Development of a Microarray system for the Rapid and Simultaneous Detection of Bacterial and Viral Foodborne Pathogens

Gebreyes, W.A. *(1), Thakur, S. *(2), Zhao S *(2), McDermott, P. *(2), White, D.G. *(2), Harbottle, H. *(2)

(1) Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, 1920 Coffey Road, 43210, Columbus, Ohio, USA
(2) Division of Animal and Food Microbiology, Office of Research, Center for Veterinary Medicine, U. S. Food and Drug Administration, 20708, Laurel, Maryland, USA

* corresponding author: gebreyes.1@osu.edu

Abstract:
Foodborne diseases are increasingly recognized as a significant global public health problem despite major advances and improvements in the quality of food, water, sanitation and hygiene. However, detection and characterization of foodborne pathogens during outbreak scenarios remains a laborious and time-consuming task. The aim of this work was to develop an oligonucleotide microarray for rapid detection and characterization of the most important infectious bacterial (Campylobacter, Salmonella and Yersinia) and viral (Noroviruses) pathogens found in swine and associated pork products. A total of 272 target regions and genes were identified that were specific for pathogen identification and characterization of specific antimicrobial resistance and virulence determinants. We designed multiple probes (up to three) per gene to increase the sensitivity and specificity of the microarray. After BLAST analysis, a total of 562 probes were finally selected to be printed on to glass slides. Appropriate control strains that were previously characterized in our laboratories by PCR were selected to test the developed arrays. Preliminary results indicated that the designed probes were highly specific and sensitive for identification of tested pathogens and known resistance and virulence genes present in the selected control strains.

Introduction:
Salmonella enterica serovars and Campylobacter spp. are two of the leading causes of bacterial foodborne illnesses in the United States (MMWR, 2006). It is estimated that the annual economic costs due to foodborne bacterial infections is $6.9 billion nationwide. Non-typhoidal Salmonella serovars are also important reservoirs for antimicrobial resistance factors. Emergence and spread of multi-drug resistance (MDR) in Salmonella has become a major concern among public health officials and the general public worldwide. Yersinia enterocolitica is another important foodborne bacterial pathogen, which causes an estimated 96,000 humans to become ill every year in the U.S. and has been listed as one of the top six priority foodborne pathogens. Pigs have been shown to be the primary reservoir for this pathogen and over 28% of the herds have been shown to carry the pathogenic species of Y. enterocolitica (Funk et al., 1998). With regards to viral pathogens, noroviruses (NoV) cause an estimated 23 million cases of acute, epidemic gastroenteritis in the U.S. annually.

Current standard protocols for the isolation and detection of these pathogens are laborious and have very low sensitivity. Further characterization to the species or strain level is also time consuming and costly. Therefore, development of a very sensitive, time-efficient, low cost simultaneous method that also enables detection of multiple antimicrobial resistance genes and other important determinants (e.g. virulence mechanisms) is essential. We proposed to achieve this by developing a multi-pathogen microarray system. This proposed research will benefit the swine industry by allowing the rapid and sensitive characterization of important foodborne bacterial and viral pathogenic strains in a short period of time thereby enabling development of efficient monitoring and tracking systems.
Materials and Methods:
After an exhaustive review of the available scientific literature, we selected a total of 272 genes/regions that were suitable for the purpose of identifying Salmonella spp. and characterizing known antimicrobial resistance genes and virulence determinants. In addition, we also selected six regions specific to the important Salmonella phage types of animal and human health significance. Probes were designed using the Allele ID software (Version 4.0, Premier Biosoft International, CA). We designed up to three probes per gene. A total of 562 probes were finally selected after BLAST analysis to be printed on to glass slides. The average length of the probes selected under this method was approximately 68 base pairs and a melting temperature of around 73°C. The spots were printed using the Omnigrid Accent printer (Genomic Solutions, MI). We designed a 2x2 array with two sub arrays within an array. Therefore, every probe was spotted eight times on the glass slide. The two arrays were at a distance of 400 nanometers both horizontally and vertically. We selected two Salmonella and two Campylobacter control strains for testing and standardization of the microarray experiment as shown below. The Salmonella isolates were tested for the presence of β-lactamase genes (bla) using a Polymerase Chain Reaction (PCR) previously (Gebreyes and Altier, 2002).

Control Strains used include:
- S. Typhimurium DT 193 (UT 30) - S. Typhimurium DT 104 (UT 8)
- AKSSuT antimicrobial resistance profile - ACSSuT antimicrobial resistance profile
- bla_{TEM-1} positive - bla_{TEM-1} negative
- bla_{PSE-1} negative - bla_{PSE-1} positive
- Campylobacter jejuni (ID: 17858) and Campylobacter coli (ID: 11129)

Antimicrobial abbreviations: A: Ampicillin; C: Chloramphenicol; K: Kanamycin; S: Streptomycin; Su: Sulfamethoxazole and T: Tetracycline.

DNA from the control strains was purified using the Purelink Genomic DNA Isolation kit (Invitrogen Corporation, CA) following manufacturer's instructions. The purified DNA was then labeled and purified with either Alexafluor dye 535 or 647 using the Bioprime comparative genomic hybridization kit following the manufacturer's instructions (Invitrogen Corporation, CA). Hybridization was done using the Pronto universal microarray system (Corning Incorporated, MI). Images were captured under the appropriate fluorescence wavelength in the Gene Pix Pro array machine (Molecular Devices Corporation, MA). Preliminary analysis of the results was done using the Excel program available in Microsoft. For analysis, we used the fluorescence reading at 535 wavelength minus the background at the same wavelength. All the values greater than 500 were considered as positive and value less than that was considered as negative.

Results:
Hybridization of the labeled DNA with the immobilized probe on the glass surface is influenced by different experimental parameters of which the hybridization temperature is the most important. Different hybridization temperatures were tested including 42°C, 55°C and 65°C to determine the ideal complementary strand binding conditions. After multiple experiments, we decided to use 42°C as the hybridization temperature since the results observed at this temperature were ideal after analysis. Slides were baked at 80°C for 30 minutes prior to cross linking at 600 MJ. Preliminary testing was conducted using the Salmonella and Campylobacter control strains. The initial focus was on the pathogen identification probes. We observed specific signal intensity with values greater than 500 generated by the binding of the labeled target with specific Salmonella and Campylobacter identification probes as shown in Figure 1. No cross hybridization signals were observed between the probe and non specific target DNA indicating the high sensitivity and specificity of the identification probes. We observed specific signal intensity with values greater than 500 generated by the binding of the labeled target with specific Salmonella and Campylobacter identification probes. Microarray analysis also identified multiple identification
genes, antimicrobial resistance genes, integrons, plasmid sequences and multidrug resistant efflux pumps in the tested control strains which were further corroborated by PCR testing.

Salmonella identification probes               Campylobacter identification probes

Figure 1. Hybridization results showing the bright green pathogen identification probes after binding with the AlexaFluor 535 labeled target DNA.

Preliminary data for characterizing select antimicrobial resistance genes using the control Salmonella strains was very encouraging. The β-lactamases genes coding for resistance against β-Lactam antimicrobials, including ampicillin, had been characterized by PCR previously in our laboratory. Microarray analysis of the scanned image corroborated the PCR results as shown in figures 2 and 3 for phage type S. Typhimurium DT 104 and DT 193, respectively. The Y-axis shows fluorescence on the graphs.

In addition to the pathogen identification and β-lactamase genes that are highlighted on the graph, additional identification genes, antimicrobial resistance genes, integrons, plasmid sequences and multidrug resistant efflux pumps were identified in the tested control strains. The list of genes/target regions that have been shown to be present are shown in Table 1. Further testing for detection of Noroviruses and Yersinia species as well as additional virulence and antimicrobial resistance genes is currently underway.
Table 1. Pathogen identification & Antimicrobial resistance genes for Campylobacter and Salmonella

<table>
<thead>
<tr>
<th>Target Gene/Region</th>
<th>Fluorescence 535-Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter calcoaceticus class A beta-lactamase blaCARB-5 (blaCARB-5) gene, complete cds. (Negative Control)</td>
<td>36.875</td>
</tr>
<tr>
<td>Campylobacter coli GlyA (glyA) gene, partial cds.</td>
<td>1013</td>
</tr>
<tr>
<td>Campylobacter jejuni GlyA (glyA) gene, partial cds.</td>
<td>1900.167</td>
</tr>
<tr>
<td>Salmonella Typhimurium LT2, section 138 of 220 genome</td>
<td>960.75</td>
</tr>
<tr>
<td>Salmonella Typhimurium LT2, section 182 of 220 genome</td>
<td>1390.25</td>
</tr>
<tr>
<td>Salmonella Typhimurium LT2, section 91 of 220 genome</td>
<td>1555.125</td>
</tr>
<tr>
<td>Campylobacter jejuni strain 81-176 cmeR (cmeR), cmeABC</td>
<td>600.5</td>
</tr>
<tr>
<td>Campylobacter jejuni plasmid pCjA13 tetracycline resistance</td>
<td>1233.083</td>
</tr>
<tr>
<td>Citrobacter amalonaticus β-lactamase CTX-M-8 (blaCTX-M-8)</td>
<td>942.625</td>
</tr>
</tbody>
</table>

Discussion:

The preliminary findings from this study have shown that the developed oligonucleotide microarray can both identify a wide range of bacterial pathogens and specific antimicrobial resistant and virulence genes present. DNA microarrays or gene chips represent the latest advance in molecular technology and offers a fast, high-throughput alternative for the parallel detection of hundreds to thousands of genes of interest simultaneously. The application potential spreads across most sectors of life sciences, including environmental microbiology and microbial ecology; human, veterinary, food and plant diagnostic, water quality control, and industrial microbiology (Bodrossy and Sessitsch, 2004). Samples were labeled with dyes that fluoresced on binding to the appropriate probe thereby generating a signal that was captured by the imager and recorded. This way, we were able to identify test probes that were not only specific for identifying different bacterial foodborne pathogens but also antimicrobial resistance and virulence genes present. In our study, we generated 562 probes from 272 target genes/regions. The preliminary data is very encouraging and we were able to differentiate between different pathogens on the basis of the target identification probes that gave us specific signal based on the pathogen tested. Microarray technology is being increasingly used in numerous scientific disciplines including drug discovery and evaluation, cancer research, bacterial pathogenesis, antimicrobial resistance, as well as genomic "fingerprinting" and detecting genetic polymorphisms of microorganisms (Call et al., 2003; Chizikov et al., 2001; Hu et al., 2002; Kato-Maeda et al., 2001).

Sensitivity and specificity of the probes are important criteria that need to be addressed whenever a new microarray chip is developed. For this purpose, we specifically selected multiple probes and then spotted the same probes multiple times (up to eight) on the glass slide in a systematic manner. The fluorescence signal strength for the probes was uniform across the slide. To further confirm the microarray results, we are in the process of designing appropriate PCR primers. For the next step we will amplify all the genes by PCR that were shown to be positive by the microarray. We are currently also comparing the results from the two different microarray methods employed in this study. This high throughput oligonucleotide microarray designed in this study will benefit the swine industry as well as interested researchers in successfully identifying and characterizing common pathogens found in swine on farm, at slaughter and retail in an efficient, cheap and quick manner.

Conclusions:

The development of rapid and accurate detection methods for pathogen detection are needed as current culture based methods are time consuming and costly. The preliminary findings of this study have shown that the oligonucleotide microarray developed can identify a wide range of bacterial pathogens as well as specific antimicrobial resistance and virulence genes simultaneously. The microarray system developed in this study enables identification of specific
strains in less than two days starting from the sample collection to the final analysis. This will enable us to monitor the presence of foodborne pathogens as well as associated antimicrobial resistance and virulence genes in animals, foods, humans and the environment.

References:
enterocolitica in groups of swine at slaughter. J Food Prot. 61(6) 677-682