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A Dot- Enzyme Linked Aptamer Sorbent Assay (Dot-Elasa) for Rapid Detection of Chloramphenicol and Kanamycin Residues

Aijun Tan[1], Yixiong Lin[1], Xunde Li[2], Quande Wei[1]*, Edward RobAtwill[2]

[1]Center for Disease Prevention and Control of Zhuhai, Zhuhai, 519000, PR China
[2]Western Institute for Food Safety and Security, University of California, Davis, CA, 95618, USA

Research Article

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***For Correspondence**

Quande Wei, Department of Microbial Analysis, Zhuhai Center for Disease Control and Prevention, 9th of Weikang Ave, Nanping, Xiangzhou, Zhuhai City, Guangdong Province, PR China, 519000, Tel: +86-756-6273902, Fax: +86-756-6273929

E-mail: quandew@hotmail.com

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ABSTRACT

A novel Dot-Enzyme linked aptamer sorbent assay (Dot-ELASA) based on aptamer and the principle of Dot-ELISA was established for detecting Chloramphenicol (CAP) and Kanamycin (KAN) residues. This novel assay is capable of detecting CAP and KAN residues in milk rapidly and at concentrations as low as 10^{-7} g/L.

INTRODUCTION

Chloramphenicol (CAP) is a broad-spectrum antimicrobial drug which is widely used in animals for the treatment of several infectious diseases because of its excellent antibacterial ability and low costs. CAP achieves the antibacterial effect by inhibiting protein synthesis, which exhibits good activity against a variety of Gram-positive and Gram-negative bacteria. However, CAP has been found to have serious side effects such as gray baby syndrome, leukemia, and aplastic anemia on human beings [1,2]. Many countries regulate the use of CAP in food-producing animals, for example, the minimum required performance limit of 0.3 μg/kg was established by the European Union (EU) [3]. Kanamycin (KAN) is another antimicrobial drug commonly used to treat a wide variety of infections. It induces mistranslation and blocks translocation by interacting with the 30S subunit of the prokaryotic ribosome [4]. KAN also can cause serious side effects including loss of hearing and toxicity to the kidney sin humans through intake with food or medicinal overprescribing. The EU has established that maximum residue limits (MRLs) of KAN in milk to be 150 μg/kg [3]. Antimicrobial drug residues in food products have become increasingly concern for public health. It is of significance to test whether food products contain residual antibiotics exceeding maximum residue limits (MRLs) before sales in the market [5,6]. Chromatographic, enzymatic, and immunological methods have been reported for detection of antimicrobial drug residues in food products but more reliable, accurate, and user friendly methods are needed [7,8].

Aptamers are mainly oligo nucleotides (ssDNA or RNA) which can combine their ligands with high affinity and specificity. Since the introduction of aptamer by [9,10] and the development of the technology called systematic evolution of ligands by exponential enrichment (SELEX) [11], a variety of aptamers against different targets including organic molecules, antibiotics, peptides, proteins, and viruses have been reported [12-14]. Compared to antibodies, aptamers have excellent stability as well as high affinity and specificity, especially to small molecules. Moreover, aptamers are easy to amplify [15,16], synthesize, modify, manipulate and characterize [17,18]. Therefore, numerous aptamer-based bioanalytical methods have been proposed and applied in the detection

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of different molecules [19-22]. Recently, aptamer-based sensors have been used to detect antimicrobial drugs, such as kanamycin, tobramycin, and tetracycline [23-25]. However, these assays need specific instrument thus difficult to be standardized as a quick and simple method. Dot enzyme-linked immunosorbent assay (Dot-ELISA) as a detection system is capable to transduce molecules into an optical signal, which requires less reagents and time to process [26,27]. Another advantage of Dot-ELISA is that results can be examined by naked eye. In the present work, using aptamer and the principle of Dot-ELISA, we establish a novel system, Dot-Enzyme linked aptamer sorbent assay (Dot-ELASA) for rapid detection of CAP and KAN residues.

MATERIALS AND METHODS

Aptamer sequences

The sequence of CAP aptamer was 5'- Biotin-ACA GTG AAA AAA GAC GTG TGA ATG TCA CAC TGA AAA [28] while the sequence of KAN aptamer was 5'- Biotin-ACC GCG GGG TTG CGG ACC GGG AGC TCC AGC [29]. The secondary structure of CAP and KAN aptamers are shown in **(Figure 1).** DNA sequences of these aptamers were synthesized by TakaRa (Dalian, China).

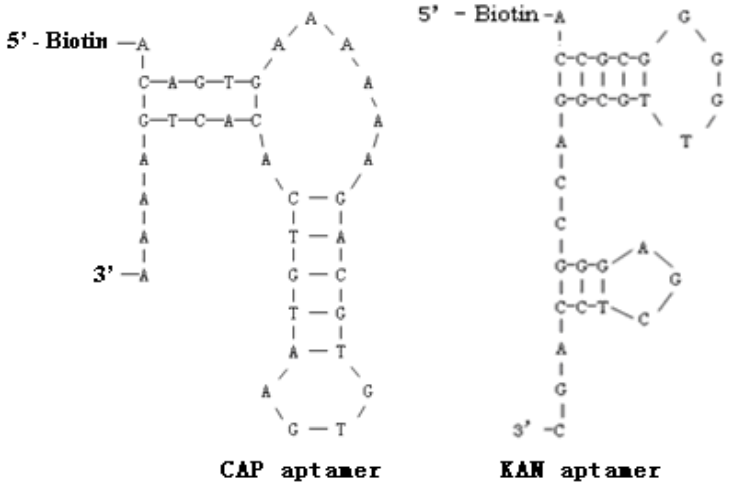

Figure 1. Predict of secondary structure of CAP and KAN aptamers.

Procedure of Dot-ELASA

Nitrocellulose (NC) membrane (0.2 μm) (Pall, USA) was cut into strip with appropriate size. Antimicrobial drugs were mixed with 10× loading buffer (Dalian, China) (pH 9.6)followed by adding bovine serum albumin (BSA) (NEB,USA) at a final concentration of 6%. The solution was spotted on the NC membrane strip (10 μl/spot) and incubated at 55°C for 1 h. Then the membrane strip was blocked with Phosphate Buffered Saline (PBS)(prepare ourselves pH 7.4) containing 4% none fat milk powder at 37°C for 1 h, followed by 3× washings with PBS-Tween 20 (PBST) (prepare ourselves pH 7.4) for 15 min each time. After that, the membrane was dried at 40°C for 30 min, soaked in 60 μM biotin-labeled apatmer at 55°C for 1 h, followed by 3× washing with PBST for 10 min each time. The membrane was dried at 40°C for 10 min and soaked in 10 mL of 500-fold diluted streptavidin labeled horseradish peroxidase (HRP) (MD, USA) for 1 h, followed by 3× washing with PBST for 10 min each time. Finally, the membrane was dried at 40°C for 5 min, followed by incubating with the 3,3'-diaminobenzidine (DAB) (Pierce, USA) substrateat 37°C for 15 min which turn positive spots into blue and negative spots colorless.

Optimizing antimicrobial drugs capturing conditions and aptamer binding temperature

Because it is critical to capture antimicrobial drugs on NC membrane and binding aptamer to antimicrobial drugs, BSA concentrations (1% ~ 10% series dilution) and temperature that antimicrobial drugs bind tobiotin-aptamer (37,40,45,50,55,60,65,70°C) were evaluated. Default concentrations of CAP and KAN, milk, aptamer and HRP were 10^{-2} g/L, 2%, 100 μM and 1:500 dilutions respectively. All the membranes were dried at 40°C and incubated at 37°C. Meanwhile, 0.01 mol/L PBS (pH 7.4) and 0.05 mol/L Tris-buffered saline (TBS) (pH 9.6) were compared as loading buffer.

Optimizing Dot-ELASA conditions

Each spot on NC membrane was spotted with 10 μL antimicrobial drugs (10^{-2} g/L) diluted with 0.05 mol TBS (pH 9.6) in 6% of BSA. A series dilution of biotin-aptamer (100,80,60,40 and 20 μM) and streptavidin-HRP (1:250, 1:500, 1:1000, 1:1500, 1:2000) were evaluated as well. The concentration of milk for blocking was 2%. All membrane was dried at 40°C and incubated at 37°C except the biotin-aptamer and antimicrobial drug combination at 55°C. A serial dilution (1, 2, 3, 4, 5%) of none fat milk for blocking was tested too.

Evaluation of sensitivity and specificity

Sensitivity of Dot-ELASA was evaluated under optimal reaction conditions by employing a serial dilution (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} g/L) of CAP and KAN. Trials with each concentration were triplicated. Spots of detectable or higher concentrations

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exhibited blue color while spots of non-detectable concentrations were colorless. CAP, KAN, neomycin and tetracycline were tested at the concentration of 10^{-2} g/L to determine the specificity of CAP specific aptamer and KAN specific aptamer.

Application of Dot-ELASA to milk samples

CAP and KAN was spiked inraw milk at serial concentrations of 10^{-2} ~10^{-8}g/L. Spiked milk sample was centrifuged at 12000 rmp for 5 min to remove fat. The supernatant was then added 2× loading buffer at ratio of 1:1 and BSA at final concentration of 6%.Each trial were triplicated.

RESULTS

Optimizing antimicrobial drug capturing conditions and aptamer binding temperature

Capture antimicrobial drugs on NC membrane is the critical step for this assay. The small molecular of antimicrobial drugs will escape from NC membrane if no protein exists. We found that BSA is beneficial to retain antimicrobial drugs on NC membrane when the concentration of BSA was >5% in sample solutions and the optimum concentration was 6%. Temperature for antimicrobial drugs to bind to biotin-aptamer is another key factor in this assay. The temperature range in which antimicrobial drugs can be detected was 50~65°C with optimum temperature of 55°C. For loading buffer, 0.05 mol/L TBS (pH 9.6) exhibited better effects than 0.01 mol/L PBS (pH 7.4).

Optimization of of Dot-ELASA conditions

In order to obtain the best sensitivity of strip test, the concentration of biotin-aptamer and amounts of streptavidin-HRP were screened in a two-dimensional checkerboard titration. The suitable concentration of biotin-aptamer was found to be 60 μM and the optimum amount of streptavidin-HRP was 1:500 dilutions for both CAP and KAN. The 2~5% low fat milk were used to block NC membrane in Dot-ELASA and optimum fat concentration was found to be 4%. No significant differences were observed between incubating at 37°C,45°C and 55°C but samples dried much faster on the membrane at 55°C. Incubation for 1 h increased detection in comparison to incubating for 15 min or 30min.

Sensitivity and specificity of Dot-ELASA

Results of sensitivity and specificity of the Dot-ELASA are shown in **(Figure 2 and 3)** respectively. As shown in **(Figure 2)**, spots on strips with >10^{-8} g/L CAP and KAN exhibited blue but other spots with 10^{-8} g/L concentrations turned colorless. The results indicated that the lowest detectable concentration of both antibiotics were 10^{-7} g/L. As shown in **(Figure 3)**, CAP specific aptamer binds to CAP only and no cross-reactions with KAN, neomycin and tetracycline were found. Similarly, KAN specific aptamer reacted to KAN only but no other antibiotics.

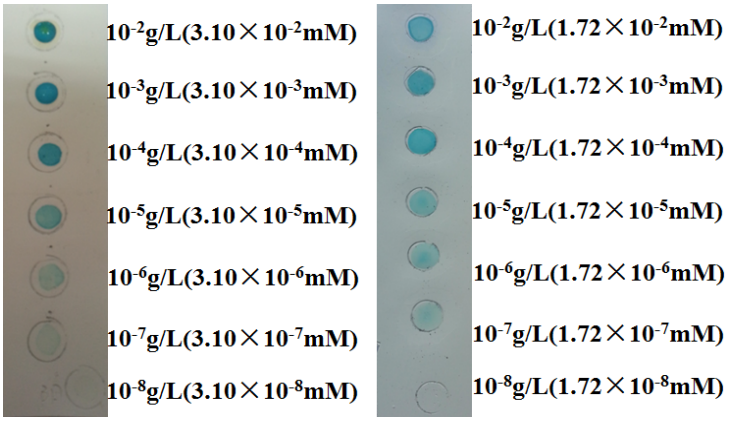

Figure 2. Sensitivity of Dot-ELASA assay for detecting CAP and KAN (left: CAP; Right: KAN).

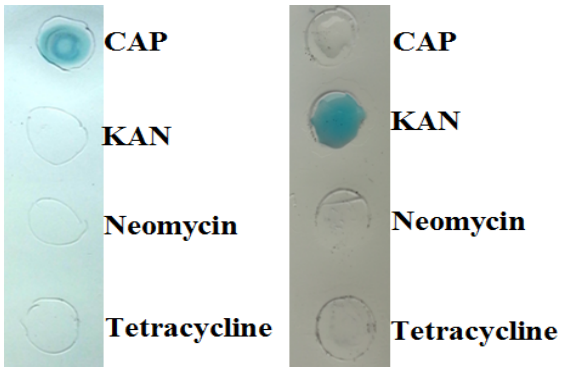

Figure 3. Specificity of DOT-ELASA assay for detecting CAP and KA N (left: CAP; Right: KAN).

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Dot-ELASA detection of CAP and KAN from milk samples

(Figure 4) shows the Dot-ELASA detection of CAP and KAN from spiked milk samples. The lowest detectable concentration of both antibiotics in spiked milk samples were 10^{-7} g/L.

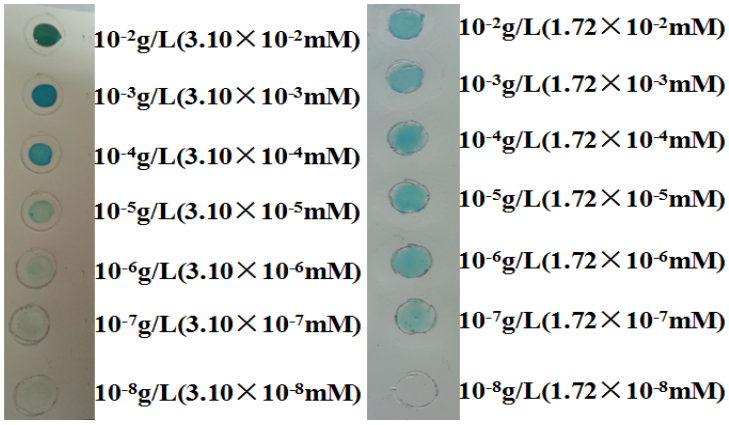

Figure 4. Dot-ELASA detection of CAP and KAN from milk samples (left: CAP; Right: KAN).

DISCUSSION

Binding temperature is a key factor in aptamer based methods. CAP and KAN were detectable at temperature of 50~65°C with 55°C as optimum at which the ssDNA aptamers maintain stable structures. Similar findings have been reported by other researchers [30]. Temperature over 60°Cmay denature the aptamer and inactivate antibiotics, thus cause changes of their conformation and subsequently invalid recognition between antibiotic and its aptamer. The limit of detection of Dot-ELASA were 3.1×10^{-7} Mm (10^{-7} g/L) for CAP and 1.72×10^{-7} mM (10^{-7} g/L) for KAN in milk samples. The limit of detection of CAP was similar to that using a sensor method [1] but lower than that in other methods previously reported [31,32]. The limit of detection of KAN was similar to that of biotin-streptavidin based immunosensor [33] but lower than that of immunosorbent assay [34] and other reported methods including colorimetric detection methods [23,30,35]. Nonetheless, the limit of detection of Dot-ELASA in the present work meets the current international standard (MRLs of EU).

Aptamers have shown many advantages in detecting small molecules, such as high affinity, specificity and easy synthesizing [17,18]. One of the challenges of aptamer based methods is the detection of aptamers after binding to targets. Unlike immunoassay based methods, it is difficult to separate un-binded aptamers from binded ones. To overcome this challenge, label free sensors [30] and aptazymes based sensors (a combination of aptamers and catalytic nucleic acids) are emerging as an alternative method for detecting antibodies [36]. Methods based on catalytic beacons [37] and gold nanoparticle aggregation [23,35] have also been reported. However, designs of these methods were based on conditions of unfixed small molecules such as antibiotics. The Dot-ELASA method described in present work was based on fixing target antibiotics first. Thus the detection procedure was very simple and results can be examined by eyes as traditional Dot-ELISA. The Dot-ELASA method was specific, rapid, economic, and feasible for detecting CAP and KAN in milk samples. Future works are warranted to evaluate this method for detecting CAP and KAN and other antibiotics in other types of food samples. Also, it is possible to develop quantitative detections after establishing standard detection curves.

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