Phenotypic and virulence genes screening of *Escherichia coli* strains isolated from different sources in delta Egypt.

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Abstract : The present study is conducted to identify the strains of *Escherichia coli* isolated from different sources in delta Egypt (diarrheic calves, clinical and subclinical mastitis cow`s milk, *Mugil capito, Oreochromis niloticus" tilapia"* and water) by phenotypic and molecular based techniques. Thirty two *E. coli* strains were isolated from the previous sources with percentage of 28.57%, 20%, 13.33%, 15%, 36%, and 80% respectively. All the *E. coli* isolates were further identified by PCR using universal primers targets the 16S rRNA gene that gave specific band for *E. coli* at 996bp. Serological identification of *E. coli* isolates was represented by eleven O-Serogroups (O86, O25, O158, O119, O78, O55, O26, O125, O127, O111 and O153). All *E.coli* strains were screened for their virulence characters phenotypically (hemolytic activity, Congo red binding activity and Vero cell cytotoxicity). The results of hemolytic activity showed that α–hemolysis was detected with percentage of 37.5% while β- hemolysis with percentage of 12.5%. On the other hand 50% of strains gave no hemolysis. All of 32 tested strains for the CR binding affinities were 100% positive while they were not able to produce cytopathic effect on the Vero cells. Further, all the isolated *E. coli* strains are subjected to screening for certain virulence genes (*eaeA, Stx1, Stx2, hylA, Sta and Stb*). The *eaeA* gene was detected in serotypes (O86, O55 and untypable strain) in calves, (O86, O55, O26 and O127) in milk, (O55, O125 and O153) in fish and (O55 and O153) in water. The *Stx1* gene was positive in O55 in calves, O55 in milk, O119, O55 and O125 in fish and O55 in water. The gene *Stx2* was positive only in case of O55 in calves, O55, O26 and O111 in milk .The *hylA* gene was detected in O86, O25, O158, O119 and O55 in calves, O55, O86 and O127 in milk, O119,O55 and O125 in fish. The *Sta* gene was positive in O158 and O55 in calves, O55 and O26 in milk, O119, O55, O125 and untypable strains in fish and O55 in water. While gene *Stb* was detected in O86, O25, O119 and O78 in calves, O111 in milk, O119, O55 and O125 in fish.
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Introduction

Escherichia coli are Gram's negative rods within the family *Enterobacteriaceae*, and represent a part of the normal micro flora of the intestinal tract of humans and warm-blooded animals. Due to their high prevalence in the gut, *E. coli* are used as the preferred indicator to detect and measure fecal contamination in the assessment of food and water safety. Pathogenic *E. coli* strains are distinguished from other *E. coli* by their ability to cause serious illness as a result of their genetic elements for toxin production, adhesion and invasion of host cells, interference with cell metabolism and tissue destruction (*Borgattaa et al., 2012*).

E. coli strains could be classified according to the presence of these virulence factors to verotoxigenic *E. coli* (VTEC) which produce a toxin that is lethal to cultured African green monkey kidney cells (Vero cells) but not to some other cultured cell types (*Holko et al., 2006*). Enterohemorrhagic *E. coli* are VTEC that possess additional virulence factors, giving them the ability to cause hemorrhagic colitis and hemolytic uremic syndrome (*Kobori et al., 2004*). Enterotoxigenic *E. coli* (ETEC) leads to watery diarrhea which lasts up to a week, but can be protracted. On infection, ETEC firstly adhere to the epithelium of the small intestine via one or more colonization factor antigens (CFA). Enteroinvasive *E.coli* (EIEC) invades the epithelial cells of the intestine and disseminate from cell to cell while Enteroaggregative *E. coli* (EAggEC) strains are characterized by their ability to aggregative adherence to tissue culture cells in a distinctive "stacked, brick-like" manner, milk and water have all been implicated in EAggEC outbreaks (*DiRita, 2007*).

Ruminants, in particular cattle are the main reservoir for VTEC. Beef has historically been most linked to VTEC infections. Epidemiological investigations have shown that cattle frequently excrete strains of Shiga toxin producing *E. coli* (STEC) in their feces and this may represent a source of infection (*Blanco et al., 1996*).

Dairy cattle with acute coliform mastitis, which caused primarily by *E. coli,* exhibit a wide

range of systematic disease which ranged from mild with only local inflammatory changes of the mammary gland to severe significant clinical signs including rumen stasis, dehydration, shock, and even death (*Wenz et al., 2001*).

Calf scour or calf diarrhea causes more financial loss to cow-calf producers than any other disease-related problem they encounter. Calf scour is not a disease; it is a clinical sign of a disease which can have many causes. In diarrheas, the intestine fails to absorb fluids and/or secretion into the intestine is increased. *Escherichia coli* has been incriminated as a major cause of diarrhea, which characterized by progressive dehydration and death may occur depends on the age of the calf when scour started and on the particular serotype of *E. coli* (*Tan et al., 2011*).

Fish is susceptible to microbial spoilage as it carries high microbial load on skin, gills and intestine. *Escherichia coli* in fish and water are considered as an indicator to sewage pollution (*Rajasekaran, 2008*).

In Egypt, poultry waste, sewage and cow dung are mostly used to fertilize fish ponds that may be the main sources of fish contamination. Therefore this study came as response to such need, to investigate the phenotypic and molecular characterization of some virulence genes of *E. coli* isolated from different sources (grazing diarrheic calves, mastitic cow milk in addition to fish and water).

2. Material and Methods

Sample collection and preparation; A total of 120 samples (diarrheic calves' rectal swabs n=35, clinical mastitic cows' milks n=20, subclinical mastitic cows' milks n=15, *Oreochromis niloticus* (*Tilapia*) n=25, *Mugil capito* n=20 and water samples n=5) were collected from Kafr El Shiek Governorate. Rectal swabs collected from diarrheic calves were inoculated into Trypticase soya broth and incubated at 37°C for 24 hours. The milk samples were collected in sterile screw capped glass bottles and immediately transferred to the laboratory in a cold chamber container to be cultured without delay. The milk samples were centrifuged at 3000 rpm for 20 minutes. The cream and the supernatant fluid were discarded and the sediment was inoculated into nutrient broth medium and incubated at 37°C for 24 hours. The fish samples were collected randomly from the farm's ponds of a private fish farm, which transported in tanks partially filled with the same water of the pond then transported to the laboratory. In the laboratory each fish was rinsed with de-ionized water and the surface of the fish was decontaminated by ethyl alcohol and lightly flamed. After opening the body wall of fish, the surfaces of organs (Liver, spleen and kidney) were sterilized by swabbing with70% ethanol before bacterial isolation. All samples were inoculated into nutrient broth tubes and incubated at 37°C for 24 hours. Water samples were taken in sterile glass bottles; dechlorinating agent (Sodium thiosulphate) was added. Water samples were concentrated by filtration through 0.4 µm pore size nitrocellulose filters (Sartorius - France), and then the filters were vortexed in peptone broth for recovering the bacteria. After removing the filters, the bacteria were cultivated at 37°C for 20 hours.

Bacterial isolation and identification; A loop-full from all previous tubes were cultivated on MacConkey agar (Oxoid), Eosin methylene blue agar (EMB) (Oxoid) and blood agar media. All of the inoculated plates were incubated aerobically at 37°C for 24 hours. The purified lactose fermenting colonies on MacConkey agar , hemolytic or non-hemolytic colonies on blood agar and colonies with metallic green sheen colonies on EMB were picked up and examined for their morphological, cultural and biochemical characters (*Cruickshank et al., 1979*). API 20E kit (Bio Merieux) was performed according to manufacturer's instruction in order to detect the biochemical profile of the isolated organisms.

Serological identification of E. coli isolates; the serological typing depended on the identification of the somatic antigens (O). O antigens were identified as described by *Guinée et al.* (*1981*). All available somatic antigens were (O1 to O185) antisera which done in Animal Health Institute laboratory. Dokki. Giza.

Virulence Assays;

Hemolysis assay; *E. coli* isolates were propagated on blood agar base supplemented with 5% washed sheep erythrocytes. The plates were incubated at 37°C for 24 hrs and hemolytic activity of the isolates were recorded.

Congo red (CR) binding test; All *E. coli* isolates were tested for their growth status on Congo red medium. The reaction was best seen after 18, 24, 48 and 72 hrs of incubation at 37°C and was then left at room temperature for an additional 2 days (not to exceed 4 days). Orange colonies were considered positive and different intensities in the dye uptake were expressed as +, ++ and +++ according to *Berkhoff and Vinal* (*1986*).

Vero cell cytotoxicity activity; the test was performed according to *Emery et al.* (*1992*) and *Giugliano et al.* (*1982*) as following;

a) *Preparation of E. coli extracts;* The tested *E. coli* isolates were first grown in brain heart infusion broth for 8 hours at 41ºC with vigorous agitation. Then 5 ml of each isolate were subcultured into 50 ml Casamino acid-yeast extract- salts (CA-YE) medium without glucose. The cells were allowed to grow aerobically at 37^{o}C with vigorous shaking. After 18-20 hours the cells were removed by centrifugation at 12,000 xg for 15 minutes at 4^{o}C. The supernatant fluid was

collected and the cell pellet was resuspended in PBS, pH 7.8. The cell pellet was washed twice in PBS and resuspended in 50 ml of PBS. The cells were ultrasonically disrupted continuously for 2 minutes in an ice bath using a sonicator. The disrupted cell suspension was centrifuged at 12,000 xg for 15 minutes and the supernatant was retained (sonis extract). Both the culture supernatants and the sonic extract were filter sterilized by membrane filtration Acro disc 0.22 µm pore size, and stored at -85^{o}C until assayed for verotoxin.

b) *Cytotoxicity assay*; two 96 well tissue culture plates containing 24 hours monolayer sheet of Vero cells were used. Each well contained 200µl of Eagles minimal essential medium EMEM (Gibco) and one hundred µl of a suspension of Vero cells containing approximately 4x10^{5} cells/ ml. A volume of 50µl of each filtered sonic extract and culture supernatants were added to duplicate wells (*Giugliano et al., 1982*). The plates were sealed with plastic film, incubated at 37^{o}C in 5%CO_2 incubator, examined under an inverted microscope after 18- 24 hour, fixed with 10% formalin, stained with crystal violet then washed and examined again under an inverted microscope to estimate the degree of destruction of the Vero cells.

Confirmation of E. coli isolates by PCR

One pair of primers, designated U1 and U2, with sequences conserved was selected. The sequence of primer U1 is 5-CCAGCAGCCGCGGTAATACG-3, corresponding to nucleotides 518 to 537 of the *E. coli* 16S rRNA gene, and that of U2 is 5-ATCGG(C/T) TACCTTGTTACGACTTC-3, corresponding to nucleotides 1513 to 1491 of the same gene according to *Jang et al*. (*2000*). PCR performed with these two primers is referred to as the universal PCR in order to amplify a portion of the 16S rRNA gene of *E. coli* in our study.

PCR amplification, A reaction mixture containing approximately 50 ng of template DNA extracted using QIA amp mini kit, Qiagen, PCR buffer (10 mMTris-HCl, pH 8.3; 50 mMKCl; 2.5 mMMgCl2; 0.001% gelatin), a 0.2 mM concentration of each PCR primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.) in a total volume of 50 ml was prepared. After a 10-min denaturation at 94°C, the reaction mixture was run through 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C, followed by an incubation for 10 min at 72°C. Five microliters of PCR product was electrophoresed on a 1% agarose gel to determine the size of the product according to *Jang et al*. (*2000*).

Detection of certain virulence genes

Specific oligonucleotide primers for certain virulence genes (*Stx1, stx2, hylA*, *sta, stb* and *eae*) were used. The sequence, specificities and the length of the amplified products were summarized in Table (1).

Table (1): Oligonucleotide primers for the studied virulence genes

Primer designation	Specificity	Sequence (5' – 3')	Amplified product size (bp)	
Stx1	**Shigatoxin 1**	**F, ACA CTG GAT GAT CTC AGT GG**	**614**	Márcia et al.(2003); Sheng et al.(2005) and Leyla and Kadri (2007)
Stx1		**R, CTG AAT CCC CCT CCA TTA TG**		
Stx2	**Shigatoxin 2**	**F, CCA TGA CAA CGG ACA GCA GTT**	**779**	
Stx2		**R, CCT GTC AAC TGA GCA GCA CTT TG**		
hylA	**Hemolysin**	**F, ACG ATG TGG TTT ATT CTG GA**	**165**	
hylA		**R, CTT CAC GTG ACC ATA CAT AT**		
Stb	**heat-stable enterotoxin b (Stb)**	**F, GCG TCC CTG CGT ATC AGT AT**	**241**	
Stb		**R, CTT TTA AGG CAA GCG TCG TC**		
Sta	**heat-stable enterotoxin a (Sta)**	**F, GCT AAT GTT GGC AAT TTT TAT TTC TGT A**	**190**	
Sta		**R, AGG ATT ACA ACA AAG TTC ACA GCA GTA A**		
eae A	**Intimin**	**F,GACCCGGCAACAAGCATAAGC**	**384**	
eaeA		**R,CCA CCT GCA GCA ACA AGA GG**		

DNA amplification and PCR running, The amplified reactions were performed in 50 µl volumes in micro-amplification tubes (PCR tubes). The reaction mixture consisted of 10 µl (200 ng) of extracted DNA template from bacterial cultures, 5 µl 10X PCR buffer, 0.5 µl $MgCl_2$ (2 mM), 1µl dNTPs (200 µM), 0.1 µl (0.5 Unit) AmpliTaq DNA polymerase, 0.1 µl (0.2 µM) from each primer pairs and the volume of the reaction mixture was completed to 50 µl using DDW**.** PCR amplifications were performed in thermal cycler (Perkin Elmer/Cetus Research**,** USA) that was adjusted with the following programs; 1. After a 3 min denaturation at 95°C, the reaction mixture was run through 35 cycles of denaturation for 1 min at 94°C, annealing for 45 sec at (58°C for *Stx1*, 60°C

for *Stx2, 52*°C for *hylA and 55*°C for *eaeA)*, and extension for 1.5 min at 72°C, followed by an incubation for 10 min at 72°C. 2. After a 3 min denaturation at 95°C, the reaction mixture was run through 25 cycles of denaturation for 30 Sec at 94°C, annealing for 45 sec at (60°C for *sta* and 57°C for *stb),* and extension for 1.5 min at 70°C, followed by an incubation for 10 min at 70°C. The PCR products were analyzed according to *Sambrook et al.* (*1989*) by using 1.5% agarose gel electrophoresis and DNA molecular weight marker of 100 base pair ladder (Bioron GmbH) (Jena Bioscience - Germany).

3. Results

A total of 32 *E. coli* strains were recovered from feces of diarrheic calves, clinically mastitic cow's milk, Subclinical mastitic cow's milk, mugil ,tilapia and water with an incidence 28.57% , 20% , 13.33% , 15% , 36% and 80% respectively as showed in Table (2).

The results of serotyping of E. coli ; the results of serological identification of 32 strains of *E. coli* showed that, 25 strains were classified and gave different somatic antigens. On the other hand 7 strains were untypable as illustrated in Table (3).

Results of hemolytic activity of E. coli isolates; the all 32 typable and untypable serotypes were examined for their hemolytic activities,12 serotypes gave α-hemolysis with an incidence of 37.5%, while 4 were β- hemolysis with an incidence 12.5%. On the other side 16 serotypes gave no hemolysis with percentage 50%. As showed in Table(4), serotype O86 gave hemolytic activities in diarrheic calves' and subclinically mastitic cow's milk, serotypes O55 revealed hemolytic activities with diarrheic calves, clinically and subclinically mastitic cow's milk, mugil and no hemolytic activities with water. O119 gave the same positive results with diarrheic calves and tilapia. O111 showed no hemolytic activities with clinically mastitic cow's milk and mugil and finally, O153 gave also no hemolytic activities with tilapia and water.

Results of Congo red binding activity of E .coli isolates; Congo red assay was used as a phenotypic marker for the invasive and non–invasive *E. coli.* In our study, all of 32 tested strains for the CR binding affinities were 100% positive. The Congo red positive (CR+) isolates were indicated by the development of bright or orange red colonies. The binding activity of the CR dye was found to be variable in their affinity according to their serovars**.**

Results of Vero cell cytotoxicity; the tested strains from different sources were not able to produce cytopathic effect on the Vero cells.

Table (2) Number and percentage of *E. coli* isolated from different sources

Type of sample	No of examined samples	No of positive samples %	Typable isolates	Untypable isolates
Diarrheic calves	35	10 (28.57%)	9	1
Clinical mastitic cow's milk	20	4 (20%)	4	0
Subclinical mastitic cow's milk	15	2 (13.33%)	2	0
Mugil	20	3 (15%)	3	0
Tilapia	25	9 (36%)	5	4
Water	5	4 (80%)	2	2
Total	120	32 (26.7%)	25	7

Table (3) The results of serological identification of *E. coli*

Type of samples	positive samples	Sero-types											
		O25	O26	O55	O78	O86	O111	O119	O125	O127	O153	O158	Un typable
Diarrheic calves	**10**	**1**	-	**2**	**1**	**1**	-	**3**	-	-	-	**1**	**1**
Clinical mastitis cow's milk	**4**	-	**1**	**1**	-	-	**1**	-	-	**1**	-	-	-
Sub clinical mastitis cow's milk	**2**	-	-	**1**	-	**1**	-	-	-	-	-	-	-
Mugil	**3**	-	-	**2**	-	-	**1**	-	-	-	-	-	-
Tilapia	**9**	-	-	-	-	-	-	**1**	**2**	-	**2**	-	**4**
Water	**4**	-	-	**1**	-	-	-	-	-	-	**1**	-	**2**
Total	**32**	**1**	**1**	**7**	**1**	**2**	**2**	**4**	**2**	**1**	**3**	**1**	**7**

Table (4) The correlation between haemolytic activity and serotypes

Sources of samples	Serotypes	Hemolytic activity on blood agar
Diarrheic calves (Rectal swabs)	O 25	+ve
	O55 (2)	+ve
	O78	-ve
	O 86	+ve
	O119 (3)	+ve
	O 158	+ve
	Untypable	-ve
Clinically mastitic milk cows	O26	-ve
	O 55	+ve
	O111	-ve
	O127	-ve
Subclinically mastitic milk cows	O 55	+ve
	O 86	+ve
Mugil	O 55 (2)	+ve
	O111	-ve
Tilapia	O119	+ve
	O125 (2)	+ve
	O153 (2)	-ve
	Untypable (4)	-ve
Water	O 55	-ve
	O153	-ve
	Untypable (2)	-ve

Results of PCR for the detection of (16S rRNA) gene

All 32 *E.coli* strains were positive and gave PCR product of the expected size at (996 bp) as showed in photo (1)

Results of PCR for the detection of certain virulence genes; Among 32 typable and untypable serotypes of *E. coli* used in PCR to detect certain virulence genes, we found that all serotypes carried at least one virulence gene except one serotype (O111) which isolated from fish and the two untypable serotypes isolated from water did not carry any virulence genes. Photo (2, 3) showed the results of *eaeA* and *Stx1* genes at the expected amplified product (384 bp and 614 bp respectively**.**

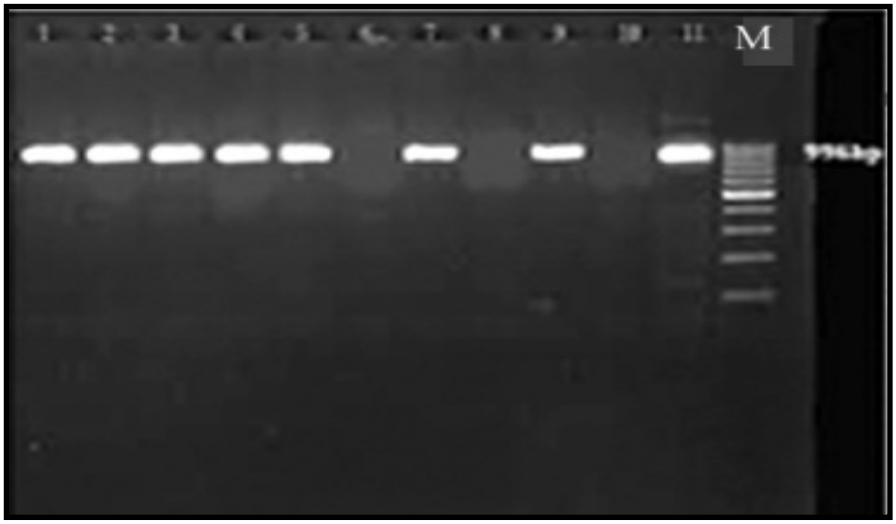

Photo (1) - Amplified PCR product of 16SrRNA gene at 996bp. Lane M: 100 bp ladder, Lanes 1, 2, 3, 4, 5, 7, 9 and 11 are positive *E.coli* strains

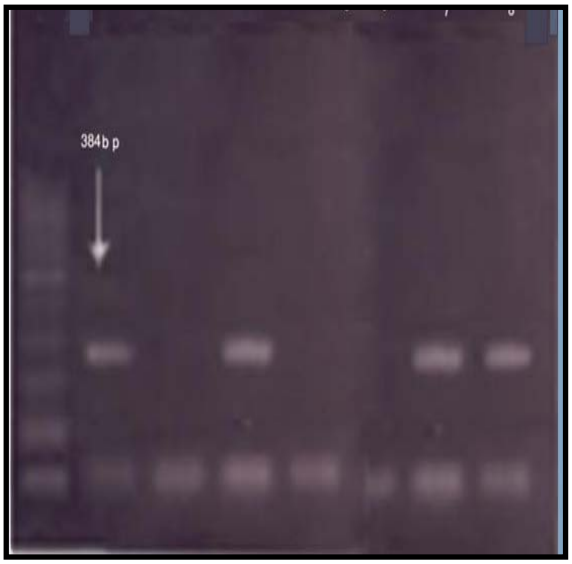

Photo (2) Amplified PCR product of (*eaeA*) gene at 384 bp .Lane M: 100bp ladder, Lane 1, 3, 6 and 7 are *eaeA* positive *E.coli* strains.

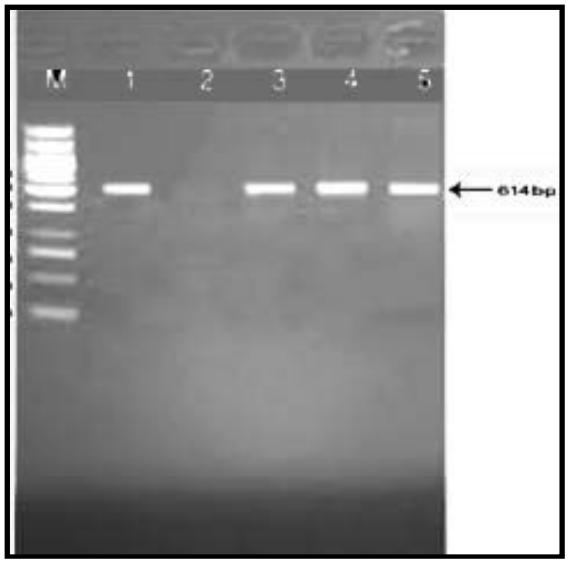

Photo (3) Amplified PCR products of (*Stx1*) gene at 614 bp., lane M: 100 bp. ladder, lanes 1, 3, 4 and 5 are stx1 positive *E. coli* strains.

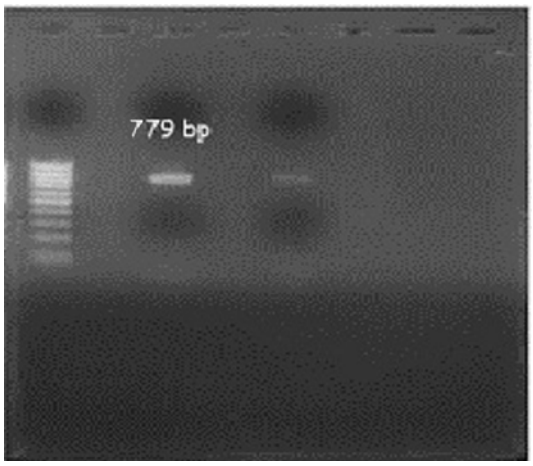

Photo (4) - Amplified PCR product of (*Stx2*) gene at 779bp .Lane M: 100bp ladder, Lanes 3 and 5 are *Stx2 positive E. coli* strains.

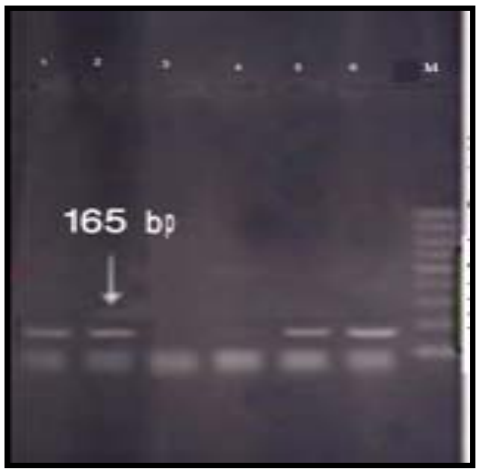

Photo (5) Amplified PCR products of (*hylA*) gene at 165 bp., lane M: 100 bp. ladder, lanes 1, 2, 5 and 6 are *hylA* positive *E. coli* strains.

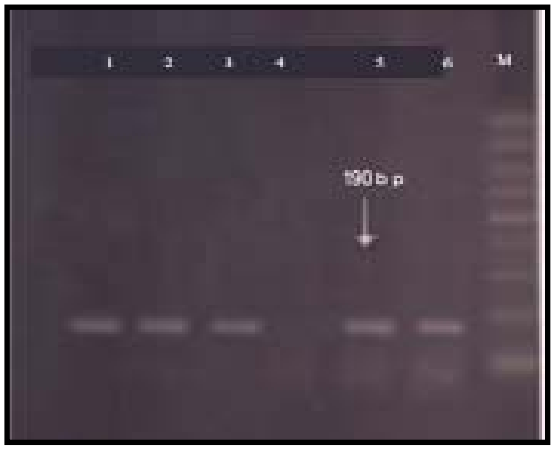

Photo (6) - Amplified PCR product of (*Sta)* gene at 190 bp .Lane M: 100 bp ladder, Lane 1, 2, 3, 5 and 6 are *sta* positive *E.coli* strains.

Photo (7) Amplified PCR products of (*stb*) gene at 241 bp., lane M: 100 bp. ladder, lanes 1, 4 and 5 are *stb* positive *E. coli* strains.

Table (5) virulence genes screening results of E. coli strains isolated from different sources

Virulence genes	calf-O25	calf O55(n=2)	calf-O78	calf-O86	calf-O119 (n=3)	calf-O158	Untyp.calf Un typable calf uuuuuuuuuuuu	cow O26	cow O55 (n=2)	cowO86	cow O111	cow O127	Fish O55 (n=2)	Fish O111	Fish O119	Fish O125	Fish O125	Fish O153 (n=2)	Untypable. Fish (n=4)
eae A	-	+	-	+	-	-	+	+	+	+	-	+	+	-	-	+	+	+	-
Stx1	-	+	-	-	-	-	-	-	+	-	-	-	+	-	+	-	+	-	-
Stx2	-	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-
hyla A	+	+	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-
Sta	-	+	-	-	-	+	-	+	+	-	-	-	+	-	+	-	+	-	+
Stb	+	-	+	+	+	-	-	-	-	-	+	-	+	-	+	+	-	-	-

4. Discussion

The incidence rate of *E. coli* isolation from calves samples was 28.57%, these results has much differences with *Osman et al.*(*2012*) who isolated *E. coli* from diarrheic calves with a percentage of 63.6%. *E. coli* were isolated from mugil and tilapia with percentage of 15% and 36% respectively. *Azza et al.* (*2012*) reported that the incidence of *Escherichia coli* from 20 *Mugil capito* and 40 tilapia samples was 42.8 %, and 27% respectively, that were to some extent close to our results. Water samples have *E. coli* strains with percentage of 80%, these results were not surprising for us as *EL-Jakee et al.* (*2009*) isolated *E. coli* with percentages 42.9, 33.3, 33.3, 25, 25 and 7.7 among water samples collected from agricultural drain, treated sewage water, well, canal, untreated sewage water and drinking underground water samples respectively. Finally, *E. coli* strains were isolated from clinical and subclinical mastitis cases with percentages 20% and 13.33% respectively. *Hassan et al.* (*2012*) isolated *Escherichia coli* from bovine mastitic milk samples (268 samples) with an incidence of 27.23% that was compatible with our results.

Vero cell cytotoxicity assay, considered to be the 'gold standard' for the detection of *stx* *Konowalchuk et al.* (*1977*). *Beutin et al.* (*1989*) noticed a close relation between cytotoxicity and production of hemolysin, *Bettelheim* (*2001*) realized that non-O157 VTEC strains are as important as O157 VTEC. However, it should be noted that not all hemolytic positive strains are VTEC. These results agreed with our study as hemolytic strains of *E. coli* did not show any cytopathic effect. All of the tested *E. coli* strains for the CR binding affinities in our study were 100% positive. The binding activity of the CR dye was found to be variable in their affinity according to their serovars. These results were in harmony with *Osman et al.* (*2012*).

The results of PCR by universal primers of 16S rRNA gene to all 32 *E. coli* strains confirmed that all strains gave amplified product at 996 bp and these agreed with *Jang et al.*(*2000*) who reported that *E. coli* bacteria which detected by the universal primer from the cerebrospinal fluid gave the same amplified product.

Shiga toxin producing *E. coli* (STEC) has been implicated as an etiological factor of calf diarrhea, (*Sandhu and Gyles, 2002*) and these animals form a principle reservoir of STEC that is pathogenic for humans (*Boerlin et al. 1999*). We have 9 *E. coli* strains from diarrheic calves with serotypes O25 n=1, O55 n=2, O78 n=1, O86 n=1, O119 n=3 and O158 n=1, in addition to one untypable *E. coli* strain. It was found that only *E.coli* serotype O55was positive for *stx* genes among all strains isolated from calves with an incidence of 22.2%. These results were disagreed with *Tan et al.* (*2011*) who found that a total of 177 isolates of calves diarrhea in Vietnam (51%) were positive for the *stx* genes. However, *Salvador et al.* (*2003*) in Brazil and *Arya et al.* (*2008*) in India detected 40% or more of *stx* gene positive *E. coli* strains in diarrheic calves. On the other hand *Osek et al.* (*2000*) have reported that less than 10% of STEC were detected in diarrheic calves.

All 10 strains of diarrheic calves showed negative results to *eae* gene (O25, O158, O119 and O78) except serotypes O86, O55 (n=2) and the untypable strain were positive with an incidence of 40% and these results are not matching with those of *Kobayashi et al.* (*2003*) who found that a significant number of strains recovered from diarrheic calves possess *eae*. While *Osman et al.* (*2013*) mentioned that *eae* was detected in one fecal sample. The low

prevalence of *eae* gene has been reported in many studies (*Hornitzky et al., 2005* and *Fremaux et al., 2006*). The importance of this data lies in the fact that *eae*-positive strains are considered more virulent for humans than *eae*-negative strains.

Sta gene was detected in serotypes O55 n=2 and O158 with a percentage of 30% while *stb* was detected in O25, O86, O78 and O119 n=3 with percentage 60% among the diarrheic calves serotypes. *Rajkhowa et al.* (*2009*) found that**,** ETEC in the fecal samples of mithun calves was low, only two isolates (3.7%) were found to harbor *ST* enterotoxin gene. Similarly, *Salvadori et al.* (*2003*) also recorded 3.9% ETEC possessing *ST* and *LT* enterotoxin from diarrheic calves by PCR in Brazil. In contrast, *Rigobelo et al.* (*2006*) reported higher prevalence rate of *E. coli* carrying genes for *ST* (25.4%) enterotoxins from diarrheic cow calves in Brazil. The finding of serogroup O55 has *ST* enterotoxin in our study was in conformity with *Rigobelo et al.* (*2006*) and *Rajkhowa et al.* (*2009*).

HylA gene was present in all *E. coli* serotypes of diarrheic calves O25, O55 n=2, O86, O119 n=3 and O158 with a percentage of 80% with exception to serotype O78 and untypable strain. On the other side combination of *hylA* and *stx* genes was presented in case of one strain O55. These finding agreed with *Osman et al.* (*2012*) who stated that *stx1* and *stx2* genes were undetected, in contrast to all previous reports, while the *hlyA* gene prevailed. *Schmidt et al.* (*1995*) reported the genetic analysis of a new plasmid-encoded haemolysin, *Ehly*, is associated with severe clinical diseases in humans. *Beutin et al.* (*1989*) studied hemolysin production in a large number of serologically diverse VT+ *E. coli* strains and found an association between enterohemolysin and verotoxin production in 89% of *E. coli* strains belonging to nine different serotypes. A suggestion was raised that enterohemolysins may complement the effects of shiga toxins enhancing their virulence (*Nataro and Kaper, 1998*).

Six *E. coli* serotypes O26 n=1, O55 n=2, O86 n=1, O111 n=1 and O127 n=1 isolated from milk were also subjected to the PCR for detection of the previously mentioned virulence genes. Our result was in harmony with *Salwa et al., 2011* and *Osman et al.* (*2012*) that isolated *E. coli* serotypes O26, O127 and O111from milk samples. We found that *stx*1 gene was negative in all strains except serotypes O55; *stx*2 was positive in case of serotypes O26, O55 n=2, and O111, *eae* gene was found to be negative only in case of serotype O111, *hylA* gene was positive in cases of serotypes O55 n=2, O86 and O127 and finally, *sta* were detected in serotypes O26 and O55n=2 and *stb* were positive in serotype O111.

There are many studies which showed that the STEC strains are the most prevalent resources for milk-poisoning, *Solomakos et al.* (*2009*). *Hassan et al.* (*2012*) showed that the milk of animals with mastitis and especially subclinical mastitis is the main source for STEC strains. *Moussa et al.* (*2010*) stated that *stx2* and *eaeA* genes were the most prevalent virulence factors in cow's environment that is contaminated by feces, and it is also a frequent cause of bovine mastitis. *Leyla* and *Kadri* (*2007*) indicated that genes encoding Shiga toxins 1 and 2 (*stx1* and *stx2*), intimin (*eaeA*) and heat-stable enterotoxin a (*sta*) were the most prevalent virulence factors which were isolated from clinical bovine mastitis cases in Turkey. Some studies indicated that, in addition to virulence genes like *stx1*, *stx2*, *eae* and *ehly* mainly accompanied by attendance of antibiotic resistance genes (*Verdier et al., 2012*). In Egypt *Osman et al.* (*2012*) revealed that all *E. coli* strains which were isolated from mastitic milk samples had *stx1*, *stx2*, *hylA*, *stb*, *sta* and *eaeA* virulence genes. These previous studies disagreed with our result that revealed milk serotypes did not carry *stx*1 gene.

All the 12 *E. coli* serotypes from fish were subjected also to virulence genes screening O55 n=2, O111n=1, O119 n=1, O125 n=2, O153 n=2 and besides 4 the untypable strains. *Stx*1, *sta*, *stb* and *hyl*A genes were positive in serotypes O55 n=2, O119 and O125, *eae* gene was positive in serotypes O55 n=2, O125 n=2 and O153. The four untypable *E. coli* isolates carried *sta* gene only. On the other hand *stx*2 gene was negative with all serotypes. *Sanath Kumar et al.* (*2001*) demonstrated the presence of STEC in fish of serotypes other than that of O157. All except one of the *stx*-positive isolates were sorbitol fermenters. The authors added that among 60 fish samples, 2 samples were positive for *stx* and *hlyA* genes by PCR. The presence of *E. coli* harboring the EHEC-*hlyA* gene is significant, since there is increasing evidence that this hemolysin gene may be the marker for Shiga toxin-producing *E. coli* (*Gyles et al., 1998*).

E. coli serotypes O55 and O153 isolated from water were positive to *eae* gene, serotype O55 carried *stx*1, *eae* and *sta* genes and untypable strains n=2 do not carry any virulence genes. *EL-Jakee et al.* (*2009*) stated that the predominant *E. coli* serotype isolated from the examined water samples was O128 followed by O157, O111 and O55 respectively. *E. coli* strains isolated from water sources were characterized by PCR and showed that 8 isolates carried *stx1* gene (verocytotoxin 1) and 4 possessed *stx2* gene (verocytotoxin2). Intimin *(eae)* and enterohemolysin *(hly),* virulence genes were detected in 21.4, 21.4 and 28.6 % of the isolates respectively. *Ram and Shanker* (*2005*) reported

the presence of varies types of virulence genes of *E. coli* from water samples.

Our study found that pathogenic serotype O119 which isolated from calves diarrheic and fish sources shared together in two virulence genes (*hylA* and *Stb*), on the other hand pathogenic serotype O55 which isolated from water, fish and calves diarrheic cases also shared the same virulence genes (*eae, stx1* and *Sta*). These results may explain a relation between the different sources. It could be a way of transmission of infection between the farms in Kafr El Shiek Governorate as a result of the proximity between the farms in the Governorate.

Conclusion

The poultry waste, sewage and cow dung that are mostly used to fertilize fish ponds constitute hazardous sources of contamination for water and fish which reflected directly in public health, this dangerous source of contamination is considering alarm for damaging the aquatic culture in Kafr El-Shikh. The severity of these sources of contamination arouses from the detection of STEC and ETEC in fish samples that constitute the main causes of food poisoning and hemorrhagic enterocolitis in man due to eating the improperly processed fish meals.

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