P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com
Research Article https://doi.org/10.47278/journal.ijvs/2022.161

# Bovine Mastitis in Egypt: Bacterial Etiology and Evaluation of Diagnostic Biomarkers

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**Article History:** 22-538 Received: 24-Feb-22 Revised: 18-Apr-22 Accepted: 13-May-22

## ABSTRACT

This study is providing new techniques for diagnosis of bovine mastitis using multiplex real-time PCR and detection of some inflammatory markers at an early stage of infection. The consequences of bacterial culture and PCR for the identification of the 8 etiological agents of mastitis clinical or subclinical were demonstrated. Some pathogens recognized by conventional culture techniques were confirmed by PCR. Corynebacterium and mycoplasma were only distinguished by PCR. These data indicated moderate accord among the positive results of bacterial culture and PCR. Mixed infection of 2 or more mastitic bacterial agents were also identified more frequently by PCR while the measured blood and milk indices could separate healthy from mastitis and subclinical mastitic animals, it could not do so either between mastitis and subclinical mastitis or between single and mixed infection. Regarding inflammatory markers, most blood indices differed significantly between healthy vs mastitis animals, except for monocyte. Haptoglobin expression in milk varied significantly altering on mastitis circumstances and type of infection with mean fold change values of 1.87 in negative cases, 7.26 in single infected, and 10.64 in mixed infection. Also, a highly significant between the type of infection and immune- histocompatibility was detected. As immune histocompatibility was strongly expressed with mixed infection and wasn't detected in negative cases. From a diagnostic point of view, TLC, lymphocyte, and neutrophil were considered good biomarkers for mastitis (Area under curve >0.9), and haptoglobin was the best biomarker for subclinical mastitis (AUC>0.9). Haptoglobin and basophils were the only markers having high AUC (being 0.6 and 0.7, respectively) discriminating single from mixed infection. Therefore, our diagnostic strategy has been deservedly proved its effectiveness as a rapid, complementary, and sensitive substitute to traditional techniques.

**Key words:** Bovine mastitis, Biomarkers, Haptoglobin, Etiological agents, Multiplex real-time PCR.

## INTRODUCTION

Mastitis is an endemic syndrome of dairy cows and is considered to be one of the most important and costly diseases to the dairy industry in many countries (Khan et al. 2013; Qayyum et al. 2016a; Du et al. 2022). It is frequently initiated by an intramammary infection and can be clinical, where the animal exhibits over the signs of disease, or more frequently subclinical at when the animal shows no external symptoms, however, has a raised somatic cell count (SCC) (Åkerstedt et al. 2007; Hussain et al. 2013). Pathogens which frequently initiate mastitis are commonly categorized as either 'contagious' or 'environmental' pathogens, relying on the bacterial source and the manner of spread. The most prevalent contagious bacteria are *Staphylococcus aureus* and *Streptococcus sp*. These pathogens have acclimatized to survive through the mammary gland and multiply from animal to animal at or in the range of the milking duration and mastitis cases are principally caused by infection with contagious pathogens (Korwin-Kossakowska 2008; Qayyum et al. 2016b). The quality of a suitable assay for confirmatory diagnosis relies on several agents, for instance specificity, price, period required and pertinence to huge quantities of milk samples. The most popular assay to distinguish the potent chronic infections is a somatic cell count however it is non-specific.

**Cite This Article as:** El-Demerdash AS, Bakry NR, Aggour MG, Elmasry SS, Mowafy RE, Erfan A, Taha MF, El-Gmaal AAAM, Mohamed AAE, Hagag N and Samir M, 2023. Bovine mastitis in Egypt: bacterial etiology and evaluation of diagnostic biomarkers. International Journal of Veterinary Science 12(1): 60-69. https://doi.org/10.47278/journal.ijvs/2022.161

Therefore, elevated detection of somatic cell count must be followed with the identification of the involved pathogens. Early diagnosis assays have been illustrated to improve the recovery ratio and minimize the period taken to obtain normal milk. Traditional methods for isolation and identification of microbial pathogens from the milk sample of an infected animal have represented as the gold criteria in mastitis diagnosis. But it has several disadvantages as, it is a comparatively slow technique, taking at least time of 24-48 hours before a microorganism can be dependably identified. The lack of fast results may lead to the use of broad-spectrum antibiotic therapy in many cases leading to antibiotic residues problem in bovine milk. Thus, nucleic acid-based PCR assays have been evaluated to reveal the incidence of several pathogens in milk and therefore, decrease time-consuming. Previous studies displayed that milk samples may be represented as the main target for amplification of specific DNA alignments using PCR (Berri et al. 2000). A multiplex polymerase chain reaction (PCR) technique was developed for instantaneous species recognition. Depending on this hypothesis we advanced a multiplex real-time PCR assay with primers sets, as many bacterial pathogens are involved in bovine mastitis.

Moreover, the acute phase response is very useful in the diagnosis of subclinical mastitis. Many studies have indicated the significance of haptoglobin which is the most important acute phase protein as a clinically beneficial parameter for measuring the occurrence and seriousness of inflammatory responses in cattle with mastitic infection. Still, some investigators demonstrated that gene expression of haptoglobin aid in the early forecast of the disease after 24 hours only of the infection (Hiss et al. 2004; Thielen et al. 2007; Lakshmi et al. 2014). Besides, the function of the major histocompatibility complex (MHC) in immune response creates an MHC catchy nominee to analyze affiliations with disease resistance or susceptibility, where the majority of them are associated with host defense and intercellular communication.

Therefore, our study was designed to detect the early generalized immune response in mastitis by expression of an important inflammatory marker (haptoglobin) and measurement of the major histocompatibility complex (MHC) in milk samples. Additionally, the enhancement of a molecular diagnostic implementation for mastitic pathogens using multiplex real-time PCR.

## MATERIALS AND METHODS

### Ethics Approval

The study was conducted with the permission of the Animal Health Research Institute (AHRI) and in line with the guidelines of the committee.

### Sampling

Animals: A total of 547 dairy cows and buffalos were examined according to the animal census from different governorates under hygienic conditions following statistics data.

Milk samples: A total of 2188 individual quarter milk samples were exposed to somatic cell count.

Whole blood samples: Total 547 samples were collected for complete blood picture examination

Serum samples: Total 547 samples were collected for haptoglobin expression examination.

### Somatic Cell Count Detection

Somatic cell count (SCC) was performed using a somatic cell counter to detect the number of somatic cells from the obtained milk samples (Thurmond 1990). The SCC range deemed normal is below 200.000 cells/mL of milk.

### Isolation and Identification of the Involved Pathogens

After counting somatic cells, the milk sample of the high count was inoculated on a blood agar and tryptic soya agar then incubated for 24 hrs. at 37°C. Further identification of specific bacterial isolates such as *Staphylococci* spp., *Streptococci* spp., *M. bovis*, and gram-negative bacteria was done according to the manufacturer designated by the National Mastitis Council and API.

### Detection of the Mastitic Pathogens from Milk Samples by Multiplex Real-time PCR

### Validation of PCR

Dual-labeled probes were designed using internet-based tools PCR primers and probes were developed for single real-time PCR techniques and verified for specificity and sensitivity previously in laboratory (Gillespie and Oliver 2005 and Keane et al. 2013). The utilized primers and probes were provided from Willowfort (UK) and listed in Table 1.

### DNA Extraction

The DNA from milk samples and bacterial isolates was extracted using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH, Cat. No. 51304) with some modifications. Concisely, 200µL of the sample suspension was incubated with 10µL of proteinase K and 200µL of lysis buffer at 56$^{O}$C for 10min. After incubation, 200µL of absolute ethanol was provided to the lysate.

### Multiplex real-time PCR amplification and cycling conditions

PCR cycling was performed in a final volume of 20µL containing 10µL of 2x Sensifast probe No-ROX buffer (Bioline, UK), 3.125µL PCR grade water, 0.25µL of each primer (5pmol conc.) and 0.125 µL of each probe (30pmol conc) and 5µL of DNA template. The establishment of primers sets for multiplex cycling conditions was as follows:

a. *M. bovis with E. coli* and *Streptococcus* species

b. Pseudomonas with Klebsiella and *S. aureus*

c. *Coagulase negative Staphylococci (*CNS) with *C. bovis*

Cycling conditions were started by primary denaturation at 94ºC for15 min, followed by 40 cycles of denaturation at 94ºC for 15s, annealing at 50ºC for 30s, and extension at 72ºC for 30s.

### Making Complete Blood Picture

The rise in the count of neutrophils is a very important indication of the presence of inflammation. Thus, we made a complete blood picture of whole blood samples using the Sysmex xt 2000v apparatus.

### Gene Expression for Haptoglobin

RNA was extracted from serum samples using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH, Cat. No. 74904). The B-actin housekeeping gene is represented as an internal control to normalize the expressional

**Table 1:** Primers and probes sequences used in the multiplex real-time PCR assay

| Primer/ probe | Sequence 5´- 3´ | Reference |
|---|---|---|
| *M. bovis*.F | GAGAATGCTTCAGTATTTTGACGG | Naikare *et al.* (2015) |
| *M. bovis*.R | CAAAAGCAAAATGTTAAATTCAGG | |
| M. bovis-Prob ROX | CAL Fluor Red 610-CATATATAAGTGAGACTA ACTTATT-BHQ2 | |
| Klebsiella.F | TGGAGCATGTGGTTTAATTCGA | Cunningham *et al.* (2013) |
| Klebsiella.R | TGCGGGACTTAACCCAACA | |
| Klebsiella-Prob ROX | CAL Fluor Red 610- CACGAGCTGACGACAACCATGCA-BHQ2 | |
| *E. coli*.F | CGCCTAATCCGCAACGTAAT | This study |
| *E. coli*.R | CGCAGCGTGATCCTGTTTAT | |
| *E. coli*-Prob FAM | FAM-TGGCGCAGATGACTGATAAAGCCA-BHQ1 | |
| Strept.F | GTACAGTTGCTTCAGGACGTATC | |
| Strept.R | ACGTTCGATTTCATCACGTTG | |
| Strept-Prob HEX | CAL Fluor Orange 560 -ACAATTGGACGAAGGTCTTGCTGGA-BHQ1 | |
| *S. aureus*.F | TCGAAATTAAATGTTGTCGTGTCTTC | Goto *et al.* (2007) |
| *S. aureus*.R | TCATTTTTGACATGRAGAGAAACATC | |
| *S. aureus*-Prob FAM | FAM- TCGCGACATTCATTATGCCCAAATTTTTAA-BHQ1 | |
| Pseudomonas.F | TGTGAAGAAGGTCTTCGGATTG | This study |
| Pseudomonas.R | CGAAGTTAGCCGGTGCTTAT | |
| Pseudomonas-Prob HEX | CAL Fluor Orange 560-AGTTGGGAGGAAGGGCAGTAAGTT-BHQ1 | |
| CNS.F | GCGGdTCCATCTATAAGTGA | Edwards *et al.* (2001) |
| CNS.R | GGGTGAGTAACACGTGGA | |
| CNS-Prob FAM | FAM-GGATAATATATTGAACCGCA-BHQ1 | |
| *C. bovis* .F | ACCTGGTGGACGAGAAGA | This study |
| *C. bovis*.R | CACCGAACTGGGCCTTAC | |
| *C. bovis*-Prob HEX | CAL Fluor Orange 560-CGTACTCCATGATCACCCAGCAGC-BHQ1 | |

**Table 2:** Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR

| Target gene | Primers sequences | Reverse transcription | Primary Denaturation | Amplification (40 cycles) | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | Secondary denaturation | Annealing (Optics on) | Extension | |
| Bhb1 | GTCTCCCAGCATAACCTCATCTC | 50˚C | 94˚C | 94˚C | 55˚C | 72˚C | Hiss *et al.* |
| | AACCACCTTCTCCACCTCTACAA | 30 min. | 15 min. | 15 sec. | 30 sec. | 30 sec. | (2004) |
| B. actin | CGTGGGCCGCCCTAGGCACCA | | | | 55˚C | | Fitzpatrick |
| | GGGGGCCTCGGTCAGCAGCAC | | | | 30 sec. | | *et al.* (2002) |

levels among samples. Primers were used in a 25µL reaction comprising 10µL of the 2x HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 1µL of RT Enzyme Mix (20X), 0.5µL of each primer of 20pmol concentration, 5µL of water, and 3µL of RNA template. The reaction was realized in a step one real-time PCR machine with certain conditions cited in the Table 2. To approximate the difference of gene expression on the RNA of the several samples, the Ct of each sample was compared with that of the positive control group following the "ΔΔCt" assay stated by Yuan et al. (2006).

**Measurement of the Major Histocompatibility Complex (MHC)**

After separation of the somatic cells, blocking of bovine separates to avoid adherent capacity will be done, followed by labeling using bovine serum leucocytic antigen (BOLA), staining by immunofluorescent dye and examination by fluorescent microscope and counting fluorescent cells (positive cells) which represent major histocompatibility complex (Filatov et al. 2007).

**Statistical Analysis of Data**

One-way ANOVA test followed by Dunnett's multiple comparison test was run to determine the significance of difference in the value of each blood parameter among healthy, mastitis and subclinical mastitis groups. To determine the diagnostic power of the analyzed biomarkers, receiver operating characteristic curve (ROC) analyses were done for each parameter comparing (1) healthy versus mastitis animals, (2) healthy versus subclinical mastitis animals and (3) single vs mixed infection. In these analyses, the area under the curve (AUC) for each marker was taken as a measure for diagnostic power and confidence intervals were calculated at 95% level using Wilson/brown method. Student unpaired, two-tailed *t*-test was done to test the difference of each markers between different animals (cow and buffalo) and between different infection model (single and mixed infections) in various conditions. In all statistical tests, a P-value was set at cut off = 0.05 with significance levels P<0.05: *, P<0.01: **, P<0.001: ***, P<0.0001:****. Principle component analyses (PCA), based Euclidean distances (ED) were used to analyze the clustering of animals under variable conditions. Bioplot was generated to visualize the contribution of each marker in the observed clustering. These analyses were done in R environment (v 3.6.3) using packages *ggplot2, vqv/ggbiplot* (Wickham 2009) and *devtools* ("https://cran.r-project.org/web/packages/devtools/index.html") and functions *dist* and *prcomp* (Borg and Groenen 2005).

## RESULTS

**Somatic Cell Count Data**

All obtained samples undergo somatic cell count revealing 538 individual samples represented the highest level of count (more than 200000 cells/mL of each milk sample) belonging to 254 animals. Descriptive statistics for results of somatic cell count per udder showed a higher count in front udders than rear ones.

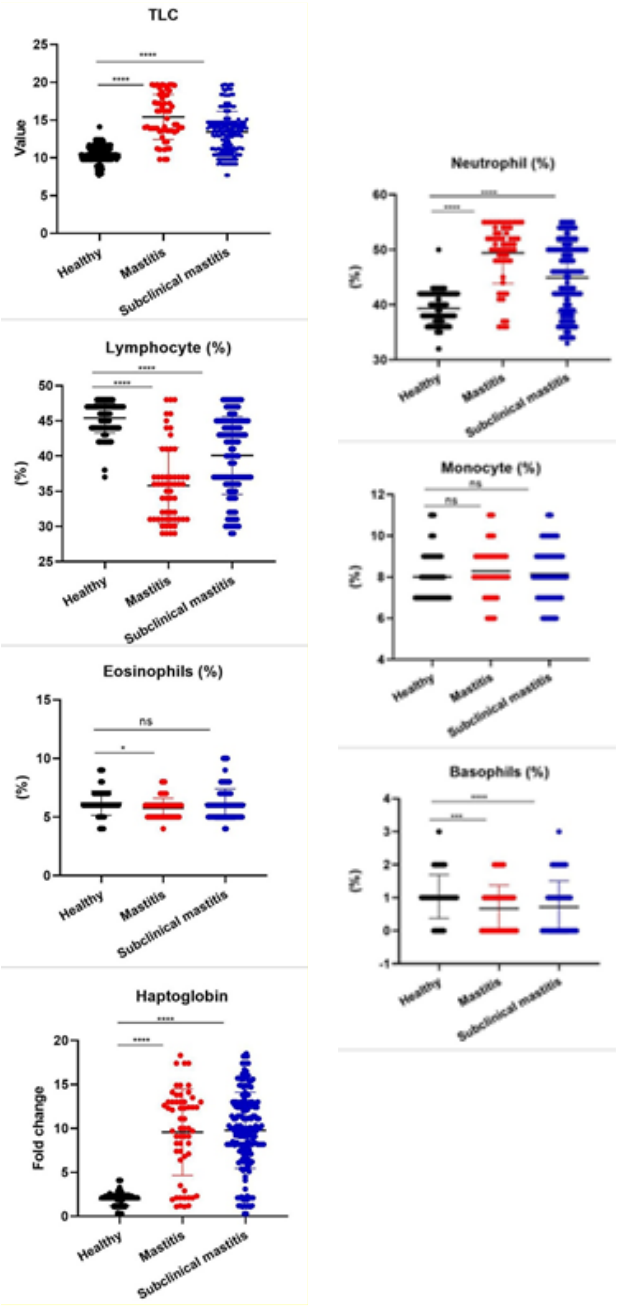


**Fig. 1:** Differences in values/percentages of various markers (shown at Y-axis) between healthy, mastitis and subclinical mastitis animals (shown at X-axis). Each dot refers to one animal. Means of values/percentages and standard error of the mean (SEM) are also shown

### Difference Between Healthy, Mastitis and Subclinical Mastitis Animals in Blood and Milk Parameters

Irrespective of the animal species, the levels of TLC, Neutrophils, lymphocytes, basophils and haptoglobin differed significantly between healthy and both mastitis and subclinical mastitis ($P<0.05$). However, monocytes percentage did not vary significantly comparing these cases. Eosinophils percentage differed significantly only when healthy animals were compared to mastitis ones (Fig. 1).

The measured parameters exhibited species-dependent differences. In healthy animals, healthy cows and buffalo differ significantly only in haptoglobin expression (Fig. 2). Furthermore, the percentage of eosinophils showed a significant difference between mastitis cow and mastitis buffalo, whereas the percentages of monocytes, eosinophils and basophils differed significantly between the two animal species when suffering from subclinical mastitis.

### Clustering of Animals in Various Disease Conditions and Infection Status

Given all markers, the healthy animals formed one separate cluster with no overlap with mastitis animal (ED=18.81) and little overlap with subclinical mastitis animals (ED=14.61) (Fig. 3A). Animals showing mastitis and subclinical mastitis overlapped to a large degree with large similarities in between (ED=13.8). Analyzing the dissimilarity within each animal category, we found high homogeneity and minimal dissimilarity among individual healthy animals (ED=5), whereas the dissimilarity increases among individual animals showing mastitis (ED=12.3) and subclinical mastitis (ED=12.8). The PCA analysis showed that PC1 was able to explain 46.2% of the variability in animals with lymphocytes (value=0.49) and neutrophils (value=-0.54) being the most important contributors in this variation. In cow or buffalo, healthy animals were also fully separated from mastitis animals but overlapped with subclinical mastitis animals (Fig. 4).

The measured markers could not separate the animals based on their infection status as animals showing a single infection overlapped with animals showing mixed infection (Fig. 3 B).

### Diagnostic Value of Blood and Milk Parameters

The ROC curve analyses revealed various AUC for each measured parameter (Table 3; Fig. 5A&B). For discriminating healthy from mastitis animals, TLC, lymphocyte, and neutrophil showed an AUC>0.9 and high significance ($P<0.0001$), followed in descending order by haptoglobin, eosinophils, basophil and monocyte. When ROC analysis was done for subclinical mastitis animals, haptoglobin was the only marker with an AUC > 0.9 ($P<0.0001$), followed in descending order by TLC, lymphocytes, neutrophils, basophils, eosinophils. Monocyte was a non-significant discriminator between healthy and subclinical mastitis conditions. For differentiating single from mixed infection, haptoglobin and basophils were the only markers having high AUC (being 0.6 and 0.7, respectively) and were significant ($P<0.001$ and $P<0.0008$, respectively).

### Major Histocompatibility Complex Results

Our results exhibited a correlation between surface protein (Major histocompatibility complex) associated with mastitis-causing pathogen bovine serum leucocytic antigen (BOLA) located on the surface of somatic cells and infection (mastitic cows). Out of five hundred and forty-seven milk, two hundred and fifty-four samples were positive with fluorescent coloration ranging from strong to weak in both mixed and single infections consequently. One hundred and eighty-six samples represented mixed infection were expressed by the positive reaction of mostly strong one while those of single infections expressed by the positive reaction with variable reaction intensity and counted 68. Negative cases as counted two hundred and eighty-three. A highly significant between types of infection and immune-histocompatibility was detected. As immune - histocompatibility was strongly expressed with

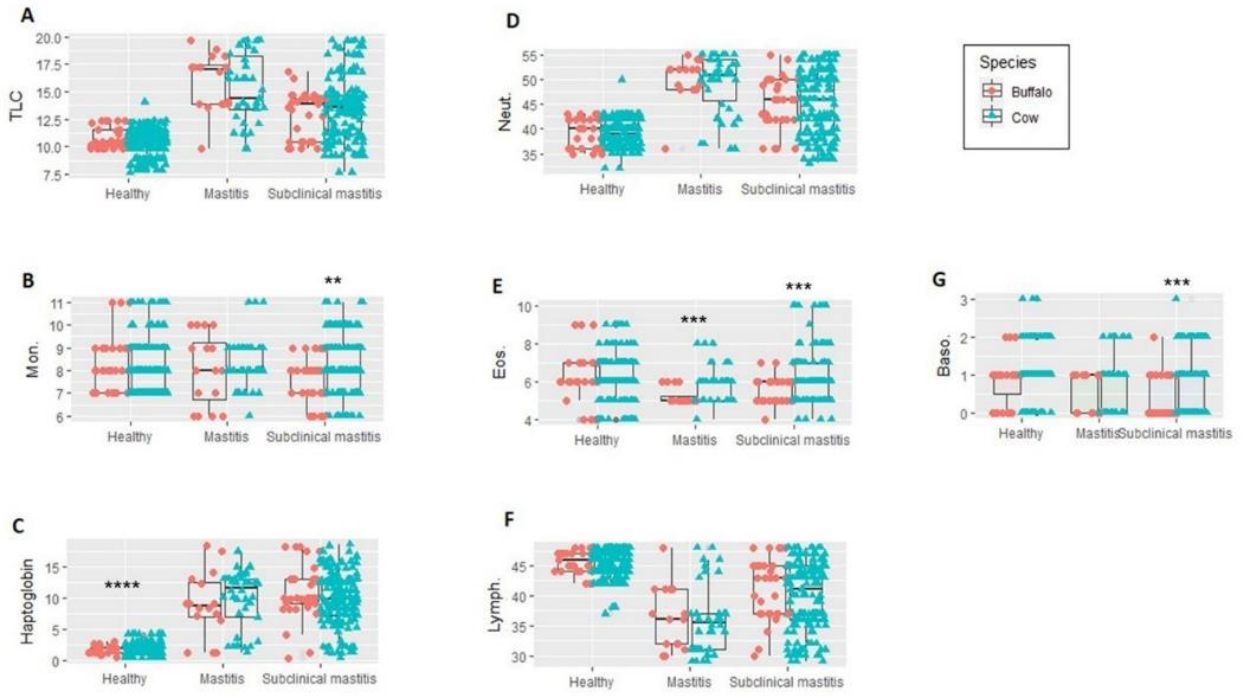


**Fig. 2:** Difference between cow and buffalo in various conditions (healthy, mastitis and subclinical mastitis) concerning all studied markers. Medians and box plot are shown for each species. Stars represent *P*-values, the degree of significant differences between both species in the respective marker and were obtained by applying student *t*-test (unpaired, two-tailed, unequal variance).

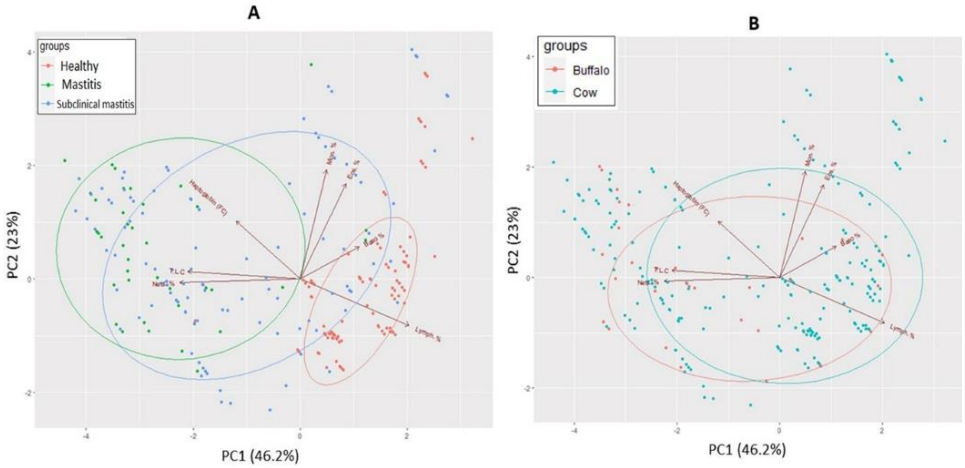


**Fig. 3:** Biplot of the Principle component analyses based on Euclidean distance showing the clustering and overlap of various subjects (animals) in different disease conditions **(A)** and species **(B)**. Each dot refers to one animal and the ellipses represent the confidence intervals. The variables are shown as arrows.

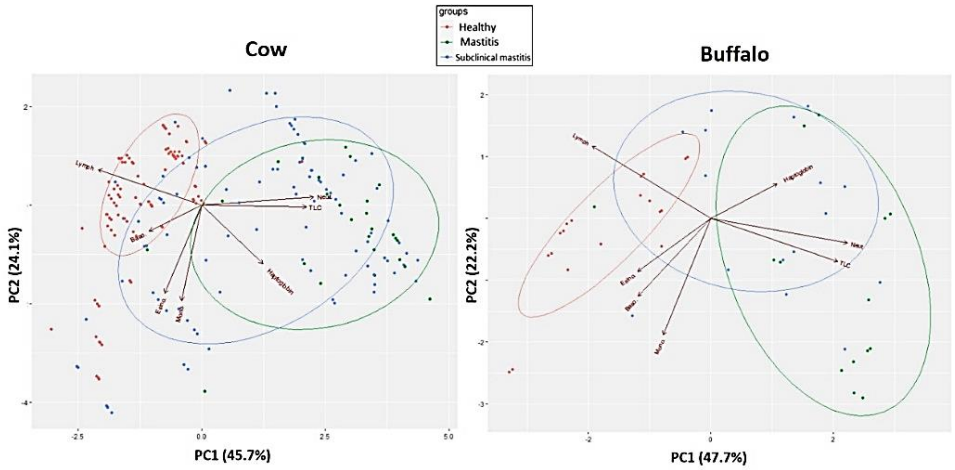


**Fig. 4:** Biplot showing the clustering and distribution of sampled animals of various conditions in the two investigated species.

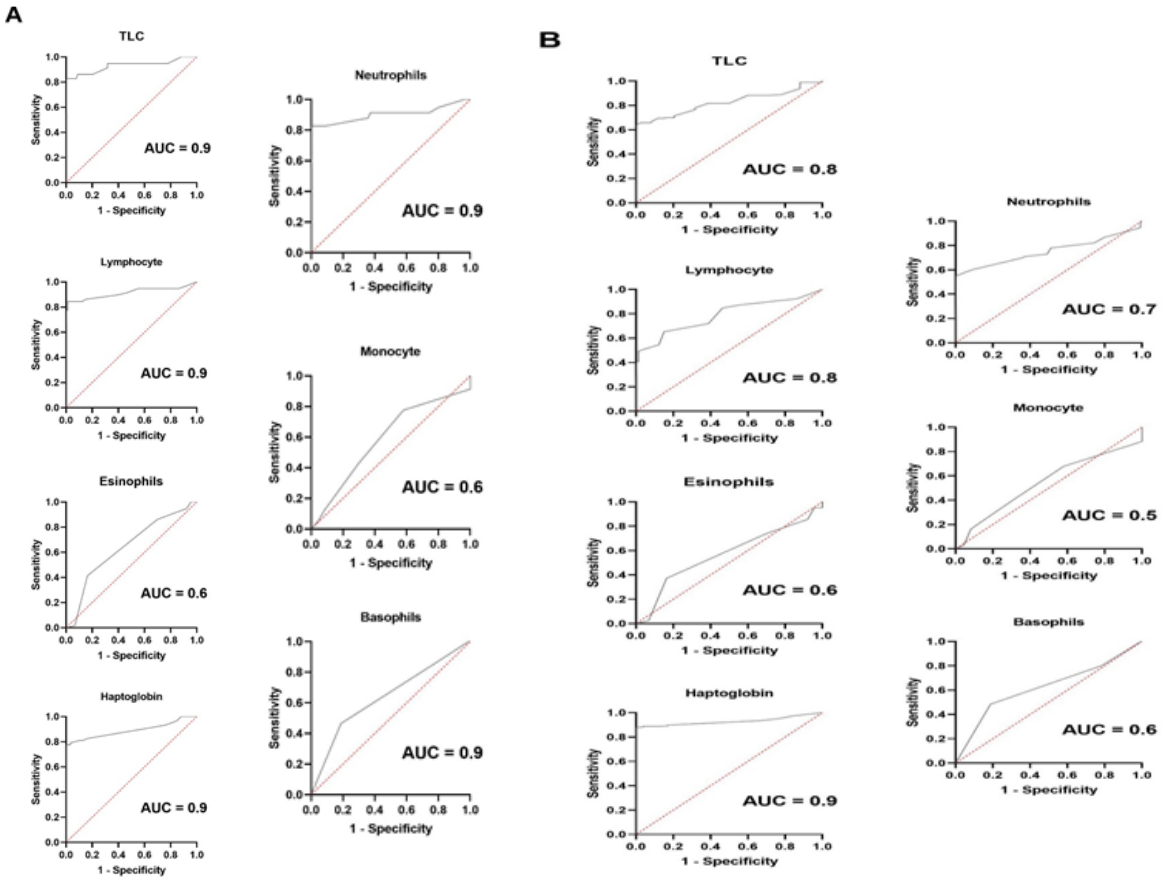


**Fig. 5:** Receiver operating characteristic curves showing the diagnostic accuracy of each parameter in mastitis **(A)** and subclinical mastitis **(B)** animals. The area under the curve (AUC) are shown on each ROC.

**Table 3:** Differences between cow and buffalo in the studied parameters in several conditions. *P*-value indicate significance of ROC analyses for each marker.

| Parameters | Units | AUC | SEM | CI (at 95%) | *P-Value* |
|---|---|---|---|---|---|
| | | | Mastitis | | |
| TLC | $10^6$/µL | 0.9288 | 0.02578 | 0.8783 to 0.9794 | <0.0001 |
| Neutrophils | g/dL | 0.9028 | 0.03203 | 0.8400 to 0.9656 | <0.0001 |
| Lymphocytes | % | 0.9141 | 0.03029 | 0.8547 to 0.9734 | <0.0001 |
| Monocytes | $10^3$/µL | 0.5882 | 0.04201 | 0.5058 to 0.6705 | 0.0338 |
| Eosinophils | $10^3$/µL | 0.6414 | 0.0394 | 0.5642 to 0.7187 | 0.0007 |
| Basophils | $10^3$/µL | 0.641 | 0.0423 | 0.5581 to 0.7240 | 0.0007 |
| Haptoglobin | Fold change (FC) | 0.8904 | 0.03291 | 0.8259 to 0.9549 | <0.0001 |
| | | | Sub-clinical Mastitis | | |
| TLC | $10^6$/µL | 0.8232 | 0.02195 | 0.7802 to 0.8663 | <0.0001 |
| Neutrophils | g/dL | 0.7516 | 0.02584 | 0.7009 to 0.8022 | <0.0001 |
| Lymphocytes | % | 0.7877 | 0.02257 | 0.7435 to 0.8320 | <0.0001 |
| Monocytes | $10^3$/µL | 0.5427 | 0.02751 | 0.4888 to 0.5966 | 0.1095 |
| Eosinophils | $10^3$/µL | 0.5681 | 0.02745 | 0.5142 to 0.6219 | 0.0107 |
| Basophils | $10^3$/µL | 0.6235 | 0.02699 | 0.5707 to 0.6764 | <0.0001 |
| Haptoglobin | Fold change (FC) | 0.9286 | 0.01581 | 0.8976 to 0.9596 | <0.0001 |

**Table 4:** Incidence of single infections using multiplex real time PCR assays

| Type of infection | Microorganism by PCR | NO. | % |
|---|---|---|---|
| Single | *E. coli* | 46 | 18.11 |
| | *Klebsiella* | 3 | 1.18 |
| | *M. bovis* | 3 | 1.18 |
| | *Pseudomonas* | 13 | 5.11 |
| | *S. aureus* | 1 | 0.41 |
| | *Streptococcus* | 2 | 0.71 |
| | Total | 68/254 | 26.7 |

mixed infection and wasn't detected in negative cases. Strong expression or reaction (strong fluorescent coloration) was expressed in that mixed infection only while a single infection was expressed by a moderate to mild reaction depending on the severity and duration of disease caused by a single pathogen. Negative expression (no fluorescent coloration) was detected in those negative cases (Fig. 6).

**Frequency of Mastitic Pathogens**

Our data demonstrated that that a high average prevalence of subclinical mastitis (35.83%, n=196) was recorded as compared to clinical mastitis (10.6%, n=58) giving an overall prevalence of 254 cases from 547 examined cases.

**Table 5:** Incidence of bacterial co-infections using multiplex real time PCR assays

| Type of infection | Microorganism by PCR | NO. | % |
|---|---|---|---|
| Mixed | *S. aureus, E. coli, Pseudomonas, CNS, Streptococcus, Corynebacteria* | 21 | 8.3 |
| | *S. aureus, E. coli, Pseudomonas, CNS, Streptococcus, Klebsiella* | 1 | 0.4 |
| | *S. aureus, E. coli, Pseudomonas, Mycoplasma, Corynebacteria* | 12 | 4.7 |
| | *S. aureus, E. coli, Pseudomonas, Streptococcus* | 1 | 0.4 |
| | *CNS, E. coli, Corynebacteria, Klebsiella* | 1 | 0.4 |
| | *CNS, Streptococcus, E. coli, Klebsiella* | 1 | 0.4 |
| | *Pseudomonas, E. coli, CNS, M.bovis* | 1 | 0.4 |
| | *S. aureus, E. coli, Klebsiella, M.bovis* | 1 | 0.4 |
| | *E. coli, Klebsiella, CNS, Pseudomonas* | 2 | 0.7 |
| | *CNS, E. coli, Corynebacteria, Pseudomonas* | 10 | 3.9 |
| | *CNS, E. coli, Streptococcus, Pseudomonas* | 1 | 0.4 |
| | *CNS, E. coli, Pseudomonas* | 4 | 1.5 |
| | *E. coli, CNS, Corynebacteria* | 2 | 0.7 |
| | *Pseudomonas, S. aureus, E. coli* | 4 | 1.5 |
| | *S. aureus, Streptococcus, E. coli* | 13 | 5.1 |
| | *M. bovis, Klebsiella, E. coli* | 2 | 0.7 |
| | *Pseudomonas, M. bovis, E. coli* | 4 | 1.5 |
| | *E. coli, CNS, Klebsiella* | 1 | 0.4 |
| | *E. coli, S. aureus, Klebsiella* | 2 | 0.7 |
| | *E. coli, Pseudomonas, Klebsiella* | 3 | 1.2 |
| | *E. coli, Corynebacteria, M. bovis* | 2 | 0.7 |
| | *E. coli, S. aureus, M. bovis* | 1 | 0.4 |
| | *E. coli, CNS, M. bovis* | 4 | 1.5 |
| | *E. coli, Corynebacteria, Pseudomonas* | 1 | 0.4 |
| | *Pseudomonas, E. coli, Streptococcus* | 1 | 0.4 |
| | *CNS, E. coli, Streptococcus* | 9 | 3.6 |
| | *E. coli, Corynebacteria* | 4 | 1.5 |
| | *Pseudomonas, Corynebacteria* | 4 | 1.5 |
| | *Streptococcus, Corynebacteria* | 1 | 0.4 |
| | *S. aureus, Corynebacteria* | 1 | 0.4 |
| | *Pseudomonas, E. coli* | 17 | 6.7 |
| | *S. aureus, E. coli* | 12 | 4.8 |
| | *Streptococcus, E. coli* | 7 | 2.8 |
| | *E. coli, Klebsiella* | 5 | 2.0 |
| | *E. coli, M. bovis* | 9 | 3.5 |
| | *S. aureus, Pseudomonas* | 1 | 0.4 |
| | *S. aureus, Streptococcus* | 3 | 1.2 |
| | *E. coli, CNS* | 15 | 5.9 |
| | *Pseudomonas, CNS* | 2 | 0.7 |
| | Total | 186/254 | 73.3 |

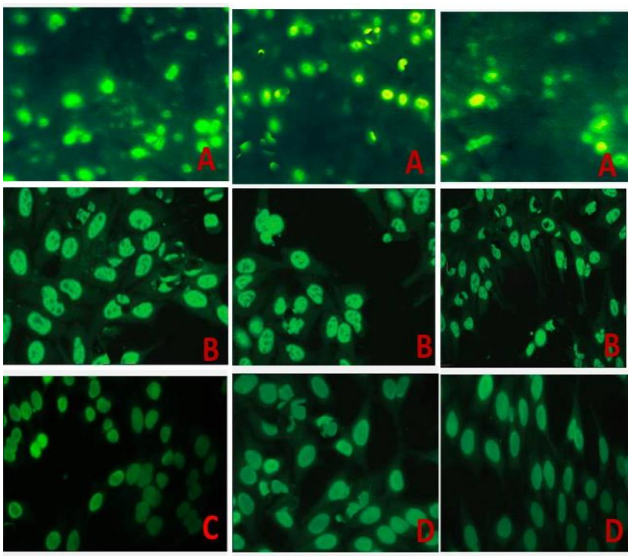


**Fig. 6:** Immunohistocompatibility complex under the fluorescent microscope: A) represents strong expression, B) represents moderate expression, C) represents weak expression, and D) represents negative expression

From the 245 clinical and subclinical mastitis cases recorded, bacteria were well cultured and only one hundred and ninety-eight (80.8%, n=198) grew pure cultures. Other rest samples gave negative results. No Corynebacteria nor *M. bovis* could be isolated.

Regarding PCR examination, multiplexing real-time PCR techniques proved their efficacy. There were bacterial populations in all examined positive samples. The most predominant microbial genera are *E. coli* (86.6%), followed by *Pseudomonas aeruginosa* with a prevalence rate of 40.6%. Additionally, almost all samples (75.9%, n=186) had mixed growth (Table 4, 5).

## DISCUSSION

Awareness of the incidence of mastitis, the microbial variety and inflammatory markers related to the disease progression would significantly develop prevention and direct the suitable therapy.

In this study, the overall incidence of mastitis detected was comparatively high (46.4%). These data were in line with the results of Abed et al. (2021). Many earlier studies

supported these results in Egypt and worldwide as (Sori et al. 2005), (Biniam et al. 2015), (Abera et al. 2010). Moreover, these results are higher than previous studies by Adane et al. (2017) 39.2%, Biffa et al. (2005) (40.40%). Nevertheless, the current finding was lower than the findings of Mekibib et al. (2010) in Ethiopia (71.05%) and Elsayed et al. (2015) in Egypt (91.48%).

The great incidence detected in this investigation could be significative of poor housing and bedding equipment, bad hygienic circumstances, preceding history of mastitis, poor milking systems and contaminated milking equipment and deficient supervision, control, and prevention measures of mastitis in the study extents (Rahularaj et al. 2019).

Concerning the results of the bacteriological assessment, out of 254 samples, only one hundred and ninety-eight (80.8%) represented positive bacterial isolation. Negative microbial isolation could be attributed to several causes: the existence of antibiotic residues that may clarify incorrectly negative bacteriological output since the withdrawal period is not appreciated in the flocks; only one milk sample may not be adequately insufficient, and more than a single bacteriological sampling is essential to detect if the quarter is influenced or not. The most prevalent microorganisms isolated from SCM of apparently healthy dairy cows were *E. coli*, followed by *S. aureus*, and *Pseudomonas aeruginosa*. These results are in the line with prior investigations distinguishing the main pathogens isolated from subclinical mastitic cases (Zeinhom et al. 2013).

Meanwhile, multiplexing PCR assay gave positive results for all examined samples. Sixty-eight samples (26.7%) represented single infection in which *E. coli* was the most frequent, followed by *Pseudomonas aeruginosa* and *S. aureus*. Meantime, a total of 177 samples (72.3%) exhibited co-infections with the co-existence of different patterns. Co-infection might be clarified as that either one organism was the etiological agent, and the rest were commensals, or one organism stimulated primary infection, and the rest were secondary invaders. The great incidence of co-infections in our investigation could be attributed to the inadequate typical sanitary and managemental routines, comprising overcapacity, and insufficient dung elimination within the dairy farms which allowed the expand of environmental and contagious infections (Rahularaj et al. 2019; Schauer et al. 2021).

The relatively low prevalence of *S. aureus* (29.1%) was also reported in Egypt (24.4%) (Awad et al. 2017), and Turkey (26.1%) (Hande et al. 2015). *S. aureus* moreover can elude and affect the cow's immune system by producing several toxins and enzymes producing injury to the mammary tissue (Abed et al. 2021). *S. aureus* can still alive on the skin and keratin layer of the teat canal of healthy cows and can overcome phagocytosis (Alekish et al. 2013). It would be a great danger for public health since the mastitic milk is often added into a bulk milk tank, principally in inhabitants where some individuals might ingest raw milk or non-heat-treated dairy products like yogurt or cheese (Awad et al. 2017).

The detection of mycoplasma and corynbacteria by PCR only may be attributed to significant changes in the electrolyte content or enzyme activity in the mastitic milk of the affected cow, which caused rapid reduction of mycoplasma and corynbacteria in the mastitic milk, thereby affecting the recognition rate by traditional assays (Weger and Stull 1978; Youssif et al. 2020; Mohsin et al. 2022).

The PCA plot based on ED is the technique of choice to simplify the complication of high-dimensional data while retaining trends and patterns. Here we make use of this to study the distribution and clustering of individual animals based on measured blood and milk biomarkers. We observed that healthy animals with or without regard to the species formed a single cluster. This initially indicates the value of these markers in diagnosing mastitis cases, which already agrees with previous studies (Lever et al. 2017) and (Carvalho-Sombra et al. 2021). The observation that healthy animals were fully segregated from mastitis but showed some overlap with subclinical mastitis animals complements the previously documented difficulties in diagnosing subclinical mastitis (Carvalho-Sombra et al. 2021). Since the measured indices represent part of the host response to udder inflammation, these results suggest that perturbation in blood and milk parameters, as a response to inflammation, is expected to be higher in mastitis than in subclinical mastitis. This also reflects the increased importance of these parameters in diagnosing clinical mastitis in bovines.

From a diagnostic point of view, the measured parameters were not a good classifier for animals infected with single or mixed infection as shown by the high degree of overlap (thus small ED) between these two categories. This indicates the low value of these parameters in discriminating animals with single or mixed infection. This agrees very well with the results of ROC analyses where most of the parameters have a moderate AUC of 0.5.

It is expected that mastitis and subclinical mastitis would differ in the degree of the inflammation being induced by the host. The current analysis further supports this idea and links this to the ramification of this inflammation on blood or milk. Here, we found differences in diagnostic power of blood or milk markers between mastitis and subclinical mastitis. While TLC, neutrophil, and lymphocytes have excellent diagnostic power (AUC=0.9) in mastitis animals, these markers were of low diagnostic value in subclinical mastitis, in which haptoglobin expression was the best indicator (AUC=0.9).

Haptoglobin scavenges hemoglobin and prevents utilization of iron by bacteria translocated into the blood systemic circulation and is considered as a significant sign of inflammation and infection (Humblet et al. 2006). Immune-histocompatibility complex is a unique marker for the detection of pathogen infection in the early stages of mastitis hence, using it as a through applying marker fluorescent immunoprecipitation analysis (FIPA) had an important step in examination positivity of subclinical mastitic cases. This suggested assay is appropriate with mass spectrometry examination and allows the recognition of immunoprecipitated proteins (Filatov et al. 2007).

Negative staining with fluorescence meaning a negative expression of bovine serum leucocytic antigen (BOLA) or absence of pathogen, while positive expression even in weak reaction means the presence of pathogen in milk sample which advanced to mastitis with the time which explained by Filatov et al. (2003) who reported the expressing negative results meaning the absence of BOLA and in turn absence of any involved pathogen. Labelling

BOLA by fluorescent stains instead of other labeling stains was our choice for its advantage in the detection of positivity and pathogen existence even in a small dose which was supported by Patton (2000).

These results hypothesize that the differences between mastitis and subclinical mastitis at the level of symptoms and inflammation could have been manifested also as similar differences in biomarkers being used to diagnose each case. Therefore, awareness, at the cellular and molecular degree, of the immune response at relative health and during the infection is essential to the early and timely diagnosis and in potential pathogenesis-based therapy.

## Conclusion

Our data represented that the effect of poor hygiene measurements within the dairy farms understudy was distinct within the great prevalence rate of udder co-infections and high prevalence ratio of environmental pathogens especially *E. coli and Pseudomonas*. Moreover, utmost of the farm sanitary systems and measurements as the sanitary circumstance of the milking environment, hygiene of the milk tanks, udder and teats washing, usage of the specific towels for separately animals, and the individual sanitation of the milkers were not completely achieved by most of the farm owners. Based on some inflammatory markers, we recommended regular monitoring of these emerging mastitic pathogens, and control parameters ought to be employed in the infected dairy ranches.

## Availability of data and material (data transparency)

All data used have been included in the manuscript. Code availability Not applicable.

## Conflict of Interest

The authors declare that they have no competing interests.

## Author's Contribution

All authors participated in the conception and design of the study, acquisition of data, practical part, writing the paper, analysis of the results, and revising it critically for important intellectual contents. All the authors have approved the final article version to be submitted.

## Acknowledgments

This paper is based upon work supported by Science, Technology & Innovation Funding Authority (STDF) under Basic & Applied Research Grants (STDF-BARG), Call 4 and ID: 26026.

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