



Short communication

Biosensor based on imaging ellipsometry for serotype-specific detection of *Riemerella anatipestifer*

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ABSTRACT

In this study, a practicable method for the detection of *Riemerella anatipestifer* (*R. anatipestifer*) using biosensor based on imaging ellipsometry (BIE) is described. The method is performed by immobilizing *anti-R. anatipestifer* egg yolk immunoglobulin (IgY) onto modified chemistry surface to form sensing layer. Antigen captured by sensing layer can then be quantitatively measured through imaging ellipsometry in grayscale format. The results demonstrate that it can detect *R. anatipestifer* as low as 5.2×10^3 CFU/mL. Furthermore, the proposed method can realize specific discrimination for multiple serotypes of 1, 2, 4, and 14 of *R. anatipestifer*. It has the advantages of rapid, simple, high sensitivity and low cost.

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1. Introduction

Riemerella anatipestifer can bring septicemia to ducklings, geese, turkeys and other birds [1–3]. Till now, 21 serotypes have already been reported [4–7] and 1, 2, 5 and 14 are the predominantly popular all of the world [8]. As flocks often infected simultaneously by several serotype *R. anatipestifer*, it is significant to discriminate virulent strain's serotype for effective control through vaccine immunization. The conventional methods, such as agar gel precipitation, slide agglutination and polymerase chain reaction, are customarily utilized to confirm this pathogen. Agar gel precipitation is routinely executed by agar plate fabrication, wells punching, samples addition, incubation and results confirmation. The procedure is not only labor-intensive especially for large-scale of samples to be analyzed but also unacceptable sensitive. Slide agglutination is an annoying process when face to much more samples to be checked repeatedly. In addition, it often disturbed by cross reactions. Polymerase chain reaction may not be suitable for manipulation in common laboratory as the expensive equipment and professional operation. Therefore, development of a detection method with advantages of rapid, simple and high throughput is necessary.

In the past decade, protein array or sensor has been successively used in proteomics for large scale analysis due to its capability of multiple samples analysis [9]. Alternatively, microfluidic systems integrated in it plays important role in chemical analysis systems such as a micro total analysis system or a lab-on-a chip [10]. Micropumps and microchannels will significantly facilitate analysis performances by reducing cycle times, reagent costs and labor intensity. Microfluidic systems combined with fabricated protein array can exert analysis in serial or parallel format, which can achieve simplicity.

As a typical label-free representative technique, biosensor based on imaging ellipsometry (BIE) is a fast, reliable and convenient assay device to measure protein surface concentration [11]. BIE is a combination of imaging ellipsometry and array fabrication together with microfluidic channel deliver system [12]. It carries the advantages of both high sensitivity of spectroscopy ellipsometry that subnanometer level (vertically) can be achieved in bioaffinity-based sensing [13] and high throughput of protein array in which at least 48 dots were already fulfilled [14]. BIE can perform multiplex assay by continuous fashion involved in sample treatment, addition, sample delivering and washing cycles within a microchannel network [15]. These merits are very appropriate for multiple serotypes analysis of pathogen. Although the detection of virus and bacteria has been proposed [16,17], BIE is not being used for multiple serotypes analysis. In this study, application of BIE as a model method to *R. anatipestifer* detection with high sensitivity and serotype-specific discrimination only requiring single experimental step is demonstrated.

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2. Materials and methods

2.1. Material

Polished silicon wafers were purchased from General Research Institute for Nonferrous Metals (China). 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde (GA, 50% aqueous solution) were from ACROS. *R. anatipestifer* isolates were originated from ducklings and geese with typical clinic symptoms. Heterologous pathogens including *Escherichia*, *Salmonella* and *Pasteurella* isolated from clinical samples in our laboratory were used as control (Table 1). All other reagents were of analytical grade. Water was obtained from a millipore Milli-Q ion exchange apparatus. Phosphate-buffered saline (PBS, 8 mM Na₂PO₄·2-H₂O, 2.68 mM KCl, 1.14 mM KH₂PO₄, 137 mM NaCl; pH 7.4) was prepared in deionized water. PBST (0.05% tween-20) was used as washing solution.

2.2. BIE principle

BIE presented here has been developed in Institute of Mechanics (Chinese Academy of Sciences, China), which consists of microfluidic array system and imaging ellipsometry (IE) [18]. The microfluidic array system is used for surface patterning and array fabrication. IE is used for reading the protein arrays. The polydimethylsiloxane (PDMS) template in micro-fluidic array system contains an 8×6 cell array. When the cell array was attached to the silicon wafer surface, 48 individual chambers were formed independently. As each chamber had two access holes, namely, an inlet and an outlet, protein solution could transfer in and out of the chamber. By such microfluidic system, protein solutions were delivered individually to different cells for immobilization so that the surface is patterned homogeneously and simultaneously in array format.

IE uses a CCD camera to image the ellipsometry response of a larger area sample, and the result was grabbed as a digital image and stored in a computer with a grayscale format (8 bits, 0–255 grayscale) for further evaluation by an image-processing program. Once imaging ellipsometer was fixed, the detected signal intensity “I” (grayscale) is the function of the layer thickness (d), $I = f(d)$, where $f(d)$ denotes the function relationship which is determined by layers with known intensity and thicknesses, then the unknown thickness of protein layer could be deduced from the detected intensity according to the function. At the same time there is a relationship between surface concentration and layer thickness: surface concentration ($\mu\text{g}/\text{cm}^2$) $\approx K \times d$ (nm), where $K = 0.12$ [19]. Thus, the grayscale value directly reflects layer thickness and surface concentration. The higher grayscale value, the thicker the layer and the higher the surface concentration. Here the angle of incidence is 75° and the optical wavelength is 632.8 nm. The configuration is illustrated in Fig. 1(A). When antibody was immobilized on substrate, two dots “a” and “b” with identical grayscale G_0 was obtained (Fig. 1B). Then, the grayscale turned to G_i of dot “b” due to antigen combination whereas grayscale of dot “a” is still G_0 (Fig. 1C).

Table 1
Reference pathogen used in this study.

Strain	Serotype	Host	Isolation time
<i>R. anatipestifer</i> (S1)	1	Ducklings	2003.08
<i>R. anatipestifer</i> (S2)	2	Ducklings	2005.10
<i>R. anatipestifer</i> (S3)	4	Ducklings	2006.02
<i>R. anatipestifer</i> (S4)	14	Ducklings	2007.01
<i>Escherichia</i>	O157:H7	Ducklings	2002.08
<i>Salmonella</i> app	Field isolate	Ducklings	2005.06
<i>Pasteurella</i> app	Field isolate	Ducklings	2006.09

2.3. Bacteria cultivation and IgY purification

R. anatipestifer grows on 5% rabbit-blood agar plate at 37 °C for 18 h. After formation of single colonies, it was transferred to 5 ml nutrient medium and incubated at 37 °C for 18 h on a shaker at 80 rpm. Later, centrifuge the culture at 12,000 g for 10 min, discard the supernatant, then wash the pellets by formaldehyde physiological saline solution (FPSS, 0.3%) several times and stored at 4 °C. IgY was harvested from eggs laid by hens (25 weeks of age, single comb white Leghorn) immunized individually by formalin-killed 1, 2, 4 and 14 serotype *R. anatipestifer* vaccine [20]. IgY purification was as follows: crude of IgY was centrifuged for 10 min, add the supernatant into PBS solution, ammonium sulfate drop by drop to 50% saturation, stir for 1 h. Centrifuge the mixture at 10,000 g for 20 min and remove the supernatant, wash the pellet several times and resuspend it in an equal volume of ammonium sulfate solution. Centrifuged again, the product was adjusted to 100.0 $\mu\text{g}/\text{mL}$.

2.4. Silicon modification

Silicon wafer was initially treated with piranha solution (30% H₂O₂:70%H₂SO₄ = 1:3, v/v) for 30 min followed by intensive rinsing with deionized water. The oxidized silicon wafer was immersed in ethanol solution containing APTES (5%) for 2 h incubation. After it washed by ethanol and deionized water repeatedly, the modified silicon wafer was placed in a mixture of glutaraldehyde (GA)/PBS (1:10, v/v) to react for 1 h, rinsed with PBS and deionized water once a time, forming the aldehyde surface. The modification qualities were conformed by contact angle measurement as the literature described [21]. Finally, the prepared silicon substrate was stored at 4 °C for later use.

2.5. IgY immobilization and *R. anatipestifer* detection

IgY immobilization procedure was illustrated in Fig. 2. Silicon wafer was firstly impressed onto the PDMS template. Then, 20 μL IgY served as capture antibody was dispensed by microfluidic channel system to the substrate to react for 20 min with a 1.0 $\mu\text{L}/\text{min}$ flow rate. In this step, the amino group of IgY molecule will be covalently linked with the aldehyde group on the silicon substrate. After the above reaction, PBST was used to wash the channels at 20.0 $\mu\text{L}/\text{min}$ for 5 min. Then, 1 M ethanolamine was dispensed to deactivate the unbound aldehyde group at 5 $\mu\text{L}/\text{min}$ for 10 min. Washed by PBST again at 20.0 $\mu\text{L}/\text{min}$ for 5 min, 30 μL bovine serum albumin (BSA) (1.0 mg/mL) in PBS was dispensed to block the channels at 1.0 $\mu\text{L}/\text{min}$ for 30 min. All channels were washed by PBST, and then sensing layer was formed.

The fabricated sensing layer can be used to capture antigen. *R. anatipestifer* from purified culture or effusion from clinic sample was exposed to the sensing layer to carry out antigen–antibody reaction at 1.0 $\mu\text{L}/\text{min}$ for 30 min. After the channels were washed by PBST at 20.0 $\mu\text{L}/\text{min}$ for 10 min, silicon wafer was taken from the template, washed with deionized water intensively, dried by a stream of N₂ and rendered to imaging ellipsometry for grayscale measurement. The total assay time was approximately 20 min.

3. Results and discussions

3.1. *R. anatipestifer* combining confirmation

IgY was immobilized on aldehyde surface by microfluidic system forming two protein dots “a” and “b” shown in the Fig. 3(A). It was found that protein dots were brighter than the ambient darker region which was formed by silicon substrate. The grayscale value in dot “a” is 93.4 whereas ambient dark region is 70.6. After dot “b” exposed to a solution containing *R. anatipestifer* with 5.2×10^7 CFU/ml, grayscale

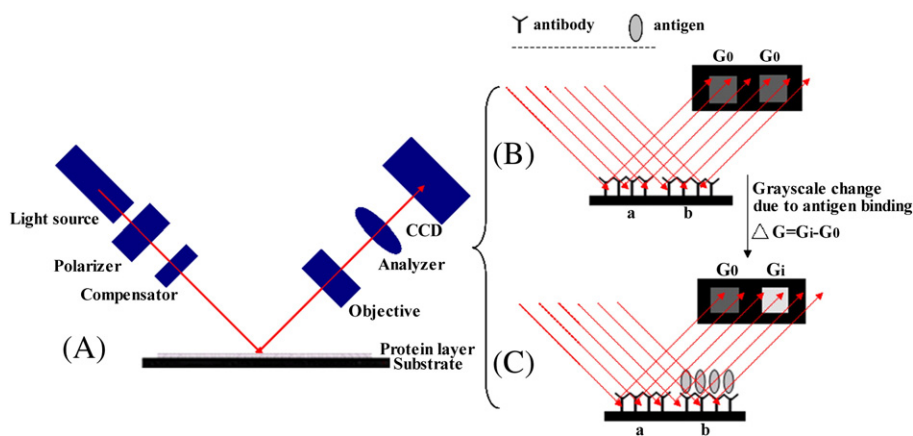


Fig. 1. Detection principle is illustrated. (A) The configuration of imaging ellipsometry, (B) fundamental grayscale value of antibody immobilized on the chemistry surface is G_0 without antigen binding, (C) which changes to G_i due to antigen binding.

value increases to 141.6. This demonstrated that the specific combination of *R. anatipestifer* onto the sensing layer was happened and the molecular surface density was accordingly increased. In order to get more in details about pathogen binding onto the sensing layer, we present dot “b” to scanning electron microscope (SEM) observation. Fig. 3(B) shows typical SEM image of dot “b”, in which captured *R. anatipestifer* displays typical rod-shaped characteristics.

3.2. Analytical sensitivity

We diluted serially *R. anatipestifer* solution from 5.2×10^7 to 5.2×10^2 CFU/mL. Each dilution titer was delivered to react with the sensing layer in triplicates. Dose–response curve was then obtained. Fig. 4 shows typical ellipsometry image and grayscale response curve. Changes in mean grayscale value were plotted to antigen concentration. According to appraisal, the minimum detection limit is 5.2×10^3 CFU/mL ($S/N = 3$, $R^2 = 0.98$). *R. anatipestifer* is rod-shaped bacterial and its general size is 0.3–1.0 μm . Therefore, low concentration of antigen can induce significant grayscale change and achieve high sensitive detection. Compared with the detection limit of 10^5 CFU/mL for *Legionella pneumophila* with surface plasmon resonance technique [22] and 2.5×10^4 CFU/mL for *E. coli* O157 with a light-addressable potentiometric sensor technique [23], the sensitivity of BIE for bacterium detection is comparatively superior.

3.3. Analytical specificity

R. anatipestifer often gives rise to mixed infection or polyinfection with *E. coli*, *Salmonella* or *Pasteurella* spp. To verify the specificity of BIE, we tested the grayscale changes by non-specific binding of *Escherichia* O157:H7 (5×10^7 CFU/mL), *Salmonella* spp (6.2×10^8 CFU/mL) and *Pasteurella* spp (5.6×10^7 CFU/mL) to the sensing layer. Fig. 5

shows the results. The grayscale change (ΔG) is 63 ± 3.2 for 5.2×10^7 CFU/mL *R. anatipestifer* binding, while ΔG is only limited to 4 ± 2.8 for *E. coli* O157:H7, 3.8 ± 2.1 for *Salmonella* spp and 4.1 ± 2.4 for *Pasteurella* spp binding. The results demonstrated that the IgY chosen for sensing layer fabrication and the strategy for immobilization in this study is effective to *R. anatipestifer* detection. Therefore, it was verified that BIE detection for *R. anatipestifer* has acceptable specificity.

3.4. Analytical reproducibility

Standardized guideline for biosensor application would be important for reliable analysis. As there are no existing guidelines for the validation of protein chip, we consult some guidelines for protein chip evaluation [24]. The reproducibility of the assay was assessed by intra-slide and inter-slide coefficients of variation (CV). CV% was defined as $CV = \sigma / \bar{x} \times 100\%$, where σ is standard deviation and \bar{x} is mean value. In this work, the intra-slide CV% were conducted by 8 tests for the same concentration on one silicon slide and inter-slide CV% were conducted by 5 tests for the same concentration of *R. anatipestifer* on different silicon slides. CV% values of intra-slide and inter-slide are 8.6%, 7.4%, 10.5% and 8.9%, 10.6%, 10.7% for 2.4×10^5 , 4×10^6 and 4×10^7 CFU/ml, respectively (Table 2).

3.5. Serotype-specific discrimination

As multiple channels available in the array, various serotypes IgY can be immobilized simultaneously so that multi-serotypes *R. anatipestifer* can be accordingly detected in parallel. Four different serotype strains (They were separately designated as S1, S2, S4, and S14.) and its corresponding IgY (IgY1, IgY2, IgY4 and IgY14) were used to explore the possibility of serotype-specific discrimination.

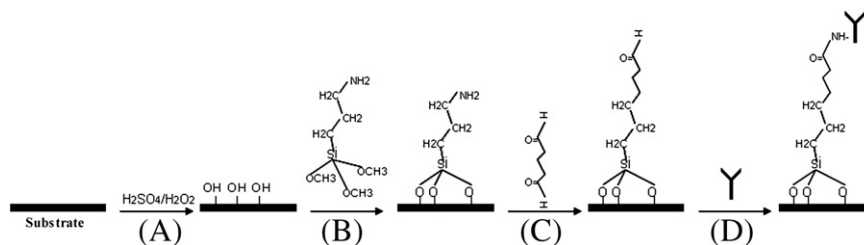


Fig. 2. Surface modification and IgY immobilization. (A) Oxidization of silicon wafer, (B) APTES coupling, (C) glutaraldehyde cross-linking reaction and (D) immobilization of IgY.

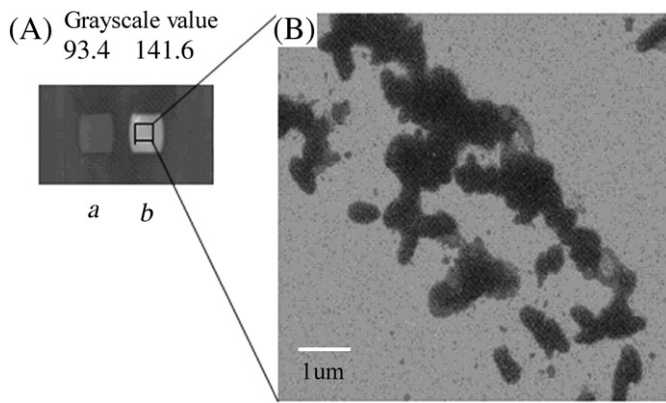


Fig. 3. Detection of *R. anatipestifer* by IE and observed by scanning electron microscope (SEM). (A) Ellipsometric grayscale image. IgY1 against *R. anatipestifer* serotype 1 was immobilized on the silicon surface and protein dot "a" (only IgY immobilized) and "b" (antigen binding) with different grayscale value was obtained. (B) SEM image on dot "b" contains captured *R. anatipestifer*.

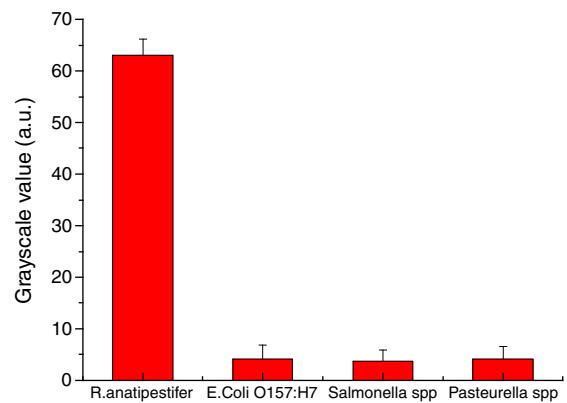


Fig. 5. Grayscale changes were evaluated by non-specific binding of *Escherichia* O157:H7 (5×10^7 CFU/ml), *Salmonella* spp. (6.2×10^8 CFU/ml) and *Pasteurella* spp (5.6×10^7 CFU/ml) with BIE.

Matrix organization design for antigen–antibody interactive reaction was developed. In this configuration, IgY can combine with corresponding serotype strain other than mismatched one due to the serotype-specific combination. In other words, IgY1 can not interact with S2, S4 and S14 but with S1, IgY2 cannot interact with S1, S4 and S14 but with S2, and IgY4 cannot interact with S1, S2 and S14 but with S4 and IgY14 cannot interact with S1, S2 and S4 but with S14.

During testing, IgYs were adjusted to identical concentration ($100.0 \mu\text{g}/\text{mL}$) and strains were adjusted to approximately 5.2×10^7 CFU/mL. The assay was performed in the sequence of IgY immobilization, PBST washing, BSA blocking, antigen binding, and PBST washing, and all of the steps were automated by microfluidic channel system. The results were shown in Fig. 6. IgY of four dots in first line (a1, b1, c1 and d1) were IgY1, the second line (a2, b2, c2, and d2) were IgY2, the third line (a3, b3, c3, and d3) were IgY4, and the fourth line (a4, b4, c4, and d4) were IgY14, respectively. After four strains (S1, S2, S4 and S14) *R. anatipestifer* react independently with the sensing layer in four dots in each line, grayscale value corresponding to its IgY is higher than the other three dots. Complying

with this fashion, grayscale values with dot a1 in line 1, b2 in line2, c3 in line3 and d4 in line4 were higher than the other three dots. This is because the antigen–antibody specific combination was happened, which were IgY1 against S1, IgY2 against S2, IgY4 against S4 and IgY14 against S14 in the detection, respectively. Their corresponding grayscale values were tabulated in Table 3. The so-called serotype-specific discrimination can also be applied to the identification of virulent strains about *E. coli* [25] and *Salmonella* [26], which have hundreds to thousands serotypes. Therefore, the proposed method is meaningful to public health.

3.6. Other considerations

The important aspect should be pointed out that immunized hens transfer high content of IgY (8–25 mg/mL) into yolks of their eggs [27], which can be non-invasively obtained by simple collection methods. The IgY cheaply obtained can decrease detection cost utmost. Compared with the detection time of agar gel precipitation, slide agglutination and polymerase chain reaction that hour to days is required, the BIE is a more rapid method. And, miniaturization of BIE

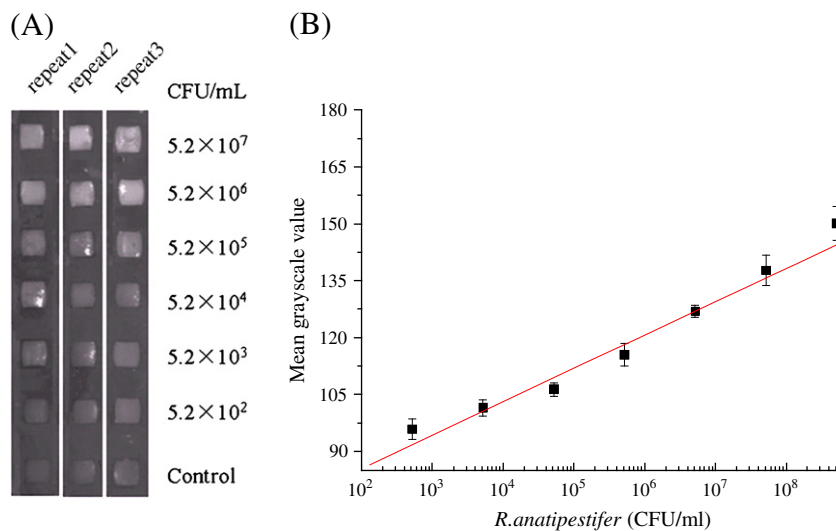


Fig. 4. Typical grayscale image for *R. anatipestifer* detection (A) and calibration curve (B). Data obtained in triplicates at room temperature under optimized conditions.

Table 2
Reproducibility analysis for different *R. anatipestifer* concentration.

Sample	<i>R. anatipestifer</i> (CFU/ml)	Intra-slide (CV%)	Inter-slide (CV%)
1	5.2×10^5	8.6% (n=8)	8.9% (n=5)
2	5.2×10^6	7.4% (n=8)	10.6% (n=5)
3	5.2×10^7	10.5% (n=8)	10.7% (n=5)

system has progressed [28] and will greatly propel its application in the future. Moreover, it is not impossible to develop a comprehensive immunoassay based on BIE platform for multiple species detection if excellent antibody is available.

4. Conclusions

To sum up, this work demonstrated that it is feasible to use BIE as a novel tool to detect *R. anatipestifer*. The chicken anti-*R. anatipestifer* IgY was used to construct a label-free assay based on biosensor technique. IgY can be covalently immobilized on chemistry surface forming sensing layer through linkage between aldehyde group of surface and lysine of IgY. When *R. anatipestifer* applied to the sensing layer, antigen specific binding is confirmed by imaging ellipsometry and scanning electron microscope observation. The proposed method can detect *R. anatipestifer* at the lower concentration of 5.2×10^3 CFU/ML and realize serotype-specific discrimination. The described method has the advantages of rapid, simple and serotype-specific detection of *R. anatipestifer*. It has the potential for application in clinic laboratory.

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Table 3
Grayscale values of protein dots in Fig. 6.

No.	Grayscale	No.	Grayscale	No.	Grayscale	No.	Grayscale
a1	171.2	b1	81.3	c1	99.5	d1	96.3
a2	95.6	b2	154.9	c2	100.2	d2	106.2
a3	106.3	b3	86.8	c3	173.6	d3	95.8
a4	116.5	b4	86.2	c4	98.8	d4	189.5

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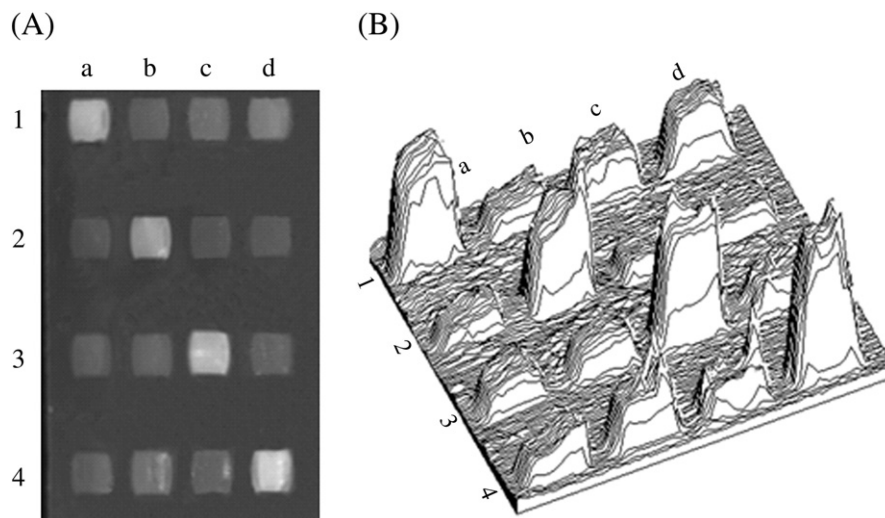


Fig. 6. Serotype-specific detection of *R. anatipestifer* using BIE in array format. (A) Ellipsometry image of the serotype-specific detection of *R. anatipestifer*. IgY in line 1 (dot a1, b1, c1 and d1) are S1, line2 (dot a2, b2, c2 and d2) are S2, line3 (dot a3, b3, c3 and d3) are S4 and line 4 (dot a4, b4, c4 and d4) are S14. The strain of line1 (dot a1, b1, c1 and d1) are serotype1, line2 (dot a2, b2, c2 and d2) are serotype2, line3 (dot a3, b3, c3 and d3) are serotype4, and line4 (dot a4, b4, c4 and d4) are serotype14, respectively. Detailed description can be consulted in the text. (B) Three-dimension map corresponding to grayscale values.