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ORIGINAL PAPER

Rapid Fluorescent Detection of *Escherichia coli* K88 Based on DNA Aptamer Library as Direct and Specific Reporter Combined With Immuno-Magnetic Separation

Zhihui Peng · Min Ling · Yi Ning · Le Deng

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Abstract Nucleic acid aptamers have long demonstrated the capacity to bind cells with high affinity so that they have been utilized to diagnose various important pathogens. In this study, a DNA aptamer library was on initial efforts developed to act as a specific reporter for rapid detection of enter toxigenic *Escherichia coli* (ETEC) K88 combined with immuno-magnetic separation (IMS). During a Whole-cell Systematic Evolution of Ligands by Exponential Enrichment (CELL-SELEX) procedure, the last selection pool against ETEC K88, which is named "DNA aptamer library" here, was selected and subsequently identified by flow cytometric analysis and confocal imaging. A K88 monoclonal antibody (mAb) with high affinity (K_{aff}: $1.616\pm0.033\times10^8$ M^{-1}) against K88 fimbrial protein was prepared, biotinylated and conjugated to streptavidin-coated magnetic beads (MBs). After the bacteria were effectively captured and enriched from the complex sample by immuno-magnetic beads (IMBs), 5′-FITC modified aptamer library was directly bound to target cells as a specific reporter for its detection. The detection system showed clearly high specificity and sensitivity with the detection limit of 1.1×10^3 CFU/ml in pure culture and 2.2×10^3 CFU/g in artificially contaminated fecal sample. The results also indicated that fluorophore-lablled DNA aptamer library as specific reporter could generate more reliable signals than individual aptamer with best affinity against target cells and implied it would have great applied potential in directly reporting bacteria from complex samples combined with IMS technology.

Keywords ETEC K88 detection · IMS · Aptamer library · Reporter

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Introduction

Some pathogens such as *Escherichia coli*, *Salmonella*, *Listeria*, *Staphylococcus aureus*, *Leishmania* and *Vibrio* from food, contaminated water and other sources are serious threats to human beings and farming animals [1, 33, 37]. For latter, especially for weanling piglets, diarrhea caused by ETEC K88 is an important factor of both mortality and reduced growth rate resulting in heavy economic losses. Swift and precise identification of ETEC K88 from infected samples is critical for the subsequent treatment of infectious diseases caused by ETEC K88 [9, 36]. K88 fimbriae, expressed on the surface of K88 ETEC strains, are responsible for their adhesion and colonization on small intestinal and producing enterotoxins, as well as being special detection target to ETEC K88 [23, 21, 35]. In fact, in clinical diagnosis, it's very hard to simply and quickly distinguish ETEC K88 from other pathogens resulting in diarrhea of early weanling piglets by the commonly used methods, which makes some very good preventive or treatment products such as specific vaccine, egg yolk antibody not in widespread use at low cost and leads to abuse of antibiotics in livestock breeding [20, 39]. The most reliable method to detect those pathogens including ETEC K88 is traditional plate incubation, however, which is known to be labor-intensive and time-consuming. To date, various new detection platforms were developed, such as enzyme-linked immunosorbent assay (ELISA) [4, 7, 19], fluorescent barcodes PCR-based techniques [27, 33], electromechanical systems [16, 18, 24], fluorescent resonance energy transfer (FRET) system [5, 6] and so on, however, few of which succeed in commercial applications in direct detection of pathogens till now.

Herein, we presented a strategy to rapidly detect ETEC K88 at low concentration based on DNA aptamer library as a direct specific reporter combined with IMS technology. Aptamers are single stranded nucleic acids that are able to fold in to well-defined tertiary structures upon target binding

and generated via SELEX [14, 28, 38]. Generally, CELL SELEX technology is utilized to select aptamers against certain cell [11, 25, 32]. Today, many aptamer biosensors have been developed to diagnose various pathogens. However, in most cases, "individual aptamer/monoclonal aptamer" or "polyclonal aptamer" composed of 2–4 individual aptamers is applied to detection system and indirectly reports targets with complicated detection strategy [10, 26, 30]. The strategies of fluorophore-lablled individual aptamer as direct reporter for bacteria cell detection are simple, however, commonly with low detection signal. Whether an aptamer selection pool composed of many aptamers that may bind different sites of target cells can generate stronger signals in a simple reporting system or not, a series experiments will be done to answer it. In our strategy, IMBs against ETEC K88 will be prepared and confirmed the ability to enrich target cells from complex sample. Meanwhile, the last selection of aptamer pool with high affinity against the same bacteria during CELL-SELEX procedures, which is so called "DNA aptamer library" in this paper, will be selected and used as a specific reporter to directly report its targets. An individual aptamer with best affinity against the bacteria will be also screened and characterized for comparison. The schematic of the detailed detection strategy is presented as Fig. 1.

Materials and Methods

Bacterial Strains

The strains of ETEC K88 (CVCC 216) and ETEC K99 (CVCC 193) were from China Veterinary Culture Collection Center (CVCC), cultured in Luria–Bertani (LB) medium and harvested at an early log growth phase. *Salmonella enteritidis* (*S. Enteritidis*, ATCC13076) and *Staphyloccocus aureus* (*S. aureus*, CMCC 26,113) were obtained from the Basic Medicine Department of Hunan University of Traditional Chinese Medicine. Bacterial concentrations were determined by serial dilutions followed by plate counts on agar medium.

Purification of ETEC K88 Fimbrial Antigen

After centrifugation at 8,000× g for 10 min at 4 °C, the sediment of bacteria was washed three times and then suspended in phosphate-buffered saline (PBS, 50 mM, pH 7.2). Fimbriae were detached from bacteria by heat shock at 60 °C for 30 min. The bacterial suspension was centrifuged at 8,000×g for 10 min and the pellet was discarded. Subsequently, K88 fimbriae were precipitated by slowly adding ammonium sulphate 60 % (w/v). Precipitated protein was collected by centrifugation at 10,000×g for 20 min, resuspended in PBS, and dialyzed for 24 h with at least two changes of the buffer. Insoluble material was removed by centrifugation at 8,000×g for 20 min. Size-exclusive (SE) chromatography was performed on a Tskgel SW3000 column for further purification (Waters 2,695 HPLC system). The chromatographic solution at right peak was collected and concentrated by Amicon Ultra centrifugal device (10 Kd, Minipore, USA). The protein samples before and after chromatography were both taken to run SDS-PAGE to check the efficiency of purification. Protein concentration was estimated by the method of Bradford with bovine serum albumin (BSA) as standard (Bradford kit, Sangon Biotech, shanghai, China).

Preparation of K88 mAb

The purified K88 fimbrial proteins were used to immune five female BALA/c mice (Yingrun Biotech, Changsha, China) at 20 day intervals, all of which developed a serum response to antigen as measured by indirect ELISA (Multiskan Ascent, Labsystems, Helsinki, Fenland). The mouse with the highest titer was intraperitoneally injected, then used in hybridoma fusions that yielded a total of 30 positive cultures having antibodies against K88 fimbrial proteins. Two times subclones were performed by limiting dilution resulting in finally five cell strains obtained that produced K88 mAb, which were subsequently identified with K88 fimbrial proteins by Western blot (WB). MAbs were accordingly obtained from the ascites of mice injected intraperitoneally with screened hybridoma

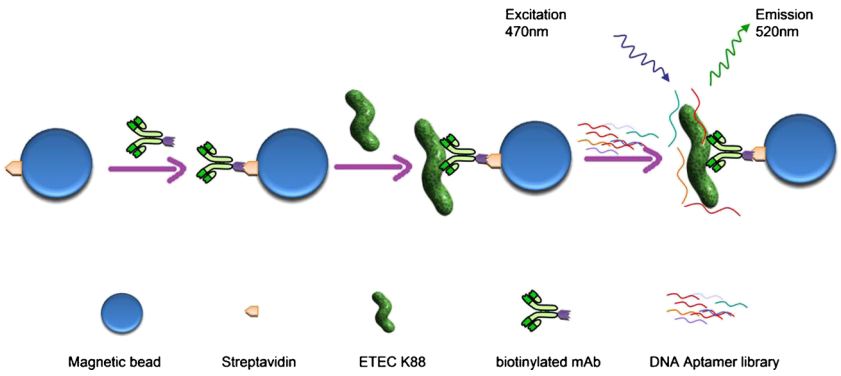

Fig. 1 Schematic of the detection strategy based on DNA aptamer library as a reporter combined with IMS technology

cells and purified by utilizing protein A/G affinity column. The purified K88 mAbs were biotinylated with BNHS purchased from Sigma and then identified via WB (K88 mAbs 1:500 diluted).

Affinity Constant (K_{aff}) Determination of K88 mAb and Preparation of IMB

Typical avidin-biotin system ELISA (ABS-ELISA) was used for K_{aff} determination of K88 mAb. A series of bidilutions of biotinylated K88 mAbs, ranging from 1:50 to 1:12,800, were respectively added to the plates to bind with ETEC K88 fimbrial antigens at dilutions of 1:250, 1:500, 1:1,000, which were coated to the plates in advance for 1 h at 37 °C. The amount of bound mAb was measured by alkaline phosphatase labeled streptavidin. The enzymatic reaction was developed with PhosphoGlo AP substrate. Three concentrations of antigen were used to calculate K_{aff} of K88 mAb.

The MBs (Dynabeads M-280 streptavidin, 6×10^8 beads/ml) coated with streptavidin were obtained from Life Technologies Corporation in this experiment. To prepare IMB, the supernatant containing biotinylated K88 mAb was added to the suspension of MBs and incubated for 30 min at 37 °C with gentle rotation in a centrifuge tube coated with PBSB over night. The washing procedure was performed by placing the plastic tube on a magnet device purchased from the same company as well. The particles were drawn toward the inner wall of the tube close to the magnet and the supernatant was discarded. Washing was repeated six times with PBS.

Various amounts of MBs were bound with K88 mAbs at the concentration of 10 g/ml respectively. An excess of FITC conjugated goat anti-mouse IgG (Biosynthesis biotechnology, Beijing, China) was added to evaluate the binding efficiency between MBs and K88 mAb. Prior to fluorescence-based detection (LS55, Perkin–Elmer, Hayman, Germany), four washings had to be carried out to remove the left goat anti-mouse IgG and the bound FITC-IgG was subsequently eluted from the beads.

Capture Efficiency of K88 IMB

MBs solution was used to prepare IMBs according to the above mentioned procedures. Bacteria calculated by plate counts were diluted to the level of 10^2 and 10^3 CFU/ml. K88 IMBs were incubated with 200 μl ETEC K88 dilution for 30 min at 37 °C following by four washings to remove bacteria not attached to the particles. Initial and final bacteria attached to IMBs were incubated on LB medium agar at a volume of 200 μl to calculate the capture efficiency. The MBs without K88 mAbs served as negative control.

Selections of DNA Aptamer Library and Individual Aptamer

The DNA oligonucleotide library contained a 35-nt random sequence flanked by two primer binding regions (5-GGGA GCTCAGAATAAACGCTCAA-N35-TTCGACAT-GAGG CCCGGATC-3′). The FITC-labeled primer (5′-FITC- GGGA GCTCAGAATAAACGCTCAA-3′) was used to measure the ratio of the ssDNA binding with target cells to initial pool in a round. The library and primers were both synthesized by Sangon Biotech.

The procedures to select aptamer library against ETEC K88 were carried out as follows: the ssDNA pools (1,300 pmol for initial round) suspended in 400 μl selection buffer were heated at 95 °C for 5 min in a selection buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 0.1 % NaN_3) and then snap-cooled on ice for 10 min. BSA and a fivefold molar excess of yeast tRNA (Invitrogen) were added to decrease the binding background. The bacteria suspended in 500 μl physiological saline (10^9 CFU/ml for the first round and 10^8 CFU/ml for subsequent rounds) were incubated with the ssDNA pools at 37 °C for 60 min with moderate shaking. Separation of bound and unbound ssDNA was done by centrifugation at 8,000×g for 10 min and washed three times with 500 μl selection buffer (with 0.2 % BSA) to remove unbound and non-specifically bound aptamers. The bound ssDNAs were eluted from the targets by heating at 100 °C for 5 min in 100 μl sterile ddH_2O. Centrifugation was performed at 22,000×g for 15 min to discard the cells and recovery the supernatant. The eluted ssDNAs were then enriched by PCR amplification (5 min at 94 °C, then 28 cycles of 30 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C, followed by 5 min at 72 °C) at the concentrations of template 5 μM, dNTP 0.2 mM, forward and downstream primers 0.5 μM, Mg^{2+} 0.75 mM, Taq Plus DNA polymerase 1 U at 20-μl volume of reaction mixture. To acquire ssDNA as the next enriched library, asymmetry PCR was run as the following procedure: an initial denaturation of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 65 °C, 30 s at 72 °C, and a final elongation period of 5 min at 72 °C. The concentration of forward and downstream primers is 1 μM, 0.01 μM, respectively. All the reagents related PCR are from Shanghai Sangon Biotech. Electrophoresis followed by DNA gel extraction (Kit purchased from Shanghai Sangon Biotech) was used to extract and purify forward-primed ssDNA from double strand sequences, simultaneously reverse-primed sequences being removed in the form of double strand sequences as well.

To improve affinity and specificity of the aptamer library, three rounds of counter-SELEX were carried out sequentially after the sixth round. In each counter-SELEX round, aptamer pool was incubated with ETEC K99, *S. enteritidis*, *S. aureus*, respectively (10^8 CFU/ml). The other procedures of counter-SELEX were the same as above mentioned. The last selection

pool will be adopted to act as "DNA aptamer library" in the detection system.

The final PCR products were subcloned into a pUCm-T vector with the TA cloning kit and sequenced (Shanghai Sangon Biotech). All sequences were analyzed, classified into several families. Typical sequences were selected as aptamer candidates, which were performed dissociation constant (Kd) determination using fluorescence assay [40] and analyzed secondary structure. The individual aptamer with best affinity against ETEC K88 was synthesized by Shanghai Sangon Biotech to be characterized further and act as reporter in the detection system.

Flow Cytometric Analysis and Confocal Imaging of Bacteria Bound With Aptamer Library or Individual Aptamer

To further identify the affinity between the selected aptamer library or individual aptamer and their targets, flow cytometric analysis and confocal imaging were performed subsequently. The FITC-labeled selected aptamer library or individual aptamer (30 pmole) was incubated with the bacteria (10^6 CFU/ml) at 37 °C for 1 h with gentle shaking in 500 μl selection buffer. The fluorescence was determined with a flow cytometry (FC 500MPL, Beckman Coulter, Brea, CA, USA) by counting 10,000 events. Cell imaging was performed by a confocal microscopy (LSM710, Carl Zeiss Corporation, Jena, Germany). The FITC-labeled selected aptamer library or individual aptamer (60 pmole) was incubated with ETEC K88 (10^8 CFU/ml) at a moderate rotation in 200 μl selection buffer at 37 °Cfor 1 h. After washing three times, the cells were immobilized by 1 % paraformaldehyde and then observed under confocal microscope.

Sensitivity and Specificity of the Detection System

MBs solution (blocked with 1 % BSA in advance) and K88 mAbs were used to prepare IMBs. Tenfold dilutions of ETEC K88, ranging from 10 to 10^5 CFU/ml, were made in PBS. The prepared IMBs and bacteria (200 μl) were incubated at gentle rotation at 37 °C for 30 min, respectively. After being washed several times with PBST, IMBs with bacteria were resuspended and incubated with DNA aptamer library or individual aptamer (60 pmole) at 37 °C for 30 min. The IMB-bacteria-ssDNA conjugates were magnetically separated, washed several times, resuspended in PBS at a volume of 200 μl, and heated subsequently at 95 °C for 5 min. The next steps involved magnetic separation and fluorescence analysis of the supernatants.

For specificity identification, after IMBs were respectively used to capture ETEC K88, ETEC K99, *S. enteritidis*, *S. aureus* (10^5 CFU/ml, 200 μl), the selected FITC-lablled aptamer library or individual aptamer (60 pmole) was incubated with IMB-bacteria conjugates. The ssDNAs were finally separated from conjugates and analyzed by fluorescence spectrophotometer. Control was set as no bacteria added.

Artificially Contaminated Fecal Sample Analysis Based on the Detection System

The fecal samples from health piglets were obtained from a stock farm in Shaodong, Hunan, China. ETEC K88 culture (10^3 CFU/ml, 1 ml) mixing with the fecal sample (500 mg) artificially stimulated real sample from diarrhea piglet. The preparation procedure of IMBs and the detection protocol were the same as mentioned above. Other bacteria such as ETEC K99, *S. Enteritidis*, and *S. aureus* were detected in the same conditions as ETEC K88, respectively. As a control, no bacteria-mixed fecal samples were detected.

Results

Purification of K88 Fimbrial Protein and Preparation of K88 mAb

SDS-PAGE of K88 fimbrial extract revealed a major protein of about 25 kD (Fig. 2a), which was in agreement with references reported [17, 21]. The purified K88 fimbrial protein

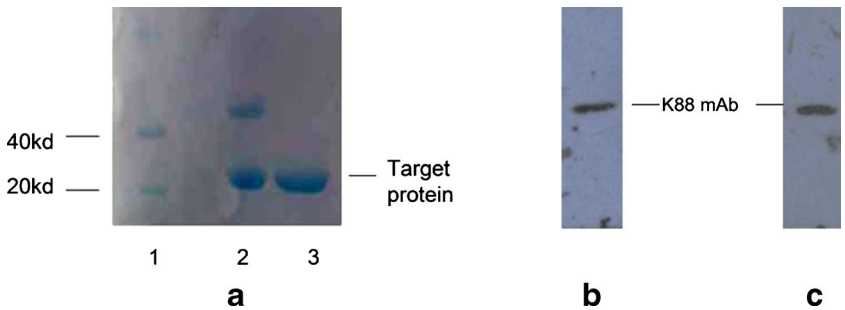

Fig. 2 **a**. SDS-PAGE of K88 fimbriae at different purification stages. Lane1: marker; Lane 2: ammonium sulphate precipitate; Lane 3: purified protein through SE-chromatograph. **b**. WB of biotinylated K88 mAb with HRP-streptavidin. **c**. WB of biotinylated K88 mAb with HRP labled goat anti-mouse IgG

was concentrated to satisfy for preparing K88 mAb. Following the protocol above, K88 mAbs were prepared and biotinylated. The result of WB analysis with HRP-streptavidin and HRP lablled goat anti-mouse IgG proved the success of biotinylation of K88 mAb (Figs. 2b and c). The concentration of the biotinylated K88 mAb is estimated to approximately 0.6 mg/ml.

K_{aff} Determination of K88 mAb

The binding activity of K88 mAb against K88 fimbriae was confirmed by typical ABS-ELISA and expressed as absorbance unit ($OD_{405\ nm}$–$OD_{450\ nm}$) (Fig. 3a). Based on Beatty's method [2, 12], K_{aff} of K88 mAb was determined to be $1.616 \pm 0.033 \times 10^8\ M^{-1}$, which indicated that K88 mAb had great binding ability against K88 fimbrial protein. The fitting curves were made by the software Origin 8.0.

Capture Efficiency of IMB

In order to verify the binding ability of MBs with prepared biotinylated K88 mAb and find out their optimal binding concentrations, FITC modified goat anti-mouse IgG was bound to IMB complex to exhibit the binding results (Fig. 3b). With the increase of MBs, the fluorescence signals gradually increased. However, much more magnetic beads (1.2×10^8 beads/ml) led to signal decrease slightly. When K88 mAb was 10 μg/ml, the optimal binding concentration of MB was 6×10^7 beads/ml because of the highest fluorescence intensity. The results revealed their good binding ability and weak background signal resulting from low fluorescence intensity of negative control.

As the efficiency of the detection system was mainly based on good capture of target, plate count was adopted to quantify bacteria before and after they were captured by IMBs. As shown in Fig. 3c, 74.5 %, 63.8 % of the initial bacteria is respectively captured when IMBs were incubated with ETEC K88 dilutions at 10^2, 10^3 CFU/ml, indicating that prepared IMBs are capable to highly efficiently capture their targets, whereas bare magnetic beads hardly could.

Selection of DNA Aptamer Library and Individual Aptamer

Cell-SELEX technology was used to generate aptamer library and individual aptamer against ETEC K88. The affinity of ssDNA aptamer was determined by the ratio of fluorescence intensity of ssDNA eluted from target cells and total ssDNA pool in each round. As shown in Fig. 4a, the ratio has a slow increase in the first six rounds whereas a great rise from the beginning of the 7th round due to counter-selection, finally reaching to ca 30 %. The 13th round selection pool with highest affinity binding to target bacteria was named "DNA

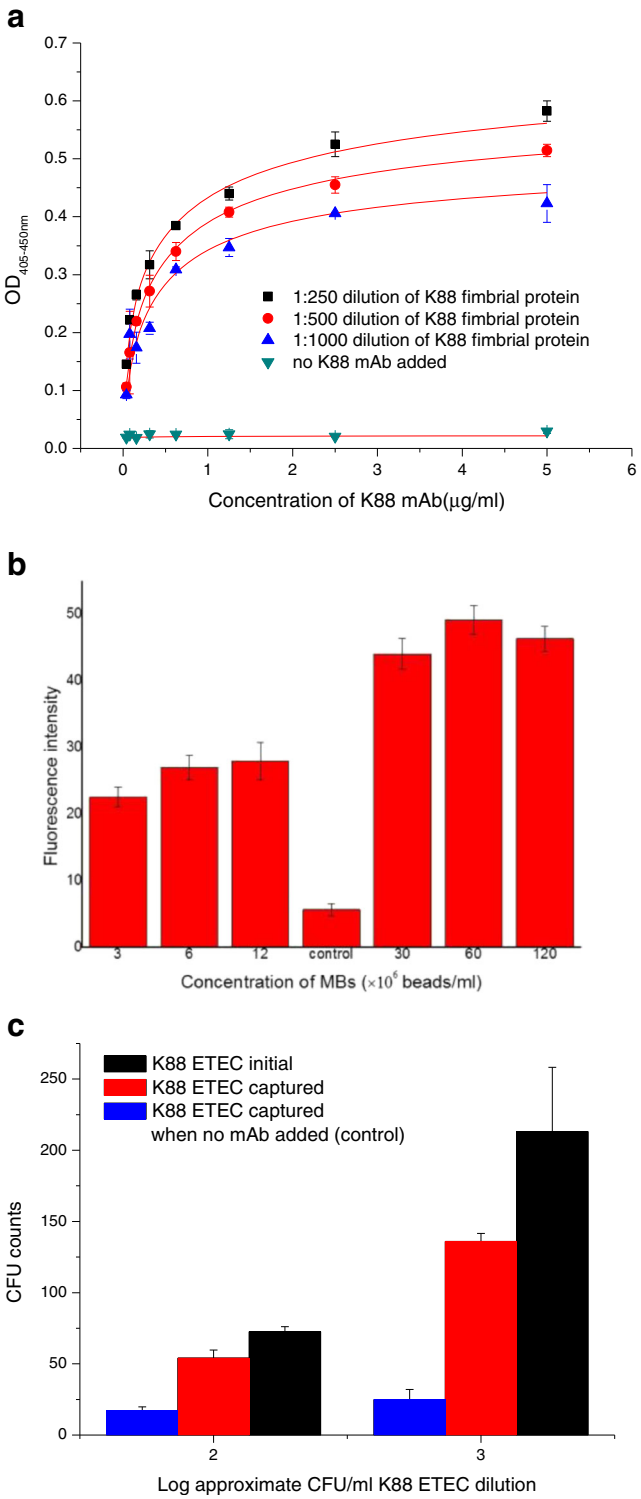

Fig. 3 **a**: Assay of K_{aff} between K88 mAb and K88 fimbrial protein. Three bidilution concentrations of K88 antigen were used to assay K_{aff} according to Beatty's method. **b**: Verification of the ability of MBs binding to K88 mAb. A series of concentrations of MBs were incubated with K88 mAbs. Control was performed with 1.2×10^7 beads/ml. **c**: Identification of capture efficiency of IMB for ETEC K88. The capture efficiency is calculated when the prepared IMBs were incubated with 200 μl of ETEC K88 dilution at 10^2, 10^3 CFU/ml, respectively. Each value is the mean ± S.D. (n=3)

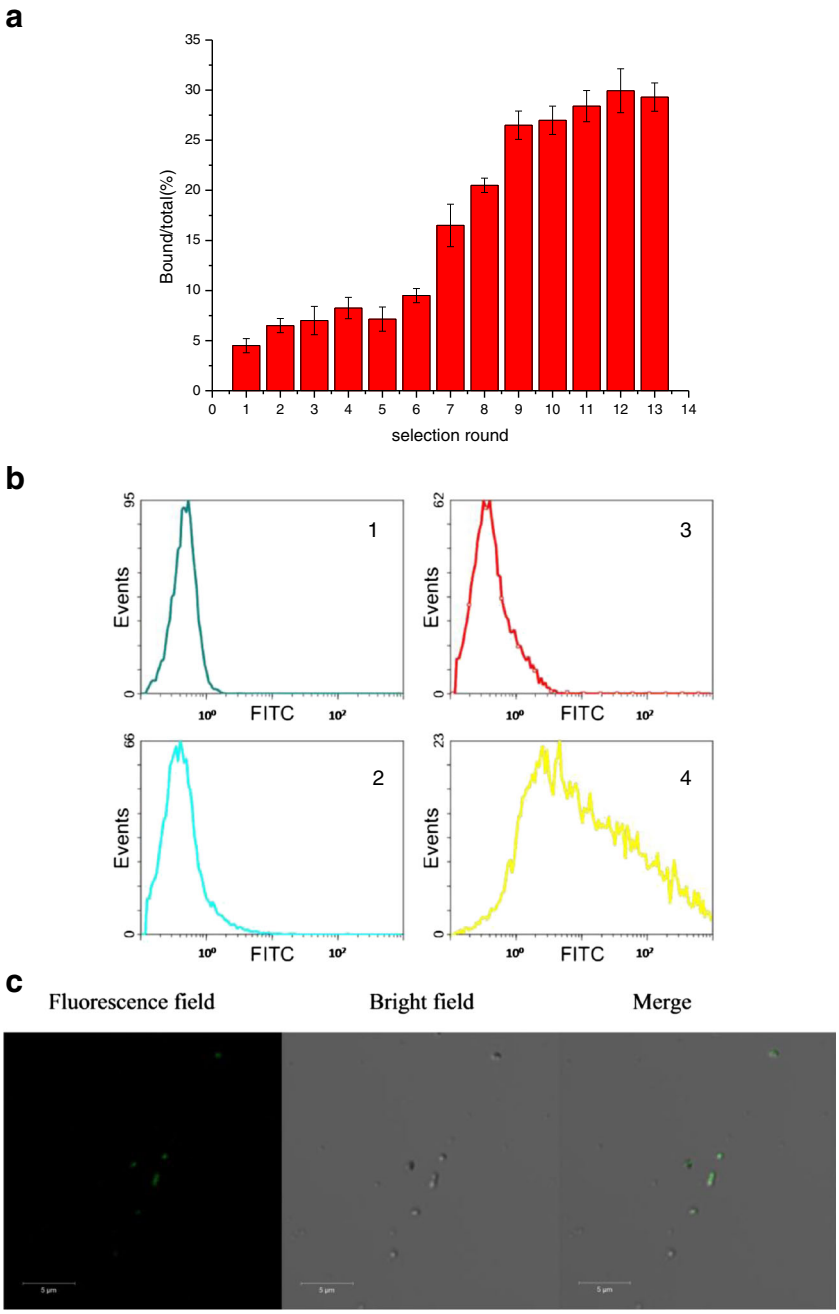

Fig. 4 **a**: Production of aptamer library with high affinity against ETEC K88 by Cell-SELEX technology. The bar graph reveals the ratio of the amounts of ssDNA bound to target cells to total ssDNA pool in each round selection. Error bars indicate S.D. (n=2). **b**: Flow cytometric analysis in ssDNA pool enrichment. The fluorescence was determined with a flow cytometry by counting 10,000 events. 1. the initial library; 2. the 3rd round pool; 3. the 6th round pool; 4. the 13th round pool. **c**: Confocal microscopy images of target bacteria binding with FITC-labled aptamer library (Excitation: 488 nm, emission: 510±10 nm)

aptamer library" and would act as specific reporter in the detection system.

The 13th round aptamer pool was subsequently subcloned into *E.coli* TOP10. 40 clones were chosen, sequenced and classified to families based on their sequence homology and secondary structure. Typical aptamer candidates in each family were performed Kd determination and some of them with relatively high affinity were showed in Table 1. An individual aptamer (Apt B12) with best affinity (Kd=15±4 nm) against ETEC K88 bacteria was achieved, FITC lablled and characterized, which finally acted as reporter in our detection system for comparison.

Flow Cytometric Analysis and Confocal Imaging of Cells Bound With Aptamer Library or Individual Aptamer

Flow Cytometric analysis and cell imaging were performed to illuminate the affinity of the selected aptamer library. The initial library, the 3rd round pool, the 6th round pool and the 13th round pool in the selection were FICTC lablled, incubated with target bacteria and monitored by flow cytometry. As a result, the fluorescence intensity of the conjugates from the last ssDNA pool was much higher than that of initial library, the 3rd round ssDNA pool and the 6th ssDNA pool, indicating that the enlargement of affinities were significant between the

aptamer library and target bacteria after 13 rounds of selection (Fig. 4b). By a confocal microscopy, the bacteria with FITC-labled aptamer library bound were easily visible in fluorescence field, further demonstrating their bindings with high affinity (Fig. 4c). It means that the selected aptamer library is likely to be a good specific reporter in the detection system.

The affinity between individual aptamer (Apt B12) and ETEC K88 was also identified by the same method as aptamer library. The individual DNA aptamer could bind to the target bacteria, which exhibits visible fluorescence under a confocal microscopy (Fig. 5). Flow cytometric analysis showed that 26.5 % of target cells are stamped by FITC-lablled individual aptamer.

Sensitivity and Specificity of the Detection Approach

Subsequently, the detection of ETEC K88 in pure culture was carried out by the presented approach. Tenfold dilution of the target cells, ranging from 10 to 10^5 CFU/ml, were captured with IMBs and marked with FITC-lablled DNA aptamer library or individual aptamer (Apt B12) at a volume of 200 μl. When the concentration of bacteria was 10^3-10^5 CFU/ml, reliable fluorescence signals were clearly presented, whereas no distinct changes at low concentration (10, 10^2 CFU/ml) compared with the control (Fig. 6). Herein, it's noteworthy that the result will be thought to be reliable when the fluorescence intensity is more than triple of its control. It was revealed that the lower detection limit was 1.1×10^3 CFU/ml in pure culture (As showed in Fig. 3C, the amount of ETEC K88 in 200 μl culture is 2.1×10^2 CFU at the dilution of 10^3 CFU/ml). However, lower fluorescence signals were displayed when individual aptamer acted as a reporter instead of aptamer library.

Table 1 Candidate aptamer sequences for ETEC K88 binding

Name	Sequence (from 5′ to 3′)	Kd (nM)
Apt B12	5′-TGGGAGCTCAGAATAAACGCTCA AGGCACACAGGACTATACAGTGTT GCAGTGTTGCTGTTCGACATGAG GCCCGGATCA-3′	15±4
Apt H11	5′-TGGGAGCTCAGAATAAACGCTCA ACCCTGCGGGGCTGCCCGATATGT GTCCAAGTGGTGTTCGACATGAG GCCCGGATCA-3′	66±7
Apt C09	5′-TGGGAGCTCAGAATAAACGCTCA ATGGCCGTGTGGATAGAGGCGTG TTGTATGGGTGTGTTCGACATGAG GCCCGGATCA-3′	52±8
Apt H12	5′-TGGGAGCTCAGAATAAACGCTCA AGGGGAGGCAGTGTGTTGTGCCG TGTGTATGCTTGGTTCGACATGAG GCCCGGATCA -3′	106±12

Numbers in name indicate different clones

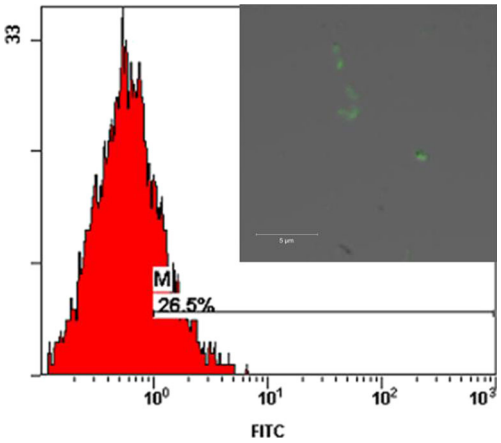

Fig. 5 Affinity identification of selected individual aptamer (Apt B12 F). The result from flow cytometric analysis is presented on the bottom left. Cell imaging (bar, 5 μm) of target bacteria binding with FITC-labled single aptamer is presented on the upper right (Excitation: 488 nm, emission: 510±10 nm)

The specificity of the approach was also tested by assaying the fluorescence intensity of the conjugates of FITC-labled aptamer library or individual aptamer (Apt B12) binding to four strains at dilution of 10^5 CFU/ml in pure culture. As shown in Fig. 7, whether aptamer library or individual aptamer acted as reporter, the fluorescence detection signals of target bacteria were much more than those of others whereas the control was lower, implying a high specificity of the approach. Obviously, higher signal intensity was shown when aptamer library is used in the experiment than individual aptamer.

Artificially Contaminated Fecal Sample Analysis Based on the Detection System

Finally, to verify whether the detection system could work or not when real sample was tested, artificially contaminated

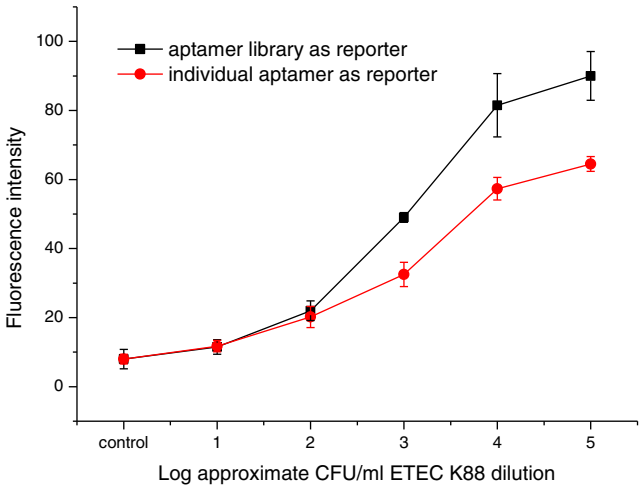

Fig. 6 Detection of ETEC K88 in pure culture by FITC-labled DNA aptamer library and single aptamer as specific reporters. The approximate concentration of target bacteria is from 10 to 10^5 CFU/ml. Each value is the mean ± S.D. (n = 3)

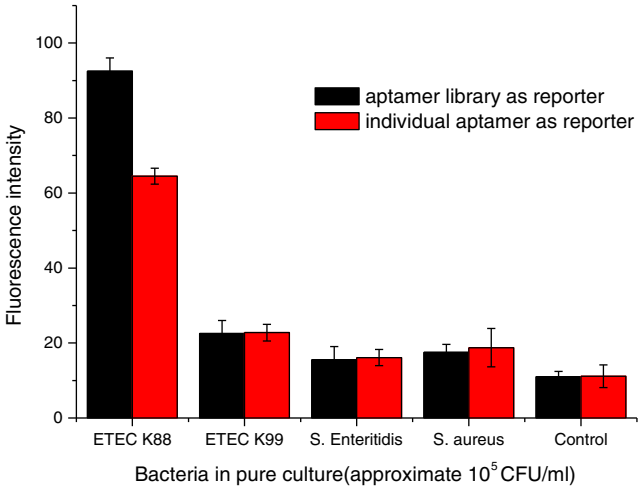

Fig. 7 Selectivity identification of the detection system. Each value is the mean ± S.D. (n=3)

fecal samples consisting of faces of healthy piglet and the target bacteria were detected by the system. Fig. 8 showed that when aptamer library acted as reporter to detect ETEC K88 even at low dilution of 10^3 CFU/ml, there was considerable increase in fluorescence intensity in contrast to other bacteria and negative control, whereas individual aptamer could not. The detection limit as low as 2.1×10^3 CFU/g in artificially contaminated fecal sample (1 ml of bacteria dilution at 10^3 CFU/ml is mixed with 500 mg fecal sample) could be achieved in the detection system. It means that it is feasible to apply the novel approach based on DNA aptamer library as a reporter combined with IMS technology to detect target bacteria.

Discussion

In this work, we put forward a strategy to detect ETEC K88 with high specificity, in which streptavidin-biotin system was used to construct IMS system due to their acknowledged high affinity [8]. High affinity of K88 mAb against K88 fimbrial protein and appropriate capture efficiency of IMB to detect ETEC K88 were confirmed to be sure of the reliability of our IMS system. Although IMS technology has been applied widely to separate and detect different target pathogens, most researches are combined with enrichment culture, plate count and PCR technology [34, 37, 29, 27], which are time-consuming or have to be aid of expensive apparatus. Hence, for the purpose of rapid detection, we prepared FITC-lablled DNA aptamer library against ETEC K88 to directly report the enrichment of target bacteria by experiment-proved reliable IMS system, which could be quickly and simply detected in the form of fluorescence intensity.

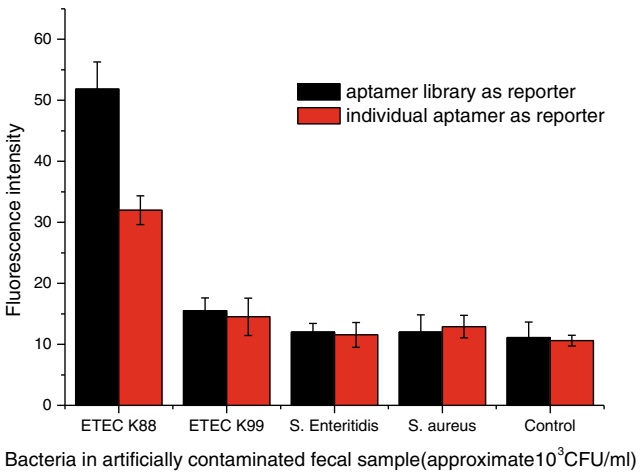

Fig. 8 Ability verification of the approach to detect artificially contaminated fecal

Aptamer pool/library was ever used to indirectly detect protein targets in complicated system such as FRET in rare cases [3]. However, no report was found that it was directly used to report bacteria combined with IMS technology up till now. In this paper, we succeeded using DNA aptamer library as reporter to detect the target bacteria by a simple reporting system. By comparison, no reliable result was achieved when an individual aptamer with best affinity was used against target bacteria to complete the same work due to rather low fluorescence signal. Aptamer library is composed of many individual aptamers and has more binding sites with target bacteria than the latter, generating stronger fluorescence signals in the reporting system. In addition, bacterial structure is more complex than that of protein. So the diversity of aptamer in an aptamer pool is very important in the design of bacterial cell detection strategy, especially aptamer acting as direct reporter. Therefore, DNA aptamer library should be superior to individual aptamer as reporter in a direct bacterial reporting system.

Nested PCR was developed to detect ETEC K88 by Hornes et al. [15] with the limit of 2×10^3 CFU/ml in pure culture. [41] reported a method of PCR combined with IMS technology to diagnose *E. coli* O157:H7, obtaining detection limit of 4.0×10^2 CFU/ml in pure culture and 7.5×10^3 CFU/g in artificially contaminated sample. IMS-associated biosensors for bacteria detection have a limit range from 3.2×10^2 CFU/ml to 1.5×10^5 CFU/ml [22, 13, 31]. Compared with the biosensors mentioned above, our approach is competitive in sensitivity with the limit of 1.1×10^3 CFU/ml in pure culture and 2.1×10^3 CFU/g in artificially contaminated fecal sample.

It should be stressed that cloning, sequencing and affinity determining of ssDNA for individual aptamer selection are unnecessary as the last pool is directly adopted as aptamer library after rounds of selections, resulting in saving much time and operation processes. Lower experiment cost is also obvious since a sum of fluorescence dye-lablled aptamer library can be quickly obtained by simple PCR. With the help of a portable fluorescence spectrophotometer, the approach

may probably be applied to detect low abundance of target bacteria in complex samples on site only after several steps of magnetic separates. By the detection system, the diagnosis for pathogens can be completed within 1.5 h without any enrichment, significantly faster than traditional methods.

In conclusion, this work described an approach to rapidly detect ETEC K88 with high selectivity and sensitivity combined with IMS technology. IMBs enriched the target bacteria and aptamer library could make more lablled aptamers bind to the cells, resulting in enhancing the detection signals. The use of double specific factors, both aptamer library and mAb, improved its specificity as well. Furthermore, DNA aptamer libraries against other bacteria can be also selected to rapidly report different pathogens by using this strategy. However, the further researches should be done to improve the sensitivity of the system and real fecal samples from diarrhea piglets should be assayed. Although it's very difficult to detect bacteria quickly and conveniently on site by methods known the art, the approach we present here may be a good alternative with greatly applied potential.

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