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RESEARCH ARTICLE

Milk microflora with *S. aureus* alike colony characteristics limits its identification over selective and differential agar media

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Abstract: *Staphylococcus aureus* is considered a significant public health concern, owing to its involvement in food poisoning outbreaks. Milk and milk products are consumed by almost all age groups and serves as a vital nutrition source. Being highly nutritious milk serves as a promising matrix for the proliferation of *S. aureus* and other potential pathogens. Rapid detection of food-borne pathogens like *S. aureus* is vital for ensuring consumer safety. Conventional methods are still considered as the gold standard for pathogen detection in food matrices. Analysis using these methods involves sample plating over selective agar media, followed by microscopic analysis and biochemical tests. Although phenotypic detection based on colour and colony characteristics over selective and differential media is indicative, its sensitivity is compromised by organisms that display colony characteristics similar to the target organism. In this study we attempted to identify organisms from milk matrix showing characteristics similar to *S. aureus* over different agar media that are claimed to be selective for *S. aureus*. Identification of these organisms depicting *S. aureus* alike colony characteristics and development of more selective media can help to expedite *S. aureus* identification from milk and milk products with more confidence.

Abbreviations: *S. aureus*, *Staphylococcus aureus*; BPA, Baird Parker Agar; MSA, Mannitol Salt Agar; VJA, Vogel-Johnson Agar; BHI, Brain Heart Infusion; MALDI-TOF MS, Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry

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Introduction

Food safety is a serious global public health concern, primarily in densely populated areas. *Staphylococcus aureus* is one of the most infamous and widely distributed bacterial pathogens, producing large number of minor skin infections each year, as well as millions of more serious, invasive infections worldwide. Its involvement in food poisoning incidences is attributed to high rate of human skin and nasal carriage, the ability of efficient air-borne spread, and strong survival in fomites. These properties also allow the organism to eradicate competing microorganisms having less ability to endure conditions like elevated temperatures, high osmotic pressure, and relatively low humidity (Le et al. 2021). *S. aureus*, classified as a high-tier priority II pathogen by the World Health Organization is linked to several health problems in both humans and animals. Furthermore, it is one of the most common causes of mastitis in cattle, sheep and goats, resulting in significant reductions in milk output and quality, as well as significant financial losses to dairy industry (Abril et al. 2020). *S. aureus* can gain access to milk either through direct excretion from infected udders with clinical or subclinical mastitis or through contamination from the environment that occurs during the processing of raw milk. Mastitis not only has a negative impact on the production and composition of milk and results in enormous economic losses, but also raises the cost of treatment, decreases the production life span, boosts the rate of elimination and is frequently accompanied by other diseases (Wang et al. 2021). In an earlier study, at least 50% of the dairy farms reported that the entire cost of mastitis were ≤ US$ 1.0/milking cow/day (Vissio et al. 2015). Keeping the above in mind, rapid and accurate identification of *S. aureus* becomes important. For *S. aureus* identification, conventional method includes sample pre-enrichment followed by plating over selective agar media. Commonly used *S. aureus* selective media include Baird Parker Agar (BPA), Vogel-Johnson Agar (VJA), and Mannitol Salt Agar (MSA). Although analysis using these selective media is quite efficient, their performance varies with sample matrices. However, some *S. aureus* alike organisms have been reported to grow well

over these selective and differential media with *S. aureus* alike colony characteristics, making its presumptive identification false and questionable. In this study, we attempted to identify microorganisms from milk matrix having phenotypic characteristics similar to *S. aureus* posing serious challenge to isolation and identification.

Materials and Methods

Sample collection and processing

Raw milk samples including mixed milk (Cattle + Buffalo milk), Goat milk (Beetle Goat), Yak milk, Sheep milk, Camel milk, and Human milk were collected hygienically and transported to laboratory under refrigerated conditions in pre-sterilized glass bottles. Milk samples were serially diluted and appropriate dilutions were spread plated over BPA and VJA for selective pick-up of *S. aureus* alike colonies. Following overnight incubation at 37°C, plates were observed for colonies with similar characteristics to *S. aureus*. The colonies with desirable characteristics (grey-black shiny over BPA and VJA) were selected and processed for further identification.

Bacterial Identification

Individual bacterial colonies with characteristics similar to *S. aureus* were picked up and transferred to Brain Heart Infusion (BHI) broth and incubated overnight at 37°C. The microbial growth from BHI broth was streaked on to BHI agar plates for identification. Individual colonies were subjected to morphological (Gram's staining, Negative staining) and biochemical identification (Catalase and Coagulase) tests (Becker and von Eiff, 2011). Species level identification was carried out by MALDI-ToF-MS facility at College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU). Briefly, pure single colony of test organism from a non-selective agar was smeared onto a spot on MALDI-ToF target plate. Smear was air dried and overlayed with 70% formic acid (1µL) and allowed to dry at room temperature. After drying, the spot was overlayed with HCCA solution (matrix) (1µL) and again allowed to air-dry at room temperature. Target plate was introduced into the biotyper for real-time analysis (Zhu et al. 2015).

Results and Discussion

A total of 6 raw milk samples were used for isolation and identification of organisms showing phenotypic characteristics similar to *S. aureus*. From every milk sample around 50 colonies (25 colonies from each media i.e. BPA and VJA) having characteristics similar to *S. aureus* were picked up and transferred to BHI broth for further analysis. All the isolates showed growth in BHI broth and were streaked on to BHI agar for identification by MALDI-ToF-MS. Apart from *S. aureus*, a total of about 90 isolates fall among six different genera and species *viz*. *Macrococcus caseolyticus*, *Lactococcus lactis*, *Lactococcus garvieae*, *Enterococcus durans*, *Klebsiella pneumoniae* and *Proteus mirabilis* from mixed, yak, goat and camel milk **(Table 1)**. However, all the colonies picked up from sheep and human milk were identified as *S. aureus*. All of the six isolates along with *S. aureus* (ATCC 700698) were analysed for morphological and biochemical properties. Out of 6 isolates, four were found to be Gram positive (*M. caseolyticus*, *L. lactis*, *L. garvieae, E. durans*); while two (*K. pneumoniae*, *P. mirabilis*) turned out to be Gram negative. Later, all the isolates were tested for their catalase and coagulase activity. Among the isolates, *M. caseolyticus*, *K. pneumoniae* and *P. mirabilis* were found to be catalase positive and *L. lactis*, *L. garvieae,* and *E. durans* gave catalase negative reaction. Isolates other than *L. garvieae* and *L. lactis* gave coagulase positive reaction **(Table 1)**. Figure 1 (a-f) presents the colony characteristics of all the target strains alongside *S. aureus* over respective agar media.

This study attempted to identify bacterial strains having colony characteristics similar to *S. aureus* over selective and differential agar media. Milk being a nutritious source for growth and propagation of microbes often contributes to episodes of food-borne infections. Identification and elimination of *S. aureus* alike organisms from milk matrix can help in early pathogen detection and also identification of scenarios like mastitis. In this study,

Table 1 List of isolates used in the study

Lab Identity	Source	Media	Colony characteristics	MALDI-ToF-MS based identification	Microscopic characteristics	Gram's staining	Catalase test	Coagulase test
M3	Mixed milk	BPA	Circular Black center with zone	*Macrococcus caseolyticus*	Cocci, in cluster	+	+	+
M10	Mixed milk	BPA	Shiny black	*Proteus mirabilis*	Rod shaped	-	+	+
C8	Camel Milk	BPA	Circular flat black	*Klebsiella pneumoniae*	Short, plump, straight rod, arranged singly, in pairs, or in short chains and sometimes in clusters	-	+	+
M6	Mixed milk	VJA	Circular flat black	*Lactococcus garvieae*	Cocci, in pairs and short chains	+	-	-
Y1	Yak Milk	VJA	Shiny black	*Enterococcus durans*	Cocci, arranged individually in pairs or short chains	+	-	+
G5	Goat milk	VJA	Pin point small black	*Lactococcus lactis*	Cocci, groups in pairs and short chains	+	-	-

M, Mixed milk; C, Camel; Y, Yak; G, Goat; BPA, Baird Parker Agar; VJA, Vogel Johnson Agar; +, positive reaction; -, negative reaction

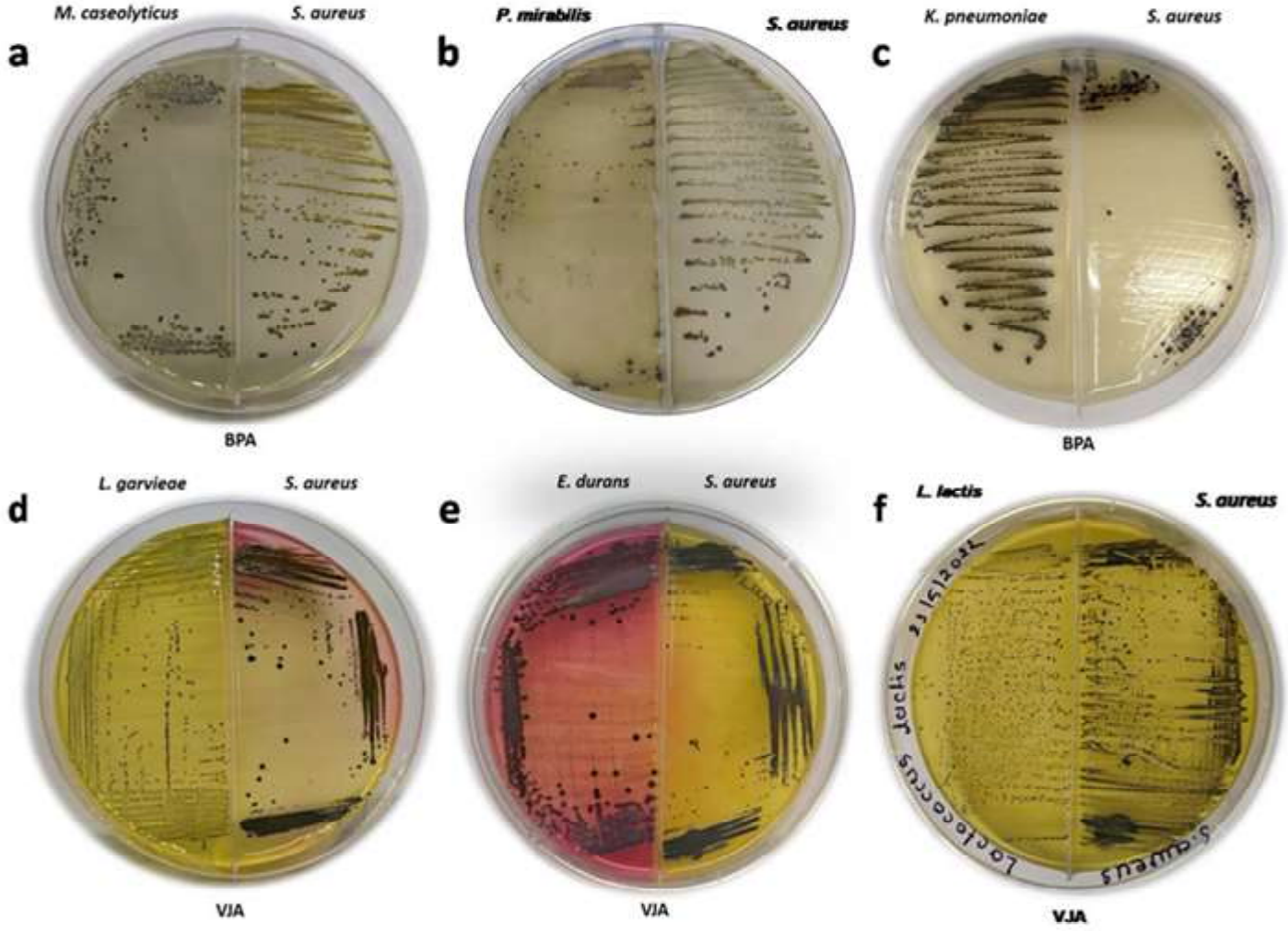

Fig 1. Colony characteristics of *S. aureus* alike strains over BPA and VJA

raw milk samples were processed using standard protocols for enumeration and detection of *S. aureus*. BPA and VJA were used in this study as these two media are considered highly selective for enumeration and detection of *S. aureus* and are recommended by various national and international standards including IS, ISO, BAM, FDA and AFNOR. Earlier, Kim et al., 2010 compared five different selective media and reported BPA and VJA to be highly sensitive and specific media for isolation of *S. aureus* from food matrices. The sensitivity and specificity of BPA was reported as 100% for isolation of *S. aureus* from agriculture products (Kim et al., 2012). After incubation, grey-black shiny colonies from BPA and VJA were selected for further identification. Baird and Lee (1995) described colony characteristics of *S. aureus* over BPA as black colonies with or without halos. The black colony color is attributed to tellurite reduction. From all the samples plated on two different selective media, 300 colonies having similar characteristics like *S. aureus* were isolated and subjected to identification using MALDI-ToF-MS. Even after being represented as selective media for growth of *S. aureus*, 90 non staphylococcal isolates falling under different genera and species were identified. Our results agree with the data presented in oxoid manual which states that organisms other than *S. aureus* can grow on BPA (Oxoid Manual 1998). Similar type of observation of getting different type organisms on BPA was made by Ledina et al. (2018). In this study cheese samples were plated over BPA and the isolates were identified using MALDI-Tof-MS. A large number of non-staphylococcal isolates were identified from BPA including organisms from genus *Macrococcus* and *Enterococcus*. Similarly, in a study targeting *S. aureus* from water samples, majority of the isolates were identified as *Proteus* spp. and *Enterococcus* spp. This study also explains that although Baird-Parker agar is most commonly used to isolate staphylococci, other bacteria known to tolerate tellurite and polymyxin B, can also grow over it (Kaseem et al. 2008). It was reported that organisms from genus *Enterococcus* were selectively isolated on BPA due to their natural resistance against tellurite present in BPA (Vandera et al. 2018). Earlier, Nelson and George (1995) observed the growth of *P. mirabilis*, *Escherichia*

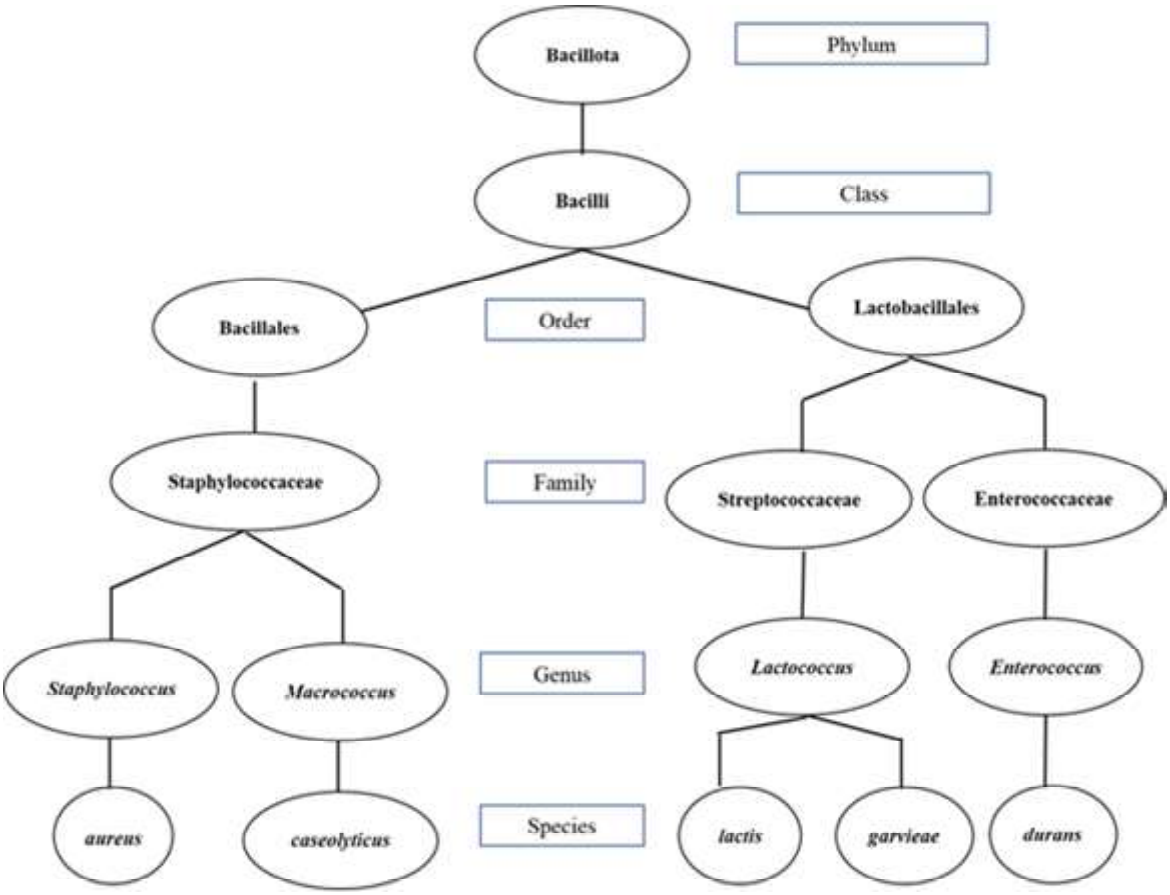

Fig 2. Phylogenetic relationship between test strains

coli and *Enterococcus faecium* over BPA. In our study, among non-staphylococcal bacteria isolated from BPA plates, *M. caseolyticus* was the most abundant. *M. caseolyticus* was formerly classified as *Staphylococcus caseolyticus* and is closely related to staphylococci. *M. caseolyticus* is known to have close evolutionarily relationship with *S. aureus.* Phylogenetic analysis based on 16S and 23S rRNA sequences indicates that the two genera share same ancestor (Tsubakishita et al. 2010). The essential biological pathways of *M. caseolyticus* were also found to be similar to those of staphylococci (Baba et al. 2009). These two microorganisms also share some of the clinically relevant resistance genes and due to presence of resistance genes carrying plasmids it was suggested that horizontal gene transfer may have occurred between *Macrococcus* and *Staphylococcus* species (Chanchaithong et al. 2019; Schwendener et al. 2020). As *M. caseolyticus* is typically considered commensal to livestock, it was also identified in earlier studies in milk samples (Giannino et al. 2009). Only a few reports are available on assessment of selectivity and specificity of VJA and majority of them reported use of this media for growth of *Listeria monocytogenes*. Up to the best of our information, this is the first report showing the growth of *L. lactis*, *L. garvieae* and *E. durans* over VJA from milk matrix.

M. caseolyticus, *L. lactis*, *L. garvieae* and *E. durans* shared colony characteristics with *S. aureus* over selective media and were interestingly identical microscopically. These four strains were cocci arranged in cluster, single or in pairs. *S. aureus* can have different arrangements depending on the source, it can occur as classic bunch like morphology or can occur in tetrad, pair or single cell arrangement. Habib et al. (2015) observed different arrangement of *S. aureus* isolated from different animal sources. The gram-negative isolates (*K. pneumoniae*, *P. mirabilis*) were observed to have rod shaped cells occurring singly or in pairs. *S. aureus* could be distinguished from *K. pneumoniae*, *P. mirabilis, L. lactis*, *L. garvieae* and *E. durans* on the basis of catalase reaction. However, *M. caseolyticus* shared similar morphology, catalase and coagulase reaction with *S. aureus.*

In order to understand the concept behind the utilization of selective components meant for *S. aureus* by other organisms, we analysed the phylogenetic information available for all the organisms as presented in Figure 2. On connecting their evolutionary footsteps, it was observed that four out of six test organisms shared phylogeny with *S. aureus*. The most closely related isolate was *M. caseolyticus*, which share same family with *S. aureus* i.e Staphylococcaceae. Other isolates including *L.*

lactis, *L. garvieae* and *E. durans* share same class Bacilli with *S. aureus*. As clearly evident that *L. lactis* and *L. gravieae* shares same genus so are very closely related to each other. These two organisms are connected to *E. durans* at order level. All of these three isolates belong to order Lactobacillales. All these evolutionary connections indicate that these isolates may have similar biochemical pathways and ability to grow on the selective media specific for *S. aureus*. Two isolates, *K. pneumoniae* and *P. mirabilis* do not share any evolutionary bond with *S. aureus*.

Conclusion

Food poisoning caused by *S. aureus* is a major public health concern around the world and studies have reported contamination of milk and milk-based products with *S. aureus*. In order to deal with the food poisoning cases, accurate identification of causative organisms is very crucial. In this study we focused on *S. aureus* like microorganisms showing similar colony characteristics and growing under similar growth conditions. Identification of these microorganisms is very important to formulate a media with improved selectivity and specificity for *S. aureus* leading to more accurate and rapid conclusive results. Knowledge in this area is steadily improving, but more research is required, not only for improved characterization of *S. aureus* but also to propose new media for inhibiting the *S. aureus* alike strains and to minimize high economic losses caused by *S. aureus* and its toxins to the dairy industry.

Conflict of Interest: None

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