Application of F0F1-ATPase immuno-biosensors for detecting *Escherichia coli* O157:H7

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Abstract. *Escherichia coli* (*E. coli*) O157:H7 is an important food-borne pathogen with a low infective threshold and high resistance to treatment. There are currently a number of detection methods available, however, the majority are time-consuming, complex and expensive, thus it is hard for these methods to be applied in routine detection. Therefore, there is urgent requirement to develop more sensitive, rapid and specific detective techniques. In the present study, an immuno-biosensor based on the interference of load to the F0F1-ATPase rotation, indicated by the fluorescence fluctuation, was constructed to detect O157:H7. The results demonstrated a good linear relationship (R^2=0.9818) between antigen concentration (range, 10^2 cfu to 10^4 cfu) and the fluorescence intensity. The detection signals of the samples containing 10^2 cfu/well and 10^4 cfu/well *E. coli* O157:H7 were significantly stronger than the signal produced by the control sample (P<0.01). Due to its higher sensibility and simplicity when compared with the current methods applied, the results of the present study indicate a promising future for the application of this technique in detecting food source pathogens.

Introduction

Escherichia coli (*E. coli*) O157:H7 is a type of pathogenic bacterium that infects humans and livestock primarily through contaminated food. It can cause abdominal pain, hemorrhagic fever or bloody stools, and can induce secondary haemolytic uraemic syndrome in infants, preschool children or weak, elderly individuals. In addition, due to its strong drug resistance, it is very hard to eliminate O157:H7 from contaminated food sources. O157:H7 contamination has now become an international food security concern (1). The American Centers for Disease Control has revealed that *E. coli* O157:H7 is one of the major pathogenic bacteria causing food-borne diseases; thus, poses a serious threat to public health. Furthermore, this strain has been detected in pork, beef and mutton in China (2).

There are several detection methods currently used for pathogenic bacteria, including culture-based, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR)-based methods (3-5). However, these methods are usually time-consuming, expensive and insensitive, which makes them unsuitable for the detection of this pathogen. Therefore, it is necessary to develop more efficient detection apparatus.

F0F1-ATPase, located in the mitochondria and/or the chloroplast thylakoid of eukaryotic organisms and the bacterial plasma membrane, catalyzes the synthesis of ATP using the transmembrane proton gradient. In *E. coli*, the soluble F1 and transmembrane F0 parts are comprised of the α3β3γδε and ab2cn subunits, respectively. These two parts are connected by the stalks of γε in the centre and b2 δ on the outside. When the downhill proton passes through F0, the c and γε subunits are rotated leading to conformational changes in F1, which promotes the formation of ATP from ADP and inorganic phosphate, and vice versa. As a result, F0F1-ATPase forms a molecule size motor, which can transform the electric chemical potential energy into chemical energy. If this process is disturbed by other factors, the rate of ATP synthesis and proton flux maybe altered; this phenomenon maybe reflected by pH sensitive substances (6-7). F1300 is a pH sensitive fluorescent probe that can be used as an indicator of changes in the pH of F0F1-ATPase. During ATP synthesis, protons are pumped out of the chromatophore and this transfer of protons is detected by F1300 (4-5,8). This concept was used to construct the immuno-rotary biosensor (IRB) for detecting specific targets, which achieved great success (4-5,9-10). Although this type

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of biosensor has been used to detect a virus (3,6-7), detection of much larger antigen such as a single bacterium has not been reported. The aim of the present study was to investigate the potential use of this method for the detection of *E. coli* O157:H7.

Materials and methods

Bacterial strains. E. coli O157:H7 (strain no. ATCC35150) was obtained from the Guangdong Microbial Culture Collection Center (Guangzhou, China) and was incubated in nutrient broth medium at 37˚C for 24 h. The bacterial suspension was then diluted to 10^{-4}, 10^{-5} and 10^{-6} in bacteria-free PBS. A total of 100 μl was transferred to a panel for further cultivation and each dilution gradient sample was tripled. The bacterial clone in the incubated sample was counted 24 h later. Surplus bacteria were in activated by heating to 80˚C for 1 h, then 10 ml was centrifuged for 30 min at 4,000 x g and 4˚C. The supernatant was discarded and the precipitate was resuspended with sterile normal saline (NS) to the original volume. This process was repeated twice to remove medium complex components and the precipitate was resuspended with sterile NS to the original volume following the third centrifugation.

Salmonella (strain no. ATCC14028; American Type Culture Collection, Manassas, VA, USA) and *Escherichia coli* (*E. coli*; strain no. CMCC-44101; China Medical Culture Collection, Beijing, China) were also subjected to the same procedure as O157:H7; they were tested with the same methodology to estimate the specificity of O157:H7.

Preparation of 'signal into components'. 'Signal into components' is a chromatophore with the pH sensitive fluorescent probe, F1300.

*Preparation of chromatophores. Thermomicrobium-roseum*wa0073 (ATCC27502) were purchased from the American Type Culture Collection and incubated at 60˚C for 24 h. The cells were harvested by centrifugation at 4,000 x g for 30 min at 4˚C and resuspended in buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 0.1 mM PMSF and 2 mM $MgCl_2$, pH 8.0) followed by ultrasonication for 10 min in an ice bath. The suspension was centrifuged at 10,000 x g for 30 min at 4˚C to remove unbroken cells and cell fractions. The cell-free supernatant was centrifuged at 40,000 x g for 1 h at 4˚C to collect membrane vesicles, termed chromatophores. The chromatophores were stored in 50% glycerol at -80˚C. The chromatophore structure is presented in Fig. 1.

Labeling of chromatophores with F1300. The chromatophores were centrifuged at 12,000 x g at 4˚C for 30 min to remove glycerol, then they were resuspended with buffer (pH 6.0, 0.1 mM tricine, 5 mM $MgCl_2$ and 5 mM KCl). A total of 1-2 μl F1300 (1 mg/ml) was added to 600 μl chromatophores prior to ultrasonication for 3 min in an ice bath, to incorporate the probe into the inner part of the chromatophores. The free F1300 fraction was purified by centrifugation at 12,000 x g for 30 min at 4˚C. The purification process was repeated three times to remove free F1300, and aliquots of the supernatant were collected to assess the level of purification. The precipitate was resuspended in tricine-NaOH buffer

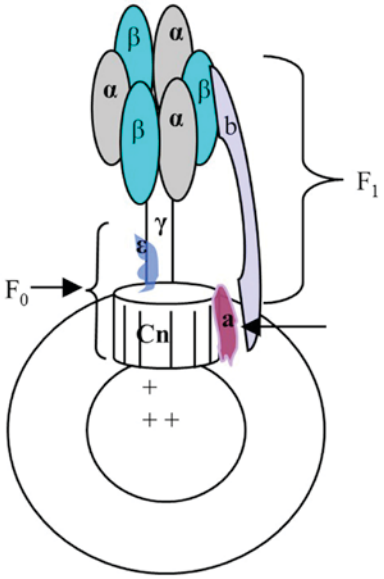

Figure 1. Schematic view of F1300 chromatophores. The arrow indicates the F0F1-ATPase carrier. The chromatophore provides H^+ for ATP-synthesis. The chromatophore and the F0F1-ATPase are the organic combination. Cn, subunit c; a, subunit a; b, subunit b.

(0.1 mM tricine, 5 mM $MgCl_2$ and 5 mM KCl, pH 6.5) and then the relative fluorescence signal was detected using the Varioskan Flash spectral scanning multimode reader (excitation, 485 nm; emission, 538 nm; Thermo Fisher Scientific, Inc., Waltham, MA, USA). When the relative fluorescence signal did not decrease further, the free F1300 was removed completely. The ATP hydrolysis activity of the labeled chromatophores was assayed using the enzyme coupling method with pyruvate kinase and lactate dehydrogenase, as described previously (4). The unit of enzyme activity was defined by hydrolyzing 1 μmol ATP per minute with 1 mg chromatophores (Fig. 2).

Constructing the O157:H7 detector. A ATPase β-subunit antibody [Homemade, as previously described (10)], biotin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and an antibody targeted against O157:H7 (cat. no. AB81131; Abcam, Cambridge, UK) were used for the following: 2 μl of 10 mM biotin was added to 500 μl of 3 mg/ml β-subunit antibody for 15-30 min at room temperature, then free biotin was removed via dialysis to produce the β-subunit antibody-biotin complex. In addition, 2 μl of 10 mM biotin was added to 500 μl of 3 mg/ml O157:H7 antibody for 15-30 min at room temperature, then free biotin was removed via dialysis to produce the O157:H7 antibody-biotin complex. To create the capture system complex, a reaction was set up that contained equal amounts ofβ-subunit antibody-biotin complex (200 μl, 50 mM) and O157:H7 antibody-biotin complex (200 μl, 50 mM). Subsequently, 200 μl 55 mM streptavidin (Sigma-Aldrich; Merck KGaA) was added and incubated for 15-30 min at room temperature. This produced the capture system complex: the β-subunit antibody-biotin-Streptavidin-biotin-O157:H7 antibody (Fig. 3). The capture system complexes and chromatophores labeled with F1300 (F1300-ch) were then mixed to a 4:1

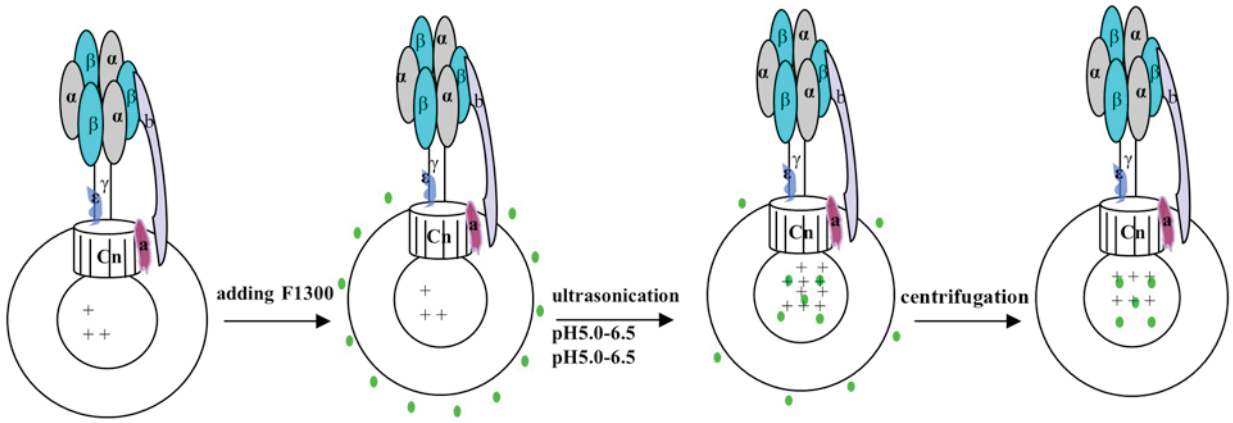

Figure 2. Schematic view of the F1300-labelled inner chromatophores (green dots). Cn, subunit c; a, subunit a; b, subunit b.

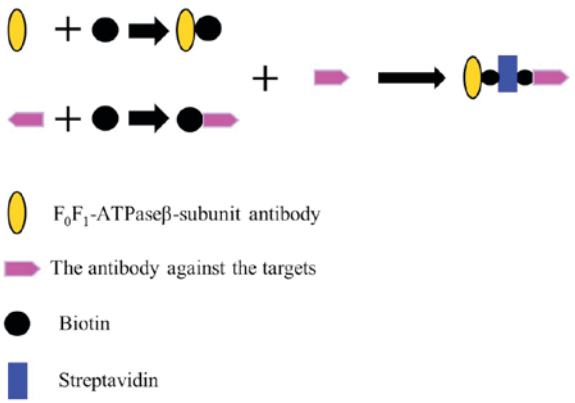

Figure 3. Schematic view of the process applied to establish the capture system complex.

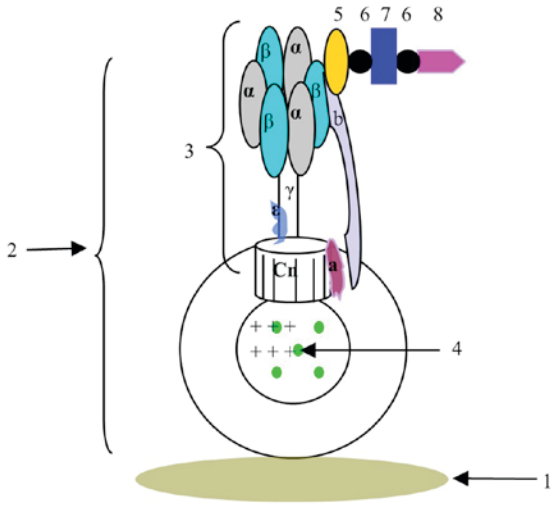

Figure 4. Schematic view of the biosensor constructed based on F0F1-ATPase. 1, detection carrier; 2, chromatophore complex; 3, ATPase; F1300-ch; 5, β-subunit antibody; 6, biotin; 7, streptavidin; 8, O157:H7 antibody; Cn, subunit c; a, subunit a; b, subunit b.

dilution, and were incubated at 37˚C for 1 h. This produced the biosensor used in the present study (Fig. 4). Three different reactions were set up, Group A, Group Band Group C, to demonstrate that the construction of the immuno-biosensor was successful, based on the fluorescence of F1300-ch with different loads: Group A, F1300-ch control; Group B, F1300-ch-β-subunit antibody-biotin-Streptavidin complex; Group C, F1300-ch-β-subunit antibody-biotin-Streptavidin-biotin-O157:H7 antibody complex.

Fluorescence assay. The concentration of the bacterial suspension was adjusted to $5x10^3$, $5x10^4$ and $5x10^5$ cfu/ml. In order to generate 10^2, 10^3 and 10^4 cfu bacteria/well, respectively, four groups were set up: Group 1, control (sterile NS); Group 2, 10^2 cfu bacteria/well; Group 3, 10^3 cfu bacteria/well; Group 4, 10^4 cfu bacteria/well. To each well, 50 μl biosensor and 20 μl of the bacterial suspension were added. Following incubation for 30 min at 37˚C, 70 μl ATP synthesis buffer (50 mmol/l tricine, 10% glycerol, 2 mmol/ADP, 5 mmol/l NaH_2PO_4 and 5 mmol/l $MgCl_2$, pH 8.0) was added to each well for further incubation at 45˚C for 15 min. The relative fluorescence signal was detected using the Varioskan Flash spectral scanning multimode reader (excitation, 485 nm; emission, 538 nm; Thermo Fisher Scientific, Inc.).

Specificity. ATCC14028 *Salmonella* and CMCC44101 *E. coli* were subjected to the same protocols in order to determine the specificity to O157:H7. The bacterial concentration for each group and pathogenic bacteria are presented in Table I.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 10 software (SPSS, Inc., Chicago, IL, USA). The correlation was assessed by linear regression and a Dunnett's T3 post hoc test was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Construction of the immuno-biosensor. The inner chromatophores were successfully labeled with the fluorescent pH indicator F1300 as a unidirectional label (Fig. 5; Table II). The fluorescence intensity of the control chromatophores was the lowest. The fluorescence intensity of the mixture of chromatophores and F1300 was much higher than the control; however,

Table I. Bacterial concentration in assay wells to estimate the specificity of the O157:H7 biosensor.

Strain of pathogenic bacteria	Bacterial concentration (cfu/well)				
	Control	Group 2	Group 3	Group 4	Group 5
E. coli O157:H7	0	27	133	265	1,325
E. coli CMCC44101	0	35	175	350	1,750
Salmonella ATCC14028	0	26	128	255	1,275

E. coli, *Escherichia coli*; cfu, colony forming units; Control, contained sterile normal saline and 0 cfu/well O157:H7.

Table II. Results of F1300 labelling in the inner chromatophores.

Group	Relative fluorescence value
Chromatophores	0.519
Fluorescence labeling	18.12
1st centrifugation	6.69
2nd centrifugation	2.82
3rd centrifugation	1.49
4th centrifugation	1.48

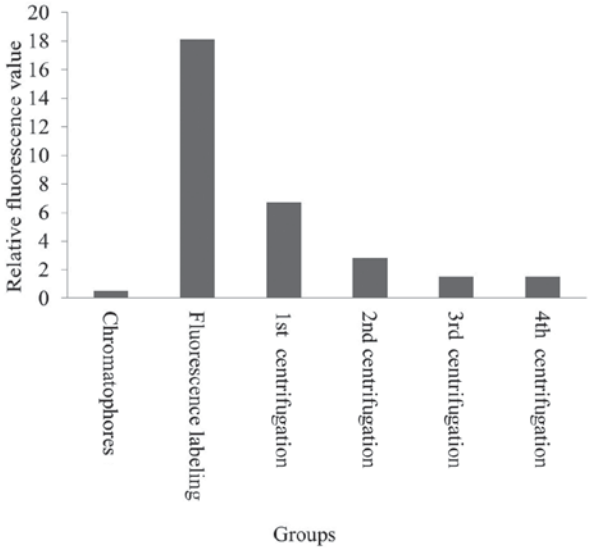

Figure 5. Relative fluorescence value of the F1300-labeled inner chromatophores. Activity of F1300-ch in the control, fluorescence labelled and the 4 *E. coli* centrifugation groups. Following the incorporation of F1300 into the chromatophore the mix was centrifuged 4 times to produce the 4 *E. coli* centrifugation groups. This was performed to verify that the inner chromatophores were labeled with F1300 successfully.

it decreased with ultrasonication and the four subsequent centrifugation steps. Initially, a part of F1300 entered the chromatophore with ultrasonication; however, the remaining free F1300 stayed out of the chromatophore, producing high fluorescence intensity. Following three centrifugation procedures, the fluorescence intensity decreased to 1.49. This indicated that the free F1300 were removed by centrifugation. The fluorescence intensity did not change following the fourth round of centrifugation; it was 2.8 times higher than that of the control. The results revealed that free F1300 was completely removed, while the fluorescent probe F1300 labeled the inner chromatophores. The synthetic activity of F1300-chisshown in Fig. 6; the enzyme activity was 106.4 μmol/mg/min.

As presented in Fig. 7, the fluorescence intensity of group A was the highest, while group C was the lowest. This indicated that the capture system complex (β-subunit antibody-biotin-Streptavidin-biotin-substrate antibody) was successfully established. In addition, it revealed that the greater the load on F0F1-ATPase, the lower the enzyme activity and thus, the lower the relative fluorescence. Therefore, the F1300-labeled chromatophores were used in the present study.

Sensitivity for O157:H7. The fluorescence value gradually decreased with the increasing concentration of O157:H7 (Fig. 8; Table III). Following statistical analysis, the results revealed that there were significant differences between the control and 10^4 cfu group ($P<0.01$), and between the 10^2 and 10^4 cfu groups ($P<0.01$). The results of O157:H7 detection using this method identified a strong positive gradient between 10^2-10^4 cfu ($R^2=0.9818$).

Specificity for O157:H7. The curve of 10^1-10^3 cfu identified a good separation, which is consistent with the positive threshold value for O157:H7, 0.063-0.075. The relative fluorescence value of the CMCC44101 *E. coli* groups was 0.035-0.043 and 0.035-0.052 for *Salmonella*. These results demonstrated that this biosensor has specificity for O157:H7 (Fig. 9; Table IV).

Time of detection. This method is short and includes only four steps: bacterium solution treatment, preparation of biosensors, loading and testing. The time required for each step was 2, 1.75, 0.5 and 0.25 h, respectively, thus 4.5 h in total. Though this method requires separation and enrichment of target bacteria when testing samples, the limit of detection was 100 cfu. In addition, by combining it with the immune magnetic separation technique, the time required for sample pretreatment was 8.5 h and the total time for testing samples was <16 h.

Discussion

F0F1-ATPase has the following two characteristics: i) it can use the H^+ gradient between the inside and outside of chromatophores to produce ATP and it can also hydrolyze

Table III. Results of the comparison between the concentration groups.

Concentration group (cfu/well)		P-value
0	10^2	0.19239
0	10^3	-0.06347
0	10^4	0.00327[a]
10^2	10^3	0.63584
10^2	10^4	0.00015[b]
10^3	10^4	0.60539

Comparisons were performed using Dunnett's T3 test. [a]P<0.01, 0 vs. 10^4; [b]P<0.01, 10^2 vs. 10^4 cfu, colony forming units.

Table IV. Results of the comparison between the O157:H7, *E. coli* and salmonella groups.

Group comparison	P-value
O157:H7 vs. *E. coli*	
Group1	0.69
Group 2	$2.81x10^{-5}$[a]
Group 3	$1.03x10^{-4}$[a]
Group 4	$1.89x10^{-7}$[a]
Group 5	$9.82x10^{-7}$[a]
O157:H7 vs. Salmonella	
Group 1	0.36
Group 2	$2.15x10^{-5}$[b]
Group 3	$2.00x10^{-4}$[b]
Group 4	$1.16x10^{-6}$[b]
Group 5	$4.92x10^{-5}$[b]

[a]P<0.01, vs. *E. coli*; [b]P<0.01, vs. salmonella.

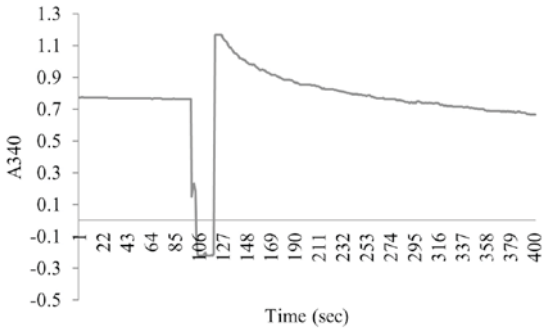

Figure 6. Synthetic activity of the F1300-labelled chromatophores (F1300-ch).

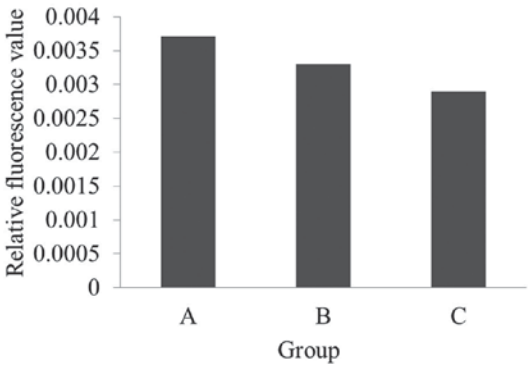

Figure 7. Effects of the biosensor based on F0F1-ATPase for O157:H7. This experiment was performed to verify that the F0F1-ATPase-based biosensor for the detection of O157:H7 was constructed successfully. Group A, F1300-ch; Group B, F1300-ch-β-subunit antibody-biotin-Streptavidin complex; Group C, F1300-ch-β-subunit antibody-biotin-Streptavidin-biotin-O157:H7 antibody complex.

ATP by reverse transporting H^+. During ATP synthesis, protons are pumped to the outside from the inside of chromatophores, which leads to a change in proton concentration inside the chromatophores; ii) F0F1-ATPase rotation speed and the loads on its subunits are positively correlated. Based on its two enzymology characteristics, the F0F1-ATPase nano-biosensor is labeled by pH sensitive F1300, a fluorospectrophotometric probe, to produce the functional unit, F1300-ch. The F1300-ch combined with the capture system achieves the molecular motor nano-biosensor, which can be used as a fast detection technique (7,11-13). Liu *et al* (8) directly observed the mechanically driven proton influx or efflux in F0 coupled with rotation of F1 at a single molecular level; the specific underlying mechanism will be studied further in the future.

F0F1-ATPase activity is regulated by external links on b subunits with different molecular weights. It is inhibited when anti-b subunit antibodies, streptavidin and H9 antibodies link on to the β subunits successively (7). The holoenzyme activity was inhibited as it linked to more external substances, including Chloramphenicol, Listeria monocytogenes, H9 virus, Clenbuterol and Deoxynivalenol (4-5,7,9,10). When the O157:H7 loads into the chromatophore, the chromatophore cannot move completely and there are no alterations in protons between the internal and external chromatophore, therefore the alteration in relative fluorescence intensity should be generated by those non-O157:H7-loaded chromatophores in each detection well. In another way, this is similar to the competition method in ELISA; the stronger the concentration, the lower the changing biosensors and so the smaller the change value (14,15).

The application of F0F1-ATPase immuno-biosensors for the detection of O157:H7 has not been reported previously. The present study used biosensors to detect O157:H7, demonstrating that this method is rapid, sensitive, simple and has a low cost.

When compared with other novel detection methods, this method is faster, more sensitive and easier to operate. In addition, the present study investigated its specificity, as well as the feasibility of this method using standard strains. The results demonstrated that it has a good specificity to *E. coli*. Table V presents a comparison of the results between the current different methods. At present, the sophisticated testing methods of pathogenic microorganisms include the microbial method, PCR and ELISA. The microbial method

Table V. A comparison of the current methods used for detection.

Method	Time	Detection limit	Specificity	Cost
F0F1-ATPase immuno-biosensor	13 h	1-5 cfu/25 g	95%	Very low
Microbial method	5-7 d	1 cfu/25 g	100%	Low
PCR	>48 h	10^2-7x10^3 cfu/ml	99% (DNA easily polluted)	High
ELISA	36-48 h	10^6 cfu/ml	95%	Low
Reverse transcription PCR	48 h	1-5 cfu/25 g	99% (DNA easily polluted)	High
SPR biosensor	>24 h	2x10^6 cfu/ml	95%	High
CEZ	>24 h	10^5-10^6 cfu/ml	95%	Low

PCR, polymerase chain reaction; SPR, Surface plasmon resonance; CEZ, capillary zone electrophoresis; cfu, colony forming units.

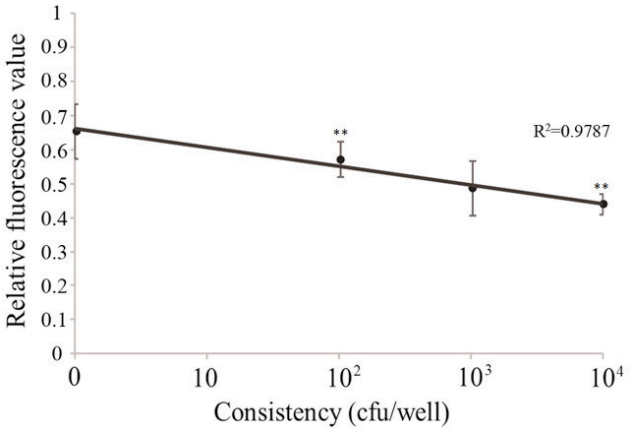

Figure 8. Sensitivity for O157:H7 in the 0 (control), 10^2, 10^3 and 10^4 cfu/well groups. R^2=0.9787, P=0.009163. **P<0.01 vs. 0 cfu/well (control).

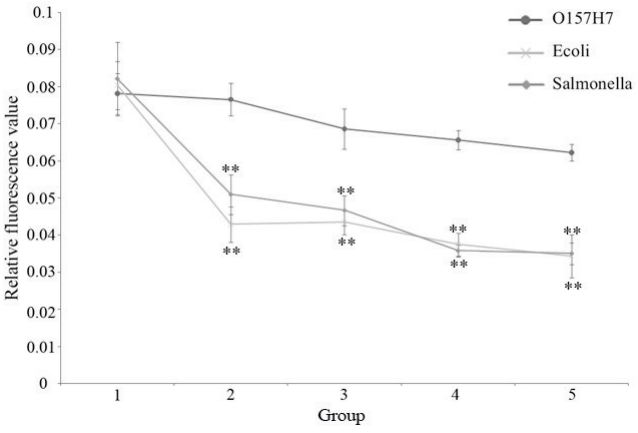

Figure 9. Specificity for O157:H7 in Groups 1 to 5, comparing O157:H7, Salmonella and *E. coli*. **P<0.01 vs. O157:H7. *E. coli, Escherichia coli*; Group 1, control (sterile normal saline); Group 2, 27, 35 and 26 cfu/well for O157:H7, *E. coli* and *Salmonella*, respectively; Group 3,133, 175 and 128 cfu/well for O157:H7, *E. coli* and *Salmonella*, respectively; Group 4, 265, 350 and 255 cfu/well for O157:H7, *E. coli* and *Salmonella*, respectively; Group 5, 1,325, 1,750 and 1,275 cfu/well for O157:H7, *E. coli* and *Salmonella*, respectively.

is time-consuming and involves complicated processes. PCR is the most mature method in the national testing methods of pathogenic microorganisms, however, its reagents are expensive and the procedure is complex. In addition, as this method is extremely sensitive, the experimental conditions, the exogenous DNA, improper controls, primer design and the target selection of sequence will all affect the results. The ELISA method is relatively time-efficient; however, it requires skilled operation and a detection limit of 10^6 cfu/l. Surface plasmon resonance, biosensors, capillary zone electrophoresis and other technologies are also being studied by the researchers, as they are quicker than the microbial method and simpler than PCR; however, they require expensive instruments and the low detection limit is 10^5-10^6 cfu/ml (16-23).

The present study performed preliminary research on the feasibility of applying F0F1-ATPase immuno-biosensors for O157:H7 detection. The detection techniques based on F0F1-ATPase can rapidly detect the disease markers in patient serum or feces, which will aid rapid clinical diagnosis. To promote its application, novel fluorescent material could be chosen as fluorescent probe to improve the sensitivity and accuracy of this method (21-25). In addition, high specificity immune magnetic beads could be used to enrich the target bacterial, which can minimize the interference of other bacterial (26-28). Due to the complexity of the sample and the variety of bacteria in different samples, the application of a biosensor for the detection of pathogens is rarely reported. Furthermore, the application of biosensors in pathogenic bacteria detection is rarely reported; therefore, sample pretreatment, and increasing bacteria and bacteria solution treatment, will require extensive research in practical sample testing.

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