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Bacteriological and molecular detection of *pseudomonas* species from raw milk sold in Port-Said City markets.

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Abstract

A total of 100 random samples of raw milk were collected from different markets in Port- Said city aseptically for isolation and identification of ***Pseudomonas*** species. The results revealed that the ***Pseudomonas*** species could be detected in a percentage of 6% from raw milk samples. Six strains were identified biochemical as ***Pseudomonas aeruginosa***. This study confirms the rapidity and sensitivity of **16SrDNA** analysis in identifying strains which contribute in early monitoring, accurate analysis and control of microbial risks in food products. The described methodology has special relevance in raw milk quality control and safety. Molecular characterizations of ***P. aeruginosa*** were confirmed using **16SrDNA** of ***Pseudomonas* spp.** and **16SrDNA** of ***P. aeruginosa*** by conventional PCR. Which were developed with specific primers for the detection of different virulence genes (***oprL***, ***toxA, lasI, rhlR, ExoU, ExoS*** and ***ecfX***) of ***P. aeruginosa***, which may be considered a significant in food safety threat. ***P. aeruginosa*** possesses a variety of virulence genes that may contribute to its pathogenicity. Our results showed that ***oprL*** and ***oxA*** genes were detected in all 6 tested strains of ***P. aeruginosa***, while ***lasI*** gene was detected in strains 1,2,3,4. But ***ExoS*** gene was detected only in 1, 2, and 3 strains. On the other hand ***Rhl1*** gene was detected in 1, 3, and 4 tested strains. ***ExoU, rhlR, and ecfX*** genes not detect in any tested strains. PCR method is rapid than other diagnostic methods for the identification of ***P. aeruginosa*** strains, without using additional biochemical tests and It is a useful technique for detection its virulence genes. The public health hazard of this microorganism, as well as recommended measures to improve quality status of raw milk was discussed.

Key words: ***pseudomonas*** species, raw milk, virulence genes, PCR, Public health**.**

INTRODUCTION

Raw milk is widely consumed since ancient times and its market demand is continuous throughout the world. Milk is a significant food of human nutrition owing to its high nutritional value. It is naturally a good medium for growth of microorganisms. Quality control of raw milk is therefore of paramount importance **(Bashir, et al., 2014)**.

Negligence of hygienic condition such as improper cleaning of bulk tank, dirty udders, milking equipments, milk handling technique and improper storage will increase the proportion of Gram-positive and Gram-negative bacteria in the bulk tank milk **(Bonfoh et al., 2003)**. These parameters play an important role in determining the characteristics of milk, which are one of the major sources of protein in a vegetarian's diet.

Bacteria in the family ***Pseudomonadaceae*** are among the most important spoilage bacteria originating in refrigerated raw milk. They are considered psychrotrophs, growing well at common refrigeration temperatures (0-15°C). The main psychrotrophic microflora encountered in raw milk are Gram negative rods with ***Pseudomonas*** spp. **(Vyletelova et al., 2000)** comprising at least 50% of the total bacteria in milk **(Champagne et al., 1994)**.

Pseudomonas species are considered the most important organisms contributing to the milk spoilage through the production of lipolytic and proteolytic enzymes **(Widemann et al., 2000)**. Although Pseudomonas bacteria are killed by pasteurization, ***Pseudomonas*** can also easily contaminate processed milk after pasteurization, thus ***Pseudomonas*** species are the most important bacterial genus responsible for spoilage of pasteurized milk during refrigerated storage **(Smithwell and Kailasapathy 1995)**.

Pseudomonas aeruginosa is an opportunistic pathogen associated with ventilator-acquired pneumonia **(Chastre and Fagon 2002),** acute lower respiratory tract infections in immune-compromised patients, chronic respiratory infections in cystic fibrosis patients **(Mowat et al., 2011)**, catheter- associated urinary tract infections (UTIs) **(Goldberg, 2010)**, skin infections **(Wu et al., 2011)**, wound infections **(Seth et al., 2012)**, and keratitis **(Zhu et al., 2002)**, among others. High incidence, infection severity, and increasing antibiotic resistance characterize ***P. aeruginosa*** infections **(Kipnise et al., 2006)**, highlighting the need for new therapeutic options.

Therefore, detection of ***Pseudomonas*** species is useful to track down the contamination sources and hygienic status of the dairy plants. By controlling the presence and activity of ***Pseudomonas***, it is possible to extend the shelf life of dairy products. The apparent importance of ***Pseudomonas*** for the dairy industry necessitates the development of a rapid method to identify **Pseudomonads**.

The rapid, accurate and reliable identification of spoilage microorganisms through PCR is very important in the efficient monitoring of microbiological qualities, especially in raw and ready-to-eat foods **(Arakawa et al., 2008)**. Molecular approaches, especially those based on the use of rRNA genes (DNA) and related techniques, have provided the opportunity to analyse complex communities on the basis of sequence diversity. Bacterial species can be identified by generating clone libraries of the **16S rDNA** followed by sequencing

and comparison with databases containing thousands of ribosomal sequences to allow a phylogenetic affiliation to cultured, as well as uncultured microorganisms **(Ercolini, 2004)**. Unlike conventional cultivation methods which are time consuming and labour intensive, molecular diagnosis tools being highly powerful, sensitive and rapid are gaining popularity for microbial identification **(Böhme et al., 2014)**.

Identification of ***P. aeruginosa*** has traditionally relied on phenotypic methods. This still is the most accurate standard when dealing with typical isolates of ***P. aeruginosa***. ***P. aeruginosa*** isolates display unusual phenotypic reactions. Moreover, biochemical testing takes long time to perform and requires extensive handson work by the technologist, both for setup and for ongoing evaluation. Molecular methods have been reported to be superior to the phenotypic methods for identification of ***P. aeruginosa*** **Qin et al., (2003)**.

Conventional microbiological methods for identifying ***P. aeruginosa*** from raw milk samples are reliable; they require several days to be completed. Rapid detection of isolates helped to distinguish ***P. aeruginosa*** in raw milk through detection the isolates and its virulence genes. It is not only highly sensitive and specific, but it also provides rapid and reliable results **(Nikbin et al., 2012)**. PCR has the potential for identifying microbial species rapidly by amplification of sequences unique to a particular organism **(Khan and Cerniglia 1994)**.

Detection of ***oprL , lasI, toxA, exoU, Exos, rhlR, rhlI*** **and** ***ecfx*** genes by PCR is recommended for molecular identification of ***P. aeruginosa***. Determination of different virulence genes of ***P. aeruginosa*** isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity. Ribotyping showed that strains with the same genetic patterns of the genes do not necessarily have similar ribotype patterns **(Nikbin et al., 2012)**.

Virulence of ***P. aeruginosa*** is multifactorial and has been attributed to cell-associated factors such as lipopolysaccharide (LPS), flagellum, and pilus and non-pilus adhesins, as well as to exoenzymes or secretory virulence factors, including protease, elastase, phospholipase, pyocyanin, exotoxin A, exoenzyme S, hemolysins, rhamnolipids, and siderophores **(Hentzer et al., 2003)**. Several of these virulence factors, acting alone or synergistically with each other, are believed to cause cell death, severe tissue damage, and necrosis in the human host **(König et al., 1996)**. If the selective pressure from bacteriophage on the host population results in alterations to any of these virulence determinants, change in the virulence of the phage-resistant variants is to be expected **(Friman et al., 2011)**.

This study was planned to :

1-Investigate the presence of ***P. aeruginosa*** among raw milk using bacteriological and biochemical methods. identify the especially ***Pseudomonas*** 2-Applicate conventional PCR depending on **16S rDNA** sequences on the isolates to identify genus- and species- specific ***P. aeruginosa*** those allow the differentiation of ***P. aeruginosa*** from other ***Pseudomonas*** species.

3- Detect some of the virulence genes responsible for initiation of pathogenesis of ***P. aeruginosa*** **(*oprL , lasI, toxA, exoU, Exos, rhlR, rhlI*** and ***ecfx*** **)** using PCR as rapid, sensitive, powerful and accurate method.

MATERIAL and METHODS

1-Collection of Samples: One hundred random samples of raw milk were collected randomly from different milk markets in Port- Said city aseptically for isolation and identification of ***Pseudomonas*** species. All collected samples were transported in an ice box to be examined bacteriological for ***Pseudomonas*** isolation.

2- Preparation of the samples (APHA, 2004): Accurately 25 ml of examined raw milk were transferred to a sterile polyethylene bag and 225 ml of 0.1% sterile buffered peptone water in a stomacher at 2000 rpm for 1-2 minutes to provide a homogenate incubated at 37°C for 24 hrs.

3- Microbiological analysis:

3-1 Isolation and Identification of *Pseudomonas* species: The milk samples were plated onto ***Pseudomonas*** agar plates at 37°C for 24 hr. The suspected colonies were purified and cultured onto nutrient agar slopes and incubated at 37°C for 24 hrs. The purified colonies were subjected for further identification either morphologically and biochemically according to **Kreig and Holt (1984)**.

3-2 Morphological characteristics: The smear was prepared from the isolated culture on clean microscopic glass slide and stained with Gram's stain.

3-3 Biochemical examination: Biochemical tests were performed to confirm ***Pseudomonas*** species using motility, methyl-red and Voges – Proskauer tests, Oxidase, Catalase, hydrolysis of casein, citrate utilization, nitrate reduction, Indole production, gelatin hydrolysis, and hydrolysis of polysaccharides and fermentation of various sugars **(Holtz et al., 2000)**.

4- Molecular examination of *Pseudomonas aeruginosa* : The ***Pseudomonas*** species isolated from raw milk samples were conferment by PCR using **(16SrDNA** of ***Pseudomonas*** **spp.)** and **(16SrDNA** of ***P. aeruginosa*****)**, also determining some virulence genes of it using specific primers (***oprL***, ***toxA, lasI, rhlR, ExoU, ExoS*** and ***ecfX***) of ***P. aeruginosa*** **(Spilker et a., 2004 and Nikbin et al., 2012)**.

4.1. DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at $56^{O}C$ for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

4.2. Oligonucleotide Primers:

Primers used were synthesized in the Reference lab. For veterinary quality control on poultry production **(Egypt)** and are listed in table (1) & (2).

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondar denaturation	Annealing	Extension		
***Pseudomonas* spp.** 16SrDNA	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	94˚C 10 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Spilker *et al*., 2004**
P. aeruginosa 16SrDNA	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	94˚C 10 min.	94˚C 1 min.	52˚C 1 min.	72˚C 1 min.	72˚C 12 min.	
ToxA	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396	94˚C 10 min.	94˚C 45 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Matar *et al*., 2002**
ExoS	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	118	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Winstanley et al., 2005**
ExoU	CCGTTGTGGTGCCGTTGAAG CCAGATGTTCACCGACTCGC	134	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
EcfX	ATGGATGAGCGCTTCCGTG TCATCCTTCGCCTCCCTG	528	94˚C 10 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Choi et al., 2013**
LasI	ATGATCGTACAAATTGGTCGGC GTCATGAAACCGCCAGTCG	606	94˚C 10 min.	94˚C 45 sec.	52˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Bratu et al., 2006**
Rhl1	CTTGGTCATGATCGAATTGCTC ACGGCTGACGACCTCACAC	625	94˚C 10 min.	94˚C 45 sec.	53˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	
RhlR	GACCAGGAGTTCGACCAGTT GGTAGGCGAAGACTTCCTTG	132	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
oprL	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504	94˚C 10 min.	94˚C 45 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Xu et al., 2004**

Table (2): Virulence genes of *P. aeruginosa* and function for conventional PCR.

Gene	Function
toxA	Exotoxin A (ETA).
lasI	required for transcription of the genes for elastase (lasB) and LasA protease (lasA), alkaline protease gene (apr), three proteases associated with virulence. The *las* system consists of the transcriptional activator.
RhLI and rhlR	The *rhl* system consists of the transcriptional activator, RhlR and of an autoinducer synthase, RhlI. RhlI directs the synthesis of N-butyoyl-1-homoserine lactone (PAI-2). PAI-2 binds to RhlR and this complex activates the transcription of *rhlI, rhlA and rhlB*, an operon coding for rhamnosyltransferase, which is required for rhamnolipid production (hemolysin).
ExoU and ExoS	Those genes are translated into protein products (toxin) related to type III secretion systems (TTSS). TTSS are sensory probe to detect the presence of eukaryotic organisms and secrete proteins that help the bacteria infect them. The secreted effector proteins are secreted directly from the bacterial cell into the eukaryotic (host) cell, where they exert a number of effects that help the pathogen to survive and to escape an immune response.
ecfX	ecfX encodes an ECF (extracytoplasmic function) sigma factor which is restricted to ***P. aeruginosa***, and might play a role in haem-uptake and virulence.
OPrL	Lipoprotein in outer membrane for detection species of ***P. aeruginosa***.

4.3. PCR amplification: DNA samples. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of

DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

4.4. Analysis of the PCR Products: Conventional PCR products. The products of PCR were separated by electrophoresis on 1.5% agarose gel stain with Athidium Promide (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A 100 bp and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Results and discussion

1-Isolation of *Pseudomonas* species from raw milk samples***:***

Six isolates of ***Pseudomonas*** species were isolated from 100 raw cow's milk in a percentage of (6%). The colonies were cultured on ***Pseudomonas*** Agar plates. ***Pseudomonas*** colonies appeared fluorescent yellow or green color on the ***Pseudomonas*** Agar plates (fig. 1).

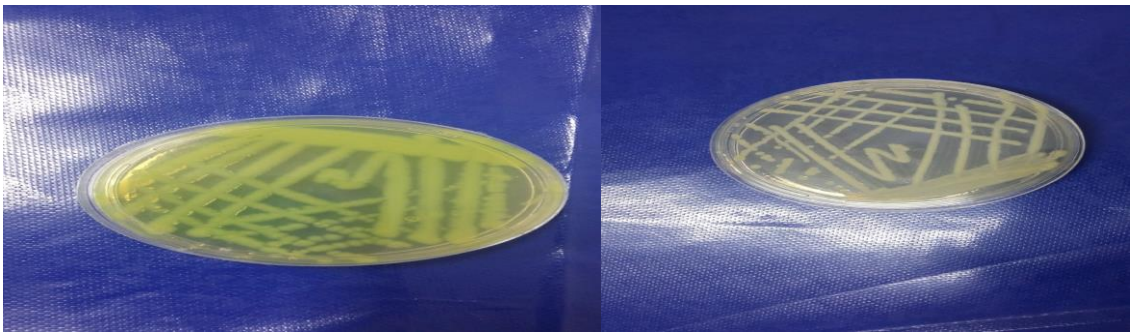

Figure 1: *Pseudomonas* strains cultured on ***Pseudomonas*** Agar plates**.**

2-Biochemical characterization: The 6 isolates were subject for further confirmation by Gram's Stain. The isolated ***Pseudomonas*** strains were biochemically characterized by IMViC. All the isolates, showed positive to Citrate utilization and Catalse test. All the isolates were found negative to Indole test, Methyl red test and VP test. All the isolates were identified as ***Pseudomonas aeruginosa***.

3-Molecular characterization of *Pseudomonas* species**:**

Table (3): Molecular characterization and some virulence genes present in *Pseudomonas aeruginosa*.

Strains	Results									
	***Pseudomonas* 16SrDNA**	***P. aeruginosa* 16SrDNA**	**oprL**	**lasI**	**toxA**	**ExoU**	**ExoS**	**Rhl1**	**rhlR**	**ecfX**
1	+	+	+	+	+	-	+	+	-	-
2	+	+	+	+	+	-	+	-	-	-
3	+	+	+	+	+	-	+	+	-	-
4	+	+	+	+	+	-	-	+	-	-
5	+	+	+	-	+	-	-	-	-	-
6	+	+	+	-	+	-	-	-	-	-

***Pseudomonas* spp. 16SrDNA**								L	***P. aeruginosa* 16SrDNA**							
Neg	6	5	4	3	2	1	Pos		Pos	1	2	3	4	5	6	Neg

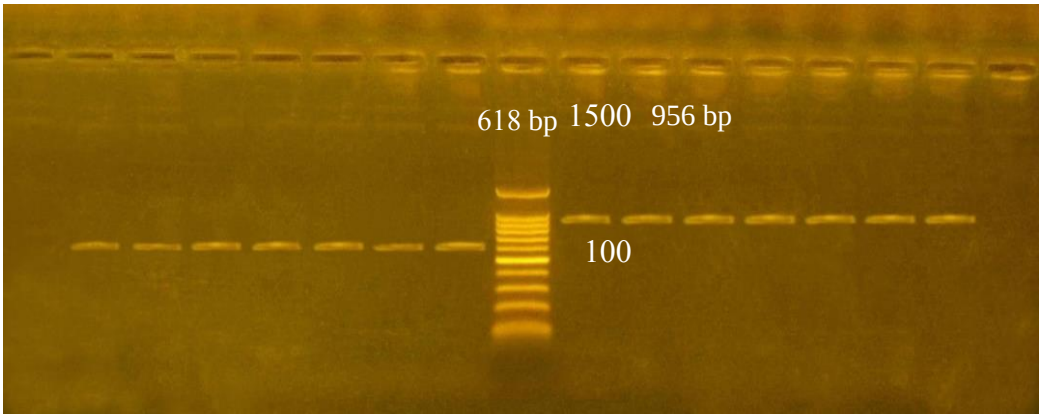

Figure 2: Agarose gel electrophoresis of PCR products after amplification of:
1- ***Pseudomonas*** spp. **16SrDNA** gene. MWM-molecular weight marker (100 – 1500 bp DNA ladder), + control (Positive, Negative) + different strains of ***Pseudomonas* spp.** (***Pseudomonas* spp. 16SrDNA** gene products at 618 bp).
2- ***P. aeruginosa* 16SrDNA** gene (***P. aeruginosa* 16SrDNA** gene products at 956 bp).

toxA								L	LasI							
Neg	6	5	4	3	2	1	Pos		Pos	1	2	3	4	5	6	Neg

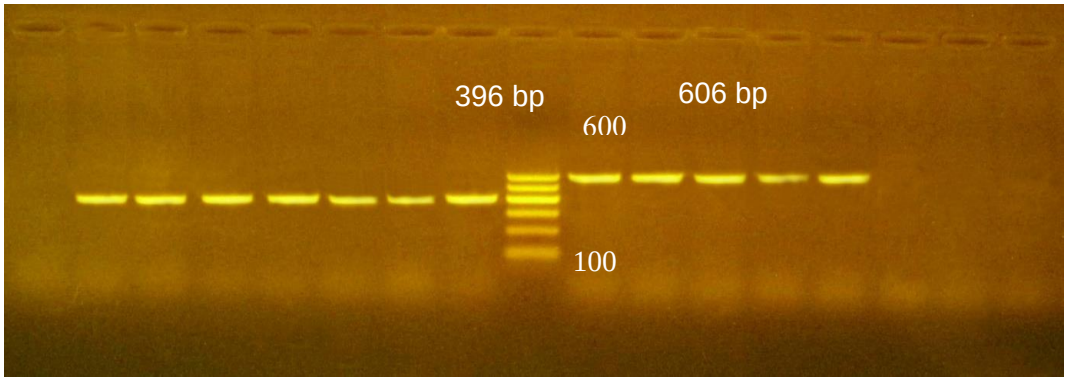

Figure 3: Agarose gel electrophoresis of PCR products after amplification of:
1- toxA gene. MWM-molecular weight marker (100 – 600 bp DNA ladder), + control (Positive, Negative) + different strains of ***P. aeruginosa*** (**toxA** gene products at 396 bp).
2- lasI gene (**lasI** gene products at 295 bp)

exoS								L	exoU							
Neg	6	5	4	3	2	1	Pos		Pos	1	2	3	4	5	6	Neg

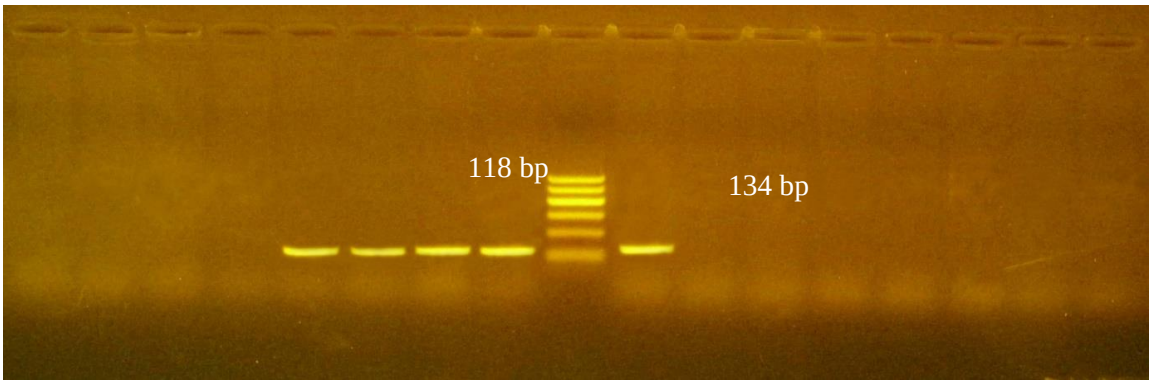

Figure 4: Agarose gel electrophoresis of PCR products after amplification of:
1- exoS gene. MWM-molecular weight marker (100 – 600 bp DNA ladder), + control (Positive, Negative) + different strains of ***P. aeruginosa*** (**exoS** gene products at 118 bp).
2- exoU gene (**exoU** gene products at 134 bp)

Rhl1								L	**rhlR**							
Neg	6	5	4	3	2	1	Pos		Pos	1	2	3	4	5	6	Neg

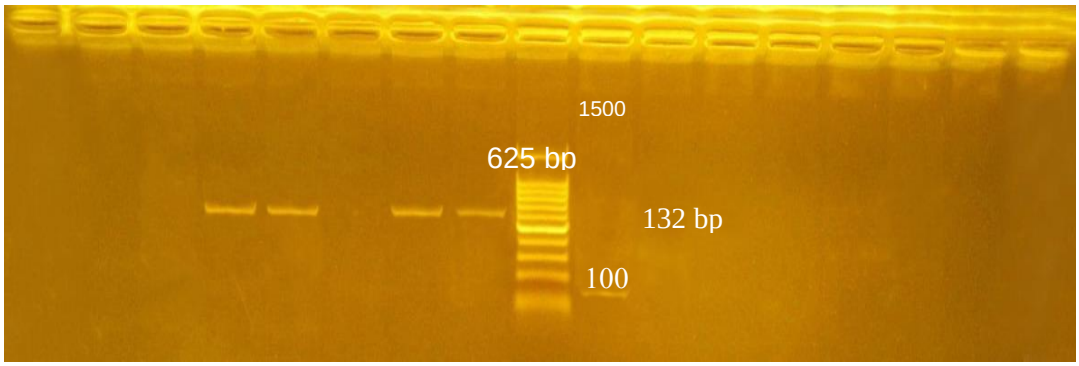

Figure 5: Agarose gel electrophoresis of PCR products after amplification of:
1- Rhl1 gene. MWM-molecular weight marker (100 – 1500 bp DNA ladder), + control (Positive, Negative) + different strains of ***P. aeruginosa*** (**Rhl1** gene products at 625 bp).
2- rhlR gene (**rhlR** gene products at 132 bp).

oprL								L	**ecfX**							
Neg	6	5	4	3	2	1	Pos		Pos	1	2	3	4	5	6	Neg

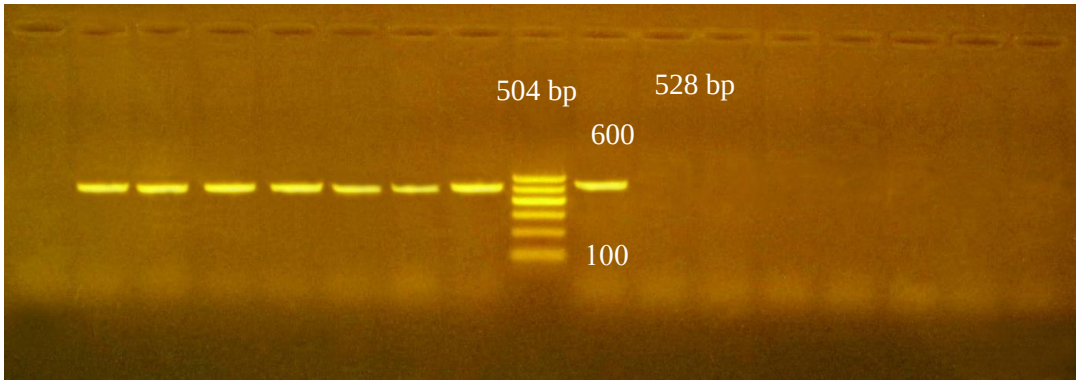

Figure 6: Agarose gel electrophoresis of PCR products after amplification of:
1- oprL gene. MWM-molecular weight marker (100 – 600 bp DNA ladder), + control (Positive, Negative) + different strains of ***P. aeruginosa*** (**oprL** gene products at 504 bp).
2- ecfX gene (**ecfX** gene products at 528 bp).

Microbial analysis of raw milk has a critical role to evaluate its quality, promoting public health safety **(Nicolaon and Goodacre, 2008).** Recent outbreaks of food-borne illnesses associated with raw milk consumption have been found to be contaminated with pathogenic microorganisms such as ***Pseudomonas*** spp. **(Nawaz and Bhattarai 2015)**.

Milk contaminated with disease-causing bacteria does not smell or look any different from non-contaminated milk, and there is no obvious way for the consumer to know if the milk is contaminated **(Julia, 2010)**.The present work was made in order to evaluate the prevalence of ***Pseudomonas*** species among raw milk, also to determine the virulence genes characteristics of ***P. aerugenosa*** using conventional PCR.

So, a total of 100 samples of raw milk were examined for presence of ***Pseudomonas*** species. The percentage of ***Pseudomonas*** species was 6 % (6 isolates). All the six isolates by biochemical identification were identified as ***P. aerugenosa***. These results were nearly similar to those reported by **El- Zubeir and El- Owni (2009)** who isolated ***P. aerugenosa*** from raw milk samples in proportion of 6.6%. However, the current results were less than those recorded by **Jyoti et al., (2014) and Aylin et al., (2012)** they showed that ***P. aerugenosa*** was isolated from 9.5%, 11.11% and 34.6% of the raw milk samples and higher than those found by **Hussein (2008)** who isolated ***P. aerugenosa*** in a percentage of 3.7% from raw milk samples. The difference between our results and the previous studies may be attributed to sampling techniques, sources of sampling, handling of samples and types of media. Bacterial identification was conducted based on morphological and biochemical tests **Holtz et al., (2000)** and **MacFaddin, (2000).**

Molecular characterizations of ***P. aeruginosa*** were confirmed by using **(16SrDNA** of ***Pseudomonas*** **spp.)** and **(16SrDNA** of ***P. aeruginosa*****)** by conventional PCR. The results proved that the isolates were ***P. aeruginosa*** table (3) and figure (2)**.**

PCR assays were developed with specific primers for the detection of different virulence genes (***oprL***, ***toxA, lasI, rhlR, ExoU, ExoS*** and ***ecfX***) of ***P. aeruginosa*** , which may be considered a significant in food safety threat. ***P. aeruginosa*** possesses a variety of virulence factors that may contribute to its pathogenicity. Our results showed that ***oprL and toxA*** genes were detected in all 6 tested strains of
P. aeruginosa , while ***lasI*** genes detected in strains 1,2,3,4. But ***ExoS*** genes detected only 1, 2, and 3 isolates. On the other hand ***Rhl1*** genes detected in 1, 3,

and 4 tested isolates. ***ExoU, rhlR, and ecfX*** not detect in any strains [table (3) and figure (3, 4, 5 and 6)]. The differences in the distributions of virulence factor genes in the populations strengthen the probability that some ***P. aeruginosa*** strains are better adapted to the specific conditions found in specific infectious sites **(Lanotte et al., 2004)** that may returned to the different environmental and geographical sources.

Conclusion:

In conclusion, identification and determination of the ***P. aeruginosa*** by conventional methods requires long period, whereas the detection of ***P. aeruginosa*** and different virulence genes by Conventional PCR assay can be done within a few hours. PCR seems that simultaneous use of specific primers as general **(16SrDNA** of ***Pseudomonas* spp.)**, specific **(16SrDNA** of ***P. aeruginosa*)** and different virulence factors genes as **(*oprL*, *toxA, lasI, rhlR, ExoU, ExoS*** and *ecfX*) of ***P. aeruginosa*** provides more confident detection of ***P. aeruginosa***. The differences in the distributions of virulence factor genes in the isolated strains need further studies for finding out the actual role of these genes of ***P. aeruginosa*** from different sources. PCR showed that all ***P. aeruginosa*** strains do not necessarily have similar virulence genes.

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