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Kalaiselvi G
Department of Animal Biotechnology, Madras Veterinary College, Central University Laboratory, TANUVAS, Chennai, Tamil Nadu, India

Tirumurugaan KG
Department of Animal Biotechnology, Madras Veterinary College, Central University Laboratory, TANUVAS, Chennai, Tamil Nadu, India

Dhinakar Raj G
Department of Animal Biotechnology, Madras Veterinary College, Central University Laboratory, TANUVAS, Chennai, Tamil Nadu, India

Vijayarani K
Department of Animal Biotechnology, Madras Veterinary College, Central University Laboratory, TANUVAS, Chennai, Tamil Nadu, India

Baranidharan R
Department of Animal Biotechnology, Madras Veterinary College, Central University Laboratory, TANUVAS, Chennai, Tamil Nadu, India

Corresponding Author:
Kalaiselvi G
Department of Animal Biotechnology, Madras Veterinary College, Central University Laboratory, TANUVAS, Chennai, Tamil Nadu, India

Preparation and characterization of streptavidin-biotin aptamer coated magnetic nanoparticle for colour based detection system of antibiotic residues in biological samples

Kalaiselvi G, Tirumurugaan KG, Dhinakar Raj G, Vijayarani K and Baranidharan R

Abstract
Aptamers are oligonucleotides that bind to a specific target molecule. Nucleic acid aptamers are usually created by selecting them from a large random sequence pool. Mostly magnetic nanoparticles are inert in nature, low toxicity and biocompatible. Functionalization of magnetic particle with an existing surface and gives a new surface with possibly new properties. Magnetic particle prepared by chemical co precipitation technique of iron salts in aqueous medium. The zeta value of -6.5Mv before coating with aptamer and 35.6 Mv after coating with OTC specific aptamer. The nanoparticle size shows 155.4nm before coating with OTC specific aptamer and 446.1nm after coating with aptamer. In case of coating, 250 µg of magnetic nanoparticle and 300 µg of streptavidin concentration shows good peroxidase activity. Polymerase chain reaction of oxytetracycline aptamer with specific primer shows amplification size 76 bp at 4% agarose gel in aptamer coated particle but negative in uncoated particle. Washing and storing of aptamer coated particle does not remove the aptamer from the surface of magnetic particle which indicates the aptamer and magnetic coupling is more stable. The DNA content of supernatant and coated magnetic particles were analysed by Nano drop which shows the coated magnetic particle have higher DNA content when compared with supernatant.

Keywords: Magnetic nanoparticle, aptamer, Oxytetracycline, streptavidin, PCR reaction

Introduction
Antibiotics are widely used for the prevention, control and treatment of diseases and infections. Improper use of antibiotics in livestock industries leads to accumulation of antibiotics in food products including meat, milk and chicken eggs (Kitazono *et al.*, 2012) [8]. Specific aptamer based detection of antibiotic residues in biological samples comparatively easy than other excising method. Aptamers are oligonucleotides that bind to a specific target molecule. Nucleic acid aptamers are usually created by selecting them from a large random sequence pool by a process called 'Systematic evolution of ligands by exponential enrichment' (SELEX). Aptamers are typically composed of single stranded DNA (ssDNA) or RNA. Aptamer can recognize a wide variety of targets, from small molecules to proteins and other macromolecules (Kim *et al.*, 2010) [7]. The specific binding of the aptamer to the target, results from structure compatibility, stacking of aromatic rings, electrostatic and van der Waals interactions and hydrogen bonding or combination of these effects. Aptamers exhibit specificity and avidity comparable to exceeding that of antibodies and can be generated against most of the targets. Aptamers can discriminate between targets using structural differences such as the presence of a hydroxyl group or a methyl group. The ability of specific oligonucleotide sequences to bind strongly and specifically to targets complementary sequence will allows oligonucleotide based detection strategies (Paramasivam *et al*., 2007) [10].
Magnetic nanoparticles are coloured and also exhibits Plasmon Rresonance will be utilized in the field of biological science. Mostly the magnetic nanoparticles are inert in nature, low toxicity and biocompatible. The surface functionalization of nanoparticle with specific bio molecules called as ligands which interact with nanoparticle surface by various forces like adsorption, van-der-Waals forces, electrostatic interaction and covalent bonding (Song *et.al* 2012) [12]. Functionalization of magnetic particle with an existing surface and gives a new surface with possibly new properties (Wang *et al*., 2006) [13].

The ligand molecules like thiol compounds, amino acids, proteins, antibodies and biological polymers confer colloidal stability. Streptavidin coated magnetic particle easily couple with biotinated aptamer. Magnetic metal nanoparticles have been used in protein/enzyme immobilization owing to their unique properties such as super para magnetism, high surface area, large surface-to-volume ratio and easy separation under external magnetic fields (Laurent *et al*., 2008) [9]. The advantages of nanoparticles such as magnetic nanoparticles ideally regulate the orientation of the proteins/enzymes on the supports. The non-porous nanoparticles doesn't have external diffusion problems, which makes the particle more competitive especially for large scale industrial usage in solid–liquid systems. However, as drawbacks compared to porous supports, proteins/enzymes immobilized on non-porous nanoparticles may suffer inactivation for soluble proteins/enzymes, especially through interaction with gas bubbles generated by strong stirring or bubbling of oxygen (Colomie *et al.*, 2001) [3] Such inactivation by interfaces might proceed and finally result in the irreversible activity loss due to the sustained effects such as destabilization of electrostatic, hydrophobic and hydrogen bonds (Wu *et al.*, 1993) [14]. The frequently utilized magnetic nanoparticles are iron oxides, superparamagnetic Fe_3O_4 nanoparticles are the most commonly used materials because they have low toxicity, good biocompatibility (Shubayev *et al.*, 2009) [11]. The bare iron magnetic nanoparticles have high reactivity and easily undergo degradation upon direct exposing to certain environment, leading to poor stability and disparity (Zhao *et al.*, 2012) [16]. Various modification methods have been developed to get the soluble and biocompatible iron magnetic nanoparticles for protein immobilization. The applications of immobilized enzymes based on iron magnetic nanoparticles very widely used in the fields of immunoassay, biosensor, bio separation, targeted drug delivery, and environmental analysis This study includes functionalization of magnetic nanoparticle with aptamer specific to oxytetracycline for the development of colour based methods for detection of oxytetracycline residues in biological samples.

Materials methods

The magnetic particle prepared by chemical co precipitation test by adding 10 ml of 1M of Ferric II chloride tetrahydrate and 20 ml of 1M ferric III hexachloride (Sigma USA). 50 ml of 1.5M sodium hydroxide taken in conical flax kept in magnetic stirrer. The ferric mixture were added drop by drop along with nitrogen perching and finally the solution were neutralized with 0.1N Hcl. The Nanoparticle pellet was collected and centrifuged at 3000rpm for 20 min and the supernatant were discarded and the magnetic pellet was washed three times with phosphate citrate buffer. The pellet was dried at 65 ºC overnight in an hybridization oven and the dried pellet were grinded in mortar and pestle and weighed. 5% suspension of magnetic particle were prepared by adding phosphate citrate buffer and sonicated for 30 min. The magnetic property of nanoparticles were checked by using magnetic stand. The pellet was washed three times by using phosphate citrate buffer. 0.3% suspension of magnetic particle were prepared and 100 µl of diamino benzidine were added and incubated at 37 ºC for 1 hr. The peroxidise activity of magnetic nanoparticle analysed by using ELISA plate reader at 650nm wavelength. The magnetic nanoparticles were stored at room temperature as well as refrigeration temperature for a month to analyse the stability. The synthesized magnetic particle characterized by the zeta potential and particles dispersion of nanoparticle preparations was recorded in Horiba SZ100 Nanopartica in the range of 400 to 700 nm using band width of 0.5 nm.

Coating the magnetic particle with streptavidin

Coating of magnetic particle with streptavidin done based on the method described by Zhang zhifing *et al*., (2007) [16] with slight modification. The unknown concentration of streptavidin determined by using known concentration BSA standard. 0.5 mg of freshly prepared magnetic particle were transferred in to 2 ml eppendorf tube and treated with 500µl of tris Hcl buffer (P^H 7.4). The particle was magnetically separated and supernatant was removed and the magnetic particle washed three times with tris Hcl buffer and 300 µg of streptavidin transferred into magnetic particle to ensure the final volume was 500 µl (0.3 mg/ml streptavidin). The mixture was kept in the shaking incubator for about 2 hrs. The magnetic particle and supernatant were separated by using magnetic stand and the coated particle subjected into peroxidise activity and the supernatant subjected into bradford assay. Pre-coupling and post-coupling Peroxidise activity of magnetic particles was determined and the coupling efficiency of streptavidin were analysed.

PCR confirmation of tetracycline specific aptamer

Aptamer are short single-stranded oligonucleotides with a three-dimensional structure that show high affinity of binding and high-specificity target molecule. The oxytetracycline biotin labelled Aptamer designed based on the method described by Jand *et al*. (2008). The nucleotide sequence of aptamer includes 5'CGT GCG GAA TTC GCT AGC CGA GTT GAG CCG GGC GCG GTA CGG GTA CTG GTA TGT GTG GGG AGC CGA GCT CCA CGT G 3' and the PCR was performed by using following primers Forward primer – 5' CGTACGCAATTCGCTAGC 3' and Reverse primer – 3' CACGTGGAGCTCGGATCC5'. 10µl PCR reaction volume includes 1µl volume of OTC biotin labelled aptamer, forward primer 1µl, reverse primer 1µl, mastermix 5 µl and 2 µl of nuclease free water. The PCR cyclic condition include initial denaturation 95 ºC for 5 min and denaturation 95 ºC for 30 second and annealing 60 ºC 30 second for 15 cycle. The agarose gel electrophoresis was performed by using 4% gel along with 50 bp ladder. The PCR reaction were performed with different concentration of OTC specific aptamer.

Immobilization of biotinated OTC specific aptamer with streptavidin coated magnetic particle: Coupling of biotinated oxytetracycline aptamer with streptavidin coated magnetic particle done based on the method described by Zhang ZhiFeng *et al.*, (2007) [15] with slight modification. The 250 µg of streptavidin coated magnetic particle transferred in to 2ml tube and washed three times with Tris Hcl buffer (P^H7.4). The coated supernatant and uncoated supernatant and aptamer coated magnetic particle and uncoated magnetic particle and streptavidin standard are subjected into PCR reaction with tetracyline specific aptamer with specific primers. The chemical-coated beads were washed five times with binding buffer and suspended in 200µl of the same buffer containing 1 µg of aptamers. The unbound aptamer was washed three times with binding buffer and pooled. The pooled aptamer (unbound and bound) was purified and measured using Nanodrop. The coated supernatant and uncoated supernatant and aptamer coated magnetic particle

and uncoated magnetic particle and streptavidin standard were subjected into dot blot assay.

Results and discussion

The magnetic particle synthesized by chemical co precipitation method. The magnetic nanoparticle shows good peroxidise activity after storing different temperature and different time duration.

Table 1: Analysing of stability and peroxidase activity of streptavidin coated magnetic particles with different temperature and different time interval

Temperature	Peroxidase activity			
	OD Value average at 0 day	OD Value average at 16 day	OD Value average after 30 day	OD Value average after 3 months
37 ºC	0.57	0.504	0.350	0.271
4 ºC	0.57	0.500	0.300	0.27

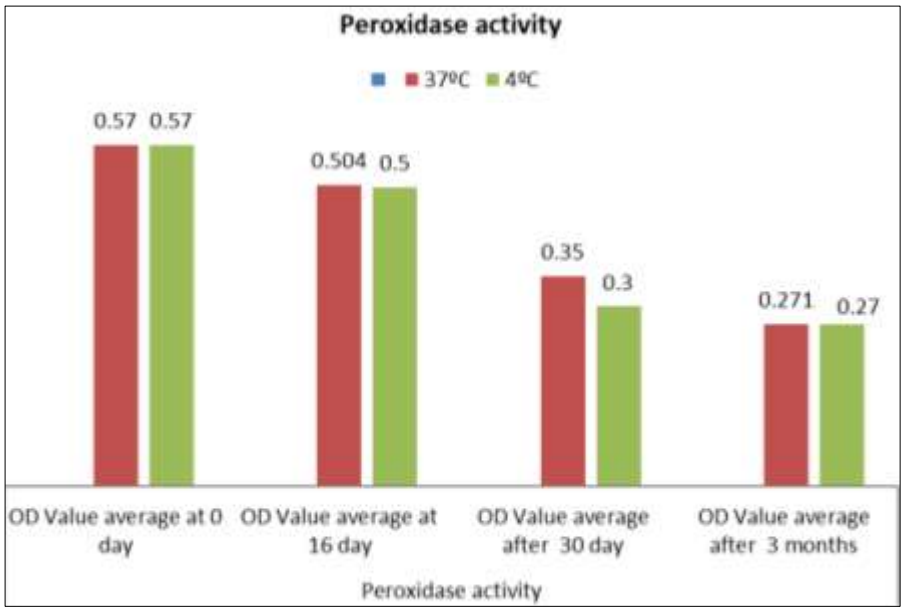

Fig 1: Analysing of stability and peroxidase activity of streptavidin coated magnetic particles with different temperature and different time interval

Magnetic particle prepared by chemical co precipitation technique of iron salts in aqueous medium might be the simplest and most efficient pathway to obtain iron magnetic nanoparticles. It has been demonstrated the particle size as well as the poly dispersity of the nanoparticles could be tailored by changing the associated factors such as Fe^{2+}/Fe^{3+} ratio base (NaOH, ammonium hydroxide, and CH_3NH_2), ionic strength ($N(CH_3)^{4+}$, $CH_3NH_3^+$, NH_4^+, Na^+, Li^+ and K^+). Slight increase of the mixing rate or temperature tends to decrease the particle size. Perching of nitrogen into the reaction system not only protects against critical oxidation of the magnetite but also reduces the particle size when compared to methods without oxygen removal.

The zeta potential and particles dispersion of magnetic nanoparticle preparations was recorded in Horiba SZ100 Nanopartica in the range of 400 to 700 nm using band width of 0.5 nm which shows zetavalue of -6.5Mv before coating with aptamer and 35.6 Mv after coating with OTC specific aptamer. The nanoparticle size shows 155.4nm before coating with OTC specific aptamer and 446.1nm after coating with aptamer.

Coating of magnetic particle with streptavidin

The effective concentration of streptavidin determined by using the known concentration BSA standard by using linear regression curve. The streptavidin coating efficiency with magnetic particle were analysed by peroxidase activity and Bradford assay and dot blot assay technique. In 250 µg of magnetic nanoparticle and 300 µg of streptavidin concentration shows good peroxidase activity than lower concentration and less peroxidise and protein concentration.

Table 2: Analysis of streptavidin coating efficiency in magnetic particle by Bradford assay

Brad ford assay	OD Value average
Coated particle supernatant	0.317
Uncoated particle supernatant	0.310
Blank	0.314

Determining peroxidise activity of streptavidin coated magnetic particle

Table 3: Comparison of peroxidase activity of streptavidin coated magnetic particles and uncoated particles

Peroxidase activity	OD Value average
Streptavidin coated particle	0.730
Streptavidin uncoated particle	1.105
Blank	0.314

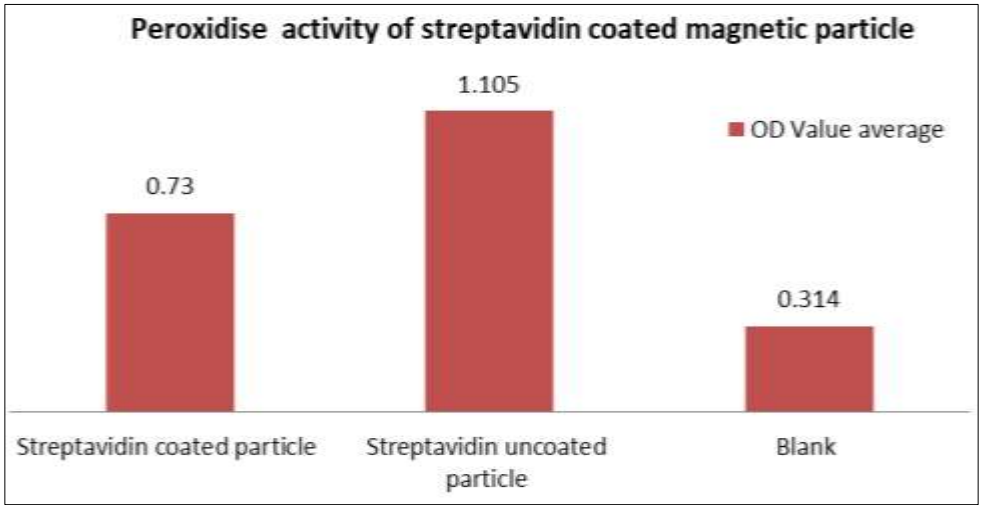

Fig 2: Comparison of peroxidase activity of streptavidin coated magnetic particles and uncoated particle

Polymerase chain reaction with OTC specific aptamer

Polymerase chain reaction of oxytetracycline aptamer with specific primer shows amplification size of 76 bp at 4% agarose gel in aptamercoated particle but negative in uncoated particle. Washing and storing of aptamer coated particle does not remove the aptamer from the surface of magnetic particle which indicates the aptamer and magnetic coupling is more stable.

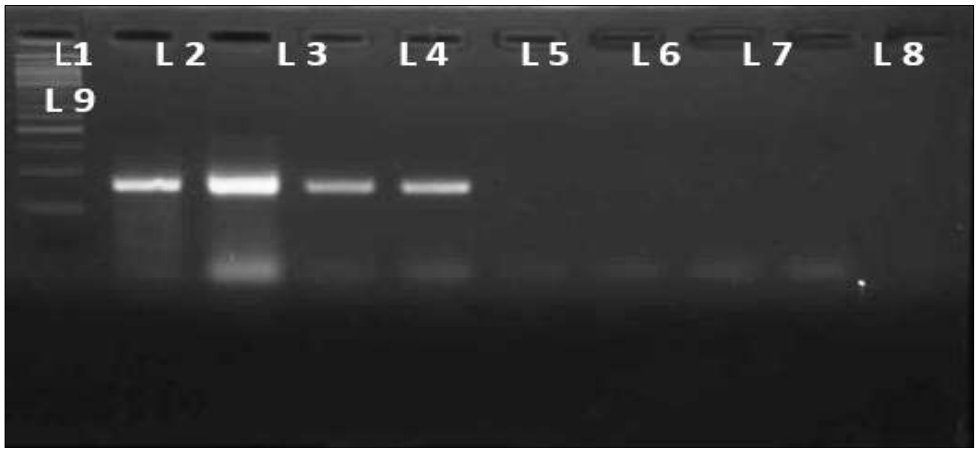

Fig 3: 4% Agarose gel shows amplicon size of 76bpL1 - 50bp ladder, L2 - OTC specific aptamer control & L3 - OTC specific aptamer as template L4 – Aptamer coated magnetic particle shows specific amplification &L5 - Aptamer coated magnetic particle supernatant shows specific amplification &L6 – Uncoated magnetic particle L7- Uncoated magnetic particle supernatant & L8 – Streptavidin control & L9 – Negative control

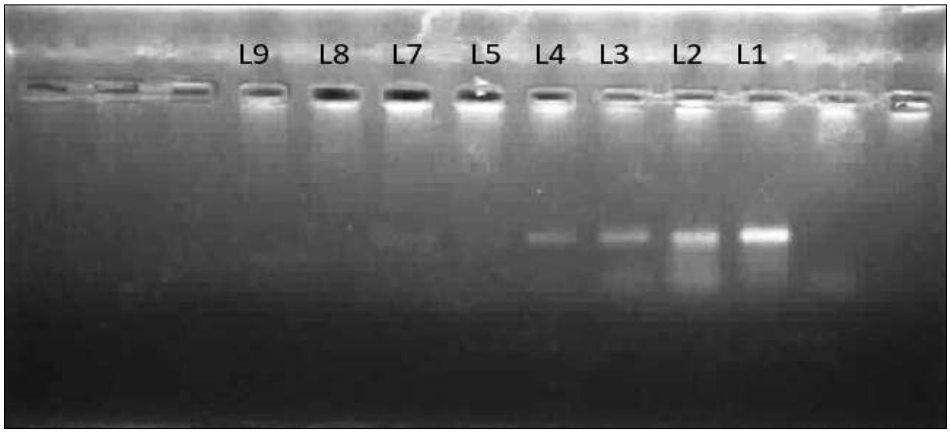

Fig 4: 4% Agarose gel shows amplicon size of 76bp L1-10pmol/µl, L2-5pmol/µl, L3-2.5pmol/µl, L4-0.5pmol/µl dilution of aptamer shows varying band intensity of amplification with specific primer

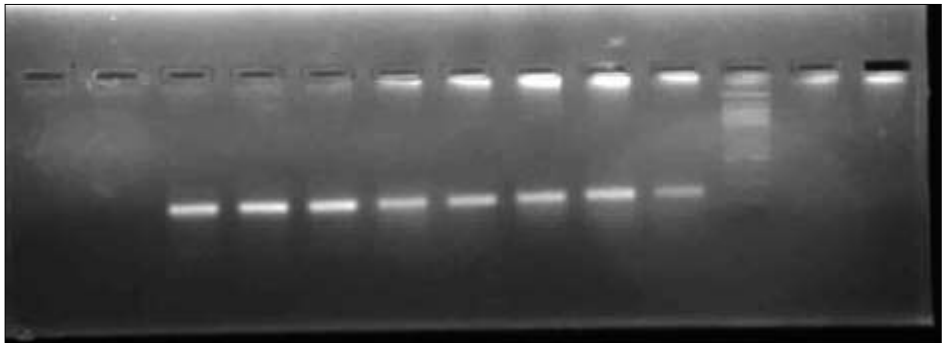

Fig 5: 4% Agarose gel shows amplicon size of 76bp and 10 pmol/µl aptamer coated magnetic particle shows same intensity of amplification with specific primer after 30 days storage at room temperature

Stability of aptamer coated magnetic particle

The streptavidin coated magnetic particle coupled with different concentration of OTC specific aptamer and stored at room temperature for 30 days shows PCR amplification with specific primer and good peroxidase activity. The DNA content of supernatant and coated magnetic particles were analysed by nanodrop which shows the coated magnetic particle have higher DNA content when compared with supernatant.

Table 4: Optimization of the different concentration of aptamer for efficient coating in magnetic particles

S. No	Concentration of aptamer for coating magnetic particle pmol//µl	DNA content in nanogram/µl	
		Supernatant of coated particle	Aptamer coated magnetic particle
1	100	3.5	10.9
2	50	2.2.	11.2
3	25	1.4	15.4
4	10	0.6	15.4
5	Uncoated magnetic particle	0.0	0.0

The peroxidase activity of different concentration of aptamer coated magnetic particles after storing at room temperature for 30 days shows the stability of particle and aptamer coating of magnetic does not changes the surface activity and stability and the peroxidase activity of magnetic nanoparticle not much affected with steptavidin and aptamer coating. We can use this magnetic particle for colour based detection system.

Table 5: Analysis of peroxidase activity of aptamer coated magnetic coated particle

S. No	Concentration of aptamer for coating magnetic particle pmol//µl	Peroxidase activity at 650nm OD value average
1	100	1.702
2	50	1.705
3	25	1.635
4	10	1.659
5	Uncoated magnetic particle	1.873
	Blank	0.453

The stability of aptamer coated magnetic particle shows good peroxidise activity after 4 weeks of storage at room temperature and the aptamer coated magnetic particle and uncoated magnetic particle shows at most similar peroxidase activity it indicates that the aptamer coated magnetic particle property doesn't changed similar to the finding of Zhang ZhiFeng *et al.* (2007) [13]. They found that the coupling efficiency is about 82.2% and the amount of oligonucleotide immobilized on 1 mg of streptavidin Goldmagnetic particles was 2839 picomol concentration and they found that the binding capacity for biotinylated oligonucleotide of these two kinds of streptavidin magnetic particles was about 6 times higher than that of Dynabeads M -270 streptavidin and finally they concluded that the streptavidin magnetic particles had high biotin binding capacity and able to bind many biotinated ligands. Biotinylated antibody, biotinylated oligonucleotide can maintain their biological activity (Zhang ZhiFeng *et al.*, 2007) [15]. Magnetic nanoparticles and gold nanoparticles are powerful tools used in biological fields such as immunoassay, nucleic acid hybridization and cell sorting.

Conclusion

The aptamer coated magnetic particle replaces many of complicated antibiotic detection system. Generally magnetic nanoparticles are inert in nature, low toxicity and biocompatible so prerequisite for every possible application is surface functionalization of nanoparticle with specific bio molecules which interact with nanoparticle surface by various forces like adsorption, van-der-waals forces, electrostatic interaction and covalent bonding. Enzyme linked aptamer based assay is one of the simple and low costly assay. In this short research we developed aptamer coated stable magnetic nanoparticle for the detection antibiotic residues in biological samples but further in depth detailed study is needed.

Declaration section

Ethical approval and consent to participate: Not applicable
Consent for publication: Yes I am agree
Availability of supporting data: Not applicable
Conflict of interest: Not interested

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