Analysis of Biofilms Formation and Associated Genes Detection in Staphylococcus Isolates from Bovine Mastitis

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ABSTRACT

Objective The objective of the study is to investigate biofilm forming ability, distribution of biofilm associated genes of the clinically isolated bovine mastitis Staphylococcus and the correlation of them. Methods Silver staining, SEM and crystal violet staining were conducted for the detection of biofilm forming ability in 24-well plates. Genes of bap, icaAD, icaBC, sar, agr, sigB, clfaA, clfaB, fnbpA and fnbpB were amplified by PCR. Results Formation of biofilm could be found macroscopically in 120 out of 137 strains by silver stainning, the biofilm formation rate of 87.6%. It showed that 5 strains didn't adhere to the surface of silica gel by crystal violet staining, while the rest 132 ones did. Bap was amplified in 57 isolates and icaAD and icaBC in 43 and 54 strains respectively; sigB, sar and agr are amplified in 73, 49 and 38 isolates, and clfaA and clfaB in 76 and 50 strains respectively; fnbpA was present in 52 strains and fnbpB in 26 isolates. Conclusion It revealed that bap, sigB, sar, icaAD and icaBC may be crucial biofilm associated genes, for these genes are present more in biofilm-positive strains than in biofilm-negative strains. There is no obvious difference between the frequency of agr in biofilm-positive strains and that in biofilm-negative strains, which may because the role of agr in biofilm developmemt is still in controversial. The dis-

tribution of clfaA, clfaB, fnbpA and fnbpB in biofilm-positive strains is not greatly different from that in biofilm-negative strains, however, the distribution of these genes in a few of isolates suggests that these genes are positive for biofilm formation.

INTRODUCTION

Staphylococcus is the most important pathogenic bacteria to cause bovine mastitis. Staphylococcus, including Staphylococcus aureus and Staphylococcus epidermidis, which belong to the opportunistic pathogenic germs, with characteristic polysaccharides and adheresion protein factors on the surface, is easiest to form bacterial biofilm. In relation to the pathogenicity of Staphylococcus biofilms, Melchior et al. (2007) proposed that biofilm is one of the major causes of recurrent and chronic mastitis in dairy cattle (Ref. 1). Staphylococcus biofilm formation mechanism is complex, with many kinds of proteins' participation. Cucarella et al. (2004) reported that bap (biofilm-associated protein)-positive Staphylococcus strains show a high capacity to infect and persist in the mammary gland, which is closely related to the biofilm formation of gram-positive bacterium (Ref. 2). In the course of biofilms formation, accumulation and the biofilm mature stage is mainly depends upon the polysaccharide adhesins which promot bacterium gathers mutually, especially PIA(Polysaecharide intercellular adhesion).The icaADBC operon, participates in biofilm formation by encoding enzymes involved in the synthesis of PIA, with regulation of sar, agr and sigB(Ref. 3). Clumping factor (Clfa) and fibronectin-binding proteins (Fnbp) mediate bacterial adherence, whether they play an influential role in biofilms formation? People focus on studying the mechanism of Staphylococcus biofilm formation abroad, and most of them use standard strains instead of clinical isolates. While, people concentrate on biofilm of Staphylococcus clinical isolates from human in China. The aim of the present study is to test the biofilm forming capability of bovine mastitis Staphylococcus qualitatively and quantitatively, and to analyze the distribution of biofilm-associated genes.

MATERIALS AND METHODS

Bacterial strains and growth condition

One hundred and thirty-seven Staphylococcus strains used in this study were isolated from milk of bovine with mastitis in Shandong province, which were identified and preserved in this laboratory. Bacteria stored at -70°C were freshly shaked on Luria-Bertani medium before each experiment.

Silicone elastomer slices biofilm formation assays and silver staining.

Silicone elastomer slices (1×1cm2) were cut from 1mm-thick medical grade silicone elastomer sheetings. Biofilm formation was conducted using a modified plate assay. One milliliter of sterile TSB and ten microliters of overnight cultured Staphylococcus strain were added to each well of a 24-well plate. Slices were placed in the bottoms of wells with sterile forceps and incubated at 37°C in biochemical incubator for 7d, the medium changed every two days. After incubation, the culture medium was discared and 1mL of sterile PBS was added into each well to remove planktonic bacteria for three times. Each Silicone elastomer slice was divided into two, one for silver staining, and the other for SEM. Each slice was observed thought an optical microscope after silver staining, slicas in wells containing uninoculated medium served as negative controls and biofilm qualitative results through SEM as positive controls(4).

Crystal violet staining

Slices were incubated as described above for biofilms formation, and 1mL of sterile 0.9%NaCl solution was added in to remove planktonic bacteria and impurity for three times. Two hundreds microliters of methanol was added in to fix biofilm and discarded 15min later .After air dried, biofilms were strained with 200 μL 2% crystal violet for 5min, rinsed under running water to remove excess stain, and air dried over night. Then stained slices were destained with 33% glacial acetic acid and measured by reading the

Table 1 *Primers used in present study*

Target Gene	Primer Sequence	Annealing Temperature (°C)	Product Size (bp)	Reference
bap	5'-CCCTATATCGAAGGTGTAGAATTG-3' 5'-GCTGTTGAAGTTAATACTGTACCTGC-3'	62	971	Cucarella et al. (2001)
icaAD	5'-CCTAACTAACGAAAGGTAGG-3' 5'-TTAGCGTTGGGTATTCCCTC-3'	55	1266	Sun et al. (Ref. 6)
icaBC	5'-ATGGTCAAGCCCAGACAGAG-3' 5'-GCACGTAAATATACGAGTTA-3'	55	1188	Sun et al. (2009)
sar	5'-CGGTACCGTTGATTTGGGTAGTATGC-3' 5'-TTGCCATGGTTAAAACCTCCC-3'	55	867	Sun et al. (2009)
agr	5'-GTGCCATGGGAAATCACTCCTTCC-3' 5'-TGGTACCTCAACTTCATCCATTATG-3'	55	976	Sun et al. (2009)
sigB	5'-CGGATCCGGTGTGACAATCAGTATGAC-3' 5'-CGGAATTCGCGACATTTATGTGGATACAC-3'	55	937	Sun et al. (2009)
fnbpA	5'- CATAAATTGGGAGCAGCATCA-3' 5'- ATCAGCAGCTGAATTCCCATT-3'	55	127	Vancraeynest et al. (2004)
fnbpB	5'- GTAACAGCTAATGGTCGAATTGATACT-3' 5'- CAAGTTCGATAGGAGTACTATGTTC-3'	55	524	Tristan et al. (2003)
clfaA	5'- ATTGGCGTGGCTTCAGTGCT-3' 5'- CGTTTCTTCCGTAGTTGCATTTG-3'	55	292	Primer 5.0 designed
clfaB	5'- ACATCAGTAATAGTAGGGGGCAAC-3' 5'- TTCGCACTGTTTGTGTTTGCAC-3'	55	205	Primer 5.0 designed

optical density at 590nm (OD590). Slices uninoculated were stained as blank controls.

The OD value can reflect the firmly degree of biofilms adhesion to contact surface. According to the critical OD value (ODc is equal to that the mean value of blanks adds on 3 times of its standard deviations) biofilms can be classified: when OD≤ODc, biofilms don't adhere to the contact surface, recorded as (0); when $OD_c<OD\leq 2OD_c$, biofilms adhere to the contact surface weakly, recorded as (+); when $2OD_c<OD\leq 4OD_c$, the adhesion between biofilm and contact surface is moderate, recorded as (++); when $OD<4OD_c$, adhesion between biofilm and contact surface is strong, recorded as (+++) (Ref. 5).

DNA extraction

Genomic DNA was prepared as followed: 5μL bacterium was inoculated in 5mL LB culture medium and shaking cultured at 37°C for 16h. Absorbed 1.5mL becteria into a 1.5mL centrifuge tube and centrifuged at 12000r/min for 3 min. The supernatant discarded and suspended the precipitation with TNE, then centrifuged as above. Discarded the supernatant again and resuspended the precipitation in 200μL TE. Following, 4μL Lysozyme was added in and fully mixed to inoculate at 37°C. After 1 h, 4μL Proteinase K was added in and reacted for 1 h at 55°C. After cooling to room temperature, they were boiled for 10 min and placed in ice for 3 min immediately. At last, centrifuged for 5 min at 12000r/min and supernatant was reserved in -20°C refrigerator.

Analysis of genes involved in biofilm formation (PCR assay).

Used the bacterial chromosomes prepared above as templates, the thermal cycling procedure consisted of a predenaturation at 94°C for 3 min, 30 cycles of denaturation 94°C for 30 s, annealing (annealing temperatures were presented in Table 1) for 20 s, and extend 72°C for 50 s, with a final step elongation at 72°C for 5 min. The

Table 2 *Quantitative detection results of bacterial biofilms*

Firmly degree of biofilms adhesion to contact surface	No adhesion (0)	Weak adhesion (+)	Moderate adhesion (++)	Strong adhesion (+++)
Number (n)	5	75	55	2

size of the PCR products was analyzed by electrophoresis on 1% (wt/vol) agarose gels. Primers used in the PCR assays, as well as the expected amplified product sizes and the references are presented in Table 1.

RESULTS

Qualitative biofilm formation detection

After silver staining, biofilm formation situation was observed by an ordinary optical microscope. Biofilms could be found macroscopically in 120 out of 137 strains after silver stainning, and the biofilm formation rate is 87.6% (Fig. 1). SEM visualization showed that cells were growing in communities and organized into a three-dimensional architecture (Fig. 2A). Fig. 2B showed a scanning electron micrograph of one biofilm-negative strain in which cells were growing scatteredly or developed into microcolonies.

Quantitative biofilm formation detection

Bacterial biofilms were quantified by OD590, biofilms of 5 out of 137 strains didn't adhere to the surface of silica gel ,75 strains adhered to the contact surface weakly, 55 strains moderately and 2 trains strongly(Table 2 and data not shown).

Genes involved in biofilm formation

57 out of 137 isolates included in this study were positive for bap; icaAD and icaBC were amplified in 43 and 54 strains respectively; 73 isolates harboured sigB, 49 isolates harboured sar and 38 isolates harboured agr; clfaA and clfaB were amplified in 76 and 50 strains respectively; 52 strains carried fnbpA,and 26 strains were positive for fnbpB (Table 3). Assays were repeated two times.

Figure 1 *Ordinary optical micrographs of Staphylococcus biofilms by silver stainning (100×): (A) A blank control; (B) A biofilm-negative strain; (C) A biofilm-positive strain*

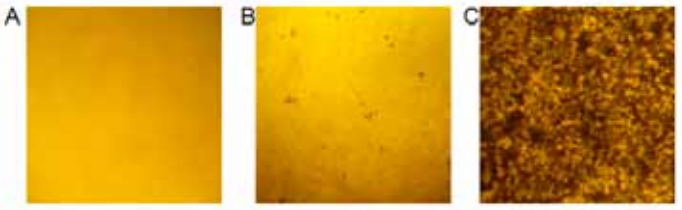
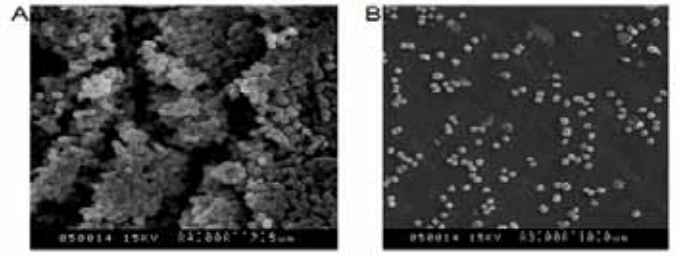


Figure 2 *Scanning electron micrograph of Staphylococcus biofilms : (A)A biofilm-positive strain(×4.00K); (B) A biofilm -negative strain(×3.00K)*



DISCUSSION AND CONCLUSION

Bacteria that attach to non-active object or vivo surfaces aggregate in a hydrated polymeric matrix synthesized by their own to form biofilms (Ref. 7). Cucarella et al. reported that biofilm associated genes must be studied firstly in order to investigate the molecular foundation of Staphylococcus variation and pathogenesis mechanism of chronic infection caused by Staphylococcus. Bap-positive isolates are able to infect and persist in the

bovine mammary gland and were less susceptible to antibiotic when forming biofilms in vitro(Ref. 2). Lasa et al.(2006) showed that, Bap, which was a member of Staphylococcus aureus biofilm formation related protein family and with a molecular weight of 254KD, play an important role in the process of biofilm formation, help neighboring bacteria form biofilms, and was related to pathogen infection(Ref. 8). Furthermore, the ability of biofilm formation lose after gene knockout (Ref. 9). Nevertheless Vautor et al. (2008) reported that, a collection of 262 isolates obtained from other species and various locations was tested by PCR, using published primers and dot-blot, and the results indicated that none of the isolates carried the bap gene (Ref. 10). In this study, bap gene was amplified only in 51 out of 116 biofilm-positive strains, indicating that bap gene was present in not all but a few some biofilm-positive strains. while 6 strains carried bap gene can generate biofilm, this is inconsistent with previous reports.

Ica operon consists the four genes (icaADBC), their coding product co-synthesize the key material PIA of Staphylococcus adhesion in the process of biofilm formation. It is reported previously that ica is commonly found in Staphylococcus aureus (Ref. 11), directly related with the formation of bacterial biofilm as an essential factor. In this study, there are only 41 strains carried icaAD and 51 strains carried icaBC respectively in 116 biofilm-positive strains instead of all. Furthermore, some few isolates carried ica can't form biofilm, it would be because ica expression is regulated by multiple accessory regulators, or due to other ica-independed biofilm formation mechanism. In addition, strains carried icaAD or icaBC alone are discovered in this study, which is inconsistent with previous reports, and it would be due to the differences between hunman and bovine Staphylococcus strains.

Ica expression is regulated by multiple genes such as sigB, sar(staphylococcal accessory regulator), agr(accessory gene regulator). They may interact each other, and regulate biofilm formation through the ica operon expression eventually. sigB, sar and agr are proved to perform important function in staphylococcus life process. sar, an staphylococcal accessory regulator , widely regulates the intracellular and extracellular protein expression, whose mutations show

Table 3 *Results of biofilm-associated genes testing*

Genes	Number of (+) isolates in biofilm-positive strains		Number of (--) isolates in biofilm-negative strains		Total number of (+) isolates	
	(n)	%	(n)	%	(n)	%
bap	51/116	44.0%	6/21	28.6%	57/137	41.6%
sigB	65/116	56.0%	8/21	38.1%	73/137	62.9%
sar	46/116	40.0%	3/21	14.3%	49/137	35.8%
agr	34/116	29.3%	4/21	19.0%	38/137	27.7%
icaAD	41/116	35.3%	2/21	9.5%	43/137	31.4%
icaBC	51/116	44.0%	3/21	14.3%	54/137	39.4%
clfaA	67/116	57.8%	9/21	42.9%	76/137	55.5%
clfaB	44/116	37.9%	6/21	28.6%	50/137	34.5%
fnbpA	42/116	36.2%	10/21	47.6%	52/137	38.0%
fnbpB	24/116	20.7%	2/21	9.5%	26/137	19.0%

Note: 116 biofilm-positive strains were positive for both silver staining and crystal violet staining, and the rest 21 strains were biofilm-negetive strains.

a significant decrease of ica operon expression and subsequently affect the synthesis of PIA (Ref. 12). agr, an assessory gene regulater of Staphylococcus aureus, controls the expression of a series of toxins and virulence factors and the interaction with the innate immune system(Ref. 13). However, the role of agr in the infection process seems to be controversial (Ref. 13). In the biofilm-related infections, its role is to reduce rather than induce biofilm formation and virulence factor expression. agr increased expression of surfactant-like peptides leads to detachment of biofilms (Ref. 14), that is cells dislodged from the biofilm structure as planktonic cells disseminate to a distant site to reform another biofilm. sigB major regulate the expression and transcription of bacteria gene under stress conditions, its levels and activity adjust to the environmental pressure(Ref. 15). In this study, sigB was amplified in 73 strains, sar in 49 strains, and agr in 38 strains. In contrast to previous reports, not all of the biofilm-positive strains carried sigB, sar and agr, and these genes could be amplified in a small amount of biofilm-negative strains.

Initial adhesion stages of Staphylococcus aureus involved in a variety of surface proteins, adhesion factors which mediate Staphylococcus aureus adhesion to the host cells is the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family, including fibronectin-binding proteins A (FnBPA), fibronectin-binding proteins B (FnBPB), collagen-binding protein (Can), fibrinogen-binding protein (Fbe), clumping factor A (ClfA) and clumping factor B (ClfB), etc. The MSCRAMM family is important to the adhesion between Staphylococcus aureus and polymer in vitro, e.g. clfaA mediates bacterial attachment to plasma clots formed in vitro and to plastic biomaterial(Ref. 16). In this study, the distribution of clfaA, clfaB and fnbpB in biofilm-positive strains is not greatly different from that in biofilm-negative strains, the former is adout 10% more than the latter; while, the former is 11.4% less than the latter, when it comes to fnbpA. There are 10 isolates contained only adhesin genes can form biofilm, showing that adhesion genes may play a certain role in promoting biofilm formation.

In the present study, silver staining and crystal violet staining were used for qualitative and quantitative detection the capacity of biofilm formation by Staphylococcus aureus involved in dairy cattle mastitis, a combination of the two methods is more effective for detection of biofilms formation. While, there are personal operation factors in the process, vigorous flushing would destroy biofilms, too small strength causes planktonic bacteria to remain, the two methods are suitable for a large amount sample's detection of biofilm-positive and biofilm-negative strains sketchily. The gene testing results reveale that bap, sigB, sar, icaAD and icaBC may be crucial biofilm associated genes, for these genes are present more in biofilm-positive strains than in biofilm-negative strains. There is no significant difference between the frequency of agr in biofilm-positive strains and that in biofilm-negative strains, which may because the role of agr in biofilm development is still in controversial. The distribution of clfaA, clfaB, fnbpA and fnbpB in biofilm-positive strains is not greatly different from that in biofilm-negative strain. However, the distribution of these genes in a few of isolates suggests that these genes are positive for biofilm formation. Staphylococcus aureus biofilm formation is a complex result of multiple genes control, to understand the role of each gene in biofilm formation and the modulation between them will provide an important basis and means for the control of Staphylococcus aureus biofilm formation. Further more studies are required, gene knockout would be used, as well as antibodies for the growth inhibition assays of biofilms to verify the associated genes after their expression in prokaryotic cells.

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