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Isolation, identification and molecular characterization of *S. aureus* from raw milk sold in Bikaner

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Abstract
The research work was conducted to isolate, identify and characterize *Staphylococcus aureus* in raw milk sold in Bikaner. Detection of *S. aureus* was performed on the basis of isolation, culture and colony characterization. Further, characterized by Polymerase Chain Reaction (PCR) by 23*s rRNA ribotyping*. One hundred milk samples were collected from various sources like dairy farms (n=30), milk pooling booths (n=30), and local milk vendors (n=40) in Bikaner, Rajasthan. Isolation of *S. aureus* was done on selective media mannitol salt agar (MSA) and identification of *S. aureus* was done by colony characteristics, Gram's staining and for biochemical tests HiStaph™ commercial kits were used. Out of 100 raw milk samples analysed, 65 samples (16 from dairy farms, 20 from pooled milk and 29 from local vendors) were found positive for *S. aureus*. The prevalence of *S. aureus* was 53.33%, 66.67% and 72.5% for raw milk collected from dairy farms, pooled milk and local vendors, respectively. 59 out of 65 isolates of *S. aureus* tested were found coagulase positive. All the 65 isolates of *S. aureus were* subjected for molecular characterization by using species-specific primer targeting *23s rRNA* gene and all were found positive for *23s rRNA* gene. The value of chi square test was found 2.82 and P= 0.244 revealed that there is no significant (P>.05) association between sources of milk samples and presence of *S. aureus.*

Keywords: milk, *staphylococcus aureus*, polymerase chain reaction, biochemical tests, chi square, p value

Introduction
Milk is the normal mammary secretion derived from complete milking of healthy milch animal without either addition there to or extraction there from. It shall be free from colostrum (Food Safety and Standards Authority of India, 2009) [6]. Milk in its natural form has a high nutritive value as it is a good source of quality proteins, fats, carbohydrates, vitamins and minerals (Neumann *et al.*, 2002) [17]. Production of milk under unsanitary conditions and poor production practices can exert both a public health and economic constraints (Swai and Schoonman, 2011) [25]. *S. aureus* considered as the third most important cause of disease in the world among the reported food borne illness is due to its capability to produce a wide range of heat stable enterotoxins. *S. aureus* can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing of raw milk (Normanno *et al.*, 2005) [18].

Materials and Methods
Collection of milk samples
A total of one hundred raw milk samples were randomly collected from dairy farms (n=30), pooled milk sources (n=30) and local vendors (n=40) of Bikaner, (Rajasthan).

Isolation and identification of *Staphylococcus aureus* (*S. aureus*)
Raw milk samples were subjected to aerobic cultivation. Each sample was streaked on nutrient agar plates in primary, secondary, and tertiary fashion in order to obtain isolated colonies of bacteria. These petri plates were incubated for 24 hr at 37^0c. After 24 hr incubation these isolated colonies were cultured on mannitol salt agar (MSA), plates for isolation of *S. aureus.* The growth was examined for the colonial morphology and pigmentation and in order to obtain pure culture different types of colonies were sub-cultured on separate nutrient agar plates. The confirmation of the isolates as *E. coli* and *S. aureus* were done using Gram's staining, coagulase test and a set of 12 biochemical tests provided in HiStaph™ Identification Kit (HiMedia, Mumbai) for *S. aureus*.

23 S rRNA* gene-based genotypic identification of *S. aureus

The genotypic confirmation was done through *23S rRNA* ribotyping as per the method described by Straub *et al.* (1999) [24] using the primers as mentioned in Table-1.

Table 1: Primers used for amplification of *S. aureus* isolates from raw milk samples

S. No.	Oligo Name	Sequence (5'-3')	Size of amplified product (bp)	Reference
1.	*23S rRNA*	F- ACGGAGTTACAAAGGACGAC R- AGCTCAGCCTTAACGAGTAC	1250 bp	Straub *et al.* (1999) [24]

The PCR was performed in Veriti Thermal Cycler (Applied biosystem) using the following cycling parameters (Table-2).

Table 2: Steps and conditions of thermal cycling for PCR of *S. aureus* isolates

Cycle	Step	Temperature (°C)	Duration
Cycle 1	Pre denaturation	95	5.0 min
Cycle 2-36	Denaturation	94	40 sec
	Primer annealing	64	1 min
	Primer extension	72	1.15 min
Cycle 37	Final extension Incubation	72	3 min

Results and Discussion

On the basis of morphology and biochemical characteristics, 65 samples (16 from Dairy farms, 20 from pooled milk and 29 from local vendors) were found positive for *S. aureus.* The prevalence of *S. aureus* was 53.33%, 66.67% and 72.5% for raw milk collected from dairy farms, pooled milk and local vendors, respectively (Table-3 and Figure-1). The value of chi square test (2.82) and P value (0.244) revealed no significant (*P*>.05) association between sources of milk and presence of *S. aureus*.

Jorgensen *et al.* (2005) [7] reported very high prevalence who found 96.2% samples being positive for *S. aureus.* On the other hand Majalija *et al.* (2020) [10]; Patel *et al.* (2018) [19]; Reta *et al.* (2016) [21]; Cortimigilla *et al.* (2015); Mistry *et al.* (2015) [12]; Fadaei (2014) [5]; Sadek *et al.* (2014) [22]; Vahedi *et al.* (2013) [26] and Chu *et al.* (2012) [2] have reported lower prevalence as 46%, 10.16%, 24.2%, 43.1%, 46.67%, 41.66%, 46%, 22% and 16.75%, respectively. De Oliveira *et al.* (2011) and Lingathurai and Vellathurai (2013) [9] examined raw milk samples for the presence of *S. aureus* in milk and 34 (68%) and 37(61.7%) samples showed the presence of *S. aureus*, respectively. The above findings are very close to present findings which is 65%. On the other hand Mehra, (2007) [11] found 78% prevalence of *S. aureus* which is higher to our findings.

Ren *et al.* (2020) [20] examined 84 raw milk samples collected from southern Xinjiang, China and found 65 isolates of *S. aureus* and in the present findings we also found 65 isolates of *S. aureus* from 100 milk samples examined.

Table 3: Number of isolates of *S. aureus* from raw milk samples collected from various sources in Bikaner

S. No.	Sources of milk samples	No. of samples	Number of isolates		
			S. aureus		
			+ve	-ve	Prevalence
1.	Dairy farms	30	16	14	53.33%
2.	Pooled milk	30	20	10	66.67%
3.	Local vendors	40	29	11	72.5%
Total		100	65	35	65%
Chi square value			2.82		
P value			0.244		
Significance			Non-significant		

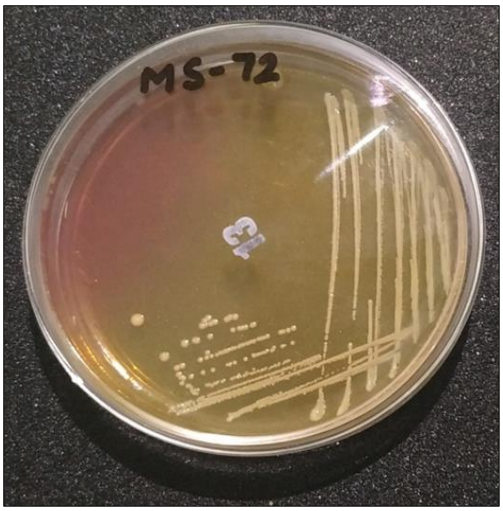

Fig 1: Isolation of *S. aureus* on mannitol salt agar from raw milk sample

Biochemical characterization of *S. aureus* isolates

In the present study, all Gram positive cocci growing on mannitol salt agar were subjected to confirmation of *S. aureus*. *S. aureus* produced glistening, smooth and round colonies with variable degree of pigmentation (golden yellow) on mannitol salt agar (Figure-1). Out of 65 phenotypically identified isolates 59 (90.77%) isolates produced coagulase. Confirmation of *S. aureus* was done by using HiStaph™ commercial kits. The results of various biochemical tests were shown in (Table-4 and Figure-2).

Table 4: Results of biochemical tests for *S. aureus* using HiStaph™ commercial kits

Sr. No.	Test	Positive		Negative	
		Number	%	Number	%
01.	Voges proskauer's	59/65	90.77	06/65	09.23
02.	Alkaline phosphatase	65/65	100	-	-
03.	ONPG	15/65	23.08	50/65	76.92
04.	Urease	65/65	100	-	-
05.	Arginine utilization	65/65	100	-	-
06.	Mannitol	42/65	64.62	23/65	35.38
07.	Sucrose	65/65	100	-	-
08.	Lactose	65/65	100	-	-
09.	Arabinose	20/65	30.77	45/65	69.23
10.	Raffinose	51/65	78.46	14/65	21.54
11.	Trehalose	65/65	100	-	-
12.	Maltose	65/65	100	-	-

Our results are in agreement to those of Kateete *et al.* (2010) [8] who investigated 29 (91%) coagulase positive isolates of *S. aureus* out of 32 phenotypically identified *S. aureus.*

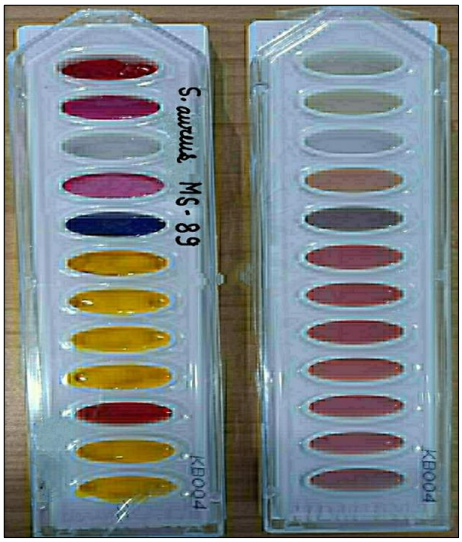

Fig 2: Results of biochemical tests for *E. coli* using HiE. Coli TM commercial kits

Genotypic confirmation of *S. aureus*

S. aureus isolates were genotypic confirmed by *23s rRNA* species specific based ribotyping. The DNA based methods, developed a *23s rRNA* gene based PCR system which producing species specific PCR product of 1250 *bp* size allowing species detection of all strains of species specific and essentially identifies *S. aureus*. Our findings were in agreement with Straub *et al.* (1999) [24].

In the present investigation, all the 65 isolates from raw milk samples were subjected to *23s rRNA* based ribotyping for genotypic confirmation. The ribotyping produced an amplicon of 1250 *bp* and all the isolates confirming them to be *S. aureus.* Nazari *et al.* (2014) [16] observed *S. aureus* in 52 samples of 246 raw milk samples and according to the results of PCR assay by amplification of the *23s rRNA* gene specific to *S. aureus*, all 52 isolates contained 1250 *bp* DNA fragments bands and showed positive PCR assay, same as in our study. Momtaz *et al.* (2010) [13] found the existence of 1250 *bp* DNA fragment in 86 samples those showed PCR assay. All the samples contained 1250 *bp* DNA fragments bands and in present study 65 isolates showed positive PCR assay having 1250 *bp* DNA fragments bands. The present study was fall in line with the studies conducted by Akinedenn *et al.* (2001) [1]; Nathawat *et al.* (2015) [15] and Salauddin *et al.* (2020) [23].

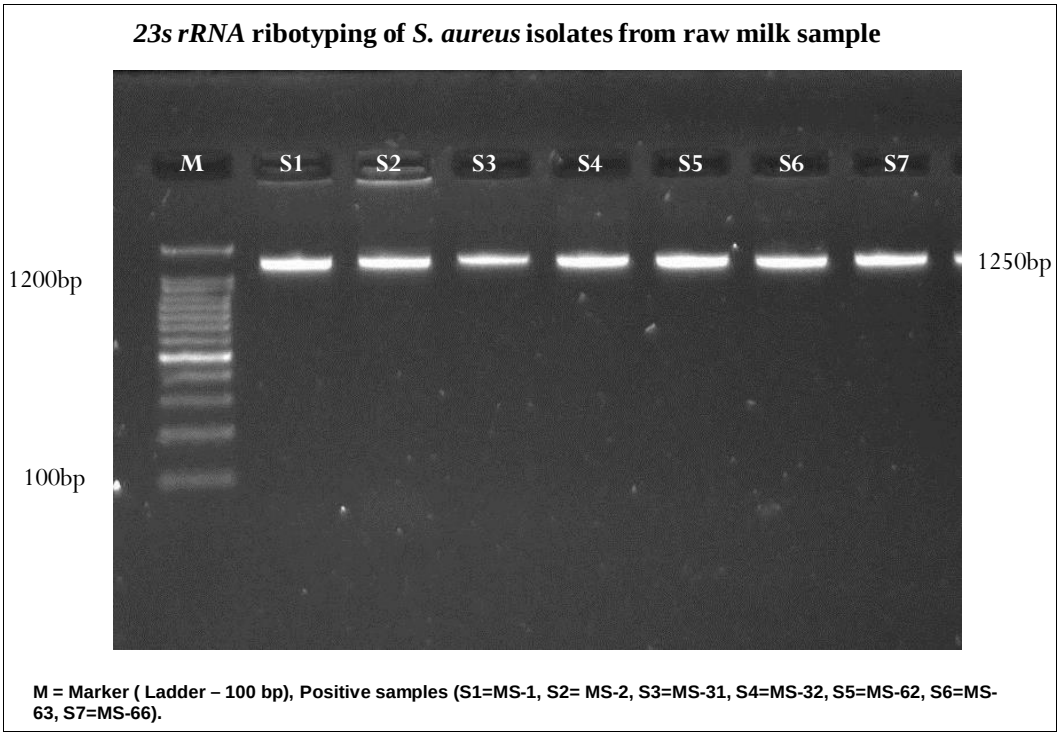

Fig 3: *23s rRNA* ribotyping of *S. aureus* isolates from raw milk samples.

Conclusion

Present study has shown evidence that *S. aureus* are frequently occurring organisms in milk. Due to food poisoning outbreaks by the ingestion of contaminated milk products, the public health significance of *S. aureus* is great. High prevalence of *S. aureus* indicates that it is very essential to adopt effective strategy for ensuring food safety by preventing the contamination of milk. This can only be done by effective implementation of hygienic practices during production, storage, processing, distribution and consumption of milk and milk products. It is necessary to implement strict hygienic measures to decrease the bacterial contamination and improve the quality of milk.

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