

Phenotypic and genotypic characterization of canine pyoderma isolates of *Staphylococcus pseudintermedius* for biofilm formation

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(Received 21 January 2015/Accepted 18 March 2015/Published online in J-STAGE 18 April 2015)

ABSTRACT. Biofilm-forming ability is increasingly being recognized as an important virulence factor in several *Staphylococcus* species. This study evaluated the biofilm-forming ability of sixty canine derived clinical isolates of *S. pseudintermedius*, using three phenotypic methods, microtiter plate test (MtP), Congo red agar method (CRA) and tube adherence test, and the presence and impact of biofilm-associated genes (*icaA* and *icaD*). The results showed that *icaA* and *icaD* genes were detected concomitantly in 55 (91.7%) of 60 isolates. A majority (88.3%) of the strains screened had matching results by the tube adherence test, MtP and PCR analysis. Better agreement (95%) was found between the PCR-based analysis and the CRA. Results of the *icaA* and *icaD* gene PCRs showed good agreement with CRA results, with a kappa of 0.7. Comparing the phenotypic methods, the statistical analysis showed that the agreement among the phenotypical tests using categorical data was generally good. Considering two classes (biofilm producer and biofilm non-producer), the percentage of matching results between the CRA method and the tube adherence test and between the CRA method and the MtP was 93.3%. A concordance of 100% was revealed between the MtP and the tube adherence test. The results indicate a high prevalence of the *ica* genes within *S. pseudintermedius* isolates, and their presence is associated with *in vitro* formation of a biofilm. A combination of phenotypic and genotypic tests is recommended for investigating biofilm formation in *S. pseudintermedius*.

KEY WORDS: biofilm, Congo red agar, *ica* genes, microtiter plate test, *Staphylococcus pseudintermedius*

doi: 10.1292/jvms.15-0043; *J. Vet. Med. Sci.* 77(8): 945–951, 2015

Staphylococcus pseudintermedius is an opportunistic pathogen of dogs and has emerged as a leading cause of skin, wound infections, urinary tract infections and otitis externa worldwide [5, 7, 28]. Biofilm-forming ability is increasingly being recognized as an important virulence factor in several staphylococcal species that make it difficult to treat chronic infections [19, 23]. The principal component of biofilms is a polysaccharide intercellular adhesion molecule (PIA) produced by the *ica* operon on the bacterial chromosome that includes four genes (A, B, C and D) [17, 27]. In *S. epidermidis*, it has been shown that co-expression of *icaA* and *icaD*, in addition to leading to an increase in the activity of PIA [17], is related to biofilm phenotypic expression [1]. Moreover, *ica*-independent biofilm formation has been reported in staphylococci [15]. Although the phenotypic and genotypic bases for biofilm production have been well characterized in *S. epidermidis* and in *S. aureus* [1, 10, 33], few studies investigating biofilm production in canine clinical *S. pseudintermedius* isolates and the association with *ica* genes have been published. A recent study has shown [13] that clarithromycin was ineffective against 20 methicillin-resistant *S. pseudintermedius* (MRSP) biofilm-producing

isolates at therapeutic doses. Another report involving 23 MRSP biofilm producers from dogs in Norway showed that the isolates belonging to sequence type (ST) 71 produced significantly more biofilm compared with other STs [25]. It should be noted that in these reports, the biofilm production in *S. pseudintermedius* isolates was evaluated exclusively by microtiter plate method. In a single report [30], the presence of *ica* genes was found in 77.9% (109/140) of *S. pseudintermedius* isolates, and the *icaD* gene was found in 75.7% (106/140) of isolates. The present study was carried out to determine the biofilm-producing ability of isolates using three phenotypic methods, microtiter plate test (MtP), Congo red agar (CRA) method and the tube adherence test, and to compare the results of these methods with the presence of *icaA* and *icaD* genes in canine clinical *S. pseudintermedius* isolates.

MATERIALS AND METHODS

Bacterial isolation and identification: A collection of 60 *S. pseudintermedius* isolates from canine pyoderma were included in this study. Bacteria were isolated from clinical specimens submitted to the bacteriology laboratory at the Department of Veterinary Medicine (University of Perugia). Upon arrival at the laboratory, specimens were cultured on 5% defibrinated sheep blood agar and mannitol salt agar and incubated aerobically at 37°C for 24 hr. Suspect colonies were identified using standard techniques: colony morphology, Gram stain, coagulase and catalase test and API Staph System (bioMérieux, Marcy-l'Étoile, France). *S. pseudin-*

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termedius isolates were identified using a polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) assay based on the *MboI* digestion pattern of a PCR amplified internal fragment of the *pta* gene as previously described [4]. The isolates were stored at -70°C in glycerol storage broth pending further analyses. The isolates were aerobically subcultured on blood agar plates overnight at 37°C before analysis.

Phenotypic characterization of biofilm-producing ability on Congo red agar (CRA): To examine bacterial slime production, which is a step for biofilm formation, all the strains were cultured on Congo red agar. CRA plates were prepared by adding 0.8 g of Congo red and 36 g of saccharose (Sigma, St. Louis, MO, U.S.A.) to 1 l of brain heart infusion agar (Oxoid, Strada Rivoltana, Italy) [2]. After inoculation, the plates were incubated for 24 hr at 37°C and subsequently overnight at room temperature. On CRA, black colonies were considered slime-producing strains, almost black colonies were considered weakly slime producing, and red colonies were classified as strains unable to produce biofilm, slightly modifying the procedure of Arciola *et al.* [1].

Phenotypic characterization of biofilm formation by tube adherence test: The qualitative assay for biofilm formation was performed according to previous authors [9] with minor modifications. Glass tubes filled with 2.6 ml of tryptic soy broth (TSB) (Oxoid) containing 1% glucose were inoculated with a loopful of a pure culture of a strain from tryptic soy agar (TSA) plates containing 1% glucose. Tubes containing only TSB were included in the test as a negative control. After overnight incubation at 37°C in air, the contents of each tube was carefully removed with a pipette, and 2 ml of a 0.25% safranin solution was added. After 1 min, the tubes were emptied with a pipette and placed upside down without a wash step. The results of the test were read after drying overnight at room temperature. The test was considered positive when there was a layer of stained material on the inner surface of the tube. Adherence was estimated as absent, weak, moderate or strong. The presence of stained material at the liquid–air interface was not considered to be indicative of biofilm formation [9].

Phenotypic characterization of biofilm formation by Microtiter plate test (MtP): The ability of the isolates to form biofilms was determined by the ability to adhere to 96-well polystyrene microtiter plates (Nunc, Rochester, NY, U.S.A.) using the method described by previous authors [31] with minor modifications. Briefly, isolates were subcultured onto blood agar, and pure 24 hr growth was used for testing. Each isolate was suspended in 5.0 ml of tryptic soy broth (TSB) supplemented with 1% glucose to achieve a turbidity equivalent to a 0.5 McFarland standard ($\sim 10^8\text{CFU/ml}$). A 200 μl bacterial suspension was transferred in triplicate into microtiter plate wells, with the negative control containing growth medium only. The plates were incubated at 37°C for 24 hr, washed with water to remove non-adhered cells and then dried at room temperature before the addition of Hucker crystal violet solution (Sigma). The plates were subsequently incubated at room temperature for 30 min before excess dye was removed by rinsing with tap water. After the

plates were air-dried, the dye bound to the adherent cells was resolubilized with 160 ml of 33% (V/V) glacial acetic acid per well. The OD of each well was measured at 570 nm. For each isolate, the result was calculated by subtracting the median OD570 of triplicate determinations for the negative control (test broth only) from the median OD570 of triplicate determination for the samples. Strains with OD values ≤ 0.065 were regarded as non-adherent, strains with OD values between 0.065 and 0.130 were classified as weakly adherent, strains with OD values between 0.130 and 0.260 were classified as moderately adherent, and strains with an OD > 0.260 were classified as strongly adherent [31].

DNA extraction and PCR for detection of *icaA* and *icaD* genes: For PCR analyses, DNA was extracted from bacterial colonies using a QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Primers for *icaA* and *icaD* (Table 1) genes were designed from the *Staphylococcus pseudintermedius* ED99 genome (GenBank accession: CP002478) using the Primer-Blast suite of the NCBI [35] in order to limit the amplification of unintended targets. Briefly, the 25 μl reaction volume consisted of 10 \times PCR buffer, 1.5 mM MgCl_2 , 200 μM of each deoxyribonucleotide triphosphate, 10 μM of each primer (Table 1), 1 U of Taq polymerase and 100 ng of template DNA. The DNA of each sample was tested in duplicate. The presence and size of the amplified products were confirmed by electrophoresis on 1.5% agarose gel. PCR products were purified using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, U.S.A.) in accordance with the manufacturer's recommended protocol and subjected to direct sequencing (PRIMM Srl, Milan, Italy). All gel-purified PCR products were sequenced using gene-specific primers. The final sequences were subjected to a BLAST analysis against prokaryotic databases (NCBI) to confirm specific amplification.

Statistical analysis: Data derived from laboratory examinations were stored in Microsoft Excel. Agreement was calculated using Cohen's Kappa values (kappa) for dichotomous data and squared weighted Kappa values for categorical data. The strength of the agreement was interpreted according to Landis and Koch [20], who classified agreement with a kappa value of 0–0.20 as poor, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as good and 0.81–1 as very good. The analysis was performed using the StatsDirect software (version 2.7, StatsDirec Ltd., Cheshire, U.K.).

RESULTS

The results for all phenotypic and genotypic tests are shown in Table 2. The tube adherence test results showed that 45 of the 60 isolates (75%) were weak biofilm producers, 11 of the 60 isolates (18.3%) were moderate biofilm producers, and 4 of the 60 isolates (6.6%) were biofilm non-producers (Fig. 1), for a total of 56 (93.3%) positive isolates and 4 (6.6%) negative isolates.

By MtP, 25 out of 60 (41.6%) were classified as weekly adherent, 21 (35%) were classified as moderately adherent, 10 (16.6%) were classified as strongly adherent, and

Table 1. Primers used in this study and PCR conditions

| Gene | Primer Name | Sequence (5'-3') | Amplicon Length | PCR Conditions |
|-------------|------------------|---------------------------|-----------------|--|
| <i>icaA</i> | <i>icaA</i> -Fw | ACTGTTTCGGGGACAAGCAT | 134 bp | 94°C×3 min, 35×(94°C×15 sec, 60°C×20 sec, 72°C×20 sec) 4°C ∞ |
| | <i>icaA</i> -Rev | ATTGAGGCTGTAGGGCGTTG | | |
| <i>icaD</i> | <i>IcaD</i> - Fw | CGTTAATGCCTTCTTTCTTATTGCG | 166 bp | 94°C×3 min, 35×(94°C×15 sec, 56°C×20 sec, 72°C×20 sec) 4°C ∞ |
| | <i>IcaD</i> -Rev | ATTAGCGCACATTCGGTGTT | | |

Table 2. Phenotypic and genotypic characterization of canine *S. pseudintermedius* isolates

| Isolate | PCR <i>icaA/icaD</i> | CRA | MtP | Tube test | Isolate | PCR <i>icaA/icaD</i> | CRA | MtP | Tube test |
|---------|----------------------|--------------|----------|-----------|---------|----------------------|--------------|----------|-----------|
| 1 | +/+ | Red | Absent | Absent | 31 | +/+ | Almost black | Weak | Weak |
| 2 | +/+ | Black | Moderate | Weak | 32 | +/+ | Black | Moderate | Weak |
| 3 | +/+ | Black | Moderate | Weak | 33 | -/- | Red | Moderate | Weak |
| 4 | +/+ | Black | Moderate | Weak | 34 | +/+ | Almost black | Moderate | Weak |
| 5 | +/+ | Black | Moderate | Moderate | 35 | +/+ | Black | Moderate | Weak |
| 6 | +/+ | Black | Moderate | Moderate | 36 | -/- | Red | Weak | Weak |
| 7 | +/+ | Black | Moderate | Weak | 37 | +/+ | Black | Weak | Weak |
| 8 | +/+ | Almost black | Weak | Weak | 38 | +/+ | Black | Weak | Weak |
| 9 | +/+ | Black | Strong | Weak | 39 | +/+ | Black | Moderate | Weak |
| 10 | +/+ | Black | Moderate | Weak | 40 | -/- | Red | Absent | Absent |
| 11 | +/+ | Black | Strong | Moderate | 41 | +/+ | Black | Weak | Weak |
| 12 | +/+ | Almost black | Weak | Weak | 42 | +/+ | Red | Absent | Absent |
| 13 | +/+ | Red | Absent | Absent | 43 | +/+ | Black | Weak | Weak |
| 14 | +/+ | Black | Moderate | Weak | 44 | +/+ | Almost black | Weak | Weak |
| 15 | +/+ | Black | Weak | Weak | 45 | +/+ | Almost black | Weak | Weak |
| 16 | +/+ | Almost black | Weak | Weak | 46 | +/+ | Black | Moderate | Weak |
| 17 | +/+ | Black | Moderate | Moderate | 47 | +/+ | Almost black | Weak | Weak |
| 18 | +/+ | Almost black | Weak | Weak | 48 | +/+ | Almost black | Weak | Weak |
| 19 | +/+ | Black | Moderate | Moderate | 49 | +/+ | Black | Moderate | Moderate |
| 20 | -/- | Red | Weak | Moderate | 50 | +/+ | Black | Strong | Moderate |
| 21 | +/+ | Almost black | Weak | Weak | 51 | +/+ | Black | Strong | Moderate |
| 22 | +/+ | Black | Moderate | Weak | 52 | +/+ | Black | Moderate | Moderate |
| 23 | +/+ | Black | Weak | Weak | 53 | +/+ | Black | Moderate | Moderate |
| 24 | +/+ | Black | Moderate | Weak | 54 | +/+ | Black | Strong | Weak |
| 25 | +/+ | Almost black | Weak | Weak | 55 | +/+ | Black | Weak | Weak |
| 26 | +/+ | Black | Weak | Weak | 56 | +/+ | Black | Strong | Weak |
| 27 | +/+ | Black | Moderate | Weak | 57 | +/+ | Black | Strong | Weak |
| 28 | -/- | Red | Moderate | Weak | 58 | +/+ | Black | Strong | Weak |
| 29 | +/+ | Almost black | Weak | Weak | 59 | +/+ | Black | Strong | Weak |
| 30 | +/+ | Almost black | Weak | Weak | 60 | +/+ | Black | Strong | Weak |

4 (6.6%) were classified as non-adherent, for a total of 56 (93.3%) positive isolates and 4 (6.6%) negative isolates. Investigation of biofilm production by CRA showed that 38 of the 60 (63.3%) *S. pseudintermedius* isolates were found to produce typical black colonies, 14 (23.3%) of the 60 strains produced almost black colonies, and the remaining 8 (13.3%) strains produced red colonies even after 48 hr of incubation (Fig. 2a–2c), for a total of 52 (86.6%) positive isolates and 8 (13.3%) negative isolates.

The presence of the *icaA* (134 bp) and *icaD* (166 bp) genes was demonstrated by amplification of the corresponding fragments (Fig. 3). *IcaA* and *icaD* genes were detected concomitantly in 55 (91.6%) of 60 isolates. Neither one of the genes studied were detected in 5 (8.3%) isolates.

Statistical analysis: The agreement between the tube adherence test and MtP (Table 3a) and between the CRA and tube adherence test (Table 3b) using categorical data was fair (kappa=0.3), while it was moderate (kappa=0.5) between the CRA and MtP (Table 3c). However, weak slime-producing strains by CRA (almost black) (n=14) were regularly classified in the same manner by tube adherence (n=14) and MtP (n=13). The agreement obtained when grouping the results as dichotomous between CRA vs tube adherence and CRA vs MtP was good (kappa=0.6) and very good (kappa=1) between tube adherence test vs MtP. The percentage of matching results (considering 2 classes: producers and non-producers (NP)) between the CRA and tube adherence test and between CRA and MtP was 93.3% (Table 3b and 3c).

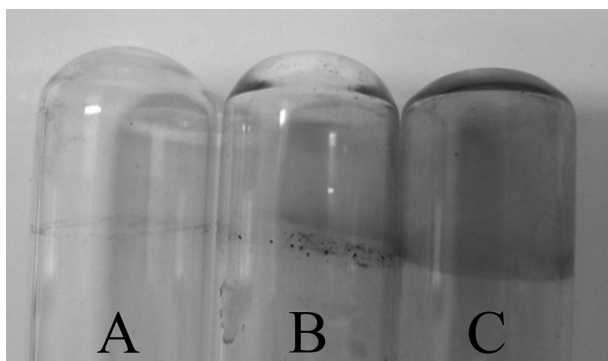


Fig. 1. Investigation of biofilm production of *S. pseudintermedius* by tube adherence test. Non-biofilm-producing isolate (A), weak biofilm-producing isolate (B) and moderate biofilm-producing isolate (C).

The percentage of matching results (considering 2 classes: producers and NP) between MpT and the tube adherence test was 100% (Table 3a).

All positive samples were classified in the same way by the three phenotypic tests, while four out of the eight negative results by the CRA were classified as positive with the other two tests. These same isolates were also negative with the *icaA* and *icaD* gene PCR. The results of the *icaA* and *icaD* gene PCRs showed good agreement ($\kappa=0.7$) with the CRA results. The percentage of matching results (considering 2 classes: producers and NP) between these two tests was 95% (Table 3d). The percentage of matching results (considering 2 classes: producers and NP) between PCR and MtP and between PCR and tube adherence was 88.3% (Table 3e and 3f).

DISCUSSION

Biofilm production by *S. pseudintermedius* may play an important role in the pathophysiology of disease and potentially colonization and could be a contributing factor in the rapid worldwide emergence of MRSP [25, 26]. The phenotypic data reported in our study demonstrate that the

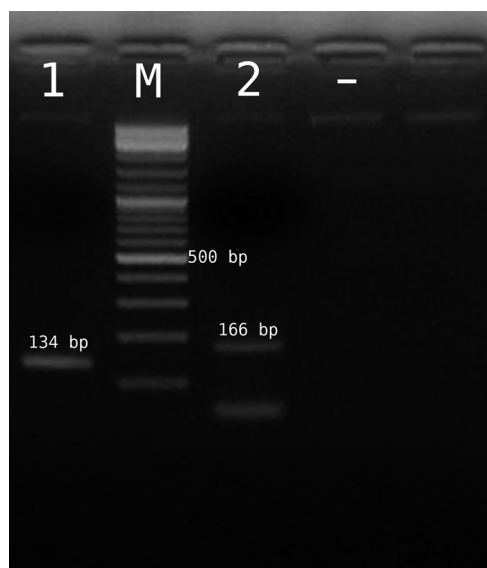


Fig. 3. Electrophoresis of PCR products. Lane 1, 134 bp (*icaA*) band from *S. pseudintermedius*; lane M, 100 bp molecular weight marker; lane 2, 166 bp (*icaD*) band from *S. pseudintermedius*.

majority of *S. pseudintermedius* isolates (96%) were able to produce biofilms. Moreover, PCR revealed the concomitant presence of *icaA* and *icaD* genes in 91% of the 60 isolates studied. All isolates had either both *icaA* and *icaD* genes or had neither gene; no strain showed the presence of one gene. These data confirm the fact that both genes are part of one operon, so the entire operon was either present or absent. These results agree with those of other studies of *S. epidermidis* and *S. aureus* that demonstrated that biofilm production occurs only when both *icaA* and *icaD* genes are expressed [1, 8, 17]. Our results differ from those reported in a study [30] in which the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs was characterized. In that study, *icaA* was detected in 109/140 (77.9%) *S. pseudintermedius* isolates, and *icaD* was detected in 106/140 (75.7%) isolates, demonstrating that some isolates

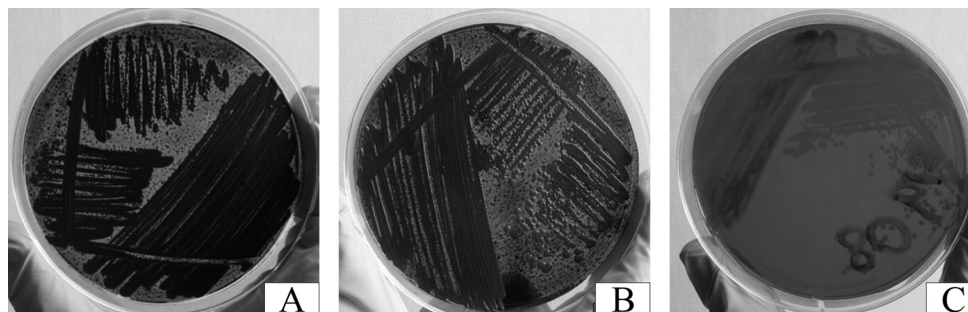


Fig. 2. CRA plate test. (A) Black colonies of biofilm-producing *S. pseudintermedius* isolates. (B) Almost black colonies of biofilm-producing *S. pseudintermedius* isolates, (C) Red colonies of non-biofilm-producing *S. pseudintermedius* isolates.

Table 3.

a. Comparison of MtP test and Tube adherence test

| MtP | | Tube adherence test | | | | Total |
|-------------------|--------|---------------------|----|----|--------|-------|
| | | Biofilm-producers | | | NP | |
| | | S | M | W | absent | |
| Biofilm-producers | S | 0 | 3 | 7 | 0 | 10 |
| | M | 0 | 7 | 16 | 0 | 23 |
| | W | 0 | 1 | 22 | 0 | 23 |
| NP | Absent | 0 | 0 | 0 | 4 | 4 |
| Total | | 0 | 11 | 45 | 4 | 60 |

S=strong, M=moderate, W=weak biofilm-producer and NP=non biofilm-producer are classified as Absent.

b. Comparison of CRA and Tube adherence test

| CRA | | Tube adherence test | | | | Total |
|-------------------|----|---------------------|----|----|--------|-------|
| | | Biofilm-producers | | | NP | |
| | | S | M | W | absent | |
| Biofilm-producers | B | 0 | 10 | 28 | 0 | 38 |
| | AB | 0 | 0 | 14 | 0 | 14 |
| NP | R | 0 | 1 | 3 | 4 | 8 |
| Total | | 0 | 11 | 45 | 4 | 60 |

S=strong, M=moderate, W=weak biofilm-producer and NP=non biofilm-producer are classified as Absent.

c. Comparison of CRA test and MtP test

| CRA | | MtP | | | | Total |
|-------------------|----|-------------------|----|----|--------|-------|
| | | Biofilm-producers | | | NP | |
| | | S | M | W | absent | |
| Biofilm-producers | B | 10 | 20 | 8 | 0 | 38 |
| | AB | 0 | 1 | 13 | 0 | 14 |
| NP | R | 0 | 2 | 2 | 4 | 8 |
| Total | | 10 | 23 | 23 | 4 | 60 |

S=strong, M=moderate, W=weak biofilm-producer and NP=non biofilm-producer are classified as Absent.

had a single *icaA* or *icaD* gene.

However, the authors found that 96% (136/140) of *S. pseudintermedius* isolates were classified as strong or moderate biofilm producers by MtP and concluded that there was no association between the presence of *ica* genes and biofilm formation. Whether the difference in results obtained can be explained by the different primers and PCR conditions used is unclear.

In the present study, 88.3% of all the strains screened had matching results by the tube adherence test, MtP and PCR analysis. Good concordance (95%) was found between the PCR-based analysis and the CRA. This association was further confirmed by the good agreement (kappa=0.7) between the CRA and *icaA* and *icaD* gene PCR results. This finding is in agreement with what was previously described by other authors [3, 34]. Comparing the phenotypic methods and considering the two classes (biofilm producer and biofilm non-producer), the percent of matching results between the CRA method and the tube adherence test and between the CRA method and MtP was 93.3%. A concordance of 100% was found between the MtP and the tube adherence test. Several

d. Comparison of PCR and CRA

| PCR (<i>icaA/icaD</i>) | CRA | | | Total |
|--------------------------|-------------------|----|----|-------|
| | Biofilm-producers | | NP | |
| | B | AB | R | |
| +/+ | 38 | 14 | 3 | 55 |
| -/- | 0 | 0 | 5 | 5 |
| Total | 38 | 14 | 8 | 60 |

B (black) colonies are classified as biofilm-producer; AB (almost black) colonies are classified as weakly biofilm-producer; R (red) as non biofilm-producer (NP).

e. Comparison of PCR and MtP

| PCR (<i>icaA/icaD</i>) | MtP | | | | Total |
|--------------------------|-------------------|----|----|--------|-------|
| | Biofilm-producers | | | NP | |
| | S | M | W | absent | |
| +/+ | 10 | 21 | 21 | 3 | 55 |
| -/- | 0 | 2 | 2 | 1 | 5 |
| Total | 10 | 23 | 23 | 4 | 60 |

S=strong, M=moderate, W=weak biofilm-producer and NP=non biofilm-producers are classified as Absent.

f. Comparison of PCR and Tube adherence test

| PCR (<i>icaA/icaD</i>) | Tube adherence test | | | | Total |
|--------------------------|---------------------|----|----|--------|-------|
| | Biofilm-producers | | | NP | |
| | S | M | W | absent | |
| +/+ | 0 | 10 | 42 | 3 | 55 |
| -/- | 0 | 1 | 3 | 1 | 5 |
| Total | 0 | 11 | 45 | 4 | 60 |

S=strong, M=moderate, W=weak biofilm-producer and NP=non biofilm-producer are classified as Absent.

authors consider the tube adherence test and MtP reliable and sensitive quantitative techniques for biofilm screening [12, 22, 24, 29].

Interestingly, three of 55 *icaA/icaD* positive strains were biofilm non-producers according to all three phenotypic methods. Similarly, a previous study [33] observed that only 24 of 35 *S. aureus* mastitis bovine isolates positive for *icaA/icaD* produced biofilms *in vitro*. Other authors [10] observed that *S. aureus* strains, despite having the *ica* locus, may fail to form biofilms *in vitro*, as biofilm formation on inert surfaces is highly sensitive to growth conditions. Another study [1] reported that CRA red variants of slime-positive *S. aureus* and *S. epidermidis* were found to lack the *icaA* and *icaD* genes as well as the whole *ica* locus. The authors suggested that the altered phenotype might be associated with deletion of the entire *ica* locus. However, using the CRA method, we observed that the red colonies of the three isolates that failed to turn black even after 48 hr of incubation carried the *icaA* and *icaD* genes. One of the common mechanisms that gives rise to slime-negative variants in *S. epidermidis* is insertional inactivation of the *ica* locus by the insertion sequence IS256,

which has been detected in *S. pseudintermedius* as well [7]. It has also been reported that failure of staphylococcal strains that possess the *ica* locus to form a biofilm *in vitro* could be due to point mutations in the locus and/or other yet unidentified factors that negatively regulate PIA production or influence biofilm formation [10].

Nevertheless, our results showed that four isolates determined to be biofilm producers by tube adherence test and microtiter plate test did not possess the *icaA* and *icaD* genes. These four strains are very interesting, since they imply the possibility that these isolates could form a biofilm *in vitro* not containing PIA. Recent studies have shown that additional components, such as accumulation-associated protein (Aap), independent of DNA and RNA or in cooperation with the *ica* operon may be important in biofilm production by staphylococci [8, 16]. Bap (biofilm-associated protein) and *ica*-independent mechanisms have been shown to be involved in the biofilm formation of *S. aureus* [11, 15]. Interestingly, Bap homologue protein (Bhp) is found in human *S. epidermidis* strains and induces an alternative mechanism of biofilm formation that does not depend on PIA [32].

The four *ica*-negative isolates could be false positives by the tube adherence test and microtiter plate test. Even though the microtiter plate method and tube adherence assay are considered reliable and sensitive quantitative methods for biofilm screening, there are certain limitations to both methods. Reading the results of the tube adherence method may be difficult, especially if the observer is not familiar with this technique. Moreover, observers frequently disagree with regard to interpretation of the results of the tube adherence test, particularly when interpreting weak reactions. Consequently, high variability has been observed, and classification as biofilm positive and negative has been difficult by this method [9, 14, 18, 21, 31]. The microtiter plate method has day-to-day and to some extent well-to-well technical variations with occasional outliers [25]. It has been demonstrated that phenotypic expression of biofilm formation is highly susceptible to *in vitro* conditions [6], and hence, variability can be detected by different methods. Therefore, a combination of various methods (phenotypic and genotypic) is useful for identifying biofilm-producing *S. pseudintermedius*. This is especially important considering the role of biofilms in evasion of the immunological defenses and resistance to antibiotic therapy and the difficulty of pathogen eradication. However, *S. pseudintermedius* biofilm production needs to be further investigated, since several authors have obtained conflicting results.

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