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# Rapid detection of *Brucella* cells using a gold nanoparticle-based aptasensor via a simple colorimetric method

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## Abstract

**Background** Brucellosis is a major worldwide zoonotic disease that is caused by *Brucella* spp. and threatens the health of communities. Novel methods for rapid detection of *Brucella* bacteria are beneficial and necessary in preventing infection and subsequent economic losses. Constructing biosensors with nanoparticles is a promising approach for identification of pathogenic bacteria in a short time. This study aimed to introduce a new detection method of *Brucella* cells using a biosensor, based on gold nanoparticles and a specific aptamer, via a colorimetric reaction. In this work, gold nanoparticles (GNPs) were synthesized and attached to the aptamer through electrostatic bonding. The binding of aptamer to gold nanoparticles was confirmed by Uv/vis spectrophotometry, FT-IR, transmission electron microscope (TEM) and zeta sizer (DLS).

**Results** In the presence of the bacterial cells, aptamers were bound to their targets, and the surfaces of the nanoparticles were depleted from aptamers resulting in intensified peroxidation activity of GNPs, and with the addition of 3, 3′, 5, 5′-tetramethylbenzidine (TMB), the color of the solution was changed from red to purple, which indicated the presence of *Brucella*. The sensitivity of the aptasensor was investigated using different concentrations of *Brucella* cells and its specificity was confirmed against several species of bacteria. The results showed that the designed aptasensor was more sensitive compared to PCR assay method with the ability to detect $1.5 \times 10^1$ CFU/mL of the bacterial cells.

**Conclusion** These findings indicate that the designed aptasensor can be used as a simple and rapid diagnostic tool to detect *Brucella* cells without need to experts and expensive laboratory equipment.

**Keywords** *Brucella*, Nanobiosensor, Colorimetric detection, Aptamer, Gold nanoparticles

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## Background

Brucellosis or Malt fever is one of the most common infectious diseases of humans and animals, which leads to economic losses in endemic areas and severe complications in affected patients. This disease is caused by *Brucella* spp that are Gram negative, facultative intracellular coccobacilli. Among them, *B. melitensis* and *B. abortus* are the most pathogenic species in humans and ruminants [1]. Symptoms of this disease in humans are undulant fever, headache, arthralgia and muscle pain, fatigue, weight loss, chills, and sweat [2]. While, brucellosis in livestock can cause a severe decrease in milk production, weak offspring, sterility, and abortion storm in herds [3]. Notably, this life-threatening disease in humans has resulted from the consumption of animal products (e.g. dairy products) as well as direct contact with infected animals or their carcass [4]. Consequently, prevention of human brucellosis is basically associated with the adoption of preventive and control programs for the infection in animals, an early step of which is diagnosis of the pathogenic agent or its infection.

Several methods including bacterial culture, polymerase chain reaction (PCR), serological tests like Rose Bengal test (RBT), serum agglutination test (SAT), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA) have been introduced for the detection of brucellosis [5, 6].

Although blood culture is the gold standard method to identify *Brucella* spp. The sensitivity of culture methods to detect *Brucella* is low, they are time consuming, and contain a risk for laboratory personnel [7]. The serologic method only detects the presence of *Brucella* antibody in serum of infected individuals, it may result in false positive/negative reactions because of cross-reactions between *Brucella* antigens with other bacteria [8]. However, molecular assays such as PCR are sensitive and capbale of detecting few *Brucella* cells in samples. These methods may not be suitable for rapid diagnosis of the disease due to limitations such as complicated sample pre-treatment, need to skilled laboratory staff, and expensive and special equipment [9, 10]. Therefore, development of rapid, simple, and inexpensive diagnostic methods/tools is desirable and will help in the prevention and control of *Brucella* infections [11].

In recent years, rapid detection has attracted the attention of researchers, especially those methods that use biosensors as detection agents [12]. Aptamer-based biosensors, called aptasensors, are considered as rapid and economical diagnostic methods compared to routine assays [13].

Aptamers are ssDNA or RNA molecules obtained by an in vitro selection process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [14]. Utilization of aptamers together with biosensors can resolve some of the problems associated with conventional methods [15–17]. Aptamers possess several advantages over antibodies: ease of synthesis, cheapness, high affinity of aptamers to target molecules, high stability to temperature, and ease of chemical modification [14, 18–20].

In association with optical biosensors, colorimetric method is one of those applicable signal transduction procedures that can be used in simple and rapid detection of pathogens by color changing which is visible to the naked eye without need for advanced equipment [21]. Various nanoparticles including magnetic, silica, gold, and $TiO_2$ nanoparticles have been used in the colorimetric method [22, 23], of which, GNPs are one of the most used due to their features such as ease of synthesis, non-toxicity, peroxidation like activity, strong plasmon resonance, and the ability to be functionalized with various biomolecules [24, 25].

Several nanobiosensors with complex structures and expensive bioreceptors have been introduced to detect *Brucella* cells. For example, Taheri et al. designed a nanobisensor based on magnetic-silica-nanoparticles and polyclonal antibody for detection of *B. abortus* [26]. In another research, Sikarwar et al. developed a Surface plasmon resonance (SPR) immunosensor based on 4-mercaptobenzoic acid modified gold (4-MBA/Au) and DNA probes for the detection of *B. melitensis* [27]. The objective of this study was to introduce a simple and rapid diagnostic method for detection of *Brucella* cells. In this work, for the first time, a new biosensor based on the GNPs and a species-specific aptamer, as a bioreceptor, was designed to detect *Brucella* via a colorimetric reaction. Considering the visual colorimetric reaction, this aptasensor is a simple and cost-effective nanobiosensor which can detect *Brucella* cells regardless of specific skills or materiel.

## Results

### Characterization of GNPs and aptasensor

UV-vis absorbance spectrum from the synthesized GNPs showed that GNPs have a strong wavelength band of around 520 nm (Fig. 1A).

As shown in Fig. 1B, attachment of the aptamers onto the GNPs caused a decreased in the absorption peak of GNPs at 520 nm. The emergence of a new peak was due to an electrostatic interaction between the citrate group of GNPs and the amine group of aptamer molecules, which confirmed binding of aptamers to GNPs [28].

The chemical modification was also confirmed by FT-IR analysis. Figure 2 illustrates the FT-IR spectra of GNPs versus GNPs-aptamers. The IR spectrum of GNPs-aptamers showed an absorption band at 3238 $cm^{-1}$ due to $NH_2$ stretching frequency of the amino groups of the aptamer, which was changed to the higher wavelength at 3519 $cm^{-1}$ after binding of aptamers to the GNPs. The

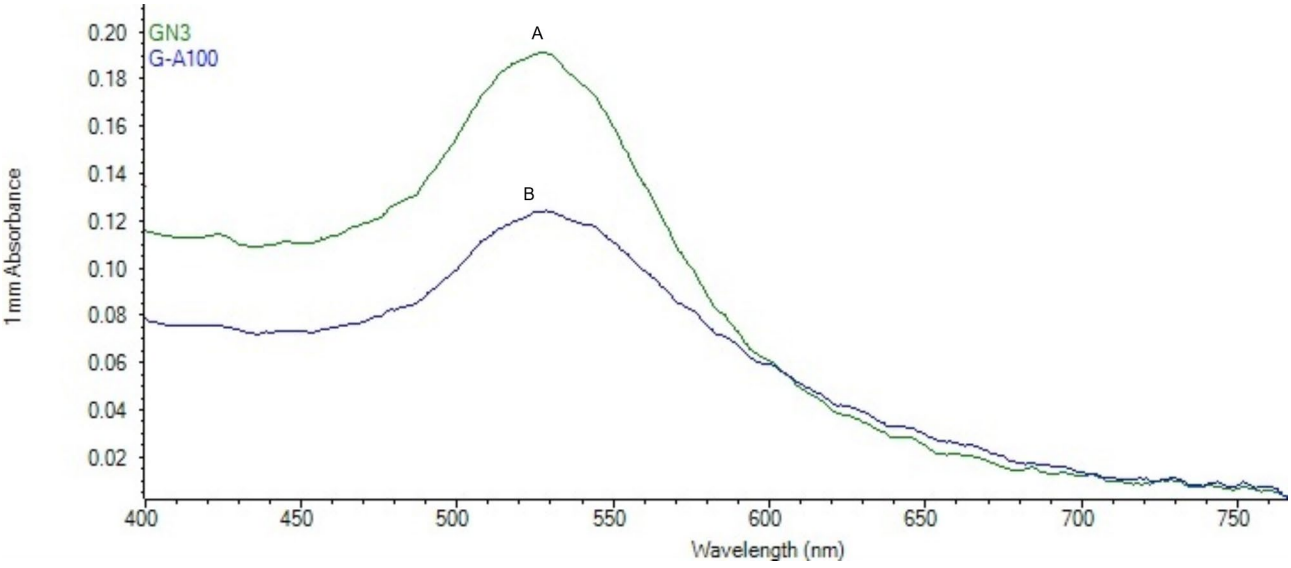


**Fig. 1** (**A**) UV-vis spectrum of the synthesized GNPs, (**B**) GNPs- aptamers

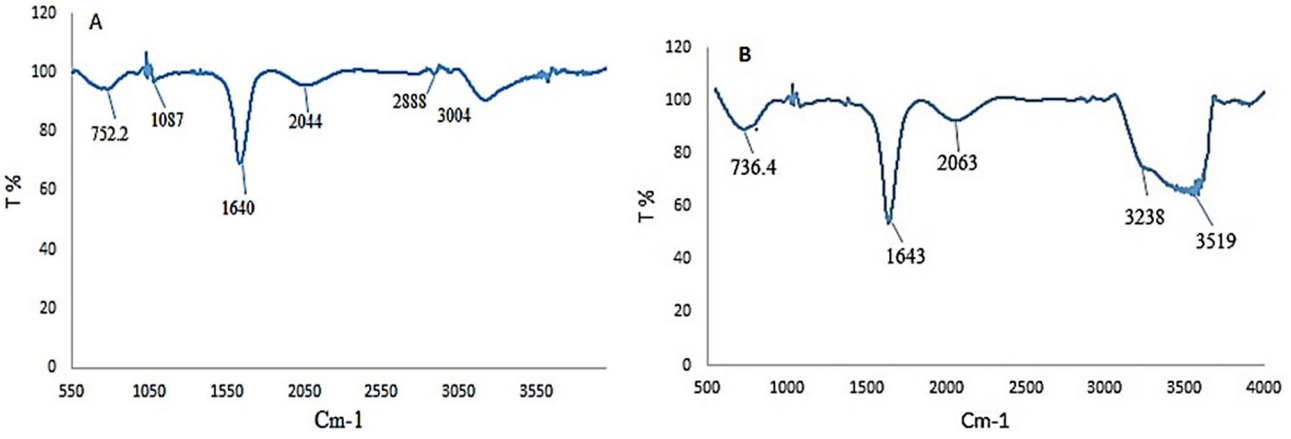


**Fig. 2** (**A**) FT-IR spectra of GNPs. (**B**) GNPs-aptamers

results indicated successful binding of nitrogen atoms of amino group of the aptamer to GNPs. The binding percentage rate for 10 µM aptamer to GNPs (100 ppm) was 46% in a period of less than 24 h of reaction.

The TEM micrograph (Fig. 3A) confirmed the production of GNPs in spherical shape in the size range of less than 40 nm. The TEM image of GNPs coated with aptamers with no undesirable change (e.g. aggregation) after the binding step is shown in Fig. 3B.

Figure 3C and D curves also present the average diameter of GNPs and GNPs-Aptamer constructs. The size and potential of the synthesized GNPs were 20 nm and −4.45 mV, respectively. However, after the binding of aptamers to GNPs, the size and surface charge were changed from 20 nm to −4.45 mV to 24 nm and 1.47 mV, which confirmed aptamer absorption on the surface of GNPs.

### Colorimetric detection of *Brucella* cells

A schematic illustration of the colorimetric method for the detection of target bacterial cells is shown in Fig. 4A. In the presence of *Brucella* cells, the aptamers are detached from the GNPs by their strong interaction with outer membrane protein (OMP) on the surface of *Brucella* cells. Hence, the GNPs exert their peroxidase-like activity on the substrate ($H_2O_2$) which leads to color change in the reaction mixture from red to purple due to TMB oxidation (as the chromogenic substance) in less than 50 min. While in the absence of the target, DNA aptamers block the surface of GNPs which prevents peroxidase activity and therefore, no color change will occur. The reactivity of aptasensor in the absence and presence of *B. melitensis* cells, as negative and positive controls respectively, is shown in the Fig. 4B.

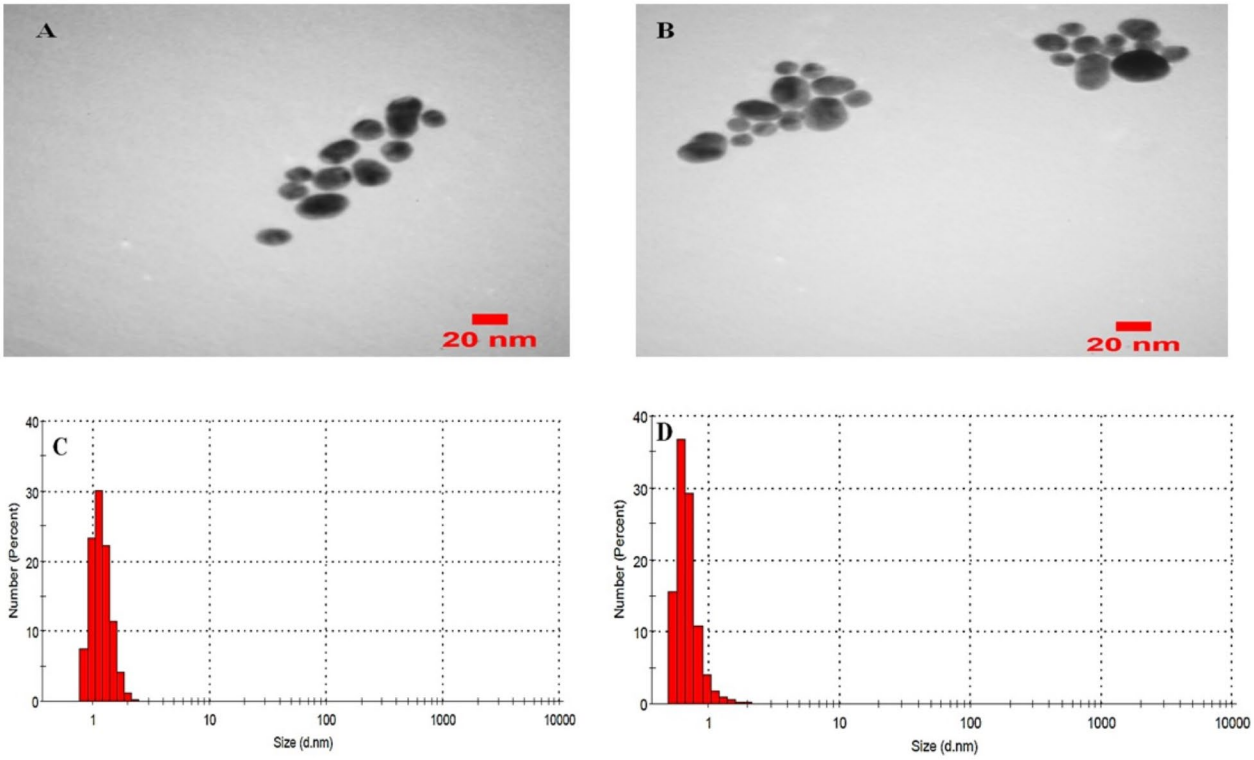


**Fig. 3** TEM micrograph: (**A**) synthesized GNPs. (**B**) GNPs coated with aptamer. DLS: (**C**) GNPs. (**D**) GNPs-aptamer

## Sensitivity

Aptasensor sensitivity assay was performed using different concentrations of *B. melitensis* from $1.5\times10^8$ to $1.5\times10^1$ CFU/mL. As shown in Fig. 5A, by increasing concentration of *B. melitensis*, the solution color gradually changed from light to deep purple. Also, an increase in absorbance values at 650 nm was observed in accordance with *B. melitensis* cell concentrations from $1.5\times10^1$ to $1.5\times10^8$ CFU/mL, which is shown in the regression equation with the calibration curve A650 nm, $Y=0.0084x+0.0935$, with $R^2=0.7245$ (Fig. 5B). Consequently, the limit of detection (LOD) of the newly designed aptasensor was determined to be $1.5\times10^1$ CFU/mL.

## Selectivity / specificity

To examine aptasensor selectivity, a panel of important bacteria species including *B. melitensis*, *B. abortus*, *E. coli*, *S.* Typhimurium, S. *aureus*, and *B. cereus* were included. None of the bacterial species showed a positive reaction except *B. melitensis* and *B. abortus*. Also, the highest rate of absorption belonged to the *B. abortus* (OD: 0.086) and *B. melitensis* (OD: 0.098) (Fig. 6).

## Real sample analysis

To study the feasibility of the designed aptasensor for the detection of *B. melitensis* in clinical samples, milk samples were spiked experimentally with *Brucella* bacterial cells. In case of milk samples, the detection limit was decreased to $10^6$ CFU/mL. This lower sensitivity could be due to the presence of natural inhibitory substances in milk e.g. high amounts of fat and proteins in which some organisms may get trapped. Nevertheless, to remove interfering materials, the spiked samples were diluted 1:10 with normal saline before the centrifugation step and the pellet was washed with normal saline as well. The absorbance rates were increased in accordance with the bacterial concentrations from $7.5\times10^1$ to $7.5\times10^7$ CFU/mL when the obtained pellet from the first spiked milk sample (concentration $7.5\times10^7$ CFU/mL) was diluted similar to the sensitivity assessment protocol indicating that appropriate laboratory procedures should be considered to eliminate milk inhibitory substances in order to maintain the aptasensor sensitivity level.

### Detection of *B. melitensis* by PCR

The results of the PCR test indicated that this method was able to identify the aforementioned amount of the extracted DNA sample from $1.5\times10^5$ CFU/mL concentration of *B. melitensis* cells (Fig. 7). Limit of detection

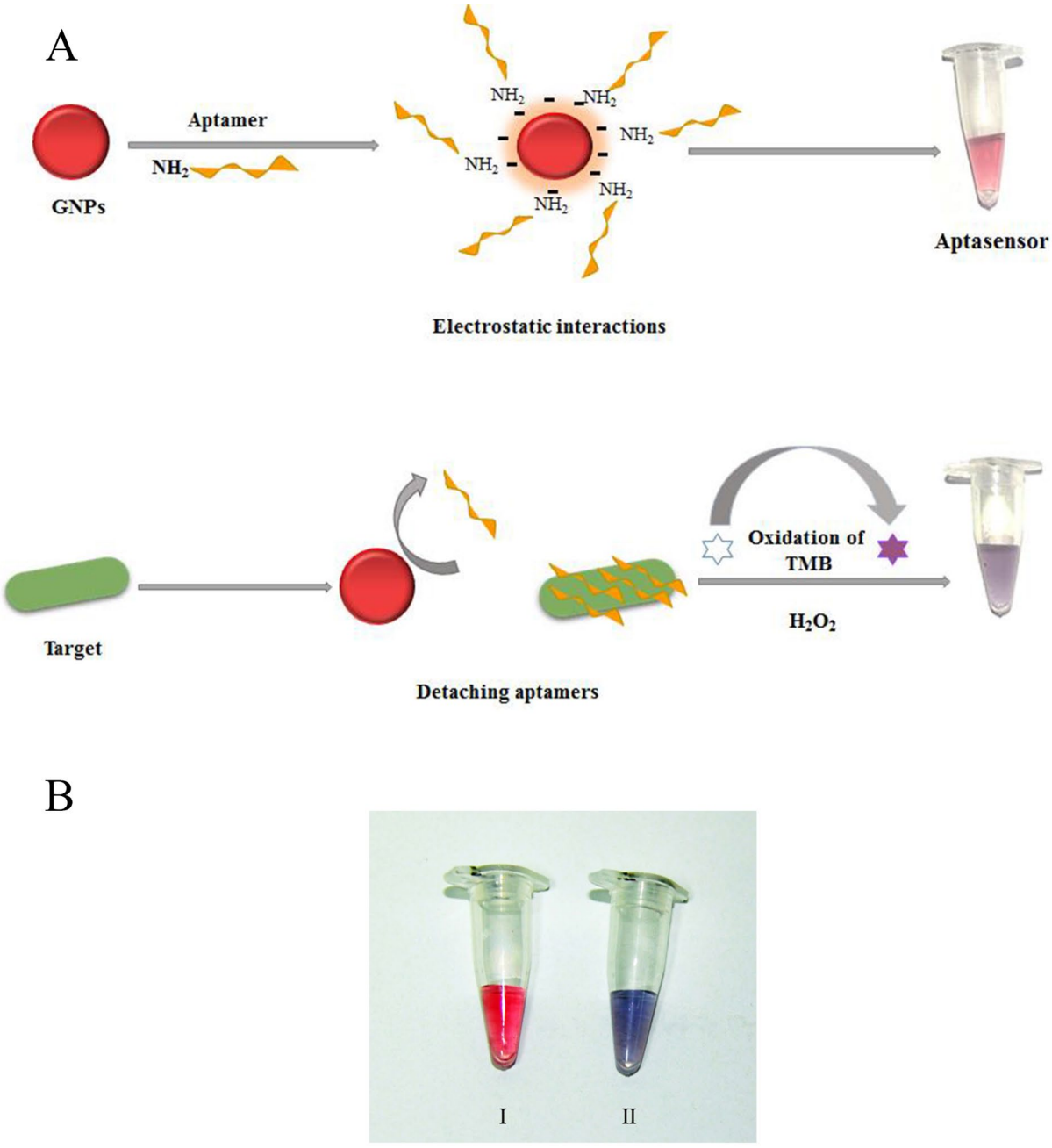


**Fig. 4** **(A)** Schematic illustration of the colorimetric reaction of the aptasensor to detect *Brucella* cell. **(B)** I: Reaction color in the absence of the bacterial cells. II: Reaction color in the present of the bacterial cells

(LOD) of several diagnostic methods for *Brucella* cells are compared in Table 1.

## Discussion

Brucellosis is one of the most important and common infectious diseases in developing countries, which leads to many economic losses and serious complications in affected patients. There are different methods to identify Brucellosis, including serological and molecular tests. Although these approaches are powerful and error-proof, most of them require a long preparation time specialized people and expensive equipment. In the following, a number of advantages and disadvantages of nanobiosensors over conventional methods for identification of *Brucella* spp are summarized in Table 2.

In this work, we introduced a simple, rapid and inexpensive method based on a new aptasensor and a colorimetric method for detection of *Brucella* cells. The

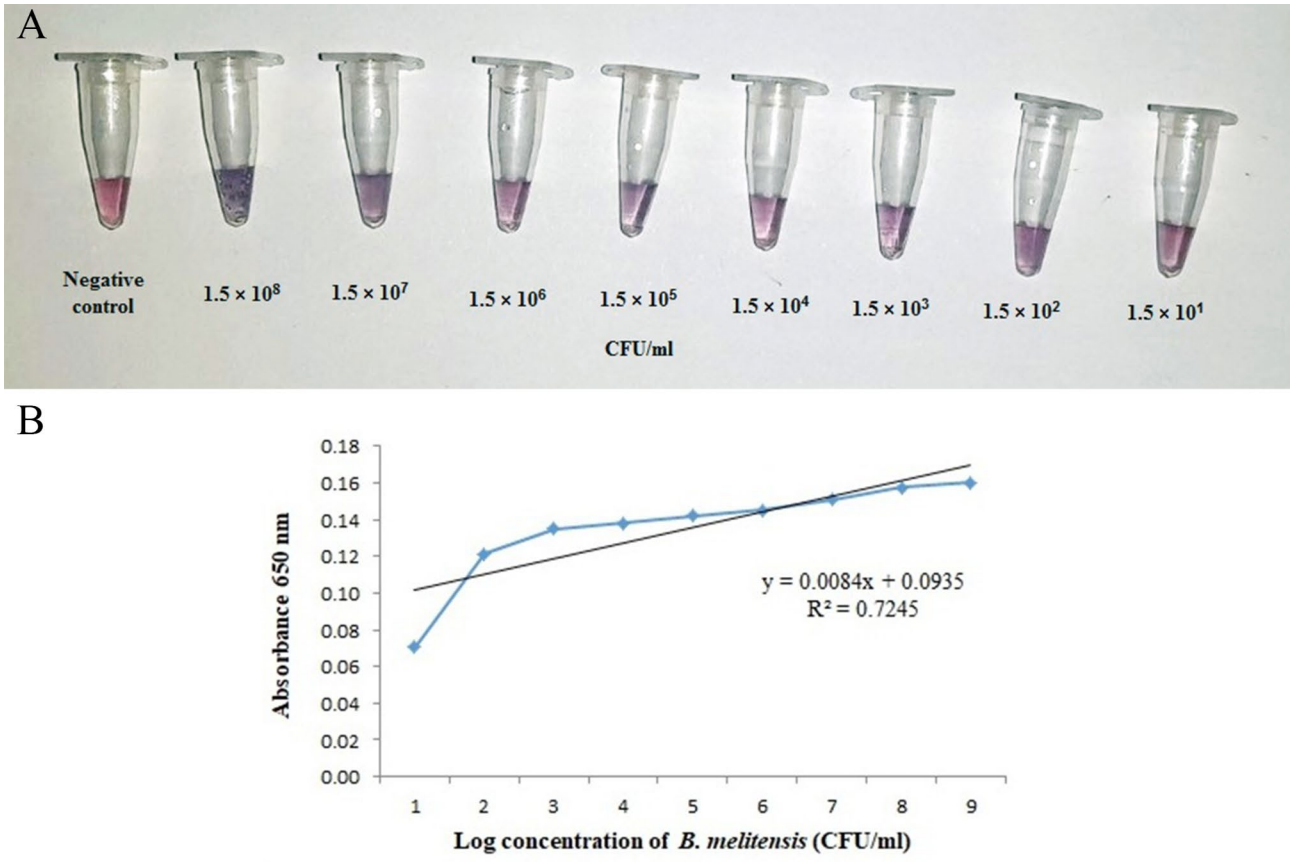


**Fig. 5** (**A**) Aptasensor sensitivity for the detection of *Brucella* cells from $1.5\times10^1$ to $1.5\times10^8$ CFU/mL. (**B**) Linear dependence between the OD values versus log *Brucella* cell numbers

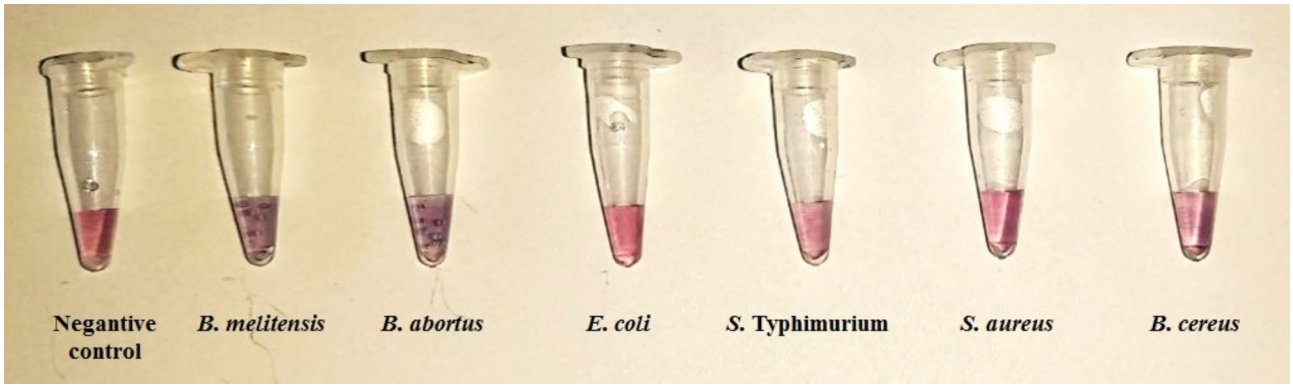


**Fig. 6** Specificity of the aptasensor against *Brucella* cells and the other bacterial species

aptamers were attached to gold nanoparticles through electrostatic bonding. In the presence of bacteria, the aptamers were separated from the gold nanoparticles surface and attached to the bacteria, which was observed to change color from red to purple in less than 50 min by adding TMB. To investigate the reproducibility of this method, the assay was repeated three times and produced exactly the same results every time. The results of the specificity test showed that the new aptasensor had a high specificity and was able to identify *B. melitensis* and *B. abortus* against other bacteria. It seems that these *Brucella* species share a common outer membrane protein (OMP) which led to the cross reaction of the aptamer molecules with surface structures of both species [36]. However, this can also be an advantage since these *Brucella* species are considered as the most important and frequent causative agents of brucellosis in humans and animals (Fig. 6). Shams et al. designed

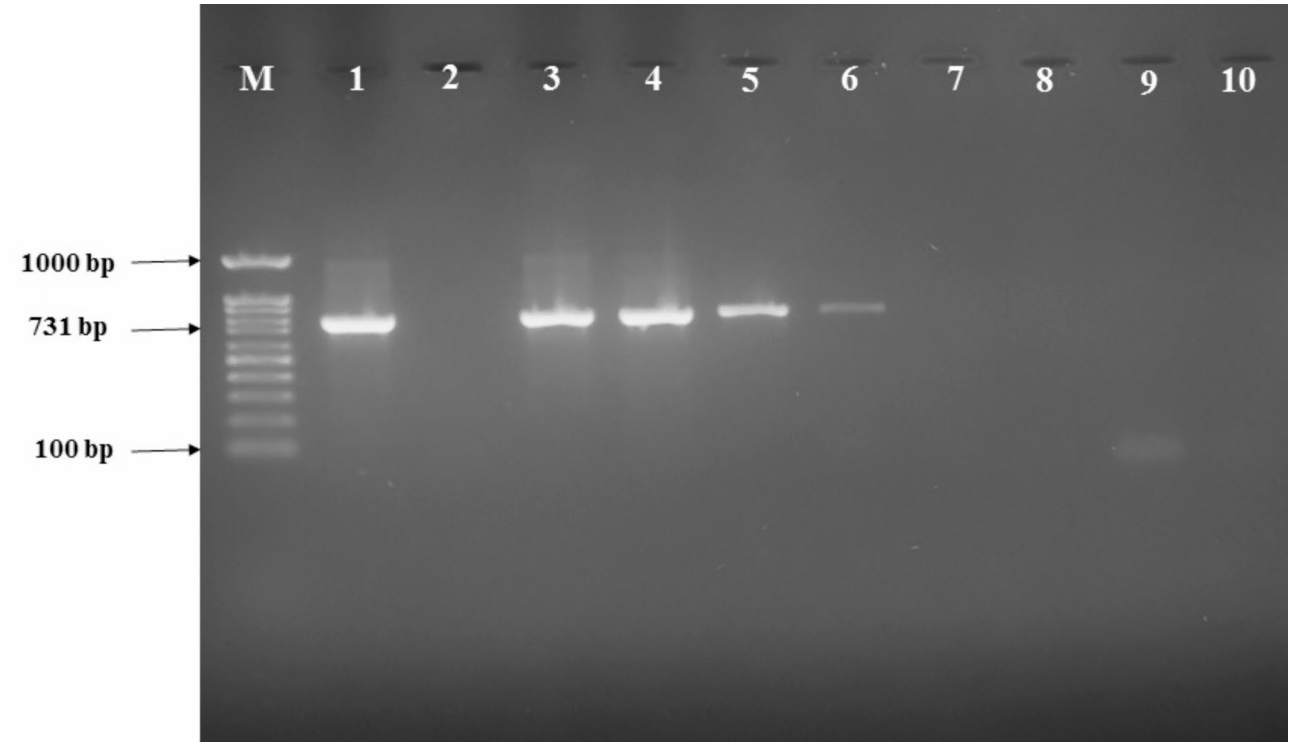


**Fig. 7** Agarose gel Electrophoresis of the species-specific PCR assay for the detection of *B. melitensis* cells. M: DNA marker 100 bp, lane 1: Positive control, lane 2: Negative control, lanes 3 to 10: PCR products of *B. melitensis* dilutions contained $1.5\times10^{8}$ to $1.5\times10^{1}$ CFU/mL

**Table 1** Limit of detection of some methods for the detection of *Brucella* cells

| Method | Recognition element | Sample matrix | Analyte | Analytical range | LOD | References |
|---|---|---|---|---|---|---|
| Electrochemical | Aptamer | Milk | *B. melitensis* | $10^{2}$ -$10^{7}$ CFU/ml | $10^{3}$ CFU/ml | [29] |
| Electrochemical | Antibody | Milk | *B. melitensis* | $4\times10^{4}$ & $4\times10^{6}$ CFU/ml | $1\times10^{4}$ & $4\times10^{5}$ CFU/ml | [30] |
| Optical (colorimetric) | DNA | Serum | *B. melitensis* | - | 3.32 pg/ml | [7] |
| Optical (colorimetric) | DNA | Semen, Urine | *B. abortus* | $10^{2}\times10^{5}$ CFU/ml | $10^{3}$ CFU/ml | [31] |
| Optical (colorimetric) | Antibody | Milk | *B. abortus* | $1.5\times10^{3}$-$1.5\times10^{8}$ CFU/ml | 450 CFU/ml | [32] |
| Fluoro-immunosensing system | Antibody | - | *B. melitensis* | 2.7–90 µg/l | 2.7 µg/l | [33] |
| PCR assay | DNA | Milk | *B. melitensis* | $1.7\times10^{3}$ -$1.7\times10^{4}$ CFU/ml | $1.7\times10^{3}$ CFU/ml | [8] |
| Real-time PCR | DNA | Meat goat | *B. melitensis* | - | 2 fg/ml | [34] |
| ELISA | Antibody | Serum | *B. melitensis* | $10^{2}$-$10^{8}$ Cell/ml | $10^{3}$ Cell/ml | [35] |
| Optical (colorimetric) | Aptamer | Milk | *B. melitensis* | $1.5\times10^{1}$-$1.5\times10^{8}$ CFU/ml | $1.5\times10^{1}$ CFU/ml | This work |

immunosensor based on the blue-silica nanoparticles and paramagnetic nanoparticles for detection of *Brucella abortus.* They used a polyclonal antibody as a receptor in the structure of the biosensor that was able to identify both species of *B. abortus* and *B. melitensis* similar to our work [32]. In the present study, aptamer was used as the receptor, which is cheaper and easily synthesized compared to polyclonal antibody [32]. In another work, Vakili et al., designed localized surface plasmon resonance (LSPR) nanobiosensor based on gold nanoparticles for identify anti-*Brucella* antibodies in the human sera [37]. Furthermore, Bayramoglu et al., designed a complex electrochemical biosensor (quartz crystal microbalance) based on magnetic nanoparticles and aptamer for detection of *B. melitensis* [29]. Nosaz et al. developed a DNA aptamer to detect *B. abortus* and *B. melitensis* through cell SELEX. They used a mixture of *Brucella melitensis* and *Brucella abortus* cells as the target. The isolated aptamers were able to identify *B. melitensis* and *B. abortus* with a remarkable binding efficiency [38]. Dursun et al. developed a Surface Plasmon Resonance (SPR) aptasensor for the detection of *B. melitensis* in milk samples. They used B70 and B46 aptamers which were immobilized on a surface of magnetic silica core-shell

**Table 2** Features of the conventional methods in comparison to biosensors for detection of *Brucella* Spp

| | Culture | PCR | ELISA | Biosensors |
|---|---|---|---|---|
| Advantages | Low cost | Specific | Easy to perform with simple procedure | Easy to use |
| | | Sensitive | High efficiency | High sensitivity |
| | Selectivity with chromogenic media | Accuracy | Labor-intensive and expensive to prepare antibody | Low cost |
| | - | Detection of small amounts of target nucleic acid | Simultaneous analysis can be performed without complicated sample pretreatment | Short response time |
| Disadvantages | Time consuming | Time consuming | Labor-intensive and expensive to prepare antibody | Pretreatment of sample may be required |
| | Low sensitivity | Possible false negative PCR results | High possibility of false positive/negative results | - |
| | Microbial contamination | No distinction between dead or alive cells | Antibody instability | - |

nanoparticle and SPR sensor chips. Their aptasensor was able to detect 27 ± 11 cells in one ml of milk sample [39]. However, the detection method developed in the present study is on the basis of a colorimetric reaction and is more simple and cheaper than the electrochemical and SPR sensors.

Pal et al. developed a nanobiosensor based on gold nanoparticles and a DNA probe as a receptor to detect *Brucella abortus* by colorimetric method. In their work, bacterial DNA was extracted and hybridized with the probe, and gold nanoparticles were aggregated by adding NaCl, and the color of the solution changed from red to purple [31]. While in our nanobiosensor, aptamer was used as a receptor. The aptamer identified the bacterial cell without DNA extraction. According to the other works, so far, a nanobiosensor based on gold nanoparticles and aptamer has not been designed to identify *Brucella* cells by colorimetric method, and this aptasensor was simple and inexpensive. The sensitivity test was compared with PCR assay. Different detection limits of the PCR for the detection of *Brucella* spp have been previously reported [6, 8]. Different factors including the effectiveness of DNA extraction protocol, size sample processed, and *Brucella* species may affect PCR sensitivity. In this study, $1.5\times10^5$ CFU/mL of *B. melitensis* was detected by the PCR assay. The results showed that the designed aptasensor was more sensitive compared to PCR assay and was able to detect $1.5\times10^1$ CFU/mL of the bacterial cells. In addition, the colorimetric method was able to identify bacterial cells within 50 min without the need for complex steps and advanced devices. Whereas, PCR assays require DNA extraction, electrophoresis, time-consuming steps, and expensive equipment.

## Conclusion

Several methods have been developed for the detection of *Brucella* cells. However they applied different strategies to sense the target cells at the first step and/or reveal the results at the final step. For example, application of aptamer molecules has previously been reported, but the bacterial identification was done using a quartz crystal microbalance (QCM) aptasensor and an oscillator. Recognition of *Brucella* cells using colorimetric reaction has also been performed but with the aid of *Brucella* specific antibodies as bioreceptors.

Using a combination of aptamers (as inexpensive bioreceptors) and a simple colorimertic reaction, a novel method for detection of *Brucella* cells has been introduced in this study for the first time. In comparison with the conventional diagnostic methods which are associated with special requirements, this method makes it possible to detect as low as $1.5\times10^1$*Brucella* cells in a very simple and rapid manner with high sensitivity and specificity in less than ~50 min. This method can be applied to identify *Brucella* infections by non-specialists and even in cases, where there is no access to an equipped laboratory.

## Methods

### Materials

Hydrogen tetrachloroaurate trihydrate ($HAuCl_4{\cdot}3H_2O$) and trisodium citrate were purchased from Sigma (USA). *Brucella* Agar, Nutrient Agar, 3,3′,5,5′-tetramethylbenzidine (TMB), $H_2O_2$, were purchased from Merck (Germany). 5′-amine-modified *B. melitensis*-specific DNA aptamer (5′$NH_2$-GAG AGT AAA GGC CAT CGG CGG CCA TTT ATG TTG TAC CC) with deltaG 55.4 Kcal/mol and affinity constant (Kd) of 5760 CFU $mL^{-1}$ for *B. melitensis* [29] was ordered to Genfanavaran Co. (Tehran, Iran). The primers used for the *B. melitensis*-specific PCR assay (forward: AACTGCTGGAGATGAATCCG and reverse: GAATGTTTGCACGCATCAAT) [40] were also obtained from Sinacolon Co. (Tehran, Iran).

### Bacterial strains

*Brucella melitensis* strain Rev. 1 vaccine and *Brucella abortus* strain RB51 vaccine, as positive controls, were prepared from Razi Vaccine and Serum Research

Institute, Iran. Beside, *Salmonella* Typhimurium ATCC 14,028, *Escherichia coli* ATCC 25,922, *Staphylococcus aureus* ATCC 49,775, and *Bacillus cereus* ATCC 11,778, as negative controls, were included in the study.

#### Bacterial culture

Vaccine strains of *B. melitensis* Rev. 1 and *B. abortus* RB51 were cultured on *Brucella* agar medium supplemented with 5% of sheep blood and incubated at 37 ˚C for 48 to 72 h. Other bacteria were cultured on nutrient agar medium and incubated at 37 ˚C for 24 h.

#### Synthesis of GNPs

GNPs were prepared by a standard citrate reduction method. Briefly, 100 mL of a $HAuCl_4$ (1 mM) solution was allowed to boil in a round bottom flask. While boiling, 10 mL of sodium citrate (38.8 mM) was quickly added to the mixture. After changing the solution color from pale yellow to wine red, the mixture was refluxed for another 15 min. Finally, the solution was cooled overnight at room temperature and stored in a cool and dark place until used [41].

The transmission electron microscopy (TEM) was used to confirm synthesis, shape, and size of the nanoparticles. To prepare the sample, a drop of GNPs was placed on a carbon-coated copper grid and dried in a vacuum desiccator. Then, imaging of the sample was done by TEM (Zeiss EM900, Sharif University of Technology).

Meanwhile, surface plasmon resonance of GNPs was characterized using an UV–vis spectrophotometer (Cary 100, Varian, Canada) at the resolution of 1 nm from 400 to 800 nm [41].

#### Preparing diagnostic aptasensor

A previously described species-specific aptamer for *B. melitensis* [29] was coated on GNPs surfaces to construct detecting aptasensor for *Brucella cells*. First, the aptamer molecules were heated at 95 ˚C for 10 min and slowly cooled down to 37 ˚C. This caused aptamer denaturation and turned them into a native tertiary structure for suitable interaction with the target bacterial cells. Then, 5 µl of the aptamer solution (10 µm) was added to 50 µl of GNPs. The mixture was shaken with a speed of 100 rpm for 30 min and incubated overnight at 37 ˚C [42]. The optimum aptamer concentration was measured using the UV-Vis spectrophotometer at 260 nm before and after aptamer-GNPs incubation ( [29] and aptamer binding rate was calculated using the following equation, the UV absorption difference before ($A_1$) and after aptamer binding ($A_2$): $(A_1- A_2) \times 100)/A_1$ (%) [42].

#### Characterization of aptamer binding to GNPs

The attachment of aptamers to GNPs was confirmed by UV–vis spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, TEM, and DLS. The wavelength of GNPs after aptamer binding was measured at 520 nm by a UV–vis spectroscopy. FT-IR analysis was performed by a FT-IR spectroscopy (Perkin-Elmer FT/IR-SDC300, USA) to confirm binding of the aptamer molecules to GNPs [42]. Zeta potential and the hydrodynamic diameters of GNPs were determined by dynamic light scattering (DLS) technique (Zetasizer Nano ZS Malvern, UK).

#### Colorimetric detection of *Brucella* cells

To detect *Brucella* cells, *B. melitensis* was selected, as the genus type species and most pathogenic sp. in humans, to perform the experiment. At first, 5 µL of *B. melitensis* suspension (concentration $1.5\times10^8$ CFU/mL) in normal saline (0.1 M) was added to 25 µL of aptamer coated-GNPs, and the mixture was incubated at 37 ˚C for 30 min or at room temperature for 45 min. Thereafter, $H_2O_2$ and TMB were added to the mixture at the final concentration of 250 mM and 500 mM, respectively followed by an extra incubation step at room temperature for 5 min. Finally, color change of the solution was investigated by naked eye and the absorbance was measured at 650 nm as well [28]. A mixture containing all compounds except bacterial cells was also considered as the negative control. The experiments were repeated at least three times.

#### Sensitivity

To investigate the aptasensor sensitivity, serial dilutions of *B. melitensis* bacterial cells containing $1.5\times10^8$ to $1.5\times10^1$ CFU/mL were prepared by diluting freshly cultured bacteria into sterile normal saline. Then, 25 µL of the constructed aptasensor was added to each dilution and left at room temperature for 45 min. $H_2O_2$ and TMB were added into the mixtures and the absorbance values were measured at 650 nm. A mixture containing all materials except the bacterial cells considered as the negative control as well [26].

#### Specificity

In order to determine the aptasensor specificity, *B. melitensis* and *B. abortus* as the main targets (positive controls), *E. coli*, *S.* Typhimurium, *S. aureus*, and *B. cereus* as negative control were considered. Hence, 5 µL of each bacterial suspension ($1.5\times10^8$ CFU/mL) were mixed with 25 µL of the aptasensor and the experiment was carried out using the same methodology [26].

#### Assessment in a real sample

Pasteurized milk samples containing *Brucella* bacterial cells were experimentally prepared to test diagnostic capacity of the constructed aptasensor. First, a serial dilution of *B. melitensis* suspensions was prepared with concentration of $1.5\times10^8$ to $1.5\times10^1$ CFU/mL, and 500 µL of each bacterial dilution was spiked and mixed into

the same volume of the milk samples to provide specimens similar to infected clinical samples (final bacterial concentrations: $7.5\times10^7$ to $7.5\times10^1$ CFU/mL). Then, 100 µL of each suspension was diluted into 900 µL of sterile normal saline and the samples were centrifuged at 12,000 rpm for 15 min, and the supernatants containing milk fats and proteins were discarded. The pellets containing bacterial cells were then dissolved in 1 mL of sterile normal saline, and the same procedure was followed to detect target cells by the designed aptasensor as described above. The assay was performed several times to confirm the reproducibility of the newly developed method [43].

### PCR amplification

Diagnostic potency of the designed biosensor was compared with a standard species-specific PCR, as a standard method [44]. To do this, different concentrations of *B. melitensis* from $1.5\times10^8$ to $1.5\times10^1$ CFU/mL were prepared and DNA samples were extracted using boiling method [45]. PCR was performed in a 25 µL reaction mixture containing 4 µL of the extracted DNA, 1 µL (10 pM) of the forward and reverse primers, 10 µL of a commercial PCR MasterMix (Ampliqon, Denmark), and 9 µL of DDW. The samples were placed in a thermocycler (Astec, Japan) and subjected to the following thermocycling process in the given order: an initial denaturation step at 95 °C for 5 min; 35 cycles at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gel and were stained with safe stain and the amplified products were visualized by UV illumination. *B. melitensis* strain Rev. 1 vaccine and a sample contained no DNA were as positive and negative controls. Each PCR was carried out in triplicate [45].

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**Author contributions**
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**Data availability**
No datasets were generated or analysed during the current study.

## Declarations

**Consent for publication**
Not applicable. No animal studies are presented in this manuscript. No human studies are presented in this manuscript.

**Competing interests**
The authors declare no competing interests.

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