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Original Article

Development of a chip-based multiplexed immunoassay using liposomal nanovesicles and its application in the detection of pathogens causing female lower genital tract infections

Wen-Hsiang Su [a,b,c,d,m], Tien-Yu Ho [b,c,m], Tsung-Shan Tsou [b,c], Wen-Ling Lee [e,f,g], Kuan-Chin Wang [e], Yuan-Yi Yu [h], Tien-Jui Chen [h], Chia-Hsuan Tan [i], Cheng-Deng Kuo [g,j], Chien-Sheng Chen [b,c,**], Peng-Hui Wang [g,k,l,*]

[a] *Department of Obstetrics and Gynecology, Da Chien General Hospital, Miaoli, Taiwan*
[b] *Institute of Systems Biology and Bioinformatics, National Central University, Tao-Yuan, Taiwan*
[c] *Institute of Statistics, National Central University, Tao-Yuan, Taiwan*
[d] *Hsin Sheng College of Medical Care and Management, Taiwan*
[e] *Department of Nursing, Oriental Institute of Technology, New Taipei City, Taiwan*
[f] *Department of Medicine, Cheng-Hsin General Hospital, Taipei, Taiwan*
[g] *School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[h] *Department of Laboratory Medicine, Yee-Zen Hospital, Tao-Yuan, Taiwan*
[i] *Peking University Health Science Center, Peking, China*
[j] *Laboratory of Biophysics, Department of Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan*
[k] *Department of Obstetrics and Gynecology, Taipei Veterans General Hospital, Taipei, Taiwan*
[l] *Immunology Research Center, Taipei Veterans General Hospital, Taipei, Taiwan*

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Abstract

Objective: Cervicovaginitis is a highly prevalent disease that is a burden on healthcare globally. Immediate and adequate treatment can eradicate the infection and block subsequent complications. The feasibility of achip-based multiplexed immunoassay using liposomal nanovesicles was tested.

Materials and Methods: A multiplexed immunoassay chip containing five antibodies for five pathogens (*Chlamydia trachomatis*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, and *Candida albicans*) was established and tested. Four patients with spiking of candidiasis were enrolled. The difference between positive and negative readings was evaluated using the paired Student *t* test.

Results: The detection threshold of *Candida* in this microarray was 100,000 CFU/mL in a vaginal sample, and the time required for the whole procedure was 3 hours. The testing of the four patients showed 100% for both sensitivity and specificity.

Conclusion: This microarray chip was a rapid, easy, inexpensive and sensitive tool for detecting female lower genital tract *Candida* infection in a one-time vaginal sampling process, although the data on the four other pathogens were still unavailable. A larger population study is encouraged to test the validity of this multiplexed immunoassay chip.

Keywords: cervicovaginitis; liposome; nanovesicle; multiplexed immunoassay chip

* Corresponding author. Department of Obstetrics and Gynecology, Taipei Veterans General Hospital, and National Yang-Ming University, School of Medicine, 201, Section 2, Shih-Pai Road, Taipei 112, Taiwan.

** Corresponding author. Institute of Systems Biology and Bioinformatics, and Institute of Statistics, National Central University, No.300, Jhongda Rd., Jhongli City, Tao-Yuan County 32001, Taiwan.

E-mail addresses: jasonchen7@yahoo.com.tw (C.-S. Chen), phwang@vghtpe.gov.tw, phwang@ym.edu.tw (P.-H. Wang).

[m] W.-H. Su and T.-Y. Ho contributed equally.

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Introduction

Lower genital tract infection in women may be one of the most common diseases in gynecological outpatient clinics [1]. The majority of lower genital tract infections are caused by mixed pathogens, although some dominant species might be identified. Since the infections are complicated, and immediate and accurate diagnoses are not always obtained, treatment with appropriate antibiotics is often delayed [2]. This might lead to progression into severe conditions, such as pelvic inflammatory diseases (PIDs) [3], or much moresevere situations, including infertility, ectopic pregnancy, preterm labor, and chronic pelvic pain [4]. Therefore, to avoid the occurrence of these severe sequelae, an early and accurate identification of the causal pathogens is important. However, many factors might interfere with the achievement of this goal, including clinical ignorance, insufficient education, poor communication between professionals, non-availability of a user-friendly detection tool, and the lag-time required for definite diagnosis [5].

The syndrome approach combined with microscopic examination and ordinary culture has traditionally been a gold standard in the diagnosis of female lower genital tract infection. In developing countries or medical resource-deficient societies, syndrome judgment is most frequently used for diagnosis of lower genital tract infections, although both sensitivity and specificity are low ($\sim$ 40%) [5−8]. To improve the low sensitivity and specificity of syndrome judgment, microscopic examination might be added. In fact, the Pap smear is a well-established tool not only for cervical cytology screening for cervical neoplasm, but also possibly for identification of infectious pathogens. Unfortunately, it is not recommended for the diagnosis of female lower genital tract infection by the World Health Organization due to its low detection rate [6]. Only ordinary culture is a standard procedure, and it is often used in comparison with other diagnostic tools, such as the Gram stain [9], and immunoassay or nucleic acid amplification tests (NAATs) [10], and of importance, it is also useful for identification of resistant strains of pathogens and provides a suggestion for the use of antimicrobial agents. However, culture is also affected by many factors [11], including the needs of the specialized culture media and the sometimes strict culture conditions, skilled staff and at least 72-hour culture time [12]. In fact, it is not easy to select specialized culture media and these various kinds of culture media might not be readily available, especially for first-line physicians.

In recent years, some molecular techniques have been developed to aid the diagnosis of lower genital tract infection when they are simultaneously used with syndrome, microscopic examination and culture methods, in order to provide prompt treatment and further antimicrobial susceptibility testing [13−15]. For example, the polymerase chain reaction method for the detection of chlamydia infection has been used frequently in fertility centers. NAATs have been reported to be a gold standard [13,14] in the detection of various lower genital tract infection pathogens, based on the near 95−100% specificity and 95% sensitivity of the tests [10]; of most importance, there have been relatively consistent results, even among different NAATs [13]. Although NAATs seem to be good diagnostic tools for diagnosis of lower genital tract infections, NAATs are not accepted in clinical practice because of their expense and complex techniques. In addition, highly trained staff and delicate and very expensive equipment are needed to achieve the high specificity and sensitivity. These factors hinder the wide use of NAATs in developing countries [16].

To overcome the high cost and complex procedures of the NAATs, a number of different immunoassay kits have been developed for the detection of lower genital tract infection [17]. The main advantage of immunoassays is that they are rapid, simple, and cheap, and of importance, microscopy is not required, although the sensitivity is low (50−85%) [17,18]. Immunoassays might outperform other standard tests in high-risk sexually transmitted disease groups, including sexual workers and/or those with low return rates in developing countries [18], and provide an appropriate therapeutic plan in the management of the infection [19,20], based on the possibility of a single approach to detect many potential pathogens [19−22].

The advances in immunoassay include high throughput assays and microarray chip fabrication [23,24]. The aim of this study was to explore the possibility of a single vaginal sampling to detect many pathogens at the same time. We used the technique of liposomal nanovesicles to build up a signal amplifying system, which has been reported to increase the sensitivity rate of immunoassays [25−28], and antibody microarray to detect many pathogens with a single test [24,29,30].

Materials and methods

Reagents

Reagents, including dipalmitoylphosphatidylethanolamine (DPPE), *N*-succinimidyl-S-acetylthioacetate (SATA), triethylamine (TEA), chloroform, methanol, dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylcholine (DPPC), cholesterol, lissamine rhodamine B (LRB), and sulforhodamine B (SRB) were obtained from Sigma, Northbrook, IL, USA.

Signal amplifying liposomal nanovesicle system

Liposome preparation

DPPE and SATA were first dissolved in a solvent consisting of 21 μL TEA and 3 mL chloroform. The mixture was kept at room temperature (RT) for 30 minutes. The DPPE-SATA solution was then repeatedly resolved by chloroform and dried with $N_{2(g)}$ at least twice. Finally, the 3.2 μmol ATA tagged DPPE was formed and resolved in 0.5 mL of chloroform. The other components of the liposome, including DPPG, DPPC, cholesterol, and LRB−DPPE were dissolved with organic solvent (0.75 mL chloroform and 0.25 mL methanol, and then 1.6 μmol of ATA-DPPE was added and mixed in a round flask

for reaction. A gel-like membrane was formed at the bottom of the flask after the total 40 μmol lipid solution was dried with $N_{2(g)}$. A proper volume of 60 μL 0.2 mM SRB solution was added into the flask to cover the entire gel-like membrane, and then the flask was placed in a 60°C water bath for encapsulation for about 1 hour.

After the SRB solution was encapsulated inside the lipid bilayers, the solution was extruded through the 50-nm membrane in-built extruder 30 times at 60°C to form a 50-nm liposome. In the end, free SRB was separated with the SRB-encapsulated liposome by size exclusion chromatography with a sepharose CL-4B (GE Protein analyzer reagents) resin column. SRB-encapsulated liposome was visually observed in the lower fraction since it was much larger than the free SRB.

The final 40 μmol liposome consisted of 5% DPPG, 45.7% DPPC, 45% cholesterol, 0.3% LRB-DPPE, and 4% ATA-DPPE with 0.2 mM SRB inside.

Conjugation of neutravidin to ATA-tagged liposome

There were five steps in this process, including: (1) derivatization of neutravidin using polyethylene glycol (PEG) compounds as spacer arms; (2) de-protection of the thiol group on the liposome surface; (3) the conjugation reaction; (4) the quench reaction to remove the excess sulfhydryl group; and, (5) size exclusion chromatography.

Derivatization of neutravidin

This step would eventually result in the combination of neutravidin and $SM(PEG)_{24}$ (succinimidyl-N-maleimido-) by removing the succinimidyl group of $SM(PEG)_{24}$, and revealing the other maleimide group end of $SM(PEG)_{24}$. Neutravidin (15.667 nmol) were dissolved with 1 mL of phosphate-buffered saline (PBS) along with 235 nmol (15-fold greater than the amount of neutravidin) of $SM(PEG)_{24}$ (Thermo, Rockford, IL) in a round-bottomed flask with a pH value adjusted to approximately 6–8, and on the shaker at 120 rpm for 2 hours. After the 7K, 5 mL of zeba desalting spin column (Pierce, USA) was prepared, the solution was loaded into the column to separate free $SM(PEG)_{24}$ from $SM(PEG)_{24}$-tagged neutravidin by centrifuge at 1000 rpm for 2 minutes.

De-protection of the thiol group on liposome

This step prepared the sulfhydryl groups on the surface of the liposome for future covalent binding. One milliliter of liposome in a concentration of 4.3 μmol/mL (about 250-fold the amount of neutravidin) was added to the round-bottomed flask with 0.1 mL of the hydroxylamine solution. The flask was then flushed with N_2 for 1 minute and immediately sealed up. It was then placed on a shaker for 2 hours until the deacetylation reaction of ATA on the liposome surface was completed.

Conjugation reaction

This step was the final conjugation of neutravidin to the ATA-tagged liposome. After both $SM(PEG)_{24}$-tagged neutravidin and the de-protected liposome were prepared, the two solutions were to be mixed together. The pH value of the liposomes was adjusted to about pH 7 (6.5–7.5) by adding aliquots of 0.5M potassium phosphate, and then the 1 mL of maleimide-derivatized neutravidin solution was added into the flask for conjugation. The mixture was put on a shaker at RT for 3.5 hours and then transferred to a shaker in a 4°C freezer overnight.

Quench reaction

This step reversed the second step and extinguished the unreacted sulfhydryl groups on the surface of the liposome. *N*-ethylmaleimide (100 mM, 0.0125 g/mL, Sigma) in potassium phosphate (0.02 M, pH 7) was prepared. *N*-ethylmaleimide in the same amount as the liposomes used for the conjugation was added into the flask and kept on the shaker for 30 minutes.

Size exclusion chromatography

This step removed the unreacted maleimide-derivatized neutravidin from neutravidin-tagged liposomes. A Sephadex CL-4B column was equilibrated with Tris-buffered saline (TBS)-sucrose buffer (51.77 g sucrose) with adjustment of the pH to 7.4. The desired fraction was eluted right after the void volume. The fractions were collected and stored in a 4°C freezer for future use.

Validation of neutravidin conjugation on liposomes

To confirm the conjugation of neutravidin on liposomes, the final product after conjugation, the original liposomes and a 96-well black plate were used. Two rows of wells with three replicates from well E1 to E3 and F1 to F3 were coated with 20 μg/mL rabbit anti-*Escherichia coli* with biotin, whereas the third row from well G1 to G3 was coated with rabbit anti-*Escherichia coli* without biotin. After a 1-hour coating procedure processed on a shaker at 50 rpm at RT, the antibody solution was cleared out and 0.2 mL of blocking buffer was added to each well and placed on a shaker for another 30 minutes. After blocking was finished, the blocking buffer was cleared out and each well washed with 0.2 mL of blocking buffer three times, and then different samples were put into the wells. Samples of neutravidin-conjugated liposome were added to the wells from E1 to E3 (Row 1) and G1 to G3 (Row 3) at a concentration of 236.364 nmol/mL. For wells F1 to F3 (Row 2), free liposome without neutravidin was added at a concentration of 78.788 nmol/mL. After incubation for 1 hour at RT on a 50 rpm shaker, the sample solutions were cleared out and the wells were washed with blocking buffer three times. Finally, the wells with blocking buffer were read with a spectrophotometer with Gen5 at OD540/590.

Fabrication of antibody chips and chips assay

Antibody chip printing

Five pathogen-specific polyclonal antibodies, including rabbit anti-*Streptococcus agalactiae* (AbD seroTec, Oxford, UK), rabbit anti-*Chlamydia trachomatis* (AbD seroTec, Oxford, UK), rabbit anti-*Neisseria gonorrhoeae* (GeneTex, Irvine, CA, USA), rabbit anti-*Escherichia coli* (AbD seroTec, Oxford, UK), and rabbit anti-*Candida albicans* (Fitzgerald,

MA, USA) with and without biotin were purchased and used to print on an aldehyde glass slide (Baio) in an eight by two cassette arrangement (ArrayIt, California, USA) with an automatic arrayer (SmartArrayer 136, CapitalBio) in a 4°C cold room. After the chips were printed, they were kept at 4°C for at least 12 hours to immobilize the proteins, and then the chips were stored in a −20°C freezer. The three controls included Cy3-labeled immunoglobulin G (IgG) (Jackson ImmunoResearch Europe Ltd., Suffolk, UK), goat anti-rabbit IgG, and biotin anti-*Escherichia coli* (Jackson ImmunoResearch Europe Ltd., UK). Various patterns with different combinations of these five antibodies and three positive controls were designed for this purpose.

Chip assay

Before the examination procedure, the chip was placed upside down in multidishes full of TBS with 5% Tween 20 (TBST) briefly without touching the printed surface to wash out the unimmobilized antibodies. A cover slide and a hybridization cassette from ArrayIt, which could contain 16 samples on one chip without contamination were then reassembled manually and 5 mL of 3% bovine serum albumin (BSA) was added to each well as a blocking buffer. Then the cassette was put on a 3D micro-shaker for 2 hours at RT. Before the chip was used, all wells were washed quickly with 1% BSA TBST, 30 μL per well. Pathogen or antigen samples of about 90 μL were added onto the side of the separate chips with a cover slide and then the chip was incubated in BioMixer at 15 rpm at RT for 1 hour. The chip was then washed with 5 mL of 1% BSA TBST quickly upside down, gently shaken by hand and then washed twice by washer with TBST, at 15 rpm for 5 minutes each time. Detecting antibody in the amount of 90 μL of 0.00025 mg/mL was added and the chip was incubated in a BioMixer at 15 rpm at RT for 1 hour. The washing procedure was repeated again. Then the chip was rinsed with 5 mL of TBS-sucrose (51.77 g) for 1 minute in the shaker at 50 rpm. Liposome solution (90 μL) was finally added to the chip, which was then incubated in a BioMixer at 15 rpm at RT for 1 hour. The chip was then washed with 5 mL TBS-sucrose in the shaker at 75 rpm, at RT, for 10 minutes, and then washed two times in the washer with TBS-sucrose (51.77 g) for 5 minutes each time at 75 rpm. The chip in the hood was stood on the side of the hood for 30 minutes to dry out.

Validation of the liposomal amplifying system on chips

The chips were composed of five different antibodies in a concentration of 0.125 mg/mL with and without biotin. Cy3 was used as the only positive control and all spots consisted of four replicates. Then the chip assay was carried out with the neutravidin-conjugated liposomes only, without the addition of a pathogen or detecting antibody.

Validation of the antibody-antigen reaction on chips

Chips composed of capture antibody in two different concentrations (0.25 and 0.5 mg/mL) and three positive controls were used to test the binding among capture antibodies, detection antibodies and antigen or pathogens. The tested antigen (pathogen) solutions were diluted in a serial concentration with a final 1% BSA TBS solution. Aside from the purchased *Chlamydia trachomatis* antigen, which was paired, all the pathogens were purchased from different sources and cultured in the laboratory, including *Escherichia coli* (ATCC 25922), *Neisseria gonorrhoeae* (from ATCC, ATCC 31426), *Streptococcus agalactiae* (BCRC, Bioresource Collection and Research Center, Taiwan, 10787, ATCC 13813) and *Candida albicans* (BCRC 22903, ATCC 90028).

Collection of clinical samples

Clinical samples from the four patients, who were present for the clinical diagnosis of vulva-vaginal candidiasis, were collected following a procedure similar to an ordinary pelvic examination. A Pap smear was performed, including one for endocervix and the other for the whole exocervix. Both swabs were dipped into a tube with 3 mL of normal saline and stirred up and down five times, and then the sticks with the brush were discarded. The tubes were stored in a −20°C freezer immediately for future assessment. The study was approved by the Institutional Review Board (VGH IRB, 2011-02-043IC).

Chip assay for clinical samples

Chips composed of both 0.25 mg/mL and 0.5 mg/mL concentrations of capture antibody (without biotin) from five pathogens and three positive controls, including Cy3-labeled IgG, goat anti-rabbit IgG, and anti-*Escherichia coli* IgG with biotin almost identical to the previous one, were used for clinical application. The specimens were added to the wells in four different conditions, with and without spiking attacks of *Candida albicans*.

Reading of the chips

After the chip was dry, it was scanned with Luxscan (CapitalBio, Beijing, China) and finally analyzed by Genpix.

Data analysis

A paired Student t test with $p < 0.05$ was considered significant.

Results

Validation of the liposomal amplifying system

Validation on a 96-well plate

Preparation of the ATA-tagged liposome took about 4 hours, and the following conjugation of neutravidin required another 4 hours and was left overnight. However, a large amount of modified liposome could be manufactured at a time and stored for future use. The average time needed to activate the microarray immunoassay chip system was 3 hours. The average ELISA fluorescence reading for three replicates was

450.7 for the system compared with 11.7 for the negative control. This neutravidin-conjugated liposome amplifying system could bind to the biotin on the detecting antibody and showed significant brightness ($p < 0.01$) on a 96-well plate (Fig. 1).

Validation on a chip

Similar validation tests were repeated using chips of pattern one, i.e., four replicates of five different antibodies with or without biotin labels and one Cy3-positive control on glass slides (Fig. 2A). Significant fluorescent signals were detected on the spots printed with biotin-labeled IgG when little or no signal was detected on the spots printed with IgG without biotin (Fig. 2B). Taking *Chlamydia trachomatis* antibody as an example, the average intensity reading was 33 and the reading of the relative negative control was zero (Fig. 4). This neutravidin-conjugated liposome amplifying system also showed a significant binding capacity to the biotin on the detecting antibodies, which were printed on the glass directly ($p < 0.01$).

Validation of the antigen-antibody reaction on chips

The best condition for detecting the pathogens cultured and stored in the laboratory was studied together with different combinations of detecting antibodies on the chips (Fig. 3A). Positive signals were recovered for every pathogen (Fig. 3B). However, better signals were obtained only when a higher concentration of antibody was used (0.5 mg/mL), especially for *Neisseria gonorrhoeae* (1.5 mg/mL). Moreover, only the study of *Chlamydia trachomatis* and *Candida albicans* showed a significant dose-dependent response for detection. Furthermore, the background on this microarray immunoassay chip for *Chlamydia trachomatis* assessment (antibody coated at a concentration of 0.25 mg/mL) was 1892 ± 56, which was a relatively noisy, intense fluorescence (3321 ± 251, $p < 0.01$) at an antigen concentration of 0.0061 mg/mL. A significant increase (4403 ± 148, $p < 0.01$) was also demonstrated when the concentration of antigen increased 10-fold (Fig. 4). However, significantly increased fluorescent intensity (4501 ± 1180 vs. 1924 ± 659, $p < 0.01$) was identified when the concentration of *Candida albicans* reached 4.54 × 10^5 CFU/mL, and the intensity of fluorescence increased proportionally (Fig. 5) when the concentrations of *Candida* increased from 10-fold (18276 ± 3177) to 100-fold (38994 ± 7452).

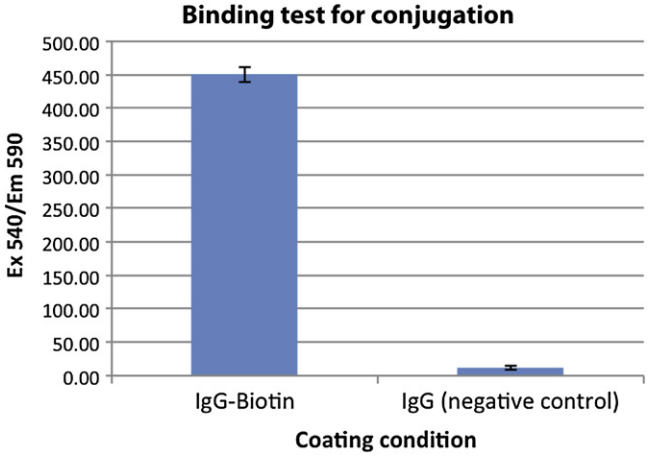

Fig. 1. Validation of conjugation of neutravidin and liposome on a 96-well plate. The wells coated with purchased immunoglobulin-biotin showed strong signal intensity (450.7) after reaction with conjugated liposomes compared with the negative controls (11.7). **$p < 0.001$. IgG = immunoglobulin G.

A

Printing/block	Antibodies				Antibodies with Biotin			
Row\column	1	2	3	4	5	6	7	8
1	*Neisseria gonorrhoeae*				*Neisseria gonorrhoeae*			
2	*Escherichia coli*				*Escherichia coli*			
3	*Chlamydia trachomatis*				*Chlamydia trachomatis*			
4	*Streptococcus agalactiae*				*Streptococcus agalactiae*			
5	*Candida albicans*				*Candida albicans*			
6	Cy3-labeled IgG							

B

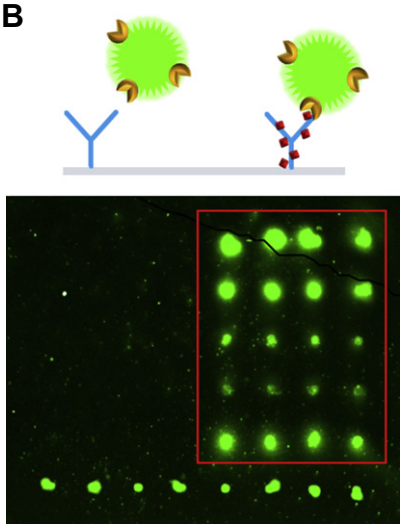

Fig. 2. (A) The designed scale of printing pattern 1. (B) Illustration of the validation principle for the detection system and the chip image showing strong signal intensity on the right side with spots coated with biotinylated antibody for every row in the red rectangle. IgG = immunoglobulin G.

Detection of pathogens in patients with and without vulvar and vaginal symptoms

Although the other four pathogens (*Chlamydia trachomatis*, *Escherichia coli*, *Neisseria gonorrhoeae*, and *Streptococcus agalactiae*) were not detected in the four patients studied, the detection of candidiasis infection was promising. When the amount of *Candida albicans* reached 2.27 × 10^7 CFU/mL, a

A

Column	1	2	3	4	5	6	7	8
1	Anti Rabbit				Biotin-anti E. coli			
2	*Escherichia coli* 0.25				*Escherichia coli* 0.5			
3	*Neisseria gonorrhoeae* 1.5				Biotin- *Neisseria gonorrhoeae*			
4	*Streptococcus agalactiae* 0.25				*Streptococcus agalactiae* 0.5			
5	*Candida albicans* 0.25				*Candida albicans* 0.5			
6	*Chlamydia trachomatis* 0.25				*Chlamydia trachomatis* 0.5			
7					100X Cy3-IgG			

B

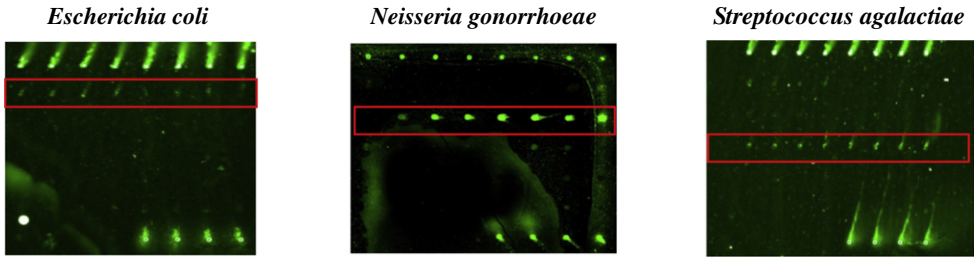

Fig. 3. Results of the antibodies and pathogens on chips binding test. (A) The pattern used in the binding test. (B) Different signal intensities were detected in the antibody-pathogen reaction chip assays with different pathogens including *Escherichia coli* (Row 2 in the left image), *Neisseria gonorrhoeae* (Row 3 in the middle image) and *Streptococcus agalactiae* (Row 4 in the right image). IgG = immunoglobulin G.

significantly increased intensity of around 1650 was shown, compared with the background fluorescence of 39 (Fig. 6). The estimated expense of this microarray immunoassay chip for five pathogens and at most 16 samples was US$ 3, if 200 chips were fabricated at the same time. The average time

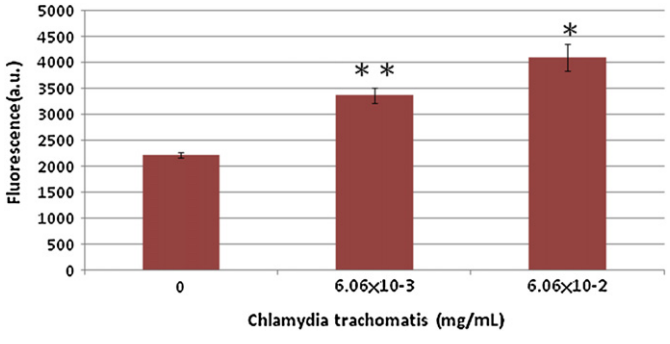

Fig. 4. The results of the antibody-antigen binding test for *Chlamydia trachomatis* in the chip assay. Significantly increased fluorescence intensity was detected when the concentration of *Chlamydia trachomatis* antigen reached 6.06×10^{-3} mg/mL. A proportional increase was demonstrated when the concentration increased 10-fold for the detection of *Chlamydia trachomatis*. $*p < 0.01$ when compared to the previous column. $**p < 0.001$ when compared to the previous column.

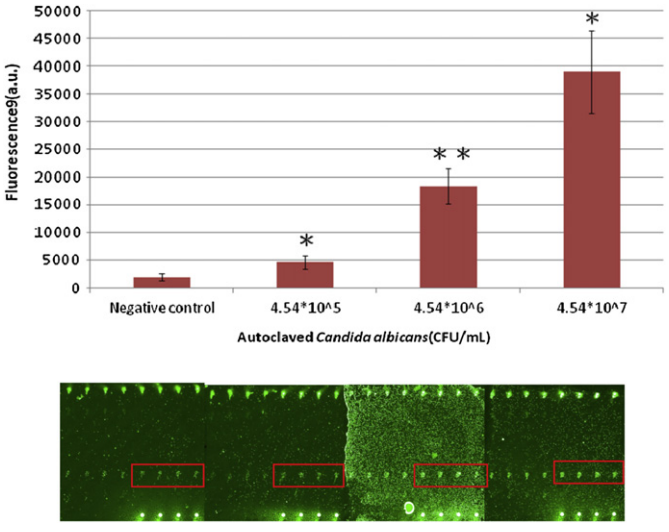

Fig. 5. The results of the antibody-antigen binding test of *Candida albicans* in the chip assay. The sensitivity level for autoclaved *Candida albicans* reached 4.54×10^5 CFU/mL. A proportional increase in the intensity of fluorescence can be seen from the upper table and the lower chip images when the concentration of *Candida albicans* increased 10-fold. The bright spots on row five circled with red lines represent the capture antibody for *Candida albicans* in a concentration of 0.5 mg/mL. $*p < 0.01$ when compared with the previous column. $**p < 0.001$ when compared with the previous column.

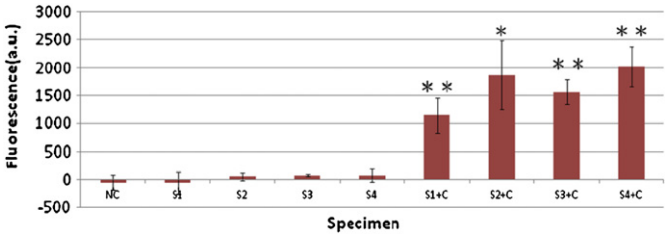

Fig. 6. The results of a chip assay for the original vaginal samples and the spiking of those samples with *Candida albicans*. Four samples were used and are shown here. Significant positive signals were demonstrated in 1:1 add-in samples. * $p < 0.01$ when compared with the original samples or NC. ** $p < 0.001$ when compared with the original samples or NC. C = *Candida albicans*; NC = negative control; S = specimen.

needed for a single test was 200 minutes. The average time needed for a single test was 200 minutes.

Discussion

The importance of the rapid and precise diagnosis of lower genital tract pathogens is well known, but it is sometimes difficult, as it is costly and the majority of the developing countries could not offer this service. For example, NAATs are too expensive and complicated to apply in routine clinical practice as a point-of-care tool [7,17]. Without prompt and accurate treatment of lower genital tract infections, subsequent severe sequelae might occur, such as tubo-ovarian abscess and infertility. To overcome this limitation, some sensitive and specific tools mediated through immunoassay have been developed. Unfortunately, only tools for certain pathogens, for example, chlamydia or human papilloma virus are available in the market [16]. In this study, we tested the feasibility of this multiplexed immunoassay chip, which can be used to detect five pathogens at the same time, and the results seem to be acceptable.

The advantages of this multiplexed immunoassay chip in this study included: (1) relatively low cost, which was only US$ 3 dollars if 200 tests were fabricated at the same time; (2) no extra need for the expensive equipment used in NAATs; (3) rapid assessment (3 hours); and, (4) high sensitivity and specificity for candidiasis diagnosis.

Although this multiplexed immunoassay chip seems very promising, some unresolved problems should be mentioned. Firstly, the antigen–antibody reaction between our chips and the real pathogens in the female reproductive tract, aside from candida, is yet to be established. In this study, only candidiasis was tested, although the results showed good correlation with a clinical situation. In addition, the *Candida* strains could not be differentiated in this chip. Secondly, since normal flora in the female lower genital tract is complicated and most importantly, infection of the female lower genital tract is often caused by multiple pathogens, it is not easy to select the correct antibodies to be printed on this multiplexed immunoassay chip. In fact, it may be necessary to identify the most common pathogens that cause female genital tract infections before the design of this multiplexed immunoassay chip, as how to select the frequent causal pathogen-related antibody for chip coating is not only one of the most critical steps, but also the most complicated and difficult one [31]. Furthermore, it is likely that rapid mutation of these microorganisms with development of drug resistance makes the selection of a sensitive antibody more and more difficult [32]. Therefore, it is reported that the discovery of a core antigen specific to the pathogen and stable from generation to generation is critical for the success of similar systems [33]; however, instead of more specific antibodies, such as antilipopolysaccharide antibody for chlamydia, only commercialized antibody reagents were applied in this multiplexed immunoassay chip, which may have contributed to the lower sensitivity of this multiplexed immunoassay chip in the detection of *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, *Chlamydia trachomatis,* and *Escherichia coli*. Thirdly, the incidence of the above-mentioned three pathogens might be too low to be detected in this very small study with only four patients.

Although our previous study showed a sensitivity of 1.0×10^5 GFU/mL in the detection of multiple pathogens in water using a liposomal amplifying system [26], the multiplexed immunoassay chip showed reduced sensitivity, partly because G-protein was not used in this protocol. G-protein induced excess noise in our original design of the multiplexed immunoassay chip. We used a more complicated biotin–antibody binding strategy, liposome-neutravidin conjugation, and biotin-neutravidin interaction than the original G-protein assay, which decreased the intensity of fluorescence in this multiplexed immunoassay chip. This was due to less fixation and the crowded space and limited amount of used substrate on this relatively small chip plain.

Specimens collection from the vagina and cervix might be another challenge. To simplify the procedure and minimize the discomfort of patients during speculum examination, a stick with a brush was used in the sampling procedure, which caused some problems. Firstly, blood contamination might have occurred. Secondly, only a minimal amount of discharge specimen was recovered, especially in the cervical sampling. Thirdly, a quantitative evaluation was almost impossible since the sampling amount varied using different brushes.

We believe that this multiplexed immunoassay chip might be of use in routine practice in the future, especially for chlamydia and candidiasis, although a further study might be required. For example, comparison between the conventional NAATs, culture and this multiplexed immunoassay chip for the same samples will enable the validation of this multiplexed immunoassay to be tested.

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References

[1] Spence D, Melville C. Vaginal discharge. BMJ 2007;335:1147−51.

[2] Lamont RF, Sobel JD, Akins RA, Hassan SS, Chaiworapongsa T, Kusanvic JP, et al. The vaginal microbiome: new information about genital tract flora using molecular based techniques. BJOG 2011;118:533−49.

[3] Donders G. Diagnosis and management of bacterial vaginosis and other types of abnormal vaginal bacterial flora: a review. Obstet Gynecol Surv 2010;65:462−73.

[4] Yang MJ, Sun PL, Wen KC, Chao KC, Chang WH, Chen CY, et al. Prevalence of maternal group B streptococcus colonization and vertical transmission in low-risk women in a single institute. J Chin Med Assoc 2012;75:25−8.

[5] Su WH, Tsou TS, Chen CS, Ho TY, Lee WL, Yu YY, et al. Are we satisfied with the tools for the diagnosis of gonococcal infection in females? J Chin Med Assoc 2011;74:430−4.

[6] WHO. A guide to essential practice. In: Research RH, editor. Sexually transmitted and other reproductive tract infections. Geneva: WHO; 2005. p. 40−9.

[7] Su WH, Tsou TS, Chen CS, Ho TY, Lee WL, Yu YY, et al. Diagnosis of *Chlamydia* infection in females. Taiwan J Obstet Gynecol 2011;50: 261−7.

[8] Wei CF, She BC, Lian HS, Ling QD, Tsai CY, Yen CW, et al. Prenatal group B *Streptococcus* test using real-time polymerase chain reaction. Taiwan J Obstet Gynecol 2009;48:116−9.

[9] Knud-Hansen CR, Dallabetta GA, Reichart C, Pabst KM, Hook 3rd EW, Wasserheit JN. Surrogate methods to diagnose gonococcal and chlamydial cervicitis: comparison of leukocyte esterase dipstick, endocervical gram stain, and culture. Sex Transm Dis 1991;18:211−6.

[10] Van Dyck E, Ieven M, Pattyn S, Van Damme L, Laga M. Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by enzyme immunoassay, culture, and three nucleic acid amplification tests. J Clin Microbiol 2001;39:1751−6.

[11] Nelson HD, Helfand M. Screening for chlamydial infection. Am J Prev Med 2001;20:95−107.

[12] Watson EJ, Templeton A, Russell I, Paavonen J, Mardh PA, Stary A, et al. The accuracy and efficacy of screening tests for *Chlamydia trachomatis*: a systematic review. J Med Microbiol 2002;51:1021−31.

[13] Masek BJ, Arora N, Quinn N, Aumakhan B, Holden J, Hardick A, et al. Performance of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by use of self-collected vaginal swabs obtained via an Internet-based screening program. J Clin Microbiol 2009;47:1663−7.

[14] Nelson HD, Saha S, Helfand M. Screening for chlamydial infection [Internet]. In: Systematic Evidence Reviews, No 3. Edited by Rockville (MD) Prepared by the Oregon Health Sciences University Evidence-based Practice Center under Contract No 290-97-0018: AHRQ Publication No. 01-S003; April, 2001.

[15] Ho MK, Lo JY, Lo AC, Cheng FK, Chan FK. Evaluation of replacing the existing diagnostic strategy for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections with sole molecular testing of urine specimens in a sexually transmitted infection clinic setting. Sex Transm Infect 2009; 85:322−5.

[16] Mahilum-Tapay L, Laitila V, Wawrzyniak JJ, Lee HH, Alexander S, Ison C, et al. New point of care Chlamydia Rapid Test−bridging the gap between diagnosis and treatment: Performance evaluation study. BMJ 2007;335:1190−4.

[17] Huppert J, Hesse E, Gaydos CA. What's the point? How Point-of-Care STI tests can impact infected patients. Point Care 2010;9:36−46.

[18] Vickerman P, Watts C, Alary M, Mabey D, Peeling RW. Sensitivity requirements for the point of care diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in women. Sex Transm Infect 2003;79:363−7.

[19] Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, et al. Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. BMC Microbiol 2005;5:61.

[20] De Backer E, Verhelst R, Verstraelen H, Alqumber MA, Burton JP, Tagg JR, et al. Quantitative determination by real-time PCR of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae* indicates an inverse relationship between *L. gasseri* and *L. iners*. BMC Microbiol 2007;7:115.

[21] Bravo AB, Miranda LS, Lima OF, Cambas AV, Hernandez ML, Alvarez JM. Validation of an immunologic diagnostic kit for infectious vaginitis by *Trichomonas vaginalis, Candida* spp., and *Gardnerella vaginalis*. Diagn Microbiol Infect Dis 2009;63:257−60.

[22] Brown HL, Fuller DD, Jasper LT, Davis TE, Wright JD. Clinical evaluation of affirm VPIII in the detection and identification of *Trichomonas vaginalis, Gardnerella vaginalis*, and *Candida* species in vaginitis/vaginosis. Infect Dis Obstet Gynecol 2004;12:17−21.

[23] Chen CS, Korobkova E, Chen H, Zhu J, Jian X, Tao SC, et al. A proteome chip approach reveals new DNA damage recognition activities in *Escherichia coli*. Nat Methods 2008;5:69−74.

[24] Chen CS, Zhu H. Protein microarrays. Biotechniques 2006;40. 423, 425, 427 passim.

[25] Chen CS, Baeumner AJ, Durst RA. Protein G-liposomal nanovesicles as universal reagents for immunoassays. Talanta 2005;67:205−11.

[26] Chen CS, Durst RA. Simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* with an array-based immunosorbent assay using universal protein G-liposomal nanovesicles. Talanta 2006;69:232−8.

[27] Ho JA, Durst RA. Detection of fumonisin B1: comparison of flow-injection liposome immunoanalysis with high-performance liquid chromatography. Anal Biochem 2003;312:7−13.

[28] Park S, Durst RA. Immunoliposome sandwich assay for the detection of *Escherichia coli* O157:H7. Anal Biochem 2000;280:151−8.

[29] Haab BB. Multiplexed protein analysis using antibody microarrays and label-based detection. Methods Mol Med 2005;114:183−94.

[30] Rucker VC, Havenstrite KL, Herr AE. Antibody microarrays for native toxin detection. Anal Biochem 2005;339:262−70.

[31] Uttamchandani M, Neo JL, Ong BN, Moochhala S. Applications of microarrays in pathogen detection and biodefence. Trends Biotechnol 2009;27:53−61.

[32] Unemo M, Palmer HM, Blackmore T, Herrera G, Fredlund H, Limnios A, et al. Global transmission of prolyliminopeptidase-negative *Neisseria gonorrhoeae* strains: implications for changes in diagnostic strategies. Sex Transm Infect 2007;83:47−51.

[33] Palmer HM, Young H, Graham C, Dave J. Prediction of antibiotic resistance using *Neisseria gonorrhoeae* multi-antigen sequence typing. Sex Transm Infect 2008;84:280−4.