

Prevalence of non-O157 Shiga Toxin-producing *E. Coli* in Children and Calves in Al- Muthanna Province, Iraq

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Abstract

The present study focus on non-O157 Shiga toxin-producing *E. Coli* (STEC), included a bacteriological study was subjected to provide additional information for non-O157 STEC prevalence in children and calves. Isolation by using selective culturing media (CHROMagar STEC and CHROMagar O157) from 127 children suffering from diarrhea and 133 calves in Al- Muthanna province. Characterization depends on culturing positive colony on MacConkey agar and Levin's Eosin Methylene blue agar, staining single colony from the growth by gram stain, biochemical tests; Indole, the Methyl Red, Voges-Proskauer, Citrate test, Oxidase, Catalase, Urease, Motility, Kligler Iron and Api-20E, were done to confirm a diagnosis of non-O157 STEC, The reliable isolation as non-O157 STEC serotyping by specific latex agglutination test for the target non-O157 STEC (big six) serogroup (O26, O45, O103, O111, O121 and O145). The current study showed the prevalence of non-O157 STEC was 20 of out 127 (15.73%) in samples collected from children and 27 / 133 (20.30%) in calves samples in conclusion the Non-O157 STEC is an important cause of diarrhea in children, and calves; finally, the calves play an important reservoir for Non-O157 STEC.

Keywords: Non-O157 STEC. Prevalence. Children. calves. and CHROMagar STEC.

انتشار بكتريا الاشيريشيا القولونية العتر المنتجة لذيفان الشيكا من غير النمط المصلي ١٥٧ في الاطفال والعجول في محافظة المثنى

الخلاصة

ركزت الدراسة الحالية على بكتريا الاشيريشيا القولونية العتر المنتجة لذيفان الشيكا من غير النمط المصلي 157 ب non-O157 STEC ، شملت الدراسة المجهرية للبكتريا توفير معلومات إضافية عن انتشار non-O157 STEC في الأطفال والعجول. تم العزل باستخدام أوساط التنمية الانتقائية (CHROMagar STEC و CHROMagar O157) شملت الدراسة 127 عينة من اطفال يعانون من الإسهال و 133 عينة من العجول في محافظة المثنى. اعتمد التشخيص البكتيري على استنبات البكتريا على الاوساط الزرع الخاصة مثل MacConkey و أجار MBE ، والتصبغ بواسطة صبغة الجرام ، وكذلك تم استخدام الاختبارات البيوكيميائية؛ حيث تم إجراء فحوصات Indole ، و Methyl Red ، و Voges-Proskauer ، واختبار السيترات ، و Oxidase ، و Catalase ، و Urease ، و Motility ، و Kligler Iron و Api-20E ، لتأكيد تشخيص non-O157 STEC ، تم تنميط العتر مصليا باستخدام اختبار اللاتكس المصلي حيث تم استهداف التنميط لل (O26 الستة الكبار) (O26 و O45 و O103 و O111 و O121 و O145). أظهرت الدراسة الحالية أن معدل انتشار non-O157 STEC كان 20 من أصل 127 (15.73%) (في العينات التي تم جمعها من الأطفال و 20.30%) 27/133 (20.30%) في عينات العجول في الختام ، يعتبر Non-O157 STEC من الأسباب المهمة للإسهال عند الأطفال والعجول. واخيرا فان العجول تعتبر خزانًا ومصدرا مهما للإصابة ب. Non-O157 STEC

Introduction

The German pediatrician and scientist, Dr.Theodor Escherich isolated organism for the first time in (1885) from the intestinal flora of infants and he called them Bacterium coli commune (1,2, 3). Later this organism was named as *Escherichia coli* or *E. coli* on his honor (4).

Escherichia coli (*E.coli*) is a member of the Enterobacteriaceae family and belongs to the genus *Escherichia*, It is a Gram-negative, facultative anaerobic, non-sporulating bacterium found in the environment, the intestine, and extra intestinal locations of mammals (5).

Bray (1945) was the first to report the link between *E. coli* strains and diarrhea in what was then known as "summer diarrhea." The pathogenic groups includes enteropathogenic *E. coli*, Enterotoxigenic *E.coli*, diffuse adhering *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E.coli*, Sepsis/meningitis *E. coli*, uropathogenic *E. coli*, and enterohemorrhagic *E.coli* or Shiga toxin-producing *E. coli*.(STEC)(4, 6), STEC are also called a vero toxic *E. coli* (VTEC) (7). STEC pathotype has emerged in the last 30 years as one of the most important causes of intestinal *E. coli* infection (8, 9). STEC infection has been reported in a variety of domestic and wild animal species, although it causes disease only in young calves, pigs, and dogs. Cattle have been identified as a major source of human infections (10, 11, 12). STEC infection in humans frequently results in serious illnesses (13, 14, 12).

Center of disease control and prevention (CDC) food net demonstrated that approximately 70% of the infections with STEC caused by non-O157 STEC infections, belong to one of these serotypes (O26, O45, O103, O111, O121 and O145) and they called it as (big six) (15, 16, 17). The Non-O157 STEC is a zoonotic pathogen that can infect both human and animals (12). Non-O157 STEC's

importance in human diseases is less well understood than STEC O157:H7(18), and its diagnosis can be a challenge because they do not have unique or distinguishable features that make them easily differentiated from other strains of *E. coli*, so it is unlike STEC O157:H7 (19). However, as the importance of non-O157 STEC grows, researchers have begun to investigate the epidemiology of these species (10, 20).

Non-O157 serotypes are more difficult to isolate and identify in the clinic than STEC O157 serotypes. Because STEC O157 is unable to ferment D-sorbitol, it is quite easy to identify (21). Because many laboratories lack the resources to isolate, identify, and describe non-O157 serotypes, they are rarely reported (22). For Shiga toxin or significant STEC strains, current detection and isolation methods include culture-based, immunoassays, and PCR-based methods.

Materials and Methods.

Isolation and identification of non-O157 STEC *E.coli*

Collection of samples

A- Collection of human stool samples

One hundred and seventy-five stool samples were obtained from children with diarrhea or bloody diarrhea, aged 1 to 10 years, of both sexes, who were admitted to the Pediatric Hospital in Al-Samawa, Iraq. Samples were collected by sterile swabs with Amies transport medium and labeled by a special number, then placed in a cool box and send to the lab.

B- Collection of animal samples

One hundred and three fecal samples were collected from calves (less than 6 months old) with and without diarrhea by recto anal mucosal swab according to (23) by Amies medium and

labeled by a special number, transported in a cool box to the lab.

Enrichment step

Enrichment step is important to increase the non-O157 STEC to a detectable levels according to (14). So that, the fecal swab put in tubes containing 10 ml of enrichment tryptone soy broth (Oxoid, England), placed in an incubator at 37°C for 6 hrs.

Culture of samples

A- CHROMagar STEC

20 µl of the enrichment tubes was streaked onto CHROMagar STEC (Paris, France) followed by 24h incubation at 37°C, then an observation of mauve color colonies indicated growth of STEC.

B- CHROM agar O157

This medium was used to differentiate between *E. coli* O157 and non-O157 STEC. By the culturing on CHROM agar O157 agar (Paris, France) and incubated at 37°C for 24 hours, mauve color colonies are indicative for *E. coli* O157, while the blue colonies are indicative for non-O157 STEC.

Gram Stain (Crescent, K.S.A.)

According to (24) a loopful of tap water was placed on a sterile slide and by the use of sterile loop a small part of colony was transferred to the drop and emulsified and dried by air, to make a dried film the later was fixed by passing it for short time to benzene flame two or three times without exposing directly to the flame, then it stained with gram stain and bacterial examined under the oil immersion lens.

Biochemical tests

Levine's Eosin Methylene Blue agar and MacConkey agar

Bacterial isolates were cultured on EMB agar (Oxoid, England) and MacConkey agar (LAB, U.K.) incubated at 37°C for 24 hrs. The presence of metallic green sheen color colonies on EMB and pink color colonies on MacConkey, is an indication for *E. coli*.

IMVIC test

It was done according to (33).

1- Indol test

Tubes containing peptone water were prepared and were inoculated with the colony of suspected bacteria and incubated at 37°C for 24 hours, and then several drops of Kovac's reagent (CDH, India) prepared according to the manufacturer's instructions, were added to the broth medium without mixing, The appearance of red ring on the surface liquid of the medium was regarded as a positive result.

2- Methyl red test

It was done by inoculation of bacterial isolates suspension into MR-VP media (Panreas, Spain) then incubated at 37°C for 24 hrs, few drops of MR reagent were added to the media, changing of the color to red referred to a positive result, while remaining the yellow color referred to a negative result.

3- Vogas-proskauer test

A loopful of bacterial cultures was inoculated into MR-VP broth (Himedia, India) and incubated for 24 hours at 37°C, and then 3:1 (v/v) of solution (A)(5% Alpha naphthol) and solution (B)(40%KOH) were added, changing of the color to the red was recorded as an indication of positive test.

4- Simmon's Citrate test

This test was done by culturing the bacterial isolates on a slant of Simmon's Citrate media (Oxoid, England) and incubated at 37°C for 24 hrs. The blue color of media was represented as a positive reaction, while the green color of media was represented as a negative reaction.

Kligler Iron medium (KI)

This test was used to differentiate the Enterobacteriaceae according to carbohydrate fermentation and hydrogen sulfide production. The organism was grown on KI slant by stabbing and streaking and then incubated at 37°C for 18

hours. The color changing of the media from orange-red to yellow was due to carbohydrate fermentation with or without gas formation at butt of slant.

Urease

This test was done by culturing the bacterial isolates on slant of urea agar (Himedia, India) base and incubated at 37°C for 24 h. this test was used to detect the ability of an organism to split urea to ammonia by the action of urease(33).

Catalase test

It was created by placing a single colony of bacteria from nutrient agar on a clean slide, followed by a few drops of hydrogen peroxide (H₂O₂) (Thomas Baker, India) were added to it, The production of O₂ bubbles referred to a positive result but if no O₂ bubbles production referred to a negative result (33).

Oxidase Test

The isolated colony was put by a wooden stick on a clean piece of filter paper then two to three drops of oxidase reagent (Panreas, Spain) were added. Production of a purple color by colonies within 10- 30 sec is positive result (25).

Api – 20E system

The Api – 20E (Analytic profile index for Enterobacteriaceae test) (BIOMÉRIEX, France) it was used to confirm the results of biochemical tests. Strip of 20 microtubes containing dehydrated substrates. These tests were inoculated by bacterial suspension. During incubation, the metabolism was under observation. Color changes were either spontaneous or by addition of the reagents, this test was performed according to manufacturer's instructions.

Serotyping by latex agglutination test for non-O157 STEC

The test was used for serotyping non-O157 STEC through application of commercial kits (Abraxis, U.K.) (The Abraxis *E.coli* rapid latex

agglutination test include (*E. coli* O26 Latex Test Kit, *E. coli* O45 Latex Test Kit, *E. coli* O103 Latex Test Kit, *E. coli* O111 Latex Test Kit, *E. coli* O121 Latex Test Kit, and *E. coli* O145 Latex Test Kit) to detect the somatic antigen for non-O157 STEC (O26, O45, O103, O111, O121 and O145).

Test Principle

The polystyrene latex particles coated with rabbit IgG specific for *E. coli* somatic antigen include (O26, O45, O103, O111, O121 and O14 serotypes). When the latex particles are mixed on a test card with fresh colonies cultured on tryptic soy agar, the bacteria will bind to the antibody causing the latex particles to agglutinate (positive reaction). Bacteria that are not *E. coli* (O26, O45, O103, O111, O121 and O145) will not bind to the antibody and will not agglutinate the latex particles (negative reaction). This test was done according to the manufacturer company.

Results and discussion

Bacteriological study included: isolation; Identification, and serotyping of *E.coli* non-O157 STEC. The bacteriological tests were conducted on the 127 stools from child and 133 fecal samples from calves to isolate and confirm *E.coli* non-O157 STEC by the following methods:

I. Isolation (Culturing on selective media) and Culturing on CHROMagar STEC

The culturing of sample after enrichment in Tryptic soy broth for 6 hours on CHROMagar STEC showed different colors and the mauve color colonies was an indication for STEC. Figure (1).

CHROMagar O157

The culturing of isolated STEC colony from CHROMagar STEC on CHROMagar O157 showed different colored colonies; the STEC serotype O157 showed mauve color, while

other serotypes of STEC were non- O157 STEC showed blue colored colonies. As in figure (2).

Our results showed that CHROMagar STEC showed a great aid in isolation and differentiation also diagnosis of STEC, STEC uses chromogenic substrates that create mauve colored colonies, whereas non-STEC *E.coli* organisms may use chromogenic substrates that produce blue to blue green colored colonies, as Gouali *et al.* (2013) and Hirvonen *et al.* (2013) have found (2012), they reported that the highest detection sensitivities were observed when stool samples are culture on CHROMagar STEC, the STEC serogroup O26 (90.0%), O111 (100.0%), O121 (100.0%), O145 (100.0%), and O157 (84.9%) (26,27), while another study done by Wylie *et al.* (2012), they found that the CHROMagar STEC had a sensitivity and specificity of 85.7% and 95.8%, respectively in isolation of STEC strain (28).

Our results showed that CHROMagar O157 was a helpful method in isolation and differentiation of STEC O157, *E. coli* O157 utilizes one of chromogenic substrates which produce mauve colored colonies, while non-O157 STEC organism utilizing other chromogenic substrates producing blue to the use of blue green colored colonies this is in agreement with Phillips *et al.* (2005) and Tavakoli *et al.* (2008), who found that using Chromogenic media has greater benefits and can be a good alternative to using traditional and routine procedures to isolate STEC.(29, 30). A similar study by Yousif and Al-Taii (2014) and Al-Dawmy and Yousif, (2013), reported that the CHROMagar O157 was useful for diagnosis of STEC O157 (31, 32).

II. Identification of STEC identity

Cultural characteristics of non-O157 STEC

Bacteriological culture revealed After a 24-hour incubation period at 37°C, diverse morphological traits of non-O157 STEC were seen on various medium. The visible colonies on MacConkey agar had a red/pink tint, whereas the visible colonies on Levin's Eosin Methylene Blue (EMB) agar had a green metallic sheen. Figure (3)

Microscopic examination:

The bacteria appeared in light microscope as gram negative, bacilli and single cells bacteria after hours post incubation at 37 oC. Figure (4) (24).

Biochemical identification

A- IMVIC test

The IMVIC tests are used to differentiate the enteric bacteria (Family Enterobacteriaceae). These tests include; Indole test, Methyl Red and Voges- Proskauer tests and Citrate test. Table (1) and Figure (5)

B-Other biochemical tests

Table (2) and Figure (6): Results of some biochemical tests of isolated non-O157 STEC

C- Api-20E

Growth of isolated strains on MacConkey and EMB agar was characteristic for *E.coli* and as indicated by Quinn et al. (2004) (33).

The biochemical results supported that the present isolates were *E.coli*, these no isolates were also) which gives diagnosis (99.7%) in the appendix as *E.coli* isolate. The biochemical assays, such as the Oxidase test, were created to discover distinct metabolic features of bacteria with the lack of color change that is indicative for *E.coli* without Cytochrome oxidase enzyme, catalase test gave a positive result with *E.coli*; so it had catalase enzyme that act to breakdown hydrogen peroxide into oxygen and water. Urease was negative because the urease enzyme was missing, resulting in unhydrolyzed urea, and simmon citrate was negative because *E.coli* did not utilise citrate as its primary carbon source,

and it was non motile in motility media. In the indole test, *E.coli* created indole from tryptophan via tryptophanase, and the result displayed as a rose ring in the upper test tube in the methyl red test; in the Kligler iron test, *E.coli* formed a mixed acid fermenter and had the ability to ferment lactose and glucose (33).

III. Serotyping test

Non-O157 STEC colonies were tested after isolation for identification and serotyping was done by Abraxis *E.coli* rapid latex agglutination test to detect the somatic antigen (O) for non-O157 STEC (26, 45, 103, 111, 121 and 145). In the presence of the specific *E. coli* cell wall antigens, the latex particles would agglutinate, or clumped together. The reaction of these on the card test appeared as red color agglutination indicating a positive result for O antigen (O26, O45, O103, O111, O121 and O145) in comparison to the clear red color of the control. Figure (8). In the present study, latex agglutination test was used as a rapid detection method to reduce the time also to identify the pathogenic non-O157 STEC which possess cultural and biochemical characters. These results are in agreement with Huang *et al.* (2001) and Wang *et al.* (2013) (34, 35).

Medina *et al.* (2012), they showed that the latex agglutination method is a simple, highly efficient, and reliable test for detection of the pathogen with 100% sensitivity and specificity for the big six of non-O157 STEC (O26, O45, O103, O111, O121, and O145) (36).

Prevalence of non-157 STEC in children and calves sample

The current study showed the prevalence of non-O157 STEC was 20 of out 127 (15.73%) in samples collected from children and 27 / 133 (20.30%) in calves samples as illustrated in table (4). The result of this study showed a high prevalence of non-O157 STEC in calves and these findings are in agreement with many other studies. In Argentina 12/75 (16%) from Rectal Swabs (37); and in Brazil 44/344(12.7%) (38); in France isolation rate was 145/415 (34.9%) (39), in Japan 227/605 (37.5%) (40); in USA 396/1189 (33.3%) (41). Similarly, Paiba *et al.* (2003); reported a high prevalence of STEC in calves in England and wales (42).

In addition, Fairbrother and Nadeau (2006); Kasper *et al.* (2010) showed that the prevalence of these organisms varies from 10% to 20%, may be reach as high as 80% to 90% (43,44).

The prevalence of non-O157 STEC in human among patients with STEC infection recorded in various region around the world; in Canada 20% (45); Japan 19 % (46), and in other country prevalence showed highest prevalence in USA 44 % (47), , Germany it was 44% (48), in Italy 34% (49), Denmark 75% (50, 51), Australia 69 % (52), Finland 53 % (53).

Conclusion

the Non-O157 STEC is important causes of diarrhea in children, and calves; the later play an important reservoir for Non-O157 STEC.

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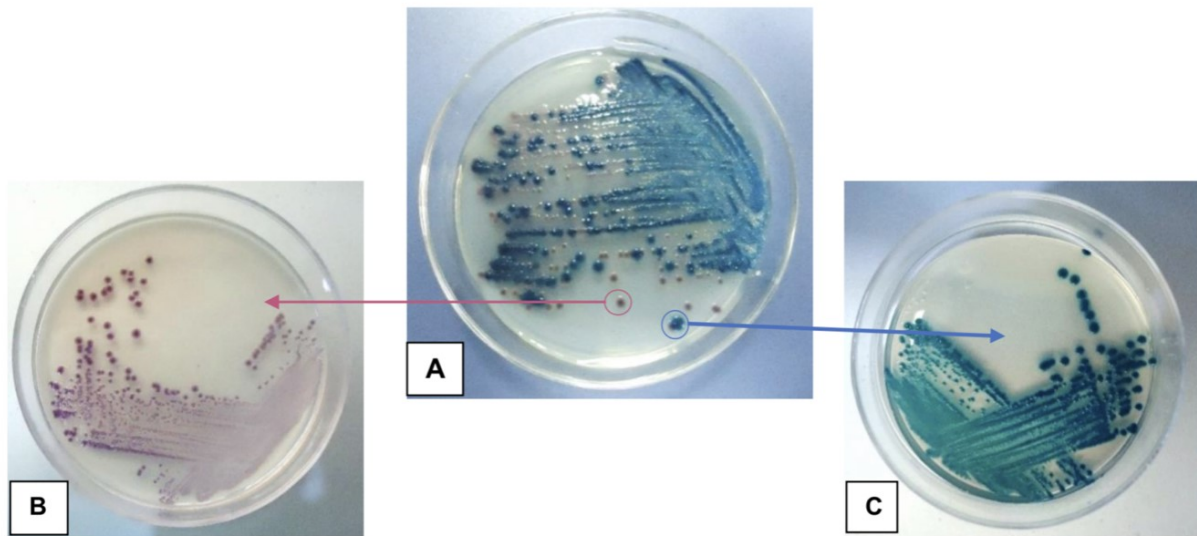


Figure (1): The cultured samples on CHROMagar STEC (A); mixed types bacteria (B); STEC bacteria and (C) other bacteria.

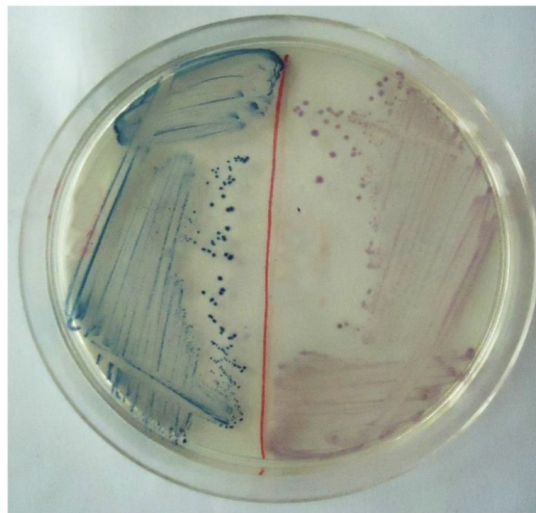


Figure (2): The isolated STEC colony on CHROMagar O157; blue color colonies (non-157 STEC) and mauve color colonies (STEC O157).

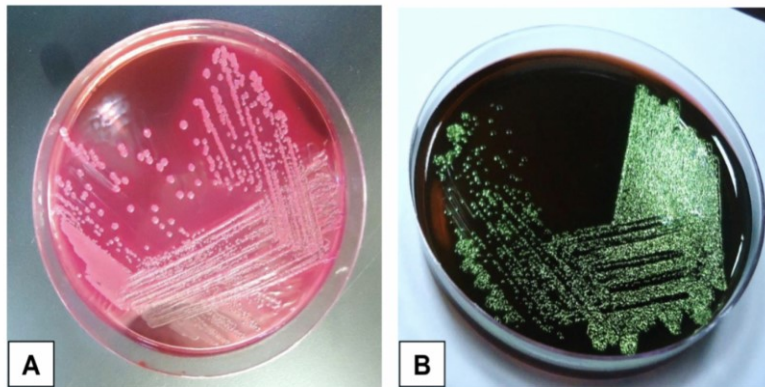


Figure (3): E.coli on MacConkey agar (A) and on EMB agar (B).

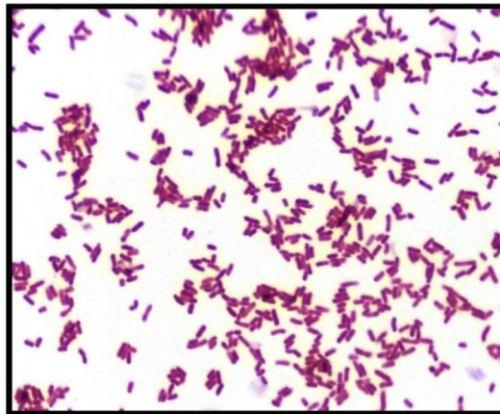


Figure (4): E.coli gram stain (x1000).

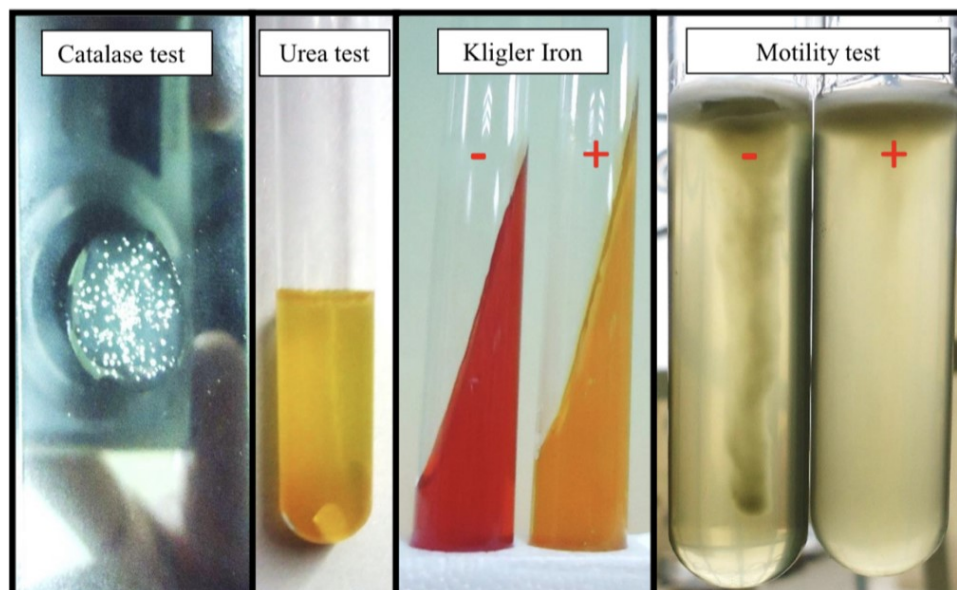


Figure (6): The results of biochemical tests of isolated non-O157 STEC.

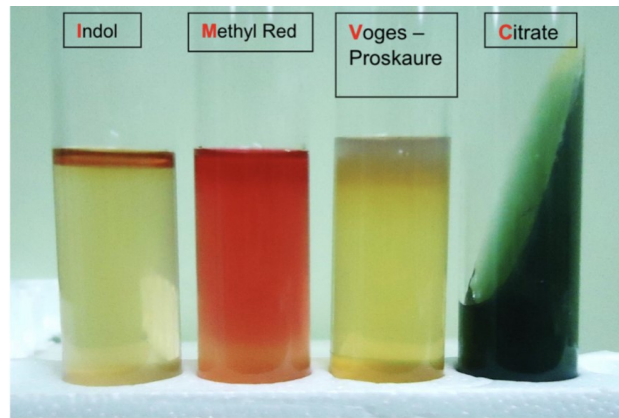


Figure (5): Tubes of IMVIC test results.

Table (2): Results of some biochemical tests of isolated non-O157 STEC.

Biochemical test	Oxidase test	Catalase test	Urease test	Motility test	Kligler Iron test
Result	-	+	-	+ (38 isolate) - (9 isolate)	Yellow /Yellow with gas production

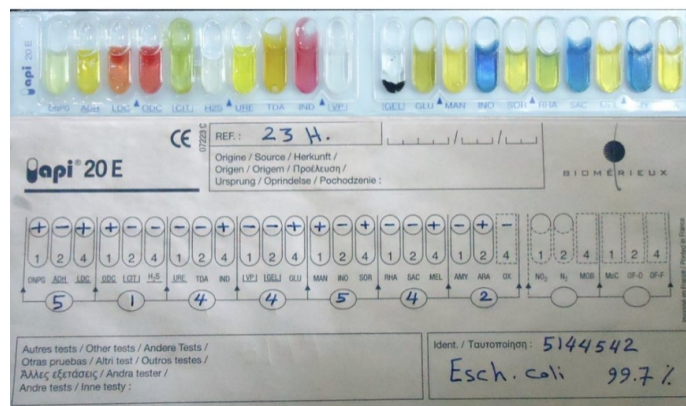


Figure (7): Api-20E of non- O157 STEC result.

Table (3): The result of Api-20E of non- O157 STEC.

Test	Active ingredients	Profile
ONPG	2-nitrophenyl-β-D-galactopyranoside	+
ADH	L-Arginine	-
LDC	L-Lysine	+
ODC	L-Ornithine	+
CIT	Irisodium citrate	-
H ₂ S	Na thiosulfate	-
URE	Urea	-
TDA	L-Tryptophane	-
IND	L-Tryptophane	+
VP	Na pyruvate	-
GEL	Gelatin (bovine origin)	-
GLU	D-Glucose	+
MAN	D-Mannitol	+
INO	Inositol	-
SOR	D-Sorbitol	+
RHA	L-Rhamnose	-
SAC	D-Sucrose	-
MEL	D-Melibiose	+
AMY	Amygdalin	-
ARA	L-Arabinose	+

Table (4): Prevalence of non-157 STEC in children and calves sample.

Source of samples	Total Number of sample	Positive for Non-O157 STEC	Percentage (%)
Children	127	20	(15.73%)
Calves	133	27	(20.30%)



Figure (8): Positive results of O26, O111, O103 and O145 antigens with latex agglutination test arrow.

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