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Isolation and identification of common bacteria causing subclinical mastitis in dairy goats

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Abstract

The purpose of the current study was to isolate and identify subclinical mastitis causing bacteria from milk samples of dairy goats. The milk samples from individual dairy goats were collected from several dairy goat farms around Kota Bharu, Kelantan. Major bacterial isolates were *Staphylococcus* spp. (73.2%). Coagulase negative staphylococci encompassing 68.3% of the isolates, whereas 4.9% was coagulase positive staphylococci. *Bacillus* spp. constituted 12.2% out of the isolates. *Listeria* spp. and *Neisseria* spp. both were represented 7.3%. There is a need to discuss the potential hazards of these bacteria in affecting milk quality, health of goats and food safety to consumers. The findings also emphasize the need to study the exact species of bacteria isolated in order to plan for their prevention as well as to assist veterinarians in prescribing correct antibiotic therapy.

Introduction

Mastitis is the inflammation of the mammary gland (specifically the udder) which eventually will cause serious health problems in dairy and non-dairy herds. It is a common disease in herds which when occurs will affect the health of the herds, milk quality and affect the farmer economically through cost of antibiotic therapy (direct cost) and decreased milk yield (indirect cost). Mastitis is categorised traditionally into clinical and subclinical. Subclinical mastitis (SCM) is the most common form of mastitis with the prevalent of about 15-40 % infected dairy goats. It is a form of mastitis without clinical signs of the mammary gland (i.e., inflammation, puss running out from the udder), but the pathogens exist in the milk and colonising the mammary gland. The bacteria enter the gland through the streak canal and multiply within the udder cells or in the teat duct (Viguier *et al*., 2009). It has been widely reported that some milk compositions changed in the event of SCM. For example, changes in protein constituents, plasmin, fat components, lactose, and mineral (Auldist and Hubble, 1998; Batavani *et al*., 2007). In addition to these effects, farmers will face economical loss due to reduce of milk yield and low milk quality (Pleguezuelos *et al.*, 2015; Gelasakis *et al*., 2016). A part from that, if the SCM left untreated, it will cause clinical mastitis and eventually increase the cost for veterinary treatment (Koop *et al.*, 2012).

The bacteria (i.e., pathogenic or non-pathogenic) which caused for SCM may originate from the farm environment, personnel or from the goats. Staphylococcus spp. are frequently isolated from cases of SCM (White and Hinckley, 1999; Marimuthu *et al.*, 2014). Others such as *Streptococcus* spp., *Pseudomonas aeruginosa, Enterobacteriaceae, Mycoplasma* spp., *E. coli, Bacillus* spp. *Listeria* spp. and other pathogens occur at lower frequencies (Contreras *et al*., 2007).

Since mastitis always related to farm management (i.e., monitoring and hygiene), therefore, by identifying the bacteria causing SCM in dairy goats, the farmers can improve prevention of the bacteria before the condition leads to clinical mastitis. The finding from this study also could assist veterinarians to prescribe the correct antibiotic treatment. Thus, the aims of the study were to isolate the bacteria from the milk causing SCM in dairy goats in small farmers in Kota Bharu, Kelantan, and further identify the bacteria using the common microbiological assays.

Materials and Methods

Eight farms that involved in this study were located in Kota Bharu District, Kelantan, Malaysia. The farms are small stakeholder dairy farms. All milk collections were conducted between March 2016

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and May 2016. The milks were collected from 51 individual dairy goats with suspected SCM. Before sampling, the first streams of milk were discarded, and teat ends were swabbed with 70% alcohol for sanitation (Tassew *et al.*, 2016). The milk samples (15 ml in each sterilised test tube) were collected and immediately chilled in the ice box. In the laboratory, all samples were kept in fridge at 4°C. The microbiological assays were done within 48 hours after sampling.

Isolation of bacteria

All samples were subjected to bacteriology assay according to the procedure suggested by UK Standard for Microbiology Investigations (2014a). The primary culture was done to detect any growth of aerobic bacteria as well as to quantify of the colonies present in the samples. The isolation of the bacteria from milk samples was performed on horse blood agar using a streak plate technique. The milk samples mixed thoroughly and a loopful of milk was streaked on blood agar plates. The sample was streaked with sterilised loop and was incubated aerobically at 37ºC for 24 hours. The individual bacterial colonies were identified based on colour and size of colony morphology (i.e., yellowish, whitish, ball point, pin point, big dry) (Cheesbrough, 2006; Becker *et al.*, 2014). The colonies identified from the primary culture were consequently subjected to the secondary culture using horse blood agar. This was done to further isolate the bacteria to get pure individual colonies of bacteria (UK Standard for Microbiology Investigations, 2013).

Identification of the bacteria

The pure colonies from secondary culture were identified according to the visual morphology on horse blood agar (i.e., colour and size of the colonies), Gram staining and microscopic characteristic (Cheesbrough, 2006; UK Standard for Microbiology Investigations, 2014b), biochemistry tests (i.e., catalase, oxidase and urease), coagulase test, salt consumption (i.e., mannitol salt agar) and ability of the bacteria to ferment lactose (i.e., MacConkey agar). The catalase test was done according to Cheesbrough (2006) with slight modifications. The tip of the wooden stick that has been smeared with the isolate was soaked with 3% hydrogen peroxide solution on the glass slide. The isolate was considered positive for catalase if there was bubble formation. Oxidase test was performed by tapping the tip of the wooden stick with the isolate onto the oxidase paper. The isolate was considered oxidase positive if the oxidase paper turns purple within 10 seconds. Urease test was conducted by streaking the agar slant with a loopful of the isolate. Then, the tube was incubated at 37°C for 24 hours. If the bacteria produces urease enzyme, the colour of the slant changes from light orange to magenta. Coagulase test using rabbit plasma was done by homogenising the presumptive individual *Staphylococcus* spp. isolates with 0.5 ml of rabbit plasma and incubated in water bath at 37°C for 4 hours. Tubes that presented plasma coagulation would be considered as coagulase positive staphylococci. The oxidase, urease, coagulase, mannitol salt agar and MacConkey tests were performed with slight modifications as described by Bridson (2006).

Statistical analysis

All tests were carried out independently in duplicate (n = 2). After analysing the isolates according to their Gram staining, morphology and biochemistry tests, they were calculated using Microsoft Excel spreadsheet. The percentages were calculated by dividing the number of presumptive isolates with total number of isolates obtained in secondary culture times 100.

Results

Isolation and identification of bacteria

Thirty three milk samples were positive in bacteria growth. From these positive samples, 41 isolates were obtained. All isolates were primarily subjected to the biochemical tests except coagulase test. Only isolates of *Staphylococcus* spp. which were selected based on their colony morphology, mannitol salt agar (MSA) and MacConkey results were proceeded to coagulase test. The results of the Gram-stain and the biochemical tests of the isolates which led to the conclusion of the presumptive organisms are presented in Table 1 and Table 2. To note, Staphylococcus spp. comprised the majority of the isolates (73.2%). Coagulase negative staphylococci (CNS) encompassing 68.3% of the isolates, whereas 4.9% was coagulase positive staphylococci (CPS). *Bacillus* spp. constituted 12.2% out of the isolates. *Listeria* spp. and *Neisseria* spp. both represented 7.3%. Table 3 summarises the bacteria that were isolated and identified to the genus level based on their microscopic examination and biochemical tests.

Discussion

To know the type of bacteria in SCM milk samples of dairy goats, the samples were subjected to microbiology assays. In this study, 80.4% of the bacteria have been isolated from SCM milks. This is

Table 1: The characteristics of Gram positive cocci isolated from the milk sample of SCM dairy goats

Isolates	Colony morphology	Coagulase test	MSA	Presumptive organism
1a	cluster	-	Y	*Staphylococcus* spp.
1b	chain to cluster	-	W	*S. epidermidis*
2a	pair to chain, cluster	-	W	*S. epidermidis*
2b	cluster	-	W	*S. epidermidis*
6	cluster	-	Y	*Staphylococcus* spp.
7	pair to chain cluster	-	Y	*Staphylococcus* spp.
7b	pair to chain, cluster	-	W	*S. epidermidis*
11a	cluster	-	Y	*Staphylococcus* spp.
11b	pair to chain, cluster	-	Y	*Staphylococcus* spp.
12b	pair tetrad to cluster	+	Y	*Staphylococcus* spp.
A2	cluster	-	Y	*Staphylococcus* spp.
A3	pair to cluster	-	Y	*Staphylococcus* spp.
A4	pair to chain, cluster	+	Y	*Staphylococcus* spp.
B3	pair to chain, cluster	-	W	*S. epidermidis*
B4	cluster	-	Y	*Staphylococcus* spp.
B5	cluster	-	Y	*Staphylococcus* spp.
B6	cluster	-	Y	*Staphylococcus* spp.
E1	chain, cluster	-	Y	*Staphylococcus* spp.
E3	cluster	-	Y	*Staphylococcus* spp.
E4	chain to cluster	-	Y	*Staphylococcus* spp.
F1	chain, cluster	-	Y	*Staphylococcus* spp.
F2	chain to cluster	-	Y	*Staphylococcus* spp.
F3	pair to chain, cluster	-	Y	*Staphylococcus* spp.
F4	chain, cluster	-	W	*S. epidermidis*
F5	cluster	-	Y	*Staphylococcus* spp.
G3	pair to cluster	-	Y	*Staphylococcus* spp.
NN1	cluster	-	Y	*Staphylococcus* spp.
NN2b	chain, cluster	-	Y	*Staphylococcus* spp.
NN3	chain to cluster	-	Y	*Staphylococcus* spp.
NN5	pair to chain, cluster	-	Y	*Staphylococcus* spp.

Y: Yellow W: White
Catalase test is positive for all isolates.
MacConkey test is positive for all isolates.
Oxidase test is positive for almost isolates except for NN2b.
Urease test is positive for almost isolates except for 1b, F4 and G3.

in agreement with Zhao *et al.* (2015), who reported that the prevalence of bacteria in SCM of dairy goats milk samples in three provinces in China were more than 95%. However, some authors got low prevalence (i.e., 38%, 29.4%) of positive samples from SCM goats (Dore *et al*., 2016; Pirzada *et al.*, 2016). Among the positive samples, 73.2% of isolated bacteria were *Staphylococcus* spp. The result is in line with Kalogridou-Vassiliadou (1991) and Marimuthu *et al*. (2014) who also found *Staphylococcus* spp. was the most prevalent (55% and 59.1%) in their SCM milk goat samples. From this *Staphylococcus* spp. isolates, CNS constituted the major bacteria (68.3%). This finding is in concordance with Contreras *et al.* (2007); Gelasakis *et al*. (2015) and Dore *et al.* (2016) who also found that CNS was the major bacteria causing SCM in goats.

Apparently, CPS was only 4.9% isolated from the *Staphylococcus* spp. isolates. This is opposite to the previous reports that reported CPS specifically *S. aureus* was the important pathogen that is responsible for the causal of SCM in goats (Ebrahimi *et al.*, 2010; Pirzada *et al*., 2016). In general, the difference between CNS and CPS is that their ability to coagulate plasma. Review by Contreras *et al.* (2003) stated that even though *S. aureus* is regarded as an important pathogen for causing subclinical and clinical mastitis, however, CNS are the most prevalent in SCM which constituted more than 50% in most of the studies. Higher incidence rate of CNS in this study could be due to poor hygiene conditions of the goats as well as the environment of the farm since CNS could infect the udder through unhygienic teat canal.

Various CNS species have been isolated from the SCM goats. Currently, Salaberry *et al*. (2015) had isolated *S. epidermidis, S. lugdunensis, S. chromogens* and *S. capitis* ss capitis. Earlier finding by Contreras *et al*. (1997) found the species of CNS namely *S. xylosus, S. chromogenes, S. caprae, S. capitis, S. warneri, S. simulans, S. epidermidis, S. lentus, S. hominis, S. sciuri* and *S. haemolyticus*. Among the species, *S. epidermidis* was the most common isolated from the SCM goat milk (Ebrahimi *et al.*, 2010; Pirzada *et al.*, 2016).

There have been few reports that SCM milk from dairy herds contains CPS species other than *S. aureus* (Phillips and Kloos, 1981; Capurro *et al*., 1999; Gandra *et al.*, 2016). Other two CPS are *S. hyicus* and *S. intermedius* (Roberson *et al*., 1992). *S. hyicus* subsp *hyicus* and *S. intermedius* have been isolated from dairy goats (Kalogridou-Vassiliadou, 1991; Salaberry *et al*., 2015), while other authors found *S. intermedius* and *S. hyicus* in the isolates of cow milk (Capurro *et al.*, 1999).

The results in this study revealed that 12.2% from the isolates was *Bacillus* spp. These bacteria are categorised as an environmental mastitis agent which contaminate the milk samples from farm surrounding. *Listeria* spp. and *Neisseria* spp. both constituted 7.3% of the isolates. *Listeria* spp. have been found in raw milk of goats (Osman *et al*., 2014) and cows (Chye *et al.,* 2004), but in low percentage. This could be due to the unhygienic milking conditions during milking process (Chye *et al*., 2004; Osman *et al*., 2014). However, it should be aware that *Listeria* spp. especially *Listeria monocytogenes* and *Bacillus* spp. are capable of causing zoonotic diseases. These pathogens are of highly importance when the milk is consumed raw by consumers. *Neisseria* spp. was the only gram negative that was isolated in this study. The presence of *Neisseria* spp. in milk is an indication that the milk samples were drawn from goats that were having *Neisseria* spp.-related diseases which were not represent any observational clinical signs as *Neisseria* spp. is not mainly considered as mastitis causal pathogens.

As shown in Table 3, there are four genus of bacteria that have been isolated from the milk samples. The identification of the bacteria was dependent on colony morphological characteristics, Gram staining morphology and biochemical tests (Table 1 and Table 2). Apparently, those tests are

Table 2: The characteristics of other bacteria isolated from the milk sample of SCM dairy goats

Isolates	Gram staining	Colony shape	Catalase	Oxidase	Presumptive organism
3	+	rod, bacillus, pair	+	-	*Bacillus* spp.
7a	+	rod, bacillus, chain	+	-	*Bacillus* spp.
B1	+	rod, bacillus , pair	+	-	*Bacillus* spp.
12a	+	rod, endospores	+	-	*Bacillus* spp.
NN4	+	rod, bacillus, pair	+	-	*Bacillus* spp.
G5	-	cocci, coffee bean shaped	+	+	*Neisseria* spp.
5a	-	cocci, coffee bean shaped	+	+	*Neisseria* spp.
5b	-	cocci, coffee bean shaped	+	+	*Neisseria* spp.
4	+	short rod	-	-	*Listeria* spp.
E2	+	short rod	+	-	*Listeria* spp.
NN2a	+	short rod	+	-	*Listeria* spp.

MacConkey and urease tests exhibited variable result which were not included in the identification of the bacteria.

not sufficient to identify the bacteria to the species level. For instance, in this study, all *Staphylococcus* spp. regardless of CNS and CPS showed similar morphology characteristics in gram staining (i.e., cluster cocci) when viewed microscopically, as well as their biochemical properties (i.e., all are catalase positive and variable results of oxidase and urease), which makes them difficult to be differentiated among species (Gandra *et al.*, 2016). Irlinger (2008) reported that the conventional biochemical tests have misidentified *S. caprae* isolates from human clinical specimens as *S. haemolyticus* or *S. hominis*. The identification for species through colony morphology colour on horse blood agar (i.e., yellow, white, grey) also is inaccurate. For example, *S. aureus* could exhibit yellow or white creamy colony on horse blood agar (UK Standards for Microbiology Investigations, 2014). MSA which is widely been used and is considered a selective medium to differentiate *S. aureus* from other *Staphylococcus* spp. also could not accurately differentiate the species. This is because, for instance, *S. saprophyticus* may ferment mannitol, thus producing yellow colonies in MSA (UK Standards for Microbiology Investigations, 2014b). This characteristic will lead to the mistaken conclusion as *S. aureus*. Suggestion has been made by Kateete *et al.* (2010) to conduct tests on MSA and coagulase plasma in order to differentiate *S. aureus*. In this study, we have done both tests as suggested. However, recent finding found that *S. chromogenes* (i.e., CNS) also is able to clot plasma (dos Santos *et al.*, 2016), which the interpretation of the coagulase test result could mislead with other CPS. This latest finding has opened our knowledge to the variable possibilities that this genus has to offer.

Table 3. Summary of bacteria isolated from the milk samples of SCM dairy goats

Genus	Number of isolates	%
Coagulase negative staphylococci	28	68.3
Coagulase positive staphylococci	2	4.9
Bacillus spp.	5	12.2
Listeria spp.	3	7.3
Neisseria spp.	3	7.3

The similar case also applies to other bacteria isolated in this study which the current tests are insufficient to identify the bacteria to the species level, unless if the identification is done by the very trained and experienced microbiologist who involves with routine analysis. Nevertheless, Irlinger (2008) has stated that routine laboratories do not usually identify CNS at species level.

Milk quality and animal health

In general, CNS is not as pathogenic as the other principal mastitis pathogens (e.g., *S.aureus, S. agalactiae*). Infection mostly remains subclinical (Pyorala and Taponen, 2009). However, if left untreated, CNS can cause persistent infections inside the mammary gland and damage udder tissue, which results in increased milk somatic cell and decrease milk production. The increment of somatic cell in milk eventually will decrease milk quality. *S. chromogenes, S. xylosus, S. cohnii* and *S. simulans* are reported to affect udder more than other CNS species (Supre *et al*., 2011).

The identification of the exact *Bacillus* spp. is unknown in this study. One of the common *Bacillus* spp. isolated from milk is *B. cereus* (Montanhini *et al.*, 2015). *B. cereus* has been widely reported to affect milk quality (Tewari and Abdullah, 2015) due to its ability to grow at refrigeration temperature (Masiello *et al*., 2014). Defects in milk that could be observed due to the bacteria is the milk turns to bitty cream and sweet curdling (Tewari and Abdullah, 2015). The presence of bacteria even in low concentration also could possibly spoil the milk in the bulk tank milk, hence will result for the economically loss to farmers due to the poor quality of milk.

Public health relevance

In Malaysia, consumption of goat milk has been increasing due to its nutritional characteristics and health benefits (Pacinovski *et al.*, 2015). In some families and culture, newborns are given the goat milk in believing to reduce neonatal jaundice. Some

consumers prefer to consume the milk in raw rather than been pasteurised based on the belief that certain components in milk will be diminished once it has been subjected to pasteurization. Even though the definition of SCM is clear theoretically, however, many small farmers in rural areas in Kelantan do not perform California mastitis test or somatic cell count as a routine test to detect SCM in their dairy herds. This ignorance might be due to lack of knowledge on the function of these tests in order to preserve the milk quality. Thus, it is possibly high that farmers will treat the milk from SCM goats similar as the milk from healthy goats. This would give potential hazards to the consumers. However, this practice need to be re-evaluated as there were findings revealed that even raw milk from healthy dairy animals could be contaminated with pathogens (Chye *et al*., 2004; Hill *et al.,* 2012; Cupakova *et al*., 2013; Jamali *et al.*, 2013).

Our study has clearly demonstrated that *Staphylococcus* spp., *Listeria* spp. and *Bacillus* spp., isolated from the subclinical mastitis goat milks are considered as potential foodborne bacteria. Even though CNS is regarded as commensal bacteria with no serious pathogenicity caused to the host and widely used in food industry specifically in meat fermentation (e.g., *S. carnosus* and *S. xylosus*) (Hugas and Monfort, 1997; Zell *et al.,* 2008) and as starter culture for making cheese (Hoppe-Seyler *et al*., 2004), however, this group of bacteria is gaining much attention when several studies reported the involvement of these bacteria in food poisoning caused by *Staphylococcus* spp. (Udo *et al.*, 1999). Basically, staphylococci food poisoning (SFP) is caused by the *Staphylococcus* spp. that could produce enterotoxins (Balaban and Rasooly, 2000; Podkowik *et al*., 2013). These enterotoxins could cause some sudden onset of clinical symptoms such as nausea, emesis, abdominal cramps and diarrhea.

Previously, *S. aureus* has been well established as the major bacteria that caused for SFP (do Carmo *et al.*, 2002; Le Loir *et al*., 2003; Argudin *et al*., 2010). However, studies by Valle *et al.*, 1990; Vernozy-Rozand *et al*., 1996; Cunha *et al*., 2007 revealed that some CNS strains also are carrying the enterotoxins genes that similar to *S. aureus*. Podkowik *et al*. (2013) have identified 23 different enterotoxins from *S. aureus*. Previously, Valle *et al.* (1990) have identified several CNS species (i.e., *S. chromogenes, S. warneri, S. sciuri, S. saprophyticus* and *S. lentus*) that produce enterotoxins from healthy goat milks. Among the enterotoxins genes, sec and sed (staphylococci enterotoxins type C and D) were detected in major quantity in CNS (Valle *et al.*, 1990; Salaberry *et al.,* 2015). Ikeda *et al.* (2005) noted that small amount of staphylococcal enterotoxin A were detected in the samples of milk causing mass outbreak of food poisoning disease in Japan, and presumed that other staphylococci enterotoxins might have contributed to the outbreak.

Apart from enterotoxins genes, genes encoding adhesions and biofilm formation (i.e., *eno, bap, ebpS, fib, fnbA*) were also detected in *Staphylococcus* spp. isolates (Salaberry *et al.*, 2015). These genes make the bacteria to adhere to the surface made from glass or plastic. Another virulence factor of CNS is to exhibit toxic shock syndrome gene (TSST-1) (Crass and Bergdoll, 1986), which is responsible in toxic shock syndrome illness. Cunha *et al.* (2007) reported that 26.7% of the CNS isolates produced some type of toxin TSST-1.

In this study, the actual identification of isolated *Listeria* spp., however, is unknown. The most significant *Listeria* spp. that is *L. monocytogenes* is of high importance since the bacteria is the source for food poisoning (Vazquez-Boland *et al*., 2001; Osman *et al*., 2014). Furthermore, this pathogen can survive and thrive in post-pasteurization, hence could promote the recontamination of the milk (Oliver *et al*., 2005). In addition, *Listeria* spp. also have virulence factors genes (i.e., *prfA, hlyA, plcA, plcB, actA, iap, mpI*) (Rawool *et al*., 2007; Osman *et al*., 2014) which possibly could contribute to variety of illness. In spite of that, *Listeria* spp. also are able to form biofilm on a glass surface and polystyrene plates (Osman *et al*., 2014). Therefore, it is of importance to aware on this ability of *Staphylococcus* spp. and *Listeria* spp. since these bacteria could be spreading easily by milking equipment made from glass and plastic.

Conclusion

The isolation and identification of the bacteria in milk causing SCM in dairy goats have been conducted. The identification of the SCM bacteria in this study is only limited to the genus level. The results showed that *Staphylococcus* spp., especially CNS were major bacteria found in SCM goat milks. This finding helps in understanding the behaviour of *Staphylococcus* spp. isolated throughout this study with regard to colony morphological characteristics, Gram staining morphology and biochemical tests. This emphasizes the importance of accurate and reliable methods for identification of the species of bacteria. The high prevalence of CNS in SCM goat milk with potential enterotoxins genes and other virulence factors suggests that the raw consumption of milk may present a risk for public health as well as

the safety of dairy products related to goat milk. The knowledge on major CNS species involved in SCM is still very limited. Hence, more accurate identification method such as on molecular aspect using multiplex polymerase chain reaction to identify the phenotypic and genotypic characteristics of bacteria would be necessary to identify the exact species of the bacteria to avoid any mistaken conclusion of the results. This is of importance to determine their pathogenic significance, virulence factors required to cause poisoning and invasion into the mammary gland, hence will help veterinarians in prescribing the correct antibiotic treatment.

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