Use of a Bacterial Antimicrobial Resistance Gene Microarray for the Identification of Resistant Staphylococcus aureus

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Impacts
- The bacterial antimicrobial resistance gene DNA microarray provides detailed relevant information on Staphylococcus aureus isolates by detecting the presence or absence of a large number of antimicrobial resistance genes simultaneously in a single assay.
- Thirty-eight antibiotic resistant S. aureus isolates possessed at least one antimicrobial resistance gene.
- The presence of more than one antimicrobial gene determinant appears rare in S. aureus isolates from bovine mastitis cases in Québec.
- No methicillin-resistant S. aureus (MRSA) were found.

Introduction
Antimicrobial resistance (AMR) in pathogenic bacteria is becoming a major public health concern as it has been linked to sepsis and other surgery complications, lengthening hospital stays at best and causing death at worst (Figueiredo-Costa, 2008). Methicillin-resistant Staphylococcus aureus (MRSA) is the leading pathogen linked to this health concern, while community-associated-MRSA cases are also on the rise in North America (Seybold et al., 2006). Numerous researchers in other countries have been reporting results on the prevalence of methicillin-resistant S. aureus (MRSA) in pigs and the risk of transmission to humans. Livestock may become an

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Summary
As diagnostic and surveillance activities are vital to determine measures needed to control antimicrobial resistance (AMR), new and rapid laboratory methods are necessary to facilitate this important effort. DNA microarray technology allows the detection of a large number of genes in a single reaction. This technology is simple, specific and high-throughput. We have developed a bacterial antimicrobial resistance gene DNA microarray that will allow rapid antimicrobial resistance gene screening for all Gram-positive and Gram-negative bacteria. A prototype microarray was designed using a 70-mer based oligonucleotide set targeting AMR genes of Gram-negative and Gram-positive bacteria. In the present version, the microarray consists of 182 oligonucleotides corresponding to 166 different acquired AMR gene targets, covering most of the resistance genes found in both Gram-negative and -positive bacteria. A test study was performed on a collection of Staphylococcus aureus isolates from milk samples from dairy farms in Canada. The reproducibility of the hybridizations was determined, and the microarray results were compared with those obtained by phenotypic resistance tests (either MIC or Kirby-Bauer). The microarray genotyping demonstrated a correlation between penicillin, tetracycline and erythromycin resistance phenotypes with the corresponding acquired resistance genes. The hybridizations showed that the 38 antimicrobial resistant S. aureus isolates possessed at least one AMR gene.
important source of community-acquired MRSA (de Neeling et al., 2007; Morgan, 2008).

In the food industry, where preventive antibiotic administration to livestock is widespread, disease causing AMR-bacteria affect livestock by slowing their growth rate thus incurring detrimental economic consequences. Detection of pathogenic bacteria in food products causes their withdrawal and destruction resulting in an economic burden to producers. When undetected, microbial contamination has been linked to several severe outbreaks in the human population (Shen et al., 2006; Srinivasan et al., 2007). Many of these pathogens are becoming increasingly resistant to antibiotics (EFSA, 2007). These events have led to recent food safety legislation in the European Union (EU, 2007) while other industrialized countries are expected to take similar measures. In addition, AMR can be encoded by genes found in mobile elements and can therefore be transmitted between species and strains through horizontal gene transfer (Kelly et al., 2009; de Vries et al., 2009). Phylogenetic studies show that AMR-resistant bacterial strains can colonize very different host species through increased association (Lowder et al., 2009). The presence of AMR genes in food products is thought to be one mode of transmission to the human population (Manges et al., 2007).

With the advent of molecular biotechnology, new genetic detection tools are available to study the spread of AMR genes among animal and human microflora. DNA microarrays have been used successfully in various genotyping and bacterial antimicrobial gene detection studies (Perreten et al., 2005; Bruant et al., 2006; Laing et al., 2009). More specifically, microarrays have been developed to target S. aureus virulence and bacterial AMR genes (Monecke and Ehrlich, 2005; Zhu et al., 2007; Spence et al., 2008) and have been used to study mastitis-associated S. aureus from Switzerland and Germany (Monecke et al., 2007). However, a number of other significant bacterial AMR genes and variants have since been characterized from Gram-positive bacteria (Weigel et al., 2003; Schmitt-Van de Leemput and Zadoks, 2007; Borbone et al., 2008).

In our study, a DNA microarray was developed to target acquired AMR genes found in a large spectrum of bacteria. The capacity of this microarray to detect AMR genes in reference strains and S. aureus isolates was investigated to get an insight into the antibiotic resistance traits of S. aureus in milk samples.

Materials and Methods

Microarray prototype preparation

We have designed, synthesized and printed 70-mer oligonucleotides on Corning Ultra GAPS slides (Corning Canada, Whitby, Ontario). Validation of >95% of the AMR probes was done using a collection of reference and well-characterized antimicrobial resistant strains and by comparison with other molecular techniques such as membrane hybridization and PCR. The oligonucleotides were designed based on bibliographic searches on AMR gene sequences as well as published PCR primers that were lengthened to 70 bases and which specificity to their target genes was tested through BLAST searches using Oligopicker 2.5.2 software (Maynard et al., 2003, 2004; Bruant et al., 2006). The 182 selected probes corresponded to 166 AMR genes and their variants found in various Gram-positive and Gram-negative strains. Table S1 lists the 66 AMR genes of Gram-positive bacteria and includes the control strains used for the microarray validation. A complete array consisted of four subarrays, in which each oligonucleotide was printed in triplicate, as previously described (Bekal et al., 2003), thus providing three technical replications per hybridized sample. Positive and negative controls, as well as printing buffer spots were added in each subarray (see Table S1 in the supplemental material). Three complete independent arrays were printed on the same slide to minimize variations resulting from fluctuations in external parameters.

Sample collection and identification

A collection of 418 S. aureus strains were isolated from milk samples taken from dairy cows in the province of Quebec during 2003–2004. The main criterion behind isolate selection was to reflect a representative coverage of farmland in the province. Specific S. aureus identification was done by a tube coagulase test (Becton-Dickinson, Sparks MD, USA) and by colony hybridization with a probe designed against the nuc gene specific to S. aureus (Baron et al., 2004).

Phenotypic AMR detection

Minimal inhibitory concentrations (MICs) were determined by microdilution using the Veterinary Bovine Sensitivity MIC plate (Trek diagnostic systems ltd, West Sussex, England), following the CLSI guidelines (formerly NCCLS, 1999, 2004). For the 418 strains of S. aureus a set of seven antimicrobials were tested individually: gentamicin, penicillin G, tetracycline, erythromycin, lincomycin, enrofloxacin, oxacillin. Penicillin G / Novobiocin and trimethoprim / sulfamethoxazole combinations were also tested. All the previous antimicrobials and combinations are used routinely on dairy cattle in Quebec, except enrofloxacin and gentamicin, which are banned and were tested only to detect possible extra-label use. Only 81 of these strains were also tested for susceptibility to...
pirlimycin by microdilution, though 415 strains were tested by Kirby-Bauer with a two ug pirlimycin disk (Pfizer, Kirkland, Canada). The CLSI thresholds were used to determine resistance of the strains to a given antimicrobial agent.

Bacterial antimicrobial resistance genes identification

Thirty-eight AMR-positive strains were tested by PCR for the presence of norA, linA, fexA, and ermC genes and validated with control strains, as well as appropriate amplification size, listed in supplemental data Table S2. Thirty-five cycles of PCR with Taq DNA polymerase (Roche Diagnostic, Indianapolis, USA) were performed on one µL of lysate with a 55°C annealing temperature for all genes and a final elongation time of five min on a Techne TC-512 thermostyler. Amplicons were visualized on a 1.5% agarose gel by ethidium bromide staining after one hour electrophoresis at five V/cm.

Hybridization experiments on the AMR microarray were performed as described previously (Bruant et al., 2006).

Briefly, bacterial DNA isolated from lysed single colonies of the 38 AMR-positive strains was labeled with Cy5-dCTP (GE Healthcare, Little Chalfont UK). Hybridizations were performed overnight, at 50°C, with one ug of labelled DNA. After washing, the slides were scanned and the hybridization results analysed using a Scanarray fluorescent scanner (Perkin-Elmer, Fremont CA, USA) and the Scanarray Express software program version 1.1.

Technical and replicates were included in the validation process.

Results

Among the 418 S. aureus isolates, 380 (90.9%) did not display any antimicrobial resistance whereas 38 (9.1%) were resistant to at least one antimicrobial (Table 1). The 38 AMR-positive isolates were found to be resistant to penicillin (68%), lincomycin (32%), tetracyclines (11%) and erythromycin (5%). Most AMR-positive isolates were resistant to a single drug (82%) while few strains showed multiple resistance to penicillin G and tetracycline (8%), penicillin G and lincomycin (5%), and to penicillin G and erythromycin (3%).

The reproducibility of the microarray was tested by performing two biological replicates (six technical replicates) which consistently produced identical results for both samples hybridized at two different times (data not shown). Probes generating false positives were redesigned and retested. The AMR genes detected by microarray hybridization with Cy5-labeled total DNA extracts are listed in Table 1. All probes giving a positive signal on the microarray were validated with hybridization controls except the linA probe. In that case, strain 178, positive for linA on the microarray, also demonstrated a high MIC for lincomamine and a subsequent PCR further confirmed the presence of the gene. In general, the microarray AMR genotyping correlated with the phenotype of the strain. Most strains were norA positive (34/38) whose presence was confirmed by PCR. No mexitilin resistance genes were found. Two strains were positive for tet genes (one tetM, the other tetK) while 22 penicillin-resistant strains were blaZ positive. Although many strains (14) were phenotypically resistant to lincomycin, only one strain was linA positive and two were ermB positive. Only three isolates carried additional AMR genes different than norA. In addition to norA, two strains carried blaZ and tet genes and another strain carried the ermA, and anti(9)-Ia (aadA9) genes.

Table 1. Comparison of the genotypes detected by microarray and PCR with phenotypes obtained by antimicrobial susceptibility testing

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Genotype</th>
<th>Phenotype (MIC ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14, 90, 99, 111</td>
<td>norA, blaZ</td>
<td>pen(4)</td>
</tr>
<tr>
<td>32</td>
<td>norA, blaZ</td>
<td>pen(64), linco(8), pirl(4)</td>
</tr>
<tr>
<td>33</td>
<td>blaZ</td>
<td>pen(64), linco(8), pirl(4)</td>
</tr>
<tr>
<td>46, 373, 422</td>
<td>norA, blaZ</td>
<td>pen(2)</td>
</tr>
<tr>
<td>55</td>
<td>norA</td>
<td>teta(64)</td>
</tr>
<tr>
<td>62</td>
<td>norA, blaZ</td>
<td>pen(1)</td>
</tr>
<tr>
<td>64, 250</td>
<td>norA, blaZ</td>
<td>pen(8)</td>
</tr>
<tr>
<td>70, 132, 252, 288</td>
<td>norA</td>
<td>linco(4)</td>
</tr>
<tr>
<td>96</td>
<td>norA</td>
<td>pen(8)</td>
</tr>
<tr>
<td>172, 257, 457</td>
<td>blaZ</td>
<td>pen(16)</td>
</tr>
<tr>
<td>178</td>
<td>norA, linA</td>
<td>linco(64), pirl(4)</td>
</tr>
<tr>
<td>180</td>
<td>norA, ermC</td>
<td>pen(32), eryth(64)</td>
</tr>
<tr>
<td>265, 304</td>
<td>norA</td>
<td>linco(8)</td>
</tr>
<tr>
<td>287</td>
<td>norA</td>
<td>pen(0,5)</td>
</tr>
<tr>
<td>318</td>
<td>norA, anti(9)-Ia(aadA9), ermA</td>
<td>eryth(64)</td>
</tr>
<tr>
<td>374</td>
<td>norA, blaZ</td>
<td>pen(64)</td>
</tr>
<tr>
<td>240</td>
<td>norA, blaZ</td>
<td>pen(0,5)</td>
</tr>
<tr>
<td>291</td>
<td>norA, blaZ, tet(M)</td>
<td>pen(8), teta(32)</td>
</tr>
<tr>
<td>382, 450</td>
<td>norA, blaZ</td>
<td>pen(32)</td>
</tr>
<tr>
<td>395</td>
<td>norA, blaZ, tet(K)</td>
<td>pen(0,5), teta(64)</td>
</tr>
<tr>
<td>400</td>
<td>norA</td>
<td>pen(64), teta(64)</td>
</tr>
<tr>
<td>412</td>
<td>norA</td>
<td>linco(32)</td>
</tr>
<tr>
<td>441, 452</td>
<td>norA, ermB</td>
<td>linco(64)</td>
</tr>
<tr>
<td>456</td>
<td>norA, blaZ</td>
<td>pen(16)</td>
</tr>
</tbody>
</table>

Microarray positive antimicrobial genes are indicated for a given strain. The microarray probes list used in this study are found in Table S1. The antimicrobial agents used in the MIC test are abbreviated as follows: pen = penicillin, linco = lincomycin, pirl = pirlimycin, eryth = erythromycin, teta = tetracycline. Only the agents above the CLSI resistance thresholds are shown for a given strain. The following agents were tested by PCR: norA, ermC, linA, fexA. All strains positive for norA by the microarray assay were confirmed by PCR. Strain 318 and strain 178 were positive by PCR for ermC and linA, respectively.

All the microarray probes and PCR primers that tested positive in this study were also positive for the corresponding control strain.
The relationship between genotypic antibiotic resistance, as determined by microarray analysis, and resistance phenotype is shown in Table 2. Microarray genotyping demonstrated a correlation between penicillin and erythromycin resistance conferring genes (85% for blaZ and 100% for ermA and/or ermC) and the resistance phenotype. The microarray proved somewhat less sensitive than MIC for lincosamide resistance mechanisms and tetracycline resistance genes. In the former case, only 25% of the lincomycin or pirlimycin-resistant strains showed positive probes for linA and ermA, and in the latter case, the genetic trait conferring resistance to two of the four Tet-resistant strains was not identified by hybridization. Interestingly, all sensitivities to the four antimicrobial families were confirmed by the absence of a given AMR gene.

### Discussion

A prototype microarray consisting of 70-mer oligonucleotide probes was designed for the specific and simultaneous detection of Gram-negative and positive bacterial antimicrobial resistance genes. To further evaluate the microarray technique, we used AMR-positive *S. aureus* isolates for comparison. The reproducibility of the microarray was excellent and the microarray hybridizations with the control strains confirmed the specificity of the microarray probes for their target gene. This technology is simple, specific and high-throughput allowing the specific identification of multiple AMR genes in a single reaction. In our collection of 418 *S. aureus* isolates, the majority were susceptible to antimicrobials with no methicillin resistant strains being found. All resistant strains possessed at least one AMR gene.

Most AMR-strains, phenotypically resistant to penicillin also carried the gene blaZ, encoding for a beta-lactamase, a correlation commonly seen in *S. aureus* (Martineau et al., 2000). Most strains were found to be norA-positive, which is expected from *S. aureus* isolates since the gene is endogenous to this species and encodes an efflux pump that confers resistance to norfloxacine but not to enrofloxacine, an antibiotic banned for dairy cattle usage within Quebec.

Twelve strains were lincosamide resistant and of these, only three hybridized with the lincosamide resistance genes, linA or ermA. Interestingly, most of the isolates negative for lincomycin resistance gene determinants have lower MICs than those with a *lin* or an *erm* gene. The apparent low sensitivity of the microarray to detect lincomamine resistance genes could be explained by the variability found among the bacterial resistance mechanisms to this antimicrobial family. Specific resistance to the lincosamides is the result of modification and inactivation by a lincosamide nucleotideyltransferase encoded by members of the *lin* (also called *lmu*) gene family. Cross-resistance to macrolide-lincosamide-streptogramin B (MLSβ) antibiotics most commonly involves N6 dimethylation of the A2058 residue of 23S rRNA and is catalysed by an *erm*-encoded rRNA methyltransferase (Bozdogan et al., 1999; Lina et al., 1999). Although the *linA* and *linB* genes and the *ermA*, *ermC* and *ermF* genes are represented on the microarray, mutated ribosomal targets of lincosamides that also confer resistance to lincomamides are not represented. In addition, a study on *Staphylococcus* isolates reported lincosamide resistance in strains while lacking any of the known lincomamide resistance genes (Novotna et al., 2005). These results suggest that other mechanisms of resistance remain to be elucidated. Even though our microarray probe collection of resistance-conferring genes is comprehensive, as microbial resistance rapidly evolves it is possible to miss some corresponding antimicrobial resistance genes in resistant strains (such as the strains showing tetracycline resistance) as these variants have not yet been characterized and consequently, are not yet available in public databases. It is also possible some variants are not detected because of thermodynamic constraints due to a given probe composition and localized mismatches although the length (70-mer) of the designed probes should reduce this to a minimum.
The presence of more than one antimicrobial resistance determinant (other than \textit{norA}) appears to be a rather rare occurrence in our collection of bovine \textit{S. aureus} strains. Among resistant isolates, 22 carried the beta-lactamase gene (\textit{blaZ}), while seven carried either macrolide/clindamycin genes (\textit{ermA} or \textit{ermB} or \textit{ermC}), tetracycline resistance genes (\textit{tetK} and \textit{tetM}) or a lincomycin (\textit{linA}) gene. The low prevalence of the MLS\textsubscript{B}-associated genes \textit{ermA}, \textit{ermC}, \textit{linA}, \textit{msrA} observed is similar to another study by Ochoa-Zarzosa et al. (2008), though a higher prevalence for \textit{ermC} and \textit{ermB} genes was reported in another study in China (Wang et al., 2008). The AMR results are consistent with the current usage of antimicrobials for treatment of mastitis in dairy cattle in Québec. These antimicrobials are tetracycline, erythromycin, lincomycin, oxacillin, penicillin \textit{G}/novobiocin and trimethoprim/sulfamethoxazole. Tetracycline and trimethoprim/sulfamethoxazole are administered systemically and the others are found in intramammary treatment veterinary products. However, this study shows no apparent direct correlation between the route of administration of a given antibiotic and the prevalence of resistance or associated AMR gene. In conclusion, the AMR microarray has the advantage of rapidly screening bacteria for the presence of known anti-biotic resistance genes in bacteria. As the presence of methicillin resistant \textit{S. aureus} in farm animals such as pigs is an important concern to the industry (de Neeling et al., 2007), the microarray should be useful for rapidly generating comprehensive information on the AMR gene content of bacteria.

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