol MacConkey agar and grown in peptone water for 6 hours. Peptone water culture was used to inoculate biochemical tests.

Serotyping of the isolated strains of E.coli was done according to Edward and Ewing (1972). E.coli somatic antigen (O157) and flagellar antigen (H7) antisera were obtained from Difco.

Antibiogram of the recovered E.coli O157:H7:

This was done by using disc diffusion standard technique according to Finegold and Martin (1982) and Quinn *et al.* (1994) using the following disc (Oxoid), including amoxicillin clavulanic acid (2: 1) (30), ampicillin (10), cefoperazone (75), cefotaxime (30), chloramphenicol (30), ciprofloxacin (5), colistin sulphate (25), enrofloxacin (5), flumequine (30), gentamicin (10), imipenem (10), maxfur (30), norfloxacin (10), pefloxacin (5) and vancomycin (30).

Hemagglutination activity of E.coli O157:H7 isolates :

According to Evans *et al.* (1979). Hemagglutination tests were performed with and without D-mannose to test for mannose resistant Hemagglutination (MRHA) and mannose sensitive Hemagglutination (MSHA) respectively by slide agglutination as follows: E.coli O157:H7 was obtained from culture grown for 18 hours at 37°C on colonization factor agar (CFA) plates. Growth was picked up with sterile wooden tooth pick and mixed with a drop of the appropriate species of blood (bovine, human, guinea pig and chicken) on microscopic

slide at room temperature and observed for 1-2 minutes. Hemagglutination was observed by the naked eye supported by the aid of hand lens. HA was recorded as mannose resistant (MR) if occurred with and without mannose and as mannose sensitive (MS) if HA was inhibited in the presence of D-mannose.

Enterotoxin assay using infant mice:

According to Pai and Mors (1978) and Robins-Brown *et al.* (1993). A volume of 0.1 ml of culture filtrate was injected through the abdominal wall into the milk filled stomach of each of the three mice (for each serotype) which were 1 to 4 days old, another 3 infant mice were injected by 0.1 ml of saline and were used as negative control. After 4 hours, the mice were killed and the entire intestine was removed. The intestine and the remaining body were weighted to calculate the ratio of (intestine weight)/ (remaining body weight). A ratio equal or greater than 0.083 was recorded as positive test for enterotoxin.

Hemolysin detection: was applied according to Beutin *et al.*(1989). E.coli O157: H7 isolates were inoculated into blood agar plates containing sheep blood 5% for the detection of β-hemolysin, after 3 hours of incubation at 37°C. Positive β-hemolysin production was indicated by inner clear zone of hemolysis.

PCR assay: for detection of shiga like toxin type 2 (Stx2) and intimin gene (eae A) in the extracted DNA of 5 E.coli O157:H7 isolates according to Paton and Paton (1998).

RESULTS

Table (1): Prevalence of sorbitol and non sorbital fermenting E.coli from buffalo-calves feacal samples.

Animal	Number of examined samples	Number of E.coli isolates	%	E.coli isolates			
				S.F.		N.S.F.	
				No.	%	No.	%
Apparently healthy buffalo-calves	100	23	23	20	20	3	3
Diarrheic buffalo-calves	115	80	69.57	76	66.08	4	3.48
Total	215	103	47.91	96	44.65	7	3.26

S.F. = Sorbitol fermenting

N.S.F= Non sorbitol fermenting.

No. = Positive number

*** The percent was calculated according to the number of examined samples.**

Table (2): Serological identification of E.coli O157:H7 isolated from buffalo-calves feacal samples.

Source of isolates	Number of E.coli isolates	S.F.								N.S.F.							
		O157 antisera				H7 antisera				O157 antisera				H7 antisera			
		+ve		-ve		+ve		-ve		+ve		-ve		+ve		-ve	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Apparently healthy buffalo-calves	23	0	0	20	86.95	0	0	20	86.95	3	13.04	0	0	3	13.04	0	0
Diarrheic buffalo-calves	80	0	0	76	95	0	0	76	95	4	5	0	0	4	5	0	0
Total	103	0	0	96	93.2	0	0	96	93.2	7	6.79	0	0	7	6.79	0	0

S.F.= Sorbitol fermenting.
N.S.F. = Non sorbitol fermenting.
+ve = Positive
-ve = Negative
No.= Number
*** The percent was calculated according to number of E.coli isolates.**

Table (3): Hemagglutination (HA) activity of E.coli O157:H7 isolated from buffalo-calves feacal samples using different RBCs.

RBCs. of	HA +ve						HA-ve	
	MRHA		MSHA		Total			
	No.	%	No.	%	No.	%	No.	%
Human	7	100	0	0	7	100	0	0
Bovine	3	42.9	2	28.6	5	71.4	2	28.6
Guinea pig	7	100	0	0	7	100	0	0
Chicken	7	100	0	0	7	100	0	0

HA +ve = Positive hemagglutination.

HA –ve = Negative hemagglutination.

MRHA = Mannose resistant hemagglutination.

MSHA = Mannose sensitive hemagglutination.

Table (4): Prevalence of enterotoxigenic E.coli O157:H7 isolated from apparently healthy and diarrheic buffalo-calves feacal samples.

Source of isolates	No. of E.coli O157: H7 isolates	+ve	%*	-ve	%*
Apparently healthy buffalo-calves	3	2	66.67	1	33.33
Diarrheic buffalo-calves.	4	3	75	1	25
Total	7	5	71.43	2	28.57

No. = Number

+ve = Positve isolates

- ve= Negative isolates.

*** Percent was calculated according to number of E.coli O157:H7 isolates**

Table (5): Detection of α and β hemolytic E.coli O157:H7 isolated from apparently healthy and diarrheic buffalo-calves feacal samples.

Source of isolates	No. of E.coli O157: H7 isolates	α	%	β	%	total +ve	%*	-ve	%*
Apparently healthy buffalo-calves	3	1	33.3%	1	33.3	2	66.67	1	33.33
Diarrheic buffalo-calves.	4	1	25%	2	50	3	75	1	25
Total	7	2	29%	3	43	5	71.43	2	28.57

α = α hemolysis
β = β hemolysis
+ve = Positive
- ve = Negative
*** The percent was calculated according to number of examined E.coli O157: H7.**

Table (6): Antimicrobial susceptibility of E.coli O157:H7 isolated from buffalo calves-feacal samples.

Antimicrobial	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Amoxicillin clavulanic A. (2:1) (30)*	7	100	0	0	0	0
Ampicillin (10)	0	0	0	0	7	100
Cefoperazone (75)	1	14.29	6	85.71	0	0
Cefotaxime (30)	0	0	4	57.14	3	42.86
Chloramphenicol (30)	5	71.43	2	28.57	0	0
Ciprofloxacin (5)	6	85.71	1	14.29	0	0
Colistin sulphate (25)	7	100	0	0	0	0
Enrofloxacin (5)	4	57.14	3	42.86	0	0
Flumequine (30)	7	100	0	0	0	0
Gentamicin (10)	0	0	4	57.14	3	42.86
Impenem (10)	7	100	0	0	0	0
Maxfur (30)	0	0	5	71.43	2	28.57
Norfloxacin (10)	6	85.71	1	14.29	0	0
Pefloxacin (5)	3	42.86	4	57.14	0	0
Vancomycin (30)	0	0	0	0	7	100

*** Concentration of antibiotic in μg.**

% = Percent was calculated according to total number of E.coli O157:H7 isolates (7).

No.= Positive number.

Table (7): Characterization of E.coli O157:H7 isolates by PCR assay.

Number of examined isolates	Stx2* gene	eae A ** gene
1	-	-
2	+	+
3	+	+
4	+	-
5	+	+

*** Shiga like toxin type-2 gene.**
**** Intimin gene.**
+: gene present
- : gene absent

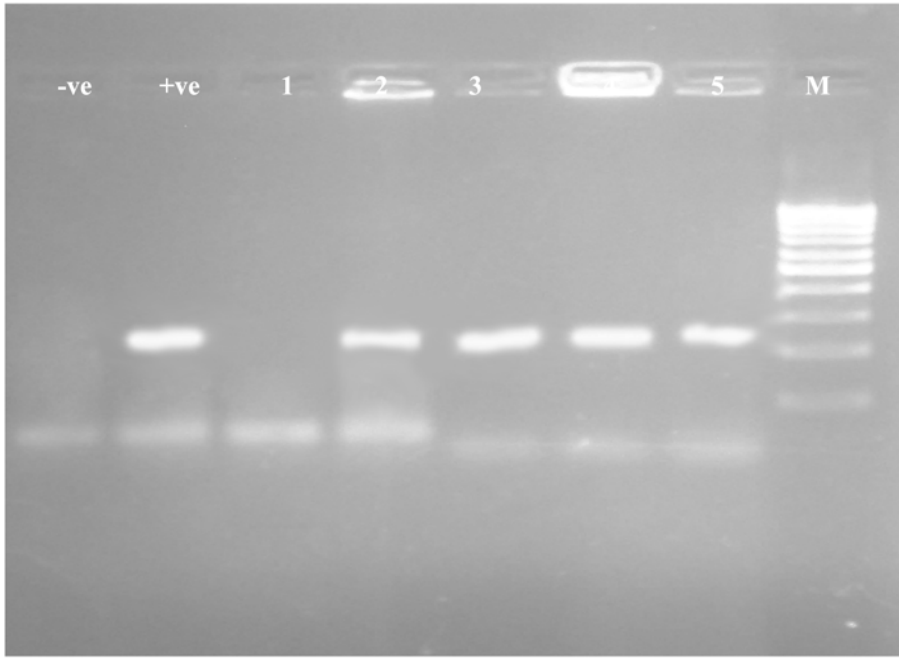

Photo (1): Shows agarose electrophoresis of PCR amplification of 255bp fragments of Shiga like toxins (Stx2) gene from the extracted DNA of *E.coli* O157: H7 isolates lane shows 100 bp ladder maker.

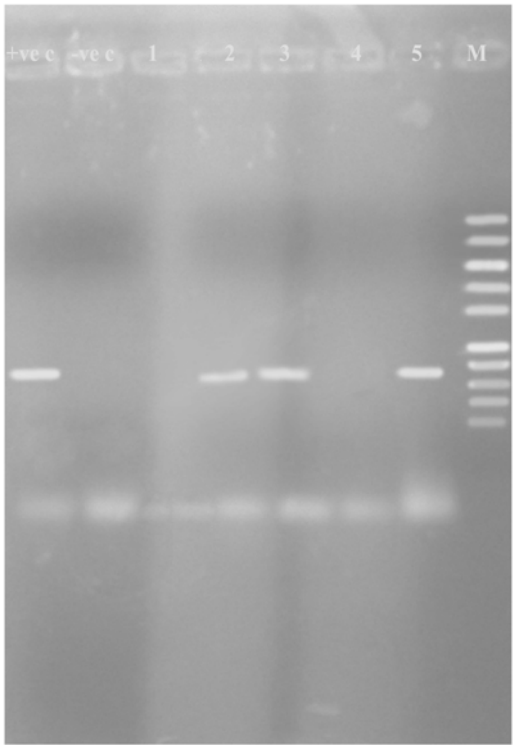

Photo (2) shows agarose gel electrophoresis of PCR amplification of 384bp fragment of intimin (eaeA) gene from the extracted DNA of *E.coli* O157: H7 isolates. Lane M shows 100 bp ladder maker.

DISCUSSION

Gyles (2007) highlighted the importance of cattle in human disease due to Shiga like toxin producing E.coli (STEC) and discussed features of STEC especially serotype O157:H7 that are important in human disease and stated that healthy dairy and beef cattle are a major reservoir of a diverse group of STEC that infects human through contamination of food and water as well as through direct contact. Therefore, this study was conducted to investigate the prevalence of E.coli O157:H7 in feacal samples collected from buffalo-calves and to study some of the virulence attributes and characteristics of E.coli O157:H7 isolates. The investigations concerned with E.coli O157:H7 in buffaloes were little in the available literature. The data presented in Table (1) indicated that the prevalence of N.S.F. E.coli was 3% and 3.48% from apparently healthy and diarrheic buffalo-calves respectively. While, the incidence of N.S.F. E.coli was 3.26% from the total examined samples (215). In this concern,

Confedera *et al.* (1997) recorded that in 3 studies conducted at slaugher, E.coli O157 was isolated in 3.6%. Also, Kang *et al.* (2004) examined 24 (9.8%) positive cases for EHEC O157 from 244 diarrheic calves and 7 (2.8%) were also positive cases from 254 non-diarrheic calves. From these data the frequency of E.coli O157 was higher in diarrheic calves than in apparently healthy ones.

It was evident from the results recorded in Table (2) that 3 isolates recovered from the apparently healthy buffalo-calves and 4 isolates from diarrheic ones, were positive by slide agglutination test using O157 and H7 antisera. These results come in accordance with that of Roopnarine *et al.* (2007) who stated that isolates of E.coli were subjected to slide agglutination test using E.coli O157 antiserum. In addition, Dunn *et al.*(2004) verified that isolates positively reacted with anti- O157 and positively reacted with anti-H7, were considered to be E.coli O157:H7.

Table (3) showed that 5 (71.4%) isolates agglutinated bovine erythrocytes while, 7 (100%) agglutinated human, Guinea pig and chicken RBCs each. The percentage of E.coli O157:H7 isolates showed MRHA with human, bovine, Guinea pig and chicken RBCs were 7(100%), 3(42.9%), 7(100%) and 7 (100%) respectively. While E.coli O157:H7 isolates showed MSHA with bovine RBCs were 2 (28.6%). In this concern, Lida *et al.* (2001) isolated E.coli O157 strain which show positive mannose sensitive hemagglutination with the Guinea pig erythrocytes. On the other hand, Moy (2004) isolated 144 E.coli strains, 31% were positive for mannose-resistant hemagglutination. Moreover, Do *et al.* (2006) examined 50 enterotoxigenic E.coli strains for in vitro hemagglutination test and they found unique mannose resistant hemagglutination activity with guinea pig, sheep, human and chicken RBCs at 37°C but not at 18°C.

From the results presented in Table (4) it is clear that 5 isolates (71.43%) were enterotoxigenic and caused accumulation of fluids in the intestinal tract of the injected infant mice. Out of 3 E.coli O157:H7 isolates recovered form apparently healthy buffalo-calves feacal samples, 2 (66.67%) were enterotoxigenic. On the other hand, 3 (75%) out of 4 E.coli O157:H7 isolates recovered from diarrheic buffalo-calves feacal samples were enterotoxigenic isolates. A ratio of the intestinal tract to the body weight at 0.083 or higher is considered positive heat stable toxin (sta^+). Sjoling *et al.* (2006) concluded that ETEC colonize the intestine by means of different host specific colonization factor (CFS) and

produce one or both of two enterotoxins, the heat stable (ST) and heat labile (LT) toxins which are both able to cause diarrhea.

Results in Table (5) showed that out of the 7 E.coli O157:H7 isolates recovered from buffalo-calves feacal samples, 5 (71.43%) isolates were hemolytic, 2 E.coli O157:H7 isolates were found to be α-hemolysin positive with a percentage of 0.29%, while 3 (0.43%) isolates were found to be β-hemolysin positive. The remaining 2 (28.57%) isolates were found to be negative hemolysis.

The chromosomal hemolysin determinats were coloned by Berger *et al.* (1982). Sanchez-Magraner *et al.* (2007) stated that α-hemolysin from E.coli is a protein toxin that targets erythrocytic cell membrane, causing loss of the permeability barrier.

Table (6) showed that antibiogram of 15 antibacterial agents on 7 E.coli O157:H7 isolates from buffalo-calves. There were variable results. All tested isolates were resistant to ampicillin and vancomycin 100% each.

On the other hand, all tested isolates were sensitive to Amoxicillin clavulanic A. (2: 1), colistin sulphate, flumequine and imipenem 100% each. Also isolates were sensitive to ciprofloxiacin, norfloxacin and cholormphenical with different percentage.

While, E.coli O157:H7 isolates showed intermediate sensitivity mainly to cefoperazone (85.7%), maxfur (71.4%), cefotaxime, gentamicin and pefloxacin (57.1% each).

In this concern, Dontorou *et al.* (2003) who recorded that E.coli O157:H7 isolates were susceptible to ampicillin, chloramphenicol, kanamycin, nalidixic acid, norfloxacin, streptomycin, sulfamethoxazole trimethoprim and tetracycline. Moreover, Vidovic and Korber (2006) who performed antimicrobial susceptibility on 194 E.coli O157 isolates and found that 10 isolates were multidrug resistant, while 73 and 5 isolates were resistant to salfisoxazole and tetracycline, respectively, we believed that isolates showed resistance to many antibiotics and this may be attributed to wrong dose, duration of drugs and route of administration or plasmid resistance.

Results achieved in Table (7), photo (1) and photo (2) revealed positive amplification of 255 bp fragment of shiga toxin 2 (stx2) gene was observed from the extracted DNA of 4 E.coli O157:H7 isolates (80%). While, no amplification could be observed with the extracted DNA of 1 E.coli O157:H7 isolate.

Moreover positive amplification of 384 bp fragment of intimin (eae A) gene from the extracted DNA of 3 E.coli O157:H7 isolates (60%). On the other hand, no amplification could be observed with the extracted DNA of 2 E.coli O157:H7 isolates. In this concern, Blanco *et al.* (1997b) indicated that eae-positive verotoxigenic E.coli (VTEC) are more commonly found among calves (22%) than among adult cows (4%), confirming that young animals are a more important reservoir of pathogenic VTEC strains. While, Vernozy-Rozand *et al.* (2000) recorded that of the three E.coli O157:H7 strains isolated from heifer fecal samples, only one strain was toxigenic, producing VT2 only. In addition, adherence of enterohemorrhagic E.coli (EHEC) to the intestinal epithelium is essential for initiation of infection. Intimin is the only factor demonstrated to play a role in intestinal colonization by EHEC O157:H7 (Torres & Kaper, 2003).

Moreover, Richards *et al.* (2006) determined the presence or absence of key pathogenic genes (stx1, stx2, and eaeA) by PCR and found that most of isolates did not contain pathogenic genes. Further more, Girard *et al.* (2007) showed colonization and attaching and effacing lesion formation on explants derived from the ileum, colon and rectum. Intimin and translocated intimin receptor (Tir) were detected at the sites of adherent bacteria; Tir was essential for colonization.

CONCLUSION

O157:H7 isolates recovered from diarrheic and apparently healthy buffaloe-calves supported the view of no association between E.coli O157:H7 infection and diarrheal illness. Perhaps animals under stress factors give chance to the opertinistic E.coli O157:H7 to produce diarrhea in animals.

It is important to mention that this investigation aimed to characterize E.coli O157:H7 isolated from Egyptian buffaloe-calves which may be a source of E.coli O157:H7 that contaminate carcasses during slaughter and processing, thereby transmit the organism to man and cause human disease.

Therefore, attempts should be made to increase the amounts of colostral immunoglobulins absorbed by calf. Also, we recommended continuous noticing of general health of animals to detect the diarrheic animals, early and rapid treatment with drug of choice in addition with fluid therapy. Also, good management should be follow and separation of diseased animals to avoid spread of the disease especially among the newly born calves and animals under stress factors.

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الملخص العربى

التعرف الجينى للميكروب القولونى ١٥٧ المعزول من عجول الجاموس

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هذه الدراسة على الميكروب القولونى ذو الأنتجين الجسمى ١٥٧ والانتجين السوطى (H7) المعزول من عجول الجاموس. تم تجميع ٢١٥ عينة براز من مزارع الجاموس، وكانت النسبة الكلية لعزل الميكروب القولونى ٤٧,٩١%. بينما كانت نسبة عزله من عجول الجاموس السليمة ظاهرياً ٢٣% وكانت ٦٩,٥٧% فى عجول الجاموس المصابة بالإسهال. كما أنه كانت نسبة الميكروب القولونى المخمر للسوربيتول ٤٤,٦٥%، والغير مخمر السوربيتول ٣,٢٦%. كما جاءت نتائج التصنيف السيرولوجى كما يلى: ٩٣,٢% سلبية للأنتيجين الجسمى ١٥٧ والأنتجين السوطى (H7)، ٦,٧٩% إيجابية لنفس الانتيجينات. وباختبار خاصية التلزن الدموى للفترات المعزولة من الميكروب القولونى ٧هـ: ١٥٧. أعطت نتائج ١٠٠% (مقاومة سكر المانوز- حساس لسكر المانوز) مع كرات الدم الحمراء للإنسان والخنزير الغينى والدجاج. بينما كانت نسبة الإيجابية ٤٢,٩%، ٢٨,٦% مع دم الأبقار. وكانت نسبة النشاط الإفرازى للسموم داخل أمعاء الفئران ٧١,٤٣%. كما كانت نتيجة اختبار ذوبان الدم بنسبة ٧١,٤٣%. كما وجدت اختلافات فى نسبة استجابة المعزولات لاختبار الحساسية فكانت استجابتها ١٠٠% للأموكسيسلين والكولستين سلفات والفلومكين والإميينم. أثبتت نتائج اختبار البلمرة المتسلسل وجود الجين الخاص بسم شيجا-٢، جين الأنتيمين فى المعزولات وكانت نسبة وجودهما (٨٠%، ٦٠%) على التوالى.