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# Identification of an unusual *Streptococcus agalactiae* growing on MacConkey Agar and its confirmation by biochemical tests, qPCR and nanopore sequencing

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**Abstract**

*Streptococcus agalactiae belongs to Group B Streptococcus (GBS), an important Gram-positive pathogen attributed to mastitis and elevated somatic cell counts (SCC) in dairy cows, which invariably exhibits complete β-haemolysis on blood agar (BA) / Polymixin Nalidixic acid Blood Agar (PNBA) and fails to grow on MacConkey agar (MCA). The present study conducted in the Advanced Agricultural Laboratories under the Almarai company, Riyadh, KSA, reports the isolation, identification and characterization of β-haemolytic Strepotococcus agalactiae which exhibited lactose fermenting colonies on MCA. Though the colony characteristics on BA/ PNBA were suggestive of Streptococcus, the observation of pink colored colonies on MCA was deemed unusual and was subjected to further confirmatory tests viz., Gram's staining, Lancefield grouping, catalase, oxidase and CAMP tests. Further, real-time PCR and Oxford nanopore sequencing of the 16S ribosomal RNA gene were performed for molecular characterization. The findings of the study would add to the existing knowledge on cultural characteristics of Streptococcus agalactiae for its presumptive identification.*

Keywords: *Streptococcus agalactiae, MacConkey agar, nanopore sequencing*

*Streptococcus agalactiae* is classified as a Group B streptococcus (GBS), a Gram-positive pathogenic bacterium and one of the major causative organisms for mastitis in cattle. Streptococcal mastitis significantly affects milk quality and yield (Keefe, 1997) resulting in a huge economic loss in dairy industry (Botelho *et al*., 2018). This organism is highly contagious in nature as it is transmitted from infected animals to healthy ones during milking, especially while using a milking machine (Crestani *et al*., 2021). In most cases, it causes subclinical mastitis without any overt signs such as abnormal milk, however, the affected milk was characterized by high SCC and bacterial count (Tamba *et al*., 2022).

*Streptococcus agalactiae* is a Gram-positive coccus, usually found in chains. The organism is catalase-negative, and facultative anaerobe which grows as glistening grey-white colonies with a narrow zone of β-hemolysis on blood agar (Whiley and Hardie, 2009). The organism is surrounded by a polysaccharide capsule belonging to Group B streptococcal antigen according to Lancefield grouping of streptococci and can be detected directly in intact bacteria using latex agglutination tests and CAMP test (Rosa-Fraile and Spellerberg, 2017). The CAMP factor produced by *S. agalactiae* acts along with the β-hemolysin produced by *Staphylococcus aureus* inducing enhanced hemolysis of

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sheep or bovine erythrocytes, forms the basis of CAMP test. Molecular techniques such as real-time PCR and sequencing have also been developed for the definitive identification of *S. agalactiae* (Rosa-Fraile and Spellerberg, 2017).

*Streptococcus agalactiae* usually grows abundantly on media enriched with blood and serum (Rosa-Fraile and Spellerberg, 2017; Filkins *et al*., 2020). Most of the *S. agalactiae* strains are unable to grow on media containing bile salts or surfactants (Davis and Pham, 1983). MacConkey agar contains bile salts and crystal violet which inhibit growth of Gram-positive organisms enabling the selective isolation of Gram-negative bacteria. The media detects lactose fermentation by bacteria with the pH indicator neutral red (Anderson *et al*., 2013). Earlier studies indicated that *S. agalactiae* cannot grow on MacConkey agar due to the presence of bile salts and absence of blood or sera components in the medium (Jokipii and Jokipii,1979).The present study was conducted in Advanced Agricultural Laboratories under Almarai Co , Riyadh, KSA as a part of the routine identification of mastitis pathogens in the dairy cows of Almarai farms. As *S. agalactiae* is an important pathogen contributing to the SCC levels and is a contagious pathogen which can be quickly transferred horizontally, identification of *S. agalactiae* harboring animals is an important management strategy in the organized dairy farms. The usual cultural characteristics for the identification of *S. agalactiae* involves the observation of greyish white β-haemolytic colonies on BA/ PNBA, which is catalase and oxidase negative and are Gram-positive cocci in short chains with no growth on MCA. Such colonies would be deemed as *Streptococcus* species and will be further tested by Lancefield grouping and CAMP test to identify it as GBS and *S. agalactiae,* respectively. Recently, in our routine mastitis pathogen identification, the observation of lactose fermenting colonies on MCA led to an ambiguity to deem these isolates as *Streptococcus* species. Nevertheless, the isolates complied with all other characteristics to be identified as *Streptococcus*. Therefore, it was decided to perform an investigation on these isolates employing real-time PCR and 16S ribosomal RNA gene sequencing. The results obtained from the real-time PCR and sequencing could prove that these colonies were *S. agalactiae* or not. Moreover, this information augments the existing understanding of cultural characteristics of the organism. For instance, certain strains of mastitis causing *S. agalactiae* can exhibit growth on MCA, which contains bile salts intended for the elimination of Gram-positive organisms and lacks growth promoting agents such as blood/serum intended for the *in vitro* growth of Streptococcus.

## Materials and methods

### *Culture of bacteria and biochemical characterization*

Milk samples obtained from mastitis cows were inoculated to BA, PNBA and MCA using sterile swabs, followed by incubation at 37$^0$C for 24-48 hours in the bacteriological incubator. After the incubation time, the BA and PNBA were observed for the colony characteristics/ hemolytic patterns and the MCA were examined to observe that the growth of the organism is inhibited. The presumptive colonies were subjected to Gram's staining followed by catalase, and oxidase tests. Those colonies presumed as *Streptococcus* spp. based on the previous tests, were subjected to Lancefield grouping and CAMP tests, as per the flowchart depicted in Fig.1.

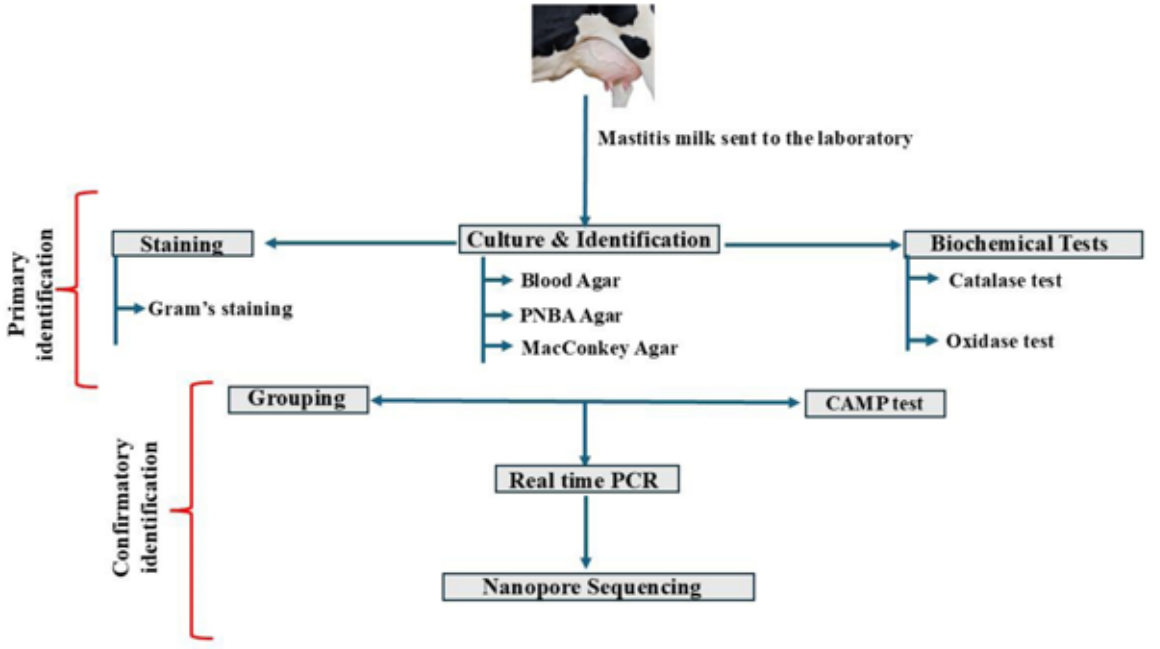


**Fig. 1:** Methods used to confirm the mastitis pathogen

Gram's staining was performed according to the manufacturer's protocol (Condalab, Spain). Catalase test was performed by mixing a loopful of the pure bacterial colony to a drop of 3% $H_2O_2$ to visualize the effervescence (Reiner, 2010). Oxidase test was performed using oxidase strips (Erba Mannheim, Germany) by rubbing a loopful of colony onto the strips and observing for any color change (Shields and Cathcart, 2010). Lancefield grouping was performed using commercially available latex agglutination test kit (Pro-Lab diagnostics, Canada) following the manufacturer's protocol. The CAMP test was carried out by streaking the colony of interest perpendicularly 2-mm apart from a narrow streak of pure *Staphylococcus aureus* inoculated at the center of the sheep blood agar plate (Hanson, 2006).

***DNA extraction and qPCR***

DNA extraction was performed using a commercial Kit (Indical Biosciences, Germany) , for which, 5-6 colonies of pure culture of *S. agalactia* were suspended in 200 µl of nuclease free water and the extraction was carried out according to the manufacturer's instructions. DNA concentration in the elute was determined by Qubit Flex Fluorometer (Themo Fisher Scientific, USA) for adjusting the input volume in the downstream processes. The extracted DNA was further used for qPCR in a real-time thermocycler (Bio-Rad, USA) using *Streptococcus agalactiae* TaqMan PCR detection kit (Norgen Biotek, Canada) following manufacturer's instruction.

***Nanopore Sequencing***

The 16S ribosomal RNA gene from the extracted DNA was amplified using the conventional PCR employing universal forward (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse (5' CGG TTA CCT TGT TAC GAC TT 3') primers. The PCR amplification was carried out using 5 µl of template DNA (5ng/µl), 2.5 µl of each primers (10 µM), 25 µl of 2X PCR master mix (Platinum SuperFi II PCR master mix, Thermo Fisher Scientific, USA) and 15 µl of NFW in a final volume of 50 µl. Thermal cycling conditions were as follows: 30 sec at 98°C followed by 40 repeats of 15 sec at 98°C and 30 sec at 60°C followed by 5 min at 72°C. The size and quality of the PCR amplified product were visualized by agarose gel electrophoresis and quantification was done using Qubit Flex Fluorometer. Subsequently, for proceeding to the generation sequencing, the amplicons were subjected to library preparation using Native Barcoding kit (SQK-NBD111.24) supplied by Oxford Nanopore Technologies (ONT) according to the detailed protocol mentioned in the brochure. Briefly, the procedure begins with end preparation followed by native barcoding, and adapter ligation. Upon completion of library preparation, the specified volume of DNA library was loaded into MinION R10.4.1 flow cell (FLO-MIN114, ONT). The sequencing process was stopped after four hours and the run parameters were QC checked, and the final consensus sequence was generated using "WF-amplicon" workflow present in the EPI2ME application (ONT, UK). The consensus sequence generated was analyzed using NCBI BLASTn tool for finding the identity match with other sequences submitted in the NCBI-GenBank database.

**Results and discussion**

After 24 hours of incubation, greyish white colonies having a diameter of 2-3mm with a narrow zone of β-hemolysis were observed on BA and PNBA plates, whereas, on MacConkey agar, on the contrary to the expectation of no growth, pink to red colonies with a diameter of 1-2 mm were also observed (Fig. 2). The PNBA agar is a growth medium used for the culture and isolation of Gram-positive bacteria, which contains polymyxin and nalidixic acid, preventing the growth of Gram-negative bacteria. The cultural characteristics on BA and a selective medium like PNBA agar was suggestive of *Streptococcus.* However, the growth observed on MacConkey agar was unusual, as *S. agalactiae* typically cannot thrive in this medium due to the presence of bile salts and absence of enrichment factors like blood or serum. Gram's staining of colonies from all the three agar plates revealed Gram positive cocci, mostly in short chains. The negative results in catalase and oxidase tests could be suggestive of streptococci (Whiley and Hardie, 2009). Further, on Lancefield grouping, the colonies were identified as GBS. Lancefield grouping is a system of classification based on the carbohydrate composition of


**Fig. 2:** Morphological characteristics in different culture media

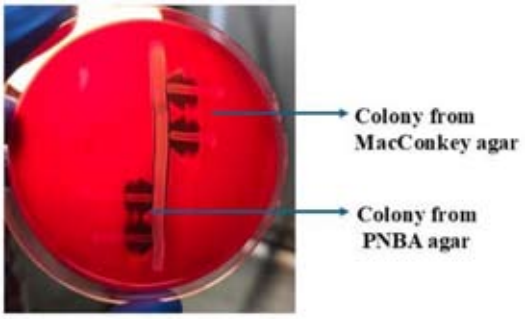


**Fig. 3:** CAMP test

bacterial antigens found on their cell walls and according to this grouping system, GBS primarily refer to *S. agalactiae* (Rosa-Fraile and Spellerberg, 2017). Enhanced hemolysis in the presence of *Staphylococcus aureus* in CAMP test was also indicative of this specific organism (Fig.3) (Rosa-Fraile and Spellerberg, 2017). All the tests used to confirm the identity of *S. agalactiae* were in full agreement, except for the observation of lactose fermenting colonies on MCA. Hence, these colonies were further subjected to real-time PCR and sequencing analysis. Real-time Taqman PCR is a highly specific and sensitive diagnostic test, by which these isolates were identified as *S. agalactiae*. Further, for carrying out the 16S ribosomal gene amplicon sequencing, the DNA extracted from the colonies of interest were subjected to conventional PCR, which yielded adequate quantity of pure single amplicon with the fragment size of ~1500 bp (Fig.4). The BLASTn results of the consensus sequence obtained after sequencing run were 100 percent identical to the previously submitted sequences of *S. agalactiae* isolates in NCBI GenBank (JQ990156.1, JQ990155.1 & CP1019995.1).

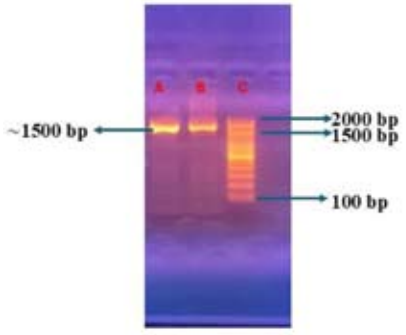


**Fig. 4:** Agarsose gel electrophoresis of PCR products

A. PCR product of bacterial DNA isolated from PNBA agar
B. PCR product of bacterial DNA isolated from MacConkey agar
C. 100 bp DNA ladder

The study confirms beyond any doubt that certain *S. agalactiae* strains can grow on MCA, which is a selective and differentiating media that typically allows the growth of Gram-negative bacteria; in which lactose-fermenting species grow as pink colonies and non-lactose-fermenting species grow as white colonies. Generally, Gram-positive bacteria like *S. agalactiae* are sensitive to bile and bile salts compared to Gram-negative bacteria. In an earlier study conducted by Jokipii and Jokipii (1979), one hundred strains of *S. agalactiae* bacteria were inoculated on MCA, but no growth was observed. However, a few reports indicate that bile tolerance is a strain-specific trait, and the tolerances of different species cannot be generalized (Chateau *et al*., 1994; Zarate *et al*., 2000). Enteric Gram-positive pathogens like *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis* and *Clostridium perfringens* have developed some mechanisms to resist the toxic action of bile salts to survive inside the intestinal tract (Alsultan and Alsallami, 2022). A recent study by Jia *et al.* (2023) found that several *S. agalactiae* strains isolated from humans exhibited bile salt resistance on tryptic soy broth with varying concentration of bile salts, which was attributed to the efflux pump activity of the bacteria. The bile salt resistance or the ability to demonstrate growth on MCA were not reported for *S. agalactiae* isolated from mastitis cases of cattle, so far.

## Conclusion

This study demonstrates the atypical growth of *S. agalactiae* on MCA generally hostile to Gram-positive bacteria. The findings highlight that certain *S. agalactiae* isolates complying with all the routine identification test parameters may also exhibit unusual growth on MCA.

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## Conflict of interest

The authors have no conflicts of interest to declare.

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