One-step multiplexed detection of foodborne pathogens: Combining a quantum dot-mediated reverse assaying strategy and magnetic separation

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ABSTRACT

A rapid and multiplexed immunosensor was developed based on a quantum dot (QD)-reverse assaying strategy (RAS) and immuno-magnetic beads (IMBs) for one-step and simultaneous detection of *Escherichia coli* O157:H7 and *Salmonella*. In a conventional QD-based immunosensor, the fluorescence signal of the “IMBs-target-QD” immunoconjugate is directly used as the assaying readout. However, the fluorescence signal is affected by IMBs due to light scattering and the “IMBs-target-QD” immunoconjugate needs multiple washing and re-suspension steps. To address these problems, we use the surplus QD-antibody conjugate as signal readout in the RAS, which prevents interference from the IMBs, increases the fluorescence signal, and avoids complex operations. Compared with conventional QD-based immunosensor, the sensitivity of QD-RSA immunosensor for detection of *Escherichia coli* O157:H7 has been improved fifty-fold, and whole analysis procedure can be finished within 1h. Therefore, this RSA strategy is promising for improving the performance of QD-based immunosensors and could greatly broaden their applications.

Keywords: Reverse assaying strategy, quantum dot, one-step detection, multiplexed detection

1. Introduction

Foodborne pathogens such as *Salmonella enterica* (*S. enterica*) and *Escherichia coli* O157:H7(*E.coli*) are main causes of epidemic disease outbreaks world wide (Jones et al. 2008; Scallan et al. 2011a; Scallan et al. 2011b). There is an urgent need for simultaneous detection of multiple types of bacteria with high sensitivity and speed to prevent outbreaks (Khan et al. 2001; Painter et al. 2013; Wolter et al. 2008; Xu et al. 2016). Conventional methods for foodborne bacteria detection mainly include colony counting, enzyme-linked immunosorbent assay (ELISA) (Thaitrong et al. 2013), polymerase chain reaction (PCR) (Kim et al. 2014; Mao et al. 2009; Wang et al. 2014) and biosensors (Chen et al. 2016; Chen et al. 2015). Colony counting is the standard method for detecting bacterial pathogens because of its accuracy, but it is labor intensive and time consuming, making it unsuitable for point-of-care testing. ELISA is an important solid-phase immunosorbent assay that is widely used in detecting pathogens because of its specificity and low cost. However, ELISA is a heterogeneous assay and needs multiple washing steps, which greatly limits its detection efficiency. PCR provides high sensitivity but requires precise instruments and professionals to perform the tests. Biosensors provide a new platform for detecting pathogen in complicated samples due to their good selectivity and high sensitivity, while the stability and repeatability should be improved. Moreover, detecting multiple types of pathogens in samples is necessary because the samples may be polluted by different bacteria. Conventional methods lack this capability.

Quantum dot (QD) is being used as a new type of fluorescent probe (Chen et al. 2016; Chen et al. 2015). QD has received increasing attention because of its advantageous optical properties over traditional fluorescent dyes, including a wide

excitation spectrum, high quantum yield, and less photobleaching (Galikowska et al. 2011; Guo et al. 2015; Wang et al. 2015c). More importantly, a single light source can simultaneously excite many different colors of QD, which makes them an ideal probe for multiplexed analysis. QD has been used to detect many types of targets, such as cancer cells (He et al. 2016; Wei et al. 2014), bacteria (Chen et al. 2012; Hahn et al. 2008; Hahn et al. 2005), viruses (Liu et al. 2016; Wang et al. 2015b), and small molecules (Chen and Xia 2014; Yu et al. 2016). Among the QD-based sensors, QD combined with immuno-magnetic separation (IMS) technology have broadened the application of QD-mediated biochemical analysis (Wang et al. 2015a; Wu et al. 2015). Homogeneous QD-immunomagnetic bead (IMB) immunosensors are a combination of IMBs for target capture and antibody-conjugated QD as a fluorescent probe (Guo et al. 2015; Zhao et al. 2012). These immunosensors have a number of advantages over traditional heterogeneous fluorescent immunosensors, such as rapid solution kinetics and target pre-enrichment (Cho et al. 2014; Jie and Yuan 2012; Wu et al. 2015). QD-antibody conjugate (QD-Ab) can be captured by the IMB-target immunocomplex, and the fluorescence signal is collected from the QD-target-IMB conjugate, which is directly related to the concentration of the target. This method is the direct assaying strategy (DAS) in traditional QD-IMB immunosensors.

However, two common problems are encountered in this method. First, the fluorescence signal of QD cannot be measured effectively due to the blocking effect of the IMBs. Because the IMBs are much larger (200 to 1000 nm) than the QD (2 to 10 nm), the volume of IMBs is six orders of magnitude larger than that of QD. Thus, when QD are captured on the surface of the IMBs to form the IMBs-target-QD conjugate, only a small fraction of all the QD can be excited to generate the

fluorescence signal. As a result, the spatially blocked QD cannot be excited, and the corresponding fluorescence signals of QD are missing and cannot be measured. Second, the sandwich IMBs-target-QD conjugate needs multiple washing and re-suspension steps that affect the speed and accuracy of the whole method. We hypothesized that the remaining QD-Ab probe could be used in a reverse assaying strategy (RAS) as a fluorescence signal readout to address these shortcomings.

We present a one-step, sensitive, and multiplex QD-RAS immunosensor to simultaneously detect *S. enterica* and *E. coli* in milk samples. RAS was used to increase the effective fluorescence signal and simplify the analysis process. As shown in Scheme 1, the amount of QD-Ab probe is kept constant, and the remaining QD-Ab probe in the solution can be used as a fluorescence signal when the QD-target-IMB conjugate is removed by magnetic separation (MS). The whole immuno-reaction can thus be finished in one step without multiple washing steps, which can largely shorten the detection time. To our knowledge, this is the first study to develop an ultrasensitive and convenient QD-based immunosensor that uses RAS for the one-step detection of multiplex pathogens.

Scheme 1. QD-RAS immunosensor for one-step and multiplex detection of *E. coli* and *S. enterica*. (A) Direct assaying strategy (DAS); (B) Reverse assaying strategy (RAS).

2. Experiment

2.1 Reagents and materials

Carboxyl QDs (2 to 10 nm in size, maximum emission wavelengths of 620 nm and 528 nm) were obtained from Ocean NanoTech (USA). Magnetic beads (MBs) (1150 nm in size, solid content: 10 mg/mL) were purchased from Shanghai Allrun Nano Science and Technology Co., Ltd. (Shanghai, China). Polyclonal antibodies against *S. enterica* (Ab1) and *E. coli* (Ab1) were used as captured antibodies and monoclonal antibodies (Ab2) as labelled antibodies to recognize *S. enterica* or *E. coli* (Abcam, USA). Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide sodium (Sulfo-NHS) were purchased from Sigma-Aldrich (USA). The common reagents and solvents were of analytical grade and purchased from Beijing Chemical Reagents Co. (Beijing, China). The water used in the experiments was deionized and ultrafiltered using a Milli-Q apparatus. All other chemicals were of analytical reagent grade, and all the aqueous solutions were prepared using distilled water.

2.2 Microorganisms

E. coli (ATCC 43888), *S. enterica* (ATCC 14028), *Shigella* spp. (ATCC 12022), *Staphylococcus aureus* (ATCC 27217), and *spirillum of cholera* (ATCC 13312) were obtained from the American Type Cell Collection (ATCC). The culture conditions for *S. enterica* and *E. coli* were the same as in previous work (Wang et al. 2015c).

2.3 Apparatus

A fluorescent microplate reader (Thermo Scientific, USA) was used to record the emission spectra of the QD. A single-photon laser confocal imaging system (Zeiss 760, Germany) was used to identify the fluorescence signal of QD. Transmission electron microscopy (TEM, JEM-2100F) was used to identify the IMBs and IMB-pathogen conjugate. Black polystyrene microliter plates were purchased from Costar (USA). A magnetic separation rack was purchased from Shanghai Allrun Nano Science and Technology Co., Ltd. (Shanghai, China).

2.4 Preparation of QD-Abs probes

Approximately 100 μL of the QDs (8 μM , 620 nm and 528 nm) was pipetted into a low-protein binding centrifuge tube, and 400 μL of PBS (0.01 M, pH=7.0) was mixed thoroughly with the QD solution. Then, 0.2 mg of anti-*S. enterica* antibody and 0.2 mg of anti-*E. coli* antibody were added and mixed well into the solutions of 528-nm QD and 621-nm QD, respectively. Next, 20 μL of fresh EDC solution (10 mg/mL) and 10 μL of NHS (10 mg/mL) were added and gently shaken at room temperature for 2 h. Then, 10 μL of quenching buffer was added, and the reaction was allowed to sit for another 15 min. This solution was transferred to a clean centrifugal ultrafiltration unit (100,000 MW), where it was centrifuged at 8000 rpm for 15 min at 4 °C. This step was repeated three times. The free non-conjugated antibody and byproduct of the conjugation reaction were removed by ultrafiltration. The two QD-Ab probes were re-dissolved using 200 μL of PBS (0.1% BSA, pH=7.4) and stored at 4 °C.

2.5 Fabrication of IMBs

First, 2 mg of magnetic beads was suspended in 2 mL of activated buffer (80 mM MES, pH=5.2). Then, 80 μ L of EDC (10 mg mL⁻¹) and 40 μ L of NHS (10 mg mL⁻¹) were added to the MB solution. After activation for about 30 min, the excess EDC, NHS, and byproducts were removed via magnetic separation using a magnetic scaffold. Subsequently, 0.2 mg of anti-*S. enterica* antibody and 0.2 mg of anti-*E. coli* antibody were simultaneously added to the activated MB solution. The mixture was gently stirred to react for 2 h at room temperature with gentle shaking at 120 rpm and then blocked with 3 % (m/v) BSA for 0.5 h. The IMB conjugate that can simultaneously capture *S. enterica* and *E. coli* in one sample was separated from the free Abs, re-suspended in 1000 μ L of PBS, and stored at 4 °C for further use.

2.6 The process of the QD-based immunosensor

The QD-RAS immunosensor procedure is illustrated in Scheme 1. First, 50 μ L of IMB solution, 50 μ L of two colors of QD-Ab probes, and 100 μ L of different concentrations of *S. enterica* and *E. coli* (10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10, 5, 1, and 0 cfu/mL) were transferred to 1.5-mL centrifuge tubes. Each mixture was gently shaken for 45 min. Subsequently, the IMB-bacteria-QD composites were separated by a magnetic scaffold, and the supernatant solution (surplus QD-Ab2 conjugate) was transferred into the wells of 96-well black plates. The fluorescence intensity of the surplus QD-Ab2 probes was then analyzed by a fluorescent microplate reader with excitation at 350 nm. The emission was simultaneously set as 528 nm and 620 nm for the QD-RAS immunosensor. The change of fluorescence intensity (ΔFI) in QD-RAS immunosensor was defined as follows: $\Delta FI = FI_{\text{blank sample}} - FI_{\text{sample}}$. To obtain the limit of detection (LOD), we employ the following formula: $LOD = 3S/M$ (S: the value of the standard deviation of blank samples; M: the slope of standard curve within the low

concentration range). Calculations were performed with Origin Pro 8.0 software. We also used TEM (200 kV JEM-2100F) and confocal microscopy to characterize the IMB-bacteria and remaining QD-Ab2 probes.

The IMB-bacteria-QD composites were re-suspended with 200 μ L of PBST solution and magnetically separated. This washing and re-suspension step was repeated three times, and the IMBs-bacteria-QDs immunocomplex was also transferred into 96-well black plates. The fluorescence signal was analyzed by a fluorescent microplate reader with excitation at 350 nm, and the emission was simultaneously set as 528 nm and 621 nm for the QD-DAS immunosensor. The change of fluorescence intensity (ΔFI) in QD-DAS immunosensor was defined as follows: $\Delta FI = FI_{\text{sample}} - FI_{\text{blank sample}}$.

2.7 Optimization of immunological reaction

Experimental factors involved in sandwich immunological reactions were optimized to improve the performance of the QD-based immunosensor. The concentrations of IMBs and QD-Ab2 conjugates were optimized to find the optimum experimental conditions for sandwich immunological recognition and the generation of the QD fluorescence signal. First, three concentration groups of IMBs (0.02, 0.05, and 0.1 mg mL⁻¹) were prepared to determine the maximum ability for capturing the target molecules. Second, four concentration groups of QD-Ab2 probe solutions (8 nM, 4 nM, 2 nM, and 1 nM) were used to optimize the fluorescence signal.

2.8 Sample analysis

Milk samples were centrifuged twice at 8000 rpm to remove supernatant, which includes fat. These samples were then re-dispersed in PBS solution and diluted tenfold for analysis.

3. Results and discussion

3.1 Characterization of QD-based immunosensor

We first investigate the change of fluorescence intensity of QDs when it conjugates the antibody, and find that this change can be ignored (**Figure S1**), which suggests that the bio-conjugation between QDs and antibody does not greatly affect the fluorescence properties of QDs. Meanwhile, the TEM images show that the IMBs can selectively capture *E. coli* in positive samples (**Figure S2**).

We also investigated the “IMBs-*E.coli*” conjugate and the fluorescence intensity of surplus QD-Ab2 probe by confocal microscopy (**Figure 1**). In the positive samples, IMBs can specifically capture *E. coli* (**Figure 1A**). In contrast, no *E. coli* was captured by the IMBs in negative samples (**Figure 1C**). The fluorescence intensity from the surplus QD-Ab2 probe in *E. coli*-positive sample is lower than that of the negative samples (**Figures 1 B and D**). This result suggests the formation of the “IMB-*E. coli*-QD” immune-complex in the positive sample, and this immune-complex can be separated in magnetic field. These results also show that the surplus QD-Abs probe can be used for the signal readout, and the QD-RAS immunosensor can be used to detect the pathogen.

Figure 1. Confocal microscopic images of *E. coli*: (A) IMBs-*E. coli* immune-complex in bright field; (B) fluorescence intensity of surplus QD-Ab probe in *E. coli*-positive sample; (C) IMBs in bright field; (D) fluorescence intensity of surplus QD-Abs probe in *E. coli*-negative sample.

To further investigate the effect of magnetic beads on the fluorescence signals of QD, we mixed the QD with different concentrations of magnetic beads (**Figure S3**). The fluorescence signal of the mixture consistently weakens as the concentration of magnetic beads increases from 0 to 500 µg/mL. This suggests that the large magnetic beads have a blocking effect on the small QD. The volume of the magnetic beads is six orders of magnitude larger than that of the QD. Excitation by diffracted or scattered light by the magnetic beads is much weaker than that by direct illumination.

Thus, the small QD cannot be effectively excited behind the large magnetic beads that block them. As a consequence, the QD-Abs probe captured on the surface of the IMBs cannot be effectively excited, and the fluorescence signal of the probe cannot be fully collected, which compromises the sensitivity of the QD-DAS immunosensor. In addition, this “IMB-target-QD” immune-complex needs multiple washing and re-suspension steps that are time consuming and result in instability. Therefore, we used the surplus QD-Ab2 conjugates to measure the signals to overcome these limitations.

3.2 Optimization of QD-RAS immunosensor

To increase the sensitivity of the QD-based immunosensor, many factors should be evaluated, including 1) the concentration of the IMBs, which influences the efficient capture of target bacteria and non-specific absorption, and 2) the concentration of the QD-Ab2 probe, which affects the fluorescence signal. We selected *E. coli* as an example to optimize the conditions of immunoreaction.

For IMB concentrations ranging from 0.02 to 0.1 mg/mL, the change in fluorescence intensity (ΔFI) increases as the concentration of *E. coli* increased from 10^4 to 10^6 cfu/mL in QD-DAS (**Figure. S4A**). ΔFI is largest at the same concentration of *E. coli* when the concentration of IMBs was 0.05 mg/mL. ΔFI also increased when the concentration of *E. coli* increased from 10^3 to 10^7 cfu/mL in QD-RAS, and the ΔFI was the most obvious when the concentration of IMBs was 0.05 mg/mL (**Figure. S4B**). At very low IMB concentrations, some *E. coli* cannot be captured completely. In contrast, at very high IMB concentrations, the background value was large due to non-specific absorption between the target bacterium and the IMBs. Thus, 0.05 mg/mL was chosen as the optimum concentration. The result shows that the lowest detectable

concentration of *E. coli* in the QD-RAS immunosensor was 10^3 cfu/mL, whereas that in the QD-DAS immunosensor was 10^4 cfu/mL. The preliminary result shows that the QD-RAS was superior to the QD-DAS, which supports our hypothesis.

We then optimized the concentration of the QD-Ab2 probe in the immunosensor. ΔFI increased remarkably as the concentration of *E. coli* increased from 10^5 to 10^7 cfu/mL when the concentration of the QD-Ab2 conjugate was 1.6 nM (**Figure. S5A**). When the QD-Ab2 probe concentration was 6.4 nM, the excess QD-Ab2 probe showed nonspecific absorbance on the surface of the IMBs, resulting in a larger background value than for the other concentrations. When the QD-Ab2 probe concentration was 0.8 nM, the ΔFI did not increase conspicuously with *E. coli* concentrations of 10^5 to 10^7 cfu/mL. This indicates that the IMBs-bacterium conjugate cannot capture enough QD-Ab2 when the concentration of *E. coli* is high. In the RAS, ΔFI is also greatly related to the concentration of *E. coli* when the QD-Ab2 probe concentration is 1.6 nM. However, this trend was not observed at QD-Ab2 probe concentrations of 6.4, 3.2, and 0.8 nM (**Figure. S5B**). The sensitivity depends on the ratio of the IMB-target-QD to the total amount of QD-Ab2 probe in RAS. When the probe concentration is too high (6.4 and 3.2 nM), much less of the probe is captured by the IMBs compared to the total amount of probe. This results in a negligible change in the trend of the fluorescence intensity of the surplus QD-Ab2 probe with respect to the *E. coli* concentrations. In contrast, when the probe concentration is too low (0.8 nM), there is not enough to be captured by the IMBs-bacterium conjugate. Based on these results, 1.6 nM of the QD-Ab2 probe was selected for subsequent steps.

3.3 Sensitivity and specificity

Under the optimized conditions, we investigated the sensitivity of the QD-based

immunosensor by measuring ΔFI of the samples with different concentrations of *S. enterica* and *E. coli*. In the QD-DAS immunosensor, ΔFI increased continuously as the *S. enterica* concentration increased from 10^3 to 10^7 cfu/mL, and the limit of detection (LOD) for detection of *S. enterica* was 720 cfu/mL (LOD=3S/M, S=0.06, M=2.5×10⁻⁴)(**Figure. 2A**). A linear relationship was observed between ΔFI and *S. enterica* concentration in the concentration range of 5×10^3 to 5×10^6 cfu/mL with an equation of $Y=0.29X-0.95$ ($X=\lg[C_{S. enterica}]$, $R^2=0.96$) (**Figure. 2B**). For detection of *E. coli*, ΔFI also increased when the *E. coli* concentration was increased from 10^4 to 10^8 cfu/mL, and the LOD was 1.5×10^3 cfu/mL (LOD=3S/M, S=0.03, M=6×10⁻⁵)(**Figure. 2C**). The relationship was also linear for ΔFI and the *E. coli* concentration in the concentration range of 10^4 to 10^7 cfu/mL ($Y=0.33X-1.14$, ($X=\lg[C_{E. coli}$, $R^2=0.98$) in the QD-DAS immunosensor (**Figure. 2D**).

In the QD-RAS immunosensor, the ΔFI increased when the *S. enterica* concentration increased from 10^2 to 10^7 cfu/mL, and the LOD for detection of *S. enterica* was 60 cfu/mL (LOD=3S/M, S=0.02, M=0.001) (**Figure. 2A**). A linear relationship was observed between ΔFI and the *S. enterica* concentration in the concentration range of 5×10^2 to 5×10^6 cfu/mL with an equation of $Y=0.36X-0.79$ ($X=\lg[C_{S. enterica}]$, $R^2=0.96$) (**Figure. 2B**). For detection of *E. coli*, the ΔFI increased when the *E. coli* concentration was increased from 50 to 10^8 cfu/mL, and the LOD was 30 cfu/mL (LOD=3S/M, S=0.05, M=0.005) (**Figure. 2C**). ΔFI was linear with respect to the *E. coli* concentration in the concentration range of 10^3 to 10^7 cfu/mL ($Y=0.39X-0.78$ ($X=\lg[C_{E. coli}]$, $R^2=0.99$) in this immunosensor (**Figure. 2D**). These results show that the sensitivity and linear range of QD-RSA immunosensor are better than those of QD-DAS immunosensor, which further prove that the RSA can improve

effective fluorescence signal in the QD-based immunosensor.

Figure 2. Sensitivity and linear range of the QD-based immunosensor for multiplexed detection of *S. enterica* and *E. coli* in PBS solution with 3% skim milk powder. (A, B) Results of QD-based immunosensor for detecting *S. enterica*. (C, D) Results of QD-based immunosensor for detecting *E. coli*. The concentrations of *E. coli* and *S. enterica* are from 10 to 10^8 cfu/mL.

Five strains were used to evaluate the selectivity of the immunosensor for detection of *E. coli* (**Figure. S6**). The ΔFI value was larger in *E. coli* group than that of other groups. In the control group, QD-Ab2 conjugate was replaced with QD to confirm that ΔFI was caused by the specific binding between the IMB-bacterium and QD-Ab2 conjugates and not from nonspecific effects. As expected, the QD did not

specifically bind to the IMB-*E. coli* conjugates, resulting in minimal changes in fluorescence intensity. This result confirms that the ΔFI originated from the antigen-antibody interaction rather than the exclusion of nonspecific interactions. The results also show that the QD-RAS immunosensor has good specificity to *E. coli*.

Compared with the QD-DAS immunosensor, QD-RAS immunosensor has two advantages: (1) the sensitivity was improved by 10 times with an enhanced linear detection range. The IMBs in the conventional QD-DAS immunosensor (200-1000 nm) are much larger than the QD (2 to 10 nm). Upon laser irradiation of the top surface of the QD-IMB conjugate, some of the QD-Ab2 conjugate that was captured on the other surfaces of the IMBs could not be excited. Thus, some of the effective fluorescence signal is lost, which compromises the sensitivity. The results in Figure 1 also support this explanation. (2) One-step detection. The fluorescence signal from surplus QD-Ab probe in QD-RAS immunosensor can avoid the multiple washing and re-suspension steps that required in conventional QD-DAS immunosensor. Multiple washing and re-suspension steps are two important factors that result in uncontrolled error and are time consuming.

ELISA was also used as a control method for detection of *E.coli* (**Figure. S7**). Compared to ELISA, the sensitivity of the QD-RAS immunosensor improved fifty- fold, and the whole analysis can be finished in one-step within 1 h. Table 1 summarizes the advantages and disadvantages of ELISA, the conventional QD-DAS immunosensor, and the QD-RAS immunosensor.

Table 1. Comparison between ELISA, QD-DAS immunosensor and QD-RAS immunosensor for detection of *E.coli*.

Approach	LOD	Linear range	Detection time
ELISA	5×10^3 cfu/mL	10^4 - 10^6 cfu/mL	4-6 h
QD-DAS	1.5×10^3 cfu/mL	10^4 - 10^7 cfu/mL	1.5 h
QD-RAS	30 cfu/mL	10^3 - 10^7 cfu/mL	1 h

3.4 Sample analysis

To determine the reliability of the immunosensors, they were used to quantify *E. coli* and *S. enterica* in milk samples simultaneously. We apply the QD-RAS immunosensor for detection of *E.coli* in different spiked concentrations to investigate the accuracy and practical applications of this method. The recovery is from 80% to 105% with the relative standard deviations (RSD) under 6% (**Table S1**), which shows that this method can detect *E.coli* in spiked samples. We further detect *E. coli* and *S. enterica* in real milk samples. Sample 9 and sample 10 were detected as negative for *E. coli* by the QD- DAS immunosensor (**Figure 3A**), while they were detected as positive by the QD-RAS immunosensor and RT-PCR (**Figure 3B**). All the samples were detected to be free of *S. enterica* using the QD- DAS immunosensor (**Figure 3C**). Samples 7 and 9 were detected as being polluted by *S. enterica* by the QD-RAS immunosensor and RT-PCR (**Figure 3D**). The QD-RAS immunosensor is more accurate than the QD-DAS immunosensor, suggesting that the RAS can greatly improve the performance of QD-based immunosensor. Thus, QD-RAS is a sensitive and reliable immunoassay for detection of pathogens in real samples.

RT-PCR is the gold-standard method for detection of pathogens because of its high sensitivity and accuracy, but it is expensive and time consuming (2 -3 h). Samples are also easily polluted, which hinders application in point-of-care testing. In contrast with RT-PCR, the QD-RAS immunosensor can be completed conveniently in one step (1 h) with high sensitivity and multiple analyses, which provides an attractive platform for the detection of pathogens.

Figure 3. Results of QD immunosensor for simultaneous detection of *E. coli* and *S. enterica* in milk samples. **(A)** QD-DAS immunosensor for *E. coli*. **(B)** QD-RAS immunosensor for *E. coli*. **(C)** QD-DAS immunosensor for *S. enterica*. **(D)** QD-RAS immunosensor for *S. enterica*. According to the RT-PCR method, samples 1 to 5 are negative samples, samples 6 to 20 are positive for *E. coli*, and samples 7 and 9 are positive for *S. enterica*.

4. Conclusion

We have developed a highly sensitive and one-step QD-RAS immunosensor to determine *S. enterica* and *E. coli* simultaneously in milk samples. RAS can effectively improve the sensitivity and avoid the multiple washing steps of a QD-based immunosensor. Combined with MS, the whole analysis can be finished in one step for multiple-pathogen analysis, which greatly improves the detection efficiency. Further studies should use this QD-RAS immunosensor and microfluidic technology to realize smart, miniaturized, and automatic detection in biochemical analysis.

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