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The Comparison of *Streptococcus agalactiae* Isolated from Fish and Bovine using Multilocus Sequence Typing

ANGELA MARIANA LUSIASTUTI[1*], HELGA SEEGER[2], AGUSTIN INDRAWATI[3], MICHAEL ZSCHÖCK[2]

[1]Research Institute of Freshwater Aquaculture, Jalan Sempur 1, Bogor 16154, Indonesia
[2]Landesbetrieb Hessisches Landeslabor, Schubert Straße 60, Haus 13, 35396 Gießen, Germany
[3]Faculty of Veterinary Medicine, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia

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Multilocus sequence typing (MLST) has greater utility for determining the recent ancestral lineage and the relatedness of individual strains. Group B streptococci (GBS) is one of the major causes of subclinical mastitis of dairy cattle in several countries. GBS also sporadically causes epizootic infections in fish. The aim of this study was to compare the evolutionary lineage of fish and bovine isolates in relation to the *S. agalactiae* global population as a whole by comparing the MLST profiles. Twenty *S. agalactiae* isolates were obtained from dairy cattle and fish. PCR products were amplified with seven different oligonucleotide primer pairs designed from the NEM316 GBS genome sequence. Clone complexes demonstrated that bovine and fish isolates were separate populations. These findings lead us to conclude that fish *S. agalactiae* is not a zoonotic agent for bovine. MLST could help clarify the emergence of pathogenic clones and to decide whether the host acts as a reservoir for another pathogenic lineage.

Keywords: multilocus sequence typing, *Streptococcus agalactiae*, bovine, fish

INTRODUCTION

Several molecular typing methods have been used to investigate the epidemiology of *Streptococcus agalactiae*. Multi locus sequence typing (MLST) differs fundamentally from pulse field gel electrophoresis (PFGE) and most other molecular typing methods, being based on nucleotide sequence data from approximately 500 bp of housekeeping genes. MLST has greater utility for determining the recent ancestral lineage and the relatedness of individual strains. MLST can be represented by a number of digits corresponding to the allelic number of each of the loci being used. This greatly facilitates inter laboratory comparisons and the study of global epidemiology (Feil & Enright 2004).

Group B streptococci (GBS) is one of the major causes of subclinical mastitis in dairy cattle in several countries (Merl *et al.* 2003). Lancefield (1934) recognized GBS as a human pathogen before 1960 but only in sporadic cases (Anthony & Okada 1977; Trijbels-Smeulders *et al.* 2004). *Streptococcus agalactiae*, GBS, is an important human pathogen, and the leading cause of septicaemia, meningitis and pneumonia in neonates, responsible for two to three cases per 1000 live birth. GBS also sporadically causes epizootic infections in fish. Today, with the intensification of aquaculture, *S. agalactiae* is become a significant cause of mortality and morbidity in both marine and freshwater cultured species, and particularly in tilapia. Several environmental factors, such as warm water temperatures, increased ammonia levels and low dissolved oxygen levels, play an important role in *S. agalactiae* outbreaks. Clinical signs of disease include anorexia, "C" shaped body posturing and erratic swimming, and many outbreaks cause considerable mortalities (Evans *et al.* 2002).

Recently, Lusiastuti and coworkers identified the isolate of *S. agalactiae* outbreak in tilapia *Oreochromis niloticus* in Indonesia and speculated GBS isolate from fish have a relationship with GBS of bovine and human origin and they can disseminate globally and cause fish disease (Lusiastuti *et al.* 2009a,b; Skov-Sorensen *et al.* 2010). In the present study, we performed MLST analyses on *S. agalactiae* isolates from fish and bovine. The aim of this study was to compare the evolutionary lineage of fish and bovine isolates in relation to the *S. agalactiae* global population as a whole by comparing the MLST profiles.

MATERIALS AND METHODS

Bacterial Isolates. In total, 20 *S. agalactiae* isolates were obtained from dairy cattle and fish.

[*]Corresponding author. Phone: +62-251-8313200, Fax: +62-251-8327890, E-mail: lusiastuti_61@yahoo.co.id

All isolates were collected between April 1999 and January 2010. Table 1 shows the origin of the milk and fish samples, source and kind of isolates.

***S. agalactiae* Culture, Identification, and Characterization.** GBS from milk was isolated by spreading 50 µl of the sample on Columbia sheep blood agar plate (Oxoid Ltd., Basingstoke, United Kingdom). Fish-isolates suspected GBS obtained from the brain, head kidney, eye, and liver were cultured on 5% Columbia sheep blood agar (Oxoid, Ltd. Basingstoke, United Kingdom). Plates were incubated at 25-30 ºC for 48 h. Haemolysis and CAMP test were performed as described by MacFaddin (2000). The biochemical test was derived from the Vitek 2 system version 04.01, following the manufacturer's instruction and results were compared with the analytical profile index of the system. Using a commercial streptococcal grouping kit with type B antisera (Oxoid) based on the Lancefield grouping (Lancefield 1934). In addition, *S. agalactiae* species-specific PCRs for the 16S rRNA gene were performed for primary species identification (Yildirim 2002) (Table 2). The individual isolates were designated as *S. agalactiae* if the 16S rRNA sequence was 100% identical to the reference sequence.

Table 1. Milk isolates, source, and origin

Samples number	Collection date	Isolated species
M262/8	11.03.1999	*S. agalactiae*
M445/81	21.04.1999	*S. agalactiae*
M693/x	23.06.1999	*S. agalactiae*
M844/x	09.07.1999	*S. agalactiae*
M1047/246	18.08.1999	*S. agalactiae*
M1387/x	12.10.1999	*S. agalactiae*
M207/x	24.03.2000	*S. agalactiae*
M544/x	19.05.2000	*S. agalactiae*
M1001/x	05.09.2000	*S. agalactiae*
M1277/x	26.10.2000	*S. agalactiae*
M1121 Tank	21.08.2002	*S. agalactiae*
M1428 Tank	30.10.2002	*S. agalactiae*
M1520/48	04.12.2002	*S. agalactiae*
M1045/81	19.08.2003	*S. agalactiae*
F1	May, 2008	*S. agalactiae*
F2	June, 2009	*S. agalactiae*
F3	September, 2008	*S. agalactiae*
F4	June, 2009	*S. agalactiae*
F5	June, 2009	*S. agalactiae*
F6	September, 2009	*S. agalactiae*

Species-Specific Identification of *S. agalactiae* by PCR. One to three colonies of each GBS isolate which previously grown freshly on Columbia sheep blood agar at 37 ºC for 24 h were resuspended in a reaction tubes containing 200 µl TE buffer (10 mmol/l of Tris–HCl, 1 mmol/l of EDTA; pH 8.0). After homogenization this suspension was heated at 90 ºC for 15 min, and centrifuged at 14.000 rpm for 10 min. The species-specific identification of the *S. agalactiae* gene was carried out by PCR protocol according to Yildirim (2002) using the primer sequences agal I 5'-ATAAGAGTAATTAACACATGTTAG-3' (forward) and agal II 5'-ACTTCGGGTGTTACAAAC-3' (reverse) targeted to amplify 1250 bp. Briefly, cycling conditions were 95 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 1 min, annealing at 50 ºC for 1 min and extension at 72 ºC for 7 min, followed by a final extension at 72 ºC for 7 min. PCR products were determined by electrophoresis of 12 µl of the amplicons in a 2% agarose gel (Biozym, Hessisch-Oldendorf, Germany) at 120 Volt in 1x Tris–acetate-electrophoresis buffer (TAE) [(0.04 mol/l Tris, 0.001 mol/l EDTA; pH 7.8)]. The molecular marker GeneRuler 100 bp DNA ladder (MBI Fermentas) was used to determine the bands size.

Multi Locus Sequence Typing (MLST) Amplification and Nucleotide Sequence Determination. The positive samples from the identification of colonies by PCR were tested with a range of primers (Table 2). PCR products were amplified with seven different oligonucleotide primer pairs designed from the NEM316 GBS genome sequence. The PCR amplification were performed in 50 µl reaction mixture comprised 5 µl DNA samples, 20 pmol/µl of each PCR primer (TIB MOLBIOL Syntheselabor, Berlin, Germany), 1 x PCR buffer with 1.5 mM MgCl2 (Qiagen, Hilden, Germany), 0.5 µl of Taq DNA polymerase (Qiagen), and 1.6 mM deoxynucleoside triphosphate mix (dNTPs, ABgene, Epsom, United Kingdom). The cycling conditions included an initial denaturation at 94 ºC for 1 min, primer annealing at 55 ºC for 45 s, and extension at 72 ºC for 1min for 35 cycles. The PCR products were resolved by agarose gel electrophoresis and photographed under UV light.

Table 2. PCR Primers

Primer name	Target gene	Primer function	PCR product (bp)	Primer sequence
16S forward	16S rRNA	PCR and sequencing	5'-GAGTTTGATCATGGCTCAG-3' (forward)	220
16S reverse			5'-ACCAACATGTGTTAATTACTC-3' (reverse)	
agal-I	16S rRNA	PCR (species identification)	5'-ATAAGAGTAATTAACACATGTTAG-3' (forward)	1250
agal-II			5'-ACTTCGGGTGTTACAAAC-3' (reverse)	

The amplification products were purified by MicroElute Cycle Pure Kit (Omega Bio-tek) as recommended by the manufacturer's instruction and their nucleotide sequences were determined on each DNA strand with Eppendorf BioPhotometer 6131. The purified product was then sequenced commercially (Gottingen Laboratories) and the sequences were compared with the gene sequences in the Gene Bank database using the National Center for Biotechnology Information Blast search tool. Each isolate was therefore designated by a seven-interger number, constituting its allelic profile. The following seven loci were selected for the MLST scheme (Table 3): alcohol dehydrogenase gbs0054 (*adhP*), phenylalanyl tRNA synthetase (*pheS*), amino acid transporter gbs0538 (*atr*), glutamine synthetase (*glnA*), serine dehydratase gbs2105 (*sdhA*), glucose kinase gbs0518 (*glcK*), and transketolase gbs2105 (*tkt*). Isolates with the same allelic profile were assigned to the same sequence type (ST), which were numbered in the order of their identification (ST-1, ST-2, etc.). The data have been deposited in a database which is accessible at http://sagalactiae.mlst.net.

Assignment to Clonal Groups. The allelic profiles and STs detected in this study, were downloaded from the *S. agalactiae* MLST website and analyzed by the eBURST approach (version 3 [http://eburst.mlst.net]). The isolates were assigned to clonal complexes named according to the inferred founding genotype: CC 17, CC 4, CC26, or CC 1 etc, as originally defined by Smith *et al.* (1993): "Clonal complexes are composed of two parts, a 'consensus group', i.e. a group of bacteria that are identical at all seven loci, and 'single locus variants' (SLVs) which are identical to a consensus group at six loci but which differ at the seventh". Isolates that did not fulfill these criteria were regarded as singletons.

RESULTS

Twenty isolates were identified and confirmed as *S. agalactiae* using Vitek 2 System. In addition all isolates were positive using 16S rRNA gene *S. agalactiae* species-specific PCR. The results of hemolytic activity showed variation between fish and bovine GBS isolates (Table 4). All of fish GBS isolates were non-hemolytic, while 14 of the bovine isolates were beta hemolytic (Figure 1).

The results of CAMP factor production revealed that all of the bovine isolates were positive while fish isolates were CAMP negative. There were no phenotypic differences between the GBS fish and bovine isolates. Molecular characterization of the GBS isolates were conducted by PCR. Positive PCR test were observed for all GBS bovine and fish isolates.

The result of clonal complexes of the bovine and fish characterized in this study using the eBurst software program showed that there were no relationship between GBS from bovine and fish isolates. MLST has demonstrated that bovine GBS isolates do not share STs with fish GBS isolates, confirming the largely divergent nature of fish and bovine GBS.

DISCUSSION

Group B streptococcus (GBS) *S. agalactiae* is the cause of severe disease affecting fish and bovine (Tomita *et al.* 2008). Different phenotypic characteristics of *S. agalactiae* have been noted

Table 3. Oligonucleotide primers for GBS MLST

Locus	Utility	Name and sequence of primer		Amplicon size (bp)
		Forward (5' to 3')	Reverse (5' to 3')	
AdhP	Amplification	GTTGGTCATGGTGAAGCACT	ACTGTACCTCCAGCACGAAC	672
	Sequencing	GGTGTGTGCCATACTGATTT	ACAGCAGTCACAACCACTCC	498
PheS	Amplification	GATTAAGGAGTAGTGGCACG	TTGAGATCGCCCATTGAAAT	723
	Sequencing	ATATCAACTCAAGAAAAGCT	TGATGGAATTGAATGGCTATG	501
Atr	Amplification	CGATTCTCTCAGCTTTGTTA	YGATGGAATTGATGGCTATG	627
	Sequencing	CGATTCTCTCAGGCTTTGTTA	AAGAAATCTCTTGTGCGGAT	501
glnA	Amplification	CCGGCTACAGATGAACAATT	CTGATAATTGCCATTCCACG	589
	Sequencing	AATAAAGCAATGTTTGATGG	GCATTGTTCCCTTCATTATC	498
sdhA	Amplification	AGAGCAAGCTAATAGCCAAC	ATATCAGCAGCAACAAGTGC	646
	Sequencing	AACATAGCAGAGCTCATGAT	GGGACTTCAACTAAACCTGC	519
glcK	Amplification	CTCGGAGGAACGACCATTAA	CTTGTAACAGTATCACCGTT	607
	Sequencing	GGTATCTTGACGCTTGAGGG	ATCGCTGCTTTAATGGCAGA	459
tkt	Amplification	CCAGGCTTTGATTTAGTTGA	AATAGCTTGTTGGCTTGAAA	859
	Sequencing	ACACTTCATGGTGATGGTTG	TGACCTAGGTCATGAGCTTT	480

Table 4. Bovine and fish isolates' phenotype properties, ST and allelic profile of GBS isolates

No. of isolates	ST type	+ Hemolysis	- CAMP	- PYR	+ Hip	- Starch	- Arg	- Esc	- Sor	- Tre	+ Rib	- Inu	- Lac	+ VP	Allelic profiles adhP	pheS	atr	glnA	sdhA	glcK	tkt
1	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	9	-	1	4	-	-
2	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	9	-	2	-	-	-
3	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	1	-	2	1	-	-
4	Non 261	+	+	-	+	-	-	-	-	-	-	-	-	+	--	9	-	13	1	-	-
5	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	--	--	1	3	--	--
6	Non 261	+	+	-	+	-	-	-	-	-	-	-	-	+	--	9	--	2	--	--	--
7	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	1	--	1	--	--	--
8	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	1	--	1	--	--	--
9	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	1	--	1	--	--	--
10	Non 261	+	+	-	+	-	-	-	-	-	-	-	-	+	--	9	--	13	--	--	--
11	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	9	--	2	1	--	--
12	Non 261	+	+	-	+	-	-	-	-	-	-	-	-	+	--	9	--	20	3	--	--
13	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	9	--	-	3	--	--
14	Non 261	+	+	-	+	-	-	-	-	-	+	-	-	+	--	9	--	2	9	--	--
15	261	-	-	-	+	-	-	-	-	-	+	-	-	+	54	17	31	4	26	25	19
16	261	-	-	-	+	-	-	-	-	-	+	-	-	+	54	17	31	4	26	25	19
17	261	-	-	-	+	-	-	-	-	-	+	-	-	+	54	17	31	4	26	25	19
18	261	-	-	-	+	-	-	-	-	-	-	-	-	+	54	17	31	4	26	25	19
19	261	-	-	-	+	-	-	-	-	-	+	-	-	+	54	17	31	4	26	25	19
20	261	-	-	-	+	-	-	-	-	-	-	-	-	+	54	17	31	4	26	25	19

- negatif, + positif, -- not detected.

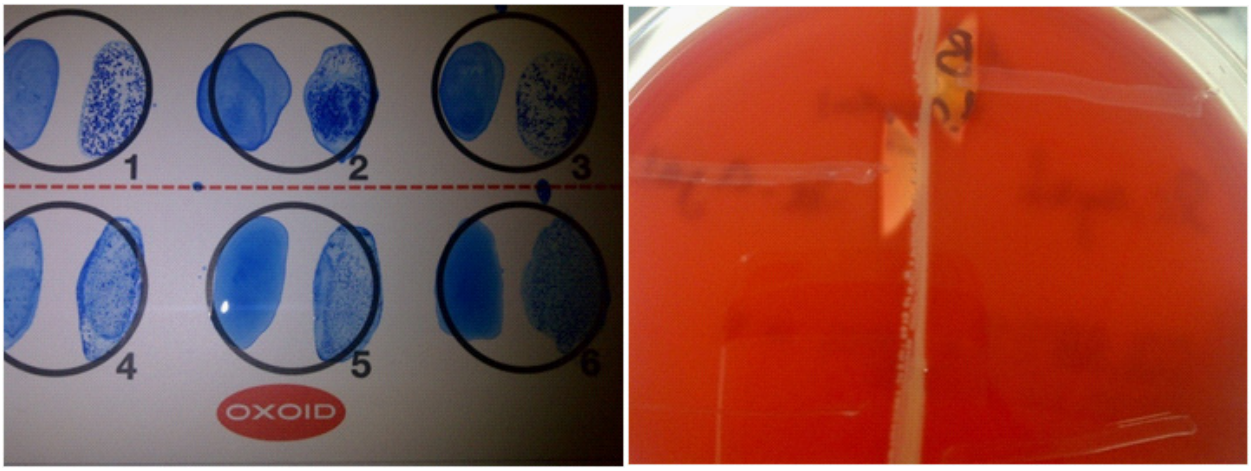

Figure 1. Fish isolates: Lancefield positive GBS (left) and negative results of CAMP test (right).

between bovine and fish isolates. The fish isolates have been considered less versatile phenotypic variants of normal β-haemolytic group B *S. agalactiae* by being non-haemolytic (Figure 1). The bovine isolates appeared more typical of group B streptococci in biochemical test than the fish isolates. However, the possibility of transmission of GBS fish isolates infecting bovine or *vice versa* has not been established. According to Pereira *et al.* (2010), streptococcal transmission between fish was most likely because of contact with carrier or infected fish and not other animals.

MLST has helped to define the phylogenetic relationship between fish and bovine strains based on their composite allele numbers which comprising each sequence type (ST) or between the concatenated sequences of the individual amplicons (Jones *et al.* 2003). Clone complexes showed that GBS fish isolates different from bovine by MLST was verified in this study. Clone complexes demonstrated that bovine and fish isolates were separate populations (Figure 2). ST 261 fish isolates and ST non-261 from bovine appeared in separate clone. E-burst did not cluster this ST 261 from

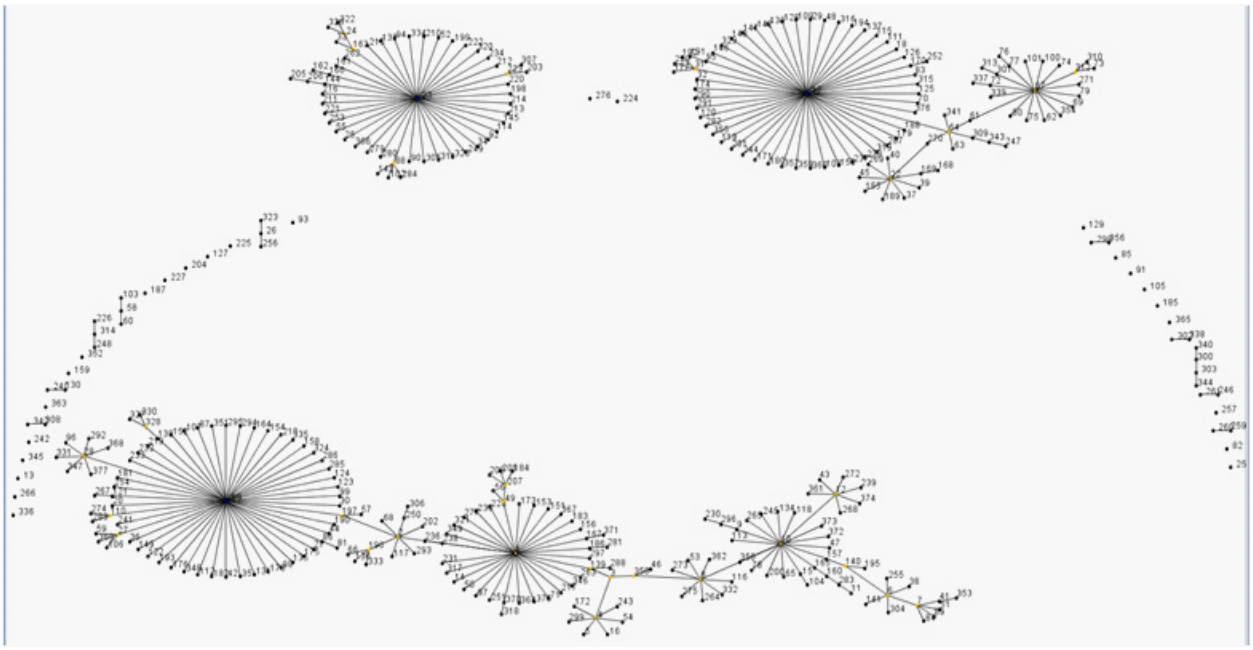

Figure 2. Clonal complexes of the bovine and fish characterized in this study using the eBurst software program.

fish with any STs from human and bovine. The six fish isolates with ST 261 are similar in that they all lacked beta hemolytic and CAMP factor. This result of MLST was support Evans *et al.* (2008) that piscine GBS isolates from Brazil, Israel, Honduras, and the USA appeared to represent a distinct genetic population of strains that were largely unrelated to bovine GBS. Previous study of genetic relationships concluded that genetic linkage is not prerequisite for *S. agalactiae* to cross the host-spesific barrier (Pereira *et al.* 2010).

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