Pathogenic E. coli Virulence Traits Regarding Quantitative Cytokine Expression in Subclinical Mastitic Cows

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ABSTRACT

The present study aimed to determine the molecular dynamics of E. coli serotypes regarding the diagnostic significance of typing virulence genes, in addition to the description of the intensity of the triggered cytokines' response by the invading pathogen to determine the severity and/or onset of the infection. The survey included 300 cows in 10 dairy farms in Egypt, characterized by low SCC bulk milk tank (≤ 4×10 ^{5}/ ml) with CMT recorded grades of (+) & (++); and the influx of neutrophils from the blood to the udder. Environmental microorganisms were isolated from 16.66% of milk samples, noting that E. coli isolates were 90% (91 isolates) of them, however, other types of bacteria (10%) were also recorded. Ten serogroups were typed; O1, O8, O20, O25, O78, O127, O146, O153, O157 & O159, with prevalence rated 9.9%, 12.1%, 11%, 13.2%, 12.1%, 9.9%, 16.5%, 8.8%, and 6.5%, respectively. Furthermore, the isolates were morphologically identified by Transmission Electron Microscope (TEM), andbiochemically characterized. The enteropathogenicity in infant mice, hemolysin patterns, Shiga-like cytotoxicity in Vero cell line, and CD50 were all studied and determined. PCR fragments of molecular sizes of 180 bp, 255 bp, 384 bp, and 534 bp distinctive for stx1, stx2, eaeA, and hlyA genes, respectively, were amplified, sequenced, and then aligned against similar GenBank records to confirm the identities. To study the influences of proteomics [9 dominant bands; 106 to109, 80 to 83, 54 to 56, 41, 37, 32, 26-25, 22, and 7.8 kDa] on bacterial pathogenicity and host immunity mRNAs expression of IL-4 and IL-10 cytokines in milk, the somatic cells were quantified, then normalized with housekeeping gene (β-Actin), finally expressed as folds of induction. qRT-PCR revealed up regulation of target cytokine genes in response to E. coli infections; the means of the folds' increase were calculated as 2.712 ± 5.178 and 11.231 ± 4.146 for IL4 and IL10, respectively (Means St. Dev. 2^{-}(ΔΔCT). The present study not only indicated diverse incidence rates of the four molecular characterizing genetic/phenotypic traits, but also, the tendency for clonality which was proved by the proteomics' variance between the pairs of the same clone of some E. coli serotypes; O1, O8, O127, O146, O153, O157 and O159, where α & β hemolysin expression were noted. Consequently, it was reflected on the udders initiating inflammatory responses. The onset and intensity of the up regulated immune cascade; especially IL4 & IL10, is an invasive pathovar dependent on the triggered cellular subset which mediated the immune responses. The adverse effects exerted by the secretory toxins on dairy herds as well as the consumers of byproducts are significant but undervalued, hence,the subclinical mastitis diagnosis is still dependent on the bacterial colonies' counts per sample not the bacterial secretory excretory toxins nor the hosts' cytokines response's quantification. In conclusion: The virulence of E. coli serotypes found dependent on the less characterized proteomics than Stx1 and Stx2, yet their role remains to be discovered.

Key Words: *Coliform, Mastitis, Cytokines, Genomics, Proteomics, qRT-PCR*

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INTRODUCTION

Mastitis is the most costly disease in dairy farms; though, the outcomes largely depend on the etiology [1-4]. The subclinical intramammary infections consumed 20% of the medical overheads in dairy herds [5-8].

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The pathogenicity is designated bythe microorganisms' proteomic-dependent phenomenon affecting the mammary glands, resulted in hosts' diverse symptoms and immune responses [3, 4, 9-11]. Following the intrusion into the lumen of the udder, the sequential host-pathogen dialogue elicited diverse responses by the onset of the bacterialflourishing in milk. The mammary gland is not considered a natural habitat for Escherichia coli (E. coli = Coliform).However, many strains are capable of surviving for short duration [12-15]. Despite that coliform pathogenesis is completely different from E. coli infectious syndromes, the udders' isolates are not characterized as separate subsets[16-18].Molecular techniques had led to various formats of the bacterial typing more rapidly and simpler than the conventional serological methods [8, 17, 19].Notwithstanding the huge information which had been collected, E. Coli's sensing of the udder immune responses is not completely understood yet, whether leading to either effective antibacterial responses or harmful inflammatory responses[4, 20].

The environmental pathogens usually induce limited mastitis with frequent eradication. They provokedynamicmotivation, characterized by up regulation of chemokines and cytokines,leading tostimulation of the local and generalized immune responses of the hosts[4, 8, 20-22]. On the other hand, the mammary epithelial cells (MEC) are designated to respond hastily to the pathogens' intrusion through the activation of numerous pattern recognition receptors (PRR) by the microbe-associated molecular patterns (MAMP) [10, 14, 23], therefore, are up regulating 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 NFκB pathways. Frequently, this activation is able to clear the infection[9, 10, 12, 16, 24-26].Nevertheless, some E. coli strains had persistence and/or recurrence in the udders [3, 11, 21, 27].

Recent investigations suggested an increase in the frequency of coliform mastitis due to epidemiological shifts by modified virulence factors enhancing the intracellular invasion [8, 14, 19, 20, 24, 28]. Bacterial intra-species polymorphism is a resultant of both mutation and lateral gene transfer with or without genomicrecombination [29-31]. E.colipossessed clonalgenetic structure with a low level of recombination. Four main phylogeneticgroupswere characterized; genotypes A, B1, B2 and D whichincluded the bulk of the species[32]. Most of the commensal and diarrheaogenicstrains belong to groups A and B1, while extra-intestinalstrains belong mainly to groups B2 and D [33-35]. Oppositely, E.coli isolates from bovinemastitis (Coliform) belong to genotype A, which mostly are non-pathogenic strains [36-38]. The virulence genes associated with invasion of E.coli enteropathogenic (EPEC) as well as the Shiga toxigenic strains (STEC) justify the invasive properties of coliform mastitis [19, 24, 39]. EPEC as well as STEC colonize the epithelial lining, and cause attaching/effacing (A/E) lesions. The intimin protein encoded by eaeA chromosomal gene, mediated adherence of attaching/effacing E. coli to epithelial cells. The eaeAgene previously detected in coliform isolates from bovine mastitis justifies the adherent colonization of bacteria on the host epithelial surfaces[19, 24, 40]. Additionally, the plasmid-encoded enterohemolysin protein was encoded by hylA gene in enterohemorrhagic (EHEC); and the pathogenic E. coli strains were isolated from animals [19, 24, 41]. Moreover, Shiga toxins types 1 and 2;stx_1 and stx_2 genes, shared the same binding specificity and biological activities between E. colibiotypes, despite stx_2genotypic variants (stx2, stx2v, stx2vha, sxt2vhb, and stx2va)detected in enterotoxigenic E. coli isolates associated with bovine diseases[19, 35].

In Egypt, the bacteriological, epidemiological and clinical studies have indicated that coliform is one of the major agents of bovine subclinical and/or subacute mastitis [17, 42, 43]. The objectives of this study is to isolate and identificate of Pathogenic E. coli from Dairy cows and to determine the epidemiological dynamics of thier serotypes in dairy farms regarding the diagnostic value of typing virulence genes. The selected genes in this study encoded putative virulence factors whose role in coliform's pathogenesis in Egyptian dairy farms has been little reported so far. In addition to, descripe the intensity of the triggered cytokines response by the invading pathogen to determine the severity and/or onset of infection.

MATERIAL AND METHOD

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 the laboratory animals (NIH Publications No. 8023, revised 1978). In addition, the adopted ethical guidelines were compiled with those of the national research center, agriculture research center, and genetic engineering research institute- university of Sadat City guidelines for the care and use of the laboratory animals in Egypt.

Studied Animal Population

Sample Collection and California Mastitis Test:

Quarter milk samples of 300 cows (n =1200) in 10 private dairy farms were collected during a representative epidemiological study held from June 2014 till July 2016, on the prevalence of mastitis in Giza governorate, Egypt. The sample collection was strictly standardized: after fore stripping about 10 ml of milk, visual inspection and evaluation of the milk by California Mastitis Test (CMT) were carried out [44].

The first milk sample of each quarter was taken for SCC evaluation; afterwards, aseptic samples of each quarter were collected for bacteriological and molecular analysis using standard procedures [45-47]. The samples were transported to the laboratory for

bacteriological tests [46] and for SCC evaluation at 4 °C [47]. Collected udder and teats of each cow were examined by visual inspection and palpation, hence, abnormal findings were recorded.

Somatic Cell Count Analysis:

The quarter milk samples were kept at 4 °C, then analyzed within 24 h from collection which were pre-warmed at 37 °C for 10 min, and then SCC were quantified by Fossomatic 5000 (Foss) [48]. Four bacteriologically negative milk samples of each herd were randomly selected as negative controls. The log10 (SCC) values had classified udders status into three categories; normal, subclinical, and mastitic, which recorded SCC ≤4×10^5, ≥4×10^5, and ≥1×10^6 cells/ml, respectively [49].

Bacteriological Characterization and Serotyping

Isolation and Identification of E. coli Isolates:

Standard procedures were used for isolation and identification of bacteria from all cultured milk samples [47]. The isolates were identified based on cultural characteristics, Transmission Electron Microscope (TEM) examination and standard biochemical tests [47]. A total of 300×4 quarter milk specimens from subclinical mastitis udders were studied. After thawing at 37 °C for 5 min, equal volumes of 10 µl of each quarter milk were simultaneously plated on Nutrient agar, Edward agar, Manitol salt agar, Salmonella/Shigella agar (SS agar), 5% Sheep-blood agar and MacConkey agar (Biolife Laboratories, Milano, Italy) and incubated aerobically at 37 °C. All 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 typed for their somatic cell antigen "O" contents by the slide agglutination test (Denka Seiken Co. Ltd., Tokyo, Japan). The test was done as outlined by [50] using the standard polyvalent and monovalent E. coli antisera.

Profiling of Shigella-Like Toxins Proteomics Produced by E. coli

The Vero Cell Lines Cytotoxicity:

The overall cytotoxic effect of serotyped E. coli isolates was studied on Vero cell line [51]. E.coli O157:H7 was used as positive control during this study. After incubation of Luria broth cultures (Oxiod) for 24 hours at 37 ^{0}C with shaking 150 rpm (Shellab), bacteria were pelleted by centrifugation; 10000 xg for 15 min at 4 ^{0}C (Jouan MR 18-12). At that point, the supernatants were collected then filter-sterilized through 0.22 µm pore-size membrane syringe filters (Sartorius). Two fold dilution of the 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 [19]. Then, 100 µl/wells were transferred in to 96-wells tissue culture plates (Coaster) seeded with Vero cells (Green Monkey Kidney Cells) monolayers (4×10^4 cells/well) which were obtained from the animal vaccines and serum production research institute, agriculture research center, abbasia, Cairo. The cytotoxic effects were determined and recorded after 12 h, 24 h, and 48 h from incubation at 37 ^{0}C in 5% CO_2 (Haraeus) by microscopic examination (Lilly) of the Vero cells, so the cytotoxic dose titer (CD_{50} unit) was defined [19].

SDS-PAGE Profiles of Pathogenicity and Virulence Traits:

The secretory protein contents of E.coli broth cultures were precipitated and concentrated for each isolate [19, 52]. The total proteincontent of each preparation was determined [53], compared to standard protein curve of bovine serum albumin fraction V (Sigma-Aldrich) ranged from 0.5 to 1000 µg/100 µl. The E. coli secretory toxins were separated in 12% SDS-PAGE matrix casted in BioRad Mini-PROTEAN II Dual Slab Cell [54], then the gel was stained with 0.5% Coomassie brilliant blue in 40% methanol (Sigma-Aldrich), finally documented and analyzed by Image Lab (BioRad).

Characterization of Virulence and Pathogenicity Phenotypic Patterns

Haemolysin Production:

All E. coli isolates were streaked on the sheep blood agar plates, incubated at 37 ^{0}C for 24 h, then examined for the developed haemolysis patterns [55].

Enterotoxin Production:

E. coli isolates were inoculated in 25 ml media/isolate [19], then the incubated (Shellab) isolates were shaken at room temperature at 200 rpm for 48 h. After 10 min centrifugation at 12000 xg, finally they were filter-sterilized through 0.22 µm pore-size membranes syringe filters (Sartorius). A 0.1 ml of each filtrate was injected intra-abdominal into milk filled stomach of 3 mice/isolate each is 2-4 days old, incubated for 4 h, then their entire intestines were removed and weighted [19]. The assay was considered indicative for enterotoxin if the ratio of the combined weight of the intestines of the three inoculated mice/isolate to combined weight of the remaining body weight was >0.083 [56, 57].

Pathogenicity Assay:

25 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 [19]. All internal organs were collected for both histopathological examination and re-isolation of E.coli isolate, followed by serotyping with the standard antisera [50].

Antibiotic Resistance and Recovery Susceptibility Patterns:

Antibiotic susceptibilities of isolates were determined by disk diffusion method on Muller Hinton agar plate [47, 58]. The following commercial antibiotic disks (Oxoid) were used: Amikacin (30 µg), Ampicillin (10 µg), Cefadroxil (30 µg), Chloramphenicol (30 µg), Colistin Sulphate (50 µg), Erythromycin (15 µg), Gentamicin (10 µg), Oxytetracycline (30 µg), Nalidixic acid (30 µg), Novofloxacin (10 µg), Trimethoprim and Sulphamethoxazol (1.25 µg and 23.75 µg). The diameter of inhibition zone of each antibiotic disk was measured and compared with the standard zone chart

according to the manual of the supplier [59]. The in vitro proved efficiency was examined for fidelity by in vivo confirmatory evaluation through the treatment scheme applied on the studied population.

The Molecular Profiling of Virulence and Pathogenicity Genomics

Oligonucleotide Primers Design:

Primers pairs used during PCR were designed with reference to annotated sequence for E.coli strain O157:H7; accession numbers AE005174v2- 1.gbk and AE005174v2-2.gbk [60].In addition to, published sequence data for stx1 [61], stx2 [62], eaeA [63], and hlyA [64] virulence proteinsgenes (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.

Table 1. Primers used in the present study

Primers	5`- Sequence -3`	Target Genes	Amplicons	References
E. coli Genes				
stx_1-F stx_1-R	5`-ATAAATCGCCATTCGTTGACTAC-3` 5`-AGAACGCCCACTGAGATCATC-3`	stx_1	180	[61]
stx_2-F stx_2-R	5`-GGCACTGTCTGAAACTGCTCC-3` 5`-TCGCCAGTTATCTGACATTCTG-3`	stx_2	255	[62]
eaeA-F eaeA-R	5`-GACCCGGCACAAGCATAAGC-3` 5`-CCACCTGCAGCAACAAGAGG-3`	eaeA	384	[63]
hlyA-F hlyA-R	5`-GCATCATCAAGCGTACGTTCC-3` 5`-AATGAGCCAAGCTGGTTAAGCT-3`	hlyA	534	[64]
Internal Quality Control of PCR				
L1091-F H1478-R	5`-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3` 5`-TGACTGCAGAGGGTGACGGGCGGTGTGT-3`	12S rRNA	400	[65]
Bovine Cytokines Genes				
IL10-F IL10-R	5`-CCAAGCCTTGTCGGAAATGA-3` 5`-GTTCACGTGCTCCTTGATGTCA-3`	Interleukin-10	42	[66, 67]
IL4-F IL4-R	5`-CATGCATGGAGCTGCCTGTA-3` 5`-AATTCCAACCCTGCAGAAGGT-3`	Interleukin-4	41	
Internal Quality Control of qRT-PCR				
βact-F βact-R	5`-CCTTTTACAACGAGCTGCGTGTG-3` 5`-ACGTAGCAGAGCTTCTCCTTGATG-3`	β-actin	47	[68]

DNA Isolation:

Five to 10 colonies of each freshly streaked isolates were suspended in 180 µl Tris-EDTA buffer (Sigma Aldrich) containing 5 µl mutanolysin (10 U/µl, Sigma Aldrich). The extraction mixture [8] was added to each bacterial sample, incubated overnight at 56°C, and then DNA isolation was done by the phase separation protocol [69]. The working DNA concentration was evaluated by NanoDrop 2000c (Thermo Scientific), then adjusted to 100 ng/µl concentration [24].

Internal Quality Control for PCR Assays:

A specific PCR assay was applied as a semi-qualitative control for the DNA extraction. During this reaction a target fragment was amplified of about 400 bp from the 12S rRNA gene of the mammals' mitochondrial genome utilizing the L1091-F and H1478-R oligonucleotides [65, 68], (Table-1).

PCR Protocol:

PCR mixture/isolate 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 PTC-100™ Thermal Cycler (MJ Research) using the following cycling protocol: initial denaturation at 95°C for 5 min and then 40 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The final extension was carried out at 72°C for 7 min [62-65]. A reagent blank containing all the components of the reaction mixture with water instead of the template DNA, and VT-negative E. coli were run as controls in every PCR procedure. The cultures showing positive results by PCR were retested on two further occasions several days later to examine the reproducibility of PCR testing. PCRs' amplified products 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) was loaded in each gel, then the gels were documented and anlyzed by Image Lab (BioRad).

Sequencing of PCRs' Products:

Each amplicon was purified for sequencing using the ExoSAP-IT PCR Product Cleanup kit (Affymetrix)

according to the manufacturer's instructions. The 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) as described by the manufacturer. Each sequencing reaction was repeated at least three times in both directions before being accepted for analysis. Then the sequences of each PCR fragment was aligned with genbank records (http://www.ncbi.nlm.nih.gov) by the multiple sequence alignment using the Clustal®W program [70].

Cytokines Expression in Mammary Glands

Isolation of Milk Somatic Cells:

RNA later (Sigma-Aldrich) was added to each milk sample after collection, then preserved until RNA isolation. The aliquots of 25ml milk were centrifuged at 1000 xg for 15 min at room temperature **[20]**. Then each pellet was collected after discarding the fat layer and supernatants. Finally, they were individually washed twice in sterile normal saline solution (pH 7.2) prepared in diethyl pyro-carbonate (DEPC) treated water. Meanwhile, 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 the manufactures' instructions. RNA integrity, purity and quantity were determined 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 [69].

RT of Total RNA to cDNA:

Total mRNAs were reversely transcriped into cDNAs copies using 1 µg of each total 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 a hot start reaction. Reverse transcription mixture contained 15 U of avian myeloblastosis virus (AMV) reverse transcriptase enzyme (Promega), AMV RT buffer (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. The diluted cDNA samples were stored at 4°C until used in qPCR reactions.

Primers' Design and qPCR Assays:

The primers of IL-4 and IL-10 genes along with β-actin housekeeping gens (Table 1) utilized in this study were designed using publicly available bovine sequences, then were purchased from Vivantis. The primers' design was considered to span an intron-exon boundary of target genes to prevent the amplification of genomic DNA [66, 67].

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 25 µl final reaction volume. The qPCR protocol was designed to include initial denaturation at 95°C/10 min, then the thermal fluctuation between 95°C /15 sec, 58°C /30 sec, and 72°C /30 sec for 40 cycles was used as described previously [71]. The fluorescence signals were measured once at the end of the extension step/cycle/gene. For each sample, a dissociation curve was generated after completion of amplification, and was analyzed to determine the specificity of qPCR reaction. The values for target genes were normalized by the internal positive control (β-actin). The normalization factors NFn and NFn+1 were calculated. The relative transcript quantification standard curves were plotted using six fold serial dilution of cDNA. The relative expression level of gene of interest was analyzed using the delta-Ct method and normalized by being divided by a proper normalization factor **[20]**. The analysis of the melting curve of the 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) [72, 73].

Data Analysis and Statistics:

A chi-square test was used to compare the prevalence of each gene profile among E. coli isolates between categories (SPSS 19). The differences between the prevalence rates were considered significant when $p< 0.05$ [74].

RESULTS

Udders Health Impression and SCC Analysis:

The visual inspection of the cows' udders did not display systemic symptoms. In addition, the milk samples were obviously normal. CMT, the cow-side test obtained a rough impression on the udders' health. The quarters scores represented four categories: 0= negative (50/1200), +1= trace (600/1200), + 2 =weak positive (400/1200), +3 =distinct positive (100/1200), and +4 =strong positive (50/1200). The survey included 10 large-scale dairy farm producing low SCC bulk milk tank (≤ 4×10^5/ml), therefore, targeted CMT positivity (+1 & +2) which were found in 1000/1200 udder quarters of the 300 cows.

E. coli identification and Serotypes Prevalence:

The results displayed small colonies that were circular in shape, entire, a diameter of approximately 0.5 mm, convex and smooth. The colonies were pink-red color on MacConkey agar. Some isolates showed alpha, beta and/or gamma -heamolysis of sheep-blood agar after 24 h while, isolates gave no growth on mannitol salt agar. Streaking E. coli on salmonella/shigella agar resulted colonies with pink color. Furthermore, Transmission Electron Microscope examination showed that cells were Gram negative, rods, motile and are arranged in clusters Figure (1).

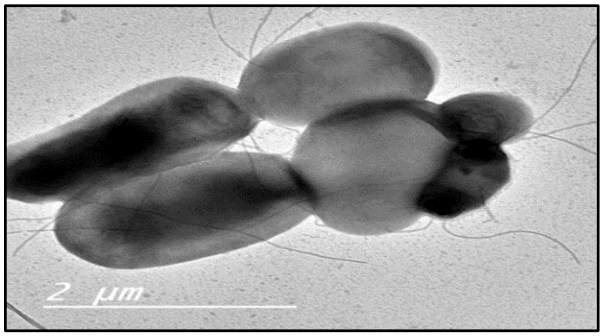

Fig 1. Electron transmission micrograph with 2µm and magnification 2000

The environmental microorganisms were isolated from 16.66% of milk samples, noting that E. coli isolates were 90% (91 isolates) of these bacterial isolates. However, other types of bacteria (10%) were also recorded. The 91 isolates of E.coli were serologically grouped under 10 serovars designed: O1, O8, O20, O25, O78, O127, O146, O153, O157, and O159 with prevalence rates of 9.9%, 12.1%, 11%, 13.2%, 12.1%, 9.9%, 16.5%, 8.8%, 8.8% and 6.5%, respectively.

Haemolysis, Enterocytotoxicity and Pathogenicity Patterns of Shigella-Like Cytotoxic E. coli:

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 (2).

Antibiogram Pattern with regards to Recovery Percentages in Dairy Cows:

The in vitro antibiotic reactivity pattern of the 10 E.coli serovars characterized during this study is illustrated in chart (1). On the other hand, the recovery profile of the lactating cows with subclinical mastitis in response to the treatment (in vivo) with those in vitro evaluated antibiotics is illustrated in chart (2). It is observed that the subclinical cases were persistent to the infections by the resistant bacteria; proved by the laboratory re-isolation of the bacteria post treatment with ampicillin and trimethoprim and sulphamethoxazol (100%), chart (2). In contrast, they were highly susceptible to recovery from infection with absolute sensitive bacteria post the treatment with gentamicin (100%), chloramphenicol (100%). However, the moderate susceptibility to recovery from infection with highly sensitive bacteria was post treated with amikacin (90%), cefadroxil (90%) and norfloxacin (90%), chart (2). While different recovery susceptibilities due to the infection with variant sensitivity bacteria were recorded post the treatment with colistin sulphate (60%), oxytetracyclin (60%), nalidixic acid (60%), and erythromycin (40%), chart (2)

Table 2. Pathogenicity, Enterocytotoxigenicity, Haemolysis, and CD_{50} Results of Isolate

E. coli Serotypes	No. of Isolates	Pathogenicity		Enterocytotoxigenicity		Haemolysis			CD_{50}
		+Ve*	%	+Ve*	%	+Ve*	Type	%	
O1	5	4	80	5	100	5	α, β	25, 75	32, 128
O8	5	5	100	5	100	5	α, β	40, 60	32, 128
O20	5	2	40	1	20	5	γ	100	64
O25	5	3	60	4	80	5	γ	100	8
O78	5	5	100	5	100	5	γ	100	4
O127	5	3	60	4	80	5	α, β	30, 70	32, 128
O146	5	3	60	3	60	5	α, β	50, 50	32, 128
O153	4	4	1	4	100	4	α, β	40, 60	32, 128
O157	6	6	100	6	100	3	α, β	50, 50	32, 128
O159	4	4	1	4	100	4	α, β	40, 60	32, 128

* The percent was calculated according to number of mice in each group

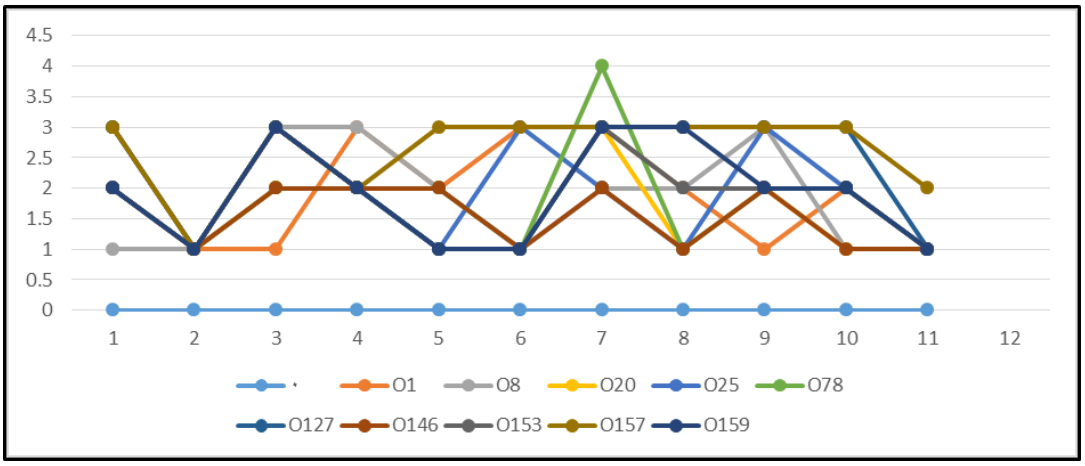

Chart 1. In vitro antibiogram pattern of the 10 E.coli serovars characterized during the study.

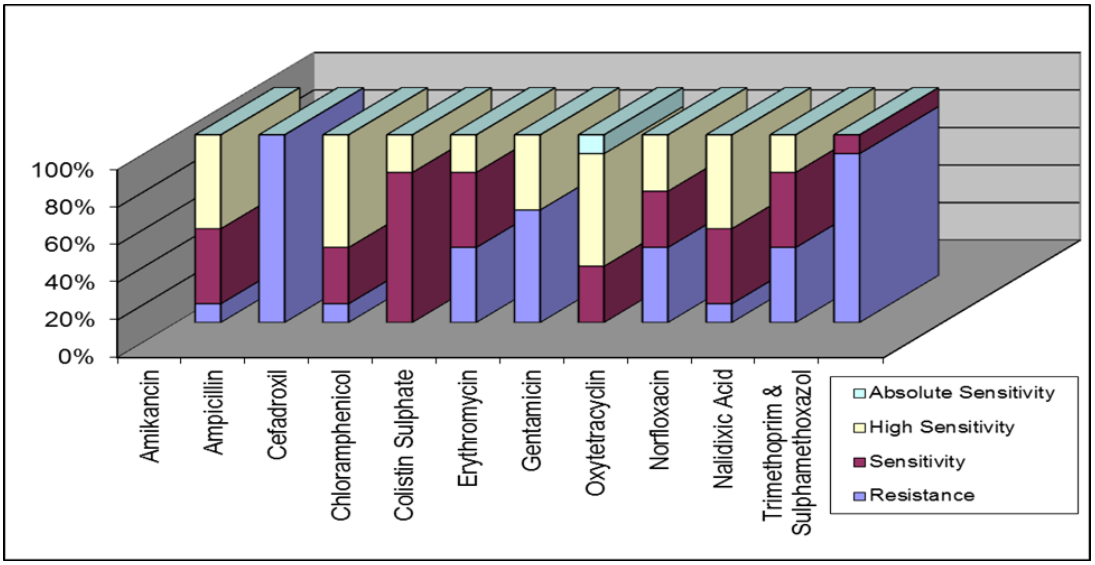

Chart 2. In vivo recovery profile of the lactating cows in response to treatment with antibiotics against predominant bacterial isolates in milk microbiota of subclinical cases.

SDS-PAGE Patterns:

The proteomic profiles of the characterized isolates are consisted of nine dominant protein bands with apparent molecular masses of 106 to109 kDa (O8, O25, O78, O127, O146, O153 and O159), 80 to 83 kDa (O8, O25, O146, and O153), 54 to 56 kDa (O25, O78, O127, O146, and O153), 41 kDa (O147 and O153), 37 kDa (O78, O146, O153 and O159), 32 kDa (O1, O8, O20, O78, O127, O146, and O157), 26-25 kDa (O8 and O78, O146, O153, O157 and O159), 22 kDa (O20, O25, O78, and O127), and 7.8 kDa (O20, O127, O157 and O159). As well, a number of low molecular mass bands was detected in the supernatants of the bacterial cultures. These bands were not uniformly expressed nor equally presented by all the serotypes used in this study.

Virulence Genomics Molecular Typing:

DNA fragments of molecular size of 180 bp, 255 bp, 384 bp, and 534 bp were documented in lanes representing the different serotypes ofE. coli.Hence, the coding sequence for stx_1, stx_2, eaeA, and hlyA genes were present with different incidence rates in 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), figure (3). On the other hand, negative control lane (reagent blank) was negative for all the coding sequences by PCR amplification (Figure 2). The identities of the amplified fragments were confirmed by sequences' alignment againstgenbank records for similar open reading frames sequences with 96-100% similarities.

Table 3. PCR amplified fragments from E. coli serotypes with different incidence rates in variant clones' population.

Sequences / E. coli		Incidence %		stx_1 gene 180 bp	stx_2 gene 255 bp	eaeA gene 384 bp	hlyA gene 534 bp
Serotype	**Clones Population**						
O20, O25, O78	One clone	30	40	+	-	+	+
O1, O8, O127	Clone A	45	50	+	+	-	+
	Clone B		20	+	+	-	-
O153, O157	Clone A	15	50	-	-	-	-
	Clone B		40	+	+	-	-
O146, O159	Clone A	10	30	+	+	-	-
	Clone B		70	+	+	+	+

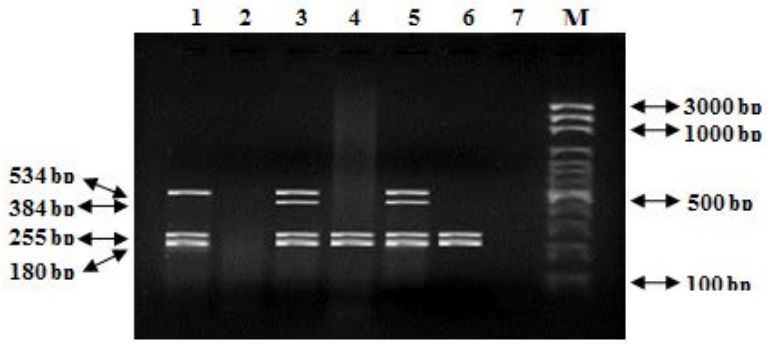

Fig 2. PCR amplification of stx1, stx_2, eaeA, and hlyA genes from E.coli serotypes, fragments sizes of 180 bp, 255 bp, 384 bp, and 534 bp, respectively. Lanes 1: E.coli serotype encoding stx_1, stx_2, and hlyA genes as case with O127 clone A. Lane 2: E.coli serotype lacking the codes for all the virulence proteins genes stx_1, stx_2, eaeA,and hlyA genes as case with O157 clone A. Lanes 3: E.coli serotypes encoding stx_1, stx_2, eaeA, and hlyA genes as case with O159 clone B. Lane 4 & 6: E.coli serotype encoding stx_1 and stx_2 genes as case with O127, O157, and O159 clones B, B, and A, respectively. Lanes 5: Control positive E.coli serotypes O157:H7 encoding stx_1, stx_2, eaeA, and hlyA genes. Lanes 7: Control negative blank reagent. Lane M: Molecular sizes 100 bp DNA Marker (100 bp-3000 bp).

Cytokines Expression in Mammary Glands with regards to E. coli serotype:

The reaction efficiency for all the reference and experimental genes of interest ranged between 0.95 and 1.03. The qRT-PCR results presented up regulation of the cytokine genes expression, both IL-4 and IL-10 in all studied cows despite the difference in E. coli serotype. However, the fold increased were higher for IL-10 than those recorded for IL-4 (Chart 3). The mean of the fold increase calculated to be 2.712 ± 5.178 and 11.231 ±4.146 (Means St. Dev. $2^{-}(\Delta\Delta CT)$.

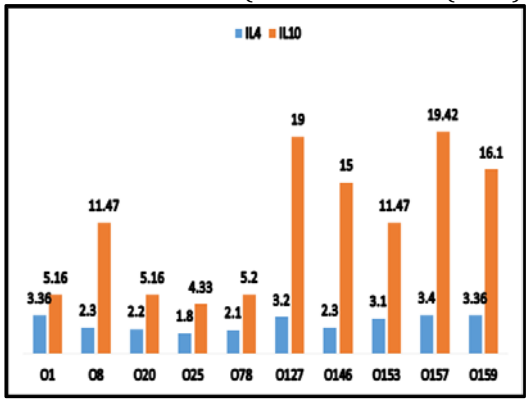

Chart 3. Up regulation mRNA expression of IL4 and IL10 cytokines genes under study in udders somatic cells quantified by qPCR.

DISCUSSION

Mastitis in lactating cows remains a serious problem,hence the environmental pathogens are flourishing in some governorates of Egypt despite the practice of teat dipping and dry cow treatment [1-4, 8, 14, 19, 20, 28]. The pathogenic E. coliinduced 90% of these infections [31] which is in agreement with the present study.It is highly adaptive organism due to its ability to acquire exogenous DNAproviding an evolutionary pathway for pathogenicity [16, 40].

In the present study, 10 serogroups were characterized, then subdivided into 17 clones by phenotypic and molecular profiling. It was previously declared that O157 STEC were responsible for the vast majority of fatality [18, 75, 76]. Nevertheless, from the presented results, it was obvious that a number of non-O157 are likely increasing in the epidemiological dynamic of E. coli worldwideas well as in Egypt [19, 24, 40]. According to the genomics and phylogenetic relatedness, veterinary E. coli isolates were classified as A and B1 genotypes mainly, yet a lower proportion belongs to B2 and D genotypes [33, 34]. Additionally, the conversion to the antibiotic resistance is recorded more in non-B2 phylogenetic groups. This statement is considered the justification for resistance of E. coli pathovars isolated in the present study. Hence, the

excessive application of the antibiotics documented in the studied farms recorded withthe simultaneous occurrence of the recurrent mastitis with A and B1 strains but not B2 strains [19, 24]. Therefore, regular screening tests involving the entire herd are an indispensable element of the professional and efficient mastitis control. Such screening should include determination of the SCC of the individual bulk,as well as, the udder-quarter milk with bacteriological examination of the quarters' macrobiotics [49]. Futhurmore, cows' SCC with subclinical mastitis increase with lactation, therefore, an effective drying-off therapy must be used to restore udder health, otherwise, cow culling based on the individual evaluation of the animals cannot be avoided [31].

Worldwide, the preference is given to genomic characterization than phenotypic or serotype profiling, hence it is associated with virulence determinant genes correlating to the pathogenesis of the infections [19, 20, 44]. In Egypt, coliform is a major inducer of mastitis [17, 42, 43]. The calculated prevalence of serotypesin the present study proved stability in infectivity, enhanced pathogenicity, and increased resistance to antibiotics of these serovars [8, 14, 19, 20, 24]. The difference in prevalence of the virulence genesdetected in the present study between herds was dependent on the differences in dairy farms' management systems [1-4]. On the other hand, several important risk factors especially the link between nutrition and mastitis in the dairy cow were also proposed [2, 3].

The isolates producing Shiga toxins type 2 were commonly more responsible for serious complications than Shiga toxin type 1 producers [19, 24]. Therefore, the fatality is superior when E. coli isolate secrets both types of Shiga toxins simultaneously. This was the comprehensive description of serotypes O125, O157, and O159 clones B, B, and A, isolated during the present study, respectively. Additionally, the plasmid-encoded enterohemolysin toxins; encoded by hlyA gene, putative accessory virulence factors characterize EHEC strains of E. coli, which was the case in the isolated O125 clone A [19, 24]. Finally, the intiminprotein encoded by conserved region of eaeA gene between STEC and EPEC, accounts for the invasive properties of mastitis associated coliform strains [19, 24, 39]. Thus, a proper diagnosis to specifically detect STEC, EPEC, and EHEC in dairy products is mandatory. Hence, it is highly advantageous to prevent the destructive effects of these toxins on udder tissues and overcome their heat resistance and persistence in byproducts even after their sanitary treatments [18]. In this study, the majority of the examined isolates were positive at least for one virulence gene which was in contrast to the previous publications. Despite the several virulence genes which have been identified, they lack most of the virulence factors in vivo [77]. Other explanations depended on mutations in coding sequences. Additionally, less availability of target genes due to less copy number of plasmids carrying coding sequences was proposed. Moreover,the release of other excretory virulence proteins that enhance the cytotoxic effects on cells is justified [19, 20, 24]. Nevertheless, the genetic constituents of the host that guide the resistance or susceptibility of udder to pathogens has priority [18, 39]. The presented results reinforced the theory of Whittam that the pathogenic E. coli strains have a clonal population structure with broad host ranges and wide geographic distribution [78, 79].

SDS-PAGE could reveal proteomics' profile of characterized species. Nine dominant protein bands with apparent molecular masses of 106 to109 kDa, 80 to 83 kDa, 54 to 56 kDa, 41 kDa, 37 kDa, 32 kDa, 26-25 kDa, 22 kDa, and 7.8 kDa were identified. Detected bands were not uniformly expressed by all serotypes, however, similar molecular weight bands were functionally characterized [19, 20, 24, 79]. P106-109, P80-83, P41, P37, P26-25 were previously designed as EspP, EspE, EspD, EspB, and EspA, respectively, of EPEC, STEC O157:H7 and other attaching and effacing E. coli [19, 20, 24, 79]. EspP (P106-109) belonged to serine protease family responsible for mucosal hemorrhage [19, 20, 24, 78]. While, EspEs (P80-83) weremembers of catalase/peroxidase enzymes responsible for attaching/effacing phenomena [19, 20, 24, 78]. EspD, EspB, and EspA presented by P41, P37, P32, and P26-25, respectively, are important signal transduction receptorsfor early bacterial attachment to mammary epithelial cells [19, 20, 24, 79]. Whereas P32 and P7.8 are believed to be the A and B subunits of Stx_1 and/or Stx_2 [19, 20, 24, 79]. On the other hand, in O78 the 32 kDa proteins bands tend to be Clostridium difficile-like toxin [19, 20, 24, 78]. The research team considered P22 and P54-56 components of the media used for toxins production, since they were not documented before and could not be justified till article was released. In contrast, all the previously mentioned protein bands need to be sequenced and aligned with homologous domain family confirming the previous conclusions.

All previously illustrated antigen and/or toxins should alter the host gene expression. Therefore, udder E. coli bacterial determinants of invasion were unclear, since they switched on udder-dependent mechanisms of innate immune response [1, 4, 36]. Quantification of transcription levels of genes responsible for immunity well justified abnormal alterations in regulation due to the infection [80]. Bovine cytokines, especially interlukine-4 (IL-4) and interlukine-10 (IL-10) had been considered useful markers in defining mammary gland defenses triggered by pathogen-dependent expression induction [81]. The milk somatic cells included several cell types; neutrophils, macrophages, lymphocytes and a small number of epithelial and natural killer (NK) cells [81]. From the obtained qRT-PCR, the bacterial toxins up regulated mRNA transcription of IL-4 and IL-10 genes in peripheral blood mononuclear cells. These cytokines induced a shift in T-cell phenotypes from CD4+ T-cells to CD8+ T-cell, as well as, the differentiation of Th1 and Th2

lymphocytes to switch on the cellular immune cascades, and enhanced the humeral immune response [80]. IL-10 was found capable to inhibit natural killer cells, so its higher expression in milk usually indicate recurrent and/or chronic infectious pathogens [82]. Moreover, IL-4 is antagonist to IFN-γ, therefore, it effectively regulated humeral IgE-mediated immune responses of the udder [80]. The dairy industry regarded the SCC as an indicator of outstanding importance among the qualification parameters of raw milk. In practice, this necessitated regular and herd level mastitis control that is quarter-specific more than being udder-dependent. Hence, even cows producing relatively low SCC milk (≤ 4×10^5/ ml) may anchorage sub-acute, subclinical or chronic mastitis in one or two of their udder quarters. In such animals, the SCC remains below the limit due to the diluting effect of milk from the healthy udder quarters. This may pose a serious risk to healthy herd mates, especially if virulent toxigenic pathogens are actively dynamic [1, 4, 19, 20, 24, 36].

Finally, there is a wide range of virulence genes which may play a role in the pathogenesis of mastitis associated with E.coli isolates, however, their inclusion was beyond the scope of the current study. The results of this study indicated that mastitis isolates of E.coli belong to different phylogroup/serogroups/clones that contained a variety of virulence associated traits. Further large scaled studies and detailed analysis of the data are necessary to identify the association between virulence factors and certain clones of E.coli affecting bovine mastitis immunity.

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