Bacteriological and molecular studies on Shiga-Toxin producing *Escherichia coli* causing cattle clinical mastitis

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A B S T R A C T

A total of 194 milk samples from clinically mastitis cattle cows were collected from Giza, Monofia, Fayoum, Ismailia, and Beni-Suef Governorates. All samples were collected during the period from December 2016 till June 2017. Bacteriological study gave a total of 29 positive strains of *Escherichia coli (E.coli)* in the rate of (14.9%) from all collected samples. Twelve *E.coli* isolates were identified from cultured samples in a single manner (6.2%) and was isolated with *Staphylococcus aureus (S.aureus) by* 8/194(4.1%), with *Streptococcus species (Strept. spp)* by 3/194(1.57%), meanwhile it was isolated with *S.aureus* and *Strept. spp.* by 6/194(3.1%). On the other hand 18 clinical mastitic milk samples were showed no growth of any pathogenic microorganisms on ordinary and specific used media of bacteriology from all investigated milk samples by (9.3%). It was observed that several serotypes were recovered from clinical cases of milk sample with different *E.coli* infection as O27, O146, O125, O126, O111, O20, and O157. Concerning the sensitivity test to choice the suitable antibacterial drug(s) for treatment clinical mastitis in cattle cows the data revealed that ,Cefiquinom, Gentamycin and Amoxicillin +Clavulinic acid were the antibacterial drugs of first choice that could be used to overcome a great number of single isolated *E.coli* causing clinical mastitis. Vice versa, the resistant antibiotics for single *E.coli* infection causing clinical mastitis were Amoxicillin, Ampicillin, and Neomycin. Studied strains were gave a positive results for virulence *E.coli* genes: *pho*A ,*omp*A and *fim*H in 5 examined strains (100 %),classified as follow :(two strains of O27/28.6%) ,(one strain of O125/14.3%) ,(one strain of O126/14.3%) , and (one strain of O146/14.3%). while *Stx1* and *Stx2* virulence genes were detected in only 2 studied strains of *E.coli* in a total percentage of 28.6 %, divided into, O111(14.3%) and O157(14.3%).

Key words: Cattle diseases - Clinical mastitis -*E.coli* infection - Molecular biology - Virulence genes - bacterial Antibiogram -Egypt Governorates.

1. INTRODUCTION

Mastitis means inflammation of the mammary gland and characterized by physical, chemical, microbiological and cellular changes in the milk as well as pathological changes in the udder (Merck Veterinary Manual, 2006). Cattle mastitis is due to different invading microorganisms that mostly found in mixed infection. The predominant organisms are *E.coli, Staphylococcus aureus* and *Streptococcus species* (Almaw et al., 2008). *E.coli* has been reported to be the most common cause of clinical mastitis in well-managed dairy herds with low milk somatic cell counts (SCC) in the United Kingdom (Bradley, 2002). *E.coli* is among the most common infectious agents isolated from severe mastitis cases in modern dairy farms (Bradley et al., 2007).*E. coli* O157:H7 is able to move through the soil profile with water after rainfall or irrigation and can even reach the groundwater (Lang and Smith, 2007). Ruminants, including cattle, are a reservoirs of *E. coli* O157:H7 Menrath et al., (2010), and may reside asymptomatically in the intestines of cattle and may be shed intermittently in feces (Caprioli et. al., 2005). *Shiga toxins* are the major virulence factors of *E.coli*, there are *Stx1* and *Stx2* that have been associated with the severity of human's infections (Madic et al., 2011). In fact, non-O157 *shiga toxin Escherichia coli* (*STEC*) pathogenesis is not fully understood (Bolton, 2011) .Many works with *E. coli* strains have been carried out especially in relation to the various virulence factors (Osman et al., 2012). Aidar-Ugrinovich et al., (2007) determined the occurrence of *STEC* from feces of dairy and beef cattle, water and feed for animals,

milk and dairy products, and ground beef. Variety of different virulence factors, individually and in combinations, has been detected in *E. coli* isolates that cause mastitis (Kaipainen et al., 2002). The most mastitis isolates have not possessed any of the virulence factors evaluated the risk Profile considering *Shiga toxin-producing E. coli* (STEC) in raw milk (Kaipainen et al., 2002; Wenz et al., 2006). Multiplex PCR is an effective method in detection of specific virulence genes of *STEC* serotypes including shiga toxins 1 and 2, intimin and enterohaemolysin A, (Bai et al., 2010). Genetic manipulation of dairy cows, to express recombinant immunomodulation proteins in their milk, would be one approach to mastitis prevention (Wall et al., 2005). Non-antimicrobial approaches for treating of *E. coli* mastitis have been studied as alternatives to antimicrobials, non-steroidal anti-inflammatory drugs (NSAID), frequent milking and fluid therapy have been commonly recommended for supportive treatment of coliform mastitis (Radostits et al., 2007). Kibret et al., (2011) resulted that *E. coli* isolates showed high rates of resistance to erythromycin, amoxicillin and tetracycline meanwhile, Nitrofurantoin, norflaxocin, gentamicin and ciprofloxacin are considered appropriate for empirical treatment of *E. coli* in the study area. Gundogan and Avci, (2014) showed that *E. coli* and *Staphylococcus aureus* exhibit resistance to ampicillin, penicillin, tetracycline, erythromycin, gentamicin and trimethoprim/sulfamethoxazole. *E. coli* isolates also showed resistance to chloramphenicol and ciprofloxacin but none of them exhibited resistance to cefotaxime. The objectives of the present study was to investigate the occurrence of *STEC* serotypes as well as to determine the frequency distribution of five virulence genes (*stx1, stx2 , pho*A , *omp*A and *fim*H) in *E.coli* isolates from cattle clinical mastitic milk, in addition to determine the drugs of choice for treatment of most *E.coli* strains causing cattle clinical mastitis.

2. Material and Methods

2.1. Clinical examination.

The studied animals were subjected to clinical examination by visual inspection; palpation of the udder for swelling, redness and pain; beside the physical changes in the milk secreted from such udders.

2.2. Samples:

According to (International Standard Organization ISO 6579: 2002) method. A total of 194 milk samples from examined clinically mastitic cattle cows were collected in sterile 30 ml containers under complete aseptic condition from some different Governorates of Egypt and transferred in ice box as soon as possible to bacteriological laboratory of Animal Reproduction Research Institute (ARRI) in Haram / Giza Governorate. All samples were collected during the period from December 2016 till June 2017 from Giza, Monofia, Fayoum, Ismailia, and Bane Suif Governorates.

2.3. Bacteriological examination.

2.3.1. Isolation of E.coli and most important bacteria causing mastitis (Quinn et al., 2002):

Milk samples were pre- incubated for 18-24 hours at 37°C, then centrifuged at 3000 rpm for 20 minutes the cream and supernatant fluid were discarded. A loopful from sediment was streaked on the surface of Nutrient agar; MacConkey's agar; Blood agar; Eosin methylene blue media (EMB), Xylose Lysine Deoxycholate (XLD) agar, Mannitol salt agar; Baird Parker agar; 7% and Modified Edward's media. All plates were incubated aerobically at 37°C for 24-72 hr. The suspected colonies were picked up and sub cultured for purification. The pure colonies were kept in Semi-solid nutrient agar for more identification.

2.3.2. Identification of suspected isolates:

2.3.2.1. Morphological identification (Quinn et al., 2002)

Smears from suspected pure colonies were stained with Gram- stain and examined microscopically.

2.3.2.2. Traditional biochemical identification

The purified isolates were examined by different biochemical reactions Indol test, Methyl Red test, Voges-Proskauer test (VP), Citrate utilization test, Urease test, H_2S production test, Catalase test, Oxidase test, Sugar fermentation tests, Nitrate Reduction test, Gelatin hydrolysis test, Coagulase test and Motility tests, according to Quinn et al., (2002).

2.3.2.3. API 20 E test for more accurate identification

(BioMérieux- France): It was used as standardized fine and more accurate identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods which uses 23 miniaturized biochemical tests and a data base.

2.3.2.4. Diagnostic E. coli antisera:

The isolates were identified serologically using diagnostic O-sera and K-sera (polyvalent and monovalent; Denka Ltd. Company, Germany).

2.3.2.5. PCR Techniques:

For more accurate identification and virulence genes detection according to Ghanbarpour and Salehi, (2010) and Hu *et al.,* (2011). Table (2): Oligonucleotide primers sequences of virulence genes. Source: Metabion (Germany).

2.3.2.6. Antibiogram assay:

The disc diffusion method was used as described by (*Nccls. 2002)*

3. RESULTS

Table (1): Number of examined clinically mastitic milk from cattle cows in different governorates of Egypt.

Governorate	No	%
Monofia	51	26.3
Giza	36	18.5
Fayoum	33	17.0
Ismailia	31	16.0
Beni-Suef	43	22.2
Total	194	100%

%were calculated according to number of all milk samples.

Table (2): Oligonucleotide primers sequences Source: Metabion (Germany).

Target MO	Target gene	Primers sequences	Amplified segment (bp)	Reference
E. coli	*Pom*A	*AGCTATCGCGATTGCAGTG* *GGTGTTGCCAGTAACCGG*	919	Ewers *et al*., 2007
	*pho*A	*CGATTCTGGAAATGGCAAAAG* *CGTGATCAGCGGTGACTATGAC*	720	Hu *et al.,* 2011
	Stx1	*ACACTGGATGATCTCAGTGG* *CTGAATCCCCCTCCATTATG*	614	Dipineto *et al.*, 2006
	Stx2	*CCATGACAACGGACAGCAGTT* *CCTGTCAACTGAGCAGCACTTTG*	779	
	*Fim*H	*TGCAGAACGGATAAGCCGTGG* *GCAGTCACCTGCCCTCCGGTA*	508	Ghanbarpour and Salehi, 2010

Table (3): The different antimicrobial discs used in the agar diffusion method and interpretation of their sensitivity.

Antimicrobial agents	Code	Concentration Of disks	Zone of inhibition Resistant	Intermediate	Sensitive
Amoxicillin +Clavulinic acid	*AMC*	30 ug	13	14-17	≥ 18
Gentamycin	*GM*	10 ug	12	13-14	≥ 15
Neomycin	*N*	10 ug	11	12-13	≥ 14
Cefiquinom	*CFQ*	30 ug	13	14-17	≥ 18
Ampicillin	*AMP*	10 ug	11	12-13	≥ 14
Chloraphincol	*C*	30 ug	12	13-17	18
Enrofloxacin	*ENR*	10 ug	≤15	16-20	21
Oxytetracyclin	*OX*	30 ug	14	15-18	≥ 19
Streptomycin	*S*	10 ug	11	12-14	≥ 15
Penicillin g	*P*	20 ug	20-27	≥ 29	20
Amoxicillin	*AM*	25 ug	11	12-13	≥ 14

Table (4): Incidence of *E.coli* isolates from 194 Samples of clinically mastitic milk of cattle cows by using culture, different biochemical test.

Culture		By traditional methods		By API 20 E.	
NO	%	NO	%	NO	%
36	18.8	31	16.0	29	149

Table (5): Incidence of isolated microorganism's form 194 examined clinical mastitic milk samples of different governorates of Egypt.

Isolated Microorganisms	Governorates										Total	
	Monofia		Giza		Fayom		Ismailia		Beni-Suef			
	No	%	NO	%	NO	%	NO	%	NO	%	NO	%
E.coli	4	33.3	1	8.3	2	16.7	2	16.7	3	25.0	12	6.2
Staph. Aureus	23		12	15,6	13	16.9	10	13.0	19	24.8	77	39.7
Strept. Spp	6	19.3	5	16.1	6	19.3	6	19.3	8	25.8	31	16.0
E.coli + Staph. aureus	2	25.0	1	12.5	2	25.0	2	25.0	1	12.5	8	4.1
E.coli + Strept. Spp	147	33.3	0	0.00	1	33.3	1	33.3	0	0.00	3	1.5
E.coli + Staph. Aureus + Strept. Spp	2	33.3	1	16.7	0	0.00	1	16.7	2	33.3	6	3.1
Staph. Aureus +Strept. Spp	9	23.1	10	25.6	6	15.4	7	17.9	7	17.9	39	20.1
No growth	4	22.2	6	33.3	3	16.7	2	11.1	3	16.7	18	9.3
Total	51	26.3	36	18.5	33	17.0	31	16.0	43	22.2	194	100%

Table (6): Incidence of *E.coli* isolates from clinically mastitic milk Samples of cattle

By serology methods N: (17)				By PCR methods N:(7)	
Typing		Un Typing			
NO	%	NO	%	NO	%
15	88.2	2	11.8	7	100%

Table (7) *E. coli* serogroups' isolated from examined milk sample

Sample NO	Poly valent	Mono valent
1	4	O27
2	2	O146
3	2	O125
4	2	O126
5	2	O125
6	4	O27
7	2	O146
8	4	O27
9	2	O126
10	4	O27
11	2	O125
12	1	O111
13	5	O20
14	3	O157
15	2	O125

Table (8): -Antibacterial sensitivity tests of *E.coli*, single and mixed infections isolated from cattle clinical mastitic milk.

Antibacterial Disks	Concentration Of disks	*E.coli* single infection(n:10) Sensitive %	*E.coli* single infection(n:10) Resistant %	*E.coli* mixed infection(n:20) Sensitive %	*E.coli* mixed infection(n:20) Resistant %
Amoxicillin +Clavulinic acid	30 ug	40	60	50	50
Amoxicillin	25 ug	0.0	100	0.0	100
Ampicillin	10 ug	20	80	50	50
Cefiquinom	30 ug	80	20	70	70
Chloraphincol	30 ug	30	70	20	80
Cloxacillin		10	90	0.0	100
Enrofloxacin	10 ug	50	50	40	60
Gentamycin	10 ug	70	30	50	50
Neomycin	10 ug	20	80	40	60
Oxytetracyclin	30 ug	30	70	50	50
Penicillin g	10 ug	20	80	20	80
Streptomycin	10 ug	0.0	100	0.0	100

Table (9):- Incidence of virulence genes from different serotypes of *E.coli*.

E.coli serotypes(N:7)	Virulence genes *phoA* NO	*phoA* %	*ompA* NO	*ompA* %	*fimH* NO	*fimH* %	*Stx1* NO	*Stx1* %	*Stx2* NO	*Stx2* %
O27	2	28.5	2	40	2	28.5	0.0	0.0	0.0	0.0
O125	1	14.3	1	14.3	1	14.3	0.0	0.0	0.0	0.0
O126	1	14.3	1	14.3	1	14.3	0.0	0.0	0.0	0.0
O146	1	14.3	1	14.3	1	14.3	0.0	0.0	0.0	0.0
O111	1	14.3	1	14.3	1	14.3	1	14.3	1	14.3
O157	1	14.3	1	14.3	1	14.3	1	14.3	1	14.3

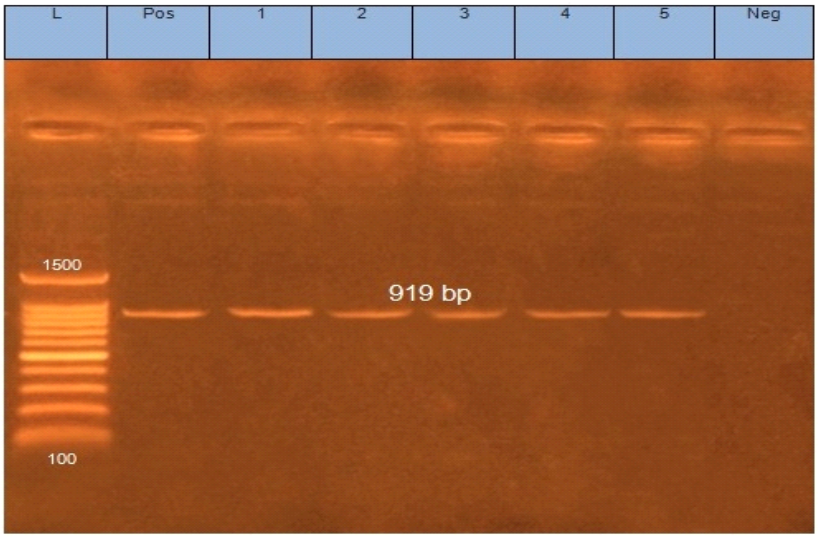

Fig. (1). Ethidium bromide stained 1.5% agarose gel representing PCR amplicons (919 bp) of the *omp*A gene from different *E. coli* isolate-genomes (lane 1; O27: lane 2; O27: lane 3; O125: lane 4; O126: lane 5; O146). Lane L: 100 bp DNA ladder, Lane Pos.: Positive control; and Lane Neg.: Negative control

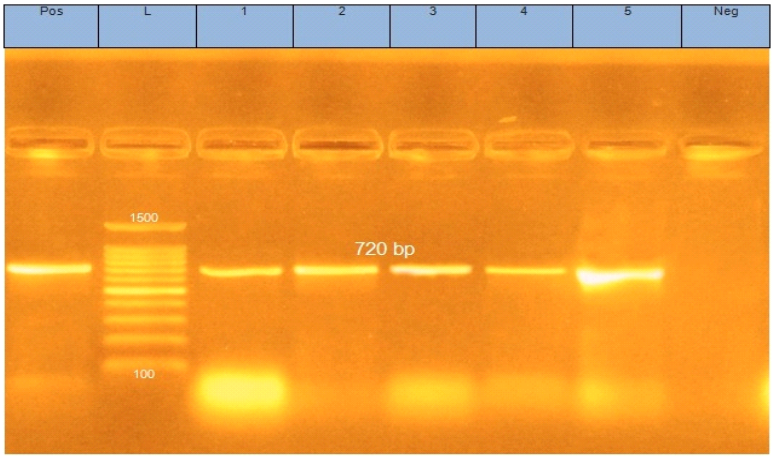

Fig. (2). Ethidium bromide stained 1.5% agarose gel representing PCR amplicons (720 bp) of the *pho*A gene from different *E. coli* isolate-genomes (lane 1; O27: lane 2; O27: lane 3; O125: lane 4; O126: lane 5; O146). Lane L: 100 bp DNA ladder, Lane Pos.: Positive control; and Lane Neg.: Negative control

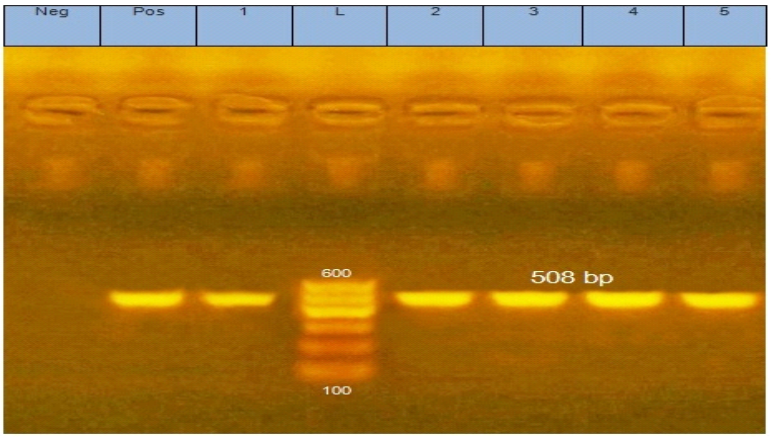

Fig. (3) Ethidium bromide stained 1.5% agarose gel representing PCR amplicons (508 bp) of the *fim*H gene from different *E. coli* isolate-genomes (lane 1; O27: lane 2; O27: lane 3; O125: lane 4; O126: lane 5; O146). Lane L: 100 bp DNA ladder, Lane Pos.: Positive control; and Lane Neg.: Negative control

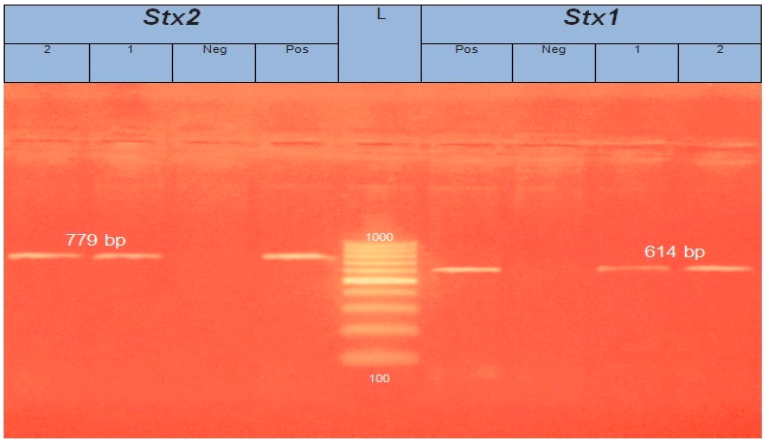

Fig. (4) Ethidium bromide stained 1.5% agarose gel representing PCR amplicons of the *Stx1* and *Stx2* genes from different *E. coli* isolate-genomes (lane 1; O111: lane 2; O157). Lane L: 100 bp DNA ladder, Lane Pos.: Positive control; and Lane Neg.: Negative control.

4. DISCUSSION

Mastitis caused by *Escherichia coli* is common in high-producing cows with a low milk somatic cell count. The severity and outcome of *E. coli* mastitis vary between cows of the same herd and between different lactation stages in the same individual. Pathogenesis of bacterial infection involves a complicated interaction between bacterial and host factors. In most *E. coli* infections, the pathogenicity of the bacterial strain is obligatory to this interaction and defines the course of the disease (Blum et al., 2017). The recorded results of clinical examination of 194 studied cows infected with clinical mastitis appeared that affected udder was warm, swollen, doughy toinful. The milk was watery, purulent or with thick clots and seven samples were tinged with blood. Most cases showed only one or two quarters affected. These results are closed to that recorded by Abd El Hameed et al., (2009). The cultural examination of *E. coli* on different media are used for initial diagnosis. MacConkey agar is frequently used to differentiate among various gram-negative bacilli that are isolated from milk samples . Thus, MacConkey agar differentiates between lactose-fermenting and non-lactose-fermenting gram negative bacteria. Fermentation of lactose by *E.coli* resulting in acid production, which causes decrease in pH and changing the color of bacteria to pink due to presence of neutral red in the media as a pH indicator as discussed by Engelkirk and Duben-Engelkirk,(2015). Table (5) showed that the culture results of *E.coli* microorganisms isolated singly or in a mixed infection from some different Governorates of Egypt and it was showed that totally 12 *E.coli* single isolates were detected in our study by percentage of 6.2%. Meanwhile *E.coli* was also isolated in a mixed infection with *S. aureus* (8 isolates, 4.1%), with *Strept. SPP*. (3 isolates, 1.5%), with *S.aureus and Strept. SPP*. (6 isolates 3.1%). These result of single *E.coli* isolates from cattle clinical mastitic milk was in agreement with El-Leboudy et al. (2014), 8%. And were disagree with Bandyopadhyay et al. (2011), 26.4%, this difference in the results were may be due to differences in management, hygiene and sanitation programs applied in other studies. In addition, that our study was applied mainly on small holders and not on organized farms. Meanwhile 18/194(9.3%) clinical mastitic milk samples were showed no growth of any pathogenic microorganisms on general and specific used media for bacteriological investigations. This negative result may be returned to limited media used for isolation i.e.it means the clinical mastitis may be due to another causative agents like fungal infection, anaerobic bacterial infections, other specific bacteria for example Mycoplasma spp. Brucella spp. Pasteurella spp. ehich need separate specific media of bacteriology or may be due to viral infection or even other non-specific causes like trauma or injures. In Table (4) the biochemical results supported that the present isolates were *E.coli* (31 isolates / 16.0%), these isolates were also identified by Api-20E system (29 isolates / 14.9%). These results were closely in agreement with Zeinhom and Abdel-Latef, (2014) was 16.7%, and disagree with El-Leboudy et al. (2014), 8%. It was observed that several serotypes were recovered from clinical cases of milk sample with different E.coli infection as O27, O146, O125, O126, O111, O20, O157, table (7). Agree with Kaspar et al., (2010).

In the present study results of antibiotic sensitivity tests of the isolated *E.coli* showed that, Cefiquinom, Gentamycin, Enrofloxacin gave high sensitivitie results, as show in table (8). These nearly similar with Ahmed *et al.,* (2006), and dis agree with EI-Mahrouk and Zaki, (2005). These unsimilar results were attributed to different types of antibiotics groups used by other researchers in addition to physiological differences between infected animals, also misused of antibiotics in treatments without applying the culture and sensitivity test play an important roles in sensitivity effect of choosing drugs. Meanwhile Streptomycin, Amoxicillin, Cloxacillin, Penicillin and Ampicillin were the most antibiotics resistant drugs used for clinical mastitis treatment these results. These results were in agreement with Ayman et al., (2012), and (Bagré et al., 2014). This difference may be due to effect of weather, locations, systems of the farms and manegment. The PCR technique showed that high virulence of *E.coli* is mostly due to its ability to produce a large number of virulence factors that can contribute to different ways to their pathogenicity (Kempf et al., 2016). The real-time PCR was used by Jenkins *et al*., (2012) for detection and characterization of verocytotoxigenic *E.coli*, they found that this test is effective, rapid, screening method for the diagnosis of *STEC* from milk specimens. Chui *et al*, (2013) reported that real-time PCR method may used as the "gold standard" for diagnosis of serotyped *E. coli* (O20, O27, O111, O125, O126, O146 and O157). *E.coli* produce shiga toxin is now one of the causes of pathogenesis worldwide, cause damage in udder tissue in mastitis infection (Lira et al., 2004) In the current study, real time PCR assay was used to confirm the diagnosis of the *E. coli* by using five genes and the results of PCR assay coincide with those of bacterial culturing and serotyping methods, which indicated that PCR

assay was more sensitive for detection of this organism. This result is compatible with Rebekka *et al*. (2006) who recorded a accuracy of real-time PCR for detection of *E. coli* O111 and O157 and they concluded that this assay was quick diagnostic methods for the presence or absence of *E. coli* strains. The molecular results for *E.coli* PCR using sets of primers was used for genotypic detection of virulence genes that may play a role in pathogenicity of *E.coli.* We studied five genes: *pho*A, *omp*A, *fim*H, *Stx1* and *Stx2*. PCR results showed that the genes: *pho*A, *omp*A and *fim*H were detected in five selected strains, while the genes, *Stx1* and *Stx2* were negative in this same strains (fig.1, 2 and 3). *Stx1* and *Stx2* gens of virulent *E.coli* were detected from another tow tested strains of *E.coli* isolated microorganism, shiga prevalence of *shiga toxin* were in (O157 and O111) (fig.4). All studied strains were gave a positive results for virulence *E.coli* genes, *pho*A ,*omp*A and *fim*H in 5 studied strains (100 %) as fallow: (tow strains of O27 / 28.6%) ,(one strain of O125 / 14.3%) ,(one strain of O126 / 14.3%) , and (one strain of O146 / 14.3%). Meanwhile *Stx1* and *Stx2* virulence genes were detected in 2 studied strains (28.6 %) represented by O111 and O157, (table 9). These results were not in agreement with Momtaz et al, (2012), *STEC* 15.06%, due to good hygiene and management and good environment conditions in their studied farms, in addition to applaye their study on a wide range of samples, which gave a low % of detected virulence genes. Meanwhile Whitelegge et al, (2014) choosed *omp*A and *pho*A virulence gene of *E.coli* bacteria and he said that this two genes are most important virulence genes of coliform infection. This idea was in agreement with our study, which concentrate on investigation of same virulence gene. *fim*H and *pho*A genes also are from an important virulence gene of *E.coli*. Our study gave a result of 100% detection from all studied strains for two examined genes .This result was similar to those detected by Nechaeva et al, (2017) and Song et al, (2017). From results of the present work it could be concluded that, clinical mastitis is a serious disease of cattle cows with economic and public health importance at Egypt Governorate. *E.coli*, mainly produce shiga toxin are the most common causes of both clinical and subclinical mastitis. Cefiquinom and Gentamycin were the most proper antibiotics with the highest in vitro efficiency against isolated *E.coli* and considered the drugs of choice for treatment of clinical mastitis. Also, PCR could indicated that, *pho*A, *omp*A, *fim*H genes was detected in all studied strains (100 %). *Stx1* and *Stx2* virulence genes were detected in 2 studied strains (28.6 %). Bacteriological and molecular studies of most important microorganisms causing clinical mastitis are very important way to approach the cattle infection agents and control this very dangerous and economic problem which effect on human health, by consuming direct milk or milk products.

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