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DEVELOPMENT OF AN ELAA KIT TO DETECT NEOMYCIN IN MILK

Nguyen Truong Giang[1,2], Tran Thi Bich Dao[1], Le Quang Huan[1,2], La Thi Huyen[1,2,✉]

[1]*Institute of Biotechnology, Vietnam Academy of Science and Technology*
[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology*

✉To whom correspondence should be addressed. E-mail: lthuyen@ibt.ac.vn

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SUMMARY

Antibiotics used in livestock production offer various benefits as an antimicrobial agent, growth promoter, and feed effective improvement. However, the abuse of antibiotics leads to antibiotic resistance which may seriously threaten human and animal welfare, and growing levels of antibiotics or antibiotic-resistant bacteria in the environment increase the numbers of drug-resistant infection outbreaks. Therefore, many detection methods have been being developed to quickly assess antibiotic content and its residues in foods. Among many analytical methods, the aptamer-based biosensor has considerable attention for its outstanding advantages such as high specificity, high sensitivity, and good selectivity. We use the ELAA (Enzyme-Linked Aptamer Assay) method - a variant of ELISA - which has a high affinity with neomycin. Firstly, we investigated different buffers to create the Neo-BSA complex. As result, 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 7 was found with the best results. Next, to help the Neo-BSA complex be fixed well on polystyrene wells, we surveyed various buffers and found the coating buffer (50mM Bicarbonate buffer, pH 9.6) rated as the most suitable for this process. In addition, the quality of the kit is also assessed through competitive ELAA reaction components. Therefore, we have investigated and optimized conditions such as aptamer concentration 25 nM in PBS buffer, and the biotinized aptamers did not need heat treatment prior to joining the reaction. From the results, we have successfully developed a calibration curve for antibiotic residue in milk using the ELAA technique, linear range 0,1 ng/mL and 100 ng/mL. Then, we initially surveyed 20 milk samples found that the ELAA method was consistent with the results from LC-MS/MS was obtained showing no difference between the two methods. We continued to test the samples to determine the kit's sensitivity and specificity. The results showed that the kit has a specificity and sensitivity of 100%. Finally, LOD and LOQ value had $x_{avg} = 0.448$; $SD = 0.22$, $LOD = x_{avg} + 3SD = 1.11$ (ng / ml); $LOQ = x_{tb} + 10SD = 2.65$ (ng / mL). We will continue to optimize the kit before being brought to the market.

Keywords: *Neomycin, aptamer, ELAA, ssDNA, Neo-BSA*

INTRODUCTION

Neomycin is an aminoglycoside antibiotic from *Streptomyces fradiae*. Neomycin is widely used in the treatment of gastrointestinal infections of domestic animals such as cattle. Also, neomycin is used for other purposes such as the promotion of animal growth. The unregulated use of antibiotics including neomycin causes the accumulation of antibiotic residual amount in meat and other products, seriously affecting human health such as kidney damage (Lakhin *et al*., 2013). Therefore, the detection and quantification of antibiotic residues in foods become a top priority. Currently, antibiotic residues in liquid samples

are detected mainly by high-performance chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and mass spectrometry (LC-MS). However, these methods are time-consuming and require modern measuring equipment and staff to have good expertise. To overcome those shortcomings, the biosensor method was one of the choices. It is a good technique for high accuracy and can guarantee a quick on-site analysis. In the specificity, the aptamer-based biosensor used in antibiotic residue detection is of interest. Aptamers are single-stranded DNA or RNA oligonucleotides, which can specifically bind to a wide range of target molecules, like nucleic acids, proteins, metal ions, and other small molecules with high affinity, selectivity, and sensitivity. Due to such advantages in comparison to antibodies, aptamers are promising alternatives for most applications. Suitable aptamers can be identified using a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX). In this approach, suitable binding sequences are first isolated from large oligonucleotide libraries and subsequently amplified (Mehlhorn *et al*., 2018). In fact, aptamers act like antibodies. Compared to antibodies, aptamer molecules are more easily absorbed and more heat-stable, especially having advantages in detecting small molecules such as antibiotics (Song *et al*., 2012). For aptamer screening, an *in vitro* screening procedure (SELEX) was used to isolate high-affinity aptamer to target molecules from a set of different aptamers. Aptamer plays an important role in many fields such as disease diagnosis and treatment, drug creation, drug delivery, food testing, toxin detection, bio-imaging, etc... (Jeong, Paeng, 2012). Around the world, in the field of food testing, aptamer in the ELAA method is widely used to detect and quantify antibiotics such as tetracycline in milk, kanamycin A (KaA) in dairy food samples, pork andchicken products.

The ELAA (Enzyme-Linked Aptamer Assay) method is derived from the ELISA method. ELISA (Enzyme-linked immunosorbent assay) is an immunoreaction based on principles linked to specific antibody-antigen. However, in the ELAA (Enzyme-Linked Aptamer Assay) method, the aptamers will be used instead of antibodies and color-producing substrates replaced with luminescent agents (Rhouati *et al*., 2013). The ELAA method using aptamer is a promising tool to detect and quantify antibiotics especially the neomycin in milk. In order to have a suitable method of analyzing neomycin residues in milk for the intended use, the development and approval of the method are necessary.

MATERIALS AND METHODS

Materials

Bovin serum albumin (BSA), antibiotic BSA conjugate (Neo-BSA) were purchased from Imgen Bio Science, Inc; Lamda exonuclease was purchased from BioLabs. Neomycin and other pure chemicals were purchased from Sigma, Merck. Streptavidin-enzyme horseradish peroxydase and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Invitrogen; PCR mastermix was purchased from Invitrogen and Promega. Other chemicals including $K_3Fe(CN)_6$, KCl, BSA, DTT, NHS, MES, H_2SO_4, etc... AptNeo6:5'-AGAGTCTGAGTAGGATGAGACTAGACA AGGTCCCAGG-3'.

The biotinized AptNeo6 has a concentration in the range 10–100 nM

METHODS

Investigation of buffers for Neo-BSA complex synthesis

Neomycin and BSA complex were modified according to the method of Lewis (Lewis *et al*., 1972) and Moryoka (Morioka *et al*., 1986) used radiological immunoassay for detection of antibiotics based on the specificity of the immunoreaction. Based on the modified Lewis' method, we dissolved 30 mg neomycin and 100 mg BSA in 6 ml of 2-(N-morpholino)

ethanesulfonic acid (MES), buffer pH 5.0. The solution was added with 15 mg 1-ethyl-3-(30dimethylaminopropyl) carbodiimide mixed in 0.5 ml of distilled water. The mixture was incubated for 18 hours at room temperature and then was dialyzed using PBS for removing salt residues and antibiotics at 4°C for 24 hours.

ELISA testing of synthetic Neo-BSA binding capacity

The wells were covered with the Neo-BSA complex (100 µL/well), incubated overnight at 4ºC. The wells were washed three times with PBS 1X to remove non-binding Neo-BSA. They were then incubated with skim milk 5% (300 µL/well) for 60 minutes at 37°C and the unbound proteins were removed by washing three times with PBS 1X. An amount of 100 µL AptNeo6 25nM was then added to each well, incubated, and shaken for 90 minutes at room temperature. After washings three times with PBS, the samples were added with streptavidin-HRP (100 µL/well) then incubated for 60 minutes at 37°C. Finally, 100 µL/well TMB solution was added and incubated for 20 minutes at room temperature until the color of the solution changed from colorless to blue. Then the coloring reaction was stopped by adding 50 µL of 0.5 M, H_2SO_4 and the color turned yellow. The absorbance was measured immediately at wave-lengths of 450 nm and 630 nm.

Investigation of buffer for fixation of BSA-Neo on polystyrene wells

We investigated two buffers PBS pH 7,4 and coating buffer (50mM Bicarbonate buffer, pH 9.6) to fix the BSA-Neo complex on to the polystyrene wells. Each well is repeated 3 times. The process is performed as ELISA testing of synthetic Neo-BSA binding capacity.

Investigation of competitive ELAA reactive ingredients

We investigated 3 concentrations of aptamer including 10, 25 and 50 nM. At the same time, we also evaluated the buffer system consisting of phosphate buffer PBS pH 7.4 and binding buffer in aptamer BB (BB: 100 mM NaCl, 20 mM, Tris, 2 mM MgCl2, 5mM KCl, pH 7.4) screening with 3 concentrations aptamer 10, 25, and 50 nM. Because the denaturation process is quite complicated, we conducted a heat treatment aptamer survey (aptamer was denatured at 95ºC within 10 minutes) and the aptamerdid not pre-heat ELAA with 3 concentrations aptamer 10, 25 and 50 nM for the purpose of evaluating whether the results are influenced by the aptamer denaturation process. The process is performed as ELISA testing of synthetic Neo-BSA binding capacity.

Determination of antibiotic residues in milk

The plates were first coated with 100 µL/well of the Neo-BSA complex and incubated overnight at 4°C. To remove the unbound Neo-BSA, the wells were washed three times with 300 µL of PBS 1X buffer. The wells were then blocked with 300 mL/well by 5% skim milk for 30–60 minutes at 37°C and the unbound proteins were removed. Next, 100 µL/well of AptNeo6 had a concentration of 25 nM (AptNeo6 in PBS) and different concentrations of neomycin: 0.01; 0.1; 10; 100; 1000 and 10000 ng/mLwere added, and binding was conducted at 90 minutes at room temperature. After washing 3X with 300 µL TPBS (Tween 0.05% in PBS), 3X with 300 µL PBS; 300 µL of streptavidin-HRP was added to the plate and incubated for 30–45 minutes at 37°C. Finally, 100 µL of TMB solution was added to each well and incubated for 20 minutes at room temperature until the color of the solution changed from colorless to blue, then the coloringreaction was stopped. Then the coloring reaction was stopped by adding 50 µL of 0.5 M H_2SO_4 and the color turned yellow. The absorbance was measured immediately at wave-lengths 450 nm and 630 nm.

Sensitivity and specificity analysis

The sensitivity and specificity of the ELAA Kit were determined based on analysis of 20 samples including samples collected from the market and market samples added with neomycin: 10 negative controls and 10 positive controls. The ELAA Kit sensitivity and

specificity would be calculated after determining the numbers false-positive (FP) and false-negative (FN), as well as true-positive (TP), and true-negative (TN) samples according to the following formula:

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}$$

Antibiotic detection in milk samples

Commercially sold milk was collected, supplemented with target antibiotics with different concentrations (done by the National Institute of Food Hygiene and Safety). Milk samples were collected and treated with trichloroacetic acid. Two (2%) solution of trichloroacetic acid was added to the milk sample in a centrifuge tube, mixed well with ultrasound for 30 minutes, then centrifuged at 10,000 rpm for 8 minutes and filtered through a 0.2 µm filter (Polyvinylidene fluoride - PVDF) to remove lipids. The pH was adjusted to neutral (pH = 7.0) with NaOH. Finally, samples were stored at 4°C to give the pH peak to neutralization.

RESULTS AND DISCUSSION

Investigation of buffers for Neo-BSA complex synthesis

The first step for the development of ELAA kit for the detection of neomycin residue in milk is the determination of a good buffer for synthesizing Neo-BSA that will be used as a standard target to adsorb tightly on the ELISA plate's wells. Accordingly, Neo-BSA complex synthesis was conducted in PBS pH 7.4 and in MES pH at 5, then dialyzed through cut-off membrane 30kDa to remove residual antibiotic. The complex was evaluated through competitive ELAA responses.

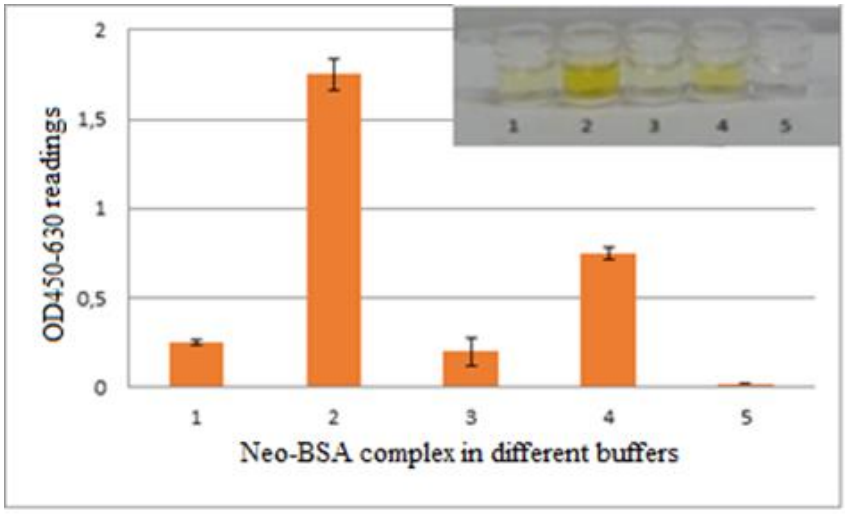


Figure 1. Diagram showing the binding capacity of the Neo-BSA complexs that were synthesized at different buffers. 1: Commercial Neo-BSA 10 X, 2: Synthetic Neo-BSA in MES buffer, 3: Synthetic Neo-BSA in PBS 10X buffer, 4: SyntheticNeo-BSAdiluted 10X by MES buffer, 5: BSA-EDC negative control.

The results showed that on the wells, the absorption of Neo-BSA/MES was the highest which means the Neo-BSA/MES complex had higher Neo content than other types. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is generally used as a carboxyl activating agent for the coupling of primary amines to yield amide bonds.

Additionally, EDC can also be used to activate phosphate groups in order to form phosphomonoesters and phosphodiesters. EDC is often used in combination with N-hydroxysuccinimide (NHS) for the immobilization of large biomolecules. In the MES buffer pH 5, the EDC helps to make the binding between Neo and BSA better because EDC worked wells at pH 4.5-6.5. In addition, the BSA-Neo complexes created in the MES absorbed better on the wells than commercial complexes (Fig. 1). Therefore, we selected MESbuffer pH 5 for preparation of the standard target complex Neo-BSA.

Investigation of buffer for fixation of BSA-Neo on polystyrene wells

We used two buffers PBS and coating buffer (50mM Bicarbonate buffer, pH 9.6) to fixthe BSA-Neo complex onto the polystyrene wells. The results showed that adhesion of BSA-Neo complexes to polystyrene surface in a coating buffer was better. In the next experiments, we used coating buffer to perform the fixation of the BSA-Neo complex.

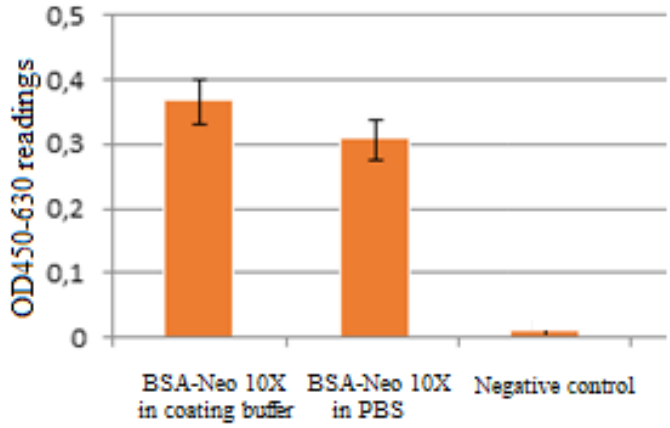


Figure 2. ELAA results for determination of BSA-Neo complex fixed buffer.

Investigation of competitive ELAA reactive ingredients

Selection of aptamer concentration

The amount of aptamer in ELAA assay is very important because it involves in the competitive ELAA reaction. If the amount is too low, it will not be sensitive enough, and if the amount is too high, it will affect on the competitive efficiency. Because it is difficult to determine the amount of Neo on BSA, we based on the articles by previous studies (Kimoto *et al*., 2019; Wildner, 2019; Wildner *et al*., 2019) with the selected three concentrations: 10, 25, and 50 nM aptamer.

The results showed that at the 10 nM and 50 nM aptamer, the obtained OD increases gradually, which means that the ability to bind to the target antibiotic increased. However, for safety and cost-saving, we selected 25 nM concentration for the next research studies.

Investigation of buffering and aptamer processing

One of the key components in the ELAA reaction is the buffering system, we investigated two buffers: Binding buffer (BB: 100 mM NaCl, 20 mM, Tris, 2 mM $MgCl_2$, 5 mM KCl, pH 7.4) and PBS pH 7.4. Also, we investigated samples of denatured aptamer and unmodified aptamer before binding to target antibiotics. Samples were surveyed at 3 concentrations of aptamer 10, 25, and 50 nM. Results were shown in Fig.4.

The obtained results were evaluated by t-test and it showed no significant difference

when using PBS buffer for the pre-heat treatment (BT) and BB buffer for the non-heat treatment (KBT) of aptamer in ELAA response when surveyed on 3 concentrations of aptamer. The obtained results were not in agreement with the resultsin previous studies (Wang *et al*., 2015; Jeong, Paeng, 2012 (Marathias, Bolton, 1999). Thus, through the obtained results, PBS buffers were used throughout the ELAA process and the biotinized aptamers did not need to heat for treatment prior to joining the reaction.

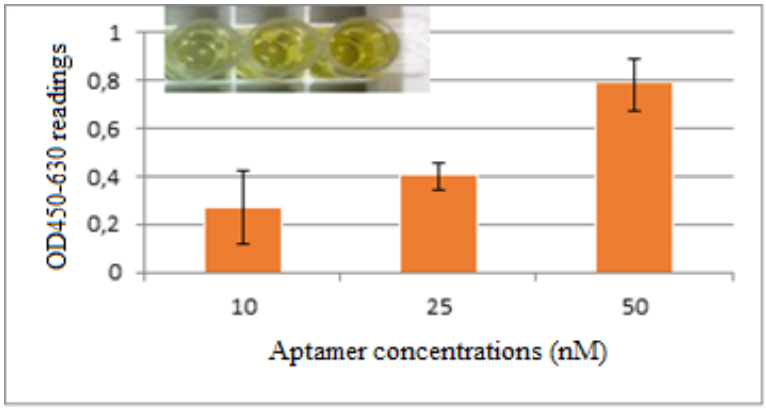
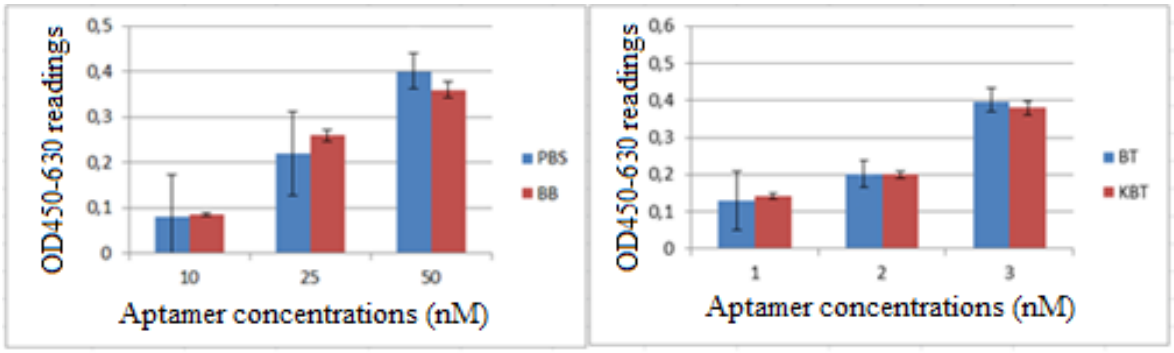


Figure 3. Results of ELAA using different concentrations of aptamer.



A B

Figure 4. Optimization of binding buffers and aptamer preprocessing with heat treatment aptamer and no heat treatment aptamer. (A): Investigation on two types of buffers: PBS phosphate buffer pH 7.4 and binding buffer in aptamer BB screening with 3 concentrations aptamer 10, 25, and 50 nM. (B): Evaluation on heat treatment of aptamer before performing ELAA with 3 concentrations aptamer 10, 25, and 50 nM. Evaluation on the difference between the absorbance values of samples at 3 aptamer concentrations by t-test showed no difference

Determination of neomycin in milk by competitive ELAA

Firstly, we applied the ELAA for the detection of different concentrations of neomycin prepared in PBS with the aim of finding a suitable concentration of neomycin for the assay. The obtained results showed that, as the antibiotic content increased gradually, the OD value decreased gradually, linearly in the range 0.1–100 ng/mL (data not shown). The results showed that it is possible to use this

competitive ELAA method to determine antibiotic residues. However, the above survey was done with antibiotics in PBS buffer. The objective of this study is to determine antibiotics in milk, so the artificial samples were prepared by adding to the milk with Neo at concentrations: 0.01; 0.1; 1; 10; 100; 1000; 10000 ng/mL. Separated milk samples were used for competitive ELAA. Results were shown in Fig.5.

The obtained results in Fig. 5 showed that the different concentration of Neo in PBS and in separated milk samples was expressed by the same partner, linear range and values statistically processed with t-test and p = 2,45> 0.05, this showed no difference between two types of samples. The results also indicated that the antibiotic extraction from milk samples was effective as well as applicable in ELAA for determining Neo residue in milk. We have also successfully developed a calibration curve for antibiotic residue in milk using the ELAA technique. Standard curve equation: y = – 23,729x + 91,702, R2= 0.987.

Determination of Neo in milk samples by competitive ELAA

In this test, milk and milk supplemented with Neo (prepared by the National Institute for Food Hygiene and Safety Test) were used.

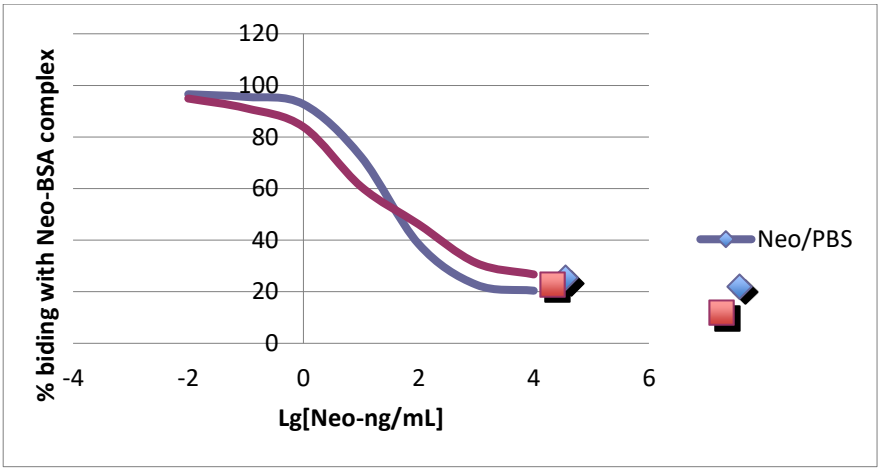


Figure 5. Results of determining antibiotic samples in milk and in PBS buffer by competitive Enzyme-Linked Aptamer Assay**.**

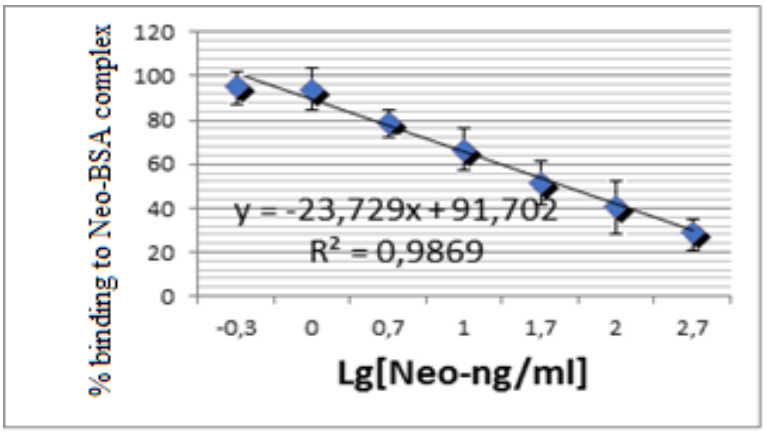


Figure 6. Result of developing a calibration curve to determine Neo antibiotic content.

Table1. Antibiotic-fortified milk samples.

Sample	Neomycin antibiotic content added (µg/kg) added	Neomycin antibiotic content (µg/kg) determined by ELAA	Neomycin antibiotic content (µg/kg) determined by LC-MS/MS
Neo100	100	63.7	101
Neo500	500	505.8	494
Neo1000	1000	963.7	926
Neo1500	1500	1458.6	1462
Neo1750	1750	1674.7	1664
Neo2000	2000	1753.6	1964
Neo2500	2500	2534.5	2468
Neo3000	3000	2779	2898
Neo4000	4000	4156.5	3904
Neo5000	5000	4777.3	4960
Fresh milk 100% with sugar Vinamilk	0	undetectable	undetectable
Strawberry fresh milk 100% Vinamilk	0	undetectable	undetectable
Love'in farm milk with sugar	0	undetectable	undetectable
UHT milk Hi-Land Moc Chau	0	undetectable	undetectable
Dutch Lady's Cao Healthy Pasteurized Milk	0	undetectable	undetectable
UHT milk strawberry milk true TH	0	undetectable	undetectable
UHT milk with sugar ADM Vinamilk	0	undetectable	undetectable
Lactose non-pasteurized milk, Vinamilk Flex	0	undetectable	undetectable
Strawberry ADM pasteurized milk Vinamilk	0	undetectable	undetectable
Cookies flavored pasteurized milk (KUN)	0	undetectable	undetectable

Comparing two numbers by the t-test, the test result $p = 0.245 > 0.05$ showed that the difference between the two numbers is not significant, so the survey within the framework of 20 samples including samples collected from the market and the antibiotic supplemented market samples showed no difference between the LC-MS/MS and ELAA method.

Preliminary result

The sensitivity and specificity of ELAA kit were determined based on analysis of 20

samples: 10 negative and 10 positive samples. Results were statistically calculated

Table2. Statistics result of ELAA.

Result		Total
Positive	**Negative**	
TP = 10	FN = 0	(+) = 10
FP = 0	TN = 10	(-) = 10
Antibiotic detected in milk (10)	Antibiotic not detected in milk (10)	20

$$\text{Sensitivity} = \frac{10}{10+0} \times 100 = 100\%$$

$$\text{Specificity} = \frac{10}{10+0} \times 100 = 100\%$$

In the study, the sensitivty and specificity of ELAA were both 100%.

LOD and LOQ determination

To compute LOD and LOQ values of competitive ELAA, we started from 10 negative milk samples (confirmed by LC-MS/MS). From there, we calculated the value of SD and xavg using Microsoft Excel software to get the value xavg = 0.448; SD = 0.22. The results obtained indicated that LOD = xavg + 3SD = 1.11 (ng / mL); LOQ = x tb + 10SD = 2.65 (ng / mL).

CONCLUSIONS

In this research, we have studied and created a test ELAA kit with high sensitivity. Besides, we also have studied and optimized the components of the kit. At the same time, we have also compared LC-MS/MS method to the test kit which showed similar results. Therefore, the kit developed by our study has given reliable results. Next to that, we have also set up a successful procedure to analyze neomycin antibiotic residue in milk by means of ELAA. In the future, we will continue to develop the kit to be used in both domestic and international markets.

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PHÁT TRIỂN BỘ KIT ELAA ĐỂ PHÁT HIỆN NEOMYCIN TRONG SỮA

Nguyễn Trường Giang[2], Trần Thị Bích Đào[1], Lê Quang Huấn[1,2], Lã Thị Huyền[1,2]

[1]Viện Công nghệ sinh học- Viện Hàn lâm Khoa học và Công nghệ Việt Nam
[2]Học viện Khoa học và Công nghệ, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

TÓM TẮT

Thuốc kháng sinh được sử dụng trong chăn nuôi mang lại nhiều lợi ích khác nhau như chất kháng khuẩn, chất thúc đẩy tăng trưởng và cải thiện hiệu quả thức ăn. Tuy nhiên, việc lạm dụng kháng sinh dẫn đến tình trạng kháng kháng sinh ngày càng tăng điều này có thể đe dọa nghiêm trọng đến sức khỏe của con người và động vật. Với mức độ sử dụng kháng sinh bừa bãi dẫn đến vi khuẩn kháng kháng sinh trong môi trường tăng lên làm bùng phát tình trạng kháng thuốc. Do đó, nhiều phương pháp hiện đại đã ra đời để đánh giá nhanh hàm lượng kháng sinh và dư lượng có trong trong thực phẩm. Trong số nhiều phương pháp phân tích, cảm biến sinh học dựa trên aptamer được chú ý nhiều nhất nhờ những ưu điểm nổi bật như độ đặc hiệu cao, độ nhạy cao và độ chọn lọc tốt. Chính vì vậy, chúng tôi đã tiến hành nghiên cứu và sử dụng phương pháp ELAA (Enzyme-Linked Aptamer Assay) - một biến thể của ELISA có ái lực cao với neomycin. Đầu tiên, chúng tôi đã khảo sát các bộ đệm khác nhau để tạo ra phức hợp Neo-BSA. Kết quả là dung dịch đệm axit ethanesulfonic acid (MES) 2- (N-morpholino) pH 7 cho kết quả tốt nhất. Tiếp theo, để giúp phức hợp Neo-BSA được cố định tốt trên các giếng polystyrene, chúng tôi đã khảo sát các bộ đệm khác nhau và nhận thấy bộ đệm phủ (đệm Bicarbonate 50 mM, pH 9,6) được đánh giá là phù hợp nhất cho quá trình này. Ngoài ra, chất lượng của bộ dụng cụ còn được đánh giá thông qua các thành phần phản ứng ELAA cạnh tranh. Do đó, chúng tôi đã nghiên cứu và tối ưu hóa các điều kiện như nồng độ aptamer 25 nM trong đệm PBS, và các aptamer sinh học không cần xử lý nhiệt trước khi tham gia phản ứng. Từ kết quả thu được, chúng tôi đã xây dựng thành công đường chuẩn cho dư lượng kháng sinh trong sữa bằng kỹ thuật ELAA, khoảng tuyến tính 0,1–100 ng / ml. Tiếp đó, chúng tôi khảo sát 20 mẫu sữa và nhận thấy rằng phương pháp ELAA phù hợp với kết quả từ LC-MS / MS. Kết quả thu được từ 2 phương pháp cho thấy không có sự khác biệt giữa hai phương pháp này. Chúng tôi tiếp tục kiểm tra các mẫu để xác định độ nhạy và độ đặc hiệu của bộ dụng cụ. Kết quả cho thấy bộ kit có độ đặc hiệu và độ nhạy là 100%. Cuối cùng, giá trị LOD và LOQ có x_{avg} = 0,448; SD = 0,22, LOD = x_{avg} + 3SD = 1,11 (ng / ml); LOQ = x_{tb} + 10SD = 2,65 (ng / mL).

Từ khóa: *Neomycin, aptamer, ELAA, ssDNA, Neo-BSA*