PCR-BASED DETECTION OF *PSEUDOMONAS FLUORESCENS* IN COWS AND BUFFALOES RAW MILK

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ABSTRACT

Investigations of the *Psuedomonas fluorescens* proteolysis activity is the basal objective of this study. To achieve this objective 16S rDNA and SM2F/SM3R primers were used in the amplification of DNA extracted from raw milk bacterial isolates. In the present study the 92 bacterial isolates , consisted of 42(cow raw milk isolates) and 50 (buffalo raw milk isolates) obtained from 240 cows and buffaloes raw milk samples (120) for each , were subjected to phenotypic and molecular identification.

The results of phenotypic identification revealed that higher ratio (41.7 and42%) for bacterial isolates plating inoculation and biochemical tests based identification respectively was observed in buffaloes raw milk compare to the ratio observed in cows milk bacterial isolates (35 and 33.3% respectively), however the differences .between cows and buffaloes raw milk in concern to bacterial identification results considered to be not statistically significant (P>0.05).

The 16SrDNA and SM2F/SM3R based PCR results, revealed that the effect of breed(cow only) and age on the amplification results of the 16SrDNA and SM2F/SM3R gene product of *P. fluorescens* in cows and buffaloes raw milk is considered to be not statistically significant (P>0.05). Depending on 16SrDNA based PCR results higher ratio of *P. fluorescens* identification (62.5 %) was observed in the native cows raw milk compare to crossbreed raw milk bacterial isolates ratio (57.7%). SM2F/SM3R based PCR results showed 33.3 and 34% of raw milk *P. fluorescens* isolates of cows and buffaloes respectively had protease activity.

INTRODUCTION

Whole milk is the milk as it came from the cow and contains about 3.5% milk fat. Research continues to expand the positive role of milk and milk products play in an individual's health. Evidence goes well beyond bone health to include its effects on immunity, mild hypertension, reducing selected cancers, (1) supporting weight management strategies and increasing satiety in dieters, among other positive effects.

Improvements in livestock and dairy technology offer significant promise in reducing poverty and malnutrition in the world (2). Milk is a good growth medium for many microorganisms. therefore the microbial content of milk is a major feature in determining its quality (3).Dairy products are very important for the food supply and have been so for thousands of years(4) .

Raw milk contains gram negative psychrotrophic bacteria (GNP) which can grow and multiply rapidly in chill-stored raw milk (5). These bacteria are killed during pasteurization, but as a result of the post-pasteurization contamination around 50% of milk can be spoiled by GNP after prolonged chill storage (6,7,8,9). Furthermore, they produce the majority of heat stable extracellular proteases, lipases and phospholipases secreted into raw milk during preprocessing storage. Many of these enzymes can survive pasteurization (72°C for 15 s) and even ultra-high-temperature treatments (138°C for 20 s or 149°C for 10 s) and can consequently reduce the eating quality and shelf life of processed dairy products (10,11,12). Some important lipases in milk are the lecithinases or other phospholipases, which are able to disrupt the integrity of the milk fat globule membrane, and expose the fat to degradation by native milk lipases, resulting in physical degradation of the emulsion in milk (13).

Pseudomonas spp. are by far the most common spoilage-causing psychrotrophic bacteria. Different *Pseudomonas* spp. have been isolated from raw milk, of which *P.fluorescens* is the most frequent (14). (6)observed that *Pseudomonas fluorescens* made up more than 55.6% of all the bacteria isolated from raw milk. (15) showed that *P. fluorescens* was present in 84% of the raw milk samples.

Milk microbiology has traditionally been performed using culture-based methods. However, in the last few decades, the emergence of molecular methods has changed the field considerably. The latest development in this area has been the introduction of gene sequencing in addition to polymerase chain reaction. These technologies allow

the massively parallel detection of bacterial DNA strands used in the monitoring milk quality and estimation of bacterial spoilage potential .

This study performed both PCR and traditional culture-based techniques to investigate the *P. fluorescens* spoilage potential within milk .Phenotypic methods have proven useful for quantifying and describing bacteria causing dairy product spoilage; however, accessible phenotypic speciation strategies for the most common dairy product spoilers, i.e., *Pseudomonas* spp., frequently yield uncertain results (13).

To solve this problem, in the current study *P. fluorescens* isolated from the of raw milk was characterized by using phenotypic and genotypic methods . the phenotyping included, Gram's stain reaction and biochemical testing while PCR analysis represnts detection and characterization of the enzymatic activity of *P. fluorescens* **.**

MATERIALS AND METHODS

Isolation of *Pseudomonas fluorescens*.

A total of 240 milk samples (cows and buffaloes,120 for each) were collected from different local markets and household animals in some regions of Basrah governorate .Each of the raw milk samples was cultured (0.1 mL aliquot) on tryptic soy agar (TSA,) modified by adding 1% dextrose tryptic soy agar (TSA, Himedia - India), violet red bile glucose agar(VRBG, Liofilchem- Italy) and pseudomonas selective agar (PSA, Liofilchem- Italy) , using the spread plate technique(16).

The plates were incubated at 26°C for 3 days to establish the pre-enrichment storage condition of raw milk . The cell morphology was determined microscopically after Gram-stain preparation. The phenotypic characteristics were determined using tests including Carbohydrate oxidative, Simmon's citrate utilization (Oxoid - UK), oxidase (1.0% tetramethyl), Urease(Himedia – India) and Gelatine liquefaction tests (16).The isolates were sub-cultured on the same medium until pure cultures were achieved. The isolated bacteria were grown in nutrient broth (Oxoid - UK) and were then stored in 15% (v/v) glycerol at−20 °C and used as stock cultures in subsequent analysis(17).

DNA Extraction and PCR

Total DNA was extracted from raw milk bacterial isolates by using commercially genomic DNA mini kit (Qiagen- Germany). following the mini spin protocol according to the manufacturer's instructions. To confirm the presence of *P. fluorescens* DNA, a standard diagnostic PCR was carried using one 16S rDNA

(850bp) (18) .F:5'- TGCATTCAAAACTGACTG -3'; R:5'- AATCACACCGTGGTAACCG -3' The proteolytic activity of this bacteria was detected by using SM2F/SM3R Primer(900pb) (19): F: 5'- AAATCGATAGCTTCAGCCAT-3';R: 5'- TTGAGGTTGATCTTCTGGTT-3

The PCR amplification mixture (25µl) includes (12.5 µl) of green master mix which contains bacterially derived Taq DNA polymerase , dNTPs , $MgCl_2$ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR (5 µl) of template DNA ,(1 µl) of each forward and reverse primers and (5.5 µl) of nuclease free water to complete the amplification mixture to (25 µl) . The PCR tubes containing amplification mixture were transferred to preheated thermocycler and start the program as follow, 2 min at $94^{O}C$ for one cycle, then 35 cycles of 45 sec at $94^{O}C$,, 45 sec at $59^{O}C$, and,$68^{O}C$ for 2 min with one final extension of 5 min at $72^{O}C$ and a subsequent hold temperature of 4°C using a thermocycler (Techne-UK).

The results of PCR were detected after the amplification process,. (10 µl) from amplification sample was directly loaded in a 1.5% agarose gel containing(0.5 µl /25 ml) ethidium bromide and DNA size marker as standard in electrophoresis and run at 70 V. The DNA was observed and photographed by using gel documentation system

Statistical analysis

To demonstrate any association between results, Pearson's chi-squared test with Yates correction were used with the limit of significance being set at 5%.

RESULTS

Morphology and Phenotypic characterization results:

Forty two and fifty isolates were obtained from cows and buffaloes raw milk respectively on the basis of colony morphology and the staining characteristics. It was observed that all isolates were gram negative rods. In Table. (1) the results of phenotypic identification revealed that higher ratio(41.7 and42%) for bacterial isolates plating inoculation and biochemical tests based identification respectively was observed in buffaloes raw milk compare to the ratio observed in cows milk bacterial isolates (35 and 33.3% respectively), however the differences between cows and buffaloes raw milk in concern to bacterial identification results considered to be not statistically significant ($P>0.05$).

Table (1): Conventional bacteriological analysis positive results of raw milk *pseudomonas* isolates

Animals species	Tested n.(%)	Conventional bacteriological analysis n.(%)	
		Plating Characterization	Biochemical Characterization
Cow	120	42(35)	14(33.3)
Buffalo	120	50(41.7)	21(42)

P>**0.05**

PCR Analysis Results

Agarose gel electrophoresis after amplification Products

To determine the presence of proteolytic *P. fluorescens* in cow and buffalo raw milk isolates, 16S rDNA and SM2F/SM3R primers were used.Genomic DNA from a *Pseudomonas* positive isolate was used as the source of template DNA. The positive results for the prescence of 16S rDNA (850 bp) and SM2F/SM3R (900 bp) genes amplified products were displayed in Figeres. 1,2 ,.

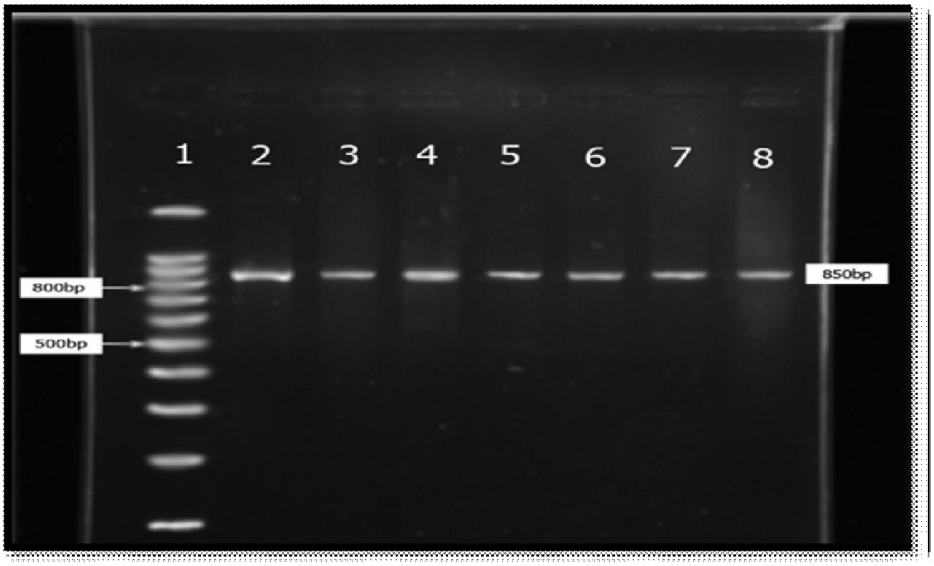

Figure (1) The amplification results of the 16SrDNA gene products of *P. . fluorescens*. Isolates(850 bp). Lane (1) is 100 bp DNA ladder marker, lanes (2 ,4 , 5 , 6, 7,8) are positive .

Figure (2) .The PCR amplification of SM2F/SM3R gene products of *P . fluorescens* isolates(900-bp) .Lane (1) is 100 bp DNA ladder marker, lanes (2 - 8) are positive.

Distribution of *P. fluorescens* in Cow And Buffalo Raw Milk

The effect of breed(cow only) and age on the amplification results of the 16SrDNA gene product of *P. fluorescens* in cow and buffalo raw milk *Pseudomonas isolates* is considered to be not statistically significant (P>0.05).By 16SrDNA based PCR analysis higher ratio of *P. fluorescens* identification (62.5 %) was observed in the native cows raw milk compare to crossbreed raw milk isolates (57.7%). In general higher *P. fluorescens* identification ratio was observed in 16SrDNA based PCR analysis compare to biochemical testing (16SrDNA based PCR overall ratio: 59.5% v 33.3% in biochemical) . (Table 2). Raw milk *Pseudomonas isolates* of cows at >1-4 years of age showed higher ratio of 16SrDNA gene based PCR and biochemical tests positive results (64.9 and 85.7% respectively) compare with that of cows at > 4-8 year of age (Table 3).On the other hand raw milk *Pseudomonas isolates* of buffalo at > 4-8 year of age identified in a higher ratio as *P. fluorescens* by16SrDNA gene based PCR (70.6%) and biochemical tests (47.1%) (Table 4).

Table (2): PCR Positive Results of Cow Raw Milk *Pseudomonas* Isolates According to Cow Breed.

Cow breed	Tested Isolates n.(%)	Biochemical Test +ve n.(%)	PCR analysis n.(%)	
			16S rDNA primer	SM2F/SM3R primer
Native n.=45	16 (35.6)	4 (25)	10 (62.5)	5 (31.3)
Crossbreed n.= 75	26 (34.7)	10 (38.6)	15 (57.7)	9 (34.6)
Total n.=120	42 (35)	14 (33.3)	25 (59.5)	14 (33.3)
P.value	P>0.05	P>0.05	P>0.05	P>0.05

Table (3): PCR Positive Results of Cow Raw Milk *Pseudomonas* Isolates According to Cow Age

Age groups (years)	Tested Isolates n.(%)	Biochemical Test +ve n.(%)	PCR analysis n.(%)	
			16S rDNA primer	SM2F/SM3R primer
>1-4 n.=101	37 (36.6)	12 85.7)(	24 64.9)(	13 (35.1)
> 4-8 n.= 19	5 (26.3)	2 (40)	1 (20)	1 (20)
Total n.=120	42 (35)	14 (33.3)	25 (59.5)	14 (33.3)
P.value	P<0.01	P>0.05	P>0.05	P>0.05

Table (4): PCR Positive Results of Buffalo Raw Milk *Pseudomonas* Isolates According to Age

Age groups (years)	Tested Isolates n.(%)	Biochemical Test +ve n.(%)	PCR analysis n.(%)	
			16S rDNA primer	SM2F/SM3R primer
> 1-4 n.=97	33 (34)	13 (39.4)	22 (66.7)	12 (36.4)
> 4-8 n.= 23	17 (73.9)	8 (47.1)	12 (70.6)	5 (29.4)
Total n.=120	50 (41.7)	21 (42)	34 (68)	17 (34)
P.value	P<0.001	P>0.05	P>0.05	P>0.05

PCR Detection of Proteolysis Activity of *P. fluorescens*

primers SM2F/SM3R used to detect the aprX gene in *P. fluorescens* as aprX gene encodes a metalloprotease. By SM2F/SM3R based PCR 33.3 and 34% of raw milk *P. fluorescens* isolates of cows and buffaloes respectively had protease activity(Table ,2,3,4).

The effects of cow breed and age only in case of buffaloes on the detection of protease activity by PCR was investigated in this study. According to breed of cows an amplification product of aprX gene (900bp) was observed in 31.3 and34.6% of native and crossbreed cows raw milk *P. fluorescens* isolates without statistical significant difference(P>0.05) (Table. 2) In general,the effect of cow and buffaloes age is considered to be not statistically significant (P>0.05) and the age group in which there was high ratio of positive 16S rDNA gene *P. fluorescens* also showed high ratio of positive SM2F/SM3R PCR based isolates(Table 3,4). Beside that higher ratio of protease activity was observed in 35.1% of younger cows(>1-4) compare with buffaloes in which older animals (>4-8 year) showed higher protease activity(29.4%).

DISCUSSION

In the current study traditional identification of *P. fluorescens,* was done using the selective plating ,morphological and biochemical characterization. Since molecular biology is providing extremely sensitive and specific tools for identifying and characterizing psychrotrophs in raw milk samples..PCR amplification of extracted bacterial DNA with specific primers was done for further confirmation of *P. fluorescens*. Many previous studies supported what was done in the current study,(20) mentioned that the population structure of psychrotrophic community is mainly characterized, but there are still many unidentified species or even genera in the population. Need for molecular tools, in combination with traditional identification methods, is therefore needed to get the full picture of raw milk bacterial community in its initial state and after cold storage.Also (21**)** indicated that PCR-ribotyping and phenotypic characterization could be helpful in tracking contamination routes in the production line for milk pasteurization.

Beside these studies (22) and (23) reported PCR-ribotyping as a rapid and accurate method for typing *Burkholderia* (*Pseudomonas*) *cepacia*. (24) used this method in genotypic characterization of *Pseudomonas* strains isolated from bulk tank milk.(25) established PCR protocol in their study reinforced the potential utility of PCR as a tool to monitor the quality of raw milk**.** (26). Monitored bacterial communities in raw milk and cheese by culture-dependent and –independent 16S rRNA gene-based analyses.

In the present study the 92 bacterial isolates of 240 cows and buffaloes(120 for each) raw milk samples previously refrigerated for 72 hr. subjected to PCR-based detection of proteolytic activity of *P. fluorescens* milk spoiler without relying on agar plating . Investigations of the *P. fluorescens* proteolytic activity was the basal objective. To achieve this objective 16S rDNA and SM2F/SM3R primers were used in the amplification of DNA extracted from raw milk bacterial isolates. The usage of these primer was according to two previous studies, the first study was the study of (25) who mentioned that psychrotrophic protease activity was correlated with the appearance of the SM2F/SM3R and 16S rDNA PCR amplification product. Both aprX and 16SrDNA genes, which were detectably amplified after 3 days of milk incubation at 7 ^{o}C, can be effective markers for monitoring milk decay provided that *P. fluorescens* is considered one of the most frequent species after prolonged storage

under refrigerated conditions..The second study (27) reported that the primers SM2F/SM3R can detect the aprX gene in several relevant species of Pseudomonas, including various members of the *P. fluorescens* group .

To increase the sensitivity of PCR method in the current study the primers that amplify larger fragments of the aprX gene (SM2F/SM3R, 900bp) gene and 16S rDNA(850 bp), according to (25) and (28) who increased the sensitivity of the PCR method by using primers that amplify larger fragments of the aprX gene(900 bp) compare to (29) who used 194 bp fragment of the target aprX gene, but amplifying a smaller fragment . This primer-dependent variation in sensitivity was confirmed by the use of PCR for the detection of *P. fluorescens* in raw milk samples, and it can be explained by the size of the amplified fragment, the efficiency of primer annealing, the amplification conditions and the number of copies of the target gene in the genome (30,31,32, 21) used the 16S rRNA gene (850 bp) as a target to obtained higher sensitivity of PCR methods.

The present results revealed that PCR with DNA isolated from cows and buffaloes raw milk bacteria led to one main product of the expected size with each primer pair(16SrDNA and SM2F/SM3R) in 59.5 and 33.3% of cows raw milk bacterial isolates respectively and in 68 and 34% of buffaloes raw milk bacterial isolates respectively. Other studies supported the current result as (13) and (24) reported differences in proteolytic and lipolytic activity of *P. fluorescens* isolates.Incontrast with current results (33) and (34) reported lower ratio(6.7 and 5.7%. respectively) of milk was contaminated with *P. fluorescens* showing proteolysis activity. But (21) found higher ratio (87%) of *P. fluorescens* isolates were proteolytic compare to that of present study..

The current discrepancy in the PCR based detection ratio of genes responsible for protease production in *P. fluorescens* might be explained by one or more points that were mentioned in the following studies: (13) (24) reported differences in proteolytic and lipolytic activity of *P. fluorescens* isolates. (35) (36) mentioned that the correct preparation of DNA samples is critical to ensure the dilution or elimination of PCR inhibitors and the success of the PCR technique . (28) observed a reduction in the PCR sensitivity in the presence of other contaminants microbiota in raw milk.

In addition, (37) reported that *Pseudomonas spp*. have showed greater genetic variability within strains belonging to the same species .On the other hand (38)

reported that the use of PCR for raw milk analysis is affected by the persistence of inhibitory substances such as proteins .and fat.Inconclusion the sensitivity of PCR facilitates the detection of *P. fluorescens* and identify their protease activity in raw milk..

اتفاعل البوليميريز المتسلسل لجرثومة الكشف المعتمد على *Pseudomonas* في حليب الأبقار والجاموس الخام*fluorescens*

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الخلاصة

الهدف الاساسي لهذه الدراسة هو التقصي عن فعالية تحليل البروتين في جرثومة *Psuedomonas fluorescens* لتحقيق هذا الهدف استعملت البادئات 16S rDNA و SM2F/SM3R في تضخيم الحامض النوويDNA المستخلص من العزلات الجرثوميه للحليب الخام وقد اخضعت للتشخيص المظهري والاحيائي الجزيئي 92 عزلة بكتيرية متكونة من42 عزلة حليب الابقار الخام و50 عزلة حليب الجاموس الخام حصل عليها من240 عزلة حليب خام للابقار و الجاموس. كشفت نتائج التشخيص المظهري عن ان اعلى نسبه 41.7) (and42% لتشخيص العزلات البكتيرية المعتمد على الزرع بالاطباق والاختبارات الكيمياحيوية لوحظت في حليب الجاموس الخام بالمقارنة مع تلك النسب التي لوحظت في عزلات حليب الابقار البكتيرية (35%) الزرع بالاطباق و(33.%) والاختبارات الكيمياحيوية مع ذلك ان الاختلاف بين الابقار والجاموس فيما يتعلق بنتائج التشخيص البكتيري لايعتبر ذو معنوية احصائية.(P>0.05). كشفت نتائج الPCR ان تاثير السلالة في حليب الابقار وتاثير العمر في الابقار والجاموس على نتائج تضخيم منتجات جينات (16SrDNA و) (SM2F/SM3R)جرثومة *P. fluorescens* في حليب الابقار و الجاموس الخام لا يعتبر ذو معنوي احصائية.(P>0.05). بالاعتماد على نتائج الPCR المعتمد على16SrDNA لوحظت اعلى نسبة (% 62.5) لتشخيص *P. fluorescens* في حليب الابقار المحليه الخام بالمقارنة مع نسب .(57.7%) العزلات البكتيرية لحليب الابقار المضربة . اظهرت نتائج الPCR المعتمد علىSM2F/SM3R ان33.3% و 34%من عزلات *P. fluorescens* لحليب الابقار والجاموس النئ على التوالي لها فعالية في تحليل البروتين.

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