



Resistant Gene of *Pseudomonas Aeruginosa* in Mastitic Cattle with Reference to some Biochemical and Immunological Parameters

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ABSTRACT

Mastitis is an important infectious disease of cattle and *Pseudomonas aeruginosa* (*P. aeruginosa*) bacteria stand out amongst those fundamental causative agents. The present study was intended to assess *P. aeruginosa* activity isolated from mastitic cattle milk samples in a veterinary hospital. It also was assessed some free radicals and immunological parameters in the milk and serum samples. Control samples were taken from apparently healthy cows, negative California Mastitis test. The results cleared that positive *P. aeruginosa* isolates were 34 of 100 milk samples. In vitro antibiotic sensitivity test indicated that 79.4%, 70.5% and 58.82% of the isolates completely resisted cefotaxime, penicillin and amikacin respectively. Also, the resistance to meropenem was 11.76% and 8.8% for carbapenem resistant isolate which completely resisted other classes of β -lactams. While enrofloxacin and gentamicin sensitivity reached to 76.47% and 73.5% respectively. The technique of the P.C.R was done for detection of MexR gene (in isolates resisted to more than three antibiotics) and Vim gene (in carbapenem resistant isolates). The biochemical results investigated that the nitric oxide and Mmalondialdehyde antioxidant levels were increased significantly while the cholesterol was decreased significantly in both serum and milk samples. Meanwhile, catalase and lysozyme were changed between groups and total protein and globulin had increased significantly in milk samples only. In conclusion, *P. aeruginosa* isolates including MexR and blaVim genes showed considerable percent of resistance to carbapenem group and other classes of β -lactam. In addition, the estimated biochemical and immunological parameters were affected in that case of mastitis in cattle. The results may encourage studies which are concerned with antioxidants treatment for mastitis in cattle. It may be a key for decreasing body resistance to antibiotics.

Key words: Mastitis, Cattle, *Pseudomonas aeruginosa*, PCR, Lysozyme

INTRODUCTION

Mastitis is one of the most predominant and expensive diseases in productive dairy cows. Almost half of all clinical cases are caused by Gram-negative bacteria, among these bacteria *P. aeruginosa* (Radostits et al., 2006; Pragasam et al., 2016). It was widely distributed in nature with an extraordinary physiological and metabolic adaptability. *Pseudomonas aeruginosa* was able to persist in both community and hospital settings for long time (Florentin et al., 2016). Moreover, its resistance for many antibiotics of clinical use, such as aminoglycosides, β -lactams and fluoroquinolones was a real problem. This reflected the great hazard to general health of public (Lister et al., 2009; Toval et al., 2015). Carbapenem was one of β -lactam class; the last therapeutic resource for control of bacterial infections and its use had been associated with emergence of carbapenem resistant bacteria (Pollini et al., 2013; Uruvoma, 2015).

The mexR gene was an important regulatory gene for multidrug resistant specially the β lactam group and was aimed to identify the carbapenem resistance by detecting β -lactams VIM gene (Suman et al., 2006). First class B of metallo- β -lactamases (MBLs) enzymes (produced by *P. aeruginosa*) were recorded in 1991 in Japan (Watanabe et al., 1991). There were main six families. However, the most frequent MBLs were Plasmid-mediated IMP-type carbapenemases (IMP) and Verona integron-encoded metallo- β -lactamase (VIM) (Toval et al., 2015; Zhao and Hu, 2015).

The normal aerobic metabolism byproducts were involved mainly with the Reactive Oxygen Species (ROS) which occurred mainly in the mitochondria. However, under stress, they increased much more body's defenses ability and oxidative stresses take place leading to cell injury (Navasa et al., 2014). In a like manner was the reactive nitrogen species (derived from nitric oxide). In sum, they impaired some biological molecules, gave rise to several diseases and changed the milk composition (Markiewicz-Keszycka et al., 2013). Then, the health of highly producing dairy cow would worsened (Sordillo and Aitken, 2009). As well as, the malondialdehyde (MDA) was the main lipid-peroxidation stress marker. It supported the ROS production subsequently damaging the mammary tissues and reducing the immune state (Rahal et al., 2014). Antioxidants are substances that successfully trapping the oxidative intermediates preventing biomolecules oxidation or reducing the oxidized ones. It well stand against the oxidative damage (Sordillo et al., 2009). In bovine milk, catalase is the index of mastitis and the lysozyme is a bactericidal agent (Fox et al., 2015).

Studying bacterium, immunity and antibiotic resistance were very important for treatment and eradication of mastitis due to *P. aeruginosa* microorganism. The disease puts a great financial stress on the dairy industry (Ventola, 2015). Hence, many researchers focused on proteins biomarkers in the biological fluids as economic tests help to diagnose and treat livestock diseases (Oskoueian et al., 2016). This study was directed to record the percent of *P. aeruginosa* including resistant gene in mastitic milk of the studied area and determined some biochemical and immunological parameters in serum and milk to evaluate the cellular damage.

MATERIAL AND METHODS

Ethical approval

Animal handling was followed by animal ethics committee guidelines, Faculty of Veterinary Medicine, Mansoura University, Egypt.

Animals and sampling

One hundred Holstein cows showing clinical signs of mastitis in different seasons at the educational veterinary hospital, Mansoura university, Dakahlia Governorate, Egypt were subjected to blood and milk sampling (in 2014 and 2015). Ten apparently healthy cattle, with negative California Mastitis test served as control. The age of the cattle ranged from 3 to 6 years. The samples were collected with hygienic precautions and were directly transported to the laboratories. The collected blood samples (in plain centrifuge tube) were placed in a slanted position (20 min at room temperature) for clotting. Milk and blood samples were centrifuged at 5000 revolutions per min (rpm) for 30 min and at 3000 rpm for ten min respectively. Serum and aqueous supernatant of milk were preserved at - 20 °C till used. The milk precipitate was used for bacteriological examination. Per the results of isolation and identification, ten milk and corresponding serum samples were randomly selected from *P. aeruginosa* mastitic samples (*P. aeruginosa* group) and were used for estimation of different biochemical and immunological parameters.

Bacteriological examination and identification of the isolates

An inoculum from each milk sample was inoculated into broth before plating on nutrient and MacConkey agar, then Pseudomonas agar base with cetrimide_nalidixic acid (C_N) supplement and blood agar. Then aerobically incubated (at 37 °C) the plates and examined after 24-48 h. Suspected colonies were described for their characteristic green pigmented colonies on nutrient and pseudomonas agar base with C_N supplement and for hemolytic activity on blood agar (Konemann et al., 1992). Gram stain and the test of oxidase were done (Macfaddin, 2000). Finally the suspected isolates were sent to the animal health research institute (Dokki, Cairo, Egypt) for further identification by VITEK2 compact system (based on biochemical identification via colorimetric technology), card 2GN (BioMérieux-France).

In vitro antimicrobial assay

Antimicrobial sensitivity test was assessed via disc diffusion method by means of Mueller-Hinton agar (Finegold and Martin, 1982). Antimicrobial discs (Oxoid) were penicillin, cefoperazone, cefotaxime, gentamicin, amikacin, enrofloxacin, meropenem and imipenem. After incubation (18-24 h), the visible clear zone of inhibition was determined (mm) following the interpretation chart guidelines (CLSI, 2014).

Extraction of the DNA

The DNA extraction has done utilizing Mini kit QIAamp DNA (Qiagen, Germany, GmbH) for manufacturer's proposals. In details, proteinase K (10 µL) and lysis buffer (200 µL) was added to the sample suspension (200 µL).

Then, it was incubated at 56 °C (10 min) at that time added the ethanol 100% (200 µL). The sample has been washed and centrifuged. Then, the elution buffer (100 µL) was eluted with the nucleic acid.

Amplification of the PCR

The used oligonucleotide primers (Metabion, Germany) were utilized in a reaction (25 µL) contained 12.5 µL of the EmeraldAmp Max PCR Master Mix (Takara, Japan), water (4.5 µL), DNA template (6 µL) and 1 µL of each primer (20 pmol concentration). This was done in an applied biosystem 2720 thermal cycler (Table 1).

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions in *P. aeruginosa* isolated from milk of Holstein mastitic cattle (3–6 years of age) in 2014-2015

Target gene	Primers sequences	Amplified segment (bp)	Prim. denat.	Amplification (35 cycles)			Final exten.	Reference
				Sec. denat.	Annealing	Exten.		
<i>P. aeruginosa</i> blaVIM	TTTGGTCGCA TATCGCAACG	500	94°C	94°C	50°C	72°C	72°C	(Amudhan et al., 2012)
	CCATTCAGCC AGATCGGCAT		10 min	45 sec.	45 sec.	45 sec.	10 min.	
<i>P. aeruginosa</i> mexR	GCGCCATGGC CCATATTCAG	637	94°C	94°C	57°C	72°C	72°C	(Sanchez et al., 2002)
	GGCATTTCGCC AGTAAGCGG		5 min.	45 sec.	45 sec.	45 sec.	10 min.	

Prim: primary, sec: secondary, denat: denaturation, exten: extension.

The PCR Products analysis

Electrophoresis was done for the PCR products separation using 1x TBE buffer had 1.5% of agarose gel (Applichem, Germany, GmbH) at room temperature by means of 5V/cm gradients. Then, analysis was done by loading the product (20 µL) into each gel slot. A gel pilot 100 bp and 100 bp, in addition to Ladder of the DNA (Qiagen, Germany, GmbH) were used for determination of the sizes of the fragment. Gel documentation system (Alpha Innotech, Biometra) was encountered for photography. The data were analyzed via computer program software.

Immunological and biochemical analysis

Serum Total Protein (TP) and serum albumin were estimated by means of readymade kits (Stanbio laboratory, USA). Globulin (TP minus albumin) and albumin globulin ratio (A/G) were calculated according to Kaneko et al., (1997). Cholesterol (Allain et al., 1974), triglycerides, (Fossati and Prencipe, 1982), catalase, Nitric Oxide (NO) and MDA were estimated using readymade diagnostic kits (Bio-diagnostic, Egypt). All parameters were calorimetrically estimated by means of semiautomatic spectrophotometer (BM-Germany 5010) according to the suppliers' kits protocols.

Catalase determination was based on the reaction of the catalase with specific quantity of H₂O₂. Stopping the reaction was done after one min by means of the catalase inhibitor (Aebi, 1984). The MDA determination based on malondialdehyde (one molecule) reaction with thiobarbituric acid (two molecules) in acidic environment (Satoh, 1978), while NO determination was done according to nitrite method. In presence of nitrite, subsequent substance coupled with N-(1-naphthyl) ethylenediamine in acidic media and the azo dye could be measured (Ignarro et al., 1987).

The turbidometric assay was done for determination of the lysozyme activity (Parry et al., 1965). In details, the lysozyme substrate was prepared using 0.75 mg/mL of micrococcus lysodeikticus (gram positive bacteria) lyophilized cells (Sigma, St. Louis, MO). Then the preparation was suspended in 0.1 M of the buffer of sodium phosphate/citric acid (pH 5.8). The substrate solution (175 mL) was placed into each microtiter plate well at 25 °C. The serum or supernatant of milk sample (25 µL) was added in duplicate. After 0 and 20 min, absorbance (at 450 nm wave length) was recorded via ELISA reader microplate (Bio TEC, ELX800G, USA). The lysozyme concentrations were recorded (µg/ml) corresponding to the standard curve obtained with lyophilized hen-egg-white-lysozyme (Sigma).

Statistical analysis

Serum and milk immunological and biochemical parameters were analyzed statistically using program, SPSS 17.0 for windows, Student's t test was used for determined the statistical significance. The difference between groups was considered significant at P < 0.05. The data were expressed as mean ± SE.

RESULTS

Prevalence and antibiotic sensitivity test of *P. aeruginosa* organism in milk samples

Out of all of the examined samples 34 (34%) were positive for *P. aeruginosa*. Isolates showed multidrug resistant to penicillin, cefotaxime and cefoperazone were 12 (35.29%). Further, the resistance for carbapenem group (meropenem and imipenem) was four (11.76%) isolates.

Antibiotic sensitivity test results were shown in table 2. There were 58.82% and 11.76% *P. aeruginosa* of the isolates were completely resist to amikacin and gentamycin respectively. Moreover 70.59%, 79.41% and 35.29% were completely resist to penicillin, cefotaxime and cefoperazone respectively. Otherwise, although about 11.76% were resistant to meropenem and 2.94% for imipenem.

Detection of mexR gene and MBL (blaVIM) gene

The results revealed positive amplification of the 637 bp of mexR from the extracted DNA of four (33.33%) isolates from 12 *P. aeruginosa* isolates which showing multidrug resistance. While three (75%) from four *p. aeruginosa* isolates (showed resistance to carbapenem group) were positive for blaVIM gene (Figure 1).

Immunological and biochemical analysis

Table 3 demonstrated the determined immunological and biochemical parameters results. Catalase, lysozyme, cholesterol, and triglycerides levels significantly (p value; 0.000, 0.001, 0.009 and 0.014 respectively) decreased in the serum of *P. Aeruginosa* group comparing with control group. Contrast to milk levels of catalase, lysozyme, TP and globulin increased significantly (p value; 0.000, 0.001, 0.197 and 0.237 respectively) however decreasing of milk A/G ratio. The oxidative stress (NO and MDA) activity increased significantly (p value; 0.006 for serum NO and 0.000 for others) in each serum and milk groups than their control ones.

Table 2. Results of antibiotic sensitivity test of milk samples (N = 34) of Holstein mastitic cattle (3–6 years of age) infected with *P. aeruginosa* in 2014-2015

Antibiotic	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Meropenem	24	70.6	6	17.65	4	11.76
Imipenem	31	91.2	2	5.88	1	2.94
Penicillin	2	5.88	8	23.53	24	70.59
Cefoperazone	3	8.82	19	55.88	12	35.29
Cefotaxime	0	0	7	20.59	27	79.41
Amikacin	6	17.7	8	23.53	20	58.82
Gentamycin	25	73.5	5	14.71	4	11.76
Enrofloxacin	26	76.5	8	23.51	0	0

Figure1. Detection of mexR gene and MBL (blaVIM) gene in *P. aeruginosa* isolated from milk of Holstein mastitic cattle (3-6 years of age) in 2014-2015.

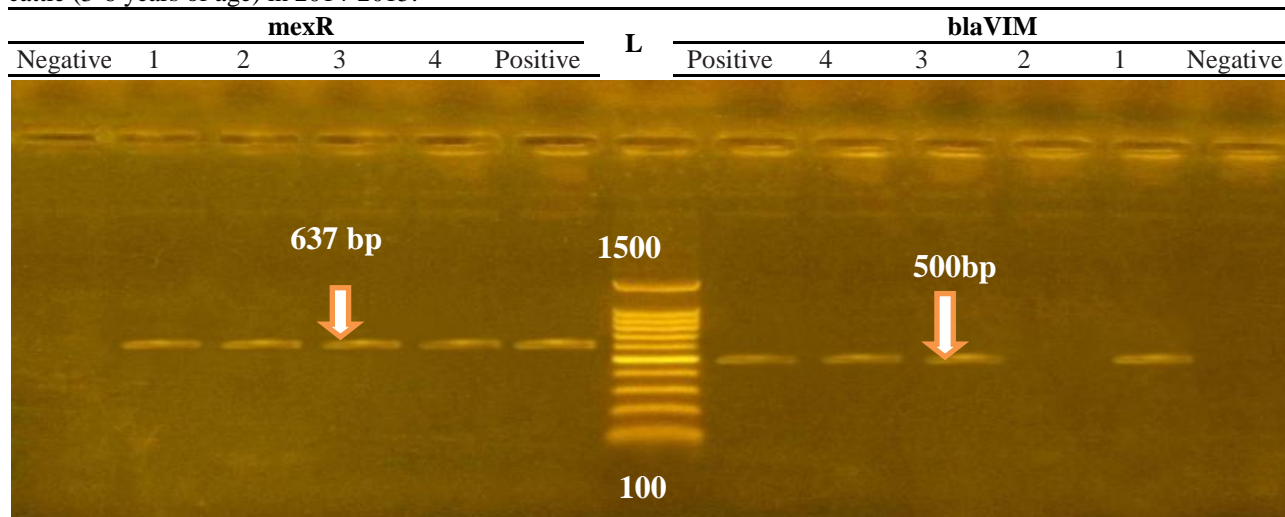


Table 3. Serum and milk biochemical and immunological parameters (Mean \pm S.E) of Holstein mastitic cattle (3-6 years of age) infected with *P. Aeruginosa* in 2014-2015

Groups (N = 10)	NO μ mol/L	Ctalase nmol/ml	MDA nmol/ml	Lyso. μ g/ml	Chol. mg/dl	Trigly. g/dl	T.P. g/dl	Alb. g/dl	Glob. g/dl	A/G ratio
Serum										
Control	1.21 \pm 0.23*	11.75 \pm 0.28*	4.41 \pm 0.73*	37.69 \pm 2.00*	117.2 \pm 17.75*	43.00 \pm 4.16*	5.88 \pm 0.21	1.53 \pm 0.10	4.35 \pm 0.24	0.37 \pm 0.04
<i>P. Aeruginosa</i>	6.65 \pm 1.71*	7.64 \pm 0.65*	10.32 \pm 1.33*	15.65 \pm 4.84*	54.83 \pm 12.06*	27.96 \pm 3.63*	6.55 \pm 0.45	1.59 \pm 0.09	4.97 \pm 0.44	0.34 \pm 0.03
P Value	0.006	0	0.001	0.001	0.009	0.014	0.197	0.685	0.237	0.569
Milk										
Control	1.26 \pm 0.08*	8.76 \pm 0.45*	1.90 \pm 0.29*	22.91 \pm 1.84*	173.0 \pm 5.66*	28.02 \pm 2.55	4.15 \pm 0.40*	1.28 \pm 0.08	2.87 \pm 0.46*	0.56 \pm 0.09*
<i>P. Aeruginosa</i>	7.74 \pm 1.06*	13.23 \pm 0.58*	23.97 \pm 9.37*	128.87 \pm 26.86*	71.8 \pm 3.01*	32.02 \pm 4.38	8.49 \pm 0.35*	1.24 \pm 0.09	7.25 \pm 0.40*	0.18 \pm 0.02*
P Value	0	0	0.03	0.001	0	0.441	0	0.754	0	0.001

NO, nitric oxide; MDA, malondialdehyde; Lyso, lysozyme; chol, cholesterol; trigly, triglycerides; T.P, total protein; Alb, albumin; Glob, globulin; A/G ratio, albumin globulin ratio. Values with asterisk (*) represent significantly difference versus control in the same column at $p < 0.05$. Values are presented as mean \pm SE.

DISCUSSION

The *P. aeruginosa* is an environmentally abundant bacterium that causes severe disease among immune compromised hosts. It is one of the common cause of mastitis (Mcvey et al., 2013). The present resistance to penicillin tend to agree with Ranjan et al. (2010). Also, the high resistance of *P. aeruginosa* to cefoperazone and cefotaxime agreed with others (Akhoon et al., 2012 and Kotwal et al., 2016).

The resistant isolates against carbapenem group were of a great concern due to it was the last therapeutic resource for controlling infections. It may attributed to carbapenemase produced by bacteria which involved in food producing animals and their surroundings (EFSA, 2013). The resistance to imipenem was agreed with Ohnishi et al., (2011) and (Kotwal et al., 2016). Contraries, *P. aeruginosa* that isolated from human, chickens, milk and calves lung were 100% sensitive to the same drug (Osman et al., 2012). Our result of multidrug resistance isolates included mexR gene may be due to mutation. That gene mutation increased the resistance to many antibiotics in samples of corneal keratitis scraping with *P.aeruginosa* in human (Suman et al., 2006). However, *mexAB* pump was not the cause of meropenem resistance (Pragasam et al., 2016).

Nitric oxide is one of the important biomarker for immunomodulation. In the same time, it has a main role in the cellular damage by means of ROS and proteolytic enzymes. This bad action was cleared in many diseases as mastitis (Lacasse et al., 2008 and Jeon et al., 2014). Present NO results were in harmony with Bastan et al. (2013) results who reported that NO concentration mainly depend on quarters infectious status by among bacterial species.

Catalase was one of the primary antioxidant enzymes that convert ROS to more stable molecules (Suntres, 2011). Lowering catalase activity in the serum may related to proliferation of consumed ROS, outcome of the toxins. Meanwhile its elevation in milk with lysozyme may attribute to the raised Somatic Cell Count (SCC). It was confirmed by increasing TP and globulins level in addition lowering of the A/G ratio. Catalase and MDA results were a reason for the oxidative damage in mastitis of dairy cows (Jhambh et al., 2013).

Lysozymes are vital constituents in the animals' innate immune system as they hydrolyzed major polymer of the bacterial cell wall. Gram negative bacteria had lysozyme inhibitors represented as virulence elements when interacting with the animal cell G (Callewaert et al., 2008). The current lysozyme results were different between serum and milk groups. The results were in concord with a case of *Staph aureus* mastitis in cow milk (Osman et al., 2010). Meanwhile serum lysozyme concentration increased in case of tuberculosis and *Staph aureus* mastitis in cow, in contrary with the level in milk whey (Ramadan et al., 2009). Though the impact of lysozyme genotypes for predicting mastitis was moderately low (Zaborski et al., 2016).

Regarding to the results of serum cholesterol and triglycerides levels may attribute to the suppressed appetite due to mastitis and the cow consuming insufficient nutrients. Triglyceride or cholesterol is dietary lipids or endogenous lipids which are produced by hepatocytes (Meyer et al., 1992). However, serum cholesterol level was decreased in two mastitic herds even with changing the feed program (Ohtsuka et al., 2006). Definite bacterial toxins increased the production of ROS, proinflammatory cytokines and biologically active lipids (Al Batran et al., 2013 and Rahal et al., 2014). Consequently, elevation of lipids, NO and MDA as well as reduction of catalase and lysozymes levels may be due to bacterial toxins.

As the resistance of *P. aeruginosa* to antibiotics increased dramatically, the maintenance of normal average of determined parameters (NO and MDA, catalase and lysozymes) could improve the immunity then the action of antibiotics will be maximized. Further studies were required for predicting the microbial mastitis from evaluating the immunological parameters.

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Competing interests

The authors have declared that no competing interest exists.

Author's contributions

All of authors participated in the idea, planning of the research, samples processing, drafting the article and approved final revision. Amany Mohamed Abd-El-Moaty and Verginia Mohamed El-Metwally Farag participated in bacteriological examination, identification and antimicrobial sensitivity test. Nermin Awad Ibrahim participated in DNA extraction, PCR analysis. Verginia Mohamed El-Metwally Farag performed immunological, biochemical, statistical analysis. Samar Magdy Atwa participated in diagnosis of the disease, samples (milk and blood) collection, collecting data and antimicrobial sensitivity test. Nermin Awad Ibrahim, Verginia Mohamed El-Metwally Farag and Amany Mohamed Abd-El-Moaty participated in writing the paper.

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