PCR BASED DETECTION OF GRAM NEGATIVE PSYCHROTROPHIC BACTERIA IN COWS RAW MILK

Amjed A. Alrazakkazal, Fawziah A. Abdullah

Department of Microbiology, Collage of Veterinary Medicine, University of Basrah, Basrah ,Iraq.

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ABSTRACT

Investigations of the proteolytic Gram Negative Psychrotrophs(GNP) bacteria was the basal objective of this study. A conventional diagnostic PCR technique based on three pairs of primers including *SerA*geneto amplify an 950 bp fragment of *Acinetobacter* sppDNA,*serA*gene for/*A. hydrophila* (650bp): and *apr*gene for/*S. marcescens* (500bps) was done.In the present study the 29 bacterial isolates obtained from 100 cows raw milk samples were collected randomly from healthy cows with different age and breed present in different farms of Thi-Qar province, previously refrigerated for 72 hr. These isolates subjected to cultural-based enrichment and PCR- based identification .The present results revealed that the raw milk GNP contamination overall ratio was 29% . *Acinetobacter* spp *were the most predominant bacteria (16%) among the studied GNP contaminants,* while *A.hydrophila* appeared in a ratio of 7% *and S. marcescens* showed the lower ratio (6%). ,the results of the studied genes product of GNP bacteria was considered to be highly statistically significant (P>0.001).The distribution of studied GNP according to age ,parturition number and breed of studied animals was investigated. The effect of these factors on the PCR-based identification results was considered to be not statistically significant(P>0.05) however, the higher overall ratio(29.1%) for cow raw milk contamination was observed in raw milk of cows between <3 - < 9 year of age. In general cows at first age group (<3 - < 9 year) showed high ratio of raw milk contamination(7.6 and6.3%) with GNP bacteria (*Serratia marscens*e and *Aremonase hydrophila* respectively)..Concerning the number of parturition, cows with high numbers of parturition(> 6-<12) showed high overall ratio(38.5%) of contamination also high ratio of *Acinetobacter* spp(23.1%) ,*Aremonase hydrophila*(7.7%) and *Serratia marscens*e

(7.7%) were observed in the same group of cows. According to breed, a high overall ratio of GNP bacterial contamination was observed in 40% of crossbred cow raw milk followed by Friesian cows(32.1%).Beside that raw milk of crossbred cows showed a high ratio of contamination with *Acinetobacter* spp (20%) and *Aremonase hydrophila*(13.3%) while *Serratia marscens*e appeared as a higher contaminant in Friesian cows raw milk with the ratio of its contamination was10.7%.

INTRODUCTION

Cow's milk has been a staple diet ever since the medical community publicized its nutritional benefits in the 1920s (1). However, health concerns over cow's milk began as early as the mid-19th century, when the public began to focus on unhygienic conditions of cows and dairy processing plants. There is a growing concern that milk, due to its wide distribution, storage in bulk tanks, rapid shelf life, and high consumption rates among humans, could be a prime target for bioterrorist attacks.(2) .

Foodborne illnesses from consuming milk were common during this time, and were mostly due to bacterial contamination (3; 4). Foodborne illnesses are often limited to ephemeral symptoms such as nausea, vomiting, and diarrhea, but can also include more serious and chronic complications, such as hemolytic uremic or Guillain–Barré syndromes; in some cases illnesses can lead to death (5).

Milk is an excellent growth medium for many microbes because it has neutral pH, the water content is high and it has a complex biochemical composition (6). Milking procedure may be contaminated from the teat surface, the udder, milking equipment, and the milking parlor environment. Psychrotrophic bacteria are defined as those that grow at 7°C, although their optimal growth temperature is higher. During cold storage after milk collection they dominate the flora, and their extracellular enzymes, mainly proteases and lipases, contribute to the spoilage of dairy products .most of the psychrotrophic bacteria found in milk are inactivated by pasteurization. However, many of these bacteria produce heat resistant lipases and proteases that degrade milk lipids and proteins (7).

The numbers of psychrotrophs that develop after milk collection depend on the storage temperature and time. The evolution of the composition of the psychrotrophicmicrobiota during the incubation of raw milk is the result of competition among the various species during the milk degradation process. (8) .

Psychrotrophic bacteria from numerous genera have been isolated from milk, both gram negative (*Pseudomonas*, *Aeromonas*,*Serratia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter*, and *Flavobacterium*) and gram positive (*Bacillus*,*Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus Streptococcus*, *Staphylococcus*, and *Lactobacillus*) (**9**). Milk microbiology has traditionally been performed using culture-based methods. 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 millions of DNA strands and represent a major development in sequencing technologies. Although conventional culture methods are still commonly used to ensure the microbiological quality of milk very few studies have been done to identify cultural microbial communities in milk by means of molecular identification tools.., (10) using the 16S rRNA gene, showed that culturable bacterial communities in raw milk were highly diversified , (8) reported the potential multiplex PCR in the detection of multiple spoilage microorganisms from the milk samples. The objective of this study was use PCR assays as an alternative, rapid method for detection of psychrotrophic bacterial contaminants in raw milk and their proteolysis activity.

MATERIALS AND METHODS

Samples collection

All studied samples were collected through period extended from November 2014 to January 2015. Raw cow's milk samples were collected randomly from 100 healthy cows with different age and breed present in different farms of Thi-Qar province ,aseptically placed into sterilized test tubes and transported with ice in cooler box to the laboratory for subsequent analysis.

Enrichment ofGNP Bacteria

The milk samples stored under refrigeration condition for 3 days. The enrichment step established in the present study was done by incubation (for 18 h at 37 ^{o}C) of 4.5 mL of Luria-Bertani broth (LB, 10 g L-1 tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) inoculated with 1% (v/v) of the raw milk samples. Culture stocks were prepared in LB broth containing 20% (v/v) sterile glycerol and were frozen at -20 ^{o}C. Before each experiment, the cells were cultured two consecutive times in LB broth. (8) .

DNA extraction and PCR amplification

Total DNA was extracted from raw milk bacterial isolatesby using Wizard genomic DNA extraction and purification kit(Promega USA)according to the manufacturer's instructions. To confirm the presence of proteolytic GNP DNA, a standard diagnostic PCR was carried using three pairs of primers designed by Machado *et al*,.(8) including a*SerA*gene to amplify 950 bp fragment of *Acinetobacter* spp DNA, F: 5'-GCGGGGTTGCCATTGAAGTA-3'; R: 5'-TGTGTATGCCGCTTCAAATGT-3'),*serA* for *A. hydrophila*at 650bp:SerAh:F:5' -TTC CTC CTA CTC CAG CGT CG-3';SerAh-R : 5'-TGA TGA TCC AGG CTC ACG GT-3' and *apr*for *S. marcescens*at 500bps ,MetS-F: 5'-CGG CGA GAT CTT CAA CCG TT-3';MetS-R: 5'- GGC GAA GGT GGT CAG AAG TC-3'

The PCR amplification of mixture (25µl) includes 12.5 µl of green master mix (which contains bacterially derived Taq DNA polymerase , dNTPs , MgCl2 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 (Techne/UK) and start the program as follow, 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 10 min.for Final extension and a subsequent hold temperature of 4°C. 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

Statistical analysis was done by using SPSS software version 11.To demonstrate any association between results, the exact Fisher test and Pearson's chi-squared test with Yates correction were used with the limit of significance being set at 5%.

RESULTS

PCR based Detection of cow raw milk GNP

The successful binding of the specific primers of *Acinetobacter* sp :SerA-F/SerA-R, *A. hydrophila* SerAh-F/SerAh-R and *S. marcescens*: MetS-F/MetS-R with the extracted DNA appeared in gel electrophoresis as single band under UV illuminator ,using ethidium bromide as a specific DNA stain .Only the band with expected size, 950bps for (*Acinetobacter sp*), 650bps for (*A. hydrophila*) and 500 bps for (*S. marcescens*) was observed in figures (1,2 and 3).

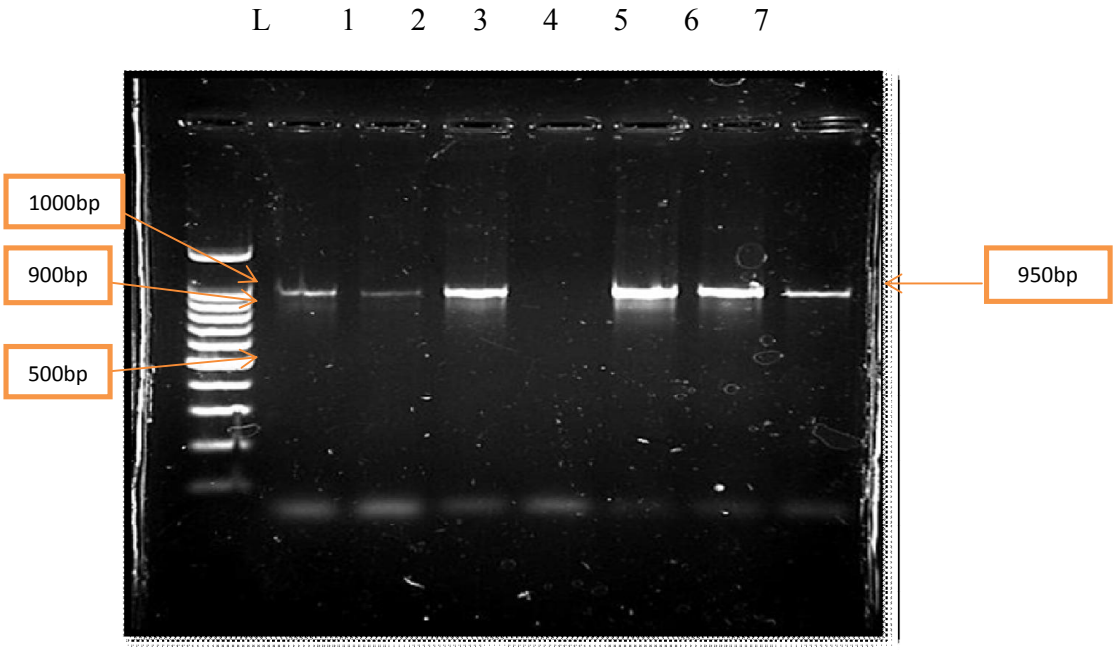

Figure (1): PCR productsband at 950 bp (*Acinetobacter* sp.), analyzed on a 1.5% agarose gel and examined under U.V. illuminator .

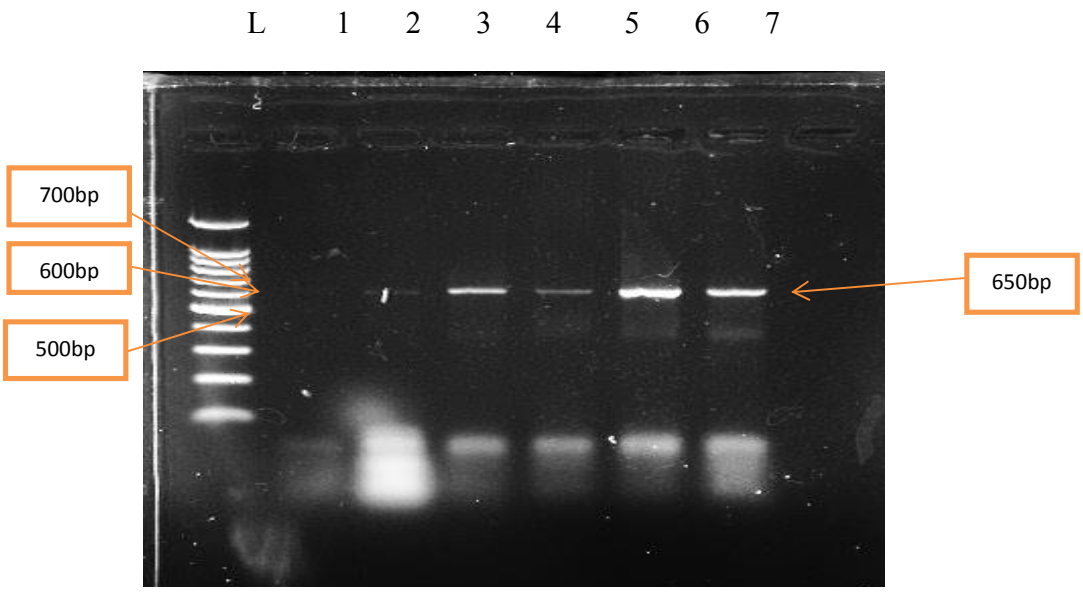

Figure (2): PCR products band at 650 bp (*A. hydrophila*.), analyzed on a 1.5% agarose gel and examined under U.V. illuminator .

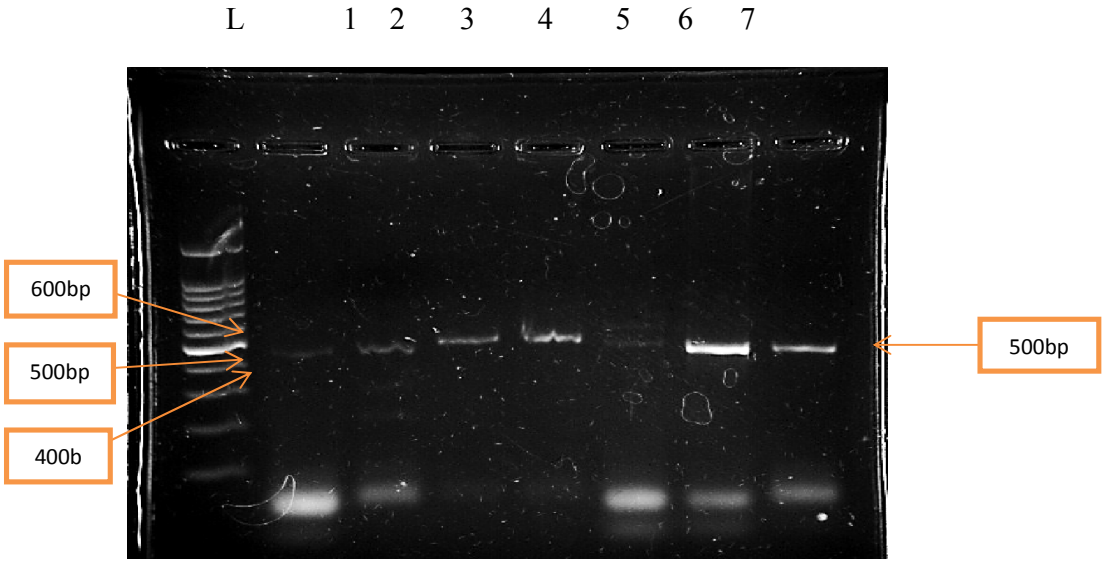

Figure (3): PCR products band at 500bp (*S. marcescens*), analyzed on a 1.5% agarose gel and examined under U.V. illuminator .

3.Prevalence ofraw milk proteolyticGNPin cow raw milk.

In general the GNP contamination ratio(29%)was observed in 100 raw milk samples collected from 100cows.*Acinetobacter* spp was the most predominant bacteria **(** 16% **)** among the studied GNP contaminants of raw milk samples of cows followed by*A.hydrophila*(7%) while *S. marcescens* showed the lower ratio (6%).The amplification results of the studied genes product of GNP in cow raw milk was considered to be highly statistically significant (P>0.001). (Table 1).

Table (1)Prevalence of raw milk proteolytic GNP in cows .

Raw milk samples	PCR results +ve n.(%)				
	Tested samples	***Acinetobacter* spp**	***Aremonase hydrophila***	***Serratia marscense***	**Total**
Cow	100(100)	16(16)	7(7)	6 (6)	29(29)

The distribution of studied proteolytic GNP in cows raw milk according to age ,parturition number and breed of cows was illustrated in table (2) .According to age the higher overall ratio(29.1%) for cow raw milk contamination by the studied proteolytic GNP was observed in cows at <3 - < 9 year of age compare to contamination ratio (28.6%) of raw milk in cows at age >9 - <15 year. cows at first age group (<3 - < 9 year) showed high ratio of raw milk contamination(7.6 and6.3%)with studied proteolytic GNP (*Serratia marscens*e and *Aremonase hydrophila* respectively) compare to the lower similar contamination ratio(4.8% for each) of these bacteria in raw milk of animals at second age group In contrast *Acinetobacter spp* appeared in higher ratio(19.04%) of second age group(>9 - <15 year) cows raw milk and lower ratio(15.2%) was observed in raw milk of cows at first age group (<3 - < 9 year).The association between age and cow raw milk proteolytic GNP contamination is considered to be not statistically significant (P>0.05).

Also in table (2) the cows were divided into two groups according to numbers of parturition. The results of this study revealed that the association between parturition and cow raw milk proteolytic GNP contamination is considered to be not statistically significant (P>0.05). However the cowswith high numbers of parturition(> 6-<12)

showed higher overall ratio(38.5%) of proteolytic GNP beside that high ratio of *Acinetobacter spp*(23.1%) ,*Aremonase hydrophila*(7.7%) and *Serratia marscens*e (7.7%) raw milk GNP contamination was observed in this group of cows compare to proteolytic GNP contamination ratio[*Acinetobacter* spp(14.9%) ,*Aremonase hydrophila*(6.9%) and *Serratia marscens*e (6.9%) with an overall ratio(27.6%)]of raw cows having low number of parturition (1 - < 6).

The effect of cows breed on PCR detection for raw milk proteolytic GNP contamination also illustrated in table(2).High overall ratio of proteolytic GNP contamination was observed in40% of crossbred cow raw milk followed by Friesian cows(32.1%).Beside that, raw milk of crossbred cows also showed a high ratio of contamination with*Acinetobacter* spp (20%) and*Aremonase hydrophila* (13.3%) while*Serratia marscens*e appeared as higher contaminant of Friesian cows raw milkwas 10.7%. However the association between the breed and cow raw milk proteolytic GNP contamination is considered to be not statistically significant (P>0.05).

Table (2):Prevalence of raw milk GNP isolates in cows

Variables		PCR results +ve n.(%)				
		Tested Samples n.=100	***Acinetobacter spp***	***Aremonase hydrophila***	***Serratia marscense***	**Total**
Age groups (year)	<3 - < 9	79(79)	12(15.2)	5(6.3)	6(7.6)	23(29.1)
	>9 - <15	21(21)	4(19.04)	1(4.8)	1(4.8)	6(28.6)
(P>0.05).						
Parturition (n.)	1 - < 6	87(87)	13(14.9)	6(6.9)	5(5.7)	24(27.6)
	> 6-<12	13(13)	3(23.1)	1(7.7)	1(7.7)	5(38.5)
(P>0.05).						
Breed	Native	57(57)	8(14.03)	4(7.02)	2(3.5)	14(24.6)
	Crossbred	15(15)	3(20)	2(13.3)	1(6.7)	6(40)
(P>0.05)	Friesian	28(28)	5(17.9)	1(3.6)	3(10.7)	9(32.1)

DISCUSSION

Multiplex PCR is an alternative detection method that can increase and broaden the detection sensitivity for the main pathogenic and deteriorative bacteria in milk and other food products (11).The usage of this method was the first basal objective of this study .The application of multiplex PCR by using the three primer pairs resulted in negative PCR amplification of extracted DNA in the current study. Therefore multiplex PCR approach has not been followed in this study because of the very high degeneracy of the primers. This assay would mean the presence of three paired of different primers in one

PCR reaction. As it was current study goal to amplify as much genes as possible out of a highly complex mixture of DNA, so that the project design was changed to work under optimal conditions for each primer pair using conventional PCR in combination with traditional identification methods.

Conventional PCR is sensitive and specific tools for identifying and characterizing psychrotrophs in raw milk samples .Many studies supported what was done in this study; (12) 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. (13); (14) indicated that the standard plate count procedure is often employed to detect psychrotrophic contamination in milk and in dairy products. however, this method is time consuming and does not allow for the rapid assessment of foods spoilage potential . (15)indicated that PCR-ribotyping and phenotypic characterization could be helpful in tracking contamination routes in the production line for milk pasteurization.(8) also reinforced the potential utility of PCR as a tool to monitor the quality of raw milk for consumption or for processing into other dairy products.

Depending on the sensitivity of PCR assays as an alternative, rapid method for monitoring the quality of refrigerated raw milk by detecting many psychrotrophs bacteria, in the current study three Gram negative psychrotrophs(GNP) were investigated including *Acinetobacter* spp, *Aeromonas hydrophila* and *Serratia marcescens*.

The use of PCR for raw milk analysis is affected by the presence of inhibitory substances such as Ca_2, fat and proteins (13; 16).To eliminate PCR antagonists and to increase sensitivity, (17) mentioned that preparation of total DNA from milk samples must be improved. Moreover, the target gene, the size of the amplified fragment and the contaminant microbiota can affect the sensitivity of PCR when using raw milk samples Therefore, improvement of DNA extraction and the use of target gene were important basal objective in the current study. To improve DNA preparation previous enrichment of milk was done by under refrigeration keeping for three days with subsequent milk

cultivation and total DNA extraction according to manufacturer information(Promega/USA).Other studies supported what was done in the present study as the study of (8) who used this method as one of three methods used to improve DNA extraction and minimize the presence of PCR inhibitors in DNA preparations and study of (18); (19) who mentioned that correct preparation of DNA samples is critical to ensure the dilution or elimination of PCR inhibitors and the success of the PCR technique.

Concerning the use of target gene and the size of the amplified fragment , (20), (21) and (22),mentioned that *Acinetobacter, P. fluorescens, Aeromonas hydrophila* and *Serratia marcescens* are the predominant populations in the microbiota of refrigerated raw milk.The simultaneous detection of these microorganisms can be carried out by amplifying protease-encoding genes from each species.In the current study to increase the sensitivity of PCR method the primers that amplify larger fragments of the *Acinetobacter* spp gene (SerA-F/SerA-R, 950bp), *A. hydrophila*(SerAh-F/SerAh-R,650) and *S. marcescens* (MetS-F/MetS-R,500 bp), were used according to (8). The sensitivity of the method can be increased with primers that amplify larger fragments of the gene. (17) achieved higher sensitivity than (23) using the same target aprX gene, but amplifying a larger fragment of approximately900 bp versus194 bp which was used by (23) .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 (24); (25).

The contaminant microbiota in raw milk, which reached numbers higher than 10^8cfu /mL after 4 days of incubation at 7 ^oC can also contribute to a reduction in the PCR sensitivity for the detection of studied psychrotrophs . (17) observed a reduction in the PCR sensitivity in the presence of other contaminants.In addition, other bacteria of the genus *Pseudomonas* can dominate the microbiota, as demonstrated by (26), who identified *Pseudomonas lundensis* and *Pseudomonas fragi*as the predominant producers of heat-resistant proteases in raw milk stored under refrigeration conditions.The primers (SerA-F/SerA-R, 950bp),(SerAh-F/SerAh-R,650) and (MetS-F/MetS-R,500 bp)used

by(8) can detect the protease-encoding genes only in the three relevant species of GNP, including *Acinetobacter sp*p, *A. hydrophila* and *S. marcescens*.

The observation of the amplified product of protease-encoding genes may indicate increase in the psychrotrophs populations and excess protease activity in raw milk as a result to three days storage of raw milk under refrigeration with subsequent enrichment of studied psychrotrophs inLuria - bertani broth medium.The present finding was supported by other researcher including, (8) who found that raw milk proteolysis was detectable on the fourth day of incubation at 7^0C, when milk casein fractions were observed by polyacrylamide gel electrophoresis. Prolonged storage of raw milk at low temperatures may result ina reduction of quality due to psychrotrophic protease activity, and such activity appears to correlate with the appearance of the SM2F/SM3R and 16S rDNA PCR amplification product. Both aprX and 16S rDNA genes, which were detectably amplified after 3 days of milk incubation at 7 ^{o}C, can be effective markers for monitoring milk decay, by *P. fluorescens*. In addition (27); (28); (29). observed degradation of raw milk due to proteolytic activity at psychrotrophs concentrations above 10^6cfu/ml.Since Protease production by psychrotrophic microorganisms occurs near the end of the logarithmic phase and progresses during the stationary phase.

The present results revealed that the GNP contamination(29%)was observed in 100 raw milk samples collected from 100cows.*Acinetobacter sp* were the most predominant bacteria **(** 16%**)** among the studied GNP contaminants of raw milk of cows, while *A.hydrophila* showed ratio(7%) and *S. marcescens* showed lower ratio (6%). The amplification results of the studied genes product of GNP in cow raw milk was considered to be highly statistically significant (P>0.001). This disagreement in the GNP PCR positivity ratio and sensitivity of the technique might be attributed to the presence of fat in whole milk . Considering that fat is a PCR inhibitor and, according to (30), it is more difficult to detect low numbers of bacterial cells by PCR in the presence of this constituent. This result demonstrates that the enrichment, step used in this study was not sufficient to minimize the presence of PCR inhibitors in DNA preparations. (31) reported the exact composition of bovine milk varies with individual animals, with breed, and with the season, diet, and phase of lactation. The percentages of the main constituents of milk

vary to a considerable extent among different species. (32) indicated that the percentage of milk fat differed according to species of animals.This result demonstrated that milk production was in poor hygiene conditions this fact confirmed by previous studies; (33) who mentioned that psychrotrophic bacteria are not part of the natural microbial population of the udder, and therefore their presence in raw milk is exclusively the result of milk contamination after milking. In addition (34) found domination of psychrotrophic bacteria in the total microbial population is even more pronounced when milk is produced in poor hygiene conditions and/or contains increased numbers of somatic cells .

The current overall ratio(29%)of GNP isolates was lower than that reported by (35); (36); (37) who mentioned that as a result of the post-pasteurization contamination around 50% of the milk packages can be spoiled by GNP after prolonged chill storage.Beside that (15) reported that 34.1 % of the milk packages showed contamination with GNP .(38) indicated that 20 different raw cow's milk samples from single farms and dairy bulk tanks were analyzed close to delivery to the dairies or close to processing in the dairy for their cultivable microbiota.Altogether, 2906 isolates have been identified as*Pseudomonas, Lactococcus* and *Acinetobacter* were the most abundant genera making up 62% of all isolates.

According to current results *Acinetobacter sp* were the most predominant bacteria **(16%)** among the studied GNP contaminants of cow raw milk samples , many previous studies confirmed this predominance. (22) said that *Acinetobacter* is one of the genera that compose a large portion of the dominant microbiota in raw milk.This study shows new microbial species which can develop during cold storage after milk treatment and contributes to identifying causes of reduced shelf life and deterioration of technological properties of milk during storage.(38) confirmed the presence of *Acinetobacter* spp as one of the predominant raw milk microbiota. (39) reported that 2,287 bulk tank milk samples were investigated ,*Acinetobacter* spp. were isolated from 7.7% of these samples.

In the present study*A.hydrophila*have been detected in 7 % of cow raw milk.In contrast (40) reported that twenty-two (15.9%) of the 138 cow's milk samples analyzed were contaminated with *A. hydrophila*. Eleven cow's milk samples (7.9%) were

contaminated with other *Aeromonas spp.* not classified. Eight (14.0%) of the 57 ewe's milk samples analyzed were contaminated with A. hydrophila.

According to present results *S. marcescens* have been detected in 6% of cow raw milk.The importance and presence of *S. marcescens* in refrigerated raw milk was confirmed by many studies including , (41) who observed six milk samples out of twenty five were contaminated with *Serratia marcescens* at a ratio of 24%.On the other hand (42); (43);(44) were in agreement with current results,as these workers have isolated psychrotrophs from refrigerated raw milk samples; about 3 to 6% of the isolated strains were *S. marcescens* .From the present study one may conclude that, refrigeration of raw milk for prolonged periods of time, at the dairy farm or at the dairy plant, can enhance its quality loss from the possibility of selection of proteolytic psychrotrophic bacteria. The potential risk of these psychrotrophic bacteria to human health reinforces the need to monitor these microorganisms in milk and dairy products.

الكشف المعتمد على تفاعل سلسلة البلمرة للبكتريا السالبة لصبغة غرام المحبه للبروده في الحليب الخام للأبقار

امجد عبد الرزاق فوزيه علي عبد الله
كلية الطب البيطري ،جامعة البصرة ، البصرة ، العراق .

الخلاصة

تقصي عن الجراثيم السالبة لصبغة غرام والمحبه للبروده كان الهدف الأساسي لهذه الدراسة .لتحقيق هذا الهدف وتأكيد وجود الحامض النووي منقوص الأوكسجينDNA للجراثيم السالبة لصبغة غرام والمحبه للبروده ، انجزت تقنية تفاعل سلسلة البلمرة التقليدي المعتمد على ثلاثة أزواج من البادئات تتضمنSerA لتضخيم قطعه من الحامض النووي منقوص الأوكسجينDNA لبكتيريا*Acinetobacter spp* بحجم950 bp و serA/*A.* hydrophila بحجم:650 bps وapr/*S. marcescens* بحجم 500 bps . في هذه الدراسة تم الحصول على29عزله بكتيريةمن عينات الحليب الخام لمئة بقره التي جمعت بصورة عشوائية ومن مختلف الأعمار والسلالات في مناطق مختلفةمن محافظة ذي قار والتي سبق تبريده لمدة72 ساعه أخضعت هذه العزلات للتشخيص المعتمد على مزارع الإغناء وتفاعل سلسلة البلمرة.كشفت النتائج الحالية عن ان النسبة الكليه لتلوث الحليب الخام بالبكتريا السالبة لصبغة غرام والمحبه للبروده كانت 29%.ومن بين البكتريا السالبة لصبغة غرام المحبة للبرودة الملوثة لعينات حليب الأبقار الخام كانت بكتريا*Acinetobacter spp* الأكثر سيادة. **(16%)**بينماأظهرت*A.hydrophila* اقل النسب (6%).اعتبرت نتائج لمنتجات الجينات قيد الدراسة للبكتريا السالبة لصبغة غرام المحبة للبروده في الحليب الخام للأبقار ذات معنويه إحصائية عاليه.(P>0.001).

تم استقصاء انتشار البكتريا السالبة لصبغة غرام المحبه للبروده في حليب الأبقار الخام اعتمادا على العمر وعدد الولادات والسلالة للحيوانات قيد الدراسة. اعتبر تأثير هذه العوامل على نتائج التشخيص المعتمد على تفاعل سلسلة

البلمرة غير معنوي إحصائيا(P>0.05) مع ذلك كانت النسبة الكليه (**29.1**%) لتلوث حليب الأبقار الخام ببكتيريا السالبة لصبغة غرام المحبه للبروده قيد الدراسةاذ لوحظت في حليب الأبقار بعمر9 > - 3> سنه. بصوره عامه أبقار الفئه العمريةالأولى(9 > - 3>) سنه أظهرت اعلى نسبة لتلوث الحليب الخام (7.6% و6.3%) ببكتيريا السالبة لصبغة غرام المحبه للبروده قيد الدراسة e*Serratia marscens*و*Aremonase hydrophila* على التوالي.

فيما يتعلق بعدد الولادات أظهرتالأبقار ذات العدد العالي من الولادات(12>- > 6) اعلى نسبه(38.5%) تلوث ببكتيريا السالبة لصبغة غرام المحبه للبروده وكذلك لوحظت في نفس المجموعة من الأبقار اعلى نسبه من *Acinetobacter spp*(**23.1**%) و*Aremonase hydrophila*(7.7%) و *Serratia marscens*e (7.7%). اعتمادا على السلالة لوحظت اعلى نسبه للتلوث ببكتيريا السالبة لصبغة غرام المحبه للبروده في 40% من حليب الأبقار الخام المضربة تليها أبقارالفريزيان 32.1%. بالإضافةإلى ذلك فان حليب الأبقارالمضربة الخام اظهر أعلى نسبة تلوث(20%) ببكتريا(13.3%)*Acinetobacter spp* , *Aremonase hydrophila* بينما ظهرت *Serratia marscens*eكأعلى ملوث لحليب أبقار الفريزيان الخام وان نسبة تلوث التلوث بها كانت .10.7%.

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