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Monitoring the prevalence of *Pseudomonas fluorescens* as a spoilage indicator in cow raw milk, teat surfaces, and milk tanks

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ABSTRACT

Background: Milk and its products are very sensitive to spoilage if they are kept under unsuitable conditions which may provide favorable circumstances for the growth of specific spoilage organisms, *Pseudomonas fluorescens* accounted as the most dominant indicator for milk spoilage.

Aim: This study highlights monitoring the prevalence of *P. fluorescens* as a spoilage indicator organism in cow raw milk and its contact surfaces represented by teat surfaces and milk tanks in Nineveh province.

Methods: A total of 150 samples from cows' raw milk, teat surfaces, and milk tank swabs were collected from different locations in Nineveh province from October 2023 till February 2024. The *Pseudomonas fluorescens* were detected by using conventional cultivation methods supported by molecular detection of the target pathogen using the polymerase chain reaction technique.

Results: Out of 150 samples, 48 (32%) were positive for the prevalence of *P. fluorescens* by traditional methods, and 39 (26%) were positive using PCR assay according to the *16SPflu* gene yielded a band at 850 bp. The *P. fluorescens* was recovered at 19 (38%) from raw milk. Teat surfaces revealed a higher isolation rate 11 (22%) compared to milk tanks 9 (18%). The mean counts of *Pseudomonas* in cows raw milk revealed 4.38, 6.29, and 7.37 log CFU/ml for the 0, 3, and 6 days of storage at chilling temperature. Results of DNA sequencing of the *16SrRNA* gene revealed 12 strains recorded in the GenBank nucleotide sequence database.

Conclusion: Our results shed light on the risk of *P. fluorescens* prevalence as a spoilage indicator in raw milk and surrounding surfaces which is inevitable to apply hygienic procedures during milk collecting, processing, and preservation to increase the shelf life of the products and ensure milk safety and consumer health.

Keywords: *Pseudomonas fluorescens,* milk spoilage, teat surfaces, milk tanks.

Introduction

Milk has a valuable nutritive value to all ages due to its beneficial nutrients of proteins, carbohydrates, fats, minerals, and vitamins which are necessary for health (Samaržija *et al*., 2012). Raw milk is very sensitive to contamination with a variety of bacteria from the surrounding environments including the cows, the contaminated milking utensils as well as the soil (Leriche *et al*., 2004; Bellassi *et al*., 2021). Usually, raw milk is kept at a low temperature till processing to reduce the multiplication of microbiota, but this is not enough to prevent the proliferation of psychotrophic bacteria groups (Kumar *et al*., 2019) which can arise aerobically at chilling temperature, especially *Pseudomonas fluorescens* (Munsch-Alatossava and Alatossava, 2006).

Pseudomonas fluorescens belongs to the Pseudomonadaceae family and is considered the most specific spoiling organisms due to their excessive proliferation, therefore it is the most common spoilage indicator in milk and milk products making them unpalatable due to discoloration, off-odor, off-flavor, and slime production (Quigley *et al*., 2013). All these deterioration signs render the products unfit for human consumption and lead to economic losses in the dairy industry (Santeramo and Lamonaca, 2021).

The spoilage activity affects the shelf life of milk due to casein and fat degradation and has been frequently isolated from cheese (Carrascosa *et al*., 2014; Al-Leboudy, et al. 2015; Stuknytė *et al*., 2016). Although some *Pseudomonas spp*. lost their activity after milk pasteurization, but their extracellular enzymes such as proteases remain stable and resist heat treatment leading to degraded milk proteins (Paludetti *et al*., 2020). Some of *P. fluorescens strains* possess the ability to form biofilm on equipment's surfaces which is hard to remove during processing (Ksontini *et al*., 2013; Rossi *et al.*, 2016). There is obvious spoilage activity when bacterial counts reach to about 10^7–10^9 CFU/g of food (Gram *et al.*, 2002).

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Nowadays milk quality assessment is done through the total bacterial counts before processing which must not exceed 300,000 CFU/ml according to the EC legislation criteria (Regulation EC, 2004). *Pseudomonas fluorescens* have frequently been used as model organisms to study spoilage mechanisms in dairy products and to evaluate the control approaches that reduce milk contamination (de Oliveira *et al*., 2015). Therefore, applying hygienic measures through the milking process is necessary to minimize the risk of contamination with psychrotolerant microorganisms (Elmoslemany et al.,2010). To the best of our knowledge, there are few studies investigating spoilage indicators in dairy products in Nineveh province. The current study was highlighted to monitor and detect *P. fluorescens* from cows' milk in Nineveh province using the polymerase chain reactions (PCRs) technique.

Materials and Methods

Samples

The research samples included 150 samples as follows, 50 raw milk samples from cows and 50 swabs from each of teat surfaces, and milking tanks. Samples were collected randomly from different regions in Nineveh province between the periods from October 2023 to February 2024. The milk was collected aseptically using sterile bottles, the teat surfaces, and milk tank swabs were taken individually using cotton swabs placed in special sterile tubes containing 5 ml of phosphate buffer solution. All samples were placed in the ice box and transported to the laboratory of the Veterinary Public Health Department, College of Veterinary Medicine, University of Mosul.

Isolation and counting

Raw milk samples were examined for isolation and counting of psychotropic *P. fluorescens* at three periods included 0 day then milk samples were cooled at 4°C for 3 and 6 days. Milk samples were diluted 10-fold in 0.1% of sterile peptone water, and 0.1 ml of each dilution was spread on *Pseudomonas* cetrimide agar (Neogen, USA). Plates were incubated at 25°C for 48 hours, then colonies were counted to calculate as log CFU/ml. The teat surfaces and milk tanks were cultivated on cetrimide agar and plates were incubated aerobically at 25°C for 48 hours.

Identifications of bacterial isolates

Identification of Pseudomonas isolates was done according to biochemical tests represented by the catalase, oxidase, production of pyoverdine, starch hydrolysis, and gelatin liquefaction (Roberts and Greenwood,2008). Vitek2 compact system (Biomerieux, France) were performed to confirm the identification of target bacterium molecular identification of *P. fluorescens* was used to confirm the diagnosis using PCR technique.

DNA extraction

Positive colonies were subjected to DNA extraction using a Bacterial DNA preparation kit (Add-bio, Korea) according to the manufacturer's protocol.

Polymerase chain reaction

The presence of *P. fluorescens* was investigated depending on PCR assay using the *16SPflu* gene, a specific primer provided by (Macrogen/Korea) (Table 1). The primer consists of set forward and reverse primers according to (Scarpellini *et al*.,2004) with a molecular weight of 850 bp. The thermal profile included an initial denaturation of 2 minutes. at 95°C after that, 35 cycles of 94°C for 45seconds, then annealing 56°C for 1 minute. next, extension at 72°C for 1 minute and a final extension of 72°C for 2 minutes. With cooling at 4°C. The products were analyzed by electrophoresis (1.5% agarose gel) provided by (AddBio, Korea) with 3 µl GelRed dye (AddBio, Korea). The pcr products were analyzed in 300mA 75 volts for 1 hour. 4 µl of DNA ladder, 100 bp provided by (GeNet Direx, Korea) was depended as standard. The specific band was identified using the Gel doc EZ image (Bio-Rad, USA).

Sequencing of the 16SrRNA gene

After the PCR products were purified the sequencing of *16SPflu* gene was assessed according to Sanger dideoxy sequencing and the Blast algorithm at the NCBI server then phylogenic analysis using ClustalX (NCBI) software programs (Tamura *et al*.,2021).

Statistical analysis

The statistical analysis system was used to detect the differences using SPSS program version 20. The Analysis of Variation was used to assess the significant differences in this study.

Ethical approval

Not needed for this study.

Results

The current study revealed that Out of 150 samples, 39 (26%) were positive for the presence of *p. fluorescens* strains using PCR techniques compared to 48 (32%) by traditional methods (Table2). From 50 samples of raw milk19 (38%) were positive for the presence of *P. fluorescens*, while out of 50 swabs to each of the teat surfaces and milk tanks revealed the recovery rate of *P. fluorescens* at 11 (22%) and 9 (18%), respectively. With conventional culture and biochemical methods

Table 1. Oligonucleotide primer sequence for *P. fluorescens* used in the current study.

Primer	Primers sequence Sequence 5"—3"	Tm ^{0}C	Product size bp	Reference
16SPflu-F	5'-TGCATTCAAAACTGACTG-3'	56	850	(Scarpellini *et al*.,2004)
16SPflu-R	5'-AATCACACCGTGGTAACCG-3'			

22 (44%), 16 (32%), and 10 (20%) of isolates were positive for the presence of *P. fluorescens* in raw milk, teat surfaces, and milk tanks, respectively (Table 3). The counts of *Pseudomonas* in cows raw milk revealed higher counts of 7.37 ± 0.286 log CFU/ml after 6 days of storage at chilling temperature compared to both of 0 and 3-days storage periods 4.38 ± 0.184 and 6.29 ± 0.192, respectively (Table 4) (Fig. 1). The *Pseudomonas*

Table 2. Total Prevalence of *P. fluorescens* in the current study by different assays.

No. examined.	Target microbes	Traditional methods		PCR	
		No.	%	No.	%
150	Positive	48	32	39	26
	Negative	102	68	111	74

Table 3. Recovery rate of *P. fluorescens among different* sources using traditional methods and PCR technique.

Source of samples	No. examined	Traditional method		PCR	
		No.	%	No.	%
Raw milk	50	22	44	19	38
Teat surfaces	50	16	32	11	22
Milk tanks	50	10	20	9	18
Total	150	48	32	39	26

Table 4. *Pseudomonas spp.* counts in raw milk stored at 4°C at different periods.

Source of samples	0 day Mean ± SE Log CFU/ml	3 days Mean ± SE Log CFU/ml	6 days Mean ± SE Log CFU/ml
Raw milk	4.38 ± 0.184	6.29 ± 0.192	7.37 ± 0.286

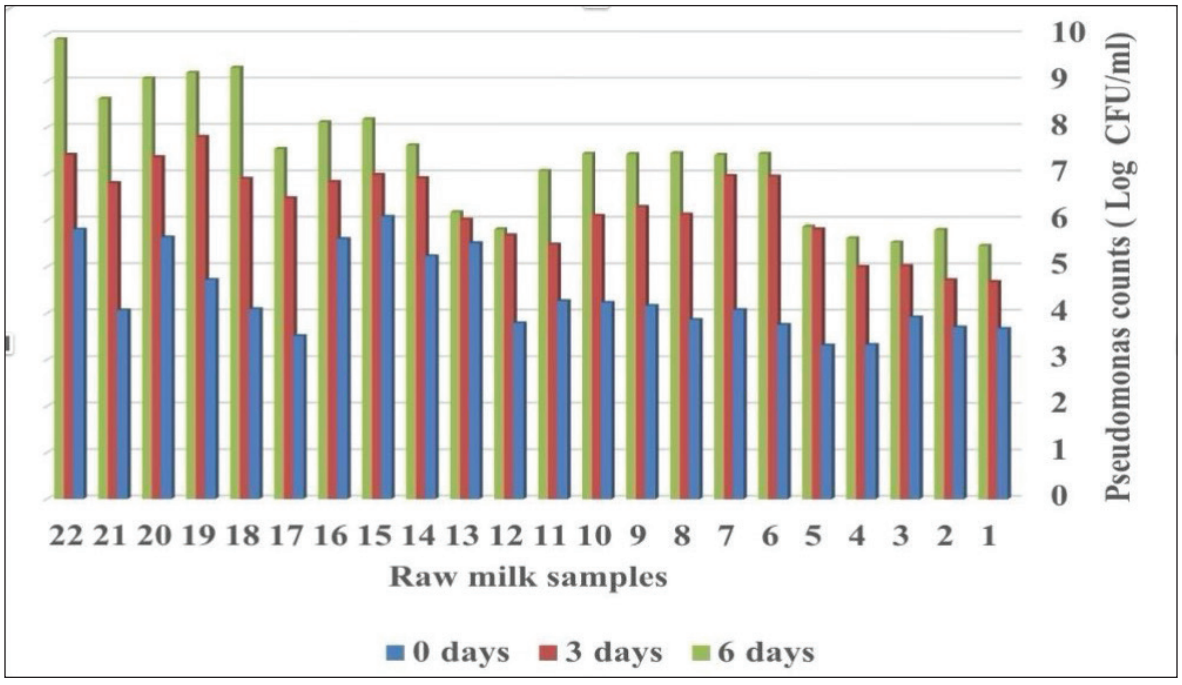


Fig. 1. Total counts of *Pseudomonas* in raw milk for different storage periods at chilling temperature.

counts in milk during storage at chilling temperatures revealed a significant increase at ($p < 0.05$) compared to the zero day. The PCR results confirmed the detection of *P. fluorescens* isolates according to the *16SrRNA* gene producing product size 850bp (Fig. 2). Sequencing of *16SPflu* gene exhibits that strains of *P. fluorescens* isolated from teat surfaces, raw milk, and milk tank have been submitted to the Genebank database with accession number PP727376. PP727377, PP727378, PP727379, PP727380, PP727381, PP727382, PP727383, PP7273784. PP727385, PP727386, and PP727387, respectively (Table 5) (Fig. 3). Also, Phylogenetic analysis revealed the sequences of our study were similar to those recorded in India, China, Nigeria, Taiwan (Fig. 4).

Discussion

Milk provides a favorable substrate for bacterial growth, *Pseudomonas* spp. is one of the important microflora of raw milk and dairy products which may be contaminated via soil and water, they are environmental mastitis-causing pathogens, the importance of *Pseudomonas spp*.

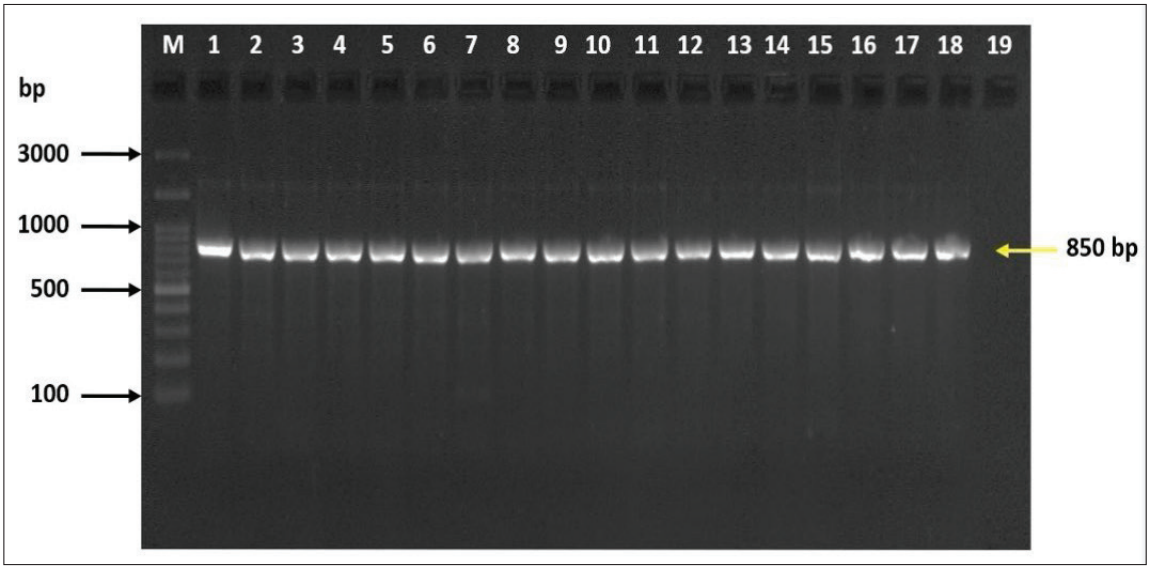
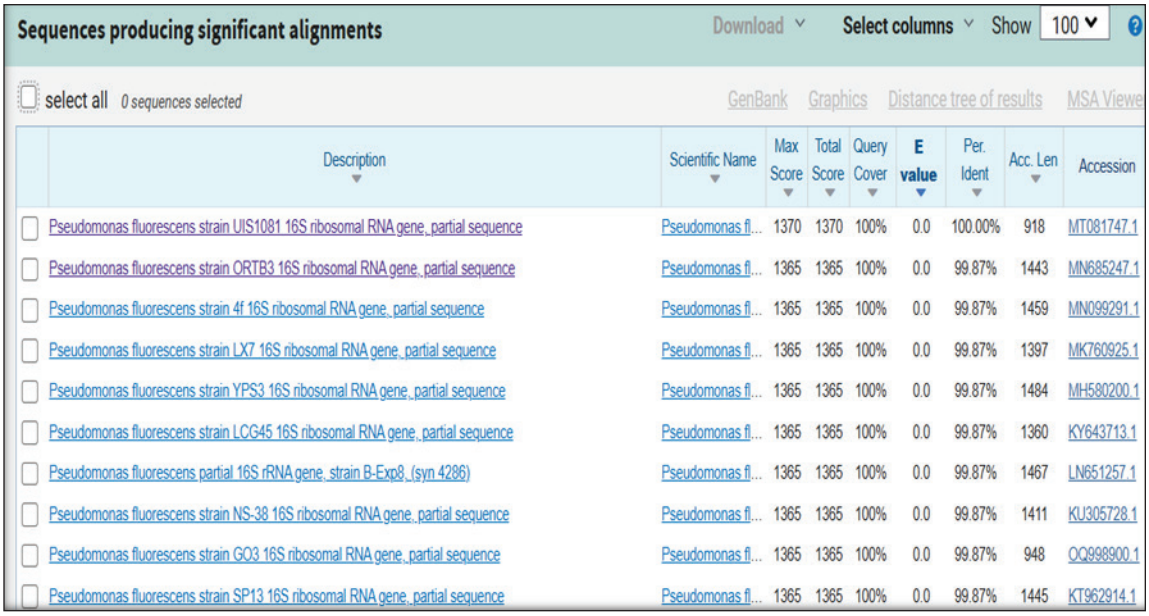


Fig. 2. Bands of Electrophoretic profile of the *16SPflu* gene of *P. fluorescens* illustrate Lanes M represent 100 bp DNA marker; lane 1–18, positive samples at 850 bp product size, lane 19 negative control.

Table 5. Distribution of *Pseudomonas fluorescens* based on 16S small subunit ribosomal RNA gene according to BLAST in Genbank of NCBI.

Local sample accession number	Identified	Query cover %	Identify %	Genebank accession number	Country identification
PP727376	*Pseudomonas fluorescens*	100	100	MT081747	Colombia
PP727377		100	99.87	MN685247	Tiawan
PP727378		100	99.87	MN099291	India
PP727379		100	99.87	MK760925	China
PP727380		100	99.87	MH580200	India
PP727381		100	99.87	KY643713	China
PP727382		100	99.87	LN651257	France
PP727383		100	99.87	KU305728	China
PP727384		100	99.87	OQ998900	Nigeria
PP727385		100	99.87	KT962914	India
PP727386					
PP727387					



Sequences producing significant alignments — Download — Select columns — Show 100

select all 0 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewe

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas fluorescens strain UIS1081 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1370	1370	100%	0.0	100.00%	918	MT081747.1
Pseudomonas fluorescens strain ORTB3 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1443	MN685247.1
Pseudomonas fluorescens strain 4f 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1459	MN099291.1
Pseudomonas fluorescens strain LX7 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1397	MK760925.1
Pseudomonas fluorescens strain YPS3 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1484	MH580200.1
Pseudomonas fluorescens strain LCG45 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1360	KY643713.1
Pseudomonas fluorescens partial 16S rRNA gene, strain B-Exp8 (syn 4286)	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1467	LN651257.1
Pseudomonas fluorescens strain NS-38 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1411	KU305728.1
Pseudomonas fluorescens strain GO3 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	948	OQ998900.1
Pseudomonas fluorescens strain SP13 16S ribosomal RNA gene, partial sequence	Pseudomonas fl...	1365	1365	100%	0.0	99.87%	1445	KT962914.1

Fig. 3. The identification of the query sample*, Pseudomonas fluorescens*, in alignment with NCBI gene bank.

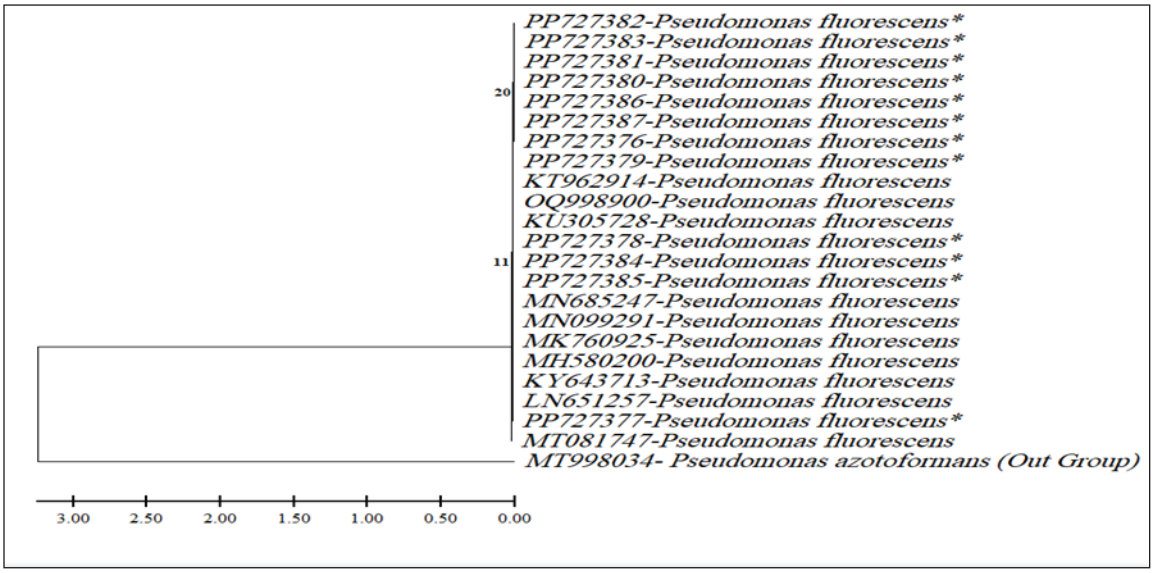


Fig. 4. Phylogenic tree of *Pseudomonas fluorescens* from milk,teat surfaces and milk tanks (*). The *P.azotoformans* (MT998034-Austria) represent the outgroup.

on animal and consumer health related to their many virulence factors as well as to their high resistance to antibiotics (Marchand *et al.*,2009). Our results showed that the recovery rate of *P. fluorescens* from raw milk was 38% which accounts high percentage affect milk quality compared to the incidence of *P. fluorescens* in raw milk from El-Menoufia Governorate 28% (Atia *et al*., 2022) while it is within the isolation rate(42.66) of *P. fluorescens* recorded by Meng *et al*. (2017) in Shaanxi province in China. The percentage of *P. fluorescens* to the total *Pseudomonas spp*. was 86.36% compared to the percentage of 75.8% recorded by Du *et al*. (2022). The isolation rate of *P. fluorescens* from teat surfaces and milk tank was 22% and 18% respectively, referred that teat surfaces with manual and mechanical milking systems in dairy farms as well as the inner surfaces of milk tank contribute to *Pseudomonas* spreading (The *et al*., 2011; Vidal *et al*., 2017). These results explain the demand for good hygienic practices to control the *Pseudomonas spp*. contamination in the raw milk stored

at chilling temperatures and improve the quality of milk and milk products by controlling the milking process (Leitner *et al.*, 2008; Santana *et al*., 2020). Also, the results exhibited the *Pseudomonas spp*. counts ranging from 4.38 to 7.37 log CFU/ml and revealed high levels of *Pseudomonas* populations in cows' milk after 6 days of storage at chilling temperatures which similar to some extent to the results obtained by (Lampugnani *et al*., 2018) in raw milk in Brazil 3–7.1 log CFU/ml while the population of *Pseudomonas* in raw milk from dairy farms reach to about 4–6 log CFU /ml (Fagundes *et al*., 2006; Almeida *et al*., 2017). These results agreed to that both storage time and temperature have a major impact on the growth of *Pseudomonas spp*. In raw milk (De Jonghe *et al*., 2011; Lin *et al*., 2016). The population of *Pseudomonas* elevated due to their evident activity at low temperatures (Rajmohan *et al*., 2002; Arslan *et al*., 2011). Therefore, the contamination of milk with psychotropic bacteria minimizes both milk quality and shelf life of milk and indicates poor hygiene during milking with faulty procedures during storage and transportation of cooled milk (Rajmohan *et al.*, 2002; Ksontini *et al*., 2013; Al-Rudha *et al.*, 2021). The gene sequencing of *P. fluorescens* isolates achieved in this study from milk, teat surfaces, and milk tanks were recorded to NCBI indicating a genetic variation and possibility of transmission within the environment (Barton *et al*., 2013). Thus, monitoring of dairy markets for specific spoilage organisms diminishes the likelihood of *Pseudomonas* proliferation and prolongs the storage period of the products (Alkhafaje *et al*., 2022).

Conclusion

Detection of *P. fluorescens* in cows' raw milk and milking surfaces indicates the possibility of spoilage arise in milk and may affect consumer health and the high recovery of *P. fluorescens* requires the application of hygienic conditions during the milking process and handling of milk as well as during milk storage.

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Conflict of interest

The authors confirm there was no conflict of interest.

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Authors' contributions

The study was designed, and the manuscript was written and revised by Muntaha Ghazi Hassan and Ahmed Hamdi Ahmed. Both authors approved the final manuscript.

Data availability

All data supporting the findings of this study are available within the article.

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