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RESEARCH ARTICLE

Direct Molecular Detection and Phylogenetic Relationship of *Staphylococcus aurous* from Mastitis Milk in Sheep and Cows

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Abstract

The present study included the direct detection of *Staphylococcus aurous* isolated from milk of sheep and cows infected by mastitis based 16SrRNA gene by PCR technique and study the phylogenetic relationship analysis based on spa gene of using DNA sequence method. The study includes collected 32 sheep and 45 cow's mastitis milk samples. These samples were submitted to milk DNA extraction, and then performed PCR technique. The PCR results were showed significant at P value: (< 0.05) in prevalence between sheep and cow *Staphylococcus aurous* positive isolates at (5/15.5%) and (14/31.1%) respectively. DNA sequencing of partial sequence of spa gene was showed *Staphylococcus aureus* sheep isolate was genetic relationship analysis different than cow isolate and related to NCBI-Blast *Staphylococcus aureus* isolate H13939 (AM407304.1) at (99%) homology sequence identity and NCBI-Blast *Staphylococcus aureus* strain AO124 (JX912490.1) at (94%) homology sequence identity total genetic change (0.005). In conclusion, *Staphylococcus aurous* isolated from mastitis milk in sheep and cows were not related genetically. Therefore, Spa gene analysis was provides an efficient method for investigating phylogenetic relationships among different clinical isolates and can be useful for monitoring distribution of pathogen related to public health.

Keywords: *Staphylococcus aureus, Milk, Sheep, Cow, PCR, 16SrRNA, spa gene.*

Introduction

The most noticeable pathogenic of Staphylococcus spp. is *Staphylococcus aureus*, numerous strains of which cause a range of diseases [1]. *Staphylococcus aureus* is the main mastitis pathogen that affecting significant sufferers in the dairy industry [2, 3]. Bovine mastitis is inflammation of the mammary glands and itis a major disease affecting dairy herds over all the world and *Staphylococcus aureus* is known as one of the most commonly isolated pathogen in cases of clinical and subclinical mastitis and recorded as one of the most contagious bacteria causing subclinical mastitis [4]. In utmost clinical laboratories, identification approaches dependent on bacterial culture of milk.

The technique of culture examination is though less sensitive method and time losses [5]. Therefore, Molecular method such as (PCR) based diagnostic method to detect various mastitis causing pathogens is a fast sensitive and dependable method to resolution bacterial causes of mastitis milk [6, 7].

The spa gene occurs in all strains of *Staphylococcus aureus*, can occupation as a genetic marker use in distinguish bacterial strains at the species level. Hence, due to these benefits, we used spa typing and the Based upon Repeat-Pattern (BURP) to assign the clonal and phylogenetic relationships of *Staphylococcus aureus* strains [8]. Spa typing as a useful method for conveying phylogenetic relationships to strains [9]. Protein A (spa) is a 42kDa protein and consider as important virulence factor complicated in the pathogenesis of S. aureus [10, 11]. The present study aimed to direct detection of *Staphylococcus aurous* study phylogenetic relationship of Staphylococcus aurous from mastitis milk in Sheep and Cows.

Materials and Methods

Samples Collections

32 milk samples from sheep and 45 milk from cow were collected from clinical mastitis infection that identified by California mastitis test (CMT) from different animal

fields in Diwanyiah city. The milk samples were collected in sterile containers after clean and washing the quarters of udder by disinfectant solution, then the milk samples transported in ice bag into laboratory and stored in 4ºC a refrigerator until use for bacterial isolation.

Milk Bacteria DNA Extraction

The bacteria isolates were subjected to bacterial nucleic acid extraction from milk according to method described by Tarate [12] by using commercial DNA extraction kit (Presto Bacteria DNA Kit. Geneaid, Biotech Ltd. Taiwan).The method was carried out depend on the instructions provided with kit using gram-negative bacteria Protocol extraction method with (10 mg/ml) lysozyme buffer.

Nanodrop UV-spectrophotometer

The extracted DNA from milk was estimated by Nanodrop UV-spectrophotometer device at two wave length 260/280nm, and then kept at freezer until used in PCR master mix.

PCR Technique

PCR technique was performed for molecular detection of *Staphylococcus aurous* isolates based on 16S ribosomal RNA gene and phylogenetic analysis based spa gene:

Primers

The PCR primers that used in this study for direct detection of *Staphylococcus aurous* based on 16S ribosomal RNA gene was designed in present study using NCBI Gene sequence data base (NR_118997.2). Wherase, spa gene specific primers were designed by Harmsen [13]. "These primers were provided from Macrogen Company, Korea as following Table (1).

Table 1: PCR primers and their nucleotide sequence

Oligonucleotide Primer	Nucleotide sequence 5'-3'	Amplicon size
16SrRNA gene	GCGGTAATACGTAGGTGGCA	914bp
	CGGCTTCGGGTGTTACAAAC	
Spa gene	TAAAGACGATCCTTCGGTGAGC	443bp
	CAGCAGTAGTGCCGTTTGCTT	

PCR Master Mix Preparation

"The reaction mix was prepared using (Maxime PCR Premix Kit, iNtRON. Korea) master mix reagent and done depend on company instructions as following Table (2).

Table 2: Company instructions of PCR master mix

QPCR master mix	Volume
Genomic DNA template 5-50ng/ µL	2.5µL
16SrRNA Forward primer (10pmol)	1µL
16SrRNA Reverse primer (10pmol)	1µL
PCR water	15.5µL
Total volume	20µL

Table 3: Company instructions of PCR master mix

qPCR master mix	Volume
Genomic DNA template 5-50ng/ µL	2.5µL
Spa Forward primer (10pmol)	1µL
Spa Reverse primer (10pmol)	1µL
PCR water	15.5µL
Total volume	20µL

"After that, the PCR mix that revealed in table above placed in Maxime PCR Premix kit that contain all other PCR components which needed to reaction such as (Taq DNA polymerase, dNTPs, 10 PCR buffer). Then, all the PCR tubes transferred into vortex centrifuge for 3 minutes. Then transferred into thermocycler (T100 Thermal cycler Bio Rad. USA)".

PCR Thermo-cycler Conditions

Table 4 PCR thermo-cycler conditions

PCR step	Temp.	Time	repeat
Initial Denaturation	95 ºC	5min	1
Denaturation	95 ºC	30sec.	30 cycle
Annealing	58 ºC	30sec	
Extension	72 ºC	1 min	
Final extension	72 ºC	5min	1
Hold	4 ºC	Forever	-

Table 5: PCR thermo-cycler conditions

PCR step	Temp.	Time	Repeat
Initial Denaturation.	95 ºC	5min	1
Denaturation.	95 ºC	30sec.	30 cycle
Annealing.	59.8 ºC	30sec	
Extension.	72 ºC	1 min	
Final extension.	72 ºC	5min	1
Hold.	4 ºC	Forever	-

PCR Product Analysis

The PCR products were examined by agarose gel electrophoresis in (1% agarose gel) using 1X-TBE buffer, stained with ethidium bromide 10mg/ml, and investigation under UV transilluminator.

DNA Sequencing Method

DNA sequencing was achieved to analysis of phylogenetic relationship between two *Staphylococcus aureus* sheep and cow isolate, also as well as NCBI-Blast *Staphylococcus aureus* isolate based on spa gene. The PCR products of spa gene were sent to Macrogen in Korea for performed the DNA sequencing. The DNA sequencing analysis was conducted by using phylogenetic tree UPGMA method (MEGA 6.0 version), Multiple alignment analysis of the partial sequences of spa gene based ClustalW alignment analysis, and NCBI-BLAST for homology sequence identity.

Statistical Analysis

Statistical analysis were achieved by using (Statistical Package for the Social Science) (SPSS) version 17. Therefore, the prevalence and significant variances performed with Chi-Square test at ($P<0.05$).

Results

A total 77 milk samples from sheep and cows were tested by California mastitis test (CMT) and then subjected to PCR based molecular detection for the samples was carried out. CMT results revealed that all samples are affected by mastitis. The PCR technique was showed direct detection of *Staphylococcus aurous* isolates from mastitis milk of sheep and cow samples. The PCR technique was based on 16S rRNA gene as showed in following Table (6).

Table 6: The PCR technique results according type of mastitis milk samples

Sample type	Total samples	Positive isolate	Percent
Sheep milk	32	5	15.5%
Cow milk	45	14	31.1%

Chi-square test showed non-significant at P value: (< 0.05)

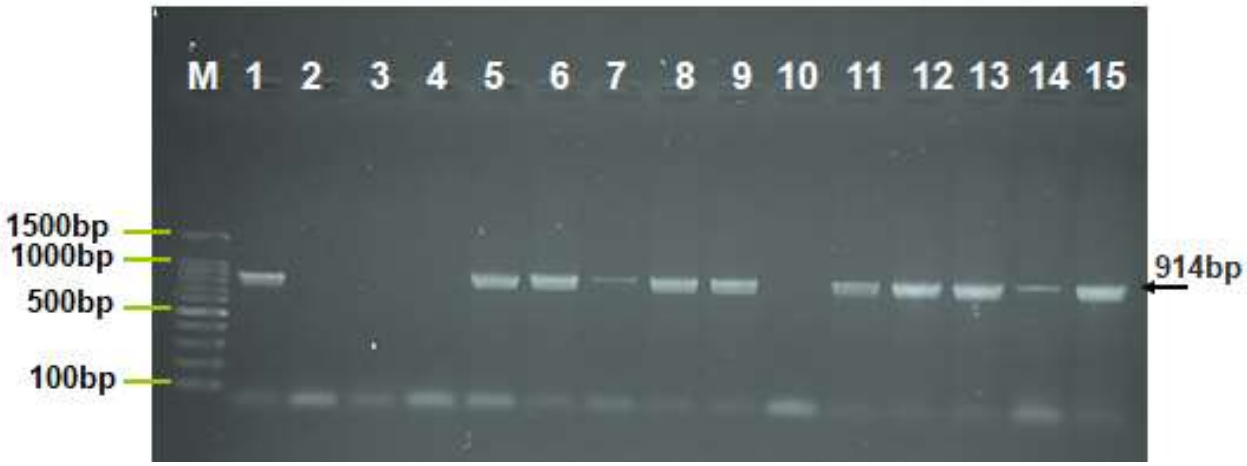

Figure 1: "Agarose gel electrophoresis of PCR assay show the some positive results of 16SrRNA gene in mastitis milk *of Staphylococcus aurous sheep and cow isolates.* Where, Lane (M) DNA marker (2000-100bp)", Lane (1-10) some positive *S. aurous cows* isolates Lane (11-15) positive *S. aurous* sheep isolates at 914bp PCR product size

The PCR technique also used for detection spa gene was showed confirmative detection in all positive mastitis milk *Staphylococcus aurous* sheep and cow isolates as showed in Figure (2).

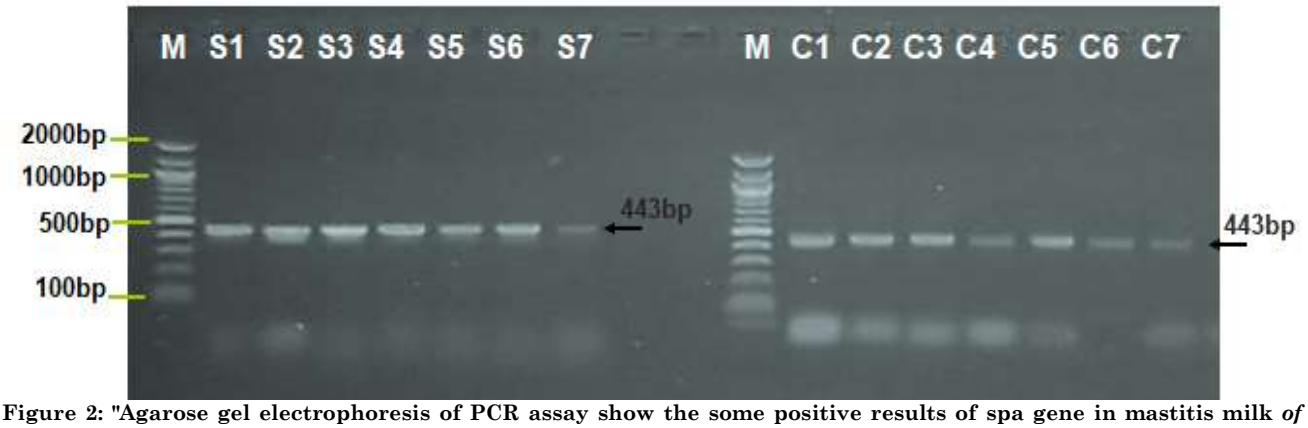

Figure 2: "Agarose gel electrophoresis of PCR assay show the some positive results of spa gene in mastitis milk *of Staphylococcus aurous sheep and cow isolates.* Where, Lane (M) DNA marker (2000-100bp)", Lane (S1-S7) positive *S. aurous sheep* isolates Lane (C1-C7) positive *S. aurous sheep* isolates at 443bp PCR product size.

DNA Sequence Analysis Results

DNA sequencing analysis based on partial sequence of 16S ribosomal RNA gene were show the phylogenetic relationship and homology sequence identity between local cattle *Escherichia coli* isolates and NCBI-BLAST isolates as show in following figures:

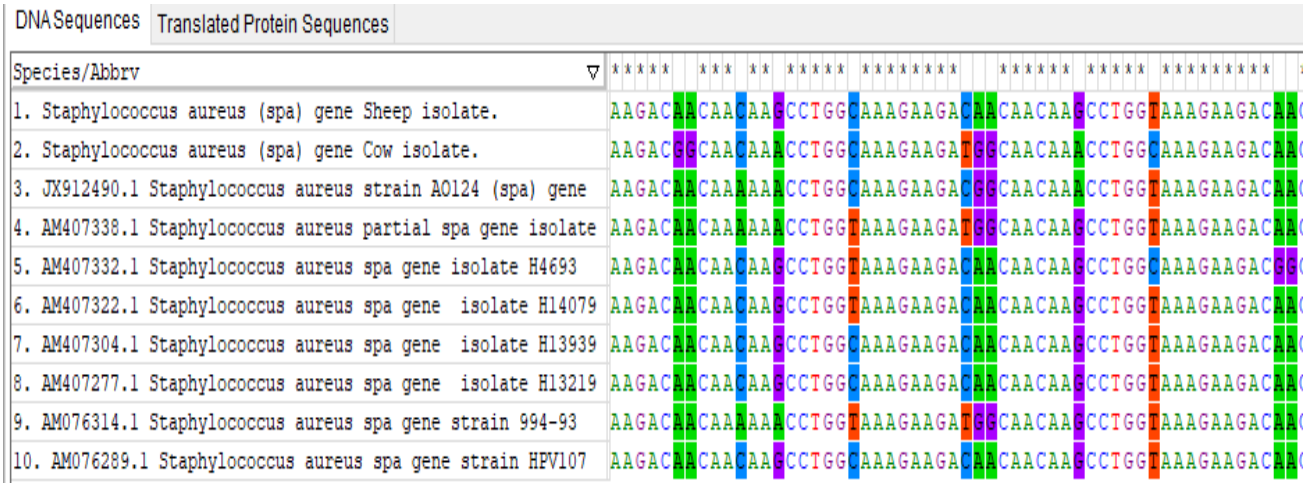

Figure3: Multiple sequence alignment analysis of the partial sequence based on (spa) gene for immunoglobulin G binding protein A in two local of *Staphylococcus aureus* sheep and cow isolate based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis was showed some similarity (*) and differences in (spa) gene nucleotide sequences

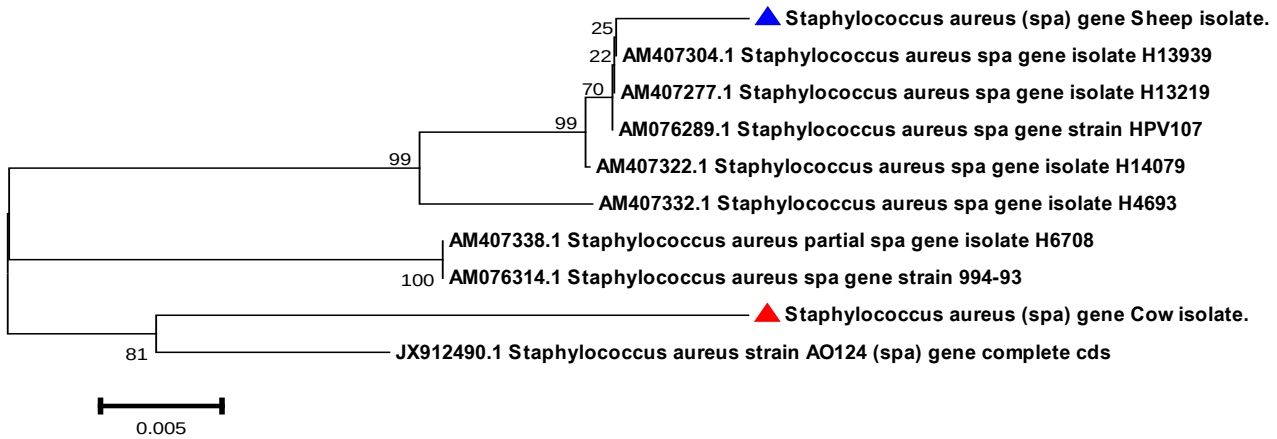

Figure 4: Phylogenetic tree analysis based on (spa) gene for immunoglobulin G binding protein A partial sequence that used for genetic relationship analysis of *Staphylococcus aureus* sheep and cow isolate. The phylogenetic tree was constructed using the evolutionary history was inferred using the UPGMA method (MEGA 6.0 version). The *Staphylococcus aureus* sheep isolate was genetically different than cow isolate and related to NCBI-Blast *Staphylococcus aureus* isolate H13939 (AM407304.1) and NCBI-Blast *Staphylococcus aureus* strain AO124 (JX912490.1) at total genetic change (0.005)

Table 7: NCBI-BLAST Homology sequence identity

Local Staphylococcus aureus	NCBI-BLAST Homology sequence identity		
	NCBI-Blast *Staphylococcus aureus*	Association number	Identity (100%)
Staphylococcus aureus sheep isolate	*Staphylococcus aureus* isolate H13939	AM407304.1	99%
Staphylococcus aureus cow isolate	*Staphylococcus aureus* strain AO124	JX912490.1	94%

Discussion

The results of this study showed that *Staphylococcus aureus* can be detected in mastitis milk samples of sheep and cows based on direct molecular PCR technique with variation in prevalence. Our results were showed highly prevalence of *Staphylococcus aureus* in sheep milk samples compared to cow milk samples (5/15.5%) and (14/31.1%) respectively**.** The prevalence of *Staphylococcus aureus* in cow milk samples at this study was consistence with many previous studies, study in Bangladesh by Jadhav (6) who revealed presence of S. aureus in (28.5%) of the total animals screened.

Study by Shome [14] "who used PCR based detection of mastitis pathogens in bulk tank milk samples revealed highest incidence rate for S. aureus followed by S. epidermidis and E. coli in areas around Bangalore dist". The less prevalence of *Staphylococcus aureus* in sheep milk samples at this study was consistence with study in Iran by Rahman [15] who revealed that prevalence of S. aureus infection (7.14%) and these observation was less than the prevalence reported in previous studies [16, 17]. This might be due to the improvements in livestock environmental management and teat health in the study areas. In this study, we used the *spa* gene of *Staphylococcus aureus* to study the phylogenetic relationship analysis between *Staphylococcus aureus* sheep and cow isolate. The *spa* typing method is sequence-based methods that presumably are useful in phylogenetic studies and can assign strains to phylogenetic relationships because this method depend on the and repeat polymorphic region in immunoglobulin G binding protein A gene nucleotide sequences [18].

DNA sequencing analysis of spa gene was showed according to phylogenetic relationship of *Staphylococcus aureus* sheep isolate was different than cow isolate and related to NCBI-Blast *Staphylococcus aureus* isolate H13939 (AM407304.1) at (99%) homology sequence identity and NCBI-Blast *Staphylococcus aureus* strain AO124 (JX912490.1) at (94%) homology sequence identity total genetic change (0.005). In conclusion, *Staphylococcus aurous* isolated from mastitis milk in sheep and cows were not related genetically. Therefore, Spa gene analysis was provides an efficient method for investigating phylogenetic relationships among different clinical isolates.

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