

Genetic detection to *Aeromonas hydrophila* proteolytic activity in milk samples (cows, buffaloes and goats) in Basra governorate

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(Received July 11, 2019; Accepted September 04, 2019; Available online 12 May 2020)

Abstract

This study aim is to determine the incidence and the virulence of *Aeromonas hydrophila* in raw milk, randomly collected from Basra governorate by using of polymerase chain reaction (PCR) technique. In this study, the total number of raw milk samples collected from cows, buffaloes and goats that kept from different the regions of Basra governorate were 90 samples. The PCR technique is modern method which regarded as a reliable tool to detect virulent gene of the *A. hydrophila* isolates. The PCR assays using the primers sets SerAh-F and SerAh-R resulted in the amplification of 650-bp bands from the targeted proteases gene of the *A. hydrophil*. The result of the present study showed that the results of PCR concerning the proteolytic activity of *A. hydrophila* in the tested raw milk samples according to animals' source. The higher percentage of the proteolytic activity was found in the cow's raw milk samples 40% and in the buffalo's milk samples was 26.7% while, the proteolytic activity did not find in the goat's milk samples. The association between the source of the milk sample and proteolytic *A. hydrophila* positive results was considered to be statistically highly significant. The higher percentage of the *A. hydrophila* isolates found in the raw cow milk was 40%, and the *A. hydrophila* isolates found in the raw buffalo milk was 26.7%, while, the *A. hydrophila* isolates did not find in the goat milk.

Keywords: Virulent factors, Psychrotrophs, Proteases, SerAh-F, SerAh-R

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الكشف الجيني للنشاط التحللي لبكتريا *Aeromonas hydrophila* في عينات حليب الأبقار والجاموس والماعز في محافظة البصرة

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الخلاصة

هدفت الدراسة الى تحديد نسبة البكتريا المحبة للبرودة الموجودة في عينات الحليب التي جمعت عشوائيا في محافظة البصرة، إضافة الى تحديد ضراوة العزلات باستخدام تفاعل البلمرة المتسلسل كأداة موثوقة للكشف عن جينات الضراوة. تم جمع ٩٠ عينة من حليب الأبقار والجاموس والماعز من مناطق مختلفة من المحافظة وتم استخدام تقنية تفاعل البلمرة للكشف عن جراثيم *Aeromonas hydrophila* وتم استخدام مزدوج من البرايمرات ذات الوزن الجزيئي بحجم ٦٥٠ قاعدة نيتروجينية. بعد عملية التضخيم للجين المرتبط بإنتاج أنزيم البروتيناز لوحظ أعلى نشاط للإنزيم قيد الدراسة في عينات حليب الأبقار بنسبة ٤٠% يتبعه عينات حليب الجاموس بنسبة ٢٦,٧% وكانت النتائج سلبية لعينات حليب الماعز. تم اعتبار العلاقة بين مصدر الحليب والنشاط التحللي للبروتين إيجابية، حيث كان من بين ٩٠ عينة هناك ٢٠ عينة أعطت نتائج إيجابية ذات دلالة إحصائية عالية بنسبة ٢٢,٢%، تمثلت بنسبة ٤٠% لعينات الأبقار يتبعها ٢٦,٧% لعينات حليب الجاموس مع وجود فروق معنوية عالية لعينات حليب الأبقار والجاموس والماعز.

Introduction

Aeromonas species are facultative anaerobic gram-negative microscopic organisms. It related to the family Aeromonadaceae, which is frequently isolated from various food and environmental sources including sea, river, fresh and ground water (1). The amassing information showing that *A. hydrophila* is the causal operator for 85% of human gastrointestinal issue (2). *A. hydrophila* is regarded the major pathogen causing extreme diarrhea and extra-intestinal contaminations in people (3). There are unassuming data on the commonness of *Aeromonas* species of mammals other than a man. *Aeromonas* has the ability to produce the various virulent factors which included the different types of hemolysins such as aerolysin, proteases, adhesions (adherence intervened by pili), invasins, a heat labile enterotoxin, phospholipase, and lipase (4,5). Lots of proteases delivered by psychrotrophic microscopic organisms are steady even under ultra-high temperature (UHT) condition and proceed actively in dairy products (6). *A. hydrophila* has a various extracellular catalyst, including proteases, hemolysins, and enterotoxins. A portion of the toxin has been biochemically portrayed, however, their particular role in the pathogenicity of *A. hydrophila* have not decided up to this point. The two noteworthy extracellular proteolytic activities of *A. hydrophila* that have been depicted similar to this, a 38-kDa thermostable metalloprotease and a 68-kDa temperature-labile serine protease are found in basically *A. hydrophila* culture supernatant (7). Isolation of *Aeromonas* spp. announced from raw milk, yogurt, and cheeses (7-9). The estimation of microbial tainting of cow's raw milk is fractional by cow's healthiness, cleanliness surroundings where dairy cows are housed, drained, strategies for udder readiness, draining system, techniques for cleaning, sanitation of draining machines and milk cisterns (10). The capacity of milk under refrigerated for the significant lot before preparing is segregating for development of psychrotrophs which may transform into the transcendent microflora (11). Beside, its developing worry as a blood borne pathogen *A. hydrophila* could take an interest in disintegration of the food store at refrigeration temperature due to its capacity to grow up and make thermo-resistant extracellular proteins (lipase, protease, amylase, and nuclease) that is ready to defiling significant milk constituent and consequently change the nature of completed dairy products (12). Any way little is perceived concerning the role of milk in the spread of these pathogens so this investigation was embrace as a component of an endeavor to decide the role of milk in the study of disease transmission of *Aeromonas* contamination, the propriety of the specific media for the restoration of the living being, the proteolytic action of detached *A. hydrophila*.

The targets of this investigation were to decide the occurrence of *A. hydrophila* in raw milk which randomly gathered from Basra governorate. As well as to determine the virulence of the isolates by using of PCR strategy as a reliable tool to detect virulent gene of some *A. hydrophila* isolates (Protease).

Materials and methods

Ninety raw milk samples were collected randomly from cows, buffaloes, and goats from different farms in Basra governorate. Aseptically placed into sterilized test tubes and transport to the research laboratory for investigation by using cooler box. The milk sample stored in refrigeration conditions for 3 days. The activation step recognized in the present study was prepared the 4.5 mL of Luria-Bertani broth inoculated with the raw milk samples at a concentration of 1% and incubation for 18 h at 37°C. Culture stocks were ready in LB broth comprising sterilized glycerol at a concentration of 20% and were cold at -20°C. Before each investigation, the isolates were cultivated two successive periods in LB broth (13).

PCR Amplification

To confirm the presence of proteolytic GNP DNA, a standard diagnostic PCR was carried using one pair of primers designed by Machado *et al.* (13) including SerAh *A. Hydrophila* (650bp) were oligonucleotide sequence for SerAh-F primer was 5-TTCCTCCTACTCCAGCGTCG-3, and for SerAh-R 5-TGATGATCCAGGCTCACGGT-3.

Proteolytic Enzymes

The manufacture of extracellular proteolytic enzymes was resolute by agar-diffusion assays on plate count agar containing nutrient agar complemented with 10% skim milk powder. The plates were incubated at 25 °C, for 18-24 h. The occurrence of clear zones around the colonies was an indication to the proteolysis.

Statistical analysis

Statistical analysis was done by using SPSS software from 11 to demonstrate any association between the results, the exact Fisher test and Pearson's chi-squared test with Yates correction were used to the limit of significance at level $P < 0.05$.

Results

PCR detection of proteolytic activity

The results of the present study showed that the higher proteolytic activity was observed in the cow raw milk samples 12 (40%), followed the buffaloes milk samples 8 samples (26.7%). While, the presence of proteolytic *A. hydrophila* in goats milk samples was not found. The

association between source of milk sample and proteolytic *A. hydrophila* positive results was considered to be statistically highly significant ($P < 0.01$). Out of 90 raw milk sample 20 (22.2%) samples were positive for proteolytic *A. hydrophila*. The positive cow milk samples represent 40% (12/30) of the total tested cow milk samples followed by 26.7% (8/30) of the buffalo milk samples with high significant difference ($P < 0.01$) among cows, buffaloes and goats milk sample (Table 1 and 2).

Table1: PCR detection of proteolytic activity of *A. hydrophila* in raw milk

Animals	Positive PCR	Negative PCR
	No. (%)	No. (%)
Cow	12 (40)	18 (60)
Buffalo	8 (26.7)	22 (73.3)
Goat	0	30 (100)
Total	20	70

Table2: Distribution of the positive PCR in total number tested milk samples

PCR	The milk samples no. (%)			
	Cow	Buffalo	Goat	Total
Positive	12(40.0)	8(26.7)	0(0)	20(22.2)
Negative	18(60.0)	22(73.3)	30(100)	70(77.8)
Total	30	30	30	90

Discussion

The responsibility of raw milk as a method of transportation of causing milk borne diseases is very much perceived (14). The microorganisms are regarded as a causative agent which causing mastitis. The milk borne pathogens can get to enormous numbers in mammary tissue and later release into milk. These microorganisms are normally present on homesteads (feed, water, faces, and soil) and would thus be able to taunt the udder and nipples of the bovine and get into milk (9).

Aeromonas and *Macrococcus* have been disengaged from the raw dairy animals' milk (14-17). *A. hydrophila* was the most overall species separated from the various types of tests 13.4% (39/292) (18). Subashkumar *et al.* (3) announced an aggregate of 105 milk sample was analyzed for *A. hydrophila* and 17.14% of the milk sample observed to be contaminated with *A. hydrophila*. The frequency and property of *Aeromonas* species in milk is the vehicle for the spread of *Aeromonas* gastroenteritis, due to the consequent treatment of the milk. A portion of the strains are difficult to separate from diarrhea-associated strains and ready to deliver exotoxin at 37°C and cling to the epithelial cells. The information announced in Ahmed *et al.* (19)

demonstrated that the distributions of virulence factor, to manage the pathogenicity of *Aeromonads*, is diverse in clinical and environmental test. The protease generation was begun all the more over and again in the samples, Ahmed likewise demonstrated that protease creation with higher bond limit, among strain separated from clinical sample (19). These are in concurrence with those revealed by Sechi *et al.* (20).

Variables of the virulence factors represent by extracellular proteases, hemolysins, and cytotoxins are produced by single virulent and a virulent strain of *A. hydrophila* to a similar inclusion (20). This reality just as the nonappearances of the relationship between the comparing action and degree of virulence may perhaps demonstrate to subjective factors as opposed to quantitative ones that include in the pathologic procedures (19). The lysis of psychotropic microbes may prompt the arrival of warmth safe proteolytic enzyme. The microflora of raw milk has been accounted for to change contingent upon the area of the herd, rearing practices, and lactation period (18). The present examination used PCR the most encouraging strategies for the quick and explicit analysis of numerous microscopic organisms in the various milk samples .

Bedeltavana *et al.* (21) showed that the PCR-ribo typing and phenotypic portrayal could steady in tracking contamination routes for the creation line of milk purification. Also, the target gene, the augmented piece measure and the contaminant microbiota can be influenced to affectability of PCR when using the raw milk sample (21). Along these lines, the promote of DNA extraction and utilize of target gene are the principle basal article in the present study. Machado *et al.* (13) showed that this technique as a one of three strategies used to promote DNA extraction and decrease the incidence of PCR inhibitor in the DNA arrangement and the investigation of Maurer (22) and Pirondini *et al.* (23) who referenced that definite planning of DNA sample is serious to ensure a mitigation or expulsion of PCR inhibitors and the achievement of the PCR procedure (13,24). These two studies bolsters what was done in the present investigation.

The contaminant microorganisms in raw milk, the all-encompassing statistic supper than 108cfu/mL following four days of hatching at 70°C can likewise add to diminish in the PCR sensitivity for recognition of considered psychrotrophs Marchand *et al.* (24) saw a decrease in the PCR sensitivity in nearness of different poisons, additionally other microbial species are probably going to be a rule microscopic organisms in the cooled raw milk (24). The primers SerAh-F/SerAh-R650bp utilized by Machado *et al.* (13) recognize the protease-encoding gene just in the *A. hydrophila*. The examination of the enhanced result of protease-encoding genes may show increment in the psychrotrophs populace and over-burden protease movement in raw milk for the outcome to three days of

refrigeration to raw milk with consequent improvement of considered psychrotrophs.

In connection to the utilization of target gene and the size of the developed part, which referenced *A. hydrophila* is the primary masses of the microorganisms in cooled raw milk. The quick acknowledgment for these microscopic organisms can be passed out by the method for expanding protease-encoding qualities from this species (13). Consequences of other examination demonstrated to all isolates of the capacity were protease makers in varying degree. The distinction in the capacity of the segregates to create protease were attributable to a hereditary variety of beginnings mindful underway of protease. Inside other investigation, it found that 100% of *A. hydrophila* detach can create protease with various degrees. Which in concurrence with our study (25). In the present investigation *A. hydrophila* identified in 40% of bovine raw milk. In distinction Melas *et al.* (26) had announced that 25 (15.9%) of (138) the dairy animals' milk sample were polite with *A. hydrophila*.

In eleven dairy animals' milk test 7.9% is tainted with different *Aeromonas* spp. Eight (14.0%) of the 57 milk tests inspected, were sullied of *A. hydrophila* (26). In studies which concurrence with current outcomes, found that psychrotrophic populace cooled at 5-10°C enhanced, especially more than at 0-5 °C. This outcomes in concurrence with the investigation by Rasolofu *et al.* (27) that discovered psychrotrophic populaces expanded inside 72h when refrigerated at 8°C not at 4°C, and that the elements of microscopic organisms indicated huge variety for samples from cow, buffalo, and goat (27,25).

Other study uncovered that the capacity of *A. hydrophila* to deliver virulence factors and attack epithelial cells while put away at refrigeration temperature for 7-10 days (28,29). Likewise, a proof (100%) of *Aeromonas* spp. cytotoxic impact was constant at refrigeration temperature (2,30). Our examination found a superior pervasiveness of protease in cow sample, among strains isolated from samples. These discoveries are in concurrence with those revealed by (19,20). Studies in addition confirmed of proteolytic activity is affected by culture medium and temperature (6). The heat resistant protease secretability of *Aeromonas* spp detailed by different scientists (13,18,31). Cooling of raw milk for delayed period, can improve the loss of excellence from the likelihood of proteolytic psychrotrophic microorganism. The hidden danger of these psychrotrophic microorganisms for human health fortifies the need to show these microorganisms in milk and dairy product. Occurrence of Gram negative psychrotrophs and their protease, lipase, and biofilm catalyst in milk implying that milk is a vehicle to transmission of these microorganisms, due to the subsequent administration of the milk. Some of strains were difficult to separate from the

diarrhea- related strains that ready to create exotoxin at 37°C and hold fast to epithelial cells.

Gomashe *et al.* (32) detailed that *Aeromonas* species had proteolytic activities at room temperature yet extreme lessening in action was distinguished at high temperatures (32). Distinguished frailty of raw milk due to proteolysis action at psychrotrophic concentration surpassing 106cfu/ml. In the interim proteolytic catalyst's generation by psychrotrophic microscopic organisms emerges close-by the completion of the logarithmic stage and advances through the stationary stage milk at a proportion of 16 and 7% individually.

The current results revealed that the gram negative psychrotrophs (GNP) contaminated overall 24.7% observed in 90 raw milk sample collection from studied animals of different species in 30 cows, 30 buffaloes, and 30 goats. Higher ratio of the GNP contamination was observed in cows 12 (40%) followed by followed by buffalo's milk samples in which 8 samples (26.7%) were positive and negative results for the presence of proteolytic *A. hydrophila* in goats milk samples.

The influence of the animal's genus on the multiplication result of the examined gene product of the GNP in cow, buffalo, and goat raw milk which was estimated to be highly statistically significant ($P \leq 0.001$). This outcome shows that there was a distinction in the PCR positivity ratio among the three types of creature's raw milk. That distinction in the GNP PCR inspiration proportion and affectability of the methodology must perceive to the participation of lipid in milk.

This study demonstrated that raw goat milk ought not be put away for over multi day at 4°C (33). When put away for in excess of 54 h in a dairy plant tank at refrigerated temperature the microbial populace moved, with *Pseudomonas* and *Acinetobacter* ending up progressively predominant. This concurred with the discoveries of this investigation about goat milk. The populace shifts which happen during long term stockpiling at refrigeration temperature at $\leq 4^\circ\text{C}$. Learns about goat milk demonstrated that microscopic organisms inside the *Lactococcus* and *Streptococcus* genera commanded the raw milk microflora when accumulated up to 24 h, yet expanded capacity brought about an outgrowth of *Pseudomonas* and *Acinetobacter*, which contained over 60% of the all-out populace at 72 hours (18). The increase of different bacterial species which not in participation to any noteworthy level in the samples and furthermore a lesson of the novel predominant species equivalent to note by (34,35).

Despite this present variety in the GNP PCR positivity proportion among the three gathering of creature's raw milk. The outcome affirmed the milk item was in decreased cleanliness condition this fact exhibited by before studies; Munsch *et al.* (36) who referenced the

psychrotrophic microorganisms are not a piece of natural bacterial populace on the udder, along these lines the occurrence in raw milk is totally the result subsequent to infectivity of after milking (37). Also virulence of gene at different in the activation related with environment (38).

The use of PCR technique require four hours after the primary isolation. Also provide analytical information, may take some days in the usual test for detection bacteria. End result show a high degree of specialty and more accurate in detection of bacteria as well as it provided the effort, speed and cost in diagnosing the pathogenic bacteria (39).

Conclusion

Psychrotrophic microbes are regularly present on homesteads and in this way can taint the outside of nipples and udder of the bovines and gets into milk. *A. hydrophila* it is the for the most part overwhelming species disengaged from exceptional types of test. Some of strains were indistinguishable from looseness of the bowels related strain and ready to create exotoxin sp. The improvement of DNA extraction and utilizations for target quality significant essential things in this investigation and we utilized. The preliminaries SerAh-F/SerAh-R, 650bp to recognize the protease-encoding quality just in the *A. hydrophila*. The examination of the intensified result of protease-encoding quality can be an indication of improve in the psychrotrophs populaces and over-burden protease movement in crude milk as result to three days stockpiling of raw milk in refrigeration by later upgrade of considered psychrotrophs. Result demonstrated that *A. hydrophila* identified in 40% of cow crude milk and this distinction in capacity of the disengages for produce protease are because of hereditary variety of beginnings mindful into the generation of protease. Likewise found that psychrotrophic populaces refrigerated at 5-10°C enhanced more than those at 0-5°C. The populace shifts which happen during long haul stockpiling at refrigeration temperature $\leq 4^{\circ}\text{C}$.

Acknowledgment

I am grateful to all of those with whom I have had the pleasure to work during this and other related articles. I would especially like to thank Dr. Fawzia Ali. As my teacher and mentor, he has taught me more than I could ever give him credit for here. He has shown me, by his example, what a good scientist (and person) should be. Also I would like to thank reviewers for assistance comments that greatly improved the manuscript.

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