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Identification and Genetic Analysis of *Pseudomonas fluorescence* in Milk and Milk Products in Mosul City

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Abstract

THE CURRENT study is aimed to study the prevalence of *P. flouresence* in milk and its products of cows and ewes at Mosul city. Fifty samples from bovine and ovine dairy products were collected from outdoor public markets in Mosul city. The samples included 20 samples from each of raw milk and cheese from both of cows and ewes and 10 yoghurt samples. *P.flouresence* were identified using 16*SrRNA* gene by conventional polymerase chain reaction technique. In order to identify the similarities and differences between the Iraqi strains and the currently recognized worldwide strains, for the sequenced *16srRNA* was examined using BLAST analysis. Results revealed the presence of *P.flouresence in (70%) and (60%) of cows' raw milk and cheese respectively whereas (20%) and (10%) of ewes' raw milk and cheese were positive for P.flouresence* . All yoghurt samples were negative for the recovery of *P.flouresence in cows and ewes. Three isolates of P.flouresence from cows' raw milk were recorded in Genebank with accession number LC745963, LC745964 and LC745965 respectively. According to* phylogenetic tree the local strains were similar to strains recorded in United Kingdom (FM872377.1).

Keywords: *16SrRNA,* Milk, cheese, *Pseudomonas flouresence*, PCR.

Introduction

The characteristics of milk components allow the production of premium dairy products as cheeses, yoghurts [1]. In order to minimize bacterial growth during storage it is crucial to keep the milk products at low temperatures during production [2]. Usually, milk is kept at low temperatures for 2 to 5 days before being heated [3,4]. Milk is considered as an ideal medium for bacterial growth, although milk is stored at low temperatures, proteolytic enzymes generated by Pseudomonas species cause milk deteriorations .Milk has been found to contain psychrotrophic bacteria from a variety of genera, including Pseudomonas, Aeromonas, Acinetobacter, Alcaligenes, Achromobacter, Enterobacter, Flavobacterium, Klebsiella, Bacillus, Clostridium, Corynebacterium, Microbacterium, Micrococcus, and Coliforms such as Escherichia, Enterobacter, Citrobacter and Mannheimia [5,6]. Microbial counts, particularly those of the psychrotrophic microbiota, can rapidly rise, resulting in low quality of milk and its dairy products [7]. The most common milk-associated psychrotrophic bacteria have been identified as Pseudomonas [8]. Milk and fresh dairy products are the best substrates for pseudomonas growth because of their high nutritious ingredient richness, water content, and pH neutrality [9]. Pseudomonas comprise a serious worry for fresh dairy products that are stored in the cold. Less than 10% of the entire microflora in fresh milk is made up of pseudomonas *spp*., included *P. fluorescens, P.gessardii, P. fragi, and P.lundensis* which are the most frequently found ones in milk and milk products [10]. Due to their metabolic adaptability and diversity, they cause non-visible defects such as protein breakdown, off-odors, and off-flavors as well as obvious spoilage features like discolorations, structure loss, and rheology changes, all of which lead to reduction in shelf-life of dairy products and poor quality [11]. The main sources of contamination are surfaces, water, and obviously exposure to air because these environmental microbes are typically found in soil, the contamination of dairy products by pseudomonads happens at every stage of production, and when bacterial cells form a biofilm, it becomes more tenacious and difficult to remove, the majority of them are regulated by quorum sensing (QS), which raises the possibility that it contributes to dairy spoiling [12,13] even though they become inactive following pasteurization and sterilization of milk. The extracellular proteases produced by these bacteria are highly thermostable and keep their ability to break down milk proteins [14]. The proteases produced in the milk cause

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casein degradation and the liberated plasminogen increase proteolysis leading to bad flavor [15]. The current study's objectives were to identify *Pseudomonas spp.* from some bovine and ovine milk and milk products. Numerous investigations found that the proteolytic varied widely due to different gene expression, capacity of milk-associated Pseudomonas species *P. fluorescens* exhibited mainly high proteolytic activity, [16,17]. Pseudomonas has the ability to produce pigments such as pyoverdin which can mediate the activities of proteases and lipases through spoilage [18]. Free amino acids are also the starting point for the production of biogenic amines (BAs, such as, histamine, cadaverine, and putrescine) by pseudomonads that can adversely affect the sensory qualities of dairy products during storage [19]. The activity of lipases can seriously impair the quality and shelf life of dairy products, particularly raw milk and those with high fat contents when they are stored at low temperatures associated with rancid flavour and odours [20]. Milk lipids and triglycerides can react to release short-chain fatty acids, keto and hydroxy acids, which can leave fluid milk tasting unpleasant [21]. Lecithinases have the ability to damage the membranes of milk fat globules, making milk fat more vulnerable to the action of lipases [22]. The likelihood for deterioration of raw milk samples before processing cannot be predicted via laborious culturing on selective media. *Pseudomonas spp*. were verified by examining a genus-specific portion of the *16S DNA* region as mentioned by [23].The innovative multiplex qPCR assay offers a precise and efficient method for determining the total Pseudomonas counts in raw milk as well as for differentiating between the most common Pseudomonas species with various proteolytic potency [24].The current work is designed to investigate the prevalence of *P.flouresence* in some milk and milk products of cows and ewes at Mosul city.

Material and methods

Sampling

The study was carried out in Mosul city/ Iraq**.** A total of fifty samples of milk and milk products were included in the current study , 20 samples of raw milk and 20 samples of cheese from cows and ewes while the study included 10 samples of yoghurt .All samples were collected using sterile container from Mosul city during the period from 1 November 2022 till March 2023, gathered randomly from the outdoor public market and transported chilled to the Veterinary Public Health Laboratory, College of Veterinary Medicine, University of Mosul for further analysis

Bacterial isolation

Ten milliliters from milk were collected and enriched on nutrient broth (Neogen /USA) overnight, then plated on Cetrimide agar (Neogen/ USA) The plates were incubated for 48-72 hours at 25°C. After that pure cultures were achieved by subculture on pseudomonas Centrimide agar and incubated for 48 hours at 25°C followed by Gram staining [25]. Biochemical tests were performed to identify *P. flouresence* isolates using VITEK® 2 Gram-negative identification cards (bioMerieux Inc, France).

Molecular detection

DNA extraction

Colonies were selected and cultured in brain heart infusion broth (Himedia. Indian) for 24 hrs at 25°C for DNA extraction. According to the manufacturer instructions (Geneaid, Germany) colonies were suspended in 1.5 ml microcentrifuge tubes. After centrifugation the bacterial sediment used to extract genomic DNA. The purity of the DNA was measured using a Nano-Drop (Thermo Fisher Scientific™ USA). DNA samples were stored at -20°C for PCR assay.

Conventional PCR

Identification of *Pseudomonas spp.* Was confirmed using PCR amplification of the *16SrRNA* using the primer listed in (Table 1). PCR mixture was 20 µL which contained 1 µL forward primer, 1 µL reverse primer, 10 µL master mix,4 µL DNA template and 4 µL of PCR grade water. The DNA were amplified using thermocycler program ((Optimus/USA) including Initial denaturation at 95°C for 6 min, 35 cycles of denaturation at 95°C for 45 s then annealing at 56°C for 1min. followed by extension at 72°C for 1min., and a final extension at 72°C for 5 min. The product run on 1% agarose gel (Promega, USA) stained with safe dye ((GeNet Bio, Korea) then electrophoresed in 50 volts 300 mA for 1.5 hr by UV Trans illumination (Gel doc EZ gel documentation system) provided by (Biometra/USA) DNA ladder(100bp) (Biolaps/England).

TABLE 1. Primer used for amplification of *16S* rRNA gene.

Gene	Primer	Sequences (5′-3′)	Amplicon size	Reference
16S rRNA	Forward	TGCATTCAAAACTGACT	1200bp	(25)
	Reverse	AATCACACCGTGGTAACC		

Bioinformatics

Sequencing and phylogenetic tree

All positive samples of P.fluorescens strains base on conventional PCR amplicons were submitted to (Immunogene Center, North Korea for sequencing). Sequences of the DNA were analyzed by using Blast analysis against Pseudomonas GenBank sequences by using NCBI Blast (BLASTn) from NCBI (https://www.ncbi.nlm.nih.gov/genbank/). In addition to similarity analysis of sequences within and between our obtained sequences were preform using online multiple sequences alignment-CLUSTALW (GenomeNet) software program (https://www.genome.jp/tools-bin/clustalw) then phylogenic tree was generated ClustalX (NCBI) software programs (https://www.genome.jp/tools-bin/clustalw),[26].

Results

Depending on molecular detection by conventional PCR the results revealed that out of 50 samples 16(32%) were positive for *P.flouresence* distributed between 13(81.25%) and 3(18.75%) for cows and ewes' dairy products respectively (Figure 1).

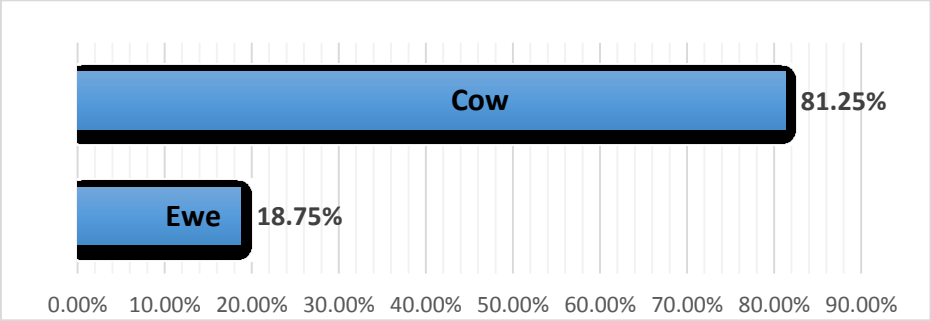

Fig. 1. Total recovery rate of *P. flouresence* from milk and cheese of cows and ewes

The recovery rate of *P.flouresence* were (70%) from cows' milk and (60%) from cheese compared to (20%) from ewes' milk and (10%) from ewes' cheese while all yoghurt samples revealed negative results for the presence of *P.flouresence* (Figure 2 & Table 2).

TABLE 2. Recovery rate of *P.flouresence* from milk and milk products of cows and ewes

Type of sample	Cows			Ewes		
	No. of samples	No. of +Ve	%	No. of samples	No. of +Ve	%
Milk	10	7	70	10	2	20
Yoghurt	5	0	0	5	0	0
Cheese	10	6	60	10	1	10
Total	25	13	52	25	3	12

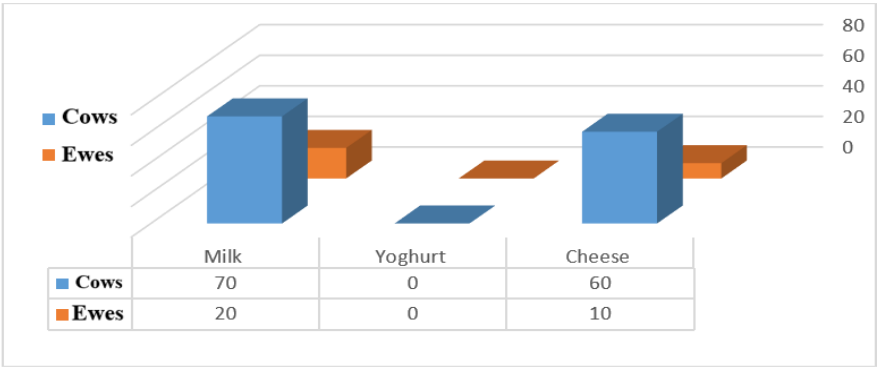

Fig. 2. Recovery rate of *P.flouresence* from milk and milk products of cows and ewes

By using PCR assay the *16SrRNA* gene of *P.flouresence* were detected producing product size was 1200 bp as shown in Figure (3).

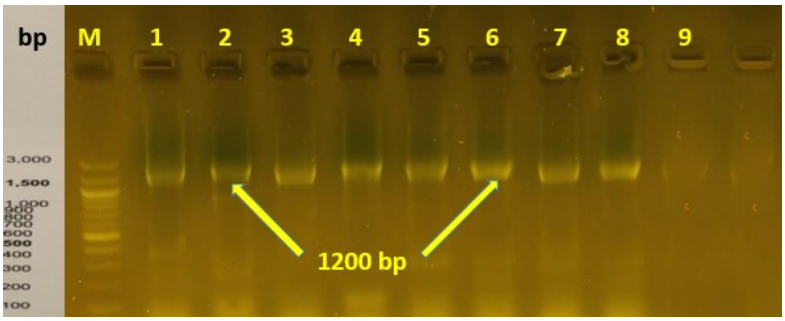

Fig. 3. Amplification of *16SrRNA* gene of *P.flouresence* Lanes M: DNA marker; Lane 1-8, *16SrRNA* gene of *P.flouresence* at 1200 bp product size;Lane 9: negative control.

DNA sequencing

The sequences of *16S rRNA* gene of *P.flouresence* isolated from cows' milk compared with (NCBI) GenBank data through BLAST program identified the following Accession numbers (LC745963.1, LC745964.1 and LC745965.1) were matched and affirmed as *P.flouresence* with 100% identity.

The study also, revealed that the homology between isolates of all *P.flouresence* strain sequences obtained from different samples and GenBank database demonstrated that *P.flouresence* strains LC745963.1 (n=5), *P.flouresence* strains LC745964.1 (n=4) and *P.flouresence* strains LC745965.1 (n=7) were highly related 100%, 100% and 99.31% identity respectively, with highly identity, ranged between 98% and 97% to those sequences previously GeneBank submitted sequences of *P.fluorescence* bv. strain LMG 1794T (FM872377.1) in United Kingdom (Figure 4, Tables 3 and 4).

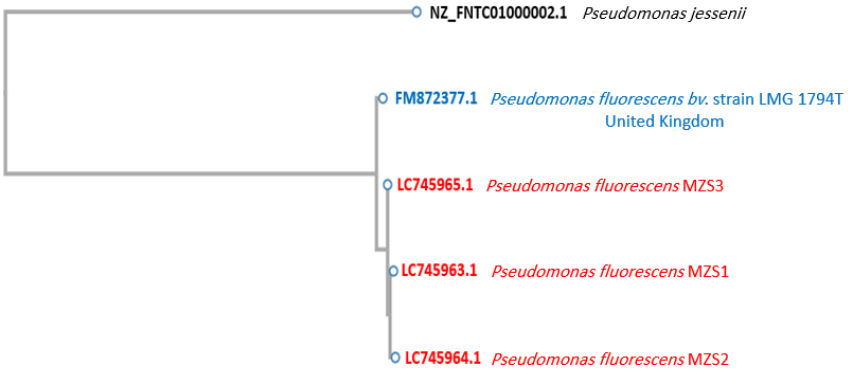

Fig. 4. Phylogenetic tree of *P.fluorescens* isolates with partial sequences of *16S rRNA* gene (Study strains, United Kingdom strain, Outer group strain).

TABLE 3. Similarity within and between *P.fluorescens* isolates strains using multiple sequence alignment-CLUSTALW

Category	Strains	No. of Samples	Similarity (%)
Current study	LC745963.1	5	100%
	LC745964.1	4	100%
	LC745965.1	7	99.31%
Reference strains	LC745963.1: LC745964.1: LC745965.1	5:4:7	98.48%

TABLE 4. Homology and identity between obtained sequences of *P. fluorescens* isolates strains and GeneBank data

Accession number	GeneBank-NCBI number	Query cover	Identity	Gap
LC745963.1	FM872377.1	98%	(497/499)99.32%	0/499(0%)
LC745964.1	FM872377.1	98%	(550/551)98.65%	0/511(0%)
LC745965.1	FM872377.1	97%	(475/488)96.93%	0/488(0%)

Discussion

The dairy industry has experienced a marked increase in the defects and deterioration of raw milk caused by psychrotrophic bacteria, pseudomonas species predominate in prolonged chilling of milk due to its very short generation time compared to other microorganisms [27]. *P.fluorescens* accounted for over 55.6% of the microorganisms found in raw milk [28]. This study findings revealed that the level of contamination with *P.flouresence* in total analyzed dairy products in Mosul city were 32% which is higher than the rate registered in previous studies. The prevalence of bacterial isolates were found in (70%) and (20%) of cows raw milk and ewes milk samples respectively these results are similar to the recovery rate of *P.flouresence* obtained from milk by [29] In Basrah city (33.3%) , it's in agreement to a lesser extent with [30] while *P.flouresence* is recorded with a ratio of 42.7 % from raw milk in china [31] and 23.1% from Raw milk in southern Brazil, [32]. Differences in isolation rate may be attributed to that cow's milk has high lactoperoxidase, but low lactoferrin and lysosome indicated their low content of antimicrobial factors [33] as well as their diverse genetic makeup and high metabolic rate which enables Pseudomonas spp. to survive in a range of environments, including soil, water, and air therefore pseudomonas able to live on the machinery employed in the dairy production chain, including pipelines, bulk tanks, milking machines, and the environment where animals are raised [34,35]. For this reason the presence of *P.flouresence* in milk used as indication of milk contamination during milking process [36,37]. Production of protease by *P.flouresence* facilitate the utilization of amino acids as a source of energy for their growth competing other microorganisms present in milk especially enteric ones [38].

[39] and [40] found that (45%) and (22.9%) of cheese samples were contaminated with *P.flouresence* respectively. Higher incidence was reported by [41] who found (70%) of cheese in Spain. High percentage of *P.flouresence* in cheese may be due to inadequate pasteurization of milk and explain the source of environmental contamination especially water used in cheese preparing process therefore after processing, these spoilage organisms most frequently contaminate products [42]. Various preservation methods, such as the direct addition of the preservative to the food product composition or the packaging material, have been suggested to prevent the growth of spoilage microorganisms [43]. Variations in geographical locations and techniques for isolation participate as other factors affecting the level of contamination of milk and cheese with *P.flouresence* whereas the development of molecular methods [44]. The risk associated with a lack of clearly defined and regulated sanitization measures is demonstrated by the pseudomonas fast environmental proliferation as biofilm communities [45]. When biofilm develops, pseudomonas bacteria are more challenging to get rid of through routine hygiene measures for surfaces that come into contact with raw milk [46,47]. Therefore, Pseudomonas spp. may be considered a significant indicator to monitor the plant environment [48] in order to apply the corrective measures to avoid economic losses.

Interestingly in our results demonstrated that *P.flouresence* strains LC745963.1, *P.flouresence* strains LC745964.1 and *P.flouresence* strains LC745965.1 were highly related with *P.fluorescens* bv. strain LMG 1794T (FM872377.1) in United Kingdom at identity rate of 98%, 98% and 97% respectively that are proven by the results of homology and phylogenetic analysis [49]. This result is inconsistent with previous studies in other countries which indicated the geographical location plays an important role in the relationship between living organisms, as the genetic types that are from similar or neighboring areas are closely related [50]. Results like this could be used in designing primers for the diagnosis of *P.flouresence* and vaccine production.

Conclusion

The importance of *P.fluorescens* is relevant to both public health and the food industry. Milk is often kept at certain temperatures. Pseudomonas spp. can develop and engage in proteolytic activity in raw milk at this temperature. It is necessary to acquire more information on Pseudomonas spp. with proteolytic activity and to develop sensitive and efficient tools to monitor for the presence of peptidases in raw milk. To prevent fresh dairy products from spoiling while being stored at low temperatures, new and sustainable solutions are required to monitoring milk quality. The detection of *P.fluorescens* and the identification of their protease activity in raw milk is made easier by the sensitivity of PCR. Also, every step in the milk processing chain must be under control to obtained safe and high-quality dairy products.

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Conflict of interest

The authors declare that there is no conflict of interest.

Funding

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Ethical approval

Not needed for this study.

Authors contributions

The study was designed, supervised and the manuscript was written by Prof. Dr. Muntaha Ghazi Hassan and Prof. Dr. Dhyaa Mohammad Taher Jwher. Zainab Qutaiba Ibrahim and Safa Bassam Yehyia participated in the work by sampling, bacterial isolation, molecular detection and analyzing the data.

Data availability

All data supporting the findings

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التشخيص والتحليل الوراثي لجراثيم الزوائف المتألقة في الحليب ومنتجاته في مدينة الموصل

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هدفت الدراسة الحالية الى الكشف عن مدى تواجد جراثيم الزوائف المتألقة في الحليب الخام واللبن والجبن لكل من الأبقار والأغنام في بعض مناطق مدينة الموصل حيث جمعت 50عينة توزعت بواقع 10عينات لكل من حليب وجبن الأبقار والأغنام و 10عينات من اللبن بواقع خمسة عينات لكل من لبن الأبقار والأغنام من مناطق مختلفة. شخصت الزوائف المتألقة بالإعتماد على الخصائص المظهرية بالإضافة الى التشخيص الجزيئي بتقنية تفاعل البلمرة المتسلسل للكشف عن وجود الجين 16S rRNA. أظهرت نتائج الدراسة وجود جراثيم الزوائف المتألقة في منتجات الأبقار من الحليب والجبن بنسبة (32%) وفي منتجات الأغنام من الحليب والجبن بنسبة (12%) وكانت أعلى نسبة لعزل الزوائف المتألقة في حليب الابقار وبنسبة (70%) وهي أعلى مقارنة بحليب الأغنام (20%) كما وتم عزل جراثيم الزوائف المتألقة من عينات جبن الأبقار والأغنام وبنسبة (60%) و (10%)على التوالي بينما أعطت عينات اللبن لكل من الأبقار والأغنام نتيجة سالبة لعزل جراثيم الزوائف المتألقة ،أظهرت نتائج التحليل التتابعي للجين 16S rRNA تطابق بنسبة (100%) مع تسلسل الجينات المثبتة في قاعدة بيانات (BLAST) والموجودة في الموقع الإلكتروني للمركز الوطني للمعلومات (NCBI) ،وقد تم تسجيل ثلاث عزلات جرثومية من حليب الأبقار الأولى بإسمMZS1 و MZS2 و MZS3 وحصلت العزلات الثلاثة على الرقم المعرف بالتسجيل(LC 745963.1) و (LC745964.1) و (LC745965.1) على التوالي ومن خلال تحليل شجرة النشوء الجيني لهذه العزلات مع العزلات العالمية المسجلة في بنك الجينات لوحظ تقاربها مع العزلات المسجلة في المملكة المتحدة والمسجلة في المركز الوطني للجينات (FM872377.1)