j.Egypt.vet.med.Assoc 78, no 4. 751 – 778 (2018)

VIRULENCE GENES PROFILES OF *ESCHERICHIA COLI* ISOLATES AND THEIR EFFECT ON QUANTITATIVE INTERLEUKINS EXPRESSION IN SUBCLINICAL MASTITIC COWS

By

Nesreen A.T. Allam[1*], Elshafaie, M. A. [2], Amany M. Mohamed[1], Doaa Sedky[1], Elgendy,A.[3], Amal A.M. Shehata[4], and Torky,H.A.[4]

[1]Parasitology and Animal Diseases Department, Veterinary Research Division, National Research Centre, Dokki, Cairo, Egypt.

[2]Mastitis and Neonatal Diseases Research Department, Animal Reproduction Research Institute, Agriculture Research Center, Haram, Giza, Egypt.

[3]Bacteriology Department, Animal Health Research Institute (AHRI), Agriculture Research Centre, Dokki, Giza, Egypt.

[4]Microbiology and Immunology Department, Faculty of Veterinary Medicine, Alexandria University, Egypt.

ABSTRACT

A longitudinal observational study was carried out in 10 Egyptian dairy herds (n= 2500) in different lactation seasons to explore the molecular dynamics of *E. coli* serotypes in subclinical mastitis with diagnostic significance of typing virulence genes. In addition to, quantification of the triggered cytokines expression as analytical biomarker to determine the severity and/or onset of infection. Subclinical mastitic cows (n= 1125) were diagnosed in two infectious forms: single (n= 635) and mixed (n=490), where 88% (n = 990/1125) of examined udders developed 280 bacterial isolates of both contagious and environmental pathogens. Environmental bacteria had higher incidences recorded 55.4% (n=155/280); 91.61% (n=142/155)and8.339% (n=13/155) for *E.coli* and *coagulase negative Staphylococcus* (CNS), respectively, while 44.6% (n=125/280) was reported for contagious bacteria. Clinically, multiple quarters infection per cow; were mostly caused by same *E. coli* clone. They were morphologically identified by Gram stain and Transmission Electron Microscope (TEM), biochemically characterized, their enter pathogenicity in infant mice determined, hemolysin patterns reported, *Shiga*-like cytotoxicity in Vero cell line and the cytotoxic dose 50% (CD_{50}) were also determined. *E. coli* isolates (n=142) were serologically grouped under 11 serotypes and 17 clones. Furthermore, serotypes were characterized by PCR with primers pairs

complementary to species-specific and virulence genes nucleotide sequences. PCR fragments of molecular sizes 1403 bp,180 bp, 255 bp, 384 bp, and 534 bp distinctive for 16S rRNA, *stx*$_1$, *stx*$_2$, *eae*A, and *hly*A genes, respectively, were amplified, sequenced, and then aligned against similar GenBank records to confirm the identities.

QRT-PCR revealed up regulation in expression of IL-6 and IL-8 cytokines mRNA in milk somatic cells in response to *E. coli* infections. The folds increased were higher for IL-8 than IL-6. The mean of the fold increase calculated to be 3.971 $\pm$ 5.178 and 15.732 $\pm$ 4.146 for IL-6 and IL-8, respectively. The onset of the up regulation is bacterial- dependent due to cellular subset count increase which mediated immune response. The presented study illustrated the capability of the investigated cytokines as biomarkers of subclinical stage of mastitis. Nonetheless, quantification of expression of additional cytokines genes upon udder subclinical infection will improve the outline of these analytical sensors of mastitis, hence, comprehensive information on onset of infection even regarding asymptomatic individuals will be developed with regulatory update on udder health condition. In conclusion, diagnosis of subclinical mastitis should not dependent on bacterial colonies count per sample, but additionally on bacterial secretory excretory toxins detection and host cytokines response estimation.

Keywords:

Subclinical Mastitis, Cytokines, 16S rRNA, *Stx*$_1$, *Stx*$_2$, *hyl*A, *eae*A, qRT-PCR, Egypt.

INTRODUCTION

Mastitis is multifactorial costly disease of dairy farms; however, controls are largely depend on the pathogen identity and hast immunity **(Sipka *et al*., 2014; Thompson-Crispi *et al*., 2014; Günther *et al*., 2016 and Younis *et al*., 2016)**. Subclinical inframammary infections reported 60% of medical treatments in dairy herds **(Hamouda *et al*., 2014 and Allam *et al*., 2017 and 2018)**.An increase in the frequency of these infections which proposed epidemiological shift due to modified virulence factors of mutant strains were reported **(Allam *et al*., 2009; Hamouda and Allam, 2011; Ibrahim *et al*., 2012; Hamouda *et al*., 2014; Allam *et al*., 2017 and Hakim *et al*., 2017)**. Moreover, *E. coli* sensing of the udder immune responses is not completely understood yet **(Günther *et al*., 2016 and Allam *et al*., 2017, 2018)**. In Egypt, bacteriological, epidemiological and clinical studies indicate that coliform is one of the major agents of bovine mastitis **(El-Rashidy *et al*., 1986; Abdel-Fattah *et al*., 2008 and El-Khodery and Osman, 2008)**. Despite the notable emergency of variant *E. coli* sub-species

in dairy problems, the higher 'stress' susceptibility in lactating herds which suppress the immune reaction, miss use of antibacterial, and the difference in animal husbandry methods between farms in Egypt exaggerated the outcomes. Neither subclinical mastitis due to variant *E. coli* sub-species regarding the regulation of interleukins expression nor the trigged immune cascade due to infections were thoroughly investigated **(Allam *et al.*, 2018 and Ishizaki *et al.*, 2018)**. *E. coli* possesses several recognized and putative virulence factors that donated to its pathogenic prospective **(Hammoda and Allam, 2011; Hammoda *et al.*, 2014; Allam *et al.*, 2009, 2018 and Ishizaki *et al.*, 2018)**. *E. coli* proteomic (soluble and particulate factors) are increased during bacterial stress, during the early stages of bacterial colonization in the mammary gland. Therefore, resulted in hosts diverse symptoms and immune responses **(Fu *et al.*, 2013; Gilbert *et al.*, 2013; Jensen *et al.*, 2013; Günther *et al.*, 2016 and Younis *et al.*, 2016)**. Despite that, the mammary gland is not natural habitat for *E. coli*, many strains still capable of surviving in the mammary gland for short duration, leading to either effective antibacterial responses or harmful inflammatory responses **(Yang *et al.*, 2008; Bonnefont *et al.*, 2012; Ibrahim *et al.*, 2012 and Porcherie *et al.*, 2012)**. The identification of *E. coli* is routinely accomplished by cultural and biochemical markers such as sugar fermentation, enterotoxigenic effects, proteolytic activities, haemolytica reactions, in addition to, Gram stain which could not overcome atypical bacterial variants in phenotype that had been reported to occur **(Hammoda and Allam, 2011; Hammoda *et al.*, 2014; Allam *et al.*, 2009, 2018 and Ishizaki *et al.*, 2018)**. Alternative analysis of species-specific stretches of the bacterial genomes were developed **(Allam *et al.*, 2018 and Ishizaki *et al.*, 2018)**. Several PCR-mediated proof of identity were previously conducted using; 16S rRNA gene sequence **(Ishizaki *et al.*, 2018)**, the 16S-23S rRNA intergenic spacer region (ISR) **(Siarkou *et al.*, 2011; Allam *et al.*, 2009; Hammoda *et al.*, 2014 and Allam *et al.*, 2018)**, and the β subunit of RNA polymerase encoding gene *ropB* as molecular targets **(González-González *et al.*, 2017 and Xiao *et al.*, 2017)**. More genetic elements were applied for molecular identification and reclassification to species level **(Ishizaki *et al.*, 2018)**. The *shiga* toxins types 1 and 2 (stx_1 and stx_2) genes **(Allam *et al.*, 2009)**, intimin protein encoded by *eae*A chromosomal gene **(Ghanbarpour and Oswald, 2010)**, in addition to, the plasmid-encoded enterohemolysin protein gene (*hyl*A) is common among pathogenic *E. coli* strains isolated from animals **(Gyles, 2007)**. Subclinical mastitis mostly lacked the contact transmission of infection, with

frequent eradication in lactating cows **(Almeida *et al*., 2011; Hogeveen *et al*., 2011; Hamouda *et al*., 2014; Günther *et al*., 2016 and Allam *et al*., 2017 , 2018)**. Notwithstanding that some *E. coli* strains had persistence and/or recurrence in the udders **(Almeida *et al*., 2011; Jensen *et al*., 2013 and Younis *et al*.,2016)**. Since, *E. coli* could provoke inflammatory vigorous stimulation. This activation is characterized by high concentrations of cytokines resulting in local and generalized immune response.Mammary immunity is nominated to react rapidly to pathogen by the activation of numerous Pattern Recognition Receptors (PRR) by the Microbe-Associated Molecular Patterns (MAMP) **(Goldammer *et al*., 2004; Ibrahim *et al*., 2012 and Gilbert *et al*., 2013)**. *Gram-negative* bacterial infection induced the expression of Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4), Tumor Necrosis Factor-α (TNF-α), Interleukin-1α (IL-1α), IL-4, IL-6, IL-8, IL-10 and the NFκβ pathway activation **(Burvenich *et al*., 2003; Yang *et al*., 2008; Hamouda and Allam, 2011; Blum *et al*., 2012; Bouchard *et al*., 2012; Fu *et al*., 2013; Gilbert *et al*., 2013; Wang *et al*., 2015; Younis *et al*., 2016 and Allam *et al*., 2018)**. In Egypt, livestock productivity is below their genetic potential because of less proper management and less controlled infections **(El-Awady and Oudah, 2011; Ibrahim *et al.*, 2012 and Abd-Elrahman, 2013)**. Improving immune response of mammary gland will help in mounting support strategies to combat the infections mainly those developed by *E. coli* the opportunistic infectious agents; hence their pathogenicity is hooked on the discrepancy in host immunity **(El-Awady and Oudah, 2011; Ibrahim *et al.*, 2012; Abd-Elrahman, 2013 and Allam *et al*., 2018)**. The main objective of present study was to characterize the differences in the response of udder to *E. coli* serotypes at the subclinical infection. In addition to, their impact on the interleukins expression in the mammary gland. Which occur prior to the detectable onset of the inflammatory response, and then determine the ultimate outcome in terms of pathogenic squeals leading to acute, subclinical or chronic mastitis (analytical biomarkers of infection).

MATERIAL AND METHODS

1-Ethical approval:

All animal experimental procedures were in accordance with the ARRIVE guidelines and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of laboratory animals **(NIH Publications No. 8023, revised 1978).** In addition, these adopted ethical guidelines are

compiled with those of the national research center (No. 11040301) and ministry of higher education and scientific research (No. 50/4/10) guidelines for the care and use of laboratory animals in Egypt offered by National Research Centre, Egypt. In addition to,

2-Animal Population and Geographical Scope of the Study:

Sample collection and California mastitis test:

The present study was carried on subclinical mastitic Holstein cows collected from 10 dairy farms from governorates in upper and Lower Egypt during 2 years, 2015 till 2017, to investigate the prevalence of mastitis pathogens in these governorates. Their age ranged between 2.5-7 years in different lactation seasons. Animals were managed similarly and fed for high production. Cows in studied farms (n= 2500) were examined periodically to monitor status of mastitis, the udder and teats of each cow were examined by visual inspection and palpation so abnormal findings were recorded. Quarter's milk samples collection were strictly standardized: after fore stripping about 10 ml of milk, visual inspection and evaluation of milk with the California Mastitis Test (CMT) was carried out **(Schalm *et al*., 1971)**.

The first milk sample of each quarter was taken for SCC evaluation; afterwards, aseptic samples of each quarter were collected for bacteriological then molecular analysis using standard procedures **(NMC, 1999)**. RNA later (Sigma-Aldrich) was added to each samples after collection then preserved until RNA isolation. The samples were transported to the laboratory at 4 °C. Samples destined for molecular testing were stored at -80 °C until further use **(Allam *et al*., 2018)**, while those for SCC evaluation at 4 °C **(Quinn *et al*., 2002)**.

After sample collection, the udder and teats of each cow were examined by visual inspection and palpation, hence, abnormal findings were recorded.

Somatic cell count analysis:

Milk samples of all quarters were kept at 4 °C, and then were analyzed within 24 h after collection. For the evaluation, they were pre-warmed at 37 °C for 10 min and then measured by a Fossomatic 5000 (Foss) **(IDF, 1999)**. For SCC controls, four bacteriologically negative milk samples of each herd were randomly selected. The log10 (SCC) values had classified udders status into three categories: Normal, subclinical, and mastitic udders recorded SCC $\leq 4\times10^5$, $\geq 4\times10^5$, and $\geq 1\times10^6$ cells/ml, respectively **(Hamann, 2003)**.

3-Bacteriological Characterization and Serotyping

Isolation and identification of *E. coli* isolates:

The specimens from subclinical bovine mastitis by CMT/SCC were studied. After thawing at 37 °C for 5 min, equal volumes of 10 µl of each quarter milk were simultaneously plated on Eosin methylene blue(EMB) agar, Xylose Lysine Deoxy cholate (XLD) agar, *Salmonella*/Shigella agar (SS agar),5% Sheep-blood agar and MacConkey agar (Biolife Laboratories, Milano, Italy) and incubated aerobically at 37 °C **(Quinn *et al*., 2002)**.

Standard procedures were used for isolation and identification of bacteria from all cultured milk samples as previously described by **(Quinn *et al*., 2002)**. The isolates were identified as *E. coli* based on colony morphology, color, Gram stain, Transmission Electron Microscope (TEM) examination **(Allam *et al*., 2018)** and standard biochemical tests **(Quinn *et al*., 2002)**. All of the *E. coli* isolates were stored in Luria-Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at -80 °C till used.

Somatic cell antigen "O" serological typing:

E. coli isolates were serotyped for their somatic cell antigen"O"contents by slide agglutination test (Denka Seiken Co. Ltd., Tokyo, Japan). Test was done as outlined by **Edwards and Ewing (1972)** using standard polyvalent and monovalent *E. coli* antisera.

4-Profiling of *Shigella*-Like Toxins.

Vero cell lines cytotoxicity:

The overall cytotoxic effect of serotyped *E. coli* isolates was studied on Vero cell line **(Giugliano *et al*., 1982)**. *E. coli* O157:H7 was used as positive control during the study. After incubation of Luria broth cultures (Oxiod) for 24 hours at 37 °C with agitation (Shellab), bacteria were pelleted by centrifugation (10000 *xg*, 15 min at 4 °C, Jouan MR 18-12), then supernatants were collected, then filter-sterilized through 0.22 µm pore-size membrane syringe filters (Sartorius).Two fold dilution of obtained filtrates by MEM cell culture medium (Earle's MEM containing 100 U/ml streptomycin, 1% nonessential amino acids, and 1 mM Na pyruvate; Sigma Aldrich) was prepared **(Allam *et al*., 2009)**. At that point, 100 µl/well were transferred in to 96-wells tissue culture plates (Coaster) seeded with Vero cell line (Green Monkey Kidney Cells) monolayers (4×10^4 cells/well) which were obtained from Animal Vaccines and Serum Production Research Institute, Agriculture Research center, Abbasia, Cairo. Cytotoxic effects were determined and recorded after 12, 24, and 48 hours of

incubation at 37 °C in 5% CO_2 (Haraeus) by microscopic examination (Lilly) of the Vero cells, where the cytotoxic dose titer (CD_{50} unit) was defined **(Allam *et al*., 2009)**.

5-Profiling of Virulence and Pathogenicity Patterns.

Haemolysin production:

All *E. coli* isolates were streaked on sheep blood agar plates, incubated at 37 °C for 24 hours then examined for the developed haemolysis **(Koneman *et al*., 1996)**.

Enterotoxin production:

A 0.1 ml of each *E. coli* isolates filtrate was injected intra-abdominal into milk filled stomach of 3 mice/isolate each are 2-4 days old, incubated for 4 hours, then their entire intestines were removed and weighted **(Allam *et al*., 2009)**.The assay was considered positive for enterotoxin if the ratio of combined weight of the intestines of the three inoculated mice/isolate to combined weight of the remaining body weight was >0.083 **(Guarino *et al*., 1987 and Erganis *et al*., 1989)**.

Pathogenicity assay:

In 40 Albino white mice aged 30-37 days weighted 18-20 grams each, 100 µl equals 9×10^8 CFU/ml of each *E. coli* isolate were inoculated intra peritoneal into each mouse **(Allam *et al*., 2009)**. All internal organs were collected for both histopathological examination and re-isolation of *E. coli* strains followed by serotyping with standard antisera **(Edwards and Ewing, 1972)**.

6-Antibiotic Susceptibility Patterns:

Antibiotic susceptibilities of isolates were determined by disk diffusion method on Muller Hinton agar plate **(CLSI, 2014)**. The following commercial antibiotic disks (Oxoid) were used in illustrated concentration: Amikacin (30 µg), Ampicillin (10 µg), Cefadroxil (30 µg), Chloramphenicol (30 µg), Colistin Sulphate (50 µg), Erythromycin (15 µg), Oxytetracycline (30 µg), Nalidixic acid (30 µg), Novofloxacin (10 µg), Trimethoprim and Sulphamethoxazol (1.25 µg and 23.75 µg). Inhibition zone diameter of each antibiotic disk was measured and compared with standard zone chart according to manual of the supplier **(CLSI, 2014)**.

7-Molecular Profiles of Virulence Genes:

Oligonucleotide primers design:

PCR amplification and sequencing of the 16S rRNA gene was carried out by primers 16SUNI-L and 16SUNI-R(Metabion International AG,Martinsried/Deutschland) which were

complementary to the 16S rRNA gene sequence of *E. coli* (NCBI accession number J01859) **(Ülbegi-Mohyla *et al*.,2010)**. The virulence genes primers pairs were designed with reference to annotated sequence for *E. coli* O157:H7 accession numbers AE005174v2- 1.gbk and AE005174v2-2.gbk **(Perna, *et al*., 2001)**, in addition to, published sequence data for *stx*$_1$ **(Jackson *et al*.,1987-b)**, *stx*$_2$ **(Jackson *et al*.,1987-a)**, *eae*A **(Yu and Kaper,1992)**, and *hly*A **(Schmid *et al*., 1995)** virulence proteins genes (Metabion International AG, Martinsried/Deutschland). These primers were used also during sequencing.

Details of the nucleotide sequence and the size of the amplified product for each primer pair are listed in (Table 1).

DNA isolation:

Five to 10 colonies of each freshly streaked isolates were subsequently suspended in 180 µl Tris-EDTA buffer (Sigma Aldrich) containing 5 µl mutanolysin (10 U/µl, Sigma Aldrich). The extraction mixture **(Hamouda *et al*., 2014)** was added to each bacterial sample, incubated overnight at 56°C, and then DNA isolation was done by phase separation protocol **(Sambrook *et al*., 1989)**. The working DNA concentration was evaluated by NanoDrop 2000c (Thermo Scientific) and then adjusted to 100 ng/µl **(Hamouda and Allam, 2011)**.

Table (1): Primers used in the present study.

Primers	5`- Sequence -3`	Target Genes	Amplicon (bp)	References
E. coli Genes				
16SUNI-F 16SUNI-R	5`-AGAGTTTGATCATGGCTCAG-3` 5`-GTGTGACGGGCGGTGTGTAC-3`	16S rRNA	1403	(Ülbegi-Mohyla *et al.*, 2010)
stx_1-F stx_1-R	5`-ATAAATCGCCATTCGTTGACTAC-3` 5`-AGAACGCCCACTGAGATCATC-3`	stx_1	180	(Jackson *et al.*, 1987b)
stx_2-F stx_2-R	5`-GGCACTGTCTGAAACTGCTCC-3` 5`-TCGCCAGTTATCTGACATTCTG-3`	stx_2	255	(Jackson *et al.*, 1987a)
*eae*A-F *eae*A-R	5`-GACCCGGCACAAGCATAAGC-3` 5`-CCACCTGCAGCAACAAGAGG-3`	*eae*A	384	(Yu and Kaper, 1992)
*hly*A-F *hly*A-R	5`-GCATCATCAAGCGTACGTTCC-3` 5`-AATGAGCCAAGCTGGTTAAGCT-3`	*hly*A	534	(Schmid *et al.*, 1995)
Internal Quality Control of PCR				
L1091-F H1478-R	5`-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3` 5`-TGACTGCAGAGGGTGACGGGCGGTGTGT-3`	12S rRNA	400	(Kocher *et al.*, 1989)
mRNA Quantification of Bovine Cytokines				
IL8-F IL8-R	5`-CACTGTGAAAATTCAGAAATCATTGTTA-3` 5`-CTTCACAAATACCTGCACAACCTTC-3`	Interleukin-8	53	(Leutenegger *et al.*, 2000 & Moussay *et al.*, 2006)
IL6-F IL6-R	5`-TCATTAAGCGCATGGTCGACAAA-3` 5`-TCAGCTTATTTTCTGCCAGTGTCT-3`	Interleukin-6	47	
Normalization of qRT-PCR				
βact-F βact-R	5`-CCTTTTACAACGAGCTGCGTGTG-3` 5`-ACGTAGCAGAGCTTCTCCTTGATG-3`	β-actin	47	(Hoorfar *et al.*, 2004)

Internal quality control for PCR assays:

Specific PCR assay was applied as a semi-qual-itative control for the DNA extraction. During this reaction a target frag - ment amplified of about 400 bp from the 12S rRNA gene of the mammals' mitochondrial genome utilizing the L1091-F and H1478-R oligonucleotides **(Kocher *et al.,* 1989 and Hoorfar *et al.,* 2004)** (Table 1).

PCR protocols:

PCR mixture/isolate/gene was prepared in 50 µl total volume. Each mix contained; 2 µl template (100 ng), 50 pM of each primer, 45 µl Ready TaqMix Complete (Alliance Bio), and nuclease free water (Qiagen) to complete the total volume of the reaction. PCRs were performed in a PTC-100™ Thermal Cycler (MJ Research) using the following cycling protocol: initial denaturation at 950C for 5 min and then 40 cycles of 940C for 1 min, 580C for 1 min, and 720C for 1 min. Final extension was carried out at 720C for 7 min. A reagent blank; containing all the components of the reaction mixture with water instead of template DNA, and VT-negative

E. coli were run as controls in every PCR procedure. Cultures showing positive results by PCR were retested on two further occasions several days later to examine the reproducibility of PCR testing. Amplified products from the PCRs were electrophoresed in 2% agarose gels, stained with ethidium bromide in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.3, Sigma Aldrich). A 100 bp ladder (Jena Bioscince, GmbH, Germany) was used with each gel which documented by Image Lab (BioRad).

Sequencing of PCRs products:

Each amplicon was purified for sequencing using the ExoSAP-IT PCR Product Cleanup kit (Affymetrix, USA) according to the manufacturer's instructions. Sequencing reactions were performed with the ABI PRISM®BigDyedye™ terminator cycle sequencing kit with AmpliTaq® DNA polymerase on an MJ Research PTC-225 Peltier Thermal Cycler (Applied Biosystems, USA) as described by the manufacturer. Each sequencing reaction was repeated at least three times in both directions before being accepted for analysis. Then sequences of each PCR product was aligned with homologous GenBank records (http://www.ncbi.nlm.nih.gov) by multiple sequence alignment using the Clustal®W program **(Thompson *et al.,* 1944).**

8-Cytokines Expression in Mammary Glands

Isolation of milk somatic cells:

Aliquots of 25ml milk were centrifuged at 1000 xg / 15 min at room temperature **(Allam *et al.,* 2017),** then each pellet was collected after discarding fat layer and supernatant. Finally they were individually washed twice in sterile normal saline solution (PH 7.2) prepared in die ethyl pyro-carbonate (DEPC) treated water. At this point, each pellet was suspended in 150 µl of normal saline solution and maintained at -80 °C until used for RNA extraction.

RNA extraction and quality assessment:

Total RNA was extracted by a double extraction method first using Trizol (Invitrogen) and then RNeasy (Qiagen) column purification according to manufactures instructions. RNA integrity, purity and quantity determination was performed using Nanodrop 2000 (Thermo scientific). The residual genomic DNA was removed by DNA digestion with RNase-free DNase-I (Qiagen) at 37°C for 10 min, then heat inactivated at 95°C for 5 min, finally chilled on ice **(Sambrook *et al.,* 1989).**

RT-PCR step:

Reverse transcription of total RNAs into cDNAs copies were carried out using 1 µg of each RNA template, incubated with 1 µg of random primers (Promega) for 10 min at 65°C and then for 5 min on ice in a final volume of 10 µl as hot start reaction. Reverse transcription mixture contained 15 U of avian myeloblastosis virus (AMV) reverse transcriptase enzyme (Promega), AMV RTbuffer(Promega),4 mM deoxynucleoside triphosphate(dNTP) (Promega), and 40 U of RNasin (Promega). Each mixture was incubated for 1.5 h at 42°C then 5 min at 95°C. Diluted cDNA samples were stored at 4°C until used in qPCR reactions.

QRT-PCR assays:

Quantitect probe which had been supplied within the qRT-PCR was designed according to the manufacturer. Primers of IL-6 and IL-8 genes along with β-actin gene housekeeping gens (Table 1) utilized in this study were designed using publicly available bovine sequences and were purchased (Vivantis). Primers design considered to span an intron-exon boundary of target genes to prevent the amplification of genomic DNA **(Leutenegger *et al.,* 2000 and Moussay *et al.,* 2006).** The reaction condition for each individual gene was optimized using Syber Green PCR kit (Roche) performed in Rotor-Gene (Qiagen). The amplification was carried out in a final reaction volume of 25 µl. The qPCR protocol designed to include initial

denaturation at 95 °C/10 min, and then thermal fluctuation between 95 °C/15 sec, 58 °C/30 sec, and 72 °C/30 sec for 40 cycles as described previously **(Bougarn *et al.*,2010).**

The fluorescence signals were measured once at the end of extension step/cycle/gene. For each sample a dissociation curve was generated after completion of amplification and was analyzed to determine specificity of PCR reaction. Values for target genes were normalized by the internal positive control (β-actin). The normalization factors NFn and NFn+1 were calculated. Relative transcript quantification standard curves were plotted using a six fold serial dilution of cDNA. The relative level of expression for gene of interest was analyzed using the delta-Ct method and was normalized by dividing by a proper normalization factor **(Allam *et al*., 2017).** Analysis of the melting curve of specific PCR products was performed by slowly raising the temperature from 60°C to 95°C by means of regular fluorescence measurements, which should be distinguished from primer dimmers (dissociation temperature < 74°C) **(Seidl *et al.*, 2011-a , 2011-b).**

9-Data analysis and statistics:

A chi-square test was used to compare the prevalence of each gene profile among isolates between categories (SPSS 19). Differences between the prevalence rates were considered significant when $p < 0.05$ **(Alan and Duncan, 2011).**

RESULTS

Udders health impression:

The score of both CMT and SCC is always indicative, semi-diagnostic and informative in monitoring the epidemiological status of the dairy farms, hence, both depends on the reaction occurs between the nuclear material of cells and specialized reagents leading to gelling of the milk (Table 2).

Table (2): Epidemiological status of clinical phases of mastitis within investigated population.

Clinical Status of the Udders in Studied Farms (n= 10)	Incidence of each Clinical Phase in Studied Population (n= 2500)	CMT & SCC Score	Types of infection	Bacterial Species
Apparently Normal	**30% (n=750)**	**0-1> $4x10^5$**	**free**	**Bacteriologically negative**
Subclinical Mastitic	**45% (n=1125)**	**$24x10^5$ to $1x10^6$**	**-Single (n= 635) -Mixed (n= 490)**	**-Environmental bacteria 55.36% (n=155/280) -Contagious bacteria 31.82% (n=125/280)**
Chronic/Recurrent Mastitic	**25% (n= 625)**	**3 - 4 < $1x10^6$**	**-Single (n= 335) -Mixed (n= 215)**	**-Gram (-) 36.36% -Gram (+) 63.64 %**

Incidences of clinical phases of mastitis regarding pathogenic bacterial species:

88% (n=990/1125) of subclinical mastitic cows revealed 280 bacterial isolates on stroked agar plates; the remaining 12% represent individuals with no bacterial growth which indicates the need for special growth medium and/or requirements. Both contagious and environmental bacterial pathogens were isolated. Environmental microorganisms had higher incidences recorded 55.36% (n=155/280); 91.61%for *E.coli* (n=142/155) and8.39% for CNS (n=13/155). While 31.82% (n=125/280) incidence of infection was reported for contagious bacteria,where 68% (n=85/125), 8.8% (n=11/125) and 23.2% (n=29/125) recorded for *S. aureus*, *Str. agalactiae* and *Corynebacterium* species, respectively.

***Escherichia coli* identification and serotypes prevalence:**

E. coli displayed small colonies that were circular in shape, entire, with diameter of approximately 0.5 mm, convex and smooth. The colonies were rounded non-mucoid, bright pink-red color (lactose fermenter) on MacConkey agar. At the same time, distinctive greenish metallic sheen color on EMB agar,and bright yellow colonies on XLD agar.

The α-and β-hemolytic colonies were seen on sheep-blood agar after 24h. On the other hand, microscopically examination of Gram stained film showed *E. coli* isolates as Gram-negative, medium sized rods, arranged singly, pairs and groups, non-spore forming. Furthermore, Transmission Electron Microscope examination showed that cells were rods, motile and are arranged in clusters Fig. (1). *E. coli* isolates were serologically grouped under 11 serotypes and 17 clones designed (Table 3): O1, O8, O20, O25, O44, O78, O127, O146, O153, O157, and O159 with prevalence rates 9.85% (n=14/142), 10.5% (n=15/142), 12% (n=17/142), 12% (n=17/142), 7.04% (n=10/142), 5.63% (n=8/142), 9.85% (n=14/142), 5% (n=7/142), 12% (n=17/142), 10.5% (n=15/142) and 5.63% (n=8/142), respectively.

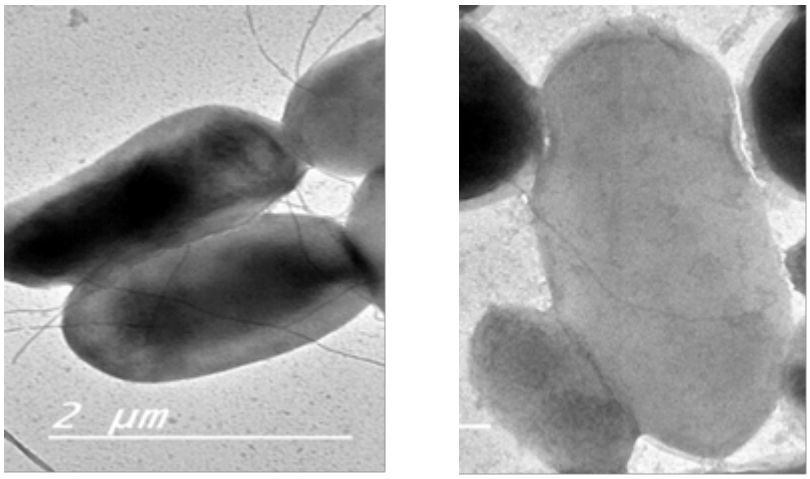

Fig. (1): Electron transmission micrograph with 2µm and magnification 2000.

Haemolysis,enterocytotoxicity,pathogenicitypatterns,and*Shigella*-likecytotoxic patterns:

All isolates were *Stxs* producers according to the recoded cytopathic effects (CPE) noted on inoculated Vero cells monolayers. Cytotoxicity of culture supernatants with CD_{50} titers, as well as other virulence and pathogenicity traits are illustrated in (Table 3).

Virulence traits molecular typing:

DNA fragments of molecular size 1403 bp, 180 bp, 255 bp, 384 bp, and 534 bp were documented in lanes representing the different serotypes of *E. coli* indicating that coding sequence for 16S rRNA, stx_1, stx_2, *eae*A, and *hly*A genes Fig. (2) are present with different incidence rates and variant clones' population as presented in (Table 3). Separate bands corresponding to the expected sizes were amplified in the control positive lane (*E. coli* O157:H7). On the other hand, negative control lane (reagent blank) was negative for all coding sequences by PCR amplification.The identities of amplified fragments were confirmed

by sequences alignment against homologous records in GenBank data for similar open reading frames sequences with 96-100% similarities.

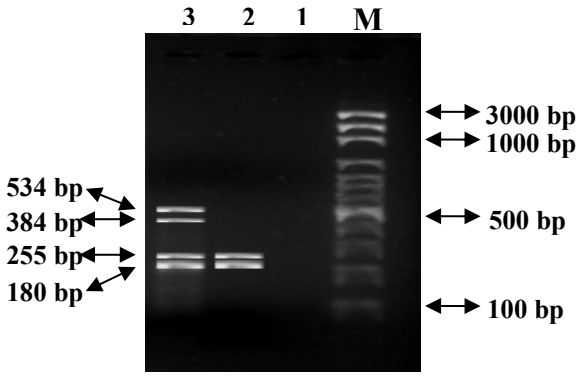

Fig. (2): PCR amplification of *stx*$_1$, *stx*$_2$, *eae*A, and *hly*A genes from *E. coli* serotypes, amplicons sizes of 180 bp, 255 bp, 384 bp, and 534 bp, respectively. Lane M: Molecular sizes 100 bp DNA Marker, Lanes 1: Control negative blank reagent, Lane 2: *E. coli* serotype encoding *stx*$_1$ and *stx*$_2$ gene, and Lanes 3: *E. coli* serotypes encoding all virulence genes.

Table (3): Pathogenicity, enterocytotoxigenicity, haemolysis, clonality and CD_{50} of *E. coli* serotypes with variant clones' and virulence genes profiles.

E. coli Isolates (n= 142)		Pathogenicity		Enterocytoxicity		Haemolysis			Clonality	CD_{50}/Clone		Virulence genes profiles				
Serotypes	Incidence %	+Ve*	%	+Ve*	%	+Ve*	Type	%		A	B	16S rRNA	*stx*$_1$	*stx*$_2$	*eaeA*	*hlyA*
O1	9.85	4/6	66.7	5/6	83.33	5/6	α, β	83.33	A, B	128	128	+	+	+	-	+
O8	10.5	5/6	83.33	5/6	83.33	5/6	α, β	83.33		32	64		+	+	-	+
O20	12	2/7	28.6	1/7	14.26	5/7	γ	71.43	one	64			-	-	-	+
O25	12	3/6	50	4/6	66.7	6/6	γ	100		8			-	-	-	+
O44	7.04	6/7	85.7	6/7	85.7	3/7	α, β	42.9	A, B	32	128		±	+	-	-
O78	5.63	6/7	85.7	5/7	71.43	6/7	γ	85.7	one	4		+	-	-	-	+
O127	9.85	3/7	42.9	4/7	57.14	5/7	α, β	71.43	A, B	64	128		+	+	-	+
O146	5	3/6	50	3/6	50	6/6	α, β	100	one	8	64		-	-	-	-
O153	12	4/6	66.7	4/6	66.7	4/6	α, β	66.7	A, B	4	128		±	-	-	+
O157	10.5	6/7	85.7	6/7	85.7	6/7	α, β	85.7	one	8	64		+	+	+	+
O159	5.63	5/6	83.33	4/6	66.7	4/6	α, β	66.7	A, B	32	128		+	-	+	+

Recovery pattern regarding the antibiogram in subclinical mastitic cows:

The *in vitro* reactivity pattern of the 11 *E. coli* serotypes against antibiotic are illustrated in chart (1). While, the recovery pattern of subclinical mastitic cows in response to treatment (*in vivo*) with antibiotics is illustrated in chart (2). Subclinical individuals were partially with resistant bacteria; proved by laboratory isolation of milk bacteria post treatment, to Ampicillin and Trimethoprim and Sulphamethoxazol (100%), chart (1,2). In addition, they were suffering isolates highly susceptible to recovery (absolute sensitive bacteria) to Chloramphenicol (100%), but moderate susceptibility (highly sensitive bacteria) to Amikacin (90%), Cefadroxil (90%), and Norfloxacin (90%), in which both groups of drugs were of choice on recommendations for treatments, charts (1 , 2). While different recovery percent due to variant bacterial susceptibility (sensitive bacteria) were recorded with Colistin Sulphate (60%), Oxytetracyclin (60%), and Nalidixic acid (60%), and Erythromycin (40%), charts (1, 2).

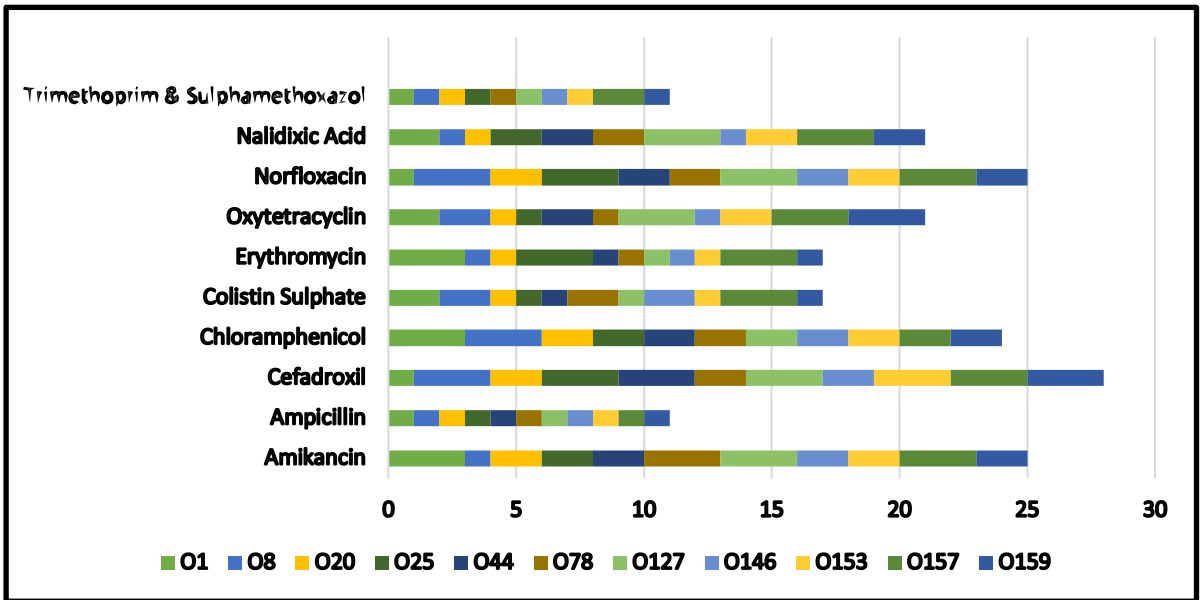

Chart (1): *In vitro* antibiogram pattern of the 11 *E. coli* serotypes characterized during the study.

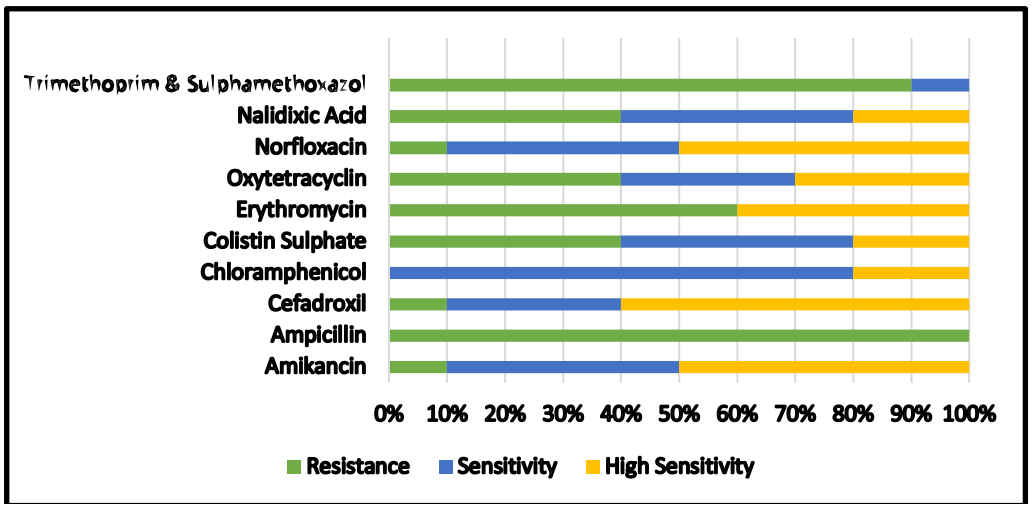

Chart (2): In vivo recovery profile in response to treatment with antibiotics against microbiota of subclinical cases.

Cytokines expression in subclinical mastitic cows'udder with regards to *E. coli* serotype:

The reaction efficiency for all housekeeping and cytokines genes of interest ranged between 0.95 and 1.03. The qRT-PCR results presented up regulation of cytokine genes expression, in both IL-6 and IL-8 in all studied cows despite the difference in *E. coli* serotype. However, the folds increased were higher for IL-8 than those recorded for IL-6 (Chart 3). The mean of the fold increase calculated to be 3.971 $\pm$ 5.178 and 15.732 $\pm$ 4.146 for IL-6 and IL-8 (Means St. Dev. 2^{-} ($\Delta\Delta$ct), respectively.

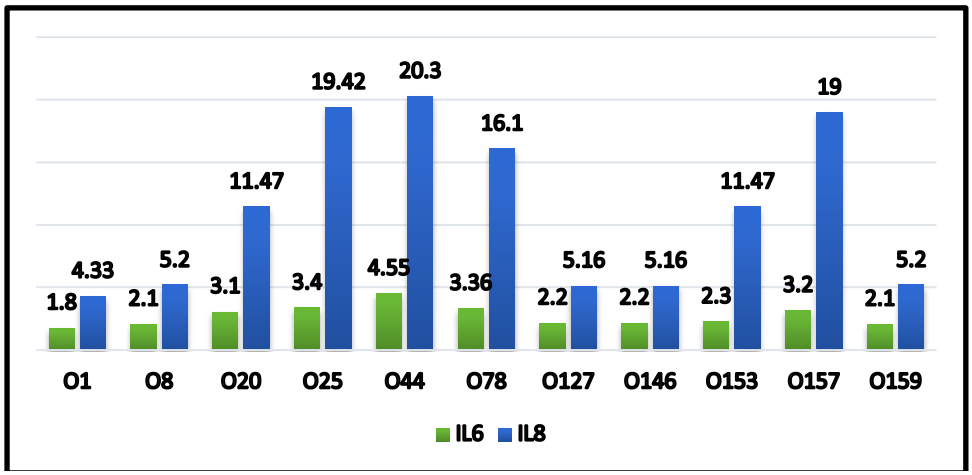

Chart (3): Level of up regulation mRNA expression of IL6 and IL8 cytokines genes under study in udders somatic cells quantified by qRT-PCR.

DISCUSSION

Mastitis in lactating cows remains a serious problem in the dairy industry **(Kościuczuk *et al.*, 2014 and Allam *et al.*,2018)**. Management procedures have decreased the incidence of contagious infections especially*Staphylococcus* and*Streptococcus* species,yet the environmental as well as opportunistic bacteria are still problematic in some Egyptian provinces **(El-Awady and Oudah, 2011)**. This is still justified in presented results. On the other hand, the obtained outcomes disagreed with previous researches on the incidence of mastitis causing pathogens, despite the practice of teat dipping and dry cow treatment **(Burvenich *et al.*, 2003;Allam *et al.*, 2009; Hamouda and Allam, 2011; Ibrahim *et al.*, 2012; Abd-Elrahman, 2013; Hammouda *et al.*, 2014; Sipka *et al.*, 2014; Thompson-Crispi *et al.*, 2014; Günther *et al.*, 2016; Younis *et al.*, 2016 and Allam *et al.*, 2017 , 2018 and Hakim *et al.*, 2017)**.

In the present study, *E. coli* species had proved their high capability to acquire exogenous virulence genes, providing an evolutionary pathway for pathogenicity **(Ghanbarpour and Oswald, 2010; Allam *et al.*, 2017 and 2018 and Hakim *et al.*, 2017)**. Hence, above 90% of the subclinical mastitis were due to pathogenic *E. coli*. On the other hand, *E. coli* isolates from dairy herds are not biochemically different from others and do not have specific O-serogroup **(Burvenich *et al.*, 2003; Abdel-Fattah *et al.*, 2008; Blum *et al.*, 2008 and Allam *et al.*, 2009, 2017 and 2018)**. *E. coli* species causing mastitis belong to a very broad range of O serogroups **(PatonandPaton,1998;Agbodaze, 1999; Quinn *et al.*,2002;Gilmour *et al.*, 2007;Blum *et al.*,2008 and Allam *et al.*, 2009, 2017 and 2018)**. Which was also confirmed in the presented results. In addition, O-antigen typing was thoughtful in the epidemiology of infections and allows differentiation between pathotypes in between farms as well as governorates. In the present study 11 serogroups were characterized then subdivided into 17 clones by phenotypic and molecular profiling. It is clearly obvious that non-O157 serogroups are prospectively growing in the epidemiological dynamic of *E. coli* worldwide as well as in Egypt **(Allam *et al.*, 2009; Ghanbarpour and Oswald, 2010; Hamouda and Allam, 2011 and Allam *et al.*, 2018)**. Moreover, veterinary *E. coli* isolates were classified as A and B1 serogroups mainly, yet a lower proportion belong to B2 and D group, which was in agreement with the obtained results **(Escobar-Paramo *et al.*, 2004a and 2004b)**.

Additionally, non-B2 phylogenetic groups of *E. coli* serotypes isolated in the present study shown their transformation to antibiotics resistance more eagerly following disproportionate

use of antibacterial. Therefore, the simultaneous occurrence of recurrent/chronic mastitis with A and B1 strains but not B2 strains was not an odd result in studied farms **(Allam *et al*., 2009; Hamouda and Allam, 2011 and Allam *et al*., 2017 , 2018)**. Therefore, regular screening for SCC involving the entire herd is fundamental for professional and efficient control, including bulk and udder-quarter milk, in addition to, the bacteriological examination of udder quarters **(Allam *et al.,* 2009; Ghanbarpour and Oswald, 2010; Hamouda and Allam, 2011 and Allam *et al.,* 2018)**.In Egypt, previous publications in addition to the present study had illustrated the incidence of *E. coli* isolated from dairy cattle **(El-Rashidy *et al*., 1986, Abdel-Fattah *et al*., 2008 and El-Khodery and Osman, 2008; Allam *et al.,* 2009; Hamouda and Allam, 2011; Ibrahim *et al*., 2012; Hamouda *et al*., 2014 and Allam *et al*., 2017,2018).** Consequently, there are increasing demands for specifically detecting STEC, EPEC, and EHEC, with preference to clone genotypic identification along with phenotypic or serotyping profiling in dairy farms. In particular sequence of the virulence genes correlating to pathogenesis of infections, hence, studied farms had different management systems which influence the clonality of detected bacterial species **(Allam *et al*.,2009; Hamouda and Allam, 2011; Sipka *et al*., 2014; Thompson-Crispi *et al*., 2014; Younis *et al*., 2016; Günther *et al*., 2016 and Allam *et al*., 2017 and 2018)**. *E. coli* fatality, pathogenicity and cytotoxicity were intensified by both types of Shiga toxins simultaneous secretion over than single type release **(Girardeau *et al.,* 2005, Allam *et al*., 2009)**. Considering that Shiga toxins type 2 encoded by stx_2 gene variants (*stx2*, *stx2v*, *stx2vha*, *sxt2vhb*, and *stx2va*), were accountable for more severe complications than those induced solely by Shiga toxin type 1 encoded by stx_1 gene **(Allam *et al*., 2009, 2017 and 2018)**. This was expressed by some of the reported serotypes-clones in the present study. In addition to, the destructive effect of the putative plasmid-encoded enterohemolysin (*hly*A gene) which is accessory virulence factor characterizing EHEC strains of *E. coli* isolated from animals **(Gyles, 2007 and Allam *et al*., 2009, 2017and 2018)**. Finally, intimin; encoded by conserved region of *eae*A gene between STEC and EPEC, which account for the invasive properties of *E. coli* isolates inducing mastitis **(Prere and Fayet, 2005; Ghanbarpour and Oswald, 2010 and Allam *et al*., 2009, 2017 ,2018)**.

Thus the capability to rapidly determine the associated type of virulence proteins responsible for pathogenicity of the infecting and/or invassing pathovars would be highly valuable in obstructing the destructive effect of these toxins on udder tissues, overcoming their heat

resistance, and persistence in milk byproducts even after their sanitary treatments **(Blum *et al.*, 2008 and Allam *et al*.,2009, 2017,2018)**. In the presented despite results the majority of reported isolates were coding at least for one virulence protein; which was in continent to previous publications **(Wenz *et al*., 2006; Hamouda and Allam,2011 and Allam *et al*., 2009, 2017,2018)**, the functionality of most of them was lost *in vivo*. Therefore, bacterial elements of invasion are still unclear. Moreover, indicated the specificity of *E. coli* invasion to mammary gland that necessitated udder-dependent mechanisms **(Dogan *et al*., 2006; Sipka *et al*., 2014; Günther *et al*., 2016 and Allam *et al*., 2017, 2018)**. In addition to, the several important risk factors that linked between nutrition and mastitis in the dairy cow **(Thompson-Crispi *et al*., 2014 and Younis *et al*., 2016)**. Furthermore, genetic constituent of the host that guide the resistance to mastitis, as well as, innate immune response of the mammary gland to various pathogens **(Prere and Fayet, 2005; Dogan *et al*., 2006; Blum *et al*., 2008; Sipka *et al*., 2014; Günther *et al*., 2016 and Allam *et al*., 2017,2018)**.Cytokines are sensitive resources to estimate the functionality of the immune responses of dairy herds. Consequently, they were suitable tool for production control and assessment of treatment and/or vaccine efficiency **(Almeida *et al.*,2011; Hogeveen *et al.*,2011; Günther *et al.*,2016 and Allam *et al*.,2017)**. Formerly, it was proved that toxins could altered host immunity, therefore, quantification of transcription levels of these genes was essential in understanding udder immunity and to justify abnormal regulation in response to infections **(Fonseca *et al.*,2009 and Allam *et al*., 2018)**. Cytokines, especially interlukine6 (IL6) and interlukine8 (IL8) were characterized by pathogen-dependent expression induction. As a result, have been considered *in vitro* useful markers in defining intramammary defenses in association to field SCC and/or CMT score **(Bannerman *et al*., 2004)**, hence, milk somatic cells comprised from neutrophils, macrophages, lymphocytes and natural killer (NK) cells **(Bannerman *et al.,* 2004)**.

The obtained qRT-PCR results indicated that subclinical mastitis due to *E. coli* had up regulated mRNA excepression of investigated cytokines; especially IL-8 over than IL-6 in udder leukocytes. These cytokines induced a shift in T-cell phenotypes from CD4+ T-cells to CD8+T-cell, as well as, differentiation of Th1 and Th2 lymphocytes to switch on the cellular immune cascades. Subsequently, enhancing the IgE humeral immune response intramammary **(Riollet *et al.*,2001 and Fonseca *et al.*,2009)**. Over and above, triggering migration of neutrophils from blood stream to the udder, despite the defined subclinical mastitis where

cows were symptomless in apparently healthy clinical phase **(Riollet *et al.*,2001 and Fonseca *et al*., 2009)**.The presented results were in agreement with **Lee *et al.* (2003)**, **and Fonseca *et al.* (2009)** who mentioned that expression of cytokines especially IL-6 and IL-8 had increased upon infections. However, further studies are fundamental to validate the obtained results and define the expression fold increase in relation to other clinical stages of mastitis. Furthermore, quantification of expression of additional cytokines upon udder subclinical infection will improve the outline of these analytical biomarkers of mastitis.

Hence, comprehensive information on onset of infection even in asymptomatic individuals will be developed, therefore, regulatory update on udder health condition. In conclusion, there is wide range of virulence genes which obviously played role in the pathogenesis of subclinical mastitis associated with *E. coli* in the investigated farms. However, their inclusion was beyond the scope of the current study. The presented study illustrated the capability of the quantified cytokines (IL6 and IL8) as biomarkers of subclinical stage of mastitis. In addition to, other cytokines genes which may play a role in the efficiency of immune reaction, which were also not investigated in the present study. SCC is regarded fundamental qualification parameters of raw milk. However, in some subclinical mastitic cows SCC remained below the limit due to the diluting effect of healthy quarters' milk. Which pretended grave risk to other herd mates, hence, toxigenic pathogen are flourishing. Therefore, regular screening for SCC involving the entire herd is fundamental for professional and efficient control, including bulk and udder-quarter milk, in addition to, the bacteriological examination of udder quarters.

Acknowledgment:

The authors would like to thank the field veterinarians who helped in collecting specimens and follow up of the animal population in investigated localities. The authors would like to thank Dr. MarwaS. Abdel-Hamid; microbial biotechnology department- genetic engineering and biotechnology institute-University of Sadat City, for her help in Electron transmission micrographing of *E.coli* isolates.

Funding:

This study was funded by the research project No. 11040301 offered by National Research Centre, Egypt. In addition to, project No. 50/4/10 offered by Ministry of Higher Education and Scientific Research, Egypt. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Abdel-Fattah AA, Amin AS, Hamouda RH. (2008): Detection of *E. coli* O_{157}:H_7 in bovine milk and fecal samples by the polymerase chain reaction assay. Journal of Egyptian Medical Association, 68 (3): 83 - 92.

Abd-Elrahman AH. (2013): Mastitis in housed dairy buffaloes: incidence, etiology, clinical finding, antimicrobial sensitivity and different medical treatment against *E. coli* mastitis. Life Science Journal, 10 (1): 532-538.

Agbodaze D. (1999): Verocytotoxins (Shiga-like toxins) produced by *Escherichia coli*: a mini review of their classification, clinical presentations and management of a heterogeneous family of cytotoxins. Comparative Immunology and Microbiology of Infectious Diseases, 22 (4):221-230.

Alan B, Duncan C. (2011): Quantitative Data Analysis with IBM SPSS 17, 18 and 19: A Guide for Social Scientists. New York: Routledge.

Allam NAT, Abd Ellatif SG, Hamouda RH. (2009): Genetic typing and antigenic characterization of Egyptian field *Shiga*-toxigenic *Escherichia coli* isolates with regard to profile of virulence proteins. Global Veterinaria, 3(6): 457- 464.

Allam NAT, Abdel-Hamid MS, Allam HA. (2018): Pathogenic *E. coli* Virulence Traits regarding Quantitative Cytokine Expression in Subclinical Mastitic Cows. International Journal of Pharmaceutical and Phytopharmacological Research, 8(2): 45-58.

Allam NAT, Sedky D, Mira EK. (2017): The clinical impact of antimicrobial resistance on she-camels recurrent mastitis with regards to genotypic divergent *Bacillus licheniformis* field isolates. Veterinary World, 10 (11): 1353-1360.

Almeida R, Dogan B, Klaessing S, and Schukken Y, Oliver S. (2011): Intracellular fate of strains of *Escherichia coli* isolated from dairy cows with acute or chronic mastitis. Veterinary Research and Communication, 35: 89 -101.

Bannerman DD, Paape MJ, Lee JW, Zhao X, and Hope JC, Rainard P. (2004): *Escherichia coli* and *Staphylococcus aureus* elicit different innate immune responses following intra mammary infection. Clinical Diagnostic and Laboratory Immunology, Li: 463 - 472.

Blum S, Heller ED, Krifucks O, Sela S, Hammer-Muntz O, Leitner G. (2008): Identification of a bovine mastitis Escherichia coli subset. Veterinary Microbiology 132, 123-148.

Blum S, Sela N, Heller ED, and Sela S, Leitner G. (2012): Genome analysis of bovine-mastitis-associated Escherichia coli O32:H37 strain P4. Journal of Bacteriology, 194 (14): 3732.

Bonnefont CM, Rainard P, Cunha P, Gilbert BF, Toufeer M, Aurel MR, Rupp R, Foucras G. (2012): Genetic susceptibility to *S.aureus* mastitis in sheep: differential expression of mammary epithelial cells in response to live bacteria or supernatant. Physiol Genomics, 44:403 - 416.

Bouchard D, Peton V, Almeida S, Le Marechal C, Miyoshi A, Azevedo V, Berkova N, Rault L, François P, Schrenzel J, Even S, and Hernandez D, Le Loir Y .(2012): Genome sequence of *Staphylococcus aureus* Newbould 305, a strain associated with mild bovine mastitis. Journal of Bacteriology, 194 (22): 6292-6293.

Bougarn S, Cunha P, Harmache A, Fromageau A, and Gilbert BF, Rainard P. (2010): Muramyl dipeptide synergizes with *Staphylococcus aureus* lipoteichoic acid to recruit neutrophils in the mammary gland and to stimulate mammary epithelial cells. Clinical Vaccine and Immunology, 17:1797-1809.

Burvenich C, Van Merrid V, and Mehrzad J, ez-Fraile A, Duchateau L. (2003): Severity of *E. coli* mastitis is mainly determined by cow factors. Veterinary Research, 34: 521-564.

CLSI (2014): Analysis and presentation of cumulative antimicrobial susceptibility data: approved guidelines (4th Ed.). CLSI document M38-A4. Wayne, PA: Clinical and Laboratory Standard Institute.

Dogan B, Klaessiga S, Rishniw M, Almeida RA, Oliver SP, Simpson K, Schukken YH. (2006): Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis. Veterinary Microbiology, 116: 270-282.

Edwards PR, Ewing WH. (1972): Identification of *Enterobacter aerogenesiaceae*. Burgess Publication, Minnecopolis Minnesota, Pp: 103-104.

El-Awady HG, Oudah EZM. (2011): Genetic and Economic Analysis for the Relationship between Udder Health and Milk Production Traits in Friesian Cows. Asian-Australian Journal of Animal Science, 24 (11): 1514 -1524.

El-Khodery SA, Osman SA. (2008): Acute coliform mastitis in buffaloes (Bubalus bubalis): clinical findings and treatment outcomes. Tropical Animal Health and Production, 40 (2):93 -39.

El-Rashidy AA, Berier L, Amin MAS, Tawfik MS. (1986): Etiology and diagnosis of subclinical mastitis among dairy herd in Egypt. Journal of Egyptian Medical Association, 48: 289 - 296.

Erganis O, Kaya O, Corlu M, Istanbulluoglu E. (1989): Hemagglutination, hydrophobicity, enterotoxigenicity and drug resistance characteristics of avian *E. coli*. Avian Diseases, 33: 631-635.

Escobar-Paramo P, Clermont O, Blanc-Potard AB, Bui H, Le Bouguenec C, Denamur E. (2004a): A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. Molecular Biology and Evolution, 21: 1085-1094.

Escobar-Paramo P, Grenet K, Le Menac'h A, Rode L, Salgado E, Amorin C, Gouriou S, Picard B, Rahimy MC, Andremont, A, Denamur, E, Ruimy R. (2004b): Large-scale population structure of human commensal *Escherichia coli* isolates. Applied and Environmental Microbiology, 70: 5698-5700.

Fonseca I, Silva PV, Lange CC, Guimarães MFM, Weller MMDCA, Sousa KRS, Lopes PS, Guimarães JD, Guimarães SEF. (2009): Expression profile of genes associated with mastitis in dairy cattle. Genetic and molecular Biology, 53 (4): 776 -781.

Fu Y, Zhou E, Liu Z, Li F, Liang D, Liu B, Song X, Zhao F, Fen X, Li D, Cao Y, Zhang X, and Zhang N, Yang Z. (2013): *Staphylococcus aureus* and *Escherichia coli* elicit different innate immune responses from bovine mammary epithelial cells. Veterinary Immunolology and Immunopathology, 155 (4): 245-252.

Ghanbarpour R, Oswald E. (2010): Phylogenetic distribution of virulence genes in *Escherichia coli* isolated from bovine mastitis in Iran. Research in Veterinary Science, 88: 6-10.

Gilbert FB, Cunha P, Jensen K, Glass EJ, Foucras G, Robert-Granié C, Rupp R, Rainard P. (2013): Differential response of bovine mammary epithelial cells to *Staphylococcus aureus* or *Escherichia coli* agonists of the innate immune system. Veterinary Research, 44: 40.

Gilmour MW, Olson AB, Andrysiak AK, and Ng L-K, Chui L. (2007): Sequence-based typing of genetic targets encoded outside of the O-antigen gene cluster is indicative of Shiga toxin-producing *Escherichia coli* serogroup lineages. Journal of Medical Microbiology, 56: 620-628.

Girardeau JP, Dalmasso A, Bertin Y, Ducrot C, Bord S, Livrelli V, and Vernozy- Rozand C, Martin C. (2005): Association of virulence genotype with phylogenetic background in comparison to different seropathotypes of Shiga toxin producing *Escherichia coli* isolates. Journal of Clinical Microbiology, 43: 6098-6107.

Giugliano LG, Mann GF, Drasar BS. (1982): Response of mammalian cell lines to the toxins of *Eschericia coli*. Journal of Medical Microbiology, 5: 531-539.

Goldammer T, Zerbe H, Molenaar A, Schuberth HJ, Brunner RM, Kata SR, Seyfert HM. (2004): Mastitis increases mammary mRNA abundance of *beta-defensin* 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clinical Diagnosis and Laboratory Immunology, 11:174-185.

González-González A, Hug SM, Rodríguez-Verdugo A, Patel JS, Gaut BS. (2017): Adaptive Mutations in RNA polymerase and the Transcriptional Terminator Rho Have Similar Effects on Escherichia coli gene expression. Molecular Biology and Evolution, 34 (11):2839 -2855.

Guarino A, Capano G, Malamisura B. (1987): Production of *E. coli sta*-like heat-stable enterotoxin by *Citobacter frundii* from humans. Journal of Clinical Microbiology, 25 (1): 110 - 114.

Günther J, Koy M, Berthold A, and Schuberth HJ, Seyfert HM. (2016): Comparison of the pathogen species-specific immune response in udder derived cell types and their models. Veterinary Research, 47: 1–11.

Gyles, C. L. (2007): Shiga toxin-producing *Escherichia coli*: an overview. Journal of Animal Science, 85 (Suppl. E): 45-62.

Hakim AS, Allam NAT, Syame SM, Sedky D, Hedia RH, Elshafaie MA. (2017): Selective pressure of resistance determinants and virulence genes on *agr* locus expression during *Staphylococcus aureus* infections in some farm animals in Egypt. Asian Journal of Epidemiology, 10(2): 89-100.

Hamouda RH, Allam NAT. (2011): Use of multiplex PCR-based molecular typing of genes encoding virulence determinants in coliform mastitis in dairy cattle. Journal of Egyptian Veterinary Medical Association, 11 (1): 245 - 256.

Hamouda, RH, Allam HA, Allam NAT. (2014): Molecular Identification of Bovine Recurrent Mastitis Status with regards to *Arcanobacerium pyogenes* (*Trueperella pyogenes*). Journal of Egyptian Veterinary Medical Association, 74 (4): 739 -754.

Hamann J. (2003): Definition of the physiological cell count threshold based on changes in milk composition. IDF Mastitis Newsletter 25, 9 -12.

Hogeveen H, Huijps K, Lam TJ. (2011): Economic aspects of mastitis: new developments. N. Z. Vet. J., 59: 16 -23.

Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. (2004): Practical considerations in design of internal amplification controls for diagnostic PCR assays. Journal of Clinical Microbiology, 42(5): 1863 -1868.

Ibrahim EA, Allam NAT, Kotb EEZ, El-Rafey GA, Alaa El-Deen, *et al.*, (2012): Sequence-based typing-study on the relationship between mastitis and *Bola-DRB3.2** allelic polymorphism in Egyptian cows. Global Veterinaria, 9 (1): 8-22.

International Dairy Federation (IDF) (1999): Suggested interpretation of mastitis terminology. Bulletin of the International Dairy Federation 338.

Ishizaki Y, Shibuya Y, Hayashi C, Inoue K, Kirikae T, Tada T, Miyoshi-Akiyama T, Igarashi M. (2018): Instability of the 16S rRNA methyltransferase-encoding *npmA* gene: why have bacterial cells possessing *npmA* not spread despite their high and broad resistance to aminoglycosides Journal of Antibiotics (Tokyo), 2018 Jun 8. Doi: 10.1038/s41429-018-0070-y.

Jackson MP, Neill RJ, O'Brien AD, Holmes RK, Newland JW. (1987-a): Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli*. FEMS Microbioliogy Letters, 44: 109 -114.

Jackson MP, Newland JW, Holmes RK, O'Brien AD. (1987-b): Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli.* Microbial pathogenesis. 2: 147-153.

Jensen K, Günther J, Talbot R, Petzl W, Zerbe H, Schuberth HJ, Seyfert HM, Glass EJ. (2013): *Escherichia coli*- and *Staphylococcus aureus*-induced mastitis differentially modulate transcriptional responses in neighboring uninfected bovine mammary gland quarters. BMC Genomics, 14: 36.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC. (1989): Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proceeding of the National Academy of Science USA, 86: 6196-6200.

Koneman EW, Allen SD, Janda WM, Schrecheneberger PC, Winn WC. (1996): Introduction to diagnostic microbiology. 6th Ed., Lippinestt Co., Philadelphia, USA.

Kościuczuk EM, Lisowski P, Jarczak J, Krzyżewski J, *et al.*, (2014): Expression patterns of β-defensin and cathelicidin genes in parenchyma of bovine mammary gland infected with coagulase-positive or coagulase-negative Staphylococci. BMC Veterinary Research, 10 (1): 246.

Lee CY, Clough EA, Yellon P, Teslovich TM, and Stephan DA, Baehrecke EH. (2003): Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila. Current Biology, 13 (4): 350-357.

Leutenegger CM, Alluwaimi AM, Smith WL, Perani L, Cullor JS. (**2000):** Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time Taq Man polymerase chain reaction. Veterinary Immunology and Immunopathology, 77: 275-287.

Moussay E, Stamm I, Taubert A, and Baljer G, Menge C. (2006): *Escherichia coli* Shiga toxin 1 enhances IL-4 transcripts in bovine ileal intraepithelial lymphocytes. Veterinary Immunology and Immunopathology, 113 (3 - 4): 367-382.

National Mastitis Council (NMC) (1999): Laboratory Handbook on Bovine Mastitis (Revised Edition). Madison, National Mastitis Council Inc.

Paton AW, Paton JC. (1998): Detection and characterization of Shiga toxigenic *E. coli* by using multiplex 23 PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfbO111, and *rfb* O157, Journal of Clinical Microbiology, 36 (2): 598 - 602.

Perna NT, Plunkett G III, Burland V, Mau B, Glasner JD, Rose DJ, Mayhew GF, Evans PS, Gregor J, Kirkpatrick HA, Posfai G, Hackett J, Klink S, Boutin A, Shao Y, Miller L, Grotbeck EJ, Davis NW, Lim A, Dimalanta E, Potamousis K, Apodaca J, Anantharaman TS, Lin J, Yen G, Schwartz DC, Welch RA, Blattner FR. (2001): Genome sequence of enterohemorrhagic *Escherichia coli* O157:H7. Nature, 409: 529-533.

Porcherie A, Cunha P, Trotereau A, Roussel P, Gilbert BF, Rainard P, Germon P. (2012): Repertoire of Escherichia coli agonists sensed by innate immunity receptors of the bovine udder and mammary epithelial cells. Veterinary Research, 43:14.

Prere MF, Fayet O. (2005): A new genetic test for the rapid identification of *Shiga*-toxins producing (STEC), enteropathogenic (EPEC) *E. coli* isolates from children. Pathologie -biologie, (Paris), 53:466 - 469.

Quinn, P.J, Markery BK, Carter ME,Donnelly WJ, Learned FC. (2002): Veterinary Microbiology and Microbial Disease. 1 Ed. Published by Blackwell Science, Ltd., UK.

Riollet C,Rainard P, Poutrel B. (2001): Cell sub populations and cytokine expression in cow milk in response to chronic *staphylococcus aureus* infection. Journal of Dairy Science, 84: 1077-1084.

Sambrook J, Fritscgh EF, Meniates A. (1989): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Schalm OW, Caroll EJ, Jain NC. (1971): Bovine mastitis complex. Leas and Febiger, Philadelphia.

Schmid H, Beutin L, Karch H. (1995): Molecular analysis of the plasmid encodid hemolysin of *Esherichia coli* O157:H7 strain EDL933. Infection and Immunity, 63: 1055-1061.

Seidl K,Chen L,Bayer AS,Hady AS, Kreiswirth BN, Xiong YQ. (2011a): Combinatorial phenotypic signatures distinguish persistent from resolving methicillin-resistant *Staphylococcus aureus* bacteremia isolates. Antimicrobial Agents and Chemotherapy, 55: 575 - 582.

Seidl K, Chen L, Abdel Hady W, Kreiswirth BN, Xiong YQ. (2011b): *agr* transcription, functionality and locus sequence profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) and correlation to vancomycin responsiveness in an experimental endocarditis (IE) model. 111th General Meeting of the American Society for Microbiology, New Orleans.

Siarkou VI, Stamatakis A, Kappas I, Hadweh P, Laroucau K. (2011): Evolutionary relationships among *Chlamydophila abortus* variant strains inferred by rRNA secondary structure-based phylogeny. PLoS One, 6 (5): *et al.* 9813.

Sipka A, Klaessig S, Duhamel GE, Swinkels J, Rainard P, Schukken Y. (2014): Impact of intramammary treatment on gene expression profiles in bovine Escherichia coli mastitis. PLoS One, 9 (1): e85579.

Thompson-Crispi K, Atalla H, Miglior F, Mallard BA (2014): Bovine mastitis: frontiers in immunogenetics. Frontier Immunolology, 5: 493.

Thompson JD, Higgins DG, Gibson TJ. (1944): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673 - 80.

Ülbegi-Mohyla H, Hijazin M, Alber J, Lämmler C, Hassan AA, *et al.* (2010): Identification of *Arcanobacterium pyogenes* isolated by post mortem examinations of a bearded dragon and a gecko by phenotypic and genotypic properties. Journal of Veterinary Science, 11 (3): 265-267.

Wang X, Ma P, Liu J, Zhang Q, Zhang Y, Ding X, *et al.* (2015): Genome-wide association study in Chinese Holstein cows reveal two candidate genes for somatic cell score as an indicator for mastitis susceptibility. BMC Genetics, 16: 111.

Wenz JR, Barrington GM, Garry FB, Ellis RP, Magnuson RJ. (2006): Escherichia coli isolates 19 serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. Journal of Dairy Science, 89:3408 -3412.

Xiao M, Zhu X, Xu H, Tang J, Liu R, Bi C, and Fan F, Zhang X. (2017): A novel point mutation in *ropB* improves osmotolerance and succinic acid production in Escherichia coli. BMC Biotechnology, 17 (1): 10.

Yang W, Zerbe H, Petzl W, Brunner RM, Gunther J, Draing C, von Aulock S, Schuberth HJ, Seyfert HM. (2008): Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF-kap-paβ in mammary epithelial cells and to quickly induce TNFα and interleukin-8 (CXCL8) expression in the udder. Molecular Immunology, 45 (5):1385-1397.

Younis S, Javed Q, Blumenberg M. (2016): Meta-Analysis of Transcriptional Responses to Mastitis-Causing *Escherichia coli*. PLoS One, 11(3): e0148562.

Yu J, Kaper JB. (1992): Cloning and characterization of the *eae* gene of enterohaemorrhagic *Esherichia coli* O157:H7. Molecular Microbiology, 6: 411- 417.