Clonal diversity and biofilm-forming ability of methicillin-resistant Staphylococcus pseudintermedius

Ane Mohr Osland, Lene K. Vestby, Hanne Fanuelsen, Jannice Schau Slettemeås and Marianne Sunde*

Section for Bacteriology—Aquatic and Terrestrial, Norwegian Veterinary Institute, PO Box 750 Sentrum, N-0106 Oslo, Norway

*Corresponding author. Tel: +47-23-21-63-81; Fax: +47-23-21-63-01; E-mail: marianne.sunde@vetinst.no

Received 26 August 2011; returned 29 October 2011; revised 9 December 2011; accepted 14 December 2011

Objectives: The aim of this study was to investigate the clonal epidemiology of methicillin-resistant Staphylococcus pseudintermedius (MRSP) isolates from dogs in Norway and to evaluate the antimicrobial resistance patterns and determine the biofilm-forming abilities of the isolates.

Methods: All MRSP index isolates from each MRSP-positive dog detected in Norway until June 2011 were included (n = 23). The MICs of antimicrobial agents were determined by the VetMIC™ microdilution method. The genetic relationship between the isolates was investigated by multilocus sequence typing (MLST) and PFGE. The isolates’ abilities to form biofilm on polystyrene were studied.

Results: The MRSP isolates investigated grouped into 11 different sequence types (STs); MRSP ST106 occurred most frequently. There were a relatively smaller number of isolates belonging to ST71, the largely predominant ST in Europe. Isolates belonging to ST71 had a significantly greater ability to produce biofilm compared with the other isolates, and especially compared with MRSP ST106.

Conclusions: A heterogeneous clonal distribution was observed among MRSP from dogs in Norway. As opposed to previous findings in Europe, MRSP clones other than ST71 have spread in Norway, such as MRSP ST106. The results also show that MRSP ST71 is possibly a good biofilm producer, and this may in turn be a contributing factor to the nosocomial character of MRSP ST71.

Keywords: sequence type, antimicrobial resistance, dogs, Norway, persistence

Introduction

Antimicrobial resistance is an emerging concern within the fields of veterinary medicine and public health, making the ability to determine genetic relatedness and knowledge of the fitness properties among important bacteria fundamental in evolutionary as well as epidemiological studies. Staphylococcus pseudintermedius is currently known to be the most common agent of bacterial skin and ear infections in dogs1,2 and, in addition, it has occasionally been isolated from humans, suggesting a possible zoonotic potential.3–6

S. pseudintermedius has previously been associated with high resistance rates, as demonstrated by the annual reports published by the Norwegian monitoring programme for antimicrobial resistance in the veterinary sector (NORM-VET),7–9 and a similar situation is found in other European countries also including methicillin-resistant isolates.10–12 Within the genus Staphylococcus, methicillin resistance is linked to the presence of the mecA gene, which encodes an alteration in the penicillin binding protein (PBP), leading to a lower affinity to all β-lactam antimicrobial agents.13

In 2008 the first case of mecA-positive S. pseudintermedius was recorded in Norway. From that time until June 2011 there have been 22 additional clinical cases in different dogs.14 Recently Perreten et al.15 reported that two major and independent clones, belonging to sequence type (ST) 71 and ST68, have disseminated in Europe and in North America, respectively. In 2009 Black et al.16 found that 37 of 38 isolates from samples collected in the United States belonged to ST68. Furthermore, in 2010 Ruscher et al.12 reported that out of 146 examined European methicillin-resistant S. pseudintermedius (MRSP) isolates, all but one isolate belonged to ST71. This implies that MRSP is spreading rapidly and that they have a common source. There are few studies that have investigated the reason for the clonal expansion of MRSP and why certain clones are more successful and predominating than others. A former study carried out in Norway reported considerable genetic diversity among methicillin-susceptible S. pseudintermedius (MSSP) from dogs. The study showed a high genetic diversity among the MSSP isolates investigated where the PFGE banding pattern from every isolate showed a distinct pattern with a high diversity and a low genetic relationship.17 The success of certain MRSP clones
seen in the USA and Europe compared with the diversity seen among MSSP clones may possibly be linked to fitness properties such as the ability to produce biofilm. This is a known capability of certain staphylococci and may perhaps cause certain clones to have an advantage compared with others.

Biofilm-producing bacteria may adhere to implants and foreign body material and have the ability to cause opportunistic infections. In addition, bacterial biofilms may also occur on mucosal and soft tissue surfaces, such as in ears, urinary and respiratory tracts, as well as skin among others. This may lead to chronic recurrent infections that are complex to treat because of the decreased antimicrobial effect often seen in connection with biofilms. Moreover, there might be a possibility for biofilm to aid in the persistence of bacteria in the environment, and in this way it may also contribute to nosocomial infections. Only a few publications are available on biofilm production properties of staphylococcal species affecting dogs. However, the ability to form biofilm is considered to be a major virulence factor for S. pseudintermedius, an important commensal of skin and mucous membranes in humans, known as a common cause of nosocomial infections. Biofilm production is one of several traits of S. epidermidis that has been suggested to be important for their survival in different environments, including hospitals, worldwide.

The purpose of the present study was to examine the genetic relationship between MRSP isolates from dogs in Norway and to investigate whether Norwegian MRSP have spread clonally. Furthermore, we have investigated the biofilm-forming abilities and the antimicrobial resistance patterns of these isolates.

Materials and methods

Bacterial isolates

MRSP isolates from 23 different dogs were collected from clinical samples submitted for routine microbiological investigation to the Norwegian Veterinary Institute (NVI) in Oslo during the period July 2008–June 2011. This collection of isolates represents all MRSP index isolates from each MRSP-positive dog detected in Norway until June 2011. Two of these isolates originated from dogs hospitalized at the same clinic, within the same time period, during the summer of 2008. The other isolates did not show any epidemiological connection. In addition, two MRSP strains from dogs in Sweden (BD19698 and AB3407) were included for comparison. These strains represent the dominant MRSP clone in Europe and have previously been shown to belong to ST71, to display the PFGE pattern J, and in this way it may also contribute to nosocomial infections.

Determination of antimicrobial susceptibility (MICs)

The MRSP isolates were examined for MICs of 12 antimicrobial agents using the VetMIC microdilution method (SV A, Uppsala, Sweden). The cut-off values used to classify the isolates as resistant or susceptible to each antimicrobial agent are the ones applied in the NORM-VET 2008 programme.

Multilocus sequence typing (MLST)

Parts of the genes agrB, tuf, hsp60, pta and 16S rDNA were amplified by PCR, purified with ExoSAP-IT (USB Corp., Cleveland, OH, USA) and subsequently sequenced. The sequences were determined using the BigDye Terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were run on a capillary sequencer (3130xl Genetic Analyzer). Resulting sequences were analysed using CLC bio Combined Workbench (CLC bio A/S, Aarhus, Denmark) and the National Centre for Biotechnology Information (NCBI) GeneBlast2 program (http://blast.ncbi.nlm.nih.gov/blast.cgi). The allele sequences were compared with the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) and ST numbers were allocated based on the key table in the database for MLST S. pseudintermedius (curator Vincent Perreten, University of Bern, Switzerland; vincent.perreten@vbi.unibe.ch). If an allelic profile of an isolate did not match a previously registered allelic profile it was submitted for registration in the same database. The phylogenetic analysis was carried out using MEGA5 software with the neighbour-joining method using the maximum composite likelihood method (www.megasoftware.net).

PFGE

PFGE was performed as illustrated by Bannerman et al. with minor modifications as described previously. The banding patterns were evaluated by visual inspection and using BioNumerics software (BioNumerics, Applied Maths, Kortrijk, Belgium).

Biofilm on polystyrene

The ability of the isolates to form biofilm was investigated by a method described by Stepanovic et al. with minor modifications, and was determined by the ability to adhere to 96-well polystyrene microtitre plates (Nunc, Nuncland, Roskilde, Denmark). In brief, the study was carried out using tryptic soy broth (TSB; Oxoid Ltd, Basingstoke, UK) as a growth medium. Overnight cultures were diluted (1:100) in the same medium and subsequently 200 μL of the diluted bacterial suspension was transferred in triplicates into microtitre plate wells, with the negative control containing growth medium only. The plates were incubated at 37°C for 24 h. Following incubation, the plates were washed with water to remove planktonic cells and dried at room temperature before the addition of 1% Crystal Violet solution (Sigma-Aldrich, St Louis, MO, USA) as a growth medium. Overnight cultures were diluted (1:100) in the same medium and subsequently 200 μL of the diluted bacterial suspension was transferred in triplicates into microtitre plate wells, with the negative control containing growth medium only. The plates were incubated at 37°C for 24 h. Following incubation, the plates were washed with water to remove planktonic cells and dried at room temperature before the addition of 1% Crystal Violet solution (Sigma-Aldrich, St Louis, MO, USA). The plates were subsequently incubated at room temperature for 30 min before excess dye was removed by washing with water. The bound dye was dissolved in 220 μL of ethanol/acetone (70/30, v/v) before optical densities at 595 nm (OD595) were measured. For each isolate, the result was calculated by subtracting the median OD595 of the triplicates of the negative control (test broth only) from the median OD595 of the
triplicates of the samples. Three independent experiments were performed and the mean of the three experiments was calculated based on the median from each experiment. All 23 MRSP isolates were included in the biofilm study, along with 2 Swedish MRSP isolates (BD19698 and AB3407) representing the dominant MRSP clone in Europe. In addition, two isolates from Nofima Mat (Norwegian Institute of Food, Fisheries and Agriculture Research) known to produce biofilm (S. aureus 50076 and 50583) were included as controls in the biofilm assay. Statistical methods are given in the Results section.

Results

Identification of MRSP isolates

All 23 isolates included in this study were mecA positive. The isolates were confirmed as S. pseudintermedius based on detection of an MboI restriction site on a PCR-amplified 320 bp internal fragment of the pta gene. Table 1 gives an overview of all isolates included in the study, the site of infection, dog breed and the geographical region the patient came from.

Resistance to antimicrobial agents

All isolates were classified as resistant to β-lactam antimicrobial agents (cefalotin, penicillin, oxacillin) based on the presence of the mecA gene. Furthermore, 20 (86.9%) of the isolates were resistant to kanamycin; 19 (82.6%) to clindamycin, erythromycin and trimethoprim; 12 (52.1%) to tetracyclines; 8 (34.8%) to fusidic acid; 7 (30.4%) to gentamicin; and 5 (21.7%) showed resistance to chloramphenical and 4 (17.4%) to ciprofloxacin. All isolates produced β-lactamase. The distributions of MIC values are shown in Table 2.

Genotyping

MRSP belonging to ST106 was most frequently found as eight isolates belonged to this ST. PFGE produced similar banding patterns, as shown in Figure 1, suggesting clonal spread of MRSP isolates belonging to this ST. Four isolates belonged to ST71, the largely predominant ST in Europe. The PFGE banding patterns produced by these isolates were similar when aligned with each other, as well as similar to the banding patterns of the two Swedish ST71 MRSP isolates. This suggests that the MRSP ST71 isolates from dogs in Norway are related to the MRSP ST71 clone disseminated in Europe. The remaining isolates included one isolate belonging to ST10, one to ST26, two to ST28, one to ST69, one to ST78, and one to ST100. The two isolates belonging to ST28 showed a difference by more than six bands on PFGE. In addition, there were three new distinct allelic profiles admitted for registration at the database for MLST S. pseudintermedius. These were denoted ST127 (two

Table 1. MRSP isolates included in the study

<table>
<thead>
<tr>
<th>Isolate number (prefix; year isolated)</th>
<th>ST (allelic profile)</th>
<th>Site of infection</th>
<th>Breed</th>
<th>Region of patient</th>
<th>Average biofilm OD595 (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009-01-2827</td>
<td>106 (1,2,13,1,2)</td>
<td>external ear canal</td>
<td>Bordeaux</td>
<td>Hamar</td>
<td>0.91 (±0.23)</td>
</tr>
<tr>
<td>2009-01-2959</td>
<td>106 (1,2,13,1,2)</td>
<td>pyoderma</td>
<td>Bordeaux</td>
<td>Sørum</td>
<td>0.90 (±0.43)</td>
</tr>
<tr>
<td>2009-01-4244</td>
<td>106 (1,2,13,1,2)</td>
<td>pyoderma</td>
<td>dachshund</td>
<td>Oslo</td>
<td>1.10 (±0.22)</td>
</tr>
<tr>
<td>2010-01-967</td>
<td>106 (1,2,13,1,2)</td>
<td>tonsil</td>
<td>Shetland sheepdog</td>
<td>Rakkestad</td>
<td>0.56 (±0.16)</td>
</tr>
<tr>
<td>2010-01-2029</td>
<td>106 (1,2,13,1,2)</td>
<td>prepuce infection</td>
<td>unknown breed</td>
<td>Nesodden</td>
<td>0.49 (±0.22)</td>
</tr>
<tr>
<td>2010-01-2096</td>
<td>106 (1,2,13,1,2)</td>
<td>urine</td>
<td>Norwegian elkhound</td>
<td>Fredrikstad</td>
<td>0.77 (±0.11)</td>
</tr>
<tr>
<td>2010-01-2839</td>
<td>106 (1,2,13,1,2)</td>
<td>tonsil</td>
<td>German shepherd</td>
<td>Oslo</td>
<td>2.00 (±0.13)</td>
</tr>
<tr>
<td>2010-01-38311</td>
<td>106 (1,2,13,1,2)</td>
<td>external ear canal</td>
<td>bullmastiff</td>
<td>Ålesund</td>
<td>0.62 (±0.18)</td>
</tr>
<tr>
<td>2008-01-2669</td>
<td>71 (1,1,9,2,1)</td>
<td>pyoderma</td>
<td>Japanese chin</td>
<td>Fredrikstad</td>
<td>3.64 (±0.28)</td>
</tr>
<tr>
<td>2008-01-3250</td>
<td>71 (1,1,9,2,1)</td>
<td>post-operative</td>
<td>Shetland sheepdog</td>
<td>Sørpsborg</td>
<td>3.64 (±0.28)</td>
</tr>
<tr>
<td>2010-01-4344</td>
<td>71 (1,1,9,2,1)</td>
<td>post-operative</td>
<td>English setter</td>
<td>Folloebu</td>
<td>2.48 (±0.32)</td>
</tr>
<tr>
<td>2011-01-14</td>
<td>71 (1,1,9,2,1)</td>
<td>post-operative</td>
<td>English setter</td>
<td>Ås</td>
<td>3.18 (±0.19)</td>
</tr>
<tr>
<td>2010-01-272</td>
<td>28 (1,2,7,1,1)</td>
<td>pyoderma</td>
<td>unknown breed</td>
<td>Fredrikstad</td>
<td>2.80 (±0.38)</td>
</tr>
<tr>
<td>2010-01-1316</td>
<td>28 (1,2,7,1,1)</td>
<td>pyoderma</td>
<td>cocker spaniel</td>
<td>Oslo</td>
<td>3.52 (±0.19)</td>
</tr>
<tr>
<td>2009-01-2723</td>
<td>127 (1,1,9,4,3)</td>
<td>pyoderma</td>
<td>boerboel</td>
<td>Åmot</td>
<td>3.06 (±0.57)</td>
</tr>
<tr>
<td>2010-01-5747</td>
<td>127 (1,1,9,4,3)</td>
<td>pyoderma</td>
<td>bullmastiff</td>
<td>Haugesund</td>
<td>1.21 (±0.33)</td>
</tr>
<tr>
<td>2009-01-3363</td>
<td>128 (1,1,2,18,1,3)</td>
<td>external ear canal</td>
<td>pug</td>
<td>Hennens</td>
<td>3.14 (±0.29)</td>
</tr>
<tr>
<td>2009-01-3736</td>
<td>100 (1,2,7,1,3)</td>
<td>tonsil</td>
<td>boxer</td>
<td>Høland</td>
<td>1.22 (±0.45)</td>
</tr>
<tr>
<td>2009-01-5870II</td>
<td>129 (1,1,24,22,3)</td>
<td>external ear canal</td>
<td>French bulldog</td>
<td>Stokke</td>
<td>2.24 (±1.18)</td>
</tr>
<tr>
<td>2010-01-181</td>
<td>78 (1,1,2,13,1,1)</td>
<td>external ear canal</td>
<td>Doberman</td>
<td>Rømedal</td>
<td>3.37 (±0.02)</td>
</tr>
<tr>
<td>2010-40-368</td>
<td>26 (1,2,10,1,4)</td>
<td>urine</td>
<td>springer spaniel</td>
<td>Sandnes</td>
<td>2.75 (±0.29)</td>
</tr>
<tr>
<td>2010-01-2845</td>
<td>10 (1,2,2,4,1)</td>
<td>pyoderma</td>
<td>unknown breed</td>
<td>Tromsø</td>
<td>3.27 (±0.13)</td>
</tr>
<tr>
<td>2010-01-3810</td>
<td>69 (1,1,25,1,1)</td>
<td>nasal cavity</td>
<td>beagle</td>
<td>Stord</td>
<td>2.70 (±0.34)</td>
</tr>
<tr>
<td>BD19698b</td>
<td>71 (1,1,9,2,1)</td>
<td>post-operative</td>
<td>unknown breed</td>
<td>Sweden</td>
<td>3.38 (±0.07)</td>
</tr>
<tr>
<td>AB3407b</td>
<td>71 (1,1,9,2,1)</td>
<td>post-operative</td>
<td>unknown breed</td>
<td>Sweden</td>
<td>3.35 (±0.26)</td>
</tr>
</tbody>
</table>

*Order of allelic profile: 16S rDNA, tuf, hsp60, pta, agrD.

*Isolates collected from dogs in Sweden included in the biofilm assay as representatives of the most common MRSP clone in Europe (MRSP ST71).
isolates), ST128 and ST129 (Table 1). The two MRSP belonging to the novel ST127 produced identical PFGE banding patterns, indicating a close genetic relationship (Figure 1). A phylogenetic tree generated by the use of MEGA5 software is shown in Figure 2.

The isolates belonging to ST71 all showed high MICs of oxacillin (>16 mg/L), while the MIC value trend within isolates belonging to ST106 was consistently lower (<8 mg/L). Moreover, the isolates belonging to ST71 were resistant to a greater number of antimicrobial agents compared with isolates belonging to ST106. Two of the isolates belonging to ST71 were resistant to all antimicrobial agents included in the panel except tetracycline, while the remaining two were resistant to all antimicrobial agents except tetracyclines, cloramphenicol and fusidic acid. On the other hand, isolates belonging to ST106 were classified as resistant to the β-lactams, erythromycin, clindamycin, kanamycin, trimethoprim and tetracycline (except two isolates) while being susceptible to the other antimicrobial agents in our panel. The two isolates belonging to ST127 were both resistant to β-lactams and fusidic acid, but were susceptible to all other antimicrobial agents tested for. The ST28 isolates (two) were resistant to β-lactams and tetracycline, and one of them was also resistant to kanamycin.

**Biofilm on polystyrene**

In this study the MRSP isolates belonging to ST71 produced the greatest amount of biofilm and showed a statistically significant difference in biofilm-forming abilities compared with the other MRSP isolates. Student’s t-test was used and the level of significance was set to P<0.05. The data were normally distributed. The highest OD595 values were observed for isolates belonging to ST71 (mean 3.28, SEM 0.17). In comparison, the other isolates in this study showed an average OD595 value of 1.93 (SEM 0.25). Isolates belonging to ST106 showed an especially moderate biofilm production (compared with the other STs in the study), with an average OD595 value of 0.92 (SEM 0.17) (Figure 3). There was no statistically significant difference in biofilm-forming abilities among the Swedish and Norwegian isolates belonging to ST71 (Student’s t-test, P=0.77).

### Table 2. MICs of MRSP isolates included in the study

<table>
<thead>
<tr>
<th>MIC value cut-off (mg/L)</th>
<th>Range tested</th>
<th>0.06</th>
<th>0.12</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>Number of resistant isolates (total = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cefalotin</td>
<td>&gt;1</td>
<td>0.06–8</td>
<td>1</td>
<td>11</td>
<td>5</td>
<td>2</td>
<td>16</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td></td>
<td></td>
<td>23b</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>&gt;16</td>
<td>0.5–64</td>
<td>1</td>
<td>11</td>
<td>6</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>&gt;0.5</td>
<td>0.06–4</td>
<td>1</td>
<td>11</td>
<td>6</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>clindamycin</td>
<td>&gt;2</td>
<td>0.25–32</td>
<td>1</td>
<td></td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
<td>19</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>erythromycin</td>
<td>&gt;2</td>
<td>0.25–32</td>
<td>1</td>
<td></td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
<td>19</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>fusidic acid</td>
<td>&gt;0.5</td>
<td>0.06–8</td>
<td>1</td>
<td></td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>gentamicin</td>
<td>&gt;2</td>
<td>0.5–64</td>
<td>1</td>
<td></td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>kanamycin</td>
<td>&gt;16</td>
<td>0.25–32</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td>20</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>oxacillin with NaCl</td>
<td>&gt;1</td>
<td>0.12–16</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td>20</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>penicillin</td>
<td>NA</td>
<td>0.06–4</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td>20</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>tetracycline</td>
<td>&gt;2</td>
<td>0.5–64</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td>20</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>trimethoprim</td>
<td>&gt;8</td>
<td>0.5–32</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td>20</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

Shaded areas indicate the range of dilutions tested. Numbers in bold denote the numbers of isolates with MIC values below or above the measured range. The ones with MIC values higher than the range tested are given as the lowest value above the range. The ones with values lower than or equal to the lowest range are given as the lowest concentration tested.

*MIC cut-off from the NORM-VET report of 2008.*

**b**Classified as resistant based on the presence of the mecA gene.

**Figure 1.** PFGE of eight MRSP isolates belonging to ST106. Lanes: M, lambda ladder; 1–8, MRSP ST106.
Figure 2. Neighbour-joining and bootstrapping analysis of concatenated sequence alignment consisting of the 16S rDNA, agrD, hsp60, pta and tuf gene sequences of all MRSP isolates included in the study. The total length of the sequence alignment was 1844 characters. Only bootstrap values larger than 75 are shown.

Figure 3. Average biofilm production of MRSP isolates. Results are given as the mean OD_{595} and SEM based on three independent experiments. Left: all isolates belonging to ST71 and all isolates belonging to ST106. Middle: all isolates belonging to ST71 and all other MRSP isolates included in the study (striped column). Right: all isolates belonging to ST106 and all other MRSP isolates included in the study.
Discussion

This study demonstrated that the clonal dissemination of MRSP in Norway is more heterogeneous than expected and that isolates belonging to ST106 occurred most frequently. It also illustrated that the representatives of the most widespread clone in Europe (ST71) included in this study produced more biofilm than the other isolates studied. Moreover, in this study the isolates belonging to ST106 had a lower average biofilm production compared with the other isolates investigated. It is important to consider the sample size as a limiting factor in this study, as MRSP has only been isolated on 23 occasions in Norway. A study with isolates from several European countries and with a larger number of isolates may possibly strengthen the present findings. This could also document the biofilm-producing properties among isolates within the two STs (ST71 and ST106) are continuously consistent. In spite of sample size as a limiting factor in this study, the relatively consistent level of biofilm formation observed within the different lineages strengthens the results.

The 23 investigated MRSP isolates grouped into 11 different STs. The great diversity of genotypes seen was interesting and unexpected, as a single MRSP clone seems to dominate in other countries. Presently the MRSP population may be more clonally diverse in Norway than what is observed elsewhere; however, as the results presented in our study are based on 23 MRSP cases only, this situation could rapidly change. In contrast to other European countries, where ST71 seems to predominate, only 4 of 23 isolates belonging to this ST were detected in this study. This is the first detection of MRSP ST71 from dogs in Norway, indicating that there is an on-going spread of this clone to new territories.

MRSP ST106 occurred most frequently, as it was isolated from eight dogs (one-third of the isolates included in the study). The similarities in PFGE banding patterns (Figure 1) indicate a close genetic relationship among the isolates. No epidemiological connection was found between the dogs with MRSP ST106, and the patients were widely dispersed in south Norway. Until now, clonal spread of MRSP has been associated with ST71, and this is the first report showing dissemination of an MRSP clone not belonging to ST71. MRSP ST106 has previously been reported from dogs on two occasions in Europe: one in Denmark and the other in the Netherlands. This suggests that MRSP belonging to ST106 may represent another successful clone with a potential for international spread. Further characterization of MRSP ST106, comparison studies and surveillance are needed in order to evaluate if this is a new successful MRSP clone with a potential for pan-European dissemination. Two MRSP isolates belonging to ST127, a new S. pseudintermedius ST, were detected. These isolates originated from dogs in geographically distinct regions, separated by more than 500 km. The PFGE banding patterns were identical, suggesting a close clonal spread of a new MRSP variant. This MRSP clone was less resistant to antimicrobial agents, as resistant was only observed to β-lactams and fusidic acid. Further monitoring of the MRSP situation will reveal if MRSP ST127 is a new successful clone. Attention should be paid to emerging non-ST71 MRSP clones, as they may play important roles in future MRSP epidemiology.

All the MRSP isolates in this study were classified as resistant to oxacillin using $\geq 0.5 \text{ mg/L}$ as a breakpoint (accepted value by the CLSI subcommittee on Veterinary Antimicrobial Susceptibility Testing). MRSP belonging to ST71 displayed oxacillin MICs $>16 \text{ mg/L}$, while MRSP ST106, although resistant, had consistently lower MICs of oxacillin. The remaining isolates (belonging to neither ST71 nor ST106) showed various MICs of oxacillin, however, a considerable proportion were found to have relatively low MICs. Low expression of mecA and/or heterogeneous expression of resistance to oxacillin may represent a challenge for diagnostic laboratories in recognizing MRSP. At the NVI, cefalexin was used as a screening agent and most of the MRSP isolates had rather large cefalexin inhibition zones (20–27 mm, susceptible $>28$ mm). The fact that these isolates were found to be mecA positive emphasizes the need to be aware of possible low-level oxacillin resistance and heterogeneous expression among MRSP. Moreover, cefalexin may not be the optimal screening agent for MRSP, and an evaluation of what is the best possible screening agent is needed, as this has not been performed on a collection containing a multitude of MRSP clones. Regarding MRSA, cefoxitin is currently recognized as the most suitable screening agent even though oxacillin was used for this purpose for several years. However, cefoxitin is known to be unsuitable for MRSP screening and oxacillin is the recommended screening agent. Nonetheless, there may theoretically be other β-lactams that can detect MRSP with higher sensitivity and specificity than oxacillin.

All the MRSP isolates included in the study were classified as resistant to β-lactams (cefalotin, penicillin and oxacillin) based on the presence of the mecA gene. The breakpoints from the NORM-VET 2008 programme misclassified a considerable proportion of the isolates as susceptible, especially to cefalexin. These breakpoints should therefore be carefully reconsidered. Previous studies have suggested that the ability to form biofilm is important for bacterial persistence and survival in the environment. We used the microtitre plate method to study the ability to produce biofilm, which is a frequently used quantitative technique that is superior for screening studies. In this study it was clear that isolates belonging to ST71 formed statistically significantly more biofilm than MRSP isolates belonging to other STs. The finding suggests that high biofilm-forming abilities could possibly be a consistent trait for this clone and a contributing factor in making it frequently connected with persistent infections in animal clinics. Even though the microtitre plate method is considered a reliable and sensitive method for biofilm screening, there are certain limitations to the method. These are the day-to-day, and to some extent also well-to-well, technical variations with occasional outliers (results not shown). Therefore, when using this method one should preferably calculate the mean of a group rather than looking at a single well, and hesitate before reporting single-strain results. Preferably one should do replicates in order to limit the error of technical variation and use the median of the replicates on one plate to take into account potential outliers.

Interestingly, in this study isolates belonging to ST106 produced lower amounts of biofilm, expressed lower MICs of oxacillin and were more susceptible to antimicrobial agents when compared with those belonging to ST71. This is an important point, as isolates belonging to this ST occurred most frequently from dogs in Norway. No epidemiological connections were found between the various clinical cases in this study, except for two MRSP ST71 isolates from dogs hospitalized at the same
Clonal diversity and biofilm-forming ability of MRSP

Clinic during the summer of 2008. In spite of clonality, a possible reason that isolates belonging to ST106 are not widely seen in hospital infections may be because of lower resistance levels and lower biofilm production abilities than what is seen among MRSP ST71.

Further studies looking at the horizontal transfer of antimicrobial resistance genes in biofilm, as well as looking at the increased tolerance to disinfectants after biofilm formation would be of interest. Bacteria in biofilm, including staphylococci, are known to be less susceptible to antimicrobial agents and disinfectants than planktonic bacteria.41,42 Because of the growing threat of increased resistance to antimicrobial agents by pathogenic bacteria, there is a continuous search for new agents to control infectious diseases, so-called anti-pathogenic drugs. One possible mode of action of these substances is by selectively blocking quorum sensing and/or biofilm formation.43–46 One such class of compounds is halogenated furanones, which have been shown to reduce biofilm formation and bacterial colonization by S. epidermidis as well as other bacteria such as Salmonella and various streptococci.20,37,62,67,68 These are also known to potentiate the effect of disinfectants and antimicrobial agents on Salmonella biofilms and can be used as a pretreatment before disinfection to gain a combined effect.37 Genes encoding antimicrobial resistance and biofilm formation are associated with accessory genes, and these characteristics are not necessarily stable. In spite of this, in this study it is demonstrated that the most resistant isolates and the ones forming the largest amount of biofilm belong to the same ST. It will be important to continue to study the potential ‘anti-pathogenic’ drugs to aid in future control and treatment of infections caused by MRSP, as, based on this study, the isolates that are largely multiresistant may also be superior biofilm producers.

In this study, in addition to the recognized diversity among genotypes, we observed the emergence of MRSP ST106, which is a non-ST71 clone. This suggests that there may be additional dominating clones contributing to future MRSP epidemiology. On top of resistance to a large number of antimicrobial agents, the widespread European MRSP clone ST71 was found to be a superior biofilm producer. In this context it is essential to consider that this is not a complete epidemiological study of the prevalence of MRSP and its STs fully comparable to the situations elsewhere. The lack of standardization of conditions for screening, as well as the large difference in sample size, makes comparison with previously described studies difficult and complex. Despite this, the study gave a clear indication of the present situation in Norway as well as opening up new areas of exploration about S. pseudintermedius and its fitness properties.

Acknowledgements
Part of this study was presented at the Fourth Symposium on Antimicrobial Resistance in Animals and the Environment, Tours, France, 2011 (poster 59). We would like to acknowledge the staff at the NVI: Bjørg Kvite, Kristin Berg, Tone Bjordal Johansen, Marianne Gillhus, Bjarne Bergsjo, Aina Steinhauk Barstad, Tormod Mark, Jarle Mikalsen, Madelaine Norstrøm and Michaela Falk. Further, we would like to thank Ulrika Grönlund-Andersen (National Veterinary Institute, SVA, Uppsala, Sweden) for the donation of MRSP BD19698 and AB3407 and Dr T. Møretø and Dr T. M. Rode from Nofima Mat (Norwegian Institute of Food, Fisheries and Agriculture Research) for the biofilm control strains S. aureus 50076 and 50583. Lastly, we thank Vincent Perreten (University of Bern, Bern, Switzerland) for help and discussions on the topic.

Funding
This work was supported by the Norwegian Veterinary Institute (NVI) and was partly funded by Dyrlege Smidts stiftelse.

Transparency declarations
None to declare.

References
5 van Duijkeren E, Houbiers DJ, Schoormans A et al. Staphylococcus pseudintermedius in Norway as well as opening up new areas of exploration about S. pseudintermedius and its fitness properties.

Acknowledgements
Part of this study was presented at the Fourth Symposium on Antimicrobial Resistance in Animals and the Environment, Tours, France, 2011 (poster 59). We would like to acknowledge the staff at the NVI: Bjørg Kvite, Kristin Berg, Tone Bjordal Johansen, Marianne Gillhus, Bjarne Bergsjo, Aina Steinhauk Barstad, Tormod Mark, Jarle Mikalsen, Madelaine Norstrøm and Michaela Falk. Further, we would like to thank Ulrika Grönlund-Andersen (National Veterinary Institute, SVA, Uppsala, Sweden) for the donation of MRSP BD19698 and AB3407 and Dr T. Møretø and Dr T. M. Rode from Nofima Mat (Norwegian Institute of Food, Fisheries and Agriculture Research) for the biofilm control strains S. aureus 50076 and 50583. Lastly, we thank Vincent Perreten (University of Bern, Bern, Switzerland) for help and discussions on the topic.

Funding
This work was supported by the Norwegian Veterinary Institute (NVI) and was partly funded by Dyrlege Smidts stiftelse.

Transparency declarations
None to declare.

References
5 van Duijkeren E, Houbiers DJ, Schoormans A et al. Staphylococcus pseudintermedius in Norway as well as opening up new areas of exploration about S. pseudintermedius and its fitness properties.
BMC Vet Res 19: 18
Futagawa-Saito K, Ba-Thein W, Sakurai N
Lønn-Stensrud J, Landin MA, Benneche T
Int J Antimicrob Agents
Staphylococcus intermedius factors in the Norwegian dog population.
Staphylococcus pseudintermedius
Norsto¨ m M, Sunde M, Tharaldsen H
conditions.
formation in
importance and implications.
J Antimicrob Chemother
agents for preventing
O’Gara JP, Humphreys H.
2010;
Staphylococcus pseudintermedius
identification of
Bannoehr J, Franco A, Iurescia M
Black CC, Solyman SM, Eberlein LC
isolates of
cluster, and novel staphylococcal chromosomal cassette in clinical isolates of mecA-containing, methicillin-resistant Staphylococcus pseudintermedius.