EUROSENSORS 2015

96 Well Microtitre Plate DNA Microarray for Fast Throughput of Bacteria Identification in Mastitic Milk Samples

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Abstract

Mastitis in cattle is an inflammation of the udder that can be caused by a number of bacterial pathogens. Standard testing requires a series of tests to accurately identify the cause. Mastitic milk cannot be sold and the treatment usually involves a course of antibiotics during which time the milk can also not be sold so a fast identification of the pathogen involved is essential to reduce lost revenue. A DNA microarray was designed to identify the pathogens that could be responsible for mastitis. A 6 x 6 grid of oligonucleotides was covalently immobilised (by use of a surface attached polymer network) onto the untreated surface of each well of a 96 well microtitre plate. DNA extracted from mastitic milk samples was amplified using Cy5 labelled primers and subsequently hybridised to the immobilised probes on the microarray. A standard ELISA plate washer was used to wash the microtitre plate and the signals from the bound PCR products were read in a commercially available reader (FLAIR). Using the species probes that give the strongest and weakest signals, S.Aure_35p (Staphylococcus aureus) and E.coli_448p (Escherichia coli) respectively the hybridisation signal variation from well to well was investigated. The microarray can be regenerated (removal of the bound amplification products) by a hot wash cycle and then reused. To test the stability of the probes over subsequent cycles of hybridisations and regenerations a Cy3 labelled oligonucleotide was added to all the spots printed on the array and the array was subjected to 10 rounds of mock hybridisation and regeneration cycles. After 10 cycles there was only a minor loss of signal.

Keywords:

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Peer-review under responsibility of the organizing committee of EUROSENSORS 2015
1. Main text

1.1. Mastitis

Mastitis is an inflammation of the milk producing organ in mammals, in the case of cattle this is the udder. It can be caused by a number of bacteria the most common of which are listed in Table 1. Infections of the udder lead to an increase in the number of white blood cells in the milk and this is monitored by measuring the number of somatic cells in the milk. Due to costs involved the somatic cell count is not measured in individual cows but a sample is taken from the bulk tank where the milk from the herd is pooled together. Cell counts of 100,000 cells/ml or less are considered to be uninfected and more than 250,000 cells/ml is considered to be infected. In Europe, levels of more than 400,000 cells/ml result in the milk being rejected from sale. The cows in question then have to be tested usually by swabbing a sample of milk on an agar plate and identifying the bacteria that grow. The correct antibiotic treatment can then be administered during which time the milk is also unfit for sale [2].

Table 1. Mastitis causing bacteria species [3]

<table>
<thead>
<tr>
<th>Gram Negative Bacteria</th>
<th>Gram Positive Bacteria</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherischia coli</em></td>
<td><em>Staphylococcus aureus</em></td>
<td><em>Mycoplasma bovis</em></td>
</tr>
<tr>
<td><em>Klebsiallapneumoniae</em></td>
<td><em>Streptococcus uberis</em></td>
<td><em>Mycoplasma bovigenitalium</em></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td><em>Streptococcus agalactiae</em></td>
<td><em>Mycoplasma californicum</em></td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus dysgalactiae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Trueperella</em></td>
<td></td>
</tr>
</tbody>
</table>

1.2. Microarray Detection of Bacteria

Using a microarray, the bacteria present in a mastitic milk sample can be identified within one day. Oligonucleotide probes that are unique for each individual bacteria of interest are immobilized onto a solid surface using a linear polymer with photo reactive groups (Figure 1). The bacterial DNA extracted from the mastitic milk is amplified by PCR using a Cy5 labeled forward primer. This PCR product is then put onto the microarray and if the DNA sequence is complimentary to a probe sequence it will hybridize and light up this probe.

![Figure 1. Depiction of linear polymer with photo reactive groups(red circles). After UV irradiation the photo reactive groups crosslink the polymer (filled red circles) to form a swellable but water insoluble polymer network. This groups are also used to bind the polymer to the surface and biomolecules.](image)

1.3. Microarray printing

The microarray is in the form of a 6 x 6 grid printed into each well of a 96 well microtitre plate (MTP) and is illustrated in Figure 2. A Cy3 labeled oligonucleotide was added to each of the spots for quality control purposes.
and a Cy5 labeled oligonucleotide was printed in the corners of the array to aid scanning in a commercially available reader (FLAIR).

The array was printed using a Scienion Sciflex arrayer and the polymer crosslinked at 254nm using a Stratalinker 2400. An example of the spots of the microarray are shown in Figure 3.

![Figure 3: Printed spots of the microarray](image)

1.4. Probe integrity over multiple uses.

To test the integrity of the probes over subsequent uses, the signal strength of the probes in the Cy3 channel was recorded after every hybridisation. The composition of the probes appeared to have an effect on the signal in the Cy3 channel. The values ranged from 0.0185 to 0.0023 arbitrary units. The average loss of signal over 10 hybridisation and regeneration cycles was 28%. This signal strength was still considered to be high enough to accurately detect hybridisation signals but a limit of 10 times reuse was set.

![Figure 4: Mean signal stability in Cy3 channel of immobilised oligonucleotide probes over ten hybridisations. The bars present the standard error of the mean.](image)
1.5. Well to well variation of hybridisation signals

To determine the variation of the hybridisation signals in multiple wells on the MTP, the species probes that give the strongest and the weakest signals, S.aure_35p (Staphylococcus aureus) and E.coli_448p (Escherischia coli) respectively were tested using pooled PCR product. The testing was carried out in one hybridisation run. Wells in rows 1, 3, 5, 8, 10 and 12 were tested with pooled PCR of product of E.coli (Figure 5), and wells 2, 4, 6, 7, 9 and 11 were tested with pooled PCR product of S.aureus (Figure 6). The mean of the signal of the 5 land lights is shown instead of the individual signals. Other relevant probes that also give a signal are presented. For E.coli this is the gram negative probe (Gneg_345p) and the eubacteria probe (euBac_352p2). For S.aureus the other relevant probes were the all Staphylococcus species probe (allStaph_95p), the gram positive probe (Gpos_274p), the alternative S.aureus probe (S.aure_43p) and the eubacteria probe (euBac_352p2). There is some variation in the signal strength of the probes, but this does seem to follow a pattern. A possible cause could be shadowing of some of the spots during crosslinking.

![Figure 5: Hybridisation signals for a pooled E.coli PCR on various arrays on the same microtitre plate.](image)

![Figure 6: Hybridisation signals for a pooled S.aureus PCR on various arrays on the same microtitre plate.](image)

Acknowledgements

The authors gratefully acknowledge funding from Safeguard Biosystems and technical assistance from Holger Frey and Suresh Banda.

References

