A Sexually Transmitted Disease (STD) DNA chip for the diagnosis of genitourinary infections

Won-young Chung, Ye Lim Jung, Ki Soo Park, Cheulhee Jung, Sung Chul Shin, Sang-Joon Hwang, Dae-Yeon Cho, Sun Ho Cha, Si Kyu Lim, Hyun Gyu Park

A R T I C L E   I N F O

Article history:
Received 15 February 2011
Received in revised form 7 April 2011
Accepted 12 April 2011
Available online 30 May 2011

Keywords:
Genitourinary infection
STD pathogen
DNA chip
Multiplex PCR

A B S T R A C T

The results of an investigation aimed at the development of a DNA chip for the detection of genitourinary infections are described. Through analysis of over 35,000 clinical cases, 14 pathogens which are most abundantly found among Koreans were selected and candidate sequences for capture probes were accordingly chosen by considering their sequences and β-globin house-keeping gene. Among this group, the most suitable capture probe sequences were selected by employing repeated chip tests in which they are immobilized on a glass chip by using a recently developed novel gold nanoparticles-based method. A multiplex PCR method was established to generate fluorescence-labeled sequences for all 14 pathogens along with the β-globin gene. By using optimized hybridization conditions, the final chip was constructed and employed to diagnose reliably both single and multiple infections in clinical human samples for 14 target pathogens. The results show that the novel chip methodology serves as a highly reliable and convenient tool for the diagnosis of Sexually Transmitted Diseases (STDs). Furthermore, this study has its great significance in that it demonstrates the entire process from statistical analysis of a large number of clinical cases to the final development of STD DNA chip just ready to be applied or commercialized in the clinical diagnostic field.

1. Introduction

Genitourinary infections are known to occur with high frequency with 78–330 million new cases diagnosed annually worldwide (Lee et al., 2007). These types of infections cause serious problems in women where they sometimes lead to premature delivery or even infertility (Lee et al., 1995). Despite this level of seriousness, most genitourinary infections in women frequently remain unrecognized and untreated because they are asymptomatic. Therefore, correct and early diagnosis of these diseases is of critical importance for timely medical treatments.

In the past, genitourinary infections were generally identified by using culturing methods. However, some of the pathogens, such as Chlamydia, are difficult to cultivate in the laboratory. Moreover, this method requires relatively long time periods and high levels of technical expertise. To overcome these limitations (McNulty et al., 2004; Stellrecht et al., 2004), the multiplex PCR method has been recently developed and widely employed for the diagnosis of these infections. Multiplex PCR consists of multiple PCR reactions on a single sample by applying a multiple set of primers, followed by gel electrophoresis analysis. This method enables simultaneous detection of multiple infections (Mahony et al., 1995; Stellrecht et al., 2004). However, since multiplex PCR relies on a gel-based post analysis several drawbacks still remain, including long assay times and the requirement for specialists to obtain correct interpretations of the results. Furthermore, tedious and complex procedures associated with gel electrophoresis make automation of multiplex PCR difficult, thus further restricting its widespread application. Therefore, a challenge exists for the development of more efficient and convenient methods for the diagnoses of genitourinary infections.

In this context, the DNA microarray method is very attractive since DNA chips are relatively cheap and easy to use in a time effective manner. Furthermore, owing to their ease of automation, DNA chip methods can be employed in real clinical environments, including hospitals that do not have clinical laboratories. Due to these advantages, a human papilloma virus (HPV) DNA chip (Biomedlab, Korea) has been developed and is currently used as the major method for the diagnosis of HPV infection. More recently, a HPV DNA chip, which can simultaneously detect 24 different HPV types, has been approved and commercialized in the European Union (EU) under the trade name PapilloCheck® (Greiner Bio-One, Germany). PapilloCheck® has been proven to be a
sensitive, reproducible and robust diagnostic tool for HPV genotyping with the potential for high throughput diagnosis in clinical settings (Dalstein et al., 2009; Jones et al., 2009). These examples for the successful commercialization of HPV DNA chip demonstrate that the DNA chip method holds promise for infectious disease diagnosis.

In a recent investigation, we have developed the first DNA chip for the effective diagnosis of genitourinary infections that cause STDs. Although several previous studies have focused on the development of DNA chips for the diagnosis of STDs, the number of pathogens that can be detected by using the chips, devised thus far, is quite small (Moon et al., 2006; Shi et al., 2005; Yoon et al., 2010). The DNA chip we have constructed in the effort described below was designed to detect 14 of the most abundant pathogens found in Koreans as determined by analysis of the statistics for over 35,000 clinical cases. Moreover, the diagnostic capability of the new DNA chip was demonstrated by its successful application to the detection of various STD infections in real clinical cases.

2. Materials and methods

2.1. Oligonucleotides

All oligonucleotides, including capture probes, primers (forward and reverse) and position markers, were synthesized and purified by GenoTech (Daejeon, Korea). Capture probes used in microarray fabrication were 35-mers and 5′ aminoethyl terminated. The reverse primers used in PCR for the preparation of target samples were 5′ Cy3-conjugated. The position marker (5′-A5-3′) used for the microarray fabrication was amine modified at the 3′ end and Cy3-labeled at the 5′ end. All nucleotides were quantitatively analyzed by measuring the absorbance at 260 nm based on the calculated molar extinction coefficient and their identities were confirmed by using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

2.2. DNA isolation from the clinical samples

All clinical samples, urine after prostatic massage or vaginal swabs, were obtained from Labgenomics Clinical Laboratories (Seoul, Korea). Thirty mL of each urine specimen was centrifuged at 14,000 × g for 2 min, washed twice with 1 mL of 1× phosphate-buffered saline (PBS; 0.2 M phosphate, 1.5 M sodium chloride [pH 7.4]) and resuspended in 400 μL of PBS for DNA extraction. Vaginal swab specimens were collected in 4 mL of PBS and 400 μL of the suspension was also used for DNA extraction. Genomic DNA was isolated from these samples using an AccuprepTM Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer’s protocol and was stored at −20°C until use. All other materials, such as stones or contaminants, which might be included in the samples were eliminated by employing these isolation procedures.

2.3. Microarray manufacturing

Six μL of 200 μM amine modified capture probes were mixed with same volume of 6.29 nM 13 nm gold nanoparticles (Jung et al., 2009) in 384-well microarray plate (Genetix, Hampshire, UK) and incubated at 30°C for 2 h. Six hundred mM sodium carbonate buffer (pH 9.5) and 1 μL dimethyl sulfoxide were added to the mixed solution. The final mixture was spotted on aldehyde-activated glass (Nuricell, Seoul, Korea) using a microspotter, VersArray ChipWriterTM Compact system (Bio–Rad Laboratories, Inc., Hercules, CA) according to the manufacturer’s instructions and then allowed to stand overnight in a humid chamber. As shown in Fig. 2(a), the duplicate spots for the two groups of 7 pathogens were immobilized in the left and right lanes, while the middle lane was used to immobilize an internal control (β-globin gene) together with a position marker (Cy3-labeled oligo A5). The glass was then cross-linked by irradiation with a UV StratalinkerTM 1800 (Strata-gene, La Jolla, CA) and washed with 0.2% SDS solution and distilled water.

2.4. Target preparation: PCR amplification

Amplifications of specific genomic regions of the 14 selected pathogens (Table 1) and the β-globin gene were first accomplished by using individual PCR reactions, carried out on a DNA Engine – Peltier thermo cycler (Bio–Rad, Hercules, CA) in a 20 μL total reaction volume containing 1 μL of DNA extracted from clinical sample, 0.25 μM of each primer, 1× PCR reaction buffer (30 mM Tris–HCl [pH 9.0], 30 mM KCl, 2 mM MgCl2, 2 mM MgCl2), 0.4 mM dNTPs (0.1 mM each), and 1.0 U i-star Taq polymerase (iNTRON Biotechnology, Gyeonggi, Korea). Two different labeling strategies were employed for target preparation. First, the target sample was labeled by using a fluorescence-labeled reverse primer during the PCR reaction. In another strategy, 0.1 mM of dATP, dGTP, dTTP, and
0.05 mM of dCTP and Cy3-dCTP were used instead of dNTP mix in order to incorporate fluorescence groups within the DNA strand.

In order to prevent primer dimer formation during multiplex PCR, the 14 pathogens and the β-globin gene were divided into three groups by considering their lengths and sequences. Multiplex PCR was carried out for each group in 15 μL total reaction volume containing 0.2 μM of each primer and 1.0 U i-star Taq polymerase under the same conditions as employed in the single PCR reactions. Genomic DNAs individually extracted from singly infected samples and properly mixed DNAs served as templates for the single and multiplex PCR reactions, respectively.

All PCRs were programmed for 5 min at 94 °C, followed by 38 cycles of 50 s at 94 °C, 45 s at 57 °C, 50 s at 72 °C, and ended with 10 min at 72 °C. After amplification, the formation of reaction products was confirmed by using agarose-gel electrophoresis.

### 2.5. Hybridization and data acquisition

The PCR products were denatured at 95 °C for 5 min and cooled on ice for 5 min. The samples were then diluted in the same volume of hybridization buffer containing 0.01% Triton X-100 and 1× SSPE (0.15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA), and allowed to stand on the microarray at 45 °C for 2–3 h. After hybridization, the array was rinsed with washing buffer containing 0.005% Triton X-100 and 6× SSPE (0.9 M NaCl, 60 mM NaH₂PO₄, 6 mM EDTA, pH 7.4) for 10 min, and then with distilled water at room temperature. The microarray was then scanned with an Axon GenePix 4000B fluorescence reader (Axon, Union City, CA). The laser power and photomultiplier tube (PMT) setting were 100 and 600, respectively.

---

**Table 1**

<table>
<thead>
<tr>
<th>Target pathogens</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Neisseria gonorrhoea (NG)</td>
<td>Escherichia coli (EC)</td>
<td></td>
</tr>
<tr>
<td>Chlamydia trachomatis (CT)</td>
<td>Klebsiella pneumoniae (KP)</td>
<td></td>
</tr>
<tr>
<td>Ureaplasma urealyticum (UU)</td>
<td>Garderella vaginalis (GV)</td>
<td></td>
</tr>
<tr>
<td>Mycoplasma hominis (MH)</td>
<td>Staphylococcus aureus (SA)</td>
<td></td>
</tr>
<tr>
<td>Trichomonas vaginalis (TV)</td>
<td>Enterococcus faecalis (EF)</td>
<td></td>
</tr>
<tr>
<td>Mycoplasma genitalium (MG)</td>
<td>Herpes simplex virus type 1 (HSV1)</td>
<td></td>
</tr>
<tr>
<td>Candida albicans (CA)</td>
<td>Herpes simplex virus type 2 (HSV2)</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2.** Microarray diagnosis of single infections. (a) Microarray fabrication format and image for verification of immobilized probes by silver enhancement. P.M.: position marker (Cy3-A5), H.K.: housekeeping gene (β-globin). (b) Fluorescence images of microarrays for the diagnosis of individual single infections for the 14 selected pathogens.
3. Results and discussion

3.1. Design and construction of microarray

Even though the main advantage of the DNA chip technology is its multiple analysis power, only a few studies have been reported in which all targets in a specific diagnostic field are covered. To fully utilize the parallel processing power associated with this methodology, we developed a DNA chip that is capable of detecting almost all the pathogens found in real clinical STD cases. For this purpose, the 14 most abundant pathogens associated with more than 99% of the genitourinary infections in Koreans were integrated into a single chip.

To construct the DNA chip, several candidate capture probes of 35 mer size were designed for all 14 of the pathogens. Two capture probes were selected in order to eliminate nonspecific bindings for each pathogen by utilizing a careful cross-check and BLAST engine (http://ncbi.nlm.nih.gov/BLAST) search. The target detecting ability of each selected capture probe was assessed by employing an actual hybridization test. This exploration uncovered some candidate probes that cause non-specific binding for nearly all of the non-target pathogens, which were then removed from the list. For some pathogens, two selected candidate probes showed almost equal responses for the corresponding target while quite different responses were obtained between two probes selected for other
pathogens. By considering both the response levels resulting from the corresponding target pathogen and nonspecific signals caused by non-target samples, we were able to identify the most suitable capture probes for the 14 target pathogens, all of which resulted in sufficiently high responses that enabled detection of all the target pathogens (Fig. S1). By employing the selected capture probes, a DNA chip consisting of three lanes (Fig. 2(a)) was constructed as described in the Materials and Methods. On the DNA chip, the internal control serves as an indicator of the proper operation of the PCR reaction and the position marker allows direct interpretation of resulting image even by using the naked eye.

Since construction of a consistent and reliable DNA chip requires precise immobilization, several efforts (Jung et al., 2008; Lim et al., 2008; Park et al., 2005) have been devoted to the development of methods to achieve high quality immobilized spots. Along this line, we have recently developed a new immobilization procedure utilizing gold nanoparticles (Jung et al., 2009), which was used to fabricate the DNA chip in this study. As a result, highly homogeneous microarrays with quite high binding capacities were fabricated. Furthermore, the integrity of the immobilized DNA probes was verified through use of the simple silver enhancement technique (Bao et al., 2005; Sun et al., 2007).

3.2. Target sample preparation

A fluorescence labeling method was employed to generate detection signals from the hybridized samples on the DNA chip. Basically, two different strategies are possible for labeling the target sample with fluorescence during the PCR reaction (Park et al., 2006). In the first, fluorescence-labeled dNTPs can be used during PCR amplification of target samples. In this case, fluorescent dyes are incorporated within the amplified strand in a random manner. On the other hand, target samples also can be labeled with fluorescence at their ends by employing fluorescence-labeled primers. Both of these labeling strategies have advantages and disadvantages arising by consideration of cost effectiveness, labeling efficiency, and fluorescence performance. In this investigation, the two methods, using Cy3-3dCTP and Cy3-labeled reverse primers, were examined and compared. Applications of fluorescence-labeled target samples, prepared by using the two methods described above, to DNA chips led to nearly identical fluorescence signal intensities (Fig. S2). However, the labeling method that employs fluorescence-labeled primers was selected for use in the DNA chip since it has the advantages of high labeling efficiency and cost effectiveness.

One of the critical issues that has to be addressed in the development of multiplexing diagnostic DNA chips is the need to minimize the number of separate PCR reactions required for target sample preparation. This results from the fact that the use of many separate PCR reactions leads to deterioration of the multiplexing performance of DNA chip and, in addition, to increasing the total cost and lengthening the time for the assay. In this effort, a multiplex PCR method was devised to amplify 14 pathogens and one housekeeping gene by employing three separate multiplex PCR reactions. This is done after the corresponding 15 sets of forward and reverse primers are divided into 3 groups based on a consideration of their lengths and sequences. This method resulted in successful ampli-
fication of all 15 target sequences in three tubes by using one PCR condition (Fig. 1).

3.3. Detection of single and multiple infections of STD pathogens

In order to verify the diagnostic capability of the new methodology, samples singly infected by 14 individual target pathogens were collected from clinical samples and tested on the DNA chip. The results show that all infections associated with the 14 individual pathogens were correctly identified through the generation of the fluorescence signals at the correct positions in the microarray without interference caused by non-specific binding or noise (Fig. 2(b)). Although real clinical samples were used in these experiments, variations in the concentrations of the samples are possible. However, these variations caused only small differences in the fluorescence signal intensities arising from the different samples. Thus, all signals were exclusively and also very clearly generated at the correct positions on the DNA chip, which is a requirement for reliable diagnosis.

Interestingly, the observation made in this effort show that genitourinary infections appear in the forms of multiple infections in many clinical cases. According to analysis of the statistics gained from diagnoses of Koreans, 41.6% of genitourinary infection patients in 2008 were simultaneously infected with two or more pathogens (Fig. 53). To demonstrate its ability to detect multiple infections, samples from the most abundant types of multiple infections were applied. As shown in Fig. 3, the DNA chip method led to the successful identification of the multiple infections by correctly generating fluorescence signals at two or more of the correct positions. The results indicate that DNA chip can be applied to detect multiple infections without causing non-specific signaling.

To assess the sensitivity of the new chip method, samples of the two selected pathogens, EF and KP, were serially diluted and subjected to analysis on the DNA chip. As shown in Fig. 4(a), the chip enables successful detection of PCR products obtained from as low as 10 copies of the initial target DNA. This sensitivity is sufficiently high to enable detection of infection caused by STD pathogens using PCR amplified samples. Finally, the specificity of the chip detection method was demonstrated by using human genomic DNA as negative control. Importantly, the chip enabled specific identification of the target pathogen without interference caused by the presence of the negative control (Fig. 4(b)).

4. Conclusions

In this study, we have developed a diagnostic chip that enables the identification of 14 major STD pathogens associated with genitourinary infections. The significance of this work is found in its demonstration of the entire process from the selection of target contents through the analysis of clinical cases to the final development of the chip, serving as a good example to realize the potential of DNA chip. Moreover, the procedures and the materials used in this methodology were selected on the basis of a full consideration of suitability and cost effectiveness and all tests of the chip were performed using real human samples. Consequently, the studies show that the developed chip can serve as a replacement for the current multiplex-PCR method for the diagnosis of genitourinary infection. Although this study is focused on Korean-specific cases, the strategy can be further extended for more universal applications because the genitourinary infections do not show much variation among the countries.

Acknowledgements

This work was supported by R&D Project in DAEDOEK INNOP-OILS (M-2007-048) and National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2009-0080602).

Appendix A. Supplementary data


References