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Title: Biofilm formation and genotyping of *Staphylococcus aureus* bovine mastitis isolates: evidence for lack of penicillin-resistance in *Agr*- type II strains

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4 **evidence for lack of penicillin- resistance in *Agr*- type II strains.**

5

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1 Abstract

2 The increasing evidence for a role of biofilm formation in bovine mastitis caused by
3 *Staphylococcus aureus* led to further investigations on biofilm formation by *S. aureus*
4 isolates from mastitis in two growth media (TSBg and bovine milk serum). The ability of 99
5 *S. aureus* strains that were recently isolated or obtained from a culture collection (historical
6 strains) to form biofilm, in both growth media as well as the correlation of biofilm formation
7 with the presence of the *ica*-, *bap*-, and *IS257* genes is described. These genes have been
8 correlated with biofilm formation by human *S. aureus* isolates. All strains were also
9 genotyped with respect to their *Agr*- type and - subtype, and for the presence of the antibiotic
10 resistance genes *blaZ* and *smr* by PCR. The prevalence of the *Agr* types and the investigated
11 genes and their correlation with biofilm formation were statistically evaluated.

12 The *Agr*-type of a strain had a marked effect on the biofilm formation, by that strain, however
13 in contrast to human isolates no significant effect of *ica*- and *IS257* genes on biofilm
14 formation was observed. The *bap* gene was not found in any of the investigated strains. The
15 presence of biofilm related genes showed a high correlation with the *Agr*-type of the strains.
16 The data give evidence for a very strong correlation of *Agr*-type I strains and penicillin-
17 resistance in the bovine *S. aureus* mastitis strains; none of the *Agr*-type II strains was found to
18 harbor penicillin- resistance genes.

19 These data indicate that the most prevalent *Agr*-types in *S. aureus* bovine mastitis, *Agr*-type I
20 and II, can be regarded as different subspecies, with different abilities for the formation of
21 biofilm in bovine milk serum. The very high correlation between *Agr*- type II and penicillin-
22 susceptibility strongly suggests that these strains are not able to accommodate *blaZ* genes.

23

24 Keywords: *S. aureus*, biofilm, bovine mastitis, genotyping, penicillin resistance, *Agr*- type.

25

1 Introduction

2 There is increasing evidence that *S. aureus* can form biofilms in the udder of cows suffering
3 from mastitis. Biofilms impair the action of both the host immune system and antimicrobial
4 agents and allow the persistence of the infection (Melchior et al., 2006b). In a study, with a
5 limited number of strains, it was shown that all strains were able to grow in a biofilm
6 (Melchior et al., 2006a). A standard CLSI susceptibility test and a biofilm susceptibility assay
7 for antimicrobials commonly used in mastitis therapy, showed that the strains are less
8 susceptible for these antimicrobials when growing in biofilm (Melchior et al., 2006a, 2007).
9 Biofilm formation by *S. aureus* is influenced by environmental factors like sugars (glucose
10 and/or lactose) or proteases present in the growth medium and depends also on the genetic
11 make-up of a particular *S. aureus* isolate. The *Agr*-(accessory gene regulator) type, *Agr*-
12 subtype and *Trap*-(target of RAP) system (Gilot et al., 2002; Gilot and van Leeuwen, 2004),
13 the *ica*-(inter cellular adhesion), *bap*-(biofilm associated protein) and *IS257* genes, all are
14 examples of
15 components of the genetic make-up of a particular strain related to biofilm formation *in vitro*
16 (Cramton et al., 1999; Cucarella et al., 2004; Kozitskaya et al., 2004). *Agr*, *Trap* and *ica* are
17 loci involved in quorum sensing and have two component regulatory systems. In human
18 medicine a correlation between *Agr* type and certain diseases caused by *S. aureus* is
19 demonstrated. (Jarraud et al., 2002). Penicillin resistance encoding *blaZ*- and *smr* loci (De
20 Oliveira et al., 2000; Bjorland et al., 2001; Sidhu et al., 2001; *MARAN*, 2003 - 2005; Bjorland
21 et al., 2005) as well as biofilm formation influence the outcome of clinical therapy and lead to
22 higher risk of persistent intramammary infections and poor antimicrobial therapy (Haveri et
23 al., 2007). A relationship of these loci with Insertional Sequences (*IS*) was demonstrated
24 (Bjorland et al., 2001; Sidhu et al., 2002; Olsen et al., 2006).

1 The aim of this study was to determine the relation between biofilm formation with the
2 presence of the *ica-*, *bap-*, *IS257* and *blaZ* genes and *Agr* types in mastitis *S. aureus* isolates.
3 The simultaneous study of biofilm production, genetic profile and resistance allowed the
4 discovery of associations between these traits. To determine the effect of growth medium on
5 biofilm formation (Melchior et al., 2006a), a comparison between an artificial growth medium
6 and serum milk was also made.

7

8 **Materials and Methods**

9 **Collection of *S. aureus* isolates**

10 The strains used in this study were selected with respect to date of isolation, since over the
11 years the genetic make-up of *S. aureus* strains from bovine mastitis and their penicillin
12 resistance has evolved (Sol, 2002), and are from three different sources:

13 *A: field strains.* From December 2005 until September 2006, cows from farms spread over the
14 Netherlands were sampled. Cow selection was based on their 4 – 6 week somatic cell count
15 (SCC) pattern. The selection criterion for a *subclinical* infection was a cow SCC > 120.000
16 cell/ ml, without any other signs of infection. Milk samples were send to the Animal Health
17 Service in Deventer (Gezondheidsdienst voor Dieren). During the same period, milk from
18 animals with *clinical* bovine mastitis, send to the Animal Health Service for diagnostic
19 reasons, was randomly collected as well. All milk samples were incubated according to
20 standard laboratory procedures of the National Mastitis Council (ISO 17025 certified
21 laboratory) and the isolated *S. aureus* strains were stored at – 70°C. From the *S. aureus*
22 positive samples all 58 strains from 54 farms were included in this study. Only one strain per
23 cow was selected. These strains are designated the **F** strains (see Table 1).

24

1 *B: Penicillin-resistant S. aureus strains:* Because of the low number of penicillin-resistant
2 strains in collection A (F strains, ~10%), which is in accordance with the current prevalence
3 of penicillin-resistance in the Netherlands (MARAN, 2003 - 2005), selected penicillin resistant
4 strains were added to this collection.

5 In total, twenty penicillin resistant *S. aureus* strains from bovine mastitis from the Dutch
6 antimicrobial surveillance program (MARAN, 2003 - 2005) collected between 2003 and 2005
7 were obtained from the Central Veterinary Institute Lelystad (CVI, Lelystad). These strains
8 are designated the **P** strains (Table 1).

9
10 *C: Historical isolates:* Preliminary results in this study revealed remarkable differences
11 between our results and those from Gilot et al.,(2002; 2004) . These strains were collected
12 from 1950 – 2000 and therefore it was decided to analyse a number of strains isolated before
13 the year 2000. Twenty-one *S. aureus* strains from bovine mastitis collected between 1988 and
14 1994 were provided by the Veterinary Medical Diagnostic Centre of the Veterinary Faculty
15 (VMDC; University of Utrecht). The strains were selected randomly from cases of bovine
16 clinical mastitis and are stored for research purposes. These strains are designated the **H**
17 strains (see Table 1).

18 Three strains were used as controls; strain BMA/FR/032/0074 was used as a high slime
19 producing, *bap* locus positive strain, strain BMA/UK/032/0112 was used as a *smr* locus
20 positive strain and strain Newbould 305 (ATCC 29740) was used as control for the *Agr*
21 typing assays (Gilot et al., 2002) (designated 0074, 0112 and N305 respectively, in this
22 study). All three strains have been used previously in biofilm antimicrobial susceptibility
23 studies (Melchior et al., 2006a).

1 The identity of all used strains as *S. aureus* was confirmed by PCR according to Martineau
2 (1998). The growth media for the quantitative biofilm assay were TSB with 1% glucose
3 (TSBg) and bovine milk serum prepared according to the procedure below.

4

5 **Production of milk serum**

6 Fresh milk was taken from a single cow with monthly somatic cell count (SCC) records of <
7 50.000 for at least four consecutive months. The SCC of this milk sample was measured in
8 duplicate for confirmation of the cell count of the sample (Delaval Cell Counter DCC). The
9 milk was centrifuged twice for 17 min at 6000 x g at 4°C and the supernatant was transferred
10 to sterile bottles. The resulting skimmed milk supernatant was centrifuged at 46.000 x g at
11 4°C for one hour (for pelleting of the casein proteins) and the supernatant was transferred into
12 a sterile bottle. Sterility of the milk and the supernatants for all centrifuge steps was tested by
13 taking 100 µl milk or supernatant in duplicate, and incubating it on sheep blood agar plates for
14 overnight growth at 37°C. The milk serum was filter sterilized (0,45 µm filters, Corning, NY)
15 and stored at -20°C. From the milk serum prepared in this way, the bulk of lipid and protein
16 contents is removed. The milk serum consists of roughly 5% lactose, milk serum proteins and
17 all the unbound enzymes (including proteases) in milk (Fox and Kelly, 2006).

18

19 **Gene typing**

20 **Agr type, Agr subtype and Trap.** Detection of the *Agr* group- specific region and the
21 variable region of the *Agr* operon was performed as described (Gilot et al., 2002; Gilot and
22 van Leeuwen, 2004). In brief, the 1070-bp variable region of the *Agr* operon was amplified by
23 PCR (for primers see Table 2), and the amplicons were electrophoresed to determine the
24 molecular weight. After digestion with *AluI* or *RsaI*, the resulting fragments were separated

1 by electrophoresis through a 3% agarose gel containing ethidium bromide and visualized by
2 transillumination under UV light.

3 The PCR amplification of the *Trap* gene was also performed according to the method of Gilot
4 et al. . In brief, the 504-bp open reading frame of the *trap* gene was amplified by PCR, and
5 the size of the amplicons was estimated by electrophoresis. The *Trap* amplicons were digested
6 with *MseI* and electrophoresed through a 3% agarose gel yielding three *Trap* types.

7 Classification of *Agr* types and the *Trap* types was based on the study of Gilot and van
8 Leeuwen (Gilot et al., 2002).

9
10 **The *ica*, *bap*, *blaZ*, *smr* locus and *IS257*.** The presence of the *icaA* and *icaD* genes of the *ica*
11 locus, the *bap* locus, the *smr* locus, the *blaZ* gene and the *IS257* was determined with primers
12 described previously (Bjorland et al., 2001; Sidhu et al., 2002; Cucarella et al., 2004;
13 Melchior et al., 2006a; Olsen et al., 2006)(Table 2).

14

15 **Quantitative biofilm assay**

16 The biofilm forming capacity of the strains was determined as previously described (Melchior
17 et al., 2006a) with slight modifications. In brief, strains were grown overnight in TSBg at
18 37°C. This overnight culture (37°C) was diluted 1:100 in TSBg or in milk serum, and an
19 aliquot of 150 µl was placed in ten wells of a sterile 96-well U bottomed polystyrene
20 microtitre plates (Costar, Corning, NY, USA). After cultivation for 24 h at 37°C, the plates
21 were carefully washed with sterile phosphate-buffered saline, air dried for one hour under a
22 hood, and stained with 0.025% safranin for 10 min. Optical density (OD) at 540 nm was
23 measured with a 96-well plate reader (Bio-Rad model 3550 plate reader). Each assay was
24 performed in duplicate on two occasions. Only strains of *Agr*-type I and- II were included in

1 the assay, *Agr*-types III- and IV were excluded because they were only present in low
2 numbers (see Table 1). Strains N305 and BMA/FR/032/0074 were used as controls.

3

4 **Statistics**

5 For calculation of the statistical significance of the differences in the measured gene locus
6 prevalence between the *Agr* typing groups, a Chi-Square test was used (SPSS for Windows,
7 release 1.0.0 Standard Version). Absorbance values from the quantitative biofilm assays were
8 compared between groups based on the *Agr*-type or presence of genes by oneway Anova
9 (SPSS).

10

11 **Results**

12 ***Agr* type, *Agr* subtype and Trap**

13 All 99 strains were tested by *Agr*-typing and *Agr*-subtyping which resulted in *Agr*-typing- and
14 restriction patterns (*Agr*-subtyping) for 99 and 95 strains respectively; from 4 strains the *Agr*-
15 subtype could not be identified. The 58 strains from the F collection showed a high
16 prevalence of the RIV-A7 restriction pattern (81%)(*Agr* subtype II), the second most
17 prevalent pattern was RIII-A1 (9%)(*Agr* subtype I) and the three remaining patterns, RI'A1
18 (*Agr* subtype I), RIV-A5 (*Agr* subtype II) and RVI-A8 (*Agr* subtype III) had a low prevalence
19 ($\leq 3\%$) (Fig 1).

20 In contrast, the strains from the P and the H collection had a high prevalence of RIII-A1
21 patterns (75 and 86%, respectively) and a low prevalence of the patterns RI'-A1, RIII-A2 and
22 RIV-A7 (all below 10%). In other words, a high prevalence of *Agr*-type II was observed in
23 the F strains, and a high prevalence of *Agr*-type I in the P and the H strains.

1 The results of the *Agr*-typing, *Agr*-subtyping and *Trap*-typing showed a strict correlation
2 between *Agr*-type, *Agr*-subtype and *Trap*-type, as strains possessing the same *Agr* restriction
3 type, also contained the same *Trap*-type(Fig 1).

4

5 **Detection of the *ica*, *bap*, *blaZ*, *smr* locus and *IS257***

6 The *ica* locus was found in 74% of all strains and subdivision of strains based on their *Agr*-
7 type showed a marked difference in occurrence of the most prevalent *Agr*-types, I and II
8 (Table 1). The *IS257* sequence was found in 33% of the strains and the highest prevalence
9 (50%) was found in *Agr*-type II strains.

10 PCR with the two primer pairs for the beta-lactamase locus, *blaZ1* and *blaZ2* (Table 2)
11 resulted in 39% and 35% *blaZ* positive strains, respectively. The *blaZ* locus was not found in
12 any of *Agr*-type II strains.

13 The *bap* locus was not found in any of the tested strains, with exception of the positive
14 control, and the *smr* locus was only found in 5% of the strains.

15 Statistic calculations comparing the presence of *ica*, *IS257*, *BlaZ1* and *BlaZ2* in *Agr*-type I
16 and II strains revealed a significant pair wise difference in prevalence of $p=0.000$, $p=0.001$,
17 $p=0.000$ and $p=0.000$ respectively (Table 1).

18

19 **Quantitative biofilm assay**

20 Quantification of biofilm production by the *Agr*-type- I and II strains in TSBg and milk serum
21 is shown in Fig. 2. When the results for all strains and for both growth media were compared,
22 it became evident that the variation in biofilm production in milk serum is clearly larger than
23 in TSBg. This larger variation is mainly due to the higher optical density values for
24 quantitative biofilm production in milk serum medium, as the results showed a maximum

1 biofilm production of 2.3 and 1.5 OD and a minimum biofilm production of 0.3 and 0.4 OD
2 for milk serum and TSBg, respectively.

3 When the correlation between *Agr*-types and quantitative biofilm production was evaluated,
4 clear differences between the two growth media (Fig 2) were observed. In general, biofilm
5 production was higher for *Agr*-I than *Agr*- II stains in TSBg, but lower for *Agr*-I than *Agr*-II
6 strains in milk serum. The *ica* locus and *IS257* have little effect on biofilm production,
7 although the variation in biofilm production is lower for *ica* negative strains, both in TSBg
8 and milk serum (data not shown).

9 Statistic calculations comparing the biofilm production by *Agr*-type- I and II strains in TSBg
10 and milk serum showed a significant difference ($p=0.000$ and $p=0.005$, respectively) in
11 optical density between *Agr*-type- I and II strains for these growth media.

12 The outcomes in means and standard deviations for control stains N305 and
13 BMA/FR/032/0074 were 1.007 ± 0.185 , 1.331 ± 0.389 and 0.705 ± 0.165 , 2.438 ± 0.178
14 respectively for TSBg and milk serum medium.

15

16 Discussion

17 Biofilm formation is influenced by numerous factors, and one of these is the growth medium.

18 In this study quantitative biofilm assays of *S. aureus* strains from bovine mastitis were
19 performed in TSBg and bovine milk serum. These growth media differ in various ways, i.e. in
20 their sugar content and concentration (glucose versus lactose) and also in the presence of
21 proteolytic enzymes (Fox and Kelly, 2006; Kelly, 2006). Both proteolytic enzymes and sugars
22 are known to influence biofilm formation (Rohde et al., 2005; Coelho et al., 2008). Milk
23 serum was chosen since it can be considered to closely mimic the *in vivo* situation. The milk
24 serum used for the experiments came from a low cell count cow, and thus mimics the
25 situation in a healthy udder that becomes infected for the first time. Proteolytic enzymes in the

1 milk come from: blood plasma, the cytoplasm of secretory cells, the milk producing
2 mammary cells and somatic cells (leucocytes) which enter the mammary gland in response to
3 bacterial infection (Fox and Kelly, 2006; Kelly, 2006; Larsen, 2006).

4 Significant differences between *Agr*-type I and *Agr*-type II strains were noted, both in milk
5 serum and TSBg medium ($p=0.005$ and $p=0.000$ respectively). Biofilm production was higher
6 for *Agr*- I strains compared to *Agr*- II strains in TSBg, but lower for *Agr*-I than for *Agr*-II
7 strains in milk serum (Fig.2). The effect of the biofilm related loci *ica* and IS257 on biofilm
8 formation by *Agr*-type I and II strains, when present in these strains (both have a prevalence
9 of ~ 50% in both *Agr*-types), did not reveal significant differences. The lower biofilm
10 production by *Agr* I strains in milk serum is not due to the absence of the *ica* locus, since *ica*
11 positive *Agr* I strains tend to produce less biofilm in milk serum than *ica* negative strains (data
12 not shown). IS257 has no effect on the biofilm production in milk serum by *Agr* II strains, but
13 the variation in biofilm quantity was larger for the IS257 positive group. It can not be
14 excluded that this is caused by the limited number of strains used. Therefore the specific
15 effect of IS257 has to be studied in more detail before its effect on biofilm formation will be
16 clear. A similar IS, IS256 modulates staphylococcal biofilm formation (Ziebuhr et al., 1999;
17 Cho et al., 2002; Conlon et al., 2004) by virtue of its localization in biofilm associated
18 genes.

19 The *ica* locus is the best understood biofilm mechanism in staphylococci and the majority of
20 clinical isolates, both from human and bovine mastitis contain the *ica* operon (O'Gara, 2007).
21 The existence of *ica* independent biofilm formation mechanisms in *S. aureus* was recently
22 shown (Rohde et al., 2005; 2007). Biofilm formation in an *ica* negative strain depends on the
23 expression of a truncated isoform of the 220 kDa accumulation-associated protein Aap
24 (Rohde et al., 2005). Expression of full-length Aap does not lead to a biofilm positive
25 phenotype. The truncated Aap function is induced by proteolytic processing through

1 staphylococcal proteases. Added serine proteases like trypsin, elastase and cathepsin G are
2 also able to induce this proteolytic processing and thus can induce biofilm formation. This
3 shows the importance of the presence of proteases in the milk serum medium.

4 Pathogenic bacteria interact with the proteinase dependent cascade systems of their host,
5 including coagulation, fibrinolysis, complement activation and phagocytosis. Bacteria can also
6 induce the release of proteolytic enzymes or their precursors from phagocytic cells
7 (Lahteenmaki et al., 2001). During infection with intramammary bacteria the SCC of the milk
8 increases simultaneously with an increase in activity of plasmin and non-plasmin proteinases.

9 Although the pattern is similar for all species, each bacterial species elicits a response in a
10 species specific manner (Leitner, 2006). The effect of these changes of proteolytic enzymes
11 during infection of the mammary gland on biofilm formation need to be addressed in future
12 studies.

13 Our data confirm that the division of bovine mastitis *S. aureus* in penicillin resistant strains,
14 and penicillin sensitive strains is dependent on more than the mere presence or absence of the
15 *BlaZ* gene as suggested by Haveri et al. (2007). We found that almost all penicillin positive
16 strains are from *Agr* type I, and none of the *Agr* type II strains contained penicillin resistance
17 genes. We also showed that these *Agr* types have a significantly different ability for biofilm
18 formation, which are not the only differences between the *Agr* types of *S. aureus*. In a recent
19 study Buzzola et al. (2007) showed that *Agr* I strains can be internalized in MAC-T cells
20 significantly better, than isolates of *Agr* type II, III and IV. It was concluded that *Agr*-type I
21 strains are not only internalized more efficiently by epithelial cells, but also persist in higher
22 numbers in mammary gland tissue than *Agr*-type II, III and IV strains in the mouse infection
23 model (Buzzola et al., 2007). These data clearly show the different virulence properties of
24 different *Agr* types of *S. aureus*, which is relevant for our studies.

1 Genotyping of the strains in our collection revealed large differences in the distribution of
2 *Agr*-types amongst the recently isolated F strains and the strains isolated between the 1950s
3 and 2000s (Gilot et al., 2002; 2004). It was also shown that the strains from the H collection
4 have an *Agr*-type prevalence similar to the strains studied by Gilot and van Leeuwen (2002;
5 2004). These results indicate a shift in *Agr* type over time, and a concomitant decrease in
6 penicillin resistance. Indeed, data from the Dutch Animal Health service show that penicillin
7 resistance decreased from the 1970's to 2002 (Sol, 2002).

8 The practical and clinical implications of all these are that it be concluded that there is
9 evidence that bovine mastitis strains of the *Agr*-types I and II are probably equipped with
10 different toolboxes to survive in the mammary gland. *Agr* I strains seem to be better adapted
11 to an intracellular survival and biofilm formation might not be an important trait. Thus
12 effective therapeutic intervention of these infections requires the use of an antimicrobial agent
13 with sufficient intracellular concentrations (the most common antimicrobials in bovine
14 mastitis, except aminoglycosides). *Agr* II strains seem to prefer an extracellular niche and
15 hence biofilm formation is an important survival mechanism for these strains. Thus here
16 therapy requires drugs with good efficacy in biofilms and *in vitro* results suggest that
17 penicillins are a good choice (Melchior et al., 2007). However, the lack of information on
18 biofilm formation in high SCC milk serum requires that one should be cautious with these
19 conclusions.

20 In conclusion, the results presented here show a strict separation in *Agr* type between
21 penicillin-susceptible and penicillin resistant strains and that these *Agr* types contain a
22 different subset of genes, which cause significant differences in biofilm formation between
23 *Agr* I and II strains, the two most prevalent *Agr* types in *S. aureus* strains isolated from bovine
24 mastitis.

1 Bovine mastitis *S. aureus Agr-II* strains were not found to be penicillin resistant. The reason
2 for this awaits further study.

3 The importance of proteolytic enzymes in bovine milk serum (Hayes and Nielsen, 2000; Fox
4 and Kelly, 2006) and the positive correlation between the milk somatic cell count (SCC) and
5 the amount of these enzymes (Ballou et al., 1995; Albenzio et al., 2004) made it necessary to
6 standardize the biofilm assay with milk serum from low SSC milk. Thereby an assay that
7 mimics the situation in a healthy udder becoming infected for the first time was developed.

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11

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25

1 **Table 1.** Correlation of *Agr* types and presence of biofilm related genes for strains in all three
2 samples. The samples F strains, P strains and H strains are subdivided according to their *Agr*
3 types, and set out against the frequencies of present genes.

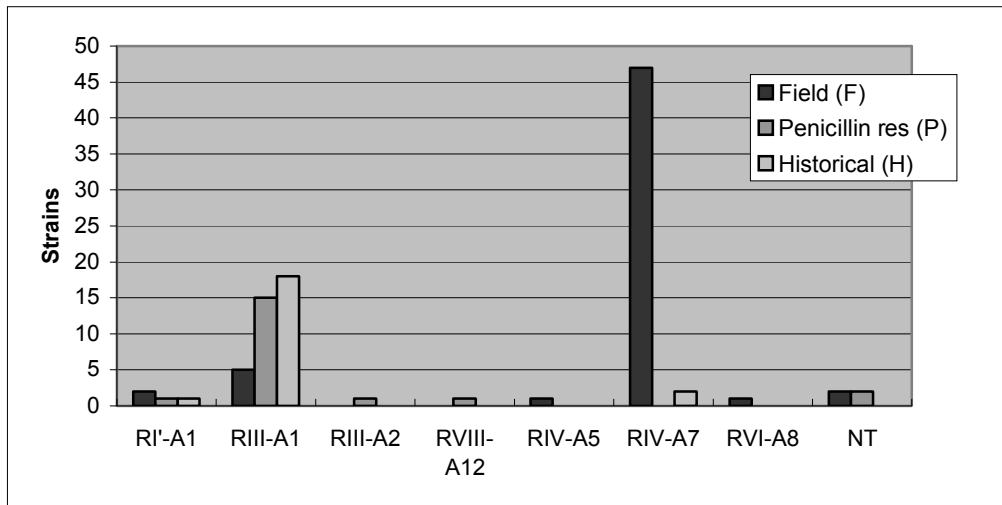
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5 **Fig 2.** Quantitative biofilm production in TSBg (A) and milk serum (B) for all tested strains,
6 and for *Agr*-type I and- II separately. Box and whisker plots with mean and 25% percentile
7 boxes. The oneway Anova statistical analysis revealed a significant difference (A: $p= 0.000$,
8 B: $p=0.005$) in optical density values between *Agr*-type I and *Agr*-type II (*).

9

10

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<i>Trap type</i>	1	1	2	3	3	2	3	1,2,3
<i>Agr-type</i>	I	I	I	I	II	II	III	I, II, III, IV

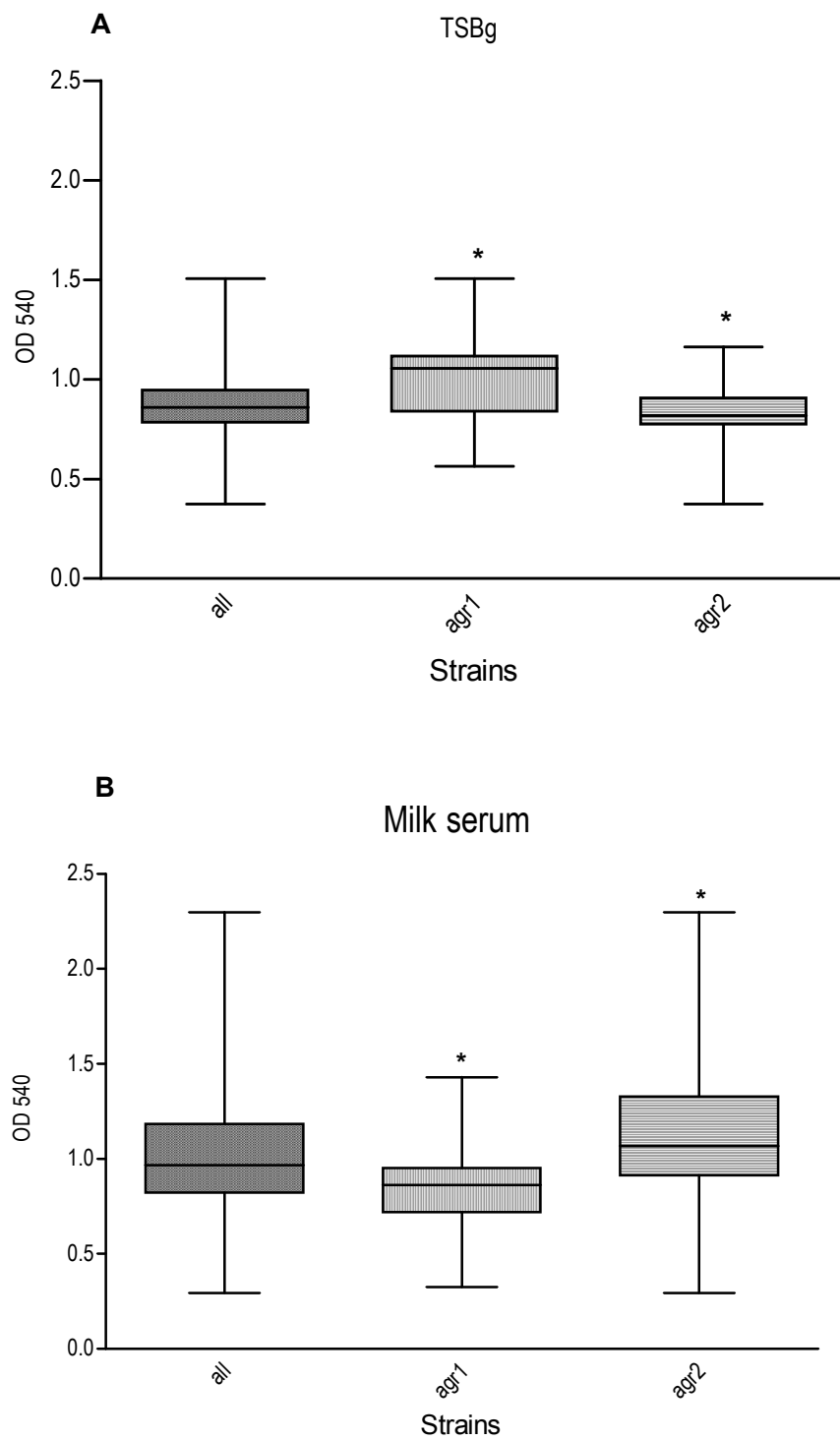


Fig 2.

Table 1. Correlation of *Agr* types and presence of biofilm related genes for strains in all three samples. The samples F strains, P strains and H strains are subdivided according to their *Agr* types, and set out against the frequencies of present genes.

		N =	Ica	IS257	BlaZ1	BlaZ2	bap	smr
F strains 2006		58						
Agr type	I	8	3	2	4	-	-	-
	II	49	49	23	-	-	-	1
	III	1	1	-	-	-	-	-
P strains 2003-2005		20						
Agr type	I	18	16	4	18	18	-	3
	III	1	1	-	1	1	-	-
	IV	1	1	-	1	1	-	-
H strains 1988-1994		21						
Agr type	I	19	1	2	17	17	-	1
	II	2	1	2	-	-	-	-
All strains		99						
Agr type	I	45	20 ^a	8 ^b	39 ^c	35 ^d	-	4
	II	51	51 ^a	25 ^b	- ^c	- ^d	-	1
	III	2	1	-	1	1	-	-
	IV	1	1	-	1	1	-	-
Total			73	33	41	35	-	5

^{a,b,c,d} Significant pair wise difference between *Agr* I and II according to Pearson Chi-Square tests.

^{a, c, d} p = .000, ^b p = .001

Table 2. Primers

Primer (5'-3')	reference
Agr B1	TAT GCT CCT GCA GCA ACT AA Gilot 2002
Agr C2	CTT GCG CAT TTC GTT GTT GA
ICAAF	CCT AAC TAA CGA AAG GTA G Cramton 1999
ICAAR	AAG ATA TAG CGA TAA GTG C
ICADF	AAA CGT AAG AGA GGT GG
ICADR	GGC AAT ATG ATC AAG ATA C
Sasp-6m	CCC TAT ATC GAA GGT GTA GAA TTG CAC Cucarella 2004
Sasp-7c	GCT GTT GAA GTT AAT ACT GTA CCT GC
487*	TAA GAG ATT TGC CTA TGC TT Olsen 2006
373*	TTA AAG TCT TAC CGA AAG CAG *(blaZ1 in table1)
486#	GTT GCG AAC TCT TGA ATA GG
488#	GGA GAA TAA GCA ACT ATA TCA TC #(blaZ2 in table1)
Smr F	ATA AGT ACT GAA GTT ATT GGA AGT Bjorland 2001
Smr R	TTC CGA AAA TGT TTA ACG AAA CTA
IS257F	TTG GGT TCA AGA ATA TGC CC Sidhu 2002
IS257R	CTT CGT TGA AGG TGC CTG AT