Comparative analysis of the virulence characteristics of methicillin–resistant and – susceptible *Staphylococcus pseudintermedius* isolates isolated from small animals: a RNA-Seq-based transcriptome analysis

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**Running title:** Transcriptome analysis in *S. pseudintermedius* by RNA-seq

**Keywords:** *S. pseudintermedius*; mecA; dogs; pathogenicity; virulence; biofilm
Abstract

*Staphylococcus pseudintermedius* is often associated with pyoderma, which can turn into a life-threatening disease. The dissemination of highly resistant isolates has occurred in the last 10 years and has challenged antimicrobial treatment of these infections considerably. We have compared the carriage of virulence genes and biofilm formation between methicillin-resistant and methicillin-susceptible *S. pseudintermedius* isolates (MRSP and MSSP, respectively) and additionally the *in vitro* gene expression profiles of MRSP and MSSP by RNA-seq.

Isolates were relatively unevenly distributed among the four *agr* groups, and *agr* type III predominated in MRSP. Five virulence genes were detected in all isolates. Only the *spsO* gene was significantly associated with MSSP isolates (p=0.04). All isolates produced biofilm in BHIB+4% NaCl. MSSP produced more biofilm on BHIB and BHIB+1% glucose media than MRSP isolates (p=0.03 and p=0.02, respectively). Virulence genes encoding surface proteins and toxins (*spsA, spsB, spsD, spsK, spsL, spsN, nucC, coa, luk-I*) and also prophage genes (encoding phage capsid protein, phage infection protein, two phage portal proteins and a phage-like protein) were highly expressed in the MRSP isolate (compared with the MSSP), suggesting they may play a role in the rapid and widespread dissemination of MRSP.

This study indicates that MRSP may upregulate surface proteins, which may increase the adherence of MRSP, especially ST71, isolates to corneocytes. MSSP may have an increased ability to form biofilm in acidic circumstances, through upregulation of the entire *arc* operon.

Complete understanding of *S. pseudintermedius* pathogenesis and host-pathogen signal interaction during infections is critical for the treatment and prevention of *S. pseudintermedius* infections.
Introduction

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) isolates have emerged as one of the leading causes of infectious diseases (including pyoderma, otitis and urinary tract infections) in companion animals, accounting for 20%-47% of all clinical *S. pseudintermedius* isolated from dogs and cats (1). Moreover, some MRSP isolates are resistant to the antimicrobials regularly used for treatment (β-lactams, fluoroquinolones, tetracyclines, lincosamides, potentiated sulphonamides) in small animal practice (1,2). The *mecA* gene, encoding resistance to β-lactams, has been acquired by several *S. pseudintermedius* clonal lineages on independent occasions, however two clones, MRSP ST68-SCCmec V and MRSP ST71-SCCmec II-III, are the dominant ones and have spread globally (1,3,4). This dissemination was rapid but the reasons for the fast emergence and success of these lineages are not fully understood (2). Genomic and proteomic studies, conducted in the last few years, are giving the first clues on the pathways by which MRSP have become successful. A recent genomic report suggested that multidrug-resistance evolved rapidly in MRSP due to the acquisition of a very limited number of mobile genetic elements and mutations (1). Thus, the use of different antimicrobial classes co-selected for the spread and emergence of the multidrug-resistant MRSP isolates (1). The frequent carriage of prophages in MRSP ST71 and ST68 genomes suggested they have a role in the fitness of MRSP and that the predominant transfer of genetic material in these isolates is through bacteriophage transduction, rather than plasmid conjugation as happens in methicillin-resistant *S. aureus* (MRSA) (1). MRSP are able to produce biofilm and MRSP ST71 isolates, in particular, are better biofilm producers than other MRSP clones (5,6). The *icaA* gene can be significantly upregulated in biofilm samples, suggesting a role in the biofilm production by *S. pseudintermedius* (7). The ability to form biofilm may play an important role in the pathophysiology of bacterial infections and can be related to survival and persistence of *S. pseudintermedius*, namely MRSP, in the environment (5,6). The MRSP ST71 isolates also show greater adherence to corneocytes than MRSP non-ST71 and MSSP, and thus it has been suggested that the enhanced adherence of ST71 might be a factor contributing to the epidemiological success of this MRSP lineage (2). Furthermore, an MRSP ST71 isolate of human origin adhered evenly well to canine and human corneocytes, implying that MRSP ST71 may also be capable of adapting to the human skin (2). Two proteins, SpsD and SpsO, can mediate adherence to canine corneocytes (8); however, the genetic factors responsible for the enhanced *in vitro* adherence of MRSP ST71 are not yet known (2).

In order to understand the epidemiological success of MRSP isolates, our goal was to understand if the phenotype (biofilm) and genotype (virulence genes) related to virulence factors...
was different between MRSP and methicillin-susceptible S. pseudintermedius (MSSP) isolates. Furthermore, we compared the in vitro transcriptional profiles by RNA-seq of one MRSP and one MSSP isolate to test the hypothesis that MRSP could have altered expression of virulence genes, by comparing to MSSP gene expression, which could have contributed to its rapid spread.

Materials and methods
Genotypic characterization of the MRSP and MSSP isolates
Twenty-one consecutive methicillin-resistant S. pseudintermedius (MRSP) isolated over a 7-year period from 2007 to 2014 were included in the study. Twenty-one matched (in terms of isolation year, isolation site and host) methicillin-susceptible (MSSP) were also included. These isolates were from 18 asymptomatic carriers (9 MRSP and 9 MSSP), 12 patients with pyoderma (6 MRSP and 6 MSSP), 6 patients with urinary tract infection (3 MRSP and 3 MSSP), 5 patients with otitis (2 MRSP and 3 MSSP) and 1 with a surgical site infection (1 MRSP). Five isolates were from cats and 37 were from dogs. Isolates were characterized by Multi-locus sequence typing (MLST) (9). The eBURST algorithm identified groups of related sequence types (ST) (10). Specific sequences for virulence genes involved in biofilm formation (bap, icaA, icaB, icaC, icaD), enterotoxin production (se-int, sec canine, seh), host adherence (ebpS, spaD, spaL, spaO), toxin production (lukS, lukF, siet, speta, expA, expB) were detected by PCR on a Mastercycle thermocycler (Eppendorf, New York, USA) with the primers, product size and annealing temperatures shown in Supplementary Table 1. The primers designed in this study were generated using the Primer-BLAST tool from NCBI. All PCR products were analyzed by electrophoresis through 1.2% agarose gels (NZYTech, Lisbon, Portugal). The primers agrD-F (5'- GGG GTA TTA TTA CAA TCA TTC -3') and agrD-R (5'- CTG ATG CGA AAA TAA AGG ATT G -3') (STABvida, Monte da Caparica, Portugal) were used as previously described to amplify a 300-bp agr fragment encompassing the 3' end of agrB, all of agrD, and the 5' end of agrC. Amplification was carried out on a Mastercycler thermocycler (Eppendorf) under the following conditions: an initial 5-min denaturation step at 94°C; followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 45°C, and 1 min of extension at 72°C; and a final extension step at 72°C for 10 min. The PCR products were purified by using the NZYGelpure (NZYTech) and sequenced with the same primers used for the PCRs (STABvida). The 42 isolates were assigned to one of the four agr groups by comparing the predicted product of AgrD and the N-terminal half of AgrC with those of four control isolates (GenBank accession numbers EU157336, EU157366, EU157334 and EU157330).
Biofilm-producing ability on polystyrene

The capacity of the isolates to form biofilm was investigated by a method described by Stepanović and colleagues (11) and Pettit and colleagues (12) with minor modifications, and was determined by the ability of *S. pseudintermedius* isolates to adhere to 96-well polystyrene microtitre plates (Greiner bio-one, Frickenhausen, Germany). In brief, the study was carried out using brain-heart infusion broth (BHIB; Biokar), BHIB with 4% NaCl and BHIB with 1% glucose as the growth media. The plates were incubated at 37ºC for 24h. Following incubation, the Alamar Blue solution was added to each well. After 30 minutes at room temperature, the optical densities at 570 nm (OD$_{570}$) were measured. *Staphylococcus epidermidis* RP62A strain (ATCC 35984) was used as a positive control. We defined the cut-off OD (OD$_C$) for the microtiter-plate test as three standard deviations above the mean OD of the negative control as described previously (11). All isolates were classified into the following categories: non-adherent (0) if the OD ≤ OD$_C$, weakly adherent (+) if the OD$_C$ < OD ≤ 2 x OD$_C$, moderately adherent (++) if the 2xOD$_C$ < OD ≤ 4 x OD$_C$, or strongly adherent (+++) if the OD ≥ 4 x OD$_C$, based upon the ODs of bacterial films (11).

RNA isolation, sequencing and gene expression analyses

To test the hypothesis that MRSP and MSSP isolates differ in their expression of virulence genes, we compared the *in vitro* transcriptional profiles of a clinical MRSP isolate and a clinical MSSP isolate using RNA-seq. We attempted to choose 2 representative of the *S. pseudintermedius* collection: isolated from skin swabs of dogs with pyoderma (the most frequent clinical specimen where *S. pseudintermedius* was isolated from); isolated in the same period of time; *agr* type III (the most frequent *agr* type found in this study); at least one ST71 (the most frequent ST found in this study); and similar virulence profiles (considering the virulence genes tested by PCR). Bacterial cells were grown until the mid-log phase growth (OD$_{600}$=0.5), since it has been shown that the majority of surface proteins are produced during this phase (13). RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). Briefly, 2x10^8 cells were removed from growing cultures and 2 volumes of RNApres® Bacteria Reagent (Qiagen) were added and incubated for 5 minutes at room temperature. Cells were then centrifuged and incubated with TE buffer (30 mM Tris.Cl, 1 mM EDTA pH 8.0, Sigma) containing 0.5-mg/ml lysostaphin (Sigma) and 15-mg/ml lysozyme (Sigma) for 20 minutes at 37ºC. Proteinase K (20 mg/ml, Sigma) was added and incubated for 10 minutes. After, the procedure was carried out according to the manufacturer’s specifications. The purified RNA was quantified using a
spectrometer (NanoDrop, ThermoScientific). RNA quality was assessed by visualization on an agarose gel. The rRNA was removed using the MICROBExpress kit (Ambion). RNA quality was then evaluated on a BioAnalyzer (Agilent). Bacterial mRNA was fragmented (yield fragments were in the size range of 200–250 bp) and the double-stranded cDNA was generated using the Ion Total RNA-seq kit v2 (Life Technologies, Thermo Fisher Scientific) according to the manufacturer’s instructions. The samples were sequenced using the Ion PGM™ (Life Technologies, Thermo Fisher Scientific) sequencer at STABvida. CLC Genomics Workbench v8.0.1. RNA-seq reads were aligned with the three available S. pseudintermedius reference genomes ED99 (ST25, agr type III, lacks spsF, spsO, spsQ), HKU10-03 (ST308, agr type III, lacks nanB) and E140 (ST71, agr type III, lacks nanB, lukF-S) (RefSeq accession numbers CP002478, CP002439 and ANOI01000001, respectively). Gene expression was normalized by calculating reads per kilobase per million mapped reads (RKPM), given by dividing the total number of reads by the number of mapped reads (in millions) times the length (kb) (14).

Differentially expressed genes were identified using the Baggerley’s test (binomial test), which compares the proportions of counts in a group of samples against those of another group of samples (15) with false discovery rate (FDR) correction applied (16). Genes with an adjusted p≤0.05 were identified as being differentially expressed. This study focused particularly in the expression of virulence genes, but expression of other relevant genes (e.g. antimicrobial resistance genes) was also evaluated.

**Statistical analysis**
All data analysis was carried out using IBM SPSS Statistics Version 20.0 (IBM, New York, USA). Differences between the two groups, MRSP and MSSP, were calculated by the Fisher’s exact test for categorical comparisons and Student’s t-test for continuous outcome. A p-value of ≤ 0.05 was considered to be statistically significant.

**Results**
The results of MLST are shown in Table 1. The MSSP isolates were divided into 21 different STs, while 15 MRSP isolates were assigned to the ST71, three to ST203; one to ST196; one to ST213, and one to ST195. Yet ST203 and ST195 belonged to the clonal complex (CC) 71, as detected by the eBURST analysis. Equally, ST196 and ST213 differed only by one allele and belonged to CC196.
All isolates were classified as part of one of the four *agr* groups, and the distribution was highly uneven, with 2 isolates belonging to *agr* group I, 7 belonging to group II, 30 belonging to group III, and 3 belonging to group IV (Table 1). There was a significant difference in the *agr* groups' distribution between MRSP and MSSP (p=0.025), with allele III being significantly more associated with MRSP than with MSSP (p=0.014).

The virulence genes detected in the MRSP and MSSP isolates are detailed in Table 2. Genes *se-int, speta, siet, spsL*, and *ebpS* were present in all 42 isolates. The genes *lukF* and *lukS*, encoding for leukocidin Luk-I, were found in all isolates except for two MRSP isolates (ST196, ST213). Gene *expB* was only detected in 3 isolates. Only two MSSP isolates carried the enterotoxin *sec*. No isolates harbored genes *seh* and *expA*. Eight isolates carried the *spsO* gene and by statistical analysis, this gene was significantly more associated with MSSP than with MRSP (p=0.04). No differences were found between clinical isolates and isolates from carriage.

Results of the biofilm-forming ability on polystyrene are shown in Table 3. All isolates produced biofilm in the BHIB+4% NaCl medium. Two and nine isolates did not produce biofilm on BHIB and BHIB+1% glucose, respectively. Biofilm production in the BHIB and BHIB+1% glucose media was significantly higher in MSSP than in MRSP isolates (p=0.03 and p=0.02, respectively), but there were no differences between clinical isolates and isolates from carriage.

The *ica* genes were detected in all 42 isolates. The number of mapped reads assigned by using each reference genome (ED99, HKU10-30 and E140) is shown in Supplementary Table 2. Of these mapped reads, the amount of *S. pseudintermedius* genes with altered expression also varied when using the three different reference genomes as shown in Figure 1. The MSSP isolate had higher expression in transcription of regulatory genes *agrB* and *agrD*. On the other hand, the MRSP isolate had higher transcription of regulatory genes *sigB*, *srrA*, *sarA*, *rot* and *saeRS* system. The signal transduction protein TRAP gene (*traP*) was also highly expressed. Considering genes encoding surface proteins, only one, *spsC* encoding an autolysin, was highly expressed in the MSSP isolate, while 6, *spsA, spsB, spsD, spsK, spsL, spsN*, were highly expressed in the MRSP isolate. The gamma-hemolysin component B gene (*hlgB*), both subunits of *luk-I* gene (*lukF-I* and *lukS-I*), the coagulase and thermonuclease genes (*coa* and *nucC*, respectively) were upregulated in the MRSP isolate. The arc genes (*arcA, arcB, arcC*, and *arcD*) were upregulated in the MSSP isolate. Several genes associated with antimicrobial resistance were highly expressed in the MRSP isolate: the *norA, gyrA* and *gyrB* genes associated with resistance to quinolones; the *aadE* and the bifunctional *aacA-aphD* genes associated with aminoglycoside-
resistance; the mecA, mecR1 and blaI genes associated with beta-lactam resistance; and tet(M) gene associated with resistance to tetracycline. The MRSP isolate upregulated several phage-associated genes (encoding phage capsid protein, phage infection protein, two phage portal proteins and a phage-like protein), and an integrase gene located in the superantigen-encoding pathogenicity islands SaPI (SPSINT_0063).

Discussion

In the last 10 years MRSP have become highly frequent in clinical samples from infected animals and as colonizers of healthy ones (1). However, it is still not clear why MRSP, especially certain lineages like ST71, have spread so quickly. To understand the rapid evolution that led to the dissemination of MRSP isolates we assessed the virulence determinants present in a collection of MSSP and MRSP isolates, and compared the ability to form biofilm in 3 different media. Finally, we performed and compared the in vitro gene expression analysis of one MSSP and one MRSP isolate.

Analysis of the virulence genotype of the MRSP and MSSP isolates revealed a strong conservation of genes: five genes (ebpS, se-int, siet, speta, spsL) were carried by all S. pseudintermedius isolates, and five genes (expB, luk-I, sec canine, spsD, spsO) were only present in some isolates. Two studies have reported the existence of some specific toxins (e.g. coa, lip, geh, htrA, nuc, clpX, hlb, se-int, speta, spsA, spsB, spsC) present in several S. pseudintermedius isolates that might be important for the canine host tropism, in particular the skin (1,17). However, variation was found in others (e.g. spsF, spsO, spsP, spsQ, luk-I, nanB), suggesting that a difference in virulence factors in the core genome was probably lineage-associated (1). For example, in one of these studies the five ST71 isolates lacked the nanB and the lukF-S genes (1). Still, in our study and in a previous study conducted in Spain (18), all the ST71 isolates carried the lukF-S genes, suggesting that variation may also be related to the region of isolation. It would be interesting to collect a large collection of ST71 isolates from different countries to study these variations. In other lineages, however, this will be difficult to ascertain, since only a few isolates in each lineage have been reported so far.

The capacity of bacteria to form biofilms is an important virulence factor not only in the development of device-related infections but also in a range of chronic infections (17). This capacity might further complicate the treatment of already challenging infections due to the decrease in effectiveness of antimicrobials on biofilms (5). In one study that all S. pseudintermedius isolates produced biofilms, suggesting that biofilm-production might be essential for the pathogenicity of S. pseudintermedius (6). Yet, the study failed to find
differences in the biofilm formation between MRSP and MSSP isolates. The number of MSSP
isolates that was studied was low and the authors suggested that further experiments with a
larger number of isolates were warranted (6). By using a larger set of isolates, we observed that
biofilm production in the BHIB and BHIB+1% glucose media was significantly higher in MSSP
than in MRSP isolates. This is a phenomenon that has been observed in S. aureus, when
comparing methicillin-resistant and methicillin-susceptible isolates, and is due to different
triggering mechanisms leading to biofilm formation, including ica-dependent and -independent
genes, suggesting this operon has a crucial role in biofilm formation. However, the mechanisms
triggering the higher biofilm production in the BHIB and BHIB+1% glucose media by MSSP
strains remain unknown. One clue to this occurrence may be related to the upregulation of the
entire arc operon in the MSSP isolate studied here. A similar operon has been found in other
staphylococcal species and, in S. aureus, arcA (belongs to the arc operon) encodes an arginine
deaminase, which allows for enhanced survival in acidic environments (20). The upregulation of
this operon may improve survival and promote biofilm formation of MSSP in acidic
circumstances, such as in BHIB medium with glucose (which has a more acidic pH than BHIB
medium alone or with NaCl).

During the early emergence of community acquired MRSA, the USA300 (ST8) lineage
disseminated rapidly and was considered hypervirulent, compared with lineages like MRSA
USA400 (ST1) (20). However, USA300 does not contain much more virulence genes than
USA400, but it does have an alteration in the expression of regulatory genes and an increased
expression of certain virulence genes (20). By microarray analysis, USA300 displayed an
increased expression of genes encoding cell envelope proteins (including lipoproteins and
superantigen-like proteins), genes residing in the prophage φSa3usa, several genes encoded in
pathogenicity islands vSAα and vSAß, proteases and the gene encoding the IgG binding protein
Sbi (20). Interestingly our MRSP isolate also had increased expression of several genes
including spsK, which encodes the IgG binding protein Sbi, the toxins nucC and coa, prophages
and several virulence regulatory genes including saeRS. The higher expression of the prophage
genes might be one of the factors contributing to the rapid dissemination of MRSP, particularly
ST71 isolates. The higher expression of the genes spsD and spsL (encoding fibronectin-binding
proteins able to adhere to the extracellular matrix) found in this study, may explain the higher
adherence of MRSP ST71 isolates to corneocytes previously detected (2). We observed a very
different expression of virulence regulatory genes between the two isolates, with agr highly
expressed in MSSP and saeRS in MRSP. This may explain the differences observed in the expression of the genes encoding surface proteins and toxins.

One of the most important bacterial defenses against uptake of foreign DNA is restriction-modification (R-M) systems (21). These systems, comprising restriction endonucleases and methyltransferases, recognize and modify specific DNA sequences, protecting “own” DNA from restriction while eliminating potentially harmful foreign DNA (21). In S. pseudintermedius, type I R-M systems have been recognized, including one that was carried on all SCCmec II-III elements of MRSP ST71 (1). One study suggested that MRSP were not more efficient or inefficient than MSSP in acquiring mobile genetic elements due to the wide distribution of Type I and Type II R-M systems in S. pseudintermedius isolates (1). In our study, however, we found that the Type I restriction-modification system restriction subunit R (hsdR) was highly expressed in the MSSP isolate, suggesting it blocks DNA horizontal gene transfer into methicillin-susceptible isolates. Lower expression of the subunit R in the MRSP isolate could also suggest a more efficient way of acquiring mobile genetic elements. In fact it has been shown that MRSP genomes carry more prophages than MSSP isolates. Our results showed that the MRSP isolate also upregulates several phage-associated genes, which could be linked to the upregulation of the integrase located in the superantigen-encoding pathogenicity islands SaPI. The upregulation of prophage particles is also concordant with the suggestion that transfer in MRSP is predominantly made by transduction (1).

In summary, this is the first study to document the global transcription differences between MSSP and MRSP isolates during in vitro growth. This study indicates that MRSP may upregulate surface proteins, which may increase the adherence of MRSP, especially ST71, isolates to corneocytes. Although MRSP and MSSP have the capacity to form biofilm, MSSP may have an increased ability to form biofilm in acidic circumstances, through upregulation of the entire arc operon. Complete understanding of S. pseudintermedius pathogenesis and host-pathogen signal interaction during infections is critical for the treatment and prevention of S. pseudintermedius infections.

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References


methicillin resistant *Staphylococcus aureus* USA300 and USA400 isolates. BMC Genomics 15:1145.


Figure 1. Number of *S. pseudintermedius* genes with altered expression identified by the Baggerley’s test with the FDR correction applied, using the three different reference genomes available (ED99 ST25-agr III, HKU10-30 ST308-agr III, E140 ST71-agr III).

Table 1. Epidemiological characteristics of MRSP and MSSP isolates used in this study. The number of isolates is shown inside parenthesis.

Table 2. Virulence traits of the methicillin-resistant and methicillin-susceptible *S. pseudintermedius* isolates.

Table 3. Overall results of the microtiter-plate test according to the pattern of methicillin-resistance in *S. pseudintermedius*. 


Figure 1. Number of *S. pseudintermedius* genes with altered expression identified by the Baggeley's test with the FDR correction applied, using the three different reference genomes available (ED99 ST25-agr III, HKU10-30 ST308-agr III, E140 ST71-agr III).
Table 1. Epidemiological characteristics of MRSP and MSSP isolates used in this study. The number of isolates is shown inside parenthesis.

<table>
<thead>
<tr>
<th>agr types</th>
<th>MSSP (n=21)</th>
<th>MRSP (n=21)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ST207 (n=1), ST215 (n=1)</td>
<td>0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>ST201 (n=1), ST205 (n=1), ST217 (n=1)</td>
<td>ST196 (n=1), ST213 (n=1)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>II</td>
<td>ST206 (n=1), ST209 (n=1), ST17 (n=1), ST197 (n=1), ST199 (n=1), ST200 (n=1), ST214 (n=1), ST379 (n=1)</td>
<td>ST71 (n=15), ST195 (n=1), ST203 (n=3)</td>
<td>0.014</td>
</tr>
<tr>
<td>III</td>
<td>ST202 (n=1), ST204 (n=1), ST210 (n=1), ST211 (n=1), ST212 (n=1), ST214 (n=1), ST216 (n=1)</td>
<td>0</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

* Differences between MRSP and MSSP.

Abbreviations: MLST, multi-locus sequence typing; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; MSSP, methicillin-susceptible *Staphylococcus pseudintermedius*; ST, sequence type.
Table 2. Virulence traits of the methicillin-resistant and methicillin-susceptible S. pseudintermedius isolates.

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th>Total (n=42)</th>
<th>MRSP (n=21)</th>
<th>MSSP (n=21)</th>
<th>p-value</th>
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<tr>
<td>expA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>expB</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>luk-I</td>
<td>40</td>
<td>19</td>
<td>21</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>sec canine</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>seh</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>spsD</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>spsO</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>0.04</td>
</tr>
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</table>

The genes ebpS, se-int, siet, speta, spsL and the ica-operon were positive in all isolates and were not included in this table.
Table 3. Overall results of the microtiter-plate test according to the pattern of methicillin-resistant in *S. pseudintermedius*.

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean OD&lt;sub&gt;570&lt;/sub&gt; and Standard Deviation</th>
<th>Number of isolates showing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No adherence (0)</td>
</tr>
<tr>
<td>MRSP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHIB</td>
<td>0.50 ± 0.052</td>
<td>1</td>
</tr>
<tr>
<td>BHIB+ 1% glucose</td>
<td>0.28 ± 0.038</td>
<td>3</td>
</tr>
<tr>
<td>BHIB+ 4% NaCl</td>
<td>0.44 ± 0.055</td>
<td>0</td>
</tr>
<tr>
<td>MSSP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHIB</td>
<td>0.57 ± 0.082</td>
<td>1</td>
</tr>
<tr>
<td>BHIB+ 1% glucose</td>
<td>0.35 ± 0.081</td>
<td>6</td>
</tr>
<tr>
<td>BHIB+ 4% NaCl</td>
<td>0.45 ± 0.098</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: BHIB, brain-heart infusion broth; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; MSSP, methicillin-susceptible *Staphylococcus pseudintermedius*; NaCl, sodium chloride.