Characterizations of *Streptococcus agalactiae* from subclinical mastitis cases in dairy cow

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**Abstract.** Subclinical mastitis (SCM) is a disease that often infects dairy cow, which then reduce milk production. *Streptococcus agalactiae* is well known as a dominant causative agent of SCM. An experiment to identify *S. agalactiae* from subclinical mastitis cases in dairy cows has been conducted. These isolates were examined using presumptive *S. agalactiae* of Christie, Atkins, and Munch-Petersen (CAMP) test and followed by Grouping test to identified it, which was cultured in soft agar (SA) and serum soft agar (SSA) to observed the capability at surface phenotype expression. Haemagglutination test of these isolates were used 1% cow erythrocyte. Fifty-seven positive Gram and negative catalase of cocci bacteria were isolated from the process of identification, of which 34 were CAMP positive. From the grouping test of these, 14 isolates of *S. agalactiae* Group B (SGB) were identified. Furthermore, 8 isolates are diffuse cultured in SA, while the remain 6 isolates are compact in SSA. Noncapsulated SGB with antigenic side would be expressed as compact culture in SA and SSA. Haemaglutynine (Hn+) property of SGB have a role in adhesive process at epithelial of mamary gland alveolus.

1. **Introduction**

Animal husbandry business has the potential to grow rapidly in Indonesia, given the adequate availability of feed and the diversity of existing livestock species. According to Subandrio and Adiarto one of the government's efforts to increase consumption of animal protein for the population of Indonesia is to develop dairy farming, which has been started since the Dutch East Indies era until now [1]. This can be seen from the increasing population of dairy cows from year to year, from 444,000 in 2013 to 519,000 in 2015, and continues to increase to 534,000 in 2016 as it is in the guideline by Direktorat Jenderal Peternakan and Kesehatan Hewan [2]. Increased public awareness about nutritional value and the need for consumption of animal protein, especially milk, also support the development of people's livestock farming. Milk is a secretion produced by mammalian glands including humans, and is a perfect food ingredient and has a high nutritional value. Until now cow's milk is still considered as the main food source that can replace breast milk (*Air Sisus Ibu*). It has been recon by I Mirdhayati and J Handoko that cow's milk is a special food for humans because of its deliciousness and ideal composition, which contains many substances needed by the body such as fat, protein especially casein, lactose, vitamins, minerals such as calcium, and others [3]. Milk consumption of the Indonesian population has increased from year to year, which is 6.50 kg / capita / year in 2004 to 16.42 kg / capita / year in 2010. The increase in milk consumption is of course followed by an increase in demand, but
the fulfillment of milk demand from domestic production can only supply 23%, and the rest comes from import substitution. National Milk Production in 2016 amounted to 852,951 tons, while milk demand was 3.7 million tons. This confirms by Direktorat Jenderal Peternakan and Kesehatan Hewan that milk production in Indonesia is still lacking [2].

The obstacle that often hinders the success of a livestock business is an attack of disease. One of the important diseases that can reduce milk production in dairy cattle is Mastitis which is also known as inflammation of the mammary glands. Most researchers C Bogni, et al agree that the economic loss due to mastitis in dairy cows is at least 70% due to a decrease in milk production and the removal of milk from mastitis cows [4]. Direktorat Kesehatan Hewan explains that Mastitis is generally divided into 2 types, first is clinical mastitis which shows symptoms of morbidity in cattle and damage to milk, and second is subclinical mastitis that does not show symptoms of inflammation in mammary glands except when tested on milk with a special test for example California Mastitis Test (CMT) [5]. As in other countries too, in Indonesia, the more common cases are subclinical mastitis Subronto [6], Supar and T Aryanti [7], M Hashemi et al [8].

Mastitis can be caused by several factors, namely incorrect milking methods, poor sanitation, unclean cages and cage floors that do not meet the requirements for dairy cows. The germs that causes mastitis from a dirty cage will enter the mammary gland through nipples and canals when milking or when cows sit on the floor of the cage. In the protocol issued by Direktorat Kesehatan Hewan potential microbes that causes mastitis are Streptococcus agalactiae, Streptococcus disgalactiae, Steptococcus uberis and Staphylococcus aureus [5]. S Estuningsih et al confirms that S. agalactiae is one of the main agents causing subclinical mastitis in dairy cows [9]. Furthermore, it is also confirmed by C Bogni et al that S. agalactiae group B (SGB) is a major bacterial cause of subclinical mastitis in dairy cows in general in the world and SGB is an obligate parasite in the mammary glands in dairy cows with subclinical mastitis as proved by AETH Wahyuni et al [10].

2. Experimental methods

*S. agalactiae* isolates derived from dairy milk showed a positive 1 / (+) reaction of California Mastitis Test/ CMT. Identification of the presence of *S. agalactiae* based on colony morphology, bacterial cell morphology by microscopic examination and Gram staining, catalase test and the presence of Christie, Atkins and Muence Petersen (CAMP) factors on blood agar media, which adopted from PJ Quin et al [11]. Sero-group identification using the Streptococcal Grouping kit (Oxoid®, England). The expression of SGB phenotype in vitro was done by inoculating SGB on Soft Agar (SA) and Serum Soft Agar (SSA) media to observe their growth patterns adapting methodology from IWT Wibawan and C Lammelr [12]. The SGB isolate was further tested for hemagglutination using 1% erythrocyte of dairy cows from AETH Wahyuni et al [13].

3. Results and discussion

From milk samples that reacted positively 1 to CMT, 57 isolates of *Streptococcus sp.* after examining the colony morphology, the morphology of the coccus chain, and the negative catalase test.

The CAMP test is a presumptive test in identifying *S. agalactiae* bacteria. The results of 57 isolates tested with CAMP found 34 isolates (59.6%) showed positive CAMP test results while 23 isolates (40.4%) showed negative CAMP test results as listed in Table 1.

| CAMP test  | Number | Proportion (%) |
|------------|--------|----------------|
| Positive (+) | 34     | 59.6           |
| Negative (-) | 23     | 40.4           |
| Total (n) | 57     | 100            |

Isolates that showed positive CAMP results formed a hemolysis zone like an arrowhead on the Blood Agar Plate / BAP media between the *Staphylococcus aureus* culture and the *Streptococcus sp.* isolates
tested, while the negative CAMP test results did not show any changes in the BAP media as seen in Figure 1.

*Figure 1.* Positive CAMP test results: a clear zone like an arrow in the middle of the Blood Agar Plate / BAP shows a positive reaction in *S. agalactiae*.

The phenomenon that occurs in the CAMP test is because of the interaction between the $\beta$-hemolysis factors produced by *S. agalactiae* isolates tested with extracellular products in the form of $\beta$-lysin staphylococcal produced by *Staphylococcus aureus*. As mentioned by SM Hansen and UBS Sorensen the hemolysis reaction on BAP media is increasingly evident because the extracellular product in the form of $\beta$-lysin staphylococcal produced by *Staphylococcus aureus* [14].

*Streptococcus agalactiae* is one of the main agents causing subclinical mastitis in dairy cows. Further study conducted by Estuningsih et al indicated that 83 bacteria isolated from 3 areas in Java, are all identified as *Streptococcus agalactiae* [9]. In accordance with the results of bacterial isolation conducted by Supar and Ariyanti from quartered milk samples in the areas of Bandung, Bogor and Sukabumi [7], 60.6% of *Streptococcus agalactiae* were obtained as the bacteria causing subclinical mastitis.

The results of grouping conducted from 34 samples that tested positive for the CAMP test found 14 isolates (41.2%) of which showed positive agglutination of latex reagent group B, while 20 isolates (57.8%) did not occur agglutination with the latex reagent group B as shown in Table 2.

| Group Test B            | Number | Proportion (%) |
|-------------------------|--------|----------------|
| Agglutinated            | 14     | 41.2           |
| Non-agglutinated        | 20     | 57.8           |
| Total sample (n)        | 34     | 100            |

Fourteen isolates that tested positive for serogrouping were pure Streptococcus group B or *S. agalactiae*, because latex reagent group B would only react to agglutination when reacted with *S. agalactiae*. It is also known, by the study of AETH Wahyuni et al [10], M Abubakar et al [15], and H Moatamedi et al [16], that *S. agalactiae* group B (SGB) is a major bacterial cause of subclinical mastitis in dairy cows and is an obligate parasite in the mammary gland.

*S. agalactiae* Group B isolates that have been obtained were inoculated in soft agar (SA) and serum soft agar (SSA) media. This test is to see the expression of *S. agalactiae* phenotype in vitro. The phenotype expression of 8 isolates on SA and SSA media showed diffuse colony growth and 6 isolates grew with compact colony growth. The results of the colony form can be seen in Table 3.

SA and SSA media are used to see the phenotypic expression of components found on the surface of bacteria, including to distinguish bacteria that form capsules from those that do not form capsules on their surface. *S. agalactiae* which have capsules on their surface will be expressed with diffuse growth patterns of colonies in SA and SSA media, while the more compact form of colonies indicates the absence of capsules on the cell wall surface as indicated by the study of IWT Wibawan and C Lammler [12].

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**Table 2. S. agalactiae isolate of Grouping Kit test.**
Table 3. *S. agalactiae* growth pattern on Soft Agar and Soft Serum Agar media.

| No | Isolate code | Colonies from media |
|----|--------------|---------------------|
|    |              | SA      | SSA       |
| 1  | CK2          | Diffuse | Diffuse   |
| 2  | EK1          | Compact | Compact   |
| 3  | EK4          | Compact | Compact   |
| 4  | M3           | Diffuse | Diffuse   |
| 5  | K2           | Diffuse | Diffuse   |
| 6  | PK1          | Diffuse | Diffuse   |
| 7  | PK2          | Diffuse | Diffuse   |
| 8  | RB4          | Diffuse | Diffuse   |
| 9  | BK2.2        | Diffuse | Diffuse   |
| 10 | BK4.1        | Compact | Compact   |
| 11 | JK3          | Compact | Compact   |
| 12 | AK4          | Diffuse | Diffuse   |
| 13 | MK1          | Compact | Compact   |
| 14 | SK2.3        | Compact | Compact   |

The capsules of most of the constituents are carbohydrates in the form of polysaccharides which cover the surface protein of bacterial cells, so that they are not expressed in SA and SSA whose constituent structures are also carbohydrates. Therefore, in SA and SSA media, capsulated bacteria will be expressed in a diffuse form of growth while noncapsulated bacteria are expressed in compact form.

Figure 2. The character of *S. agalactiae* without capsules is shown by the compact growth pattern on Soft Agar (SA) media on the right-side and capsulated with a diffuse pattern on the left.

The form of colony growth on SA and SSA media can also determine the presence of R and X protein antigens. IWT Wibawan and C Lammler confirm that the surface of bacteria covered by protein antigens R or X will be expressed by growing into a compact colony on SA and SSA media while those without protein antigens will grow diffuse [12].

In general, the components found on the cell surface are virulent factors that help bacteria in the infection process. The capsule building material is a polysaccharide which is an antigen that is not immunogenic to the host causing PMN cells do not recognize it as a foreign material to the body. However, bacteria that do not have capsules will have a higher adhesion ability to adhere to host cells.
than those with capsules. Previous study by IWT Wibawan and C Lammler shows the presence of R or X protein antigens gives rise to the cell surface having a high hydrophobicity and has an important role on the adhesion ability of \textit{S. agalactiae} to adhere to its host epithelial cells [17].

In the Hemagglutination test, the reaction is positive if agglutination occurs at the bottom of the tube and negative if there is sedimentation as in erythrocyte control. SGB without capsules obtained showed a positive reaction in the Hemagglutination test. Hemagglutinin has a role in the adhesion process. The results study of IWT Wibawan in 1998 indicated that \textit{S. agalactiae} which has hemagglutinin has greater adhesion ability than those who do not have hemagglutinin in udder epithelial cells [18]. Further confirmation of AETH Wahyuni et al in subclinical mastitis the ability of adhesion has a more important role than the ability of invasion [13]. Most strains of bacteria that react with hemagglutinin have X-type antigen proteins P Rainard et al. With this adhesion ability, \textit{S. agalactiae} is free from the effects of washing away the secretory organs, so as to avoid the effects of washing the milk flow [19]. This condition answers the reason these bacteria cause subclinical mastitis.

4. Conclusion
Bacteria isolated from dairy cows with subclinical mastitis are \textit{Streptococcus agalactiae} Group B (SGB). Fifty-seven positive Gram and negative catalase of cocci bacteria were isolated from the process of identification, of which 34 were CAMP positive. From the grouping test of these, 14 isolates of \textit{S. agalactiae} Group B (SGB) were identified. Furthermore, 8 isolates are diffuse cultured in SA, while the remain 6 isolates are compact in SSA. Noncapsulated SGB with antigenic side would be expressed as compact culture in SA and SSA. Haemaglutynine (Hn+) property of SGB have a role in adhesive process at epitheial of mamary gland alveolus. Both of these SGB character (noncapsulated and Hn+) explain that there are no capsules that cover the surface of the SGB antigen and the adhesion process causes subclinical mastitis. Further research utilizing SGB is needed for disease control such as making vaccines for the prevention of subclinical mastitis.

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