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Short communication

Helicase dependent OnChip-amplification and its use in multiplex pathogen detection

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ABSTRACT

Background: The need for fast, specific and sensitive multiparametric detection methods is an ever growing demand in molecular diagnostics. Here we report on a newly developed method, the helicase dependent OnChip amplification (OnChip-HDA). This approach integrates the analysis and detection in one single reaction thus leading to time and cost savings in multiparametric analysis.
Methods: HDA is an isothermal amplification method that is not depending on thermocycling as known from PCR due to the helicases' ability to unwind DNA double-strands. We have combined the HDA with microarray based detection, making it suitable for multiplex detection. As an example we used the OnChip HDA in single and multiplex amplifications for the detection of the two pathogens *N. gonorrhoeae* and *S. aureus* directly on surface bound primers.
Results: We have successfully shown the OnChip-HDA and applied it for single- and duplex-detection of the pathogens *N. gonorrhoeae* and *S. aureus*.
Conclusion: We have developed a new method, the OnChip-HDA for the multiplex detection of pathogens. Its simplicity in reaction setup and potential for miniaturization and multiparametric analysis is advantageous for the integration in miniaturized Lab on Chip systems, e.g. needed in point of care diagnostics.

1. Introduction

Amplification of DNA material is evident in analytical and diagnostic settings. The polymerase chain reaction set the gold standard for this task and is successfully applied in molecular diagnostics. However, one drawback of the PCR is its need for thermocycling. The need for thermocycling renders difficulties for certain environments where mobility is in demand, e.g. point of care diagnostics. Therefore alternatives have been sought since many years. One solution to avoid thermocycling is an isothermal amplification.

A promising isothermal amplification method that was recently described by Vincent et al. [1] uses the *Escherichia coli* UvrD helicase in unwinding the DNA double-strand prior to the amplification by a DNA polymerase I Klenow fragment. This mesophilic helicase dependent amplification (mHDA) was successfully carried out at 37 °C for the amplification of pathogenic genomic DNA from human blood samples. Further improvements to the HDA could be shown by An et al. [2] extending the mesophilic system to a thermophilic HDA system by using a thermostable UvrD helicase taken from *Thermoanaerobacter tengcongensis*. The possibility to perform the HDA reaction at a higher temperature (60–65 °C) improved the sensitivity and specificity. An et al. described the sensitivity of as few as 10 copies of bacterial genomic DNA. This thermophilic HDA system was further developed for diagnostic applications by Goldmeyer et al. [3,4]. Goldmeyer et al. reported that the HDA is suitable for the rapid detection of *Staphylococcus aureus* and the determination of methicillin resistence by the detection of the *mec A* gene.

The advantage of the HDA is its PCR like reaction scheme. As in the PCR the amplification is initiated by two primer oligonucleotides but instead of the denaturation step at 95 °C the HDA uses the helicase to unwind the DNA double-strand, eliminating the need for thermocycling.

Here we report a further step into the direction of the integration of amplification and detection by combining the HDA with the microarray technology. Microarrays have been proven tools for diagnostic applications [5–9]. They have great multiplex capabilities and can be utilized in point of care diagnostics, e.g. as detection area in microfluidic chips.

We have already described successful PCR [10,11], transcription [12] and RT-PCR [13] on solid surfaces where oligonucleotides were immobilized and used as templates for polymerases, transcriptases, etc. We have named this concept “active arrays”. By using the HDA we expanded the “active array” concept with an isothermal amplification method.

The HDA is carried out directly on the surface of a glass slide by immobilizing one primer on the glass substrate and leaving the

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corresponding primer in solution. With the helicase unwinding the DNA template the resulting DNA single-strand will be able to anneal at the immobilized primer and will be subsequently elongated by the DNA polymerase. By labeling the corresponding primer with a reporter, e.g. a fluorochrome like Cy5, successfully amplified targets are detected for example by laser scanning or TIRF technologies.

Here we report on the adaption of the HDA on a microarray by using two previously described assays for the detection of methicillin resistant *S. aureus* (MRSA) and *N. gonorrhoeae*. Both pathogens have been described to be successfully detected by HDA [2,4]. Our approach is, to our knowledge, the first successful application of HDA technology for single- and duplex-detection of pathogens on a microarray.

2. Materials and methods

2.1. Materials

Bacterial genomic DNA of MRSA was used with courtesy of Congen Biotechnologie GmbH (Berlin, Germany), genomic DNA of *N.gonorrhoeae* was used with courtesy of the Max Planck Institute für Infektionsbiologie (Berlin, Germany) and the Landesgesundheitsamt Baden-Württemberg, Stuttgart. For the HDA amplification the IsoAmp II tHDA Kit (Biohelix Corporation, Beverly, MA) was used. Primer sequences for amplification of the methicillin resistance gene *mec A* of *S. aureus* were used as published by Goldmeyer et al. [4]. For the piv_{Ng} gene of *N. gonorrhoeae* primer sequences were used as published by An et al. [2]. In case of the amplification of both genes in the OnChip-HDA the aforementioned primer systems were used in parallel. Reverse primers were 5′-labeled with Cy5 and forward primers were 5′-labeld with a C6-Aminolinker. All primers were obtained from Biomers.net GmbH (Ulm, Germany). Corning Epoxy coated Slides were obtained from Sigma Aldrich GmbH (Munich, Germany).

2.2. Methods

2.2.1. Spotting and coupling of primers

C6-Aminolinker labeled reverse primers were spotted with a SciFlex Arrayer (Scienion, Berlin, Germany) at a concentration of 15 µM on epoxysilane microarray slides. Additionally Cy5-labeled immobilization controls and specificity control oligonucleotides with no sequence homologies with the target templates were spotted on each array. A negative control with spotting buffer served as control for background signals that may occur due to salt deposit. The spotted glass chips were incubated overnight in a moisture chamber at 4 °C. Immediately before application the slides were blocked by overlaying the biochip surface with 100 mM gamma-amino-butyric acid (Sigma-Aldrich Chemie GmbH, Munich, Germany) for 60 min. The chips were finally rinsed with water and dried by centrifugation at 500 rpm for 5 min.

2.2.2. OnChip-HDA

OnChip-HDA reactions were carried out on a programmable hybridization station (Epigenomics AG, Berlin, Germany). Each slide is placed in a hybridization chamber which is sealed with a silicone o-ring. Each chamber is temperature adjustable and features an individually programmable active mixing system which results in an even distribution of the reaction mix over the array.

For the OnChip-HDA the hybridization station was programmed to hold a temperature of 65 °C for 120 min and a mixing program that was set to a mixing interval of back and forth pumping for 8 min followed by a 1 min hold.

The reaction mix contained 2x Annealing buffer II (Biohelix Corporation, Beverly, MA), 4 mM $MgSO_4$, 40 mM NaCl, 400 µM dNTPs, 0.2 µM Cy5-labeled reverse primer and 4 µl of IsoAmp enzyme mix in a final volume of 50 µl. In the case of the multiplex detection the reaction mix was set up with 0.2 µM Cy5-labeled reverse primer for the detection of *S.aureus* and *N.gonorhoeae*, respectively. Due to the design of the hybridization station the minimal reaction volumes are set to 150 µl, therefore the mentioned reaction mix was prepared as 3x reaction mix.

After amplification slides were washed with an automatic washing program. The program was set to wash each slide three times for 5 min at first with 2x SSC and 0.2% SDS at 30 °C followed by a wash step with 2x SSC and a final wash step with 0.2x SSC buffer at 20 °C.

2.2.3. Data analysis

The detection and data analysis of OnChip-HDA products were carried out with a laser scanner (Axon 4200A-Scanner, MDS, Sunnyvale, USA). After washing the slides Cy5-labeled products were scanned at 682 nm and the data analysis was performed with Genepix 6 software (MDS, Sunnyvale, USA). On each slide 10 subarrays were spotted in total. The array layout per subarray was set to 6×4 spots. For quantification of the fluorescence signals the contrast values of each spot signal were used. Contrasts were calculated according to the formula:

$$C = 100 \times (S - Bg) / (S + Bg)$$

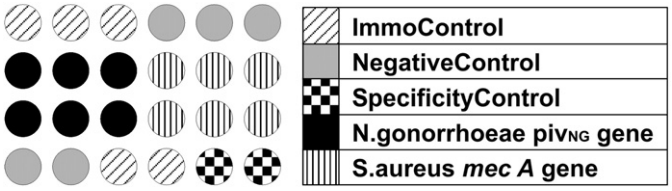

Fig. 1. Array layout for OnChip-HDA. The array layout is set to 6×4 spots per subarray. Each subarray comprises gene specific primers for *mec A* and piv_{NG} and 3 different types of controls: negative control, specificity control and immobilization control. On each slide 10 subarrays are spotted in total.

where *C* is the contrast value (given in %), *S* is the primary fluorescence signal, and *Bg* the average over the signals of the negative controls and the specificity controls. The signal is defined as the integral of the fluorescence intensity over the whole spot. The limit of detection was calculated according to the formula:

$$\mathrm{LOD} = 100 \times \left(\left(Bg + 3 \times \mathrm{SD}_{Bg}\right) - Bg\right) / \left(\left(Bg + 3 \times \mathrm{SD}_{Bg}\right) + Bg\right)$$

Bg is the average over the signals of the negative controls and the specificity controls and SD_{Bg} is the standard deviation of the background signal.

3. Results

For our OnChip-HDA experiments we took genomic DNA of the two important pathogens *S. aureus* and *N. gonorrhoeae* as an example for single and multiplex detections. The array-design (see Fig. 1) comprised a negative control, a specificity control, an immobilization control and gene specific primers for both the detection of *mec A* gene (*S. aureus*) and the piv_{NG} gene (*N. gonorrhoeae*). Forward primers were immobilized via a C6-Aminolinker on an epoxysilanized glass slide. The corresponding Cy5-labeled forward primer was in the aqueous reaction mix covering the array.

3.1. OnChip-HDA for simplex detection of mec A gene of S. aureus

The detection of the *mec A* gene was carried out with serial dilutions of genomic MRSA-DNA. The positive detection of *mec A* gene by OnChip-HDA was achieved with a minimum of 250 pg genomic MRSA-DNA which was equivalent to 5×10^4 *S. aureus* cells (Fig. 2).

OnChip-HDA slides were evaluated by scanning at 635 nm with a laser scanner at the same gain and laser power settings. Quantification of signal intensities (Fig. 2 right) verified the qualitative data (Fig. 2 left) and showed clearly the distinguishable signals at the site of *mec A* specific immobilized primers. Specificity was further confirmed by calculating a dynamic limit of detection (LOD) based on the background of a slide.

3.2. OnChip-HDA for simplex detection of piv_{NG} gene of N. gonorrhoeae

Detection of the piv_{NG} gene was carried out under the same conditions as described for the *mec A* gene. Results are shown in Fig. 3. The quantitative analysis verified the qualitative results from the laser scan. Signals both for the immobilization control and the piv_{NG} gene appeared clearly above the calculated LOD and showed no signals for negative controls or at the site of the specificity controls. Cross contamination or unspecific amplification at the site of *S. aureus* specific oligonucleotides did not occur. Positive results for detection of the piv_{NG} gene were obtained by using a minimum of 1 ng, an equivalent of $1{,}32 \times 10^5$ *N. gonorrhoeae* cells.

3.3. OnChip-HDA for multiplex detection of S. aureus and N. gonorrhoeae

After a successful amplification of single targets we further integrated detection processes by establishing parallel amplification of both the *mec A* gene of *S. aureus* and the piv_{NG} gene of *N. gonorrhoeae* by HDA directly on the chip surface. Positive results with