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International Journal of Current Research
Vol. 9, Issue, 09, pp.57647-57651, September, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

DETECTION OF *PSEUDOMONAS AERUGINOSA* FROM NORMAL RAW AND MASTITIC COW'S MILK IN BAGHDAD*

***Rasheed Badi Ermish Abo Bader and Nadia Ibrahim Abdulaal**

Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad

ARTICLE INFO

Article History:
Received 12th June, 2017
Received in revised form
20th July, 2017
Accepted 23rd August, 2017
Published online 30th September, 2017

Key words:

Pseudomonas aeruginosa,
Raw milk,
Mastitic milk,
Cows,
Biofilm.

* The research was sheathed partially from MSc. Candidate Thesis of corresponding author Rasheed.

ABSTRACT

Biofilm producing *Pseudomonas aeruginosa* is a highly versatile opportunistic food borne pathogen that contaminate food and causing serious problems in man, animals and food processing-manufacturing cycles. This research focused on isolation and Identification of *P. aeruginosa* from normal raw and mastitic cow's milk from some regions in Baghdad. Study design including collection and processing of sixty milk samples (thirty samples for each type: normal & mastitic) from regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya (twenty samples from each region: ten samples for each type) during period December (2016) to February (2017), in which they collected and processed according to modified dairy microbiological methodology in veterinary public health laboratory using gold standard Cetirmide-Naldixic acid chrome agar (CNP), Electronic RapID™ ONE (4 hours) biochemical panel micro-tubes strep identification system compendium with reference colors chart and online confirmation microcodes data base software and gold standard double staining technique, Microtiter Plate Assay for biofilm formation with methylene blue and safranin dyes. Antibiotics Susceptibility Pattern by Kirby-Bauer technique or disk diffusion method was done according to instructions of clinical laboratory standards institute (CLSI) or national committee for clinical laboratory standards (NCCLS) by using a Muller-Hinton agar and McFarland opacity tubes for checking resistance profile of isolates. Data were analyzed for significant differences by statistical package for social sciences software (IBM SPSS) in which a Chi-square was used. The results revealed isolation and identification of nine strains of *P. aeruginosa* out of sixty samples (15%): three strains from Abu-Ghraib (5%): two from mastitic (3.33%) and one from normal raw milk (1.66%), two strain from Al-Fudhaliyah (3.33%): one from mastitic (1.66%) and one from normal raw milk (1.66%), and four strains from Al-Sadrya (6.66%): three from mastitic (5%) and one from normal raw milk (1.66%). In conclusion: data revealed contamination of raw milk and infection of dairy mammary glands with *P. aeruginosa* from some regions in Baghdad, thus we recommend monitoring of milk producing animals and their environment with milk production, transportation and storage stages through application of good management practices and hazard analysis critical control points (HACCP) strategies to overcome or reduce these public health problems.

Citation: Rasheed Badi Ermish Abo Bader and Nadia Ibrahim Abdulaal, 2017. "Detection of *Pseudomonas aeruginosa* from normal raw and Mastitic cow's milk in Baghdad*", *International Journal of Current Research*, 9, (09), 57647-57651.

INTRODUCTION

Biofilm producing *Pseudomonas aeruginosa* is a highly versatile opportunistic foodborne pathogen that contaminate food and causing serious problems in man, animals and food processing-manufacturing cycles from inadequate food processing to problems in dairy products manufacturing to food spoilage due to heat-stable complex enzymatic machine system that survive pasteurization and UHT treatments to mastitis and food poisoning ending with fatal case of cystic fibrosis especially in immunocompromised individuals due to genetic makeup and polymorphism of this eco-pathogen due to phenazine pigments and exopolysaccharide mucoid secretion (alginate-biofilm) and antibiotics resistant profile (Rehm, 2008; De Bentzmann and Plésiat, 2011; El-Leboudy *et al*., 2015; Al-Shammary, Ali 2015). Because of its minimal growth requirements and nutritional flexibility, *P. aeruginosa* is particularly able to adapt to changing ecological conditions. It is also capable of surviving for long periods, especially in moist environments. In addition, the poor permeability of its outer membrane makes it intrinsically resistant to many disinfectants and antimicrobial agents, and it is therefore well adapted to compete with antibiotic-secreting microorganisms in its natural ecosystem, the soil, and to exploit the selective advantages provided by hospital environments (Rehm, 2008; Campa *et al*., 1993). The quest of nosocomial, community and livestock distribution and frequency of *P. aeruginosa* infections was hard to treat because of both the intrinsic resistance of the species (it has constitutive expression of *AmpC*beta lactamase and efflux pumps, combined with a low permeability of the outer membrane), and its remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents, including beta lactams, aminoglycosides and fluoroquinolones. *P. aeruginosa* represents a phenomenon of bacterial resistance, since practically all known mechanisms of antimicrobial resistance can be seen in it, thereby conferring multi-resistant phenotypes (Rehm, 2008; Al-Shammary, Ali 2015; Lambert, 2002; Rossolini and Mantengoli, 2005;

Corresponding author:* **Rasheed Badi Ermish Abo Bader,
Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad.

Strateva and Yordanov, 2009; Nagaveni *et al*., 2010; Kirk and Mellenberger, 2013; Xu *et al*., 2013). Contagious and environmental mastitis of dairy farms was evident primarily in low hygienic environments due to poor sanitary conditions as part of inadequate policies of good management practices as well as, this psychrotrophic pathogen can spread and persist for long period of time due to biofilm progeny dissemination, therefore contaminate many areas and forming resident foci of infection. Direct and indirect transmission of infection can occurs due to ubiquitous nature of this eco-pathogen. Contamination of milk can occurs at any stage of processing from animals to handlers and even in retailing markets due to complex scenarios from insufficient pasteurization to post heat treatment contamination, as well as the milk producing animals and handlers continues active and passive carrier stages of ascending and descending infections especially under stress interconnected factors. Nutritional deficiencies and Malfunctioning milking equipment would increase the risk on new *P. aeruginosa* infections and other bacteria due to teat end trauma (Rehm, 2008; Al-Shammary, Ali 2015; Kirk and Mellenberger, 2013; Daly *et al*., 1999). Low information about quality of raw milk as well as environmental hygienic conditions of milk producing animals and their milk production and processing cycles under these circumstances linked with the case of subclinical and clinical mastitis in dairy farms in Iraq, and obscure information about prevalence and epidemiology of *P. aeruginosa*, leading to and aiming the investigation of the occurrence and distribution of this pathogen in milk samples from regions of Abu-Ghraib,Al-Fudhaliyah and Al-Sadrya.

MATERIALS AND METHODS

Collection & Processing of Samples

Study design with pilot training procedures including collection and processing of sixty milk samples (thirty samples for each type: thirty normal raw cow's milk samples plus thirty abnormal clotted viscous mastitic cow's milk samples) from regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya (twenty samples from each region: ten samples for each type) during period December (2016) to February (2017), in which they collected and processed according to modified dairy microbiological methodology and bacteriological analytic manual (BAM) in veterinary public health laboratory (British Standards Institution, 1984; Marshall, 1993; Food and Drug Administration, 2000; Quinn, 2004; Food Safety and Inspection Services, 2013; Bacteriological Analytical Manual, 2017). Samples were collected from milk cans pools and infected mastitic cows via test tubes and containers carefully to minimize contamination and processed adequately until transportation to work lab by ice box, then refrigerated at 4 $^{©}$C as critical control point in isolation and identification procedure of *P. aeruginosa*.

Isolation & Identification Procedure

All samples were processed according to food microbiological techniques in which they refrigerated at 4 $^{©}$C for 48 hours for resuscitation of psychrotrophic *P. aeruginosa*, then mixed thoroughly by vortex to emulsifying fatty globules and clots that hide the pathogen and to inshore redistribution of it in whole sample. Processed samples were diluted and inoculated in buffered tryptone soya yeast extract broth (one part sample (10 ml) to nine part diluent (90 ml) and incubated at 37 $^{©}$C for 24 hours, then inoculated in Cetirmide-Naldixic acid chrome agar (CNP) by loop (dilution inoculation) in three replicates for each sample, then incubated at 37 $^{©}$C for 48 hours (Marshall, 1993; Bacteriological Analytical Manual, 2017; Flint and Hartley, 1996). Pure large mucoid and small polymorphic colonies with yellow-green to blue pigmentation and fruity odour were picked up and recultured in tryptone soya yeast extract brothto proliferate and revive stressed isolates, then cultured in tryptone soya yeast extract agar for further classification. Pure seedswere cultured on McConkey and Eosin methylene blue agars for more details. Gram stain and oxidase was done. Electronic RapID™ ONE (4 hours) biochemical panel micro-tubes strep identification system compendium with reference colors chart and online confirmation microcodes data base software was used for identification procedure according to company leaflet instructions (Oxoid – Remel, 2016).

Biofilm Formation Assay

Double staining technique, Microtiter Plate Assay with methylene blue and safranin dyes was used for detection of biofilm formation and secretion. Quantitavely and Qualitative detection of slime producer strains was determined by culturing the bacteria on modified Tryptone Soy Yeast Extract Broth (TSBYE) using adherence assay on large U-shape 24 well tissue culture plates as described previously by Christensen *et al*. (Christensen *et al*., 1985; O'Toole, 2011; Welch *et al*., 2012). An overnight culture grown in TSBYE at 37°C was transferred and diluted in microtiter plate as 0.1ml/0.5ml freshly prepared TSBYE inoculated for each well. Each isolate was tested in triplicate. Wells with sterile TSBYE alone was served as controls. The plates were incubated for 24 hours at 37°C. Furthermore, the culture was removed and plates were washed three times with phosphate-buffered saline to remove non-adherent cells and dried in an inverted position. Adherent biofilm was fixed with 2% sodium acetate and was stained with of 10% crystal violet and safranin for 5 min. Then, unbound stain was removed and the wells were washed three times with PBS. Plates were settled 2-3 hours for dryness then stained layers and dots of biofilm in bottom and around internal rims of wells were photographed, measured and scored according to the degree of formation, type of stain and type of isolate.

Antibiotics Susceptibility Pattern (Resistance Profile)

A Kirby-Bauer technique or disk diffusion method was dependent according to instructions of clinical laboratory standards institute (CLSI) or national committee for clinical laboratory standards (NCCLS) by using a Muller-Hinton agar and McFarland opacity tubes (Bauer *et al*., 1966; Clinical and Laboratory Standards Institute, 2009a; Clinical and Laboratory Standards Institute, 2009b). A test procedure was done by selecting well-isolated (4-5) colonies of *P. aeruginosa*from freshly inoculated overnight TSAYE, touched tops of these colonies by a loop then, transferred to freshly prepared (4-5) ml TSBYE tubes and incubated for 2 hr. at 37 $^{©}$C in order to reach a standard 0.5 opacity of McFarland tubes or approximately 10^4-10^5 cfu/ml standard inoculum broth. Preparation of freshly agar plate's cultures of Muller-Hinton then dried in incubator before testing procedure. A sterile cotton swab was dipped into the adjusted suspension and rotated several times and pressed firmly on the inside wall of the tube above the fluid level for removing excess inoculum from the swab. Streaking the surfaces of Muller-Hinton agars (4-5) times with the rim by a

swabs then left inoculated agars for (10-15) minutes to absorb the inoculum before applying selected antimicrobial disks by pressing down to ensure complete contact with the agar surface and distributed evenly. The plates were inverted and placed in an incubator at 37 $^{©}$C for (18-24) hrs. Then reading the plates and interpretation the results (Lalitha, 2004; Jorgensen and Ferraro, 2009). If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone was related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug were interpreted using the criteria published by CLSI. The results were qualitative as susceptible, intermediate or resistant isolate derived from the test rather than minimum inhibitory concentration (MIC) (Korgenski and Daly, 1998).

Statistical Analysis

Results and data were analyzed for significant differences by statistical package for social sciences software (IBM-SPSS, 2016) in which a Chi-square ($æ^2$) was used with the formula of

$$æ^2 = (O-E)^2/E, \text{ in which:}$$

O = Observed positive isolates of *P. aeruginosa* from total samples (+ve).
E = Expected negative samples free from *P. aeruginosa* from the original total samples (-ve).

RESULTS AND DISCUSSION

Detection Profileand Isolation Percentages

The results revealed isolation and identification of nine strains of *P. aeruginosa* out of sixty samples (15%): three strains from Abu-Ghraib (5%): two from mastitic (3.33%) and one from normal raw milk (1.66%), two strain from Al-Fudhaliyah (3.33%): one from mastitic (1.66%) and one from normal raw milk (1.66%), and four strains from Al-Sadrya (6.66%): three from mastitic (5%) and one from normal raw milk (1.66%). Detection Profile and isolation percentages was summarized in tables (**1**,**2**&**3**):

Table 1. Isolation ratio of *P. aeruginosa* from raw milk

Region	Number of Samples	Isolation % (10)	Isolation % (30)
Abu-Ghraib	10	1 (10%) ^{A}	1 (3.33%) ^{A}
Al-Fudhaliyah	10	1 (10%) ^{A}	1 (3.33%) ^{A}
Al-Sadrya	10	1 (10%) ^{A}	1 (3.33%) ^{A}
Total	30		3 (10%)

A: Indicate none significant differences ($æ^2$) vertically at level (P≤0.05).

Table 2. Isolation ratio of *P. aeruginosa* from mastitic milk

Region	Number of Samples	Isolation % (10)	Isolation % (30)
Abu-Ghraib	10	2 (20%) ^{AB}	2 (6.66%) ^{AB}
Al-Fudhaliyah	10	1 (10%) ^{A}	1 (3.33%) ^{A}
Al-Sadrya	10	3 (30%) ^{B*}	3 (10%) ^{B*}
Total	30		6 (20%)

*: Indicate highest isolation ratio from Al-Sadrya.
A,B,AB: Indicate significant differences ($æ^2$) vertically at level (P≤0.05).

Table 3. Isolation ratio of *P. aeruginosa* from total milk

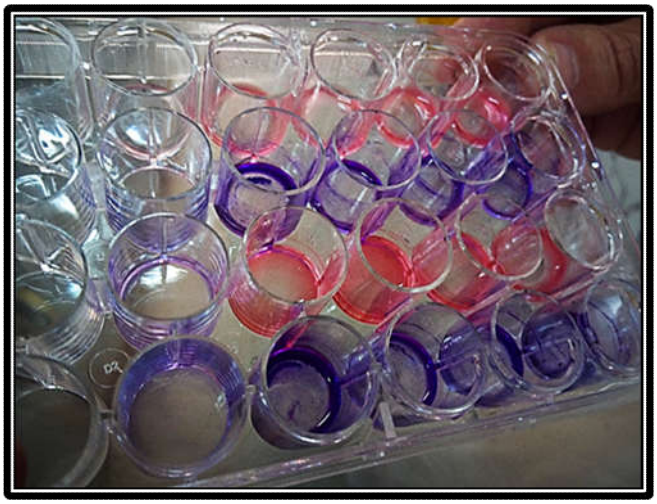
Region	Number of Samples	Isolation % (20)	Isolation % (60)
Abu-Ghraib	20	3 (15%) ^{AB}	3 (5%) ^{AB}
Al-Fudhaliyah	20	2 (10%) ^{A}	2 (3.33%) ^{A}
Al-Sadrya	20	4 (20%) ^{B*}	4 (6.66%) ^{B*}
Over all Total	60		9 (15%)

*: Indicate highest isolation ratio from Al-Sadrya.
A,B,AB: Indicate significant differences ($æ^2$) vertically at level (P≤0.05).

Up to date diagnostic facilities such as chrome agar CNP and ERIC compendium aid in isolation and identification of polymorphicstrains of *P. aeruginosa* from milk samples in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya. Isolation percentages indicate presence of infectious foci of this pathogen in these areas due to poor sanitary conditions during and after milk production as well as climatic conditions and inadequate management practices for milk producing animals and milk cans as well as infective individuals and infective carriers with flies and other interconnected factors and stressors encourage epidemiological distribution and frequency pattern of these opportunistic psychrotrophic bugs. Contamination and pollution can occurs at any stage of milk production, handling, transportation and storage (Franzetti, and Scarpellini, 2007; Hossain et al., 2013). Isolation profile and epidemiological pattern of mastitis causes may fluctuate according to different factors from hidden unrecognized subclinicalto immunocompromised cases to careless adulteration during processing of mastitic milk especially during unsanitary cleaning of milk cans that contains resident adherent biofilm infectious foci of *P. aeruginosa* in the bottom of these containers. Low number of milk samples in this project with high ratio of isolation was unacceptable quest in this time, but it's indicate the real face of truth coin in Iraqi environment in spite of presence of high quality of clean milk in Iraq, but in low number and areas. Mass production of milk with clean sanitary profile was absent in Iraq due to careless supporting of milk producers, coasty animal feeds, adulteration behavior, social factors and problems of personal safety as well as presence of mass importation of different types of retail heat processed milk from different unknown countries with low price aid in diminish depending on local production except in some situations like Arabic flavored cream production. Risk factors of these foodborne pathogens were evident through transportation from livestock to community and nosocomial relationship which facilitate distribution of biofilm producing and multidrug resistant pathogens in Iraqi environment, but actually in low number and cases and these situations may be some kind of mercy from Allah to Iraqi people especially those under poor social circumstances in spite of many unknown cases of urinary tract infections, pneumonia and cystic fibrosis in Iraqi hospitals.

Biofilm formation

Gold standard microtiter tissue culture assay with double staining technique bymethylene blue and safranin dyesplus CNP agar results (large viscous mucoid colonies) obviously indicate biofilm formation and secretionespecially in mastitic strains. Red and blue adherent layers and dots of biofilm around and in the bottom of microtiter plate tubes indicate presence of biofilm producing isolates as in photograph (**1**):



Photograph 1. Gold standard double staining technique, Microtiter Plate Assay for biofilm formation with methylene blue and safranin dyes (Christensen *et al*., 1985), revealing red and blue adhesive deposit and circles of biofilm around and in the bottom of microtiter tubes

Table 4. Pattern of Resistance, Intermediate and Susceptible isolates of *P. aeruginosa* (total 9 isolates) from normal raw and mastitic cow's milk in Baghdad

Antibiotics	Resistance %	Intermediate %	Susceptible %
Piperacillin (PRL100 µg)	6 (66.7)^{Ba}	None (0)^{Cc}	3 (33.3)^{Bb}
Cefotaxime (CTX30 µg)	5 (55.6)^{Ba}	None (0)^{Cb}	4 (44.4)^{Ba}
Aztreonam (ATM30 µg)	5 (55.6)^{Ba}	1 (11.1)^{Bc}	3 (33.3)^{Bb}
Imipenem (IMI10 µg)	None (0)^{Dc}	8 (88.9)^{Aa}	1 (11.1)^{Cb}
Meropenem (MEM10 µg)	8 (88.9)^{Aa}	1 (11.1)^{Bb}	None (0)^{Dc}
Ciprofloxacin (CIP5 µg)	7 (77.8)^{Aa}	1 (11.1)^{Bb}	1 (11.1)^{Cb}
Ticarcillin (TIC 75 µg)	1 (11.1)^{Cc}	2 (22.2)^{Bb}	6 (66.7) ^{Aa}
Carbencillin (CAR 100 µg)	1 (11.1)^{Cc}	2 (22.2)^{Bb}	6 (66.7) ^{Aa}

A,B,C,D: Indicate significant differences (æ ^{2}) among selected antibiotics for total isolates (20) vertically at level (P≤0.05).
A,b,c: Indicate significant differences (æ ^{2}) among total isolates (20) for selected antibiotic horizontally at level (P≤0.05).

Biofilm producing isolates detection was another very important and dangerous risk evident in this study because of persistent source of contamination and pollution of environment, peoples, animals, foods, feeds, etc. from these infectious foci and active and passive carriers. Gold standard double staining technique, Microtiter Plate Assay for biofilm formation with sensitive methylene blue and safranin dyes vitally assist in detection of biofilm producing isolates especially from mastitis cases because strong relationship with antibiotic resistant profile and pyocyanin-pyoverdine secretion as indicated in another study of Al-Shammary in Iraq (Al-Shammary, 2015). Production of biofilm or slim exopolysaccharides protect the isolates from harsh conditions during their life cycle and supply new virulent strains in environment. Genetic makeup of these strains may need more interpretation along with the restudy the relationship among environment, milk producing animals and handlers in distribution of these infectious fociin regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya. Risk of biofilm multidrug resistant strains incriminate in difficult to treatment cases of pneumonia, cystic fibrosis and urinary tract infections.

Antibiotic Susceptibility Pattern

Resistance Profile of most biofilm producing isolates were evident for selected antimicrobials and interpreted according to tables of CLSI (2009) as indicated in table (4):

Antibiotic resistant pattern may be partially linked to the ability of isolate to the formation of biofilm and secretion of phenazine pigments (pyocyanin-pyoverdine) due to genetic makeup of these versatile polymorphic colonies of *P. aeruginosa* as noticed in this study and linked to the study of Al-Shammary in Iraq (Al-Shammary, 2015), that show increased secretion of green discoloration with highly fruity odour and more enlarged biofilm colonies in media of CNP in those isolates, partially may be due to the surviving and reviving power of these isolates to resist these harmful situations during culturing on Muller-Hinton agar with antibiotic discs. Most study isolates were resistant to all antibiotics except ticarcillin and carbencillin, this may indicate development of resistance profile especially from biofilm producing mastitis strains. The ability to resist antibiotics may reflect the inelegant strategy and behavior of *P. aeruginosa* to overcome, modulating and buffering environmental and inoculation conditions according to their life cycle *in vivo* and *in vitro*. Risk of biofilm multidrug resistant strains incriminate in difficult to treatment cases of pneumonia, cystic fibrosis and urinary tract infections.

Conclusion and Recommendation

Data revealed contamination of raw milk and infection of dairy mammary glands with biofilm producing and multidrug resistant strains of *P. aeruginosa*from some regions in Baghdad, thus we recommend to monitoring hygienic measurements of milk producing animals and milk production especially milk cans in Iraqi environment and application of up to date diagnostic tools for reducing dangerous foci of *P. aeruginosa* and their transmission as soon as possible with treating of diseased cases carefully, monitoring active and passive carriers, and designing future hazard analysis critical control points strategies to controlling the epidemiological distribution and frequency pattern of *P. aeruginosa*in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya.

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