RESEARCH REPORT
PORK SAFETY

Title: Development of a Microarray for the Rapid and Simultaneous Detection and Tracking of Bacterial and Viral Foodborne Pathogens (**NPB-05-072).**

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Abstract:

Foodborne diseases are increasingly recognized as a significant global public health problem despite major advances and improvements in hygiene, the quality of food, water and sanitation. Detection and characterization of the pathogens during outbreak scenarios usually takes a lot of time. The main aim of the proposed research program was to develop oligonucleotide based microarray for rapid, sensitive and definitive detection, diagnosis and characterization of the most important infectious bacterial (*Campylobacter*, *Salmonella* and *Yersinia*) and viral (Noroviruses) pathogens found in swine and pork. We also compared two different microarray techniques based on probe design, assembly and probe conformation on the slide. The second aspect of this project involved development of an efficient method to concentrate and purify low levels of these pathogens from fecal and environmental samples in the pork production environment.

A total of 272 target regions and genes were identified that were specific for pathogen identification and characterization of the 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 of the probes, 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 new arrays. Preliminary results indicated that the probes designed were highly specific and sensitive for identification of the pathogen and the known resistance and virulence genes present in the selected control strains. Both the microarray methods employed gave results that attested the sensitivity and the specificity of the probes selected.

Introduction:

In the US, known pathogens including bacteria, viruses, fungi and parasites cause an estimated 14 million illnesses, 60,000 hospitalizations and 1,800 deaths. Among bacterial pathogens, *Salmonella enterica* serovars and *Campylobacter spp* of are two of the leading causes of foodborne illnesses in the United States. Nationwide, it is estimated that the annual economic costs due to foodborne bacterial infections is $6.9 billion. 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. Another important foodborne bacterial pathogen, *Yersinia enterocolitica*, 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 to cause human infections and over 28% of the herds have been shown to carry the pathogenic species of *Y. enterocolitica.* Noroviruses (NoV) cause an estimated 23 million cases of acute, epidemic gastroenteritis in the U.S. annually. Recently, viruses genetically related to Noroviruses in humans have been detected in fecal samples from swine in raising public health concerns for potential cross-species transmission to humans from a swine reservoir.

Swine and pork are important reservoirs of both bacterial (pathogens *Salmonella, Campylobacter* and *Yersinia*) and potentially viral (Noroviruses) foodborne pathogens and have been implicated in transmitting the pathogens to humans. Current standard protocols for the isolation and detection procedures of these pathogens are laborious and have very low sensitivity. In addition, characterizing to specific strain level is time consuming and very costly. Therefore, development of a very sensitive, low detection limit, time-efficient, low cost simultaneous detection method that also enables monitoring of presence of multiple antimicrobial resistance genes and other important determinants is essential. We proposed to achieve this by developing a multipathogen microarray system. This research proposed 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.

Objectives:

The main aim of the proposed research program is to develop new and improved diagnostic methods for rapid, sensitive and definitive detection, diagnosis and characterization of the most important infectious bacterial and viral pathogens found in swine and pork. The specific objectives are: 1) Identification of primers/probes targeting specific gene alleles for designing a microarray for the detection and characterization of specific foodborne pathogens in swine, 2) Development and evaluation of methods for concentrating pathogenic strains and nucleic acid materials for sensitive, specific and high resolution detection level and 3) Development of oligonucleotide microarray system for the simultaneous identification and characterization of multiple bacterial and viral foodborne pathogens important to the pork industry.

Materials and Methods:

The first step was the selection of suitable target genes and regions in the genome of the pathogens selected in this study. After an exhaustive review of available literature, we selected a total of 272 genes/regions that were suitable for the purpose of identifying the pathogen and characterizing the antimicrobial resistance and virulence determinants in the pathogen. In addition, we also selected six regions specific to the important phage types of *Salmonella* genus. The total number of genes selected specific for the different pathogens are shown in Table 1. Probes were designed using the Allele ID software (Version 4.0, Premier Biosoft International, CA). We designed up to three probes per/gene. The use of multiple genes arrayed in triplicates was done to enhance the sensitivity and evaluate the reproducibility of the microarray and to avoid non-specific results generated due to the genetic variability and single nucleotide polymorphisms.

A total of 562 probes were finally selected after BLAST analysis to be printed on to glass slides. The probes were spotted on the glass slides using two different methods. In the first methods, the probes were

spotted on the glass slides in a horizontal conformation. The average length of the probes selected under this method was approximately 68 base pairs and a melting temperature of around 73^{o}C. Under the second method, the probes were synthesized on the slide itself during printing (*de novo* synthesis) and were attached vertically to the glass surface. The average length of the probe here was approximately 25 base pairs. This method of de novo synthesis of probes during printing has the added advantages of using the same slide multiple times after stripping the target from the slide. In addition, upto seven samples can be tested at one time thereby saving a lot of time and money.

For the horizontal probe layout microarray, 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 extended spectrum beta lactamases (ESBLs) enzymes using Polymerase Chain Reaction (PCR).

Control Strains used include:

- *S.* Typhimurium DT 193 (UT 30)
  - AKSSuT antimicrobial resistance profile
  - *bla*$_{TEM-1}$ positive
  - *bla* $_{PSE-1}$ negative

- *S.* Typhimurium DT 104 (UT 8)
  - ACSSuT antimicrobial resistance profile
  - *bla*$_{TEM-1}$ negative
  - *bla* $_{PSE-1}$ positive

- *Campylobacter jejuni* (ID: 17858)
- *Campylobacter coli* (ID: 11129)

The abbreviations used for the different antimicrobials are as follows:
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 the 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.

Pathogen	Target Gene/ Sequence			
I) *Salmonella*	**Identification**	**Virulence**	**Antimicrobial Resistance**	**Phage Type**
S. enterica, *S.* Typhimurium, *S.* Enteritidis	4	*23*	179	6
II) *Campylobacter*	7	*10*	*30*	
C. jejuni, C. coli, C. upsaliensis and C. lari				
III) *Yersinia*	4	*3*	*4*	
Y. enterocolitica, Y. pseudotuberculosis				
IV) Noroviruses	2			

Table1. Table showing the number of target genes selected for designing the probes

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 hybridizations temperatures were tested including 42^0C, 55^0C and 65^0C to determine the ideal complementary strand binding conditions. After multiple testing, we decided to use 42^0C as the hybridization temperature since the results observed at this temperature were ideal after analysis. Preliminary testing has been done 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. There was no cross hybridization between the probe and non specific target DNA indicating the high sensitivity and specificity of the identification probes.

Preliminary data for characterizing the antimicrobial resistance genes using the control *Salmonella* strains was very encouraging. The ESBLs genes coding for resistance against beta Lactams including ampicillin had been characterized by PCR previously in our lab using PCR. The PCR results have been shown in the control strains used section previously. Microarray analysis of the scanned image corroborated the PCRfor phage type DT 104 and DT 193, respectively.

In addition to the pathogen identification and ESBLs that are highlighted on the graph, there were multiple other identification genes, antimicrobial resistance genes, integrons, plasmid sequences and multidrug resistant efflux pumps that were identified in the control strains tested. The list of genes/target regions that have been shown to be present by the microarray result are shown in Table 2 (pathogen identification) and 3 (antimicrobial resistance/putative target genes). Further testing for identifying Noroviruses and *Yersinia* species and characterizing the virulence and antimicrobial resistance genes is currently being carried out in the lab. We will start working on developing methods for pathogen concentration and DNA purification from samples soon.

Target Gene/Region	Fluorescence at 535-Background
Campylobacter coli cdtA, cdtB, cdtC genes for cytolethal toxin	1207.667
Campylobacter coli GlyA (glyA) gene, partial cds.	1013
Campylobacter jejuni GlyA (glyA) gene, partial cds.	1900.167
Salmonella typhimurium LT2, section 138 of 220 genome	960.75
Salmonella typhimurium LT2, section 182 of 220 genome	1390.25
Salmonella typhimurium LT2, section 91 of 220 genome	1555.125
Salmonella typhimurium LT2, section 98 of 220 of genome	1862.5
Salmonella typhimurium partial g8 gene, strain V66/01.	1361.25
Campylobacter jejuni subsp. jejuni NCTC 11168 genome	5958.625

Table 2. Pathogen identification genes for *Campylobacter* and *Salmonella*

Target Gene/Region	Fluorescence at 535-Background
Campylobacter coli partial gyrA gene for DNA gyrase subunit A, allele 16.	1316.833
Campylobacter coli strain CIT 382 CmeR (cmeR) gene, efflux pump gene	1056.333
Campylobacter jejuni CIT134C class I integron aminoglycoside (aadA2)	675.5833
Campylobacter jejuni partial gyrA gene for DNA gyrase subunit A	3652.833
Campylobacter jejuni plasmid pCjA13 tetracycline resistance (tetO)	1233.083
Campylobacter jejuni strain 81-176 CmeR (cmeR), CmeABC gene cluster	600.5
Campylobacter jejuni TetO (tetO) gene, complete cds.	2219.75
Citrobacter amalonaticus beta-lactamase CTX-M-8 (blaCTX-M-8)	942.625
Cloning vector p34S-Tp2, complete sequence.	2053.375
Escherichia coli class I integron integrase (intI) gene, partial cds;	2560.917
Escherichia coli insertion sequence ISEcp1 disrupted tnpA gene	737.75
Escherichia coli plasmid pMSP071 class I sulI-type integron In60	1041.5
Escherichia coli plasmid pOZ174 beta-lactamase CTX-M-14	1407
Flavobacterium johnsoniae beta-lactamase JOHN-1 (blaJOHN-1)	1092.167
Klebsiella pneumoniae plasmid E3 beta-lactamase TEM-1 gene	723.875
Klebsiella pneumoniae plasmid pMG252 putative integron Qnr (qnr) gene	961.125
Pseudomonas aeruginosa integron In56 integrase (intI1)	981.25
Salmonella enterica subsp. enterica serovar Cubana transfer plasmid	619.5
Salmonella typhimurium plasmid incFI integron type 1 (In-t1)	560.6667
Serratia marcescens extended-spectrum beta-lactamase BES-1	538.9375
Shigella flexneri transposon TnSF1 putative transposase (tnpA)	4204
Artificial vector pLoxCat2.	641.75
Janthinobacterium lividum blaTHIN-B gene for THIN-B protein.	1386.5
Klebsiella pneumoniae plasmid bla-ACC-1 gene.	1036.417

Table 3. Antimicrobial resistance genes, integron sequences and efflux pumps that were Positive based on signal intensity

Discussion

The preliminary findings of this study has shown that the oligonucleotide microarray developed can identify a wide range of bacterial pathogens and help in the characterizing the antimicrobial resistance and virulence genes present. 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 and test probes that were not only specific for identifying different bacterial foodborne pathogens but also for characterizing them for their antimicrobial resistance and virulence genes.

Compared to the standard microbiological and molecular methods that can take more than a week, the microarray developed by us in this study can serve the same purpose in less than two days starting from the sample to the final analysis. Proper selection of the target regions and design of the probes is very important for developing a successful microarray. This is followed by BLAST analysis to determine the specificity of the probes to the target pathogen. 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.

Sensitivity and specificity of the probes are important criteria's that needs 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 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 researchers to successfully identify and characterize the pathogens found in swine at farm, slaughter and retail meat in an efficient, cheap and quick manner.

Lay Interpretation

We developed a microarray for the rapid detection and characterization of three important bacterial pathogens including *Campylobacter, Salmonella* and *Yersinia* and a viral pathogen including Noroviruses. Probes that were printed on the glass slide were selected after analysis and through testing using control strains. The results from this preliminary study show that we can identify and characterize the pathogen of interest in a very short period of time when compared to the standard laboratory methods used for this purpose. Current standard protocols for the isolation and detection procedures of these pathogens are laborious and have very low sensitivity. In addition, characterizing to specific strain level is time consuming and very costly. Therefore, development of this microarray that is very sensitive, having a low detection limit, time-efficient and a low cost method will enable us to monitor the presence of multiple antimicrobial resistance genes and other important determinants in animals, humans and the environment.

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