RESEARCH ARTICLE

STUDIES ON ECHERICIA COLI ISOLATED FROM MASTITIC CATTLE AND COMPARATIVE REVEALANCE TO HUMAN.

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A total of 50 clinical mastitic milk samples, 42 sub clinical mastitic milk samples (positive with California Mastitis Test) and 40 hand swabs from contact human were collected from different dairy farms at Gharbia governorate and investigated bacteriologically to isolate Ecshericia coli. Among clinical mastitic samples a total of 5 isolates E. coli (10%), from sub clinical mastitic samples 3 isolates (7.4%) were detected, while 3 isolates (7.5%) were recovered from contact human hand swabs. E. coli isolates were serotyped under 6 different O serotypes (O₂7 and O₅₅) isolated from contact human while (O₆, O₈₆, O₁₁₄, O₂₇ and O₁₅₇) isolated from mastitic milk. Antibiotic sensitivity revealed that all isolates were fully susceptible to enrofloxacin, ciprofloxacin, while all isolates were fully resistant to penicillin. E. coli serogrouped isolates were subjected to PCR for detection of Stx1 and Stx2 genes. 3 out of 7 serogrouped isolates (42.85%) were carried Stx2 gene (O₅₅ and O₂₇ from contact human and O₈₆ from mastitic milk) while Stx1 gene was not detected. phylogenetic analysis for the sequence data of the Stx2 gene of E. coli serogroupes revealed that Stx2 gene isolated from mastitic milk of cattle is closely identical (100% identity) to Stx2 gene isolated from contact human. In Conclusion, isolation of STEC from cattle might have potential pathogenicity for human. So that contact human should use sound hygienic measures during milking and management of these animals to avoid zoonotic infection.

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Introduction:-

Mastitis is known by an inflammation of the mammary gland and is the leading one, that can contribute to reduce milk production and it is one of the common problems of dairy (Fekadu, 1995; Mekonnen et al., 2005.) . It is obviously an important factor that limits dairy production. Mastitis resulted in financial loss due to reduction of milk yield, discarded milk following antibiotic therapy, veterinary expense and culling of mastitic cows (Radostitis et al., 2007).
It is primarily caused by an invasion of mammary tissues by pathogenic microorganisms through the teat canal resulting in physical, chemical, pathological changes in glandular tissues and milk (Quinn et al., 2002; Radostitis, 2007).

*Ecshericia coli* is one of the most common causes of bovine clinical mastitis. The incidence of *E. coli* mastitis has increased in some countries in recent years (Green et al., 2005). It is a major problem in lactating dairy cows (Kobori et al., 2004).

Environmental contamination with faces is the main source of mastitis-causing *E. coli* bacteria (Nemeth et al., 1994). The controls of mastitis in dairy herds are accomplished in part with the aid of Antibiotics (NMC, 1999). Public hazards associated with the consumption of antibiotic contaminated milk results in allergic responses, changes in intestinal flora and development of antibiotic resistant pathogenic bacteria (Thirapatsakun, 1999).

Virulence factors of the bacterial strain can give it a chance for colonization, multiplication and survival in udder in the face of host defense mechanism (Kaipainen et al., 2002). The shiga toxin producing *E. coli* (STEC) strains can cause mastitis in bovine and reduce milk quality for human consumption (Montaze et al., 2012). Many studies concluded that the STEC strains are the most prevalent resources for milk poisoning (Solomakos et al., 2009). About 82% of the STEC strains of animal origin belong to similar serotypes detected in humans, and 51% of these belong to serotypes related to human infection with HUS (Blanco et al., 2004a). Also, other infection routes may occur through direct contact with carrier animals and indirect contact with contaminated environments (Keen et al., 2006).

The objective of this study was to apply bacteriological and molecular studies on *Ecshericia coli* isolated from mastitic cattle and comparative revealance to human contact.

**Material and methods:-**

**Sampling:**
A total number of 50 mastitic milk samples, 42 positive California mastitis milk samples and 40 hand swabs from contact human were collected from different dairy farms from Gharbia governorates.

**Milk samples:-**
Mastitis milk samples were collected aseptically into screw capped bottles and kept at 4°C until microbiological examination. Twenty five ml from each sample were homogenized with 225 ml of buffered peptone water (BPW) for pre-enrichment and incubated at 37°C for 24 h (Addis et al., 2011a).

**Contact human hand swabs:-**
Moistened sterile swabs were rolled over the palm of hands, finger tips, nails and area between fingers. Each swab was inserted in tubes containing BPW for pre-enrichment.

**Bacterial isolation by cultivation:-**
A loopful from the pre-enriched culture homogenate in BPW was streaked onto the surface of MacConkey’s agar, Eosin Methylene blue (EMB) agar media. The inoculated plates were incubated at 37°C for 24 to 48 hours then examined for bacteriological growth. Greenish metallic shiny colonies on the plates were purified on nutrient agar slants and incubated at 37°C for 18-24 hours for further identification. (Ojo et al., 2010).

**Serological identification of *E. coli* isolates:-**
The isolated strains of *E. coli* were identified serologically by using polyvalent and monovalent antisera for diagnosis of pathogenic serotypes according to Varnam and Evans, (1991 ).

**Antimicrobial susceptibility testing:-**
according to (Quinn et al., 1994) and (Winn et al. 2006) *E. coli* isolates were examined in vitro for their susceptibility to the following antimicrobial discs: enerofoxacin (Enr 10 ), ciprofloxacin (Cip 5), penicillin (P 10), amoxicillin/Clavulanic acid(Amc 10), oxytetracycl (OT 30), gentamicin (Gen 30) and sulpha trimethoprim (Sxt 25).

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Extraction of bacterial DNA:
DNA was purified according to QIAamp DNA mini kit instructions.

duplex PCR for identification of Shiga toxin genes (Stx1 & Stx2):
Purified DNA of E. coli isolates was subjected to a duplex PCR for the identification of Shiga toxin genes (Stx1 & Stx2) using specific oligonucleotide primers according to (Dipineto et al., 2006) as shown in the table (1) and agarose gel electrophoreses according to (Sambrook et al., 1989) with agarose gel (1.5 g). The PCR condition for amplification was conducted according to (Dipineto et al., 2006). Briefly, initial denaturation was performed at 94˚C for 5 min followed by Secondary denaturation at 94˚C for 30 sec., annealing at 58˚C for 45 sec. and extension at 72oC for 45 sec. No. of cycles (35) and the final extension was carried out at 72oC for 10 min.

Table (1): Designing of Shiga toxin genes primers for E. coli

| Target gene | Sequence                  | Amplified product | Reference     |
|-------------|---------------------------|-------------------|---------------|
| Stx1        | F  ACACTGGATGATCTCAGTGG   | 614 bp            | Dipineto et al., 2006 |
|             | R  CTGAATCCCCCTCCATTATG   |                   |               |
| Stx2        | F  CCATGACAACGGACGACAGTT  | 779 bp            |               |
|             | R  CCTGTCAACTGAGCAGCATTTG |                   |               |

DNA Sequencing for Stx2 gene isolated from mastitic cattle and contact human:-
A purified RT-PCR product was sequenced in the forward and/or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817. A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was initially performed to establish sequence identity to GenBank accessions.

Phylogenetic analysis:-
A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Laser gene DNAStar software Pairwise, which was designed by (Thompson et al., 1994) and Phylogenetic analyses were done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

Results:-
Incidince of E. coli isolates on the examined samples:
A total of 11 E. coli isolates were isolated from the examined samples. From clinical mastitic milk samples E. coli were 5 isolates (10%), from sub clinical mastitic milk samples 3 isolates (7.4%), while from contact human hand swabs E. coli were 3 isolates with percentage of (7.5%). E. coli isolates were serotyped under 6 different O serotypes (O 27, O 55, O6, O86, O114 and O 157 ). O 27, O 157, O86, O114 from mastitic milk samples and O 27 and O 55 from contact human hand swabs.

Antibiotic sensitivity determination
All E. coli isolates were fully susceptible to enerofloxacin (100%), followed by ciprofloxacin (90%) and sulphamethoxazole and gentamicin (70%). While moderate sensitivity to oxytetracyclin (40%). On the other hand all isolates were fully resistant to penicillin and amoxicillin/Clavulanicacid.

3.3. Detection of Shiga toxin genes in E. coli serogroups
E. coli serogrouped isolates were examined for detection of Shiga toxin virulence genes by duplex PCR. stx2 gene was detected in STEC isolates (O27, O55 ) from contact human and in O86 from mastitic milk while stx1 gene was not detected at all as shown in figure (1) and (2).

Results of sequencing of Sxt2 gene of E. coli isolated from cattle mastitis and contact human hand swabs:
Figure (3) demonstrated the identity and the diversion percent against the selected sequences , it revealed that Sxt2 gene (MG656983) isolated from mastitic milk of cattle (sample 1) was closely identical (100% identity) to Sxt2 gene (MG656984) isolated from contact human (sample 2). However they were showed identity percentage of (99.7%) with E. coli strain SWUN4041 Sxt2 (KP120720.1), E. coli strain SWUN4110 Sxt2 (KP120725.1), E. coli
strain SWUN4061 Stx2 (KP120721.1) and E. coli strain SWUN4035 Stx2 (KP120719.1) which were isolated from Yak animal. Also the percentage of identity reached (99.6%) with E. coli strain G5101 Stx2 (EF441604.1) associated with disease outbreaks in human and E. coli strain RM10058 Stx2 (KF932368.1) which isolated from brown headed cow bird. The phylogenetic tree from the nucleotide sequences (Figure 4) and the amino acid sequences (Figure 5) which represent hypotheses about the evolutionary relationships among a group of sequences. In which the length of the horizontal line connecting one sequence to another was proportional to the estimated genetic distance between the sequences. The phylogenetic analysis in this study revealed that Stx2 gene (MG656983) isolated from mastitic milk of cattle (sample 1) and Stx2 gene (MG656984) isolated from contact human (sample 2) were found in the same very short branch as they were closely related to each other and that indicate identical sequences. On the other hand they were highly related to Stx2 gene of Yak origin (KP120720.1), (KP120725.1), (KP120721.1) and (KP120719.1) and Stx2 gene of human origin (EF441604.1), (KF932368.1).

Discussion:-
The incidence of E. coli mastitis in the present study was (10%) in clinical mastitis and (7.4%) in subclinical mastitis. Our findings are in accordance with the finding of Baloch et al., 2011; Chen et al., 2012; Sylejmani et al., 2015 and Mekonnin et al., 2016 who isolated E. coli with percentage of (10.0%), (12.4%), (13.4%) and (14.29%) respectively. Higher incidence of E. coli mastitis was reported by (Nadeem et al., 2013) with percentage of (37.50%), (Rafik et al., 2014) with (25.5%) and (Chandrasekaran et al., 2014) with percent of (40.4%). Lower incidence was recorded by (Cervinkova et al., 2013) with (6.6%) and (Abera et al., 2013) with (5.71%). Incidence of E. coli mastitis is quite high. It could be due to poor hygienic conditions as it is environmental pathogen and infect the udder through the teat canal. In this study there was no predominant serogroup among the serotyped strains of E. coli and this agree with (Linton et al., 1984; Valenete et al., 1988) also agree with (Amira et al., 2013) in Egypt, and this emphasized that serotyping of E. coli not of high significance in mastitis cases characterization. This observation had been reported by (Bradley et al., 2000; El-Mahronki et al., 2006). E. coli isolates detected in this study were serotyped to different serogroups as O27, O55, O157, O86, O114 and O6. O86 and O114 are enteropathogenic E. coli (EPEC) strains and were also isolated from lactating cow milk by (Abdallah et al., 2014; Abdel kerim et al., 2015). While (Salwa et al., 2011) detected O114 and O157 E. coli serogroups from mastitic cases. Another study (Osman et al., 2012) reported that, E. coli isolates from mastitic milk samples, belonged to four different O serogroups (O26, O86, O111, and O127). O157 was among the serotyped E. coli strains recovered from mastitis cases and this serogroup is considered as enterohemorrhagic E. coli (EHEC) is the predominant and most virulent serotype in a pathogenic subset of STEC (OIE, 2004). Cattle are considered the main reservoir of this serotype. O157 is one of the most important STEC that causes severe disease in human and was reported by many other authors as a cause of mastitis (Lipman et al., 1995; Aly, 2006). In the present study E. coli was isolated from contact human hand swabs with percentage (7.5%). It was isolated from dairy workers in Egypt with percentages (11.1%) by (Awadallah et al., 2016), 16% by (Zeinhom and Abdel-Latif, 2014) and 20% by (Gwida and El-Gohary, 2013).

Isolation of E. coli from the hand of contact human and dairy workers may be attributed to poor hygienic measures during milking and poor personal hygiene practices in dairy workers. These results indicate the possibility of transferring E. coli to milk and consequently increasing the risk of infection for milk consumers (El-Gedawy et al., 2014). Regarding to E. coli isolated from contact human serotypes O27 and O55 were isolated. Strain O55 is considered as enteropathogenic E. coli (EPEC) strain and was isolated from contact workers in Egypt by (Merwad et al., 2014). Also O55 and O27 were isolated from dairy cows, and hand swabs of dairy workers by (Awadallah et al., 2016). Severe gastrointestinal diseases in humans and complications such as the haemolytic uremic syndrome can caused by Shiga toxin-producing E. coli (STEC) and it is considered as an important group of food-borne pathogens. Chern et al. (2004) and Kobori et al., (2004). Also several authors recorded that Shiga toxins genes (Stx1, Stx2) and eae (intimin) gene are the most important virulence genes in E. coli strains isolated from bovine mastitic milk Kobori et al. (2004). Wieler et al. (1996) and Paton et al. (1998). So that in the current study multiplex PCR protocol used for detection of stx1 and stx2 genes on isolated E. coli serogroups to confirm their pathogenicity. stx2 gene was detected in STEC isolates from contact human and mastitic milk. While stx1 gene was not detected. Similar results was mentioned by (Singha et al., 2013) who reported that only one isolate was positive for shiga toxin gene (stx2), and none were harbouring stx1 gene from dairy cattle suffering from clinical/subclinical mastitis. While
Merwad et al., 2014) detected the presence of stx1, stx2, both stx1 and stx2 in E. coli isolated from contact workers and milk. On the other hand, (Murinda et al., 2004; Carneiro et al., 2006; Wenz et al., 2006) mentioned that E. coli strains isolated from cows with mastitis are negative for stx2 gene by PCR. (Osman et al., 2012) found that STEC isolates were not found in bovine mastitic milk in Egypt. While (Farhad et al., 2012; Bean et al., 2004) showed that the most common virulence gene detected in mastitic milk samples was stx1. In the present study the phylogenetic analysis revealed that Sxt2 gene (MG656983) isolated from mastitic milk of cows (sample 1) and Sxt2 gene (MG656984) isolated from contact human (sample 2) were found in the same very short branch as they were closely related to each other and that indicate identical sequences. On the other hand they were highly related to Sxt2 gene of Yak origin (KP120720.1), (KP120725.1), (KP120721.1) and (KP120719.1) and Sxt2 gene of human origin (EF441604.1), (KF932368.1). This result agreed with (Asakura et al., 2000) who recorded that Stx of STEC isolated from cattle, seagulls and flies were closely related to those of human-origin STEC. (Murinda et al., 2004) investigated that E. coli isolates from cattle and human disease shared similar toxigenic profiles. These findings suggesting that the toxin of STEC from cows might have potential pathogenicity for human. So that contact human should use sound hygienic measures during milking and management of these animals to avoid zoonotic infection.

Conclusion:
Detection and treatment of E. coli mastitis appear to be important for animal health and has a public health for contact human. No association between strain serotype and the presence of shiga toxin genes and clinical disease severity. Enerofloxacin and ciprofloxacin were the most effective antibiotics on treatment of E. coli mastitis. Phylogenetic analysis revealed that Sxt2 gene isolated from mastitic cattle is closely identical (100% identity) to Sxt2 gene isolated from contact human. These findings suggesting that the toxin of STEC from cattle might have potential pathogenicity for human. So that contact human should use sound hygienic measures during milking and management of these animals to avoid zoonotic infection.

Figure (1): Gel electrophoresis pattern for detection of Shiga toxin genes in E. coli serogroups: Lane 2: amplification of (Stx2) gene at 779 bp in sample 2 (O55) from contact human hand swabs. L: ladder. Pos: positive control (Stx1 at 614 bp, Stx 2 at 779 bp). N: Negative control.

Figure (2): Gel electrophoresis pattern for detection of Shiga toxin genes in E. coli serogroups: Lane 5, 7: positive amplification of (Stx2) gene at 779 bp in serogroupe (O86) and (O27) from contact human hand swabs. L: ladder.
Pos: positive control (Stx1 at 614 bp, Stx 2 at 779 bp). N: Negative control

Figure (3):- Identity and diversion percent of amino acid sequence of sample 1 (Sxt2 gene of E. coli MG656983) mastitic milk of cattle and sample 2 (Sxt2 gene of E. coli MG656984) isolated from contact human hand swabs with 30 of the most similar Sxt2 gene amino acid sequences from Gene bank.
**Figure (4):** Phylogenetic tree for amino acids sequence of sample 1 (Sxt2 gene of *E. coli* MG656983) isolated from mastitic milk of cattle and sample 2 (Sxt2 gene of *E. coli* MG656984) isolated from contact human hand swabs with 30 of the most similar Sxt2 gene amino acid sequences from Gene bank.

**Figure (5):** Phylogenetic tree for nucleotide sequence of sample 1 (Sxt2 gene of *E. coli* MG656983) isolated from mastitic milk of cattle and sample 2 (Sxt2 gene of *E. coli* MG656984) isolated from contact human hand swabs with 30 of the most similar Sxt2 gene amino acid sequences from Gene bank.
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