MOLECULAR DETECTION OF SHIGA TOXIN (*stx1 and stx2*) AND INTIMIN (*eaeA*) GENES IN *ESCHERICHIA COLI* ISOLATED FROM FECAL SAMPLES OF CATTLE, SHEEP, AND HUMAN IN BASRAH GOVERNORATE

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ABSTRACT

The present study aims to isolate and identify *Escherichia coli* from fecal samples of farm animals and human, also, it aims to molecular detection of shigatoxin and intimin genes in isolates. A total of (264) fecal samples and swabs were collected from different parts of Basrah in the period extending from September 2018 to January 2019. These samples were composed of (85) samples from cows, (94) samples from humanand (85) samples from sheep. Different techniques were used in this study to detect the presence of *E. coli*; these techniques included conventional microbiological assays and molecular techniques (amplification of *uidA* gene by using polymerase chain reaction).

The results of these techniques indicated 50 (18.9%) were *E. coli* from the tested samples. These isolates were subjected to PCR to detect Shiga toxins and intimin genes (stx*1*, *stx2*, and *eaeA*). The results of PCR confirmed all (50) isolates were harbor at least one virulence gene. Out of 50 isolates 20 (40%) carried *stx2* gene alone, the percentages of the carrier were (66.7 %, 41.7% and 23.5%) from human, sheep and cattle samples, respectively. The genes (stx*1 and stx2)* were detected together in 9/50 (18%), represent (52.9%) of cattle isolates. The intimin gene (*eaeA*) alone was detected in 2/50 (4%), represent (11.8%) of cattle isolates. (28%) of isolates harbor (*stx2 and eaeA)* genes, the isolates belong to human and sheep isolates (33.3%) and (45.8%), respectively. Presence of the genes (stx*1, stx2,* and *eaeA)* were discovered in (10%) of isolates, (11.8%) of cattle and (12.5%) of sheep.

The isolates were resistant to ampicillin, tetracycline (92%, 74%), respectively. However, the isolates were susceptible to imipenem, gentamycin, chloramphenicol, ciprofloxacin, and cefotaxime with a ratio of 100%, 92%, 78%, 68%, and 58%, respectively.

INTRODUCTION

Escherichia coli is a Gram-negative, facultative anaerobe, non-sporulating rod within the family Enterobacteriaceae. It can ferment different sugars, but lactose fermentation (with the production of acid and gas) is a characteristic of the species (1).

Shiga toxin-producing *E. coli* (STEC) appears to be widespread in the gastrointestinal tracts of wild and domestic animals and, not surprisingly, meat and other animal products are the significant sources of human infections (2). STEC has emerged as a group of foodborne pathogens that can cause severe human disease, such as hemolytic uremic syndrome (HUS) (3). The STEC strains are characterized by their ability to produce either one or both of these cytotoxins, referred to as Stx1 [first described as Shiga-like toxin I], Stx2 or [first described as Shiga-like toxin II] (4**).**

The STEC strains were isolated from a variety of animals. However, cattle are considered the main reservoir (5). Other studies have indicated that small domestic ruminants, including sheep and goats, are also key reservoirs of STEC (6 and 7). Moreover (8), stated that, the STEC remains a significant cause of foodborne- related disease in humans.

This study aimed to isolate and identify *Escherichia coli* form fecal samples and swabs from Humans and domestic animals (cattle and sheep) also, molecular detection of *E. coli* isolates that carrying gene (stx*1*, *stx2,* and *eaeA*).

MATERIALS AND METHODS

-Samples collection

The fecal samples and swabs were collected from different parts of Basrah province**.** A total of 264 fecal samples and swabs were collected from Cattle, Sheep, and Human in the period extending from September 2018 to January 2019. Table (1)

Table (1): Source, type, and numbers of samples

Source of samples	Types of sample		No. of samples
	Fecal sample	Swabs	
Cattle	80	5	85
Human	10	84	94
Sheep	75	10	85
Total	165	99	264

Microbiological techniques

Isolation and identification of bacteria

The samples were treated according to (9). Briefly, all the collected samples were transported immediately to the laboratory of Veterinary college by using an icebox. In the laboratory, the samples were inoculated in nutrient broth and incubate at 37°C for overnight. The preincubated samples were subcultured on MacConkey's agar (Micromedia / Iran) and again incubated at 37° C for overnight. Next day 2–3 rose pink colonies randomly picked and were subcultured on to EMB agar (Himedia / India) followed by overnight incubation at 37° C. The colonies which observed as a metallic sheen, single colony were subject to Gram's stain (10), oxidase test; indole test and methyl red (M.R) and voges – proskauer (V.P) Tests (11), and Citrate utilization test using Simmons citrate agar (12).

Molecular techniques

The suspected isolates of *E. coli* by using conventional microbiological techniques were submitted to conformation by amplification of *uidA* gene by using PCR technique. Bacterial DNA was extracted according to the manufacturer of bacterial extraction kit (Genaid, Korea). The primers of *uidA* gene (housekeeping gene of *E. coli*) was designed by using online software (GenScript Online PCR Primers Designs Tool), the sequence of *E. coli* from NCBI, the accession No. (CU928164.2.)

Table (2): Sequence of primer for designed *uidA* with their manufacture

Primers	Primer sequences (5 → 3)	Length	Product size	Source	Manufacturer
uid A	F: 5’- CGTTGAACTGCGTGATGCGG -3′	20	203 bp	This study	Bioneer / Korea
	R: 5’- ACTGTTCGCCCTTCACTGCC- 3’	20			

The reaction mixture of PCR was prepared in a total volume of 50µl for *uidA* gene. 25µl of Green master mix (Promega / USA), 2µl of each oligonucleotide primer, 10µl of DNA template and 11µl of Nuclease-free water.

Table (3): Amplification conditions of the *ds.uid A* gene.

Stage	Step	Temperature	Time	No. of cycle
I	Initial denaturation	94^{o}C	3 min.	1
II	Denaturation	94^{o}C	1 min.	30
	Annealing	56^{o}C	40 sec.	
	Extension	72^{o}C	1 min.	
III	Final extension	72^{o}C	3 min.	1

-Antimicrobial Susceptibility Test

The confirmed isolates of *E. coli* were subjected to antimicrobial susceptibility test. This test was done according to the method of (13). The antibiotic disks were from (Bioanalyse/ Turkey), including Ampicillin (10µg), Cefotaxime (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg) Gentamycin (10 µg), Imipenem (10 µg) and Tetracycline (10 µg).

-Molecular detection of Shiga toxins (stx*1* and *stx2*) and intimin (*eae A*) genes

Oligonucleotide primers for PCR amplification:

Primers used for detection of virulence genes (*stx1,stx2,* and *eae A)* in *E. coli* isolated from fecal samples of farm animals and human were adopted from (14), Table (4).

Table (4): Sequences of primers for stx, *stx2* and *eae A* genes

Primers	Primer sequences (5 → 3)	Length	Product size	Source	Manufacturer
stx1	F: 5′- AAATCGCCATTCGTTGACTACTTCT-3″	25	366 bp	(14) Momtaz *et al.*, 2012	Bioneer / Korea
	R : 5′-TGCCATTCTGGCAACTCGCGATGCA-3″	25			
Stx2	F: 5′-CGATCGTCACTCACTGGTTTCATCA -3″	25	282bp		
	R: 5′-GGATATTCTCCCCACTCTGACACC -3″	24			
eae A	F: 5′-TGCGGCACAACAGGCGGCGA-3″	20	629 bp		
	R:5′-CGGTCGCCGCACCAGGATTC -3″	20			

The Amplification conditions

Amplification conditions of the genes (*stx1 and stx2*) were optimized and illustrated in the table (5). However, the optimized amplification conditions of *eae A* gene listed in the table (6).

Table (5): Optimized amplification conditions for *stx1*and *stx2* genes

Stage	Step	Temperature	Time	No. of cycle
I	Initial denaturation	94^oC	3 min.	1
II	Denaturation	94^oC	30 sec.	34
	Annealing	56^oC	45 sec.	
	Extension	72^oC	1 min.	
III	Final extension	72^oC	5 min.	1

Table (6): Optimized amplification conditions for *eae A* gene

Stage	Step	Temperature	Time	No. of cycle
I	Initial denaturation	94°C	3 min.	1
II	Denaturation	94°C	1 min	34
	Annealing	57°C	45 sec.	
	Extension	72°C	1 min.	
III	Final extension	72°C	3 min.	1

RESULTS

The total number of collected samples was 264; the samples included fecal samples and swabs from farm animals (cows, sheep) and human. The isolation rate of *E. coli* identified by using conventional microbiological and molecular techniques, as shows in Table (7). The suspected isolates were confirmed as *E. coli* via detection of. *uid A* gene by using the PCR technique, the size of product 203 bps, Figure (1). Of 264 samples 53 (20%) were identified by using conventional biochemical tests, however, of 53 suspected isolates 50 (94%) were confirmed as *E. coli*.

Table (7): Number of *E. coli* isolates which Identified by using conventional microbiological and molecular techniques.

Source of samples	Total No.	Suspected Isolates by using conventional microbiological techniques		Confirmed isolates by using Molecular detection *ds. uidA*	
		No.	%	No.	%
Cattle	85	17	20	17	20
Human	94	10	10.6	9	9.6
Sheep	85	26	30.6	24	28.2
Total	264	53	20	50	18.9

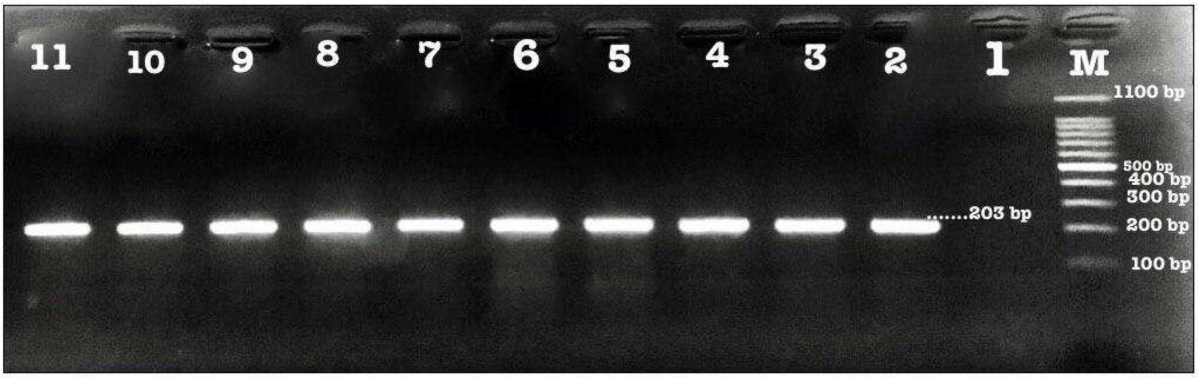

Figure (1): Electropherogram of *uidA* amplification products.

The mixture was run on1.5% agarose gel, stained with ethidium bromide. M: marker, and *ds. uidA* product size (203 bp).

Note: The sample in well No. 1 was negative control, and (2-11) were positive samples.

Antimicrobial susceptibility testing

The *E. coli* isolates were subjected to seven antimicrobials as in a table (8).

Table (8): Antibiogram of 7 antimicrobials were tested against (50 isolates) of *Escherichia coli.*

Antimicrobial susceptibility	Cattle			Humans			Sheep			Total					
										NO	%	NO	%	NO	%
	R	I	S	R	I	S	R	I	S	R		I		S	
Ampicillin (AM)	15	0	2	9	0	0	22	2	0	46	92	2	4	2	4
Cefotaxime (CTX)	6	1	10	6	1	2	6	1	18	18	36	3	6	29	58
Chloramphenicol (C)	5	0	12	2	0	7	4	0	20	11	22	0	0	39	78
Ciprofloxacin (CIP)	6	2	9	0	0	9	5	3	16	11	22	5	10	34	68
Gentamycin (Gn)	3	0	14	0	0	9	1	0	23	4	8	0	0	46	92
Imipenem (IMP)	0	0	16	0	0	9	0	0	25	0	0	0	0	50	100
Tetracycline (TE)	13	1	3	7	1	1	17	3	4	37	74	5	10	8	16

The isolates were resistant to ampicillin, tetracycline, cefotaxime, chloramphenicol, and ciprofloxacin in percentage (92%, 74%, 36%, 22%, and 22%), respectively. Whereas the isolates were susceptible to imipenem, gentamycin, chloramphenicol, ciprofloxacin, and cefotaxime with a ratio of 100%, 92%, 78%, 68%, and 58%, respectively, Table (8).

Molecular detection of *stx1, stx2* and *eaeA* genes

All isolates of *E. coli* were submitted to molecular detection of genes (*stx1*, *stx2,* and *eaeA*) by using primers. Table (10), figures (2) and (3).

Table (10): Distribution of *stx1, stx2* and *eaeA* genes in *E. coli* isolates from different sources

Samples source	only *stx1*		only *stx2*		*stx1* & *stx2*		only *eae A*		*eaeA* & *stx1*		*eaeA* & *stx2*		*eaeA, stx1*& *stx2*	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Cattle	0	0	4	23.5	9	52.9	2	11.8	0	0	0	0	2	11.8
Human	0	0	6	66.7	0	0	0	0	0	0	3	33.3	0	0
Sheep	0	0	10	41.7	0	0	0	0	0	0	11	45.8	3	12.5
Total	0	0	20	40	9	18	2	4	0	0	14	28	5	10

The total isolates which confirmed as *E. coli* were subjected to PCR detection of genes *stx1, stx2 and eaeA.* Out of 50 isolates 20 (40%) carried *stx2* gene alone, the percentages of the carrier were (66.7 %, 41.7 and 23.5) from human, sheep and cattle samples, respectively. The genes (stx*1 and stx2)* were detected together in 9/50 (18%) only in cattle samples 9/17 (52.9%). The intimin gene (*eaeA*) alone was detected in 2/50 (4%), the gene found in cattle isolates 2/17(11.8%). Of 50 isolates 14 (28%) were found as a carrier of (*stx2 and eaeA)* genes, the isolates belong to human and sheep isolates, 3/9(33.3%) and 11/24 (45.8%), respectively. Presence of the three genes (stx*1, stx2,* and *eaeA)* were discovered in 5/50 (10%) isolates, composed of 2/17 (11.8%) and 3/24 (12.5%) in cattle and sheep isolates respectively.

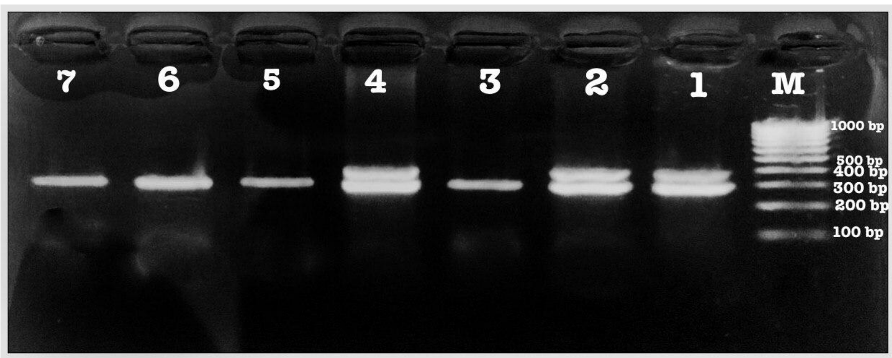

Figure (2): Electropherograms of Shiga toxin (stx*1 and stx2)* genes amplification

The mixture was run on 1.5% agarose gel stained with ethidium bromide. Lanes: M, Marker. 1,2 and 4 have both [stx1(366 bp) and stx2 (282 bp) genes] while 3,5,6and 7 has the only stx2 gene.

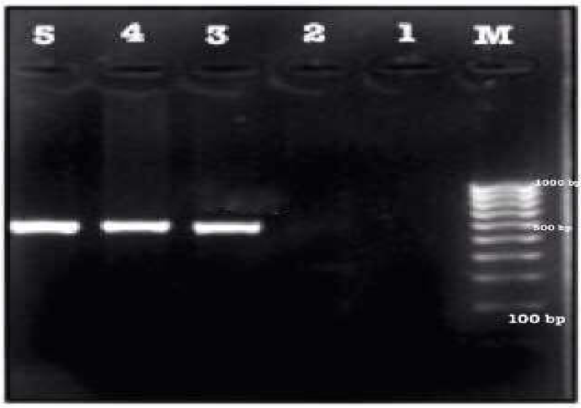

Figure (3): Electropherograms of Amplification of *eaeA.*

The mixture was run on 1.5% agarose gel stained with ethidium bromide. Lanes: M, Marker, 3-5 have *eaeA* (629 bp) genes, while 1,2 were negative control**.**

DISCUSSION

Escherichia coli is a part of healthy enteric microbiota in humans and different animals. The characteristics of phenotypic and genotypic allow the identification of *E. coli* infective strains or pathovars (15). A source of human infection for some diarrheagenic *E. coli* group (DEC) strains are animal feces, particularly those capable of producing Shiga-like toxin which have been termed as Shiga toxin-producing *E. coli* (STEC) and especially EHEC which constitute the frequent cause of severe hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (16).

Isolation rates of *E. coli*

The conventional microbiological methods used for *E. coli* detection relies on the enrichment of the sample on nutrient broth, then differentiating on MacConkey agar followed by culturing on selective media Eosin methylene blue and submitting to biochemical confirmation and molecular method according to (9).

The net isolation rate *E. coli* from total number of samples was (18.9%). The isolation rate of *E. coli* of human samples was 9.6 %. This result was lower than that, reported by (`17), who found *E. coli* in (21.4%) of stool culture. The isolation rate of *E. coli* from cattle in this study was

(20%), this rate was higher than (10.9%) which reported by (18) in Basrah province. On the other hand, the isolation rate of *E. coli* from sheep was (28.2%).

Antimicrobial susceptibility

Antimicrobial agents have primarily been used to cure infectious diseases caused by bacteria. The use of antibiotics is a significant risk factor for extension of resistance to these agents (19). Multidrug resistance in *Escherichia coli* has become a worrying issue that is increasingly observed in human but also in veterinary medicine worldwide (20).

By using the disc diffusion method, 50 isolates of *Escherichia coli* were submitted to antimicrobial susceptibility test toward 7 antimicrobial agents. The results of the current study clarified that *E. coli* isolate resistant to Ampicillin (92%), and 100% susceptible to imipenem. Table (8), these results are in agreement with (21) who found that the resistance to ampicillin was 100% and all of *E. coli* isolates were susceptible to imipenem. The reported susceptibility to gentamycin in this study was 92 %; this result is higher than 66.9% recording by (22). From the obtained result, the rate of resistance to tetracycline was 74%; this result is lower than 84.76% reported by (23) and the rate of resistance for chloramphenicol in this study was 22 %, this result is lower than 33.3%, found by (24). Furthermore, in this study, the resistance against cefotaxime and ciprofloxacin were found (36%, 22%) respectively. These results are similar to (22), who found that the resistance against Cefotaxime and ciprofloxacin were (36.6% and 18.3%), respectively.

The slight differences in resistance may result from the source of isolates; also, the reason behind continuous increasing in resistance to these antibiotics may attribute to over usage of these antibiotics. Consuming antibiotics without prescription has been assumed as one of the causes of reducing bacterial sensitivity to the antibiotics (25), moreover, in Iraq, antibiotics can be easily obtained without a prescription. All of the previous reasons might be responsible for such a high prevalence of resistance.

Molecular detection of *stx1*, *stx2*, and *eaeA* genes

Shiga toxin-producing *Escherichia coli* (STEC) are globally known pathogens that cause diarrhea, hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans. STEC are predominately shed within the feces of healthy meat-producing animal species and aren't considered to be pathogens of ruminant species except once infections occur in young (pre-weaned) animals (26).

For 50 *E. coli*, isolates were submitted to PCR targeting the characteristics of virulence genes (stx*1, stx2,* and *eaeA)*. All isolates were potentially pathogen hence harbor at least one specific virulence trait. This study revealed that the detection rate of *stx2* gene in cattle was 23.5%. This result is lower than 35% who found by (27), while the percentage of *stx2* gene in human was 66.7 %, this result was higher than 24% reported by (28). Moreover, the *stx2* ratio in sheep was 41.7%; this result was much higher than 14% found by (29), Table (10). Furthermore*, stx1*, no isolates have harbored this gene alone, this result is in agreement with (30), who has not detected the gene (*stx1*) in a study in the USA.

For both *stx1* and *stx2* genes were detected together (52.9%) only in cattle. This result was similar to (27), who noted that *stx1* plus *stx2* genes were harbored by 45.8%. Some studies have suggested that strains which possessing only *stx2* are potentially more virulent than strains harboring *stx1* or even strains carrying both *stx1* and *stx2* (31). Moreover, in mouse models، *Stx2* is 100 times more potent than *Stx1* (32).

Regarding *eaeA* gene, it was detected in 4 % of isolates. This result is in agreement with (33), who reported that *eae A* gene was 4.1% isolates. Moreover, the distribution of the *stx2* and *eaeA* genes of human in this study was 33.3 %; this result was higher than 14.3% reported by (34). While in sheep, the distribution of the *stx2* and *eaeA* genes was 45.8 %, this result disagreed with (35) who found 8.7% of fecal samples were positive for both genes.

In the present study, the presence of the three genes (*stx1*, *stx2*, and *eaeA*) in both cattle and sheep was similar in percentage 11.8%,12.5 %, respectively, this result was comparable to (36), who found the presence of three genes in cattle and sheep was (6.52% and 11.42), respectively, Table (10).

Conclusions

All *E. coli* isolates were potentially pathogen; hence, harboring specific virulence genes. The plurality of isolates were resistant to ampicillin followed by tetracycline, whereas, all the isolates were susceptible to imipenem followed by gentamycin.

الكشف الجزيئي لجينات ذيفان الشيغا (*stx1* و *stx2*) وجينات الانتمين (*eaeA*) في الإشريكية القولونية المعزولة من عينات البراز المأخوذة من الأبقار والأغنام والبشر في محافظة البصرة

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الخلاصة

تهدف الدراسة الحالية الى عزل وتحديد الايشريكا القولونية المعزولة من براز الحيوانات الحقلية (الابقار والاغنام) والانسان . وايضا تهدف الى الكشف الجزيئي لجينات الشيغا والانتمين في العزلات .اذ تم جمع (٢٦٤) عينة براز ومسحات من اماكن مختلفة من مدينة البصرة للفترة من ايلول ٢٠١٨ لغاية كانون الثاني ٢٠١٩ .العينات التي تم جمعها تكونت من (٨٥) عينة من الابقار ، (٩٤) عينة من الانسان و (٨٥) عينة من الاغنام . تم استخدام تقنيات مختلفة للكشف عن وجود الايشريكا القولونية وهذه التقنيات شملت الاختبارات البكتيريولوجية التقليدية والتقنيات الجزيئية والتي تتضمن (تضخيم جين ***uidA*** باستخدام تقنية تفاعل البلمرة المتسلسل) .نتائج هذه التقنيات تشير الى تحديد ٥٠ (١٨.٩%) عينة تم اعتبارها ***E.coli***. خضعت هذه العزلات الى تفاعل البلمرة المتسلسل للكشف عن جينات ذيفان الشيغا) والانتيمين (*eaeA*، and ***stx1,stx2***) . نتائج البلمرة اكدت ان كل العزلات (٥٠) تمتلك على الاقل جين ضراوة واحد . من بين ٥٠ عزلة وجد ٢٠(٤٠%) من العزلات تحمل جين **stx2** فقط ،وكانت نسبة العزل (٦٦.٧%،٤١.٧% و% ٢٣.٥) من عينات الانسان ، الاغنام و الابقار ،على التوالي .كما تم كشف جينات ***stx1*and *stx2***) معا بنسبة٥٠/٩ (١٨%)، تمثل (٥٢.٩%) من عزلات الابقار. تم كشف جين **eaeA)**) لوحده بنسبة ٥٠/٢ (٤%) ، ويمثل ١١.٨ % من عزلات الابقار. (٢٨%) من العزلات تمتلك جينات (***eaeA*** ، **and *stx2*,**) ، هذه العزلات تنتمي الى عزلات الانسان والاغنام (٣٣.٣ %) و(٤٥.٨%) على التوالي .تم كشف وجود الجينات (***eaeA*، and *stx1,stx2***) في ١٠% من العزلات ،(١١.٨%) من الابقار و(١٢.٥%) من الاغنام .

العزلات التي تم تاكيدها بتفاعل البلمرة تم اختبارها باستخدام الحساسية الدوائية ضد ٧ من المضادات الحيوية وبينت النتائج ان اعلى مقاومة كانت ضد الامبيسيلن والتتراساكلين(٩٢% ،٧٤%) على التوالي ، في حين كانت العزلات حساسة الى الايميبينيمن ،الجنتامايسين ، الكلورامفينيكول، سيبروفلوكساسين و سيفوتاكسيم بنسبة (١٠٠%،٩٢%،٧٨%،٦٨ و٥٨%) على التوالي .

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