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Use of MALDI TOF MS for Diagnosis of *Pseudomonas aeruginosa* Causing Mastitis in Cattle with Special Reference to The associated Immunological Parameters Changes

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Abstract

BOVINE MASTITIS caused by *P. aeruginosa* has received more attention in the recent years as it causes significant economic losses to dairy farms and to dairy industry as a whole. *P. aeruginosa* mastitis is a multifactorial disease needs interaction of pathogen, host and environment. The emergence and spread of antimicrobial resistance in P. aeruginosa is one of the most important health issues worldwide. This study aimed to isolate *P. aeruginosa* from mastitic milk samples, study its associated virulence markers and studying their AMR either phenotypically or genotypicaly by detection of ARG in addition to studying immunological parameters in milk and serum samples. 200 milk samples were collected from mastitic cows and buffaloes and examined for existence of *P. aeruginosa*, the recovered isolates were identified by conventional bacteriological procedure then confirmed by MALDI-TOF revealing a recovery rate (7%) in the examined samples. Antimicrobial susceptibility test showed that isolates exhibited high resistance rates to ampicillin, amoxicillin erythromycin and SXT (100%), followed by ceftriaxone and cefidizime, (92.8%), moderate resistance to chloramphenicol (64.2%) and doxycycline (57.4%). Meanwhile isolates showed high susceptibilities to ciprofloxacin, gentamycin, amakacin, impenim and colistin. PCR results showed amplification of oprL, toxA and pslA virulence genes in all examined isolates. ARG detected by PCR were ampC, blaTEM, blaCTX, sul1 and ermB. Result showed significant increase in gene expression of Bhb1, IL6 and in lysozyme activity in serum samples, meanwhile milk samples showed increase in Bhb1 and decrease in IL10 with no change in IL6 gene expression when compared with the control.

Keywords: MALDI TOF MS; *Pseudomonas aeruginosa*; Mastitis; Cattle.

Introduction

Mastitis is the predominant disease of dairy herds leading to massive financial losses due to decreased milk yield, increased production costs, deteriorated milk quality and increased culling rates [1]. *P. aeruginosa* mastitis rate in dairy herds usually ranges from (1-3%) and usually involves high producing cows in early lactation, although outbreaks can happen all over stages of lactation [2].

For treatment and eradication of *P. aeruginosa* mastitis there is a great need for studying 3 main involving factors (bacterium, immunity and environment) as the disease is multifactorial [3], this bacterium is an opportunistic pathogen in which disease usually occur after debilitating situation, stress factors and teat injuries giving a chance to organism attacking the udder [4].

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The extensive empirical use of antibiotics results in treatment failure and enhances the emergence of antimicrobial resistance (AMR), Consequently there is a persistent need to guide the choice of the most effective antibiotics [5], this relies mainly on monitoring bacterial etiologies and their AMR profiles to enable proper adjustment of prevention and control strategies [6].

As an alternative to conventional diagnostic methods, matrix-Assisted laser desorption ionisation time flight mass spectrometry (MALDI-TOF-MS) has proven to be rapid and dependable in the routine analysis and diagnosis of mastitis pathogens in developed nations [7].

The MALDI-TOF method, which is a novel approach to microbial identification, has sparked significant advancements in the field due to its validity, speed, simplicity, accuracy, and comparatively low cost [8]. It is based mainly on accurate determination of bacterial protein mass and comparing it to available profiles stored in a software database identifying the species in a few minutes [9].

There are three primary mechanisms by which *P. aeruginosa* acquires antimicrobial resistance: intrinsic, acquired, and adaptive resistance. Due to the production of antibiotic-inactivating enzymes, low outer membrane permeability, and the expression of efflux pumps, *P. aeruginosa* possesses intrinsic resistance [10]. Horizontal transfer of variable resistance genes, such as aminoglycoside resistance genes, β-lactamases, and metallo-β-lactamases, can establish acquired resistance [11].

Several virulence factors constituting extracellular metabolites (proteolytic and lipolytic enzymes, exotoxins, and phenazine pigments) sustain the pathogenicity of *P. aeruginosa*. These metabolites are regulated by a quorum sensing system [12].

Mastitis may present as clinical or subclinical manifestations. Recent research indicates that the impact of the infection is contingent upon the host's response during the initial phases of the condition, which may involve the influence of secreted cytokines [13]. Mammary gland inflammation severity is impacted by the presence of invasive microorganisms and the ensuing immune response [14]. The activation of certain cytokines—namely interleukin-1, interleukin-6, interleukin-8, and tumor necrosis factor-α—contributes to the initiation of inflammation [15]. Additional cytokines, such as IL-10 and IL-1 receptor antagonist (IL-1ra), are involved in the regulation of inflammation [16]. The innate immune response to *P. aeruginosa* is distinguished by the production of cytokines that promote inflammation [17]. Acute phase proteins are a class of glycoproteins whose synthesis is stimulated by the cytokine interleukin-6, which is secreted in response to inflammation or tissue damage. Haptoglobin, which is classified as an acute phase protein, is primarily synthesized in response to interleukin-6. This is due to the fact that interleukin-6 acts as the primary mediator between hepatocytes and damaged tissue cells by stimulating B and T cells [18].

Therefore the present investigation aimed to detection of *P. aeruginosa* as a mastitis-related pathogen with studying its AMR with special reference to its virulence and antibiotic resistant genes (ARG) in addition to detecting some immunological parameters in serum and milk for cellular damage evaluation.

Material and Methods

Ethical approval

The samples of milk were obtained using a routine milking process that did not compromise the well-being of the cattle. This procedure is efficacious and does not cause any harm to the health of the animals involved in the research; therefore, ethical approval was not necessary for its implementation.

Animals and Sampling

In Sharkia governorate, a total of 200 milk samples were obtained from cows and buffaloes in various localities. Of these, 110 were clinically mastitic cases and 90 were apparently healthy cases suspected of having subclinical mastitis according to CMT; five apparently healthy cattle with a negative California mastitis test served as a control. Each individual sample is a composite of all quarter samples contained in a single falcon tube. All specimens were appropriately labeled and promptly conveyed in an ice tray to the Microbiology laboratory at AHRI, Zagazig.

Serum samples

A volume of around 10 mL of blood was extracted from the jugular vein of the cows in an aseptic manner. For clotting purposes, the collected blood samples (in a simple centrifuge tube) were slanted for twenty minutes at room temperature. Thirty minutes were spent centrifuging blood samples at 5000 revolutions per minute (rpm), followed by ten minutes at 3000 rpm. The serum remained at -20°C until it was utilized.

Bacterial Isolation

Following incubation at 37°C, a loopful of each milk sample was distributed onto nutrient agar and MacConkey's agar media. These media were subsequently aerobically incubated at 37°C for duration of 24-48 hours. On EMB medium, suspected colonies were cultivated. Biochemical methods were utilized to identify members of the Enterobacteriaceae (urease, catalase, IMVC, and Oxidase assays) [19].

Identification and isolation of P. aeruginosa

Following the inoculation of broth with an inoculum derived from each milk sample, plates were prepared on nutrient, Cetramide, Pseudomonas agar base with supplement, and blood agar. Following incubation, the plates were examined after a period of 24-48 hours. Gram's staining and additional biochemical identification (catalase, oxidase, IMVC, TSI, sugar fermentation, and motility assays) were performed on the suspected colonies according to MacFadden [19].

MALDI-TOF MS

Utilizing MALDI-TOF MS, isolated *P. aeruginosa* was confirmed at (57357 Children's Cancer Hospital, an outsourced facility). Colonies were cultivated on solid media in accordance with bacterial isolation standards. The identification of bacterial species was conducted in duplicate, employing the MALDI-TOF Vitek MS (bioMérieux) to conduct experiments simultaneously on the same target slide. The results were analyzed on the Vitek MS IVD system (bioMérieux; Marcy l'Etoile, France) in accordance with the guidelines provided by the manufacturer.

Serological identification

The serological identification of *P. aeruginosa* was performed using slide agglutination in accordance with the method described before [20] in the Food Analysis Centre, Faculty of Veterinary Medicine, Benha University.

Antimicrobial susceptibility testing

P. aeruginosa isolates were subjected to antimicrobial susceptibility testing against thirteen antimicrobial agents (Oxoid, England, UK) using the standard disc diffusion method [21]. Colistin (CT), Ciprofloxacin (CIP), Chloramphenicol, Doxycycline (DO), Amoxicillin (AML), Gentamycin (CN), Amikacin (AK), Imipenem (IMP), Erythromycin (E), Ceftriaxone (CRO), Ceftazidime (CAZ), Ciprofloxacin (CIP), and Chloramphenicol were among the antimicrobials that were evaluated. The dimensions of the inhibition zones were interpreted in accordance with the criteria outlined by the CLSI [22]. The calculation for the MAR index for each isolate was as follows: The ratio of the number of antimicrobials to which the isolate exhibited resistance to the total number of antimicrobials tested [23].

Molecular detection of virulence and AMR genes

For the molecular detection of virulence and AMR genes, DNA was extracted in accordance with the manufacturer's instructions using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Amplification of target genes utilizing a pair of specifically designed primers (Table 1). The primary denaturation was done at 94°C /5min for all cycles, and a cycling and amplification protocol detailed for each gene in Table 1. Each amplicon was inserted into a 1.5% agarose gel containing 0.5µg/ml ethedium bromide, using an aliquot. Ladder of 100 bp was used as a molecular weight marker. At 100v/60min, amplified PCR products were electrophoresed.

Detection of lysozyme activity

Schltz [24] established an agarose gel cell lysis assay for the measurement of lysozyme activity in fourteen serum samples extracted from animals confirmed to be infected with *P. aeruginosa*.

RT-PCR detection of immunological parameters: Bhb1, IL-10, and IL-6 gene expression were detected in fourteen serum and milk samples obtained from animals that were confirmed to be infected with *P. aeruginosa*.

RNA extraction was performed on the samples utilizing the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). To accomplish this, 600 µl of RLT buffer containing 10 µl β-mercaptoethanol per 1 ml was added to 200 µl of the sample, and the mixture was incubated at room temperature for duration of 10 minutes. Following the addition of one volume of 70% ethanol to the lysate that had been purified, the procedures outlined in the Purification of Total RNA protocol for the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) were carried out. The primers utilized were provided by Metabion, a German company, as detailed in Table 1.

SYBR green RT-PCR

The 20-µl reaction utilized SYBR Green RT-PCR primers. It comprised the following: 10µl of the 2x HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 1µl of RT Enzyme Mix (20X), 1µl of each primer with a concentration of 20 pmol, 3µl of water, and 5µl of RNA template. The StepOneTM real-time PCR machine was utilized to conduct the reaction at the Biotechnology Unit of AHRI, Zagazig Branch, Egypt.

Examination of the SYBR green RT-PCR results

The CT values and amplification curves were analyzed using the Step One software. In order to determine the extent of gene expression variation on the RNA of the various samples, the CT of each sample was compared to that of the positive control group using the ratio (2-DDct) and the "ΔΔCt" method as described by Yuan et al. [25].

Statistical analysis

Microsoft Excel (Microsoft Corporation) was utilized to modify the data. To assess normality, a Shapiro–Wilk test was performed, as outlined by Razali and Wah [26]. The statistical analysis employed the T-test (PROC T test; SAS Institute) to determine whether there were significant differences between the control and infected groups. It was considered statistically significant if $p < 0.05$.

Results

Recovery rate of P. aeruginosa from milk samples

In the present study, 15 *P. aeruginosa* isolates were recovered from 200 analyzed milk samples comprising 9 from clinical mastitis, 6 from subclinical mastitic cases. *P. aeruginosa* isolates were presumptive identified by colonial, morphological and biochemical characteristics, in addition to the isolation of other Gram's –ve bacteria representing members of Enterobacteriaceae with variable recovery rates. The overall prevalence of *E. coli*, Klebsiella and *E. aerogens* were 19%, 8% and 3% from examined milk samples, respectively.

MALDITOF-MS

All recovered *P. aeruginosa* isolates by conventional technique were confirmed via MALDI-TOF MS technique and results showed that 14/15 was identified as *P. aeruginosa* with a total recovery rate (7%) with high agreement rate (93.3%) between MALDI-TOF and conventional method and showing specific protein profile matrix as shown in (Fig.1).

Antimicrobial susceptibility results

The in-vitro antibiogram test of 14 *P. aeruginosa* isolates against 13 antibiotic discs was illustrated in Table (2). *P. aeruginosa* showed absolute resistance to AMP, AMX, SXT, E (100%), each followed by resistance to CRO and CAZ (92.8%), each, C, DO estimated by (64.2%, 57.14%), respectively. Moreover isolates displayed high susceptibility to CN (71.42%), AK (78.5%), CIP (85.7%), CT, and IMP (100%) each. Isolates showed 7 different antimicrobial resistance patterns and the most prevalent pattern was identified by (4 isolates) as clarified in Table (3).

MAR index:

Frequency of MAR indices were illustrated in table (3) as isolates showed resistance to (4 to 10) of tested antimicrobial agents that ranged from (0.30-0.76).

Detection of virulence genes:

Molecular detectionof virulence associated genes (*tox*A, *opr*L and *psl*A) and AMR genes were carried on 8 *P. aurignosa* isolates and results showed postive amplifictaion of target virulence genes in all isolates (Fig. 2A-C). Whereas results diplayed the detection of (*amp*C, *bla*TEM and sul1) genes in all isolates and postive amplification of (*erm*B) and *bla*CTX) genes in 3 and 2 isolates, repectively. (Fig. 3, 4)

Correlation between serogroups, virulence and AMR genes existence was presented in Table (4).

Detection of immunological parameters by rt-PCR:

With regard to the expression of Bhb1, IL-10 and IL-6 in milk, the transcription of Bhb1 gene was significantly up-regulated in infected group compared to the control group ($p<0.05$) (Table 5 and Fig. 4A). Meanwhile the expression of IL-10 gene was significantly lower in infected group than that in the control group ($p<0.05$) (Table 5 and Fig. 4B), non-significant differences were detected between the control and infected groups for the expression of IL-6 ($p>0.05$) (Table 5 and Fig. 4C). Regarding the expression of aforementioned genes in serum, they showed the same trend (Table 5 and Fig. 5) except the transcription of IL-6 gene was significantly up-regulated in infected group compared to the control group ($p<0.05$; (Table 5 and Fig. 5C).

Detection of lysozyme activity:

Serum lysozyme activity was significantly higher ($p<0.05$) in infected group by 184.17% compared to the control group Fig. (6).

Discussion

Mastitis is a complex and multifactorial disease results from the interaction between pathogen, host and environment [27]. *P. aeruginosa* has a tendency to cause clinical mastitis outbreaks with marked swelling of the udder, high body temperatures and abnormal watery milk with flakes, clots or blood that can end with toxemia and animal death [2].

MALDI-TOF MS has recently been used in microbiological laboratories since it gives results in just few hours. Although the device is expensive but the reagent costs are inexpensive and bacterial identification can be done automatically. Protein profiles provide the basis for microbial identification in which mass spectrum of a tested organism is compared to reference spectra in the databases to find the closest match [28].

Herein MALDI-TOF MS was used for confirmation of *P. aeruginosa* isolates and obtained results had matched results of conventional method (93.3%) this is in accordance to Marwa et al. [29] who used the same technique in identification of *P. aeruginosa* and found it to be more accurate, additionally their research has stated that there were no previous studies in MALDI-TOF MS applied on *P. aeruginosa* diagnosis and that this technique is not common in Egypt owing that to its relatively high cost and unavailability of apparatus in most laboratories. However, MALDI-TOF MS can act as good alternative for conventional method [29].

Herein *P. aurginosa* was recovered from 7% of mastitic milk samples, similar isolation rates of *P. aurginosa* were recorded from bovine mastitic cases in previous studies conducted by Amel Ghazy *et al*. [30] 10%, Saleh *et al*. [31] 11.45% and Banerjee et al. [32] 5.4%. On the other hand, a lower recovery rate of *P. aeruginosa* from milk samples was detected (0.61%) and (4.1%) by Yadav *et al*. [33] and Abdelgawad and Bkheet [34], respectively. Previous studies recorded higher isolation rates

estimated by 45% [35] and 15% [36], respectively. The difference in prevalence rates among studies may be attributed to alteration in localities, seasonal variation, immunological status, farm management practice and sanitary measures during milking.

Exotoxin A elaborated by most *P. aeruginosa* isolates represents a key virulence marker as it plays a critical role in *P. aeruginosa* pathogenesis as it is very toxic to cells [37] and inhibit protein synthesis of the bacterial cell via the ADP-ribosylation of cellular elongation factor 2 inhibition causing cell death, local tissue injury and facilitate bacterial invasion [38]. This toxin synthesis is encoded by chromosomal *toxA* gene [37].

The intrinsic antibiotic resistance of Pseudomonas aeruginosa is attributed to the outer membrane lipoprotein, which is controlled by the oprL gene [39]. L-lipoproteins, which are outer membrane proteins linked to P. aeruginosa, are specific to Pseudomonads. As such, they can be utilized in clinical specimens for both the identification and pathogenesis assessment of Pseudomonads [40]. The genes pslA and pelA are significantly involved in the development of the biofilm matrix's carbohydrate-rich structure [41].

In the current study, eight strains of *P. aeruginosa* were analyzed in order to identify the presence of virulence-associated genes, as indicated by the data previously mentioned. 100% of the isolates exhibited the toxA, oprlA, and pslA genes, as determined by PCR.

The amplification of the toxA gene in every isolate analyzed is consistent with the findings of Mona Salem et al. [36], who reported the detection of toxA in every *P. aeruginosa* strain extracted from bovine mastitis. Banerjee et al. [32] and Raziq [42], meanwhile, have identified the toxA gene in 63.2% and 84% of *P. aeruginosa* isolates, respectively. The presence of the toxA and oprL loci in *P. aeruginosa* isolates was determined to be 95.4% and 100%, respectively [43]. Additionally, Algammal et al. [44] studied the distribution of virulence genes in retrieved *P. aeruginosa* strains and detected both genes commonly. Concerning the existence of *psl*A gene in all isolates this result matched results of previous investigation performed by Amira Lameay et al. [45] who found the same gene at 100% of the examined *P. aeruginosa* isolated from cattle considering it as one of most fundamental genes implicated in biofilm formation. Meanwhile, Aslantaş et al. [46] has detected the same gene in *P. aeruginosa* isolated from bovine mastic cases but with lower levels.

Complicated regulatory mechanisms govern the relationship between antibiotic resistance and virulence factors; at times, these mechanisms are regarded as distinct phenomena. Regardless of the perspective from which genetics is viewed, these regulatory mechanisms are interconnected [47, 48]. The oprL protein, which is present in *P. aeruginosa*, is known to have a substantial impact on the bacterium's intrinsic resistance to antibiotics and antiseptics. Biofilm is considered to be a highly concentrated area where AMR genes accumulate and are transmitted [39, 49]. These findings provide support for the hypothesis that P. aeruginosa's virulence is associated with its antibiotic resistance.

The rapid dissemination of antimicrobial resistance (AMR) is a concerning emerging concern in both the human and veterinary sectors. Egypt, for instance, has implemented a five-year National Action Plan on AMR (2017–2022). This plan seeks to ensure consistent monitoring of AMR and optimize the management of antibiotic drugs in human medicine and animal health in accordance with the One Health concept established by the World Health Organization. Despite this, antibiotics continue to be haphazardly incorporated into animal feed, either as feed additives or growth promoters [50].

P. aeruginosa infections are difficult to treat due to the microorganism's inherent resistance to antimicrobials and the emergence of multidrug-resistant (MDR) isolates, which render antibiotic treatment ineffective. ARG against phenicol, beta lactam, fluoroquinolones, aminoglycosides, and sulphonamides has been produced [51]. Moreover, it has ability to form biofilms, complicating condition and make it difficult to be treated [45].

Results of antibiotic susceptibility test revealed that examined isolates had displayed high resistance rates to most of the tested antibiotics with exception of CN, AK, IMP, CIP and CT. Our finding agree with Schauer et al. [35], Tartor and El Naenaeey [52] and Mona Salem et al. [36] who detected that *P. aeruginosa* isolated from mastic milk samples displayed high resistance to many antibiotic groups and exhibited high susceptibilities to only these antibiotics (CIP, IMP, CT, CN.). Additionally, Lambert [53] stated that 90% of *Pseudomonas* species displayed resistance to all antimicrobials with exception of Amikacin, gentamicin and piperacillin and owing that to the low permeability of its cell wall and other acquired resistance mechanisms. Agrawal et al. [54] stated that polymyxin-B, colistin, amikacin and carbapenem can act as main drugs to treat multidrug resistant *P. aeruginosa*. It can be observed that isolates showed marked resistance to some antibiotics that are permitted for cattle mastitis treatment and used as feed supplement, while resistance to impenim, meropenem, colistin and ciprofloxacin was low and this can be attributed to these antibiotics are not approved for veterinarian usage

AmpC beta-lactamases that belongs to molecular class C, are bacterial enzymes that hydrolyze 3rd

generation extended spectrum cephalosporins and cephamycins inducing resistance to these antibiotic classes in addition to resistance to ESBLs inhibitors (clavulanaic acid). AmpC β-lactamases is a main cause of multiple β-lactam resistance in *P. aeruginosa* isolates, is encoded by the chromosomal gene (ampC) [55].

The exponential growth of ESBL-producing gastrointestinal (GIV) bacteria from animals to humans is one of the most severe emergent resistance issues on a global scale and poses a potential threat to public health. The rising prevalence of ESBL *P. aeruginosa* associated with livestock is an alarming development that increases the likelihood of antibiotic therapy failure in both animals and humans. As a result, surveillance studies that incorporate veterinary medicine are critical for identifying these dangerous pathogens [56].

ESBLs are primarily responsible for *P. aeruginosa* resistance to β-lactam antibiotics, such as penicillin and cephalosporin. The primary ESBL genes responsible for inducing this form of resistance are blaCTX-M and blaTEM. ESBLs are typically encoded by plasmid-mediated bla genes; blaSHV, blaTEM, and blaCTX-M are three main clinically relevant β-lactamase genes [57].

In the current study EBLS genotypes were determined based on PCR screening test for *bla*CTX-M and *bla*TEM genes in addition to *amp*C gene detection and results revealed positive amplification of *bla*TEM in all isolates, *bla*CTX in 2 isolates whereas *amp*C gene was detected in 100% of isolates.

Regarding *bla*TEM gene, we found that 100% of isolates harbored blaTEM gene, this was corresponding to Abdelgawad and Bkheet [34] and Algammal et al. [44] who detected this gene in 100% of *P. aurginosa* isolated from mastitic milk samples and poultry farms, respectively. Meanwhile it somewhat agrees with another investigation which found that *P. aurginosa* strains harbored *bla*TEM gene with a total prevalence of 83.3% [58] but that was dissimilar with that recorded in previous studies [56, 57] who detected the same gene at 23.8% and 26.6% in EBLS *P. aurginosa*, respectively.

Concerning *bla*CTX gene, its detection rate was relatively low (20%) the same was previously stated in other investigation which found that 71.5% of extended spectrum cephalosporin resistant *P. aurginosa* isolates were negative for ESBL genes as a general and specifically *bla*CTXM gene was detected in only 17.3% of resistant *P. aurginosa* isolates, and ascribed that to other resistance mechanisms such as overproduction of chromosomal cephalosporinase (AmpC), up-regulation of efflux systems or decreased outer membrane permeability [57]. Furthermore earlier study in Brazil detected *bla*CTXM gene in 19.6% of carbapenem resistant *P. aeruginosa* isolates screened for ESBLs genes [59]. Meanwhile it was lower than that reported by Elhariri et al. [56] and Algammal et al. [44] who found the same gene in 38% and 88% of *P. aurginosa* isolates, respectively.

In the present study, we checked the presence of AMR genes, (*sul*1, and *erm*B) as isolates showed absolute resistance to both antibiotics phenotypically, and results showed detection of *sul* genes in all isolates whereas *erm*B gene was detected in 3 out of 8 isolates, sul1 gene the has been previously detected extensively in *P. aurginosa* isolates and that can be attributed to the frequent usage of sulphonamides and macrolides in livestock animals [60]. Human populations may be exposed to a significant risk due to the close proximity of animal and human populations [61]. Pseudomonas is one of the most prevalent *sul*-positive genera in soil fertilized with animal manure, indicating a potential risk to human health.

Mastitis in cows is intricately linked to the immune response that is stimulated by inflammatory cytokines. Cytokines are essential components of the immune systems of dairy cows as they regulate a wide range of cellular physiological processes. Furthermore, they function as cellular communication transmitters during the immune response and inflammation processes [62]. Interleukin-6 (IL-6) is a class of glycoproteins that are secreted by lymphocytes, activated macrophages, and epithelial cells. Numerous inflammatory and immunologic cells in the body are capable of expressing the IL-6 gene and generating IL-6 when dairy cows have bacterial infections such as mastitis and endometritis [63]. In this study, serum IL-6 levels increased while milk IL-6 levels remained unchanged. Likewise, Shaheen et al. [62] found no significant difference in IL-6 levels between healthy and subclinical mastitis-affected heifers. Hagiwara et al. [64] identified increased concentrations of IL-6 in the whey and serum of mastitic cows. They hypothesized that the production of IL-6 in milk varies depending on the pathogens that infect the cows and the stage of lactation.

Interleukin-10 (IL-10) regulates the immune response to pathogens as an anti-inflammatory cytokine. Ileumaocytes, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells are all capable of having their proliferation and differentiation regulated by IL-10 [65]. The infected group exhibited a notable decrease in IL-10 gene expression both in serum and milk, in comparison to the control group. This observation may suggest that the afflicted udder's anti-inflammatory protection was diminished [66]. Additionally, both serum and milk samples from healthy cows contained substantially more IL-10 than those from infected cows [66]. An *E. coli* infection resulted in a notable

elevation in the concentration of IL-10 in milk, whereas *S. uberis* infection induced a more gradual increase in IL-10 levels [67]. Variations in the response to intramammary infections induced by distinct bacterial species were observed in this context.

Khoshvaghti et al. [68] reported that serum and milk Hp are sensitive diagnostic factors for inflammatory conditions in cattle. Similar to Zeng et al. [69], our findings demonstrated a significant increase in the transcription of the Bhb1 gene in the milk and serum of the infected group relative to the control group ($p<0.05$). Blood serum lysozyme and bactericidal activity is a significant indicator of livestock's natural resistance [70]. The infected group exhibited a substantially higher ($p<0.05$) concentration of serum lysozyme activity (184.17%) than the control group. This finding contradicts the results of Nermin Ibrahim et al. [71] who documented a notable reduction in the serum lysozyme activity of mastitic cows infected with *P. aeruginosa*.

Conclusions

P. aeruginosa mastitis has attracted great attention in the recent years as it can end by animal death. This problem not stand at this limit but it is a foodborne pathogen owing to its prominent prevalence in several parts of the food chain. Although the golden method for its diagnosis is culturing but MALDITOF MS has proved to be rapid, sensitive and accurate technique. Conventional PCR has proved the detection of 3 of the most important associated virulence genes mainly related to biofilm (*psl*A), toxin production (*tox*A) and *opr*L genes. The appearance of multidrug resistant bacterial strains is alarming indicating random use of antibiotics and detection of ARG with high rate is a disaster as they can be transmitted from animal to human via food chain or to other bacteria via mobile genetic element. This bacterium has direct effect on animal health status and milk quality, as examined serum samples showed significant increase in gene expression of Bhb1, IL6 and in lysozyme activity, meanwhile milk samples showed increase in Bhb1 and decrease in IL10 with no change in IL6 gene expression when comparing with control. So there should be great efforts to eliminate this bacterium in animal's environment to avoid infection and control the random use of antibiotics in mastitis treatment**.**

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Conflicts of interest

The authors declared no competing interests.

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TABLE 1. Primers and cycling protocols for virulence, antibiotic resistant genes and immunological parameters

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)			Reference
			Secondary denaturation	Annealing	Extension	
pslA	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	656	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	**Ghadaksaz et al. [42]**
ermB	GAAAAAGTACTCAACCAAATA AATTTAAGTACCGTTACT	639	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	**Nguyen et al. [76]**
oprL	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	**Xu et al. [77]**
toxA	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	**Matar et al. [78]**
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	**Colom et al. [79]**
ampC	TTCTATCAAMACTGGCARCC CCYTTTTATGTACCCAYGA	550	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	**Srinivasan et al. [80]**
BlaCTX-M	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	**Archambault et al. [81]**
Sul1	CGG CGT GGG CTA CCT GAA CG GCC GAT CGC GTG AAG TTC CG	433	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	**Ibekwe et al. [82]**

Target gene	Primers sequences	Amplified segment (bp)	Amplif-cation (35 cycles)			Reference
β-actin	CGTGGGCCGCCCTAGGCACCA					**Fitzpatrick et al. [73]**
	GGGGGCCTCGGTCAGCAGCAC	50˚C 30 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	
Bhb1 haptoglobin	GTCTCCCAGCATAACCTCATCTC					**Hiss et al. [74]**
	AACCACCTTCTCCACCTCTACAA					
IL-6	GGCTCCCATGATTGTGGTAGTT					**Mallikar et al. [75]**
	GCCCAGTGGACAGGTTTCTG					
IL-10	AAAGCCATGAGTGAGTTTGACA					**Mallikar et al. [75]**
	TGGATTGGATTTCAGAGGTCTT					

TABLE 2. Result of antibiotic susceptibility test

ID	AMP	AML	CRO	CAZ	SXT	E	C	DO	CN	AK	CT	CIP	IMP
1	R	R	R	R	R	R	R	R	R	R	S	S	S
2	R	R	R	R	R	R	R	R	R	S	S	R	S
3	R	R	R	R	R	R	R	R	S	R	S	R	S
4	R	R	R	R	R	R	R	R	R	R	S	S	S
5	R	R	R	R	R	R	R	R	R	S	S	S	S
6	R	R	R	R	R	R	R	R	S	S	S	S	S
7	R	R	R	R	R	R	R	R	S	S	S	S	S
8	R	R	R	R	R	R	R	R	S	S	S	S	S
9	R	R	R	R	R	R	S	S	S	S	S	S	S
10	R	R	R	R	R	R	S	S	S	S	S	S	S
11	R	R	R	R	R	R	S	S	S	S	S	S	S
12	R	R	S	S	R	R	S	S	S	S	S	S	S
13	R	R	R	R	R	R	R	S	S	S	S	S	S
14	R	R	R	R	R	R	S	S	S	S	S	S	S

Ampicillin (AMP), Amoxycilline (AML), Cefotrioxone (CRO), Ceftazidime (CAZ), Erythromycin (E), sulphamethoxazole/trimethoprime (SXT), Chloramphenicol C, Doxycycline (DO), Gentamycin (CN), Amikacin (AK), Colistin (CT), Ciprofloxacin (CIP) and Imipenem (IMP), S: sensitive R: resistant

TABLE 3. Antimicrobial susceptibility profile with their respective MAR indices

Sample ID	Antimicrobial resistance profile	MAR index	*amp*C	*bla* TEM	*bla* CTX	*erm*B	sul
1	AMP,AML,CRO,CAZ,E,SXT,DO,C,CN,AK	0.76	+	+	-	-	+
2	AMP,AML,CRO,CAZ,E,SXT,DO,C,CN,CIP	0.76	+	+	+	-	+
3	AMP,AML,CRO,CAZ,E,SXT,DO,C,AK,CIP	0.76	+	+	+	-	+
4	AMP,AML,CRO,CAZ,E,SXT,DO,C,CN,AK	0.76	+	+	-	+	+
5	AMP,AML,CRO,CAZ,E,SXT,DO,C,CN	0.69	+	+	-	-	+
6	AMP,AML,CRO,CAZ,E,SXT,DO,C	0.61	+	+	-	+	+
7	AMP,AML,CRO,CAZ,E,SXT,DO,C	0.61	+	+	-	+	+
8	AMP,AML,CRO,CAZ,E,SXT,DO,C	0.61	+	+	-	-	+
9	AMP,AML,CRO,CAZ,E,SXT	O.46	NE	NE	NE	NE	NE
10	AMP,AML,CRO,CAZ,E,SXT	0.46	NE	NE	NE	NE	NE
11	AMP,AML,CRO,CAZ,E,SXT	0.46	NE	NE	NE	NE	NE
12	AMP,AML,E,SXT	0.30	NE	NE	NE	NE	NE
13	AMP,AML,CRO,CAZ,E,SXT,,C,	0.53	NE	NE	NE	NE	NE
14	AMP,AML,CRO,CAZ,E,SXT	0.46	NE	NE	NE	NE	NE

NE: not examined **MAR:** multiple antibiotic resistance

TABLE 4.Correlation between serogroup, virulence associated genes and antibiotic resistant genes

ID	Serogroup	oprL	pslA	toxA	ampC	blaTEM	blaCTX	sul	ermB
1	O6	+	+	+	+	+	-	+	-
2	O11	+	+	+	+	+	+	+	-
3	O6	+	+	+	+	+	+	+	-
4	O2	+	+	+	+	+	-	+	+
5	O11	+	+	+	+	+	-	+	-
6	O11	+	+	+	+	+	-	+	+
7	O2	+	+	+	+	+	-	+	+
8	O6	+	+	+	+	+	-	+	-

TABLE 5. Gene expression of studied genes *Bhb1, IL-10,* and *IL-6* in milk and serum of control and infected group (means ±SE).

Source	Items	Control-ve	Infected	*p*-value
Milk	***Bhb1***	1.00±0.00	3.32±0.79	0.0461
	IL-10	1.00±0.00	0.42±0.19	0.0398
	IL-6	1.00±0.00	1.86±0.68	0.3385
Serum	***Bhb1***	1.00±0.00	27.40±6.73	0.0212
	IL-10	1.00±0.00	0.24±0.03	0.0001
	IL-6	1.00±0.00	2.87±0.44	0.0158

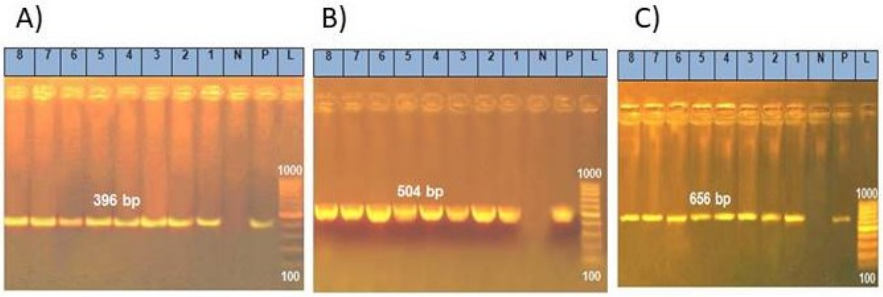

Fig. 1. Agarose gel electrophoresis of A) oprL, B) toxA and C) pslA genes lanes (1-8): positive amplification of target genes at 396, 504 and 656 bp, respectively. M: 100 bp DNA marker. P: positive control. N: negative control

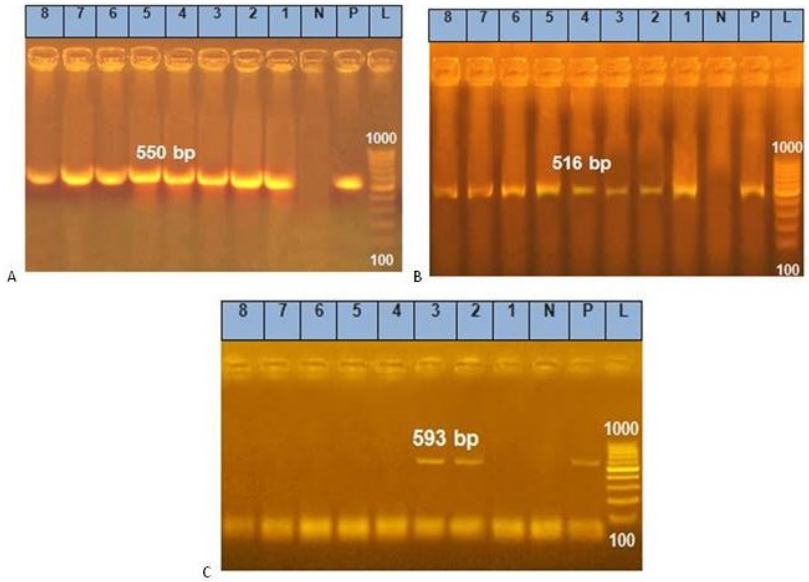

Fig. 2. PCR product electrophoresis of *A) amp*C, *B) blaTEM*, and *C)blaCTX* genes of *P. aeruginosa* isolates. Lanes (1-8), Lanes (2, 3): positive amplification of target genes at 550, 516 and 593 bp, respectively. L: 100-bp marker (ladder). P: positive control. N: negative control

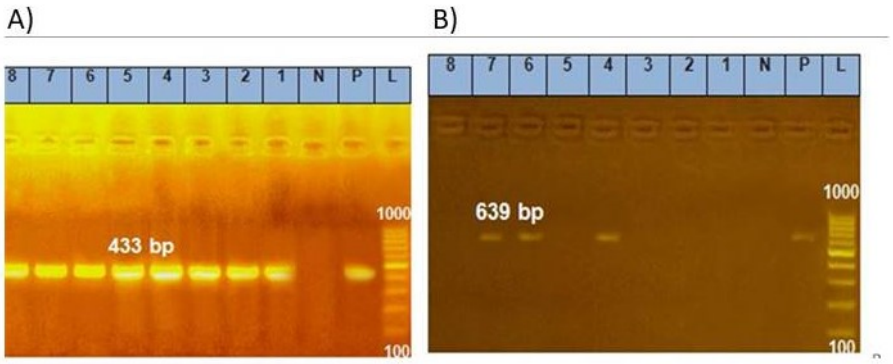

Fig. 3. Electrophoretic banding pattern PCR electrophoresis of *A) sul1 and B) ermB*, genes of *P. aeruginosa* isolates. Lanes (1-8) and Lanes (4, 6, 7): positive amplification of target genes at 433 and 639 bp, respectively. L: 100-bp marker (ladder). P: positive control. N: negative control

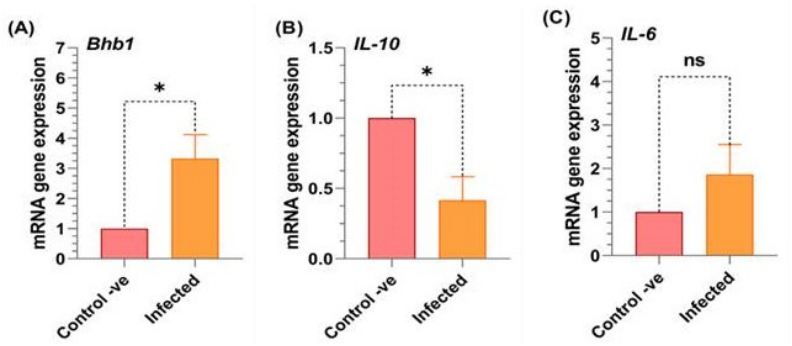

Fig 4. mRNA gene expression of studied genes including, *Bhb1* (A), *IL-10* (B), and *IL-6* (C) in milk of control and infected group (means ±SE).

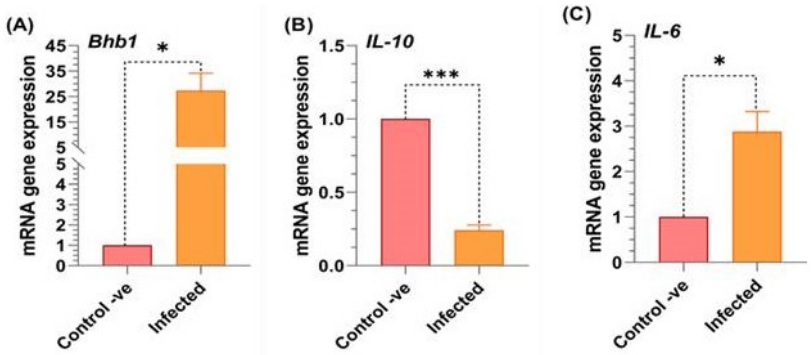

Fig. 5. mRNA gene expression of studied genes including, *Bhb1* (A), *IL-10* (B), and *IL-6* (C) in serum of control and infected group (means ±SE).

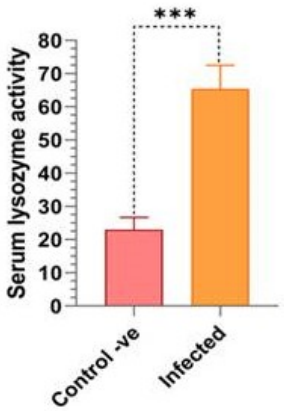

Fig. 6. Serum lysozyme activity of control and infected group (means ±SE).

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استخدام تقنية المالدي توف ماس في تشخيص السيدوموناس ايروجينوزا المسببة لالتهاب الضرع في الابقار مع مرجعية خاصة لبعض التغيرات المناعية المتصلة

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الملخص

لقد حظي التهاب الضرع الناجم عن السيدوموناس ايروجينوزا بمزيد من الاهتمام في السنوات الأخيرة لأنه يسبب خسائر اقتصادية كبيرة لمزارع الألبان وصناعة الألبان ككل. التهاب الضرع الناتج عن السيدوموناس ايروجينوزا هو مرض متعدد العوامل يحتاج إلى تفاعل بين العامل الممرض والمضيف والبيئة. يعد ظهور وانتشار مقاومة مضادات الميكروبات في السيدوموناس ايروجينوزا أحد أهم القضايا الصحية في جميع أنحاء العالم. هدفت هذه الدراسة إلى عزل السيدوموناس ايروجينوزا من عينات اللبن التي تم تجميعها من حيوانات مصابة بالتهاب الضرع ودراسة علامات الضراوة المرتبطة بها ودراسة مقاومة مضادات الميكروبات لها سواء ظاهريا أو وراثيا عن طريق الكشف عن جين ARG بالإضافة إلى دراسة المؤشرات المناعية في عينات الحليب والسيرم. تم جمع 200 عينة حليب من الأبقار والجاموس من حيوانات مصابة بالتهاب الضرع وتم فحصها للتأكد من وجود بكتيريا السيدوموناس ايروجينوزا ، وتم التعرف على المعزولات عن طريق الإجراء البكتريولوجي التقليدي ثم تأكيدها بواسطة مالدي توف ماس لتكشف عن معدل تواجد السيدوموناس ايروجينوزا بنسبة (7٪) في العينات المفحوصة. أظهر اختبار الحساسية للمضادات الحيوية أن العزلات أظهرت معدلات مقاومة عالية للأمبيسلين والأموكسيسيلين والإريثرومايسين بنسبة 100% ، يليها السيفترياكسون والسيفيديزيم بنسبة 92.8%، ومقاومة متوسطة للكلورامفينيكول (64.2%) والدوكسيسيكلين (57.4%). في حين أظهرت العزلات حساسية عالية للسيبروفلوكساسين والجنتاميسين والأماكاسين والإميبنيم والكوليستين. أظهرت نتائج تفاعل البلمرة المتسلسل وجود لجينات الضراوة oprL وtoxA وpslA في جميع العزلات المفحوصة. أظهرت النتائج وجود زيادة معنوية في التعبير الجيني لـ Bhb1 و IL6 وفي نشاط الليزوزيم في عينات السيرم، في حين أظهرت عينات الحليب زيادة معنوية في Bhb1 وانخفاض في IL10 مع عدم وجود تغيير في التعبير الجيني IL6 عند المقارنة مع العينات الحاكمة

الكلمات الدالة: مالدي توف ماس ، السيدوموناس ايروجينوزا ، التهاب الضرع ، الابقار.