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RESEARCH ARTICLE

Biofilm Formation and Biofilm-Associated Genes Assay of *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis in China

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ABSTRACT

Staphylococcus aureus is one of the most common pathogens responsible for contagious bovine mastitis. Genes involved in biofilm formation is a special defensive mechanism of this pathogen to combat the host immune response and remain stable in hostile environment. The present study was designed with objectives to investigate strategies involving biofilm formation and biofilm associated genes (BAGs) of *S. aureus* strains, and to assess the consistency of two phenotype test methods. One hundred and two *S. aureus* strains were isolated from bovine subclinical mastitis cases from 32 commercial dairy farms in nine provinces of China. These isolates were screened for biofilm-producing capacity by Congo Red Agar (CRA) and Semi Quantitative Adherence Assay (SQAA) methods. Thirteen BAGs including *rbf*, *SigB, SasG, icaA, sarA, icaR, icaD, clfA, clfB, fib, fnbpB, bap* and *fnbpA* were amplified by PCR assay. The results of current study revealed that *rbf* (95.1%) and *SigB* (94.1%) were the most prevalent BAGs, followed by *SasG* (89.2%), *icaA* (88.2%), *sarA* (87.3%), *icaR* (84.3%), *icaD* (82.5%), *clfA* (64.7%), *clfB* (45.1%), *fib* (43.1%) and *fnbpB* (19.6%). However, *bap* and *fnbpA* genes were not detected in any strain. By CRA method, 78.4% strains of *S. aureus* produced biofilm and 48.0% of strains were biofilm-positive by SQAA. Therefore, the data concluded that majority of *S. aureus* strains were capable to produce biofilm, controlled by eleven associated genes, and CRA detection rate was higher than SQAA for biofilm producing capacity of *S. aureus*.

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INTRODUCTION

Bovine mastitis is an important and costly disease of the dairy animals and mostly caused by *Staphylococcus* bacteria (Khan and Khan, 2006; Hussain *et al*., 2012). In staphylococcal infections, *Staphylococcus aureus* are the major organisms causing mastitis. *S. aureus* belongs to opportunistic pathogenic bacteria, possess polysaccharides and adhesion protein factors on cell surface, having special ability to construct and maintain biofilms in the host tissues. Biofilm gives special strength to *S. aureus* to contest wide range of adverse circumstances in the host, and is considered a major virulence factor in the pathogenesis of mastitis, evade the host immune response and craft multidrug resistance (Vergara-Irigaray *et al.,* 2009; Brady *et al.,* 2011; Kenar *et al.,* 2012). It has been reported that the high incidence and treatment difficulty of *S. aureus* mastitis may be related to biofilm formation in China (Li *et al.,* 2011), and biofilm formation is mostly responsible for recurrence and chronic mastitis in dairy herds.

Biofilm is composed of polymeric N-acetylglucosamine and its formation process includes initial attachment, cellular aggregation, clumping, exopolysaccharide production and detachment of planktonic cells (Melchior *et al.,* 2006). Adhesion is the first step for biofilm formation which is regulated by adhesion genes such as *fnbpA*, *fnbpB* (encoding fibronectin binding proteins A and B), *fib* (encoding fibrinogen binding protein), and *clfA* (encoding clumping factors A), etc. *FnbpA* and *fnbpB* encode fibronectin-binding proteins whereas *fib* (fibrinogen binding protein) and *clfA* encode fibrinogen-binding proteins (Tsompanidou *et al.,* 2012). To date, biofilm associated protein (*bap*) gene has only

been found in bovine mastitis isolates and its expression could enhance the intra-mammary adherence and biofilm formation. *SarA* (Staphylococcal accessory regulator A) is involved in production of a staphylococcal surface protein called *Bap* by utilization of its associated gene (*bap*). The transcription of the *icaADBC* gene operon is negatively regulated by an adjacent five-nucleotide base *icaR* gene sequence, which itself codes for a transcriptional regulator that binds to the *icaADBC* promoter (Rumi *et al.,* 2013). *S. aureus* surface protein G (*SasG*) binds to the extracellular matrix; involved in biofilm formation (Geoghegan *et al.,* 2010).

The prevalence of biofilm formation and the distribution of various BAGs in *S. aureus* from bovine subclinical mastitis have been reported by many researchers in different regions (Fox *et al.,* 2005; Oliveira *et al.,* 2006; Seo *et al.,* 2008; Vautor *et al.,* 2008; Wiśniewska *et al.,* 2008 Szweda *et al.*, 2012). To investigate molecular basis of *Staphylococcus* variation and pathogenesis mechanism of chronic infection caused by *S. aureus*, the study of biofilm associated genes is necessary (Cucarella *et al.,* 2004). However, little is known regarding biofilm formation and comprehensive detection of biofilm associated genes involved in the formation of biofilm by *S. aureus* strains isolated from bovine mastitis in China. Therefore, the present study was designed to investigate the frequency of biofilm formation and distribution of various biofilm associated genes in *S. aureus* isolates from mastitis in nine provinces of China.

MATERIALS AND METHODS

***Staphylococcus aureus* strains:** In total, 102 *S. aureus* strains were isolated from bovine subclinical mastitis cases during June, 2008 to July, 2012 from 32 dairy herds scattered in nine provinces (Table 1). The geographical distribution is shown in Figure 1. All strains were identified as *S. aureus* by morphological characteristics, coagulase test, biochemical test and *nuc* gene detection (Kenar *et al*., 2012). The identified strains were stored at -80°C in Luria-Bertani (LB) Broth (Invitrogen, Beijing, China).

Assays for biofilm detection: *S. aureus* biofilm-producing capacity was detected using Congo Red Agar (CRA) method and Semi Quantitative Adherence Assay (SQAA) as described by Kouidhi *et al.* (2010), with minor modification. (1) CRA method: briefly, CRA plate made by mixing 36 g saccharose with 0.8 g Congo red (Tianjin Kemiou Reagent Co., Ltd, China) in 1 L of brain heart infusion (BHI) agar, and inoculated and incubated at 37°C for 24 h under aerobic conditions. (2) SQQA: briefly, *S. aureus* cultures were grown in BHI overnight at 37°C and were diluted to 1:100 using BHI with 2% glucose (W/V). A total of 200 µl of cell suspension was transferred into wells of U-bottomed 96-well microtiter plate. Each strain was tested in triplicate. Wells with sterile BHI alone served as negative control. The plates were incubated aerobically at 37°C for 24 h. The cultures were then removed and microtiter wells were washed twice using PBS to remove non-adherent cells and dried in an inverted position. Adherent bacteria were fixed with 100 µl of 95% ethanol and stained with 100 µl of 1% crystal violet

Fig. 1: The geographical distribution of *S. aureus* isolated strains. *S. aureus* were mainly distributed in five regions: North China including Beijing, Hebei and Inner Mongolia; Northwest China including Gansu, Ningxia and Xinjiang; Central China including Henan; East China including Shanghai and South China including Guangxi.

Table 1: The origin of 102 *S. aureus* isolated strains from nine provinces of China

Origin of isolates	Number of dairy farm(s)	Number of *S. aureus*
Hebei	12	37
Beijing	9	14
Ningxia	4	11
Xinjiang	2	8
Inner Mongolia	1	7
Guangxi	1	7
Gansu	1	7
Henan	1	6
Shanghai	1	5

(Tianjin Kemiou Reagent Co., Ltd, China) for 5 min. The microtiter plates were air-dried and the optical density (OD) of each well was measured at 570 nm (OD_{570}) using an automated Multiskan reader (DNM-9602, PERLONG, China). Biofilm formation was interpreted as biofilm positive strains ($OD_{570} \geq 0.1$) and biofilm negative strains ($OD_{570} \leq 0.1$).

DNA extraction procedure: Frozen suspension of *S. aureus* was revived in Tryptose Soy Broth (TSB) at 37°C for 24 h. Template DNA was extracted from 1 ml of TSB cultures using Starspin Bacterial DNA Kit (Genstar Biosolutions Co., Ltd, China) with the modification of adding lysostaphin in cell lysis step. The genomic DNA samples were stored at -20°C until further use.

Biofilm-associated genes detection (PCR assay): Biofilm-associated genes (BAGs) of *S. aureus* strains (*rbf*, *SigB, sarA, icaA, SasG, icaR, icaD, clfA, fib*, *clfB, fnbpB, bap* and *fnbpA*) were detected by polymerase chain reaction (PCR). Nucleotide sequences of primers and expected sizes of PCR products are listed in Table 2. The PCR system contained 1 µl of forward and reverse primers respectively, 7 µl of dd H_2O, 10 µl of Taq Mix (Genstar Biosolution Co., Ltd, China) and 1µl of DNA template. The PCR conditions were as follows: initial denaturation step at 95°C for 8 min, followed by 30 cycles of 95°C for 30 s, annealing temperature of each primer pair for 30 s and 72°C for 30 s, and completed with a 10 min final extension at 72°C. The sizes of PCR products were analyzed by electrophoresis on 1.5% (wt/vol)

agarose gels stained with ethidium bromide (0.5 µg/ml), and visualized under ultraviolet illuminator gel documentation system (UVITEC, Cambridge).

RESULTS

Biofilm-producing capacity: The Congo Red Agar (CRA) method showed that 80 out of 102 *S. aureus* strains (78.4%) produced biofilm and 44 strains (48.0%) were capable to form biofilm by SQAA method. While 44 strains (43.1%) were detected as biofilm positive by both CRA and SQAA assays, and 17 strains (16.7%) were unable to produce biofilm by both methods (Table 3). Some discordance was also recorded between the CRA and SQAA: 36 strains (35.3%) were found biofilm positive by CRA but they were biofilm negative by SQAA, while 5 strains (4.9%) were able to produce biofilm by SQAA and were biofilm negative by CRA (Table 3). The results suggested that CRA detection rate is higher than SQAA for biofilm-producing capacity of *S. aureus* strains. Table 4 demonstrated the detection of biofilm positive strains of *S. aureus* in 9 provinces by CRA, SQAA, and combined methods (CRA and SQAA). The results investigated that the prevalence was 72.97, 64.86 and 54.05% in Hebei, 92.86, 21.43 and 14.29% in Beijing, 54.55, 18.18 and 18.18% in Ningxia, 87.50, 62.50 and 62.50% in Xinjiang, 85.50, 57.14 and 57.14% in Inner Mongolia, 71.43, 28.57 and 42.86% in Guangxi, 100, 57.14 and 85.71% in Gansu, 100, 50 and 33.33% in Henan and 60, 40 and 40% in Shanghai province by CRA, SQAA and combined methods (CRA & SQAA), respectively (Table 4).

Biofilm-associated genes in *S. aureus* strains: The distribution of different biofilm associated genes (BAGs) in 102 strains of *S. aureus* is showed in Table 5. In overall, the *rbf* was the most prevalent genes (95.1%), followed by *SigB* (94.1%), *sasG* (89.2%), *icaA* (88.2%), *sarA* (87.3%), *icaR* (84.3%), *icaD* (82.5%), *clfA* (64.7%), *clfB* (45.1%), *fib* (43.1%) *and fnbpB* (19.6%). However, *bap* and *fnbpA* genes were not amplified in any strains (Table 5). Differences were noted in the prevalence rate of detected genes in biofilms tested by the two methods. In 80 strains, which produced biofilm by CRA method, the most prevalent genes were *rbf* (97.5%), followed by *SigB* (96.3%); whereas, in 49 biofilm positive strains tested by SQAA, the *SigB* genes were at the highest rate (98.0%), followed by *rbf* (95.9%) genes. Table 6 showed the distribution of various detected BAGs in nine provinces of China. Generally, five regions in nine provinces indicated similar tendency of 13 detected genes (Table 6). However, sasG was seldom distributed in centre of China.

DISCUSSION

Biofilm formation is an important defensive mechanism of the pathogenic *S. aureus* to combat the host immune response and to remain stable in the hostile environment. This study indicated that a considerable proportion of *S. aureus* strains have the capability to form

Table 2: PCR primers used in this study

Primers	Oligonucleotide sequences (5'-3')	T_a value (°C)	Product Size (bp)	References/ Genbank code
Nuc	ATATGTATGGCAATCGTTTCAAT-GTAAATGCACTTGCTTCAGGAC	56	395	AP009324
rbf	ACGCGTTGCCAAGATGGCATAGTCTT-AGCCTAATTCCGCAAACCAATCGCTA	62	164	Cue *et al.,* 2009
SigB	GTTCAAGTTGGTATGGTTGGTT- GTCATAATGGTCATCTTGTTGC	56	395	CP003033
sarA	TTTTTTTACGTTGTTGTGCATTAACA-CATTTAAACTACAAACAACCACAAGTTG	56	135	Rode *et al.,* 2007
icaA	CCTAACTAACGAAAGGTAG-AAGATATAGCGATAAGTGC	56	1315	Vasudevan *et al.,*2003
SasG	CGGATCCGGTGTGACAATCAGTATGAC-CGGAATTCGCGACATTTATGTGGATACAC	55	937	Li *et al.,* 2011
icaR	CAATAATCTAATACGCCTGAG-AGTAGCGAATACACTTCATCT	54	246	Chaieb *et al.,* 2005
icaD	ATGGTCAAGCCCAGACAGG- CGTGTTTTCAACATTTAATGCAA	56	198	Chaieb *et al.,* 2005
clfA	ATTGGCGTGGCTTCAGTGCT- CGTTTCTTCCGTAGTTGCATTTG	55	292	Li *et al.,* 2011
fib	CTACAACTACAATTGCCGTCAACAG- GCTCTTGTAAGACCATTTTCTTCAC	56	404	Tristan *et al.,* 2003
clfB	ACATCAGTAATAGTAGGGGGCAAC- TTCGCACTGTTTGTGTTTGCAC	55	205	Li *et al.,* 2011
fnbpB	GTAACAGCTAATGGTCGAATTGATACT-CAAGTTCGATAGGAGTACTATGTTC	55	524	Li *et al.,* 2011
bap	CCCTATATCGAAGGTGTAGAATTG- GCTGTTGAAGTTAATACTGTACCTGC	60	971	Cucarella *et al.,* 2004
fnbpA	CATAAATTGGGAGCAGCATCA- ATCAGCAGCTGAATTCCCATT	55	127	Li *et al.,* 2011

Table 3: Biofilm-producing capacity in *S. aureus* strains by CRA and SQAA methods

Methods	Results (strains)	CRA				SQAA			
		Positive	%	Negative	%	Positive	%	Negative	%
CRA	Positive	80	78.4	0	0	44	43.1	36	35.3
	Negative	0	0	22	21.6	5	4.90	17	16.7
SQAA	Positive	44	43.1	5	4.9	49	48.0	0	0
	Negative	36	35.3	17	16.7	0	0	53	52.0

Table 4: Geographic distribution of biofilm-positive strains using Congo Red Agar (CRA), Semi Quantitative Adherence Assay (SQAA) and both methods

Area	CRA		SQAA		CRA and SQAA	
	Samples examined	Positive %	Samples examined	Positive %	Samples examined	Positive %
Hebei	27	72.97	24	64.86	20	54.05
Beijing	13	92.86	3	21.43	2	14.29
Ningxia	6	54.55	2	18.18	2	18.18
Xinjiang	7	87.50	5	62.50	5	62.50
Inner Mongolia	6	85.71	4	57.14	4	57.14
Guangxi	5	71.43	2	28.57	3	42.86
Gansu	7	100	4	57.14	6	85.71
Henan	6	100	3	50.00	2	33.33
Shanghai	3	60.00	2	40.00	2	40.00

Table 5: Distributions of BAGs in *Staphylococcus aureus* strains

genes	Biofilm positive strains				Biofilm negative strains				The total of strains (102)	
	CRA (80)		SQAA (49)		CRA (22)		SQAA (53)			
	No.	%	No.	%	No.	%	No.	%	No.	%
Rbf	78	97.5	47	95.9	19	86.4	50	94.3	97	95.1
SigB	77	96.3	48	98.0	19	86.4	48	90.6	96	94.1
sarA	73	91.3	44	89.8	16	72.7	45	84.9	89	87.3
icaA	73	91.3	44	89.8	17	77.3	46	86.8	90	88.2
sasG	72	90.0	41	83.7	19	86.4	50	94.3	91	89.2
icaR	71	88.8	43	87.8	15	68.2	43	81.1	86	84.3
icaD	69	86.3	41	83.7	15	68.2	43	81.1	84	82.5
clfA	58	72.5	47	95.9	8	36.4	19	35.8	66	64.7
Fib	40	50.0	38	77.6	4	18.2	6	11.3	44	43.1
clfB	39	48.8	39	79.6	7	31.8	7	13.2	46	45.1
fnbpB	18	22.5	19	38.8	2	9.1	1	1.9	20	19.6
Bap	0	0	0	0	0	0	0	0	0	0
fnbpA	0	0	0	0	0	0	0	0	0	0

Table 6: Distribution of BAGs in five regions of China

Genes (bp)	No (%)	Regions in China				
		North (Beijing, Hebei and Inner Mongolia)(58)	Northwest(Gansu, Ningxia and Xinjiang)(26)	Centre (Henan) (6)	East (Shanghai) (5)	South (Guangxi)(7)
rbf (164)	97(95.1)	53(91.4)	26(100)	6(100)	5(100)	7(100)
SigB (395)	96(94.1)	54(93.1)	24(92.3)	6(100)	5(100)	7(100)
sarA (135)	89(87.3)	50(86.2)	23(88.5)	5(83.3)	4(80.0)	7(100)
icaA (1315)	90(88.2)	50(86.2)	23(88.5)	5(83.3)	5(100)	7(100)
sasG (937)	91(89.2)	51(87.9)	24(92.3)	4(66.7)	5(100)	7(100)
icaR (246)	86(84.3)	49(84.5)	22(84.6)	5(83.3)	4(80.0)	6(85.7)
icaD (198)	84(82.5)	47(81.0)	23(88.5)	5(83.3)	3(60.0)	6(85.7)
clfA (292)	66(64.7)	40(69.0)	18(69.2)	3(50.0)	2(40.0)	3(42.9)
fib (404)	44(43.1)	30(51.7)	8(30.8)	2(33.3)	1(20.0)	3(42.9)
clfB (205)	46(45.1)	31(53.4)	9(34.6)	3(50.0)	1(20.0)	2(28.6)
fnbpB (524)	20(19.6)	13(22.4)	4(15.4)	1(16.7)	1(20.0)	1(14.3)
bap (971)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
fnbpA (127)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

biofilm. Although, the current proportion of biofilm formation by *S. aureus* strains was recorded slightly lower than another study in China (Li *et al.,* 2011), they reported 87.6% biofilm formation. However, all these data suggested that biofilm producer strains of *S. aureus* from bovine mastitis are highly prevalent in China. Fox *et al*. (2005) reported from Washington, 41.4% of bovine *S. aureus* strains were proficient to form biofilm. They reported a high proportion of biofilm positive strains in dairy farms of developed region. Kenar *et al*. (2012), by using CRA method, found 55.2% of Coagulase negative *Staphylococcus* to produce biofilm and 44.8% strains were biofilm negative.

In this study, we used two important assays *viz.* CRA and SQAA to detect biofilm formation in *S. aureus.* The combinations of qualitative and quantitative phenotypic tests have been adopted by researchers (Vasudevan *et al.,* 2003). Phenotypic results showed some variations between CRA and SQAA methods. The proportion of biofilm producing strains tested by CRA (78.4%) was obviously higher than that by SQAA assay (48%). Similarly, in agreement with this report, another study from America demonstrated that 91.4% *S. aureus* strains isolated from bovine mastitis were biofilm positive using CRA method, while 68.6% isolates were found positive by quantitative assay (Vasudevan *et al.,* 2003). Although tests results have relatively larger differences through different detection methods, but still have positive predictive value (Seo *et al.,* 2008). However, some reports are not in accordance with our study, they detected a lower rate of CRA method for biofilm formation than the quantitative methods (Wiśniewska *et al*., 2008; De-Castro Melo *et al*., 2013). The possible explanations of difference between the two methods might be the presence of the discrepancy of incubation time, medium category, dilution, measurement of wavelength and determination criteria. The high prevalence of biofilm producing *S. aureus* isolates by both CRA and SQAA confirmed that this kind of virulent characteristic distributed frequently and widely within herds. Each method has its own advantages. CRA method is rather easy to perform, less time consuming, sensitive and specific (Kwon *et al.,* 2008), and it has been recommended by many studies (Oliveira *et al.,* 2006; Jain and Agarwal, 2009; Kouidhi *et al.,* 2010). For quantitative methods, such as SQAA method, it has also high specificity, sensitivity, and positive predictive value, but time-consuming and complicated to operate (Mathur *et al.,* 2006). As for as, which method is more accurate, all kinds of reports are not consistent. According the report of Oliveira *et al.* (2006), in CRA and in optical density measurement (a semi-quantitative method), 37.5% (six) and 18.5% (three) *S. aureus* isolates showed the ability to form biofilm, respectively; but only two strains were found positive by two methods, simultaneously. The current study revealed that detection rate of semi-quantitative method was much lower than CRA method. In addition, both of the two assays could be chosen as applicable tools for detecting biofilm production.

Since phenotypic characteristic may arise from different genetic determinants, assessment of biofilm formation at the genetic level is important. The correlation between the phenotypic biofilm production and the existence of various BAGs was found considerably good.

In this study, we found at least one BAG in biofilm producing phenotypes of *S. aureus* strains. Additionally, our results also suggested that BAGs have imperative role in *S. aureus* pathogenesis and biofilm formation. Among thirteen BAGs, *rbf* and *sigB* were the most prevalent, suggesting that the two genes could be potentially important for virulent characteristics in bovine *S. aureus* strains in China. This report presented a relationship between existence of various BAGs and capability of biofilm production by CRA and SQAA methods. It observed that *rbf* and *SigB* were consistent with SQAA positive results. The *rbf* (transcriptional regulator) gene in *S. aureus* is considered to be required for biofilm development in critical conditions (Lim e*t al.,* 2004). Recently, it has been documented that an alternative factor sigma B (*SigB*), an important component of the stress response of *S. aureus* required for coping with oxidative, alkaline, heat and salt stress, is involved in the regulation of virulence factor expression (Mitchell *et al.,* 2013). In particular, *SigB* directly or indirectly influences the production of alpha-toxin, various proteases, lipases, clumping factor (*Clfa*), coagulase (*coa*), and fibronectin binding protein (*fnbp*) A (Mitchell *et al.,* 2013; Khan *et al.,* 2013). Ote *et al.* (2011) reported higher (96.9%) occurrence of *clfA* genes in *S. aureus* isolates. Polymeric N-acetylglucosamine is synthesized by the *ica* operon including four genes, *icaA, D, B* and *C*. The *icaADBC* operon, also encodes enzymes involved in the synthesis of Polysaccharide Intercellular Adhesion (PIA), with regulation of *sar*, *agr* and *sig*B. PIA has an essential role in the formation of biofilm. Genetic disruption of these four genes (*ADBC*) results in the loss of biofilm formation in some strains (Rode *et al.,* 2007; Cue *et al.,* 2009). In this study, the *icaA* and *icaD* genes were widely distributed among *S. aureus*, which is in accordance with the findings of other researchers (Vasudevan *et al.,* 2003; Ote *et al.,* 2011). Several reports (Melchior *et al.* 2006; Seo *et al*., 2008; Vautor *et al.,* 2008) from different geographical areas supported our study and reported the absence of *bap* and *fnbpA* genes in biofilms formed by *S. aureus* isolates. Biofilm formation by *S. aureus* is a complex mechanism controlled by multiple genes. The modulations between these genes will offer an important basis and means for the control and prevention of *S. aureus* biofilm formation in bovine mastitis.

Conclusion: The data concluded that biofilm formation in *S. aureus* strains isolated from bovine subclinical mastitis in nine provinces of China for the first time reported, the majority of *S. aureus* strains produced biofilm, and 11 associated genes including *rbf*, *SigB, sarA, icaA, SasG, icaR, icaD, clfA, fib, clfB* and *fnbpB* were detected but *bap* and *fnbpA* genes were found absent. CRA detection rate was higher than SQAA for biofilm producing capacity of *S. aureus*. The study suggested that *rbf* and *SigB* were the most important biofilm associated genes along with *icaA* and *icaD* in China.

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