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DETERMINATION OF ENROFLOXACIN IN BOVINE MILK BY A NOVEL SINGLE-STRANDED DNA APTAMER CHEMILUMINESCENT ENZYME IMMUNOASSAY

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Enrofloxacin, a widely used fluoroquinolone antibiotic, may be a cause of bacterial drug resistance and is forbidden in poultry. Consequently, a sensitive and rapid method is required for its determination. Aptamers, which are more stable and easily synthesized than antibodies, may serve as alternatives in the development of methods for rapid detection. Six single-strand DNA aptamers binding to enrofloxacin were selected by in vitro selection. Aptamer number 17 showed the highest affinity for enrofloxacin with a dissociation constant of 188 nM and the highest guanine concentration (35%), which was predicted to be crucial for strong affinity of the aptamer to enrofloxacin, and successfully distinguished enrofloxacin from its structure analogs. Using aptamer number 17, a novel chemiluminescent enzyme immunoassay associating with biotin-streptavidin was developed that allowed the determination of enrofloxacin to 2.26 ng/mL. Due to its capability to determine enrofloxacin in bovine milk, this newly selected aptamer may find broad application in food and environmental monitoring.

Keywords: Binding affinity; Chemiluminescence enzyme immunoassay; DNA aptamer; Enrofloxacin; SELEX

INTRODUCTION

Fluoroquinolones are an important class of synthetic antibiotics related to nalidixic acid that are widely used to treat infections in human and livestock (Carlucci 1998). Enrofloxacin is a second generation of fluoroquinolone and was
used in food-producing animals (Mi et al. 2013). However, the widespread utilization of enrofloxacin has already caused serious bacterial drug resistance (Drlica et al. 2012; Rice 2012) that may affect healthy animals and human beings, and the antibiotic-resistant capacity is expected to increase (Cabello et al. 2013). To control the spread of resistant bacteria, in 2005, U.S. Food and Drug Administration announced that enrofloxacin was a forbidden substance in poultry (Docket 00N-1571. 2004). Therefore, the development of methods for determining enrofloxacin in livestock and the environment has become an important goal.

Instrumental techniques, such as high performance liquid chromatography (HPLC) or liquid chromatography–tandem mass spectrometry (HPLC–MS–MS), have been used to determine enrofloxacin (Gratacos-Cubarsi, Garcia-Regueiro, and Castellari 2007; Schneider et al. 2007; Stubbings and Bigwood 2009). However, these methods require sophisticated equipment. Compared with these methods, affinity-based assays based on antibody are less time consuming and offer easy preparation (Tao et al. 2013; Wu, Lin, and Wang 2009; Jiang et al. 2013). Chemiluminescent enzyme immunoassays (CLEIA), which employ horseradish peroxidase (HRP), soybean peroxidase, or alkaline phosphatase with antigens or antibodies to catalyze reagents to emit light (Sakharov, Alpeeva, and Efremov 2006; Vdovenko et al. 2010; Šrámková et al. 2014), have been shown to be more sensitive and effective than conventional enzyme-linked immunosorbent assays (ELISA) (Marquette and Blum 2009). However, to the best of our knowledge, there are few published studies regarding the determination of enrofloxacin with antibody-based chemiluminescent enzyme immunoassays (F. Yu et al. 2012; S. Yu et al. 2014; F. Yu et al. 2014).

Aptamers are single-strand deoxyribonucleic acid (ssDNA) and ribonucleic acid (RNA) that can specifically bind to targets (Ellington and Szostak 1990). The method to find target-specific aptamers is called systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak 1990). The aptamers, which have been produced by numerous researchers, can act as an alternatives to antibodies. Compared with antibodies, aptamers are animal friendly products because they are selected in vitro experiments and can be copied more rapidly which makes them cost-effective and time-saving (Rimmele 2003).

In this study, a modified SELEX method that used magnetic beads as a supporter of enrofloxacin was employed with several ssDNA aptamers. The aptamer with the highest affinity was used to develop a direct competitive chemiluminescent enzyme immunoassay (CLEIA) for detecting enrofloxacin in milk. This novel, rapid, and cost-effective method has a high potential for monitoring enrofloxacin in animal husbandry.

MATERIALS AND METHODS

Apparatus

The Spectro Max 5 fluorescence microplate reader was from Molecular Devices, Inc. (Downingtown, USA). The Veritas microplate luminometer was from Turner Biosystem (Madison, USA). A Nanodrop 1000 spectrophotometer was from Nanodrop Technologies (Wilmington, USA). A 96-well white Nunc microplate MaxiSorp was purchased from Thermo Fisher Scientific (Roskilde, Denmark).
Dynabeads M-270 Amine and Ultra-0.5 centrifugal filter devices (10 K) were purchased from Invitrogen (Carlsbad, USA).

**Reagents**

Standards of ciprofloxacin, enrofloxacin, ofloxacin, norfloxacin, enoxacin, ofloxacin, and marborfloxacin were purchased from the China Institute of Veterinary Drug Control (Beijing, China). Bovine serum albumin (BSA) and streptavidin were obtained from Promega (Madison, USA). Other reagents (analytically pure) were supplied by Beijing Reagent (Beijing, China). The DNA library and primers were synthesized by Invitrogen (Carlsbad, USA). The chemiluminescence substrate solution (containing 3-(10⁻¹⁰-phenothiazinyl) propane-1-sulfonate, luminol and hydrogen peroxide) was provided by WDWK Biotech (Beijing, China). The following buffers were used: binding buffer, 2 mM MgCl₂, 20 mM tris(hydroxymethyl)amino methane (Tris-HCl), 100 mM NaCl, 1 mM CaCl₂, 5 mM KCl, and 0.02% Tween 20, pH 7.6 and elution buffer, 40 mM Tris-HCl, 3.5 M urea, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.02% Tween 20, pH 8.0.

**Preparation of DNA Library and Primers**

The ssDNA sequence (96-nucleotides) contained a 60-base central randomized sequence (N₆₀, the capacity was 4⁶₀) and had primer sites on both sides:

5'-CGTACGGTCGACGCTAGC-N₆₀-CACGTGGAGCTCGGATCC-3'. The primers used to prepare single strand nucleotides were 5'-GGATCCGAGCTCAGTG-3' and 5'-CGTACGGTCGACGCTAGC-3' (Li et al. 2011). The library and primers were dissolved in deionized water and stored at −20°C.

**In Vitro Selection of Aptamers for Enrofloxacin**

In order to select the most appropriate high-affinity aptamer, enrofloxacin-coated magnetic beads (for coating procedure, see supplementary information) were used in a semiautomatic selection procedure, and ofloxacin-coated magnetic beads were introduced to perform counter selection. Generally, in each SELEX round, the ssDNA pool (15 μg for initial round) was dissolved in 200 μL binding buffer and heated at 95°C for 7 min, then quickly cooled on ice for 10 min and incubated at room temperature for 2 min. The ssDNA was added to the prepared ofloxacin-coated magnetic beads; the number was 2 x 10⁸. After incubation for 30min at 25°C with shaking, the supernatant (containing unbound ssDNA) was incubated with enrofloxacin-coated magnetic beads (the number of the beads were 2 x 10⁸) for 30 min, and the supernatant was discarded. To elute the ssDNA from the enrofloxacin-coated magnetic beads, 200 μL of elution buffer were added to the beads and incubated at 80°C for 10 min with mild shaking. This process was repeated four times. The eluted ssDNA was pooled through ethanol precipitation. The sediment was dissolved in 20 μL deionized water, and the concentration was determined using a Nanodrop 1000 spectrophotometer (Niazi et al. 2008). The ssDNA recovered after each selection round was calculated as the percentage of bound ssDNA from the added ssDNA pool. The pooled ssDNA bound to
enrofloxacin was then amplified by an asymmetric polymerase chain reaction (asymmetric PCR). Each 20 μL of parallel PCR mixture contained 0.01 μM forward primer and 1 μM reverse primer. The PCR products (three-fifths of ssDNA pool in the last round) were denatured as previously described and used for the next selection round. After the last round, the PCR products were cloned and sequenced.

**Determination of Dissociation Constants**

The sequenced aptamer clones were investigated for their compatibilities against free enrofloxacin via binding assays. The equilibrium filtration (Jenison et al. 1994) method was used to perform binding assays. A series of concentrations, ranging from 0 to 0.8 μM, of selected aptamers were dissolved in 200 μL binding buffer, followed by heated at 95°C for 7 min, and rapid cooling on ice. A specified amount of enrofloxacin was added to those prepared aptamer solutions and incubated at 25°C for 30 min. The mixture was then loaded on a centrifuge (Ultra-0.5 Centrifugal Filter Device) for 5 min at 12,000 rpm, allowing approximately 100 μL of the solution to flow through the membrane. Similar to equilibrium dialysis, the solution that remained above the membrane contained free enrofloxacin, free ssDNA, and ssDNA-bound enrofloxacin. The filtrate contained enrofloxacin at equivalent concentrations as the free compound in the initial solution. An 50 μL filtrate sample was determined using the fluorescence microplate reader. To calculate the dissociation constants (K_d), the percent of bound enrofloxacin and unbound enrofloxacin (the degree of saturation) was incorporated into the following equation:

\[
Y = \frac{B_{\text{max}} \times X}{K_d + X}
\]

where \(B_{\text{max}}\) is the number of maximal binding sites, \(Y\) is the degree of saturation, and \(X\) is the concentration of ssDNA (Jenison et al. 1994; Li et al. 2011).

**Specific Tests of Individual Aptamer by Indirect Competition Assays**

The three highest binding aptamers were tested for their capacity with enrofloxacin, ciprofloxacin, and ofloxacin. The indirect competition assay was introduced to test the specificity and the distinguishing abilities from structural analogs (Mehta et al. 2011). Magnetic beads (2 × 10^5) coated with enrofloxacin were prepared to conduct this specificity assay. Aptamer (5 μg, approximately 180 pmol) was dissolved in 200 μL binding buffer and denatured as previously described. The solution was then incubated with each of three antibiotics (2 μM) for 30 min at 25°C in individual assays. This mixture contained free antibiotic, free aptamer, and aptamer-antibiotic complex. The prepared enrofloxacin-coated beads were incubated with the mixture for 30 min at 25°C. The ssDNA in the supernatant was removed for analysis by the NanoDrop assay. The reference assay was performed using binding buffer without antibiotic to incubate with aptamers. The difference of ssDNA concentrations between the individual antibiotic assays and the reference assays were obtained to verify the specificity of selected aptamers. Student’s t-test was employed to determine if the results were statistically significant.
Sample Treatment

Milk (containing 3.6 g fat per 100 g milk with high-temperature sterilization) was obtained from a grocery store in Beijing. The milk was confirmed to be fluoroquinolone-negative by high performance liquid chromatography – tandem mass spectrometry according to the National Standard of the People’s Republic of China (GB/T 21312. 2007). Appropriate enrofloxacin standards (in binding buffer) were added in 0.5 mL aliquots, shaken for 2 min, and centrifuged for 20 min (14,000 rpm at 4°C). The supernatants were diluted 10-fold with binding buffer.

Immunoadassay Procedure

To develop the novel aptamer-based chemiluminescent enzyme immunoassay, enrofloxacin-spacer-HRP (for synthetic process, see supplementary information) and the streptavidin-coated white microplates (for coating procedure, see supplementary information) were employed. First of all, 50 μL of sample or calibrator were added in the mixture of denatured biotin-aptamer (2 pmol, 25 μL) and enrofloxacin-spacer-HRP (1:125, 25 μL), and incubated for 30 min at 25°C. The binding system was added into the streptavidin-coated white microplate. The enrofloxacin and enrofloxacin-spacer-HRP that bind with the biotin-aptamer were captured by streptavidin on the microwell in a 30 min at 25°C. The plate was washed with binding buffer five times and 50 μL of the chemiluminescence substrate solution was added to each well. After incubation for 5 min at room temperature, the luminescence (relative luminescence units, RLU) was measured, and data analysis was performed according to Zhu et al. (2012). The mean intensity values were processed as the inhibition rate:

\[
\text{Inhibition rate} = 1 - \frac{RLU_{\text{sample}}}{RLU_{\text{max}}}
\]  

A standard curve was obtained by plotting the inhibition rate against the logarithm of analyte concentration and fitting to a four-parameter logistic equation using Microcal Origin (version 8.0, Microcal, USA).

RESULTS AND DISCUSSION

Selection of Enrofloxacin Aptamers

Enrofloxacin-specific aptamers were selected from the random library of ssDNA. After selection round eight, there were about 60% ssDNA recovered from the ssDNA pool (Figure 1). An enrichment of ssDNA specific binding to enrofloxacin was cloned and characterized. Six different sequences (No.11, No.17, No.19, No.21, No.27, and No.49) were selected for characterization (Table 1).

Determination of Dissociation Constants of Individual Aptamers

Binding assays were performed to determine whether the selected oligonucleotides were high affinity aptamers. Table 1 shows that the dissociation constants (K_d) of the aptamers were in the nanomolar range. Number 17 was quite remarkable, for
it had the largest G-richness (35%) and showed the best binding ability. The results showed that the lower the values of $K_d$, the higher the concentration of guanine in the sequence, and were consistent with previous studies that showed the guanine concentration (%)

Table 1. Flanked 60-nucleotide region ($N_{60}$) of the sequenced aptamers (5'-3') and the analysis of their guanine content

| Aptamer | Variable sequences of $N_{60}$ region in 96-nt aptamers (5'-3') | Dissociation constants, $K_d$ (nM) | Guanine concentration (%) |
|---------|---------------------------------------------------------------|-----------------------------------|---------------------------|
| No.11   | GGCGACGATGGGCACAGTCC-GAATCCTGACATCCCCCCACGA-TACCCCTGCGCTACTACA | 726                               | 21.7                      |
| No.17   | CCCATCAAGGGGGCTAAGGTTCGGCTCTCCTGAGGGCCCGGTATTGAGCGGA       | 188                               | 35.0                      |
| No.19   | CCCTAAGCCGGGGCTCTCCCA-GATGACAGGCTGCCAAGATAGCTGTTTCCACCTG   | 355                               | 28.3                      |
| No.21   | GGAGACCCTACCTGCTCTCTTCCCACAGCCTCCTACATTTATTCATGAGTAAGCTAATG | 1324                              | 16.7                      |
| No.27   | ACCGCAGGTGTCTGCTCCCCAAATCACAAGCCTGCTAATGCTGCTACCCGGATA      | 870                               | 18.3                      |
| No.49   | GGCGGGCTACAAATGAAAG-GATTGAGCTTAGAGAAGAAGCCTGTCCTTACCTGAGCT | 459                               | 23.3                      |

Note: Dissociation constants (Kd) of these aptamers were estimated by nonlinear regression analysis.
concentration in the aptamers influenced the binding affinity (Mehta et al. 2011; Davis and Spada 2007). The $K_d$ values for aptamers number 11, number 21, and number 27 were relatively high (0.726–1.324 $\mu$M), suggesting that their binding capacity were not strong enough to quantitatively isolate enrofloxacin from the binding buffer. Thus, these aptamers were discarded from further experiments.

The $K_d$ values for the selected aptamers were estimated by nonlinear regression analysis. Saturation curves (Figure 2) were obtained by plotting the degree of saturation vs. the concentrations of aptamers. $R^2$ values were used to indicate how well the data points fit a statistical model. The values for the aptamers were between 0.828 and 0.996, suggesting excellent fits (Mehta et al. 2011), and so the calculation of $K_d$ values was considered reliable.

**Specificity of Aptamers Using Indirect Competition Assays**

To verify the specificity of the aptamers for enrofloxacin, indirect competition assays were conducted. The difference of aptamer concentrations between the individual antibiotic assay and the reference assay reflected the relative affinity of each aptamer (Mehta et al. 2011). Values $> 0$ suggested that the concentration of aptamers in solution of the antibiotic assay were higher than that in reference assay; the larger the values, the higher the affinity of the aptamer for free antibiotic. On the contrary, values $\approx 0$ indicated that the antibiotic in the solution did not inhibit binding between aptamers and the enrofloxacin-coated beads. Therefore, the concentration of aptamers remaining in the solution of antibiotic assay had little effect compared with the reference assay.

According to Figure 3, aptamers number 17 and number 19 were better than number 49 in differentiating enrofloxacin from ciprofloxacin and ofloxacin. The

![Figure 2](image-url)  
*Figure 2.* Binding assays with single-strand DNA aptamers to enrofloxacin. Saturation curves were obtained by plotting the degree of saturation vs. the concentration of single-strand DNA aptamers. The dissociation constant was calculated by nonlinear regression analysis ($n = 3$).
difference in the values of $K_d$ may explain this phenomenon. $K_d$, the dissociation constant, shows the strength of binding. The lower the value of $K_d$, the stronger the strength of binding. Meanwhile, many studies reported that the stronger the binding between aptamer and target, the less probability of aptamer bonding to other molecules (Carothers, Oestreich, and Szostak 2006). Interestingly, aptamer number 17 exhibited a weak affinity towards cipfloxacin. It is speculated that one part of structure in aptamer number 17 may form a low-affinity bond with cipfloxacin (nonspecific binding) (Davis and Spada 2007). However, since aptamer number 17 differentiated enrofloxacin from cipfloxacin and ofloxacin ($P < 0.05$), it was selected for further study.

**Analytical Parameters of the Optimal Immunoassay**

Aptamer number 17 was used in the direct competitive chemiluminescent enzyme immunoassay with the biotin-streptavidin system because it allowed more enzyme molecules to catalyze the substrate with signal amplification (Lin et al. 2008). A competitive curve was obtained (Figure 4). Analyte was fortified to levels from 0.55 ng/mL to 1200 ng/mL in the binding buffer. The limit of detection (LOD, IC$_{10}$) and the IC$_{50}$ were calculated as the concentration of enrofloxacin inducing 10% and 50% inhibition, respectively. As shown in Figure 4, the LOD of this method was 2.26 ng/mL and the IC$_{50}$ was 24.27 ng/mL. The linear dynamic range was 6.43–89.99 ng/mL, which was determined by the values of IC$_{20}$ and IC$_{80}$.

The specificity of this method was evaluated by comparing enrofloxacin with cipfloxacin, norfloxacin, enoxacin, ofloxacin, and marborfloxacin. 2 µg/mL of each
fluoroquinolone were measured by the chemiluminescent immunoassay. The chemiluminescence intensity units were measured and presented as $\text{RLU}_{\text{max}} / \text{RLU}_{\text{sample}}$. In this assay, the concentration of fluoroquinolones was much higher than the maximum concentration of the standard curve, and, therefore, the maximal inhibition occurred in the presence of $2\mu g / mL$ enrofloxacin. If there were no significant inhibition by the other fluoroquinolones under the same condition, the cross-reactivity for these chemicals would be definitely classified as low. Figure 5 shows that there was no significant inhibition when high concentrations of the fluoroquinolones

Figure 4. Standard curve obtained by plotting inhibition rate versus concentration of enrofloxacin.

Figure 5. Specificity of the optimized chemiluminescent immunoassay using $2\mu g / mL$ of ciprofloxacin, enoxacin, norfloxacin, enrofloxacin, ofloxacin, and marbofloxacin.
(except enrofloxacin) were added. Therefore, this method was suitable for screening enrofloxacin.

Compared with the chemiluminescent enzyme immunoassay (F. Yu et al. 2014; F. Yu et al. 2012) or enzyme-linked immunoassay (Kato et al. 2007) based on antibodies, the values of IC₁₀ and IC₅₀ in this study were relatively high. However, the linear dynamic range of the immunoassay was below 100 ng/mL, which was the maximum residue limit for enrofloxacin (Wu, Lin, and Wang 2009), and met requirements for the detection of enrofloxacin.

Analysis of Enrofloxacin-Fortified Bovine Milk

Aptamers may be influenced by ions or proteins (Hianik et al. 2007); therefore, matrix interferences were a significant challenge for the assay for food analysis. G-quadruplexes are an important structural component of aptamers (Niazi et al. 2008), which are stabilized by hydrogen bonding and base stacking interactions between guanine bases (Huizenga and Szostak 1995). In this study, K⁺ and Na⁺ in the binding buffer were considered to facilitate the stabilization of G-quadruplexes because they would assemble into the quadruplex structure (Sen and Gilbert 1992). If the added matrix changed the concentrations of K⁺ and Na⁺, aptamers would not fold into the correct structure and prevent recognition. Similarly, if another region of the folded aptamer formed a bond to a compound (like a protein) in matrix, the accuracy would be influenced. Consequently, a comparison curve formed from the binding buffer and matrix at different fortified levels was used to assess the effects of the milk matrix (Figure 6). The results showed that the curve of 10-fold diluted matrix fit the standard curve well. Furthermore, it suggested that the assay is suitable for enrofloxacin in milk.

![Figure 6. Milk matrix effects on the optimized chemiluminescent immunoassay by comparison of calibration curves obtained from standard solutions prepared in binding buffer and in different matrix dilutions (1:2.5, 1:5, and 1:10).](image-url)
The recoveries of bovine milk fortified at 100, 200, and 400 ng/mL were investigated. Each sample was evaluated five times to evaluate the precision. The results are shown in Table 2. The recoveries of enrofloxacin in bovine milk were between 89.7% and 108.6%. The precision was satisfactory, with the coefficient of variation (CV) between 14.0% and 20.7%. These results indicated that enrofloxacin in bovine milk may be detected by this method with good accuracy and precision.

CONCLUSIONS

Several 96-nucleotide aptamers with a 60 nucleotide variable region were selected for enrofloxacin by a modified SELEX method. The dissociation constants of these aptamers were between 188 and 1324 nM, with number 17 showing the highest affinity for the enrofloxacin and high specificity compared to its structural analogs (ofloxacin and ciprofloxacin). Furthermore, this aptamer was used as a biological recognition tool for the determination of enrofloxacin. With the help of the biotin-streptavidin system, enrofloxacin was determined by a chemiluminescent enzyme immunoassay with a linear dynamic range of 6.43 to 89.99 ng/mL, which was below the maximum residue limit (100 ng/mL in the European Union). Enrofloxacin was determined with satisfactory accuracy and precision in fortified milk, demonstrating this method was applicable for routine analysis. Although the sensitivity of this method was not superior to some previous techniques, this approach did not require antibodies, eliminating the need for animals and the time required for antibody production, and may allow rapid determination of enrofloxacin.

Supplemental data for this article can be accessed on the publisher’s website.

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