Cytokines Expression Associated with *E. coli* Infection In bovine mammary glands

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**ABSTRACT**

In order to characterize the expression of genes associated with immune response mechanisms to mastitis, we quantified the relative expression of the IL-4, IL-6, IL-8 and IL-10 genes in milk cells of healthy cows and cows with clinical mastitis. Gene expression was analyzed by real-time PCR. IL-8 gene expression was higher than another interleukin. *E. coli* is among the major mastitis pathogens responsible for clinical mastitis in dairy cows, but the infection are normally cleared by the immune system within a few days. Mastitis tolerance/susceptibility is difficult to measure directly and hence milk somatic cell count (SCC) or milk somatic cell score (SCS) is used as an indicator trait for mastitis as both traits are highly positively correlated. Thus, understanding of mastitis pathogen and somatic cell count associated with cell mediated immune response useful for future control of mastitis.

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1. **INTRODUCTION**

Mastitis is the most costly disease for dairy farmers and industry (Hujips et al., 2008; Hogeveen et al., 2011). Which are mainly caused by the entry of bacteria to teat canal affecting up to 40 percent of cows with in a herd at any given time. Bovine mastitis is defined as an inflammatory condition of the mammary gland in response to injury, which serves to destroy and neutralize infectious agents and promote healing and return to normal function. Escherichia coli are the most important environmental pathogen (Smith and Hogan, 1993). Enumeration of the somatic cell count (SCC) of milk has long been used as a tool for measuring milk quality (Dohoo and Leslie 1991). Serogrouping of *E. coli* was carried out to give an idea about the most predominant serogroups associated with clinical mastitic cases. *E. coli* recovered from mastitis cases belonged to different serogroups and varied greatly in O groups and may not be attributed to epizootic strains (Moussa et al., 2006 and Lamey et al., 2013). Cytokine production and leukocyte adhesion play important roles during bacterial infection however, the relative contributions of these factors to the pathogenesis of mastitis are not yet fully determined and will require more extensive studies. In addition, the contributions of various lymphoid and myeloid subsets to host defense in the mammary gland have not been extensively evaluated with naturally infected cows (Park et al., 1994).

The objective of the present study to isolation of bacteria and characterize the expression of genes associated with immune response mechanisms to mastitis by expression of interleukin 4 (IL-4), IL6, IL8 and IL10.

2. **MATERIALS and METHODS:**

2.1. Animal selection and milk collection

A total number of 170 lactating Holstein-Friesian cow from 4 dairy herds located in El Bohera Governorate were studied in this investigation. A 50-mL aliquot of milk was collected from one quarter of each cow into sterile tubes. All animals were submitted to clinical examination of the udder before collection of the samples. The milk samples from cows with mastitis were collected from the quarter with clinical mastitis immediately after the onset of clinical signs and before drug treatment, thus excluding artificial infection of the cows. In addition, all samples were submitted to microbiological analysis.
2.2. Bacteriological identification

The milk samples were incubated for 18-24 hour at 37 C then loopful from the samples were cultured on MacConkeys agar media, Eosine methylene blue (EMB) agar media and Blood agar media. All plates incubated at 37 C for 24-48 hours and examined for bacterial growth (Koneman et al., 1993). Suspected colonies of E. coli appeared as pink lactose fermentating colonies 3mm or more on MacConkeys agar media and melatic black colonies on EMB media. Suspected colonies of E.coli on MacConkeys agar media and EMB agar media were picked up on to semisolid media and incubated at 37 C for 24 hours for being subjected for further identification.

2.3. Isolation of milk somatic cells (Lee et al., 2003)

Fifty milliliter of aseptically collected milk samples were diluted with an equal volume of sterile PBS and centrifuged at 700xg for 20 min at 20 C. After the fat layer and the supernatant were discarded, the cell pellet was washed twice and suspended in sterile PBS. A small portion of the cell suspension was properly diluted and cytospin centrifuged for differential counting. The remaining portion was enumerated and spun down for total RNA extraction.

2.4. Serotyping of E.coli: (Animal Research institute Dokki, Cairo.) (Sakazaki, 1992)

2.5. Cytokine detection and quantification (cytokes mRNA fold change) using real time PCR relative quantification:

2.5.1. RNA extraction:

Total RNA was isolated from milk cells (4 samples for each groupe) using the RNeasy mini RNA Purification kit RNeasy Mini Kit (Qiagen, Germany) Cat. No. 74104 (50 reaction) and 74106 (250 reaction) following manufacturer’s instructions.

Cells washed 3 times with sterile saline with centrifugation at full speed.

600 µl of prepared lysis buffer (prepared by add 6µl of 2-ME ) were added directly to the cell pellet, mixed gently by pipetting for several times then collected at 1.5 ml tube. Ethanol 70% (600 µl) were added to each sample and mixed well by vortexing and short spin. Six hundred and fifty µl from the previous step were transferred to the Rneasy mini spin column and centrifugation at 10,000 rpm for 15 sec. Fluid in the collection tube were discarded and remaining 650 µl added to the column and centrifugation at 10,000 rpm for 15 sec .then fluid was discard. Buffer RW1 (350 µl) was added and centrifugation at 10,000 rpm for 15 sec .then fluid discarded. Ten µl of DNase I stock solution were added to 70 µl buffer RDD and mixed by gently inverting the tube and spin down. Eighty µl of reconstituted DNase I were added directly to the RNeasy spin column membrane and remained at room temp. (20-30 C) for 15 min.

Washing step 5 was repeated. Five hundred µl RPE were added and centrifugation at 10,000 rpm for 15 sec and then fluid discarded.500 µl of RPE were added again and centrifugation at 15,000 rpm for 2 min. to get rid of all fluid .Optionally: the column was transferred in to new 2 ml collection tube and centrifugation at 10,000 rpm for 1 min. The column was transferred in to a new RNA grade 1.5ml tube then 50µl RNase free water were added and dropped in the center of the filter. Centrifugation at 10,000 rpm for 1min then RNA is was collected in the tube.

Table 1: According to following Denka Seiken co., LTD.

<table>
<thead>
<tr>
<th>Polyvalentes Serum</th>
<th>Monovalentes serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyvalent1</td>
<td>O1 O26 O86a O111 O119 O127a O128</td>
</tr>
<tr>
<td>Polyvalent2</td>
<td>O44 O55 O125 O126 O146 O166</td>
</tr>
<tr>
<td>Polyvalent3</td>
<td>O18 O114 O142 O151 O157 O158</td>
</tr>
<tr>
<td>Polyvalent4</td>
<td>O6 O27 O78 O148 O159 O168</td>
</tr>
<tr>
<td>Polyvalent5</td>
<td>O20 O25 O63 O153 O167</td>
</tr>
<tr>
<td>Polyvalent6</td>
<td>O8 O15 O115 O169</td>
</tr>
<tr>
<td>Polyvalent7</td>
<td>O28ac O112ac O124 O136 O144</td>
</tr>
<tr>
<td>Polyvalent8</td>
<td>O29 O143 O152 O164</td>
</tr>
</tbody>
</table>
2.5.2. Amplification of cytokine’s mRNA by Real-Time RT-PCR:

Table (2) Real-Time RT-PCR Reaction Mix Volumes for one reaction using QuantiTect SYBR® Green PCR Kits Cat No (204141) for quantitative of cytokines mRNA was described as following:

<table>
<thead>
<tr>
<th>Reagent used</th>
<th>Volume/1 Reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2XQuantiTect Probe RT-PCR Master</td>
<td>12.5 µl</td>
<td></td>
</tr>
<tr>
<td>primer A</td>
<td>1 µl</td>
<td>10 pmol</td>
</tr>
<tr>
<td>primer B</td>
<td>1 µl</td>
<td>10 pmol</td>
</tr>
<tr>
<td>Probe</td>
<td>0.5 µl</td>
<td>10 pmol</td>
</tr>
<tr>
<td>Thermo RT Mix</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Thermo RT enhancer</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>RNase-free water</td>
<td>5.5 µl</td>
<td></td>
</tr>
<tr>
<td>Template RNA</td>
<td>3 µl</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
<td></td>
</tr>
</tbody>
</table>

Table (3): PCR cycling protocol:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp and time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>50˚C/30 min</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95˚C/10 min</td>
</tr>
<tr>
<td>No. of cycles</td>
<td>No. of cycles 40</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95˚C/15 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58˚C/30 sec for B actin, IL6, IL8. And 54C/30 sec for IL4,IL10</td>
</tr>
<tr>
<td>Elongation</td>
<td>72˚C/30 sec.</td>
</tr>
<tr>
<td>Melting curve</td>
<td>95˚C/15 sec,54˚C/30 sec, 95˚C/15 sec</td>
</tr>
</tbody>
</table>

Table (4) Primers Sequences of for bovine cytokines and B-actin in real time PCR (Sigma):

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Sequences (5'-3')</th>
<th>Length</th>
<th>Tm˚C</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>IL-4f206</td>
<td>CATGCATGGAGCTGCTGCTGTA</td>
<td>41</td>
<td>60C</td>
<td>NM173921</td>
</tr>
<tr>
<td></td>
<td>IL-4r288</td>
<td>AATTCCAACCTGCAAGGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6f209</td>
<td>TCATTAAGCGCATGTTGACAAAA</td>
<td>47</td>
<td>61C</td>
<td>NM173923</td>
</tr>
<tr>
<td></td>
<td>IL-6r313</td>
<td>TCAGCTATTTTCTGCCAGTGTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>IL-8f251</td>
<td>CACTGTGAAAATTTCAGAAATCATTGTTA</td>
<td>53</td>
<td>64C</td>
<td>NM173925</td>
</tr>
<tr>
<td></td>
<td>IL-8r355</td>
<td>CTTCAAAATACCTGCACAACCTTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>IL-10f240</td>
<td>CCAAGCCTTGTCGGAATG</td>
<td>42</td>
<td>62C</td>
<td>NM174088</td>
</tr>
<tr>
<td></td>
<td>IL-10r330</td>
<td>GTTCACGTGCTCTTGTGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-actin</td>
<td>B-actin.f363</td>
<td>CCTTTTACAACAGCAGCTGGTG</td>
<td>47</td>
<td>65C</td>
<td>NM001033618</td>
</tr>
<tr>
<td></td>
<td>B-actin.r753</td>
<td>ACCTAGCAGAGTCTCTCTTGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Alluwaimi et al., 2003; Lee, J. and Zhao, X. 2000)
3. RESULTS

3.1. Isolation and identification of *E. coli*:

Table (5): The incidence of *E. coli* in mammary gland infected with *E. coli* in cow:

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Incidence</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>170</td>
<td>35</td>
<td>20.5</td>
</tr>
</tbody>
</table>

Fig (1): Gene expression of cytokine marker in milk samples

3.2. Serotyping of *E. coli*:

6 Isolates were serotyping using monovalent and polyvalent sera, isolate were O sero groups, and different O serotype, 2 un tyable strain were obtained, the predominant 4 serotype were (Poly4 O148).

3.3. Gene expression of cytokine marker in milk samples:

Results represented at graphic calculated based on the mean of 4 samples for each group.

Cytokine marker in bovine mammary gland infected with *Ecoli* with positive control B-actin occur upregulation of interleukin-6, interleukin-8 and interleukin-10 and downregulation of interleukin-4. In this study interleukin-8 was highly expressed than another interleukin. *E. coli* was able to up regulate interleukin-8 which responsible for attraction of other inflammatory cells to site of inflammation. In *E. coli* infected cows, IL8 were significantly higher (upregulated) compared to B.actin .Other interleukin did not differ.

3.4. Statistical analysis:

Groups were compared using analysis of variance (SAS, 2011).

Table (6) Relationship between different interleukin:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ct (<em>E. coli</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.actin</td>
<td>28.57 ± 0.56 BC</td>
</tr>
<tr>
<td>IL4</td>
<td>27.52 ± 0.22 AC</td>
</tr>
<tr>
<td>IL6</td>
<td>28.87 ± 0.69 BC</td>
</tr>
<tr>
<td>IL8</td>
<td>30.91 ± 0.89 A</td>
</tr>
<tr>
<td>IL10</td>
<td>29.50 ± 0.40 C</td>
</tr>
</tbody>
</table>

Values are means ± standard errors.

Means in a row with a common capital letter do not differ significantly (P>0.05).
4. DISCUSSION

Bovine mastitis is a large-scale infectious disease with significant impact on the economy of milk production (Halasa et al. 2007; Awale et al. 2012). Bovine mastitis is initiated by the entry of bacteria through the teat canal and soon after is characterized by an important inflammatory response. Shortly after entry of the invading pathogen, the resident leukocytes together with epithelial cells initiate the inflammatory response necessary to eliminate the invading bacteria (Rainard and Riollet, 2006; Aitken et al., 2011).

Cells release chemo attractants for the rapid recruitment of polymorph nuclear neutrophil leukocytes to the site of infection and consequently the Somatic Cell Count (SCC) increases, which represents different cells types present in milk, including leukocytes and epithelial cells (Souza et al., 2012).

The marked increase in milk SCC during infection is mainly due to influx of neutrophils from blood to the mammary gland, which neutrophils can represent over 90% of leukocyte population in milk from infected udder quarters in contrast to low numbers of this cell population in uninfected ones (Pyorala, 2003; Souza et al., 2012).

Shiga-like toxin producing E. coli (STEC) also known as verotoxin producing E.coli. The most E. coli serotypes isolated from mastitic cows and buffaloes produced verotoxin and this result consistent with the hypothesis that verotoxin play a major role in the pathogenesis of mastitis caused by E. coli. The pathogenicity of this disease probably results from the production of verotoxin or Shiga-like toxin which efficiency inhibits protein synthesis in mammalian cell free system (Dalia and Amany, 2007). E. coli O148 strain isolated was associated with haemorrhagic colitis and HUS, and possessed the stx2c gene, which confirms its characterization as an STEC strain (Espie´ et al., 2006). O148 Shiga-like toxin producing E. coli (STEC) strain isolated from mastitis cases in this result and that agreed with Moussa et al. (2006); Wenz et al. (2006); Fernandes et al. (2011) and Amira et al. (2013). They recorded that E. coli strains recovered from mastitis cases belonged to different serogroups and varied greatly in O groups and may not be attributed to epizootic strains. This indicates that E. coli mastitis is not caused by a limited number of specific pathogenic strains, but seems to be associated with environmental fecal contamination and be multifactorial (Rangel and Marin, 2009).

Interleukin-8 is a well-known neutrophil chemotactic cytokine that is produced by stimulated monocytes, T lymphocytes, macrophages, endothelial cells and a number of tumor cell lines (Matsushima and Oppenheim, 1989). The biological role of IL-8 in attracting neutrophils to infected bovine mammary gland was revealed by blocking the neutrophil chemotactic activity with anti-IL-8 antibodies in mastitic mammary secretions (Barber and Yang, 1998).

Ecoli able to up regulate IL8 (which responsible for attraction of other inflammatory cells to the site of inflammation). The expression of IL-8 increased of infected mammary gland of Pathogen of E.coli compared with uninfected ones and these agreed with (Guntler et al., 2010) and (Lee et al., 2003) and (Lahouassa et al., 2007) who said In vitro, mammary epithelial cells demonstrated greater mRNA expression of IL-1β, IL-8 and TNF-α24 h after infection with E. coli than S. aureus.

Interleukin-6 is one of the proinflammatory cytokines incriminated in the development of signs of acute septic shock in coliform mastitis (Riollet et al., 2000a; Shuster et al., 1993, 1997). In naturally occurring coliform mastitis, IL-6 has been detected in serum and milk, higher in the latter except in severe coliform mastitis (Nakajima et al., 1997).

In this study interleukin-6 expressed in milk somatic cell infected with Ecoli and this agreed with (Taylor et al., 1997) who said Interleukin-6 has been detected in cells from mastitic gland using RT-PCR. This result agreed with (Shuster et al., 1997) who said in mammary gland infected with E. coli, IL-6 expression was detected as early as 14 h pi.

In this study interleukin-10 expressed in milk somatic cell infected with E.coli. The present results agree with the findings of (Bannerman et al., 2004a, 2004b, 2005) who observed various studies have shown that
IL-10 is expressed in milk cells of udders infected with different pathogens.

In this result IL4 not occur expression to it and these result agree with (Fonseca., 2009) expression of the IL-4 gene showed a trend to be lower in cows with mastitis compared to healthy animals. On the other hand this result not agree with (Asai et al., 1998) marked increase in the mRNA expression of interleukin-2 (IL-2) and interleukin-4 (IL-4) was recorded.

In this result, we found that in comparison with different interleukin such as IL-4, IL-6, IL-8, IL-10 found that IL-8 highly expression than another Interleukin in milk somatic cell infected with E.coli in mammary gland of cows.

5. REFERENCES


