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RESEARCH NOTE

TETRACYCLINE RESISTANCE GENE IN *Streptococcus agalactiae* ISOLATED FROM BOVINE SUBCLINICAL MASTITIS IN SURABAYA, INDONESIA

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ABSTRACT

The aim of this research was to isolate, identify and determine tetO resistance genes in tetracycline-resistant *Streptococcus agalactiae* isolated from cows with subclinical mastitis in Surabaya and surrounding areas of Indonesia. Milk samples from cows with subclinical mastitis in six dairy farms were collected. *S. agalactiae* was isolated and antibiotic resistance was determined. Results showed that out of 173 samples analyzed, 131 (75.7%) were positive for California Mastitis Test. *S. agalactiae* was isolated in 36 out of the 131 CMT-positive samples. Antibiotic sensitivity test revealed that out of 36 *S. agalactiae* samples, nine were resistant to tetracycline. PCR analysis showed that six of the nine tetracycline resistant *S. agalactiae* isolates were positive for the *tetO* resistance genes.

Key words: *Streptococcus agalactiae,* subclinical mastitis, *tetO* gene, tetracycline resistance

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INTRODUCTION

Streptococcus agalactiae is an important cause of chronic, contagious bovine mastitis. It also causes mastitis and invasive disease in camels and is an occasional cause of disease in dogs, cats, fish and hamsters. Its presence is frequently associated with high somatic cell counts in milk and decreased milk yield (Jain *et al.,* 2012).

There are two kinds of mastitis: clinical mastitis with clearly defined clinical signs and subclinical mastitis with unobservable clinical signs (Hashemi *et al.,* 2011). Subclinical mastitis is the most dominant form in Indonesia (Effendi and Harijani, 2017) and can be found in Bogor (76%), Boyolali (91%) and Malang (81%). Differences in incidence rate of subclinical mastitis by area are also observed, in which Yogyakarta has 72%; Central Java, 65%; and East Java, 44.46% (Sudarwanto and Sudarnika, 2008; Wahyuni, 2005).

S. agalactiae and *Staphylococcus aureus* are common causes of bovine mastitis. Although there are plenty of research on *Staphylococcus* in Indonesia, research on *S. agalactiae* is limited. Therefore, it will be useful to do research on this pathogen for guidance on the prevention and control of mastitis and also for public health awareness. According to Dogan *et al.* (2005) and Songer and Post (2005), *S. agalactiae* can cause various diseases to humans, such as bacterial sepsis, pneumonia, meningitis, Scarlet fever and tonsillitis (Duarte *et al.,* 2005).

S. agalactiae often causes subclinical mastitis in dairy cattle causing economic loss for the industry (Alemu *et al.*, 2014). Dairy farmers ranked mastitis as a major disease problem in their farms (Carvalho-Castroa *et*

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al., 2017). Veterinarians are often asked to provide information for herd level control and eradication of *S. agalactiae*. Farmers are often involved with veterinarians in the treatment using antibiotics, especially tetracyclines, to solve mastitis problem (Jain *et al.*, 2012).

Tetracycline is one of the most commonly used antibiotic in many developing countries, both in human and veterinary medicine. The main reasons are its relatively low cost and availability (Zibandeh *et al.*, 2016). This class of antibiotics is still used in developed countries for prophylactic and therapeutic purposes. The widespread use of tetracycline in dairy farming could result in horizontal transfer of resistance from bovine to humans as well as to the environment. Treatment with intramammary infusion of antibiotics is the main approach to deal with mastitis, and a number of *in vivo* and *in vitro* trials to assess the antibiotic sensitivity/resistant pattern have been documented. However, there are few reports focusing on the genes involved in resistance especially for *S. agalactiae* isolates of bovine origin. The present study aims to identify tetracyline resistant gene in *S. agalactiae* isolated from subclinical mastitis cases.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from six dairy farms in Surabaya, namely Kaliwaron, Sutorejo, Wonocolo, Sepanjang, Taman and Wonoayu (Table 1). The six farms were visited during the afternoon milking. Complete herd size (including calves and young stock) varied between 14-83 animals and number of lactating cows (only counting the animals being milked at the time of the visit) varied between 10-65 cows. In total, 173 animals were examined. Before sampling, the teats were scrubbed with cotton soaked in 70% ethanol and the first squirt of milk was discarded. Approximately, 10 ml of milk was collected from each teat and samples from one cow were pooled together as one sample (Effendi and Harijani, 2017). Milk samples were placed in sterile tubes and stored in ice box during transport. A total of 173 milk samples were collected from individual cows.

California mastitis test

Cases of subclinical mastitis based on California mastitis test (CMT) were investigated. CMT is a simple indicator of the somatic cell count in milk. Positive test reactions were graded by visually - Grades 0, +1, +2 and +3). Grade +1 shows formation of solid gels; Grade +2 shows formation of solid thick gels at the *paddle* center; and Grade +3 shows large number of solid gels with convex surface (Björk, 2013).

Identification of *Streptococcus agalactiae*

Milk samples were streaked in nutrient agar (NA) (E. Merck, Darmstadt, Germany) and incubated for 24 h at 37°C. The isolates were subcultured in blood agar (BA) (E. Merck, Darmstadt, Germany) to identify the *Streptococcus* with characteristic ά-hemolysis, ß-hemolysis or without hemolysis/γ-hemolysis. Suspected *Streptococcus* spp. was characterized using gram staining and catalase test. *Streptococcus* colonies with ß-hemolysis

Table 1. Microbiological analysis and AST results of milk samples from Surabaya, Indonesia.

Name of farm	Number of population	Number of samples	CMT-Positive	*S. agalactiae* positive	Tetracycicline resistant
Kl farm	35	20	15	7	1
Wn farm	83	50	42	10	2
St farm	14	8	6	4	none
Sp farm	47	30	21	6	3
Tm farm	75	35	24	4	1
Wy farm	62	30	23	5	2
TOTAL	316	173	131	36	9

were characterized using Christie-Atkins-Munch-Peterson test (CAMP) to identify *S. agalactiae* strains (Ahmadi *et al.,* 2009).

Antibiotic sensitivity test

To detect for antibiotic resistance, the disc diffusion method, as described by Lopez-Lazaro *et al.,* (2000) was employed and the interpretation was made according to the zone size interpretation chart provided by the disc manufacturer.

Polymerase chain reaction

DNA extraction was carried out as described by Rato *et al.* (2013) with minor modifications: doubling the time of centrifugation, the amount of enzymes and addition of a final step for DNA precipitation by ethanol. Briefly, 1 ml of each sample was transferred to a microtube and centrifuged at 14,000 rpm for 4 min. The supernatant was discarded, and the pellet was re-suspended and washed 2-3 times with Tris-EDTA buffer (Tris-HCL 10 mM, EDTA 1 ml, pH 8.8) until a clear solution was obtained. The pellet was washed with PCR buffer (Buffer 10X: Tris- HCl 100 mM, KCl 500 mM, pH 8.8) and finally resuspended in 100 µl of PCR buffer. Thereafter, lysozyme (Merck, Germany) was added to each sample at a concentration of 2 mg/ml, and the sample was incubated for 20 min at room temperature. After this, proteinase K (Fermentas, Germany) was added at a concentration of 400 µg/ml and the sample was incubated at 56°C for 1 h. The sample was then boiled for 15 min and centrifuged at 14,000 rpm for 45 sec.

Approximately, 5 µl of DNA extract was used as template for the PCR amplification of the *tetO* gene fragment. In brief, 20 µl of PCR reaction consisted of 12.5 µl master mix, 0.5 µl distilled water, 1 µl of forward and reverse primers (Table 2) and template DNA. Thermocycling conditions were as follows: prewarming at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1 min 30 sec (Jain *et al.,* 2012). Electrophoresis was performed at 110V for 30 min. PCR products were stained by ethidium bromide and observed under ultraviolet light.

RESULTS AND DISCUSSION

Analysis of milk samples showed that 131 out of the 173 samples (75.7%) were positive for CMT. This result showed was similar with other reports showing high evidence rate of subclinical mastitis in East Java area. Previous reports showed that the prevalence rate of mastitis in dairy farms around East Java was at 80-86%; Nongkojajar at 82.7%; Batu at 83.1%; Surabaya at 86.4%; and Grati at 79.5% (Effendi, 2008). This study showed that the majority of subclinical cases of mastitis were due to contagious pathogens such as *S. aureus* and *S. agalactiae*. This might be related to poor milking and mastitis control practice seen in the studied farms. In the absence of hygienic milking practice, pathogens from either infected cow or dirty hands (from milking) can easily spread.

Bacterial isolation was performed on CMT positive samples using morphology, gram staining and CAMP tests (Fig. 1). Thirty-six samples were positive for *S. agalactiae*. Furthermore, antibiotic sensitivity test showed that nine isolates were resistant to

Table 2. Primers for *tetO* gene for milk samples from cows with subclinical mastitis.

Gene	Primer sequence	Position	Size amplification (bp)
tetO	F: 5′-GCGTCAAAGGGGAATCACTATCC-3′	146-169	1723 bp
	R: 5′-CGGCGGGGTTGGCAAATA-3′	1851-1868	

Source: Jain *et al.* (2012).

tetracyclines (30 ug).

Tetracycline is a family of broad-spectrum antibiotics often used in livestock production. The first generation tetracyclines, such as tetracycline, chlortetracycline and oxytetracycline, have been widely used as a growth promoter for decades and the second generation, such as minocycline and doxycycline, is commonly used both in prophylaxis and therapeutics in humans and animals (Eliopoulos and Roberts, 2003). Tetracycline is used for all animal food production species, mainly because of its wide-spectrum activities, price and availability. However, extensive use of tetracyclines can lead to the emergence of resistant bacteria (Chopra and Roberts, 2001). The extended use of tetracycline may result to selection pressure and, ultimately, resistance.

PCR results showed that six out of the nine tetracycline resistant *S. agalactiae* samples were positive for *tetO* genes, with PCR bands

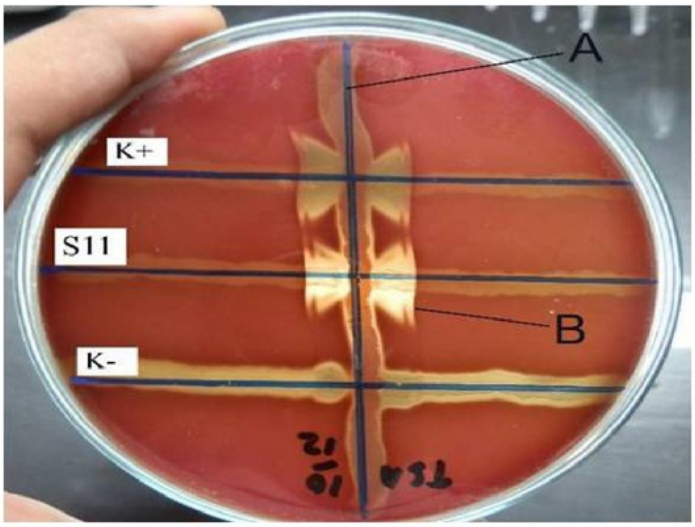

Fig 1. Christie-Atkins- Munch-Peterson (CAMP) test result of milk sample from cow with subclinical mastitis. A: *Stapylococcus aureus* bacteria; B: arrow marks that show CAMP test results; K+: Positive control, *Streptococcus agalactiae*; S11: sample; K-: negative control, *Streptococcus pyogenes*.

of 1723 bp (Fig. 2). Out of the nine tetracycline resistant isolates, six isolates were found positive for tetracycline resistance gene (*tetO*); three isolates were negative (Table 3).

Streptococcus tetracycline resistance genes were *tetL, tetM, tetO, tetQ* and *tetT*. The genes of *tetO* and *tetM* were identified as the dominant tetracycline resistance encoding gene, where *tetM* is found in *S. agalactiae* from human isolates and *tetO* gene in *S. agalactiae* from dairy isolates (Dogan *et al.*, 2005). Research by Duarte *et al.* (2005) showed that the major tetracycline gene is the *tetO* gene from 27 of 38 milk samples (71%).

The mechanism of action of tetracyclines has been reviewed by Velhner and Milanov (2015). Mainly, tetracyclines inhibit reversible protein synthesis of bacteria by binding to the ribosomal complex, preventing the aminoacyl-tRNA association with bacterial ribosomes. This results to weakened interaction of the ribosome-tRNA, thus halting protein synthesis.

Bryan *et al.* (2004) reported that environment, human and animal exposure to tetracyclines, as well as to other antibiotics may contribute to the development and spread of antibiotic resistance through horizontal gene transfer. *S. agalactiae* infections in both humans and bovines are treated by

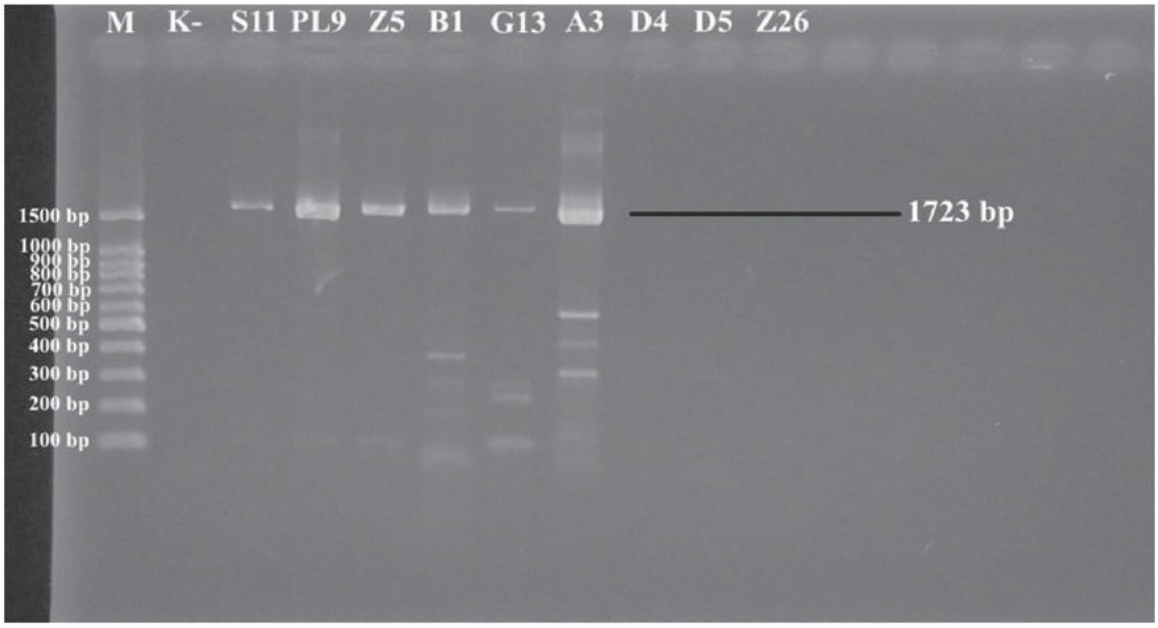

Fig. 2. PCR analysis of *tetO* gene in tetracycline resistant *S. agalactiae* from milk sample in cow with subclinical mastitis; *tetO* gene is indicated by the DNA band at 1723 bp.

Table 3. Determination of *tetO* resistance genes in tetracycline resistant *S. agalactiae* from milk sample in cow with subclinical mastitis.

Name of farm	Tetracycline resistant *S. agalactiae*	*tetO* gene
Kl farm	1	1
Wn farm	2	1
St farm	none	none
Sp farm	3	2
Tm farm	1	none
Wy farm	2	2
TOTAL	9	6

administration of antibiotics (Jake *et al.*, 2013). Extensive use of antibiotics in medicine and animal husbandry results to increased antibiotic resistance among bacterial populations (Gao *et al.*, 2012). Several studies have suggested that antimicrobial use in animals causes the development of antibiotic resistance among pathogens in humans (Dogan *et al.*, 2005). Therefore, an effective information campaign is needed to create awareness on the spread of antimicrobial resistance and the requirements on proper hygiene and adoption of other preventive measures by rural farmers to reduce losses due to subclinical mastitis.

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