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Original Article

Production of *Brucella abortus* Antiserum in Goats and its Comparison with Conventional Rabbit Antiserum

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HIGHLIGHTS

- This study presents a robust and time-saving method for the production of an efficient antiserum.
- Goat is a better and more appropriate host when higher amount of serum is required.
- The amount of serum produced in goat is approximately 8 times greater than that of rabbit.

ABSTRACT

Brucellosis, caused by *Brucella* species, is common among humans and animals, and is one of the most common infectious diseases in Iran. Several assays are available to detect brucellosis, but serological tests may be the only method used in many laboratories. In Iran, different kits are produced in the Pasteur Institute based on agglutination, such as Rose Bengal and 2-mercaptoethanol (2-ME). The positive antiserum control used in these kits is produced in rabbits. The purpose of this study was to produce the antiserum in goats and to compare the titer, quality, and quantity with the antiserum produced in rabbits. The goat immunization was performed by intramuscular injection. Seven days after the last injection, sera were collected. The produced antibody was used in slide and tube agglutination tests with different antigens. The results indicated that the antiserum, produced by the goats had a high quality and quantity. Slide agglutination test showed positive results at 1/6400 dilution with goat antiserum (4+) and 1/1600 dilution with rabbit antiserum (1+). The application of the goats is a better and more appropriate choice, in terms of both cost and quantity, when a high concentration of serum is required. In addition, one goat can provide a higher amount of antiserum compared to several rabbits.

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Introduction

Brucellosis is a zoonotic disease, which is caused by *Brucella* genus (Alizadeh et al., 2018). The most common species worldwide are *Brucella melitensis*, *Brucella suis,* and *Brucella abortus* (Franco et al.,

2007). In humans, the disease is associated with nonspecific clinical symptoms such as fever, arthritis, fatigue, and weakness. It manifests as acute and chronic phases. In livestock, the most important consequences of the disease are infertility, abortion, and reduced milk production (Franc et al., 2018). Brucellosis is endemic in most places worldwide, and Iran is one of the most important endemic areas in Mediterranean countries. In Iran, the incidence is variable between 1.5 and 107.5 cases per 100,000 people in different parts of the country (Mirnejad et al., 2017). Another study has indicated that the mortality rate of the disease increased from 244 cases in 2009 to 578 in 2015 (Moradi et al., 2019). *Brucella melitensis* is the most prevalent among all spices in Iran (Zowghi et al., 2009). Therefore, rapid detection and identification of the pathogen is essential to provide the disease control and treatment .

Brucella can be identified by different molecular and serological procedures (Moradi et al., 2019). Furthermore, microbiological tests, such as blood culture, which is considered as a gold standard (Franco et al., 2007; Wang et al., 2016) can also be utilized. Although molecular methods like Polymerase Chain Reaction are sensitive, serological tests are routinely performed in clinical laboratories in Iran. These tests include i) Rose Bengal test (RBT), which is applicable in the screening of the infection, ii) standard agglutination test (SAT), and iii) 2-Mercaptoethanol (2ME) (Zowghi et al., 2009). In serological tests, antibodies are important components, which are usually polyclonal. There are two main steps in polyclonal antibody production: 1) selecting a species for immunization and 2) immunization procedure (Hanly et al., 1995). Different studies compared monoclonal and polyclonal antibody production in various hosts. Generally, rabbits are utilized for polyclonal antibody production (Stills, 2012). Goats are suitable candidates since it is possible to take a large volume of blood for antiserum. (Hanly et al., 1995, Leenaars and Hendriksen, 2005). Maximum volume of blood that can be withdrawn from a rabbit is 25 mL, but goats provide 800 mL (Howard, 2001).

Various sites for immunization have been described in polyclonal antibody production (Stills, 2012). Antigens delivered intravenously (IV) are metabolized very rapidly. IV Immunization is principally useful for particulate antigens like whole bacteria. However, intramuscular and intradermal injection decelerate the delivery of immunogens to the immune system (Howard, 2001). Thus, immunization methods also could influence antibody production.

Diagnostic kits used for brucella identification are currently developed at the Pasteur Institute of Iran. Positive and negative anti-serum in the kits are produced in rabbits and is accompanied by several challenges such as i) the volume of collected serum in each blood sampling, ii) the number of rabbits used, and iii) the quality of the produced antibody. Therefore, this study aimed to compare produced anti-serum in goats and rabbits quantitatively and qualitatively.

Materials and Methods

Chemicals, kits and microorganism

Brucella abortus S99 strain was provided by the production and research complex of Pasteur Institute of Iran. All the chemicals were supplied from MerckChemicals, Germany. RBT antigen kit and Wright Rapid antigen kit were supplied from Pasteur Institute of Iran, production and research complex; Karaj, Iran. Filter with cut-off of 100-KD was purchased from Millipore (Pellicon® XL50 with Biomax® 100 kDa Membrane, Cat. No. PXB100C50) and PBS 0.07 M was supplied from Gibco (Cat. No.18912014). Protein ladder was purchased from SinaClon BioScience, Cat. no. SL7001 (PR901641).

B. abortus culture

Brucella abortus S99 strain was cultured on Brucella agar base (HIMEDIA, Cat. no. M074) incubated at 37°C for 72 hours. Harvesting was performed using phosphate buffer (NaH_2PO_4, Cat. no. 106346; Na_2HPO_4, Cat. no. 106585; NaCl, Cat. no. 106400, pH 6.4), and the resulting biomass was stored at 4°C until use.

Antigen preparation

The biomass was inactivated using 10% phenol followed by incubation at 90°C for 1 hour. Then, it was centrifuged for 60 minutes at 4000 g and 4°C. The pellet was suspended and washed in Phosphate Buffer Saline (PBS). The concentration of the bacterial suspension for injection was then adjusted to McFarland standard 3.

Immunization of animals

A 6-month-old goat, which was negative for *brucella* (based on the Rose-Bengal test, RBT), was used. Immunization was performed using intravenous injection of 2 mL inactivated *brucella* (1 mL in each ear vein) four times with one-week interval. Furthermore, two New Zealand rabbits were also injected intravenously using similar bacterial antigen. Blood collection was implemented seven days after last injection, from the jugular vein of goat and the heart of

rabbits. blood sampling were continued in defined intervals for 4 weeks. After each sampling, the samples were centrifuged at 2500 g at 4 ℃ for 25 min to obtain the sera. The sera were then stored at -20 ℃ until use.

Rose-Bengal Test (RBT)

The Rose Bengal test (RBT) is a rapid slide-type agglutination assay performed with a stained *B. abortus* suspension at pH 3.6–3.7 and plain serum. Because of its simplicity, it is often used as a screening test in human brucellosis and would be optimal for small laboratories with limited equipment (Díaz et al., 2011). Slide agglutination test was performed using 30 µL (one drop) of serum with an equal volume of RBT antigen kit and mixed using a toothpick. Furthermore, rabbits' and negative sera were also used as controls. The results were observed after four minutes. Agglutinations are visualized as clumps. Weakly reactive agglutinations may require an adequate light source for proper visualization, while strongly reactive agglutinations are easily seen. The result of the tests are scored from 0 to 4+, *i.e.*, 0 (no agglutination), 1+ (25% agglutination), 2+ (50% agglutination), 3+ (75% agglutination) or 4+ (100% agglutination). The smallest quantity of serum that exhibits a 2+ or 50% agglutination is considered as the end-point of serum activity or titer (Olopoenia and King, 2000).

Rapid Slide Agglutination Test

Anti-serum produced in the goats and rabbits were diluted 1 to 5 with sodium chloride solution (0.85%). Then, 2, 5, 10, 20, 40, and 80 µL of the diluted goat and rabbit serum was added on a glass slide and mixed with 30 µL of Wright Rapid antigen kit (Pasteur Institute of Iran, production and research complex; Karaj, Iran). The results were observed after one minute.

Brucella tube agglutination test

The sera of the goat, rabbits, and the negative serum were diluted with sodium chloride solution (0.85%) in hemolysis tubes and different serial dilutions (1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, 1/5120) were prepared. Then, they were mixed with an equal volume of Wright tube antigen kit. The tubes were incubated at 37 °C for 48 h, and then the samples were checked for the presence of agglutinin particles.

Antibody purification

Produced anti-serum in the goat was also purified for application in immunofluorescence and ELISA-based kits. Ammonium sulfate (Merck, Cat. No. 101217) was used to precipitate the antibodies. Briefly, equal volumes of the serum and 0.15 M NaCl were mixed and placed on a stirrer at 200 rpm and 4℃. Then, an equal volume of saturated ammonium sulfate was added to the mixture dropwise and stirred for 30 min. The final mixture was centrifuged at 1500 rpm for 30 min at 4 ℃. The supernatant was discarded and the precipitate was washed with semi-saturated ammonium sulfate. The centrifugation step was repeated, and the final precipitate was resuspended in one-third of the initial 0.15 M cold NaCl. Tangential flow filtration system (TFF) (Labscale TFF System, Millipore) was used to remove ammonium sulfate and to concentrate the sample, using a filter of 100-KD cut-off and PBS 0.07 M, pH 6.3. Ion-exchange chromatography was then used to remove remaining impurities. The column was equilibrated using 50 mL PBS, pH 7.6. Then, the samples were applied to the column. Protein was eluted from the column using the same buffer. The quality and quantity of the purified antibodies were evaluated using SDS-PAGE and Bradford assays.

Protein assay

Protein quantification was performed using optical density and the Bradford method. NanoDrop (Thermo Fisher Scientific, NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer) was used to measure Optical Density at 280 nm. In the Bradford assay, 800µL of each standard (Bovine Serum Albumin) and sample solutions was pipetted into test tubes. 200 µL of Coomassie Brilliant Blue G-250 reagent was added to each tube and vortexed. After incubation (RT and at least 5 min), absorbance was measured at 595 nm. The protein concentration of the unknown samples was calculated using a standard curve (Bovine Serum Albumin). All tests were performed in triplicate.

SDS-PAGE

Fifteen microgram of the purified goat anti-serum was suspended in 5X sample buffer (Tris 50mM, pH 8.6, SDS (2%), beta mercaptoethanol (1%), Glycerol (10%), EDTA 12,5mM, and 5mL bromophenol blue (5%) with reducing agents) and boiled for 5 minutes. Electrophoresis was performed using a vertical system (Paya Pajuhesh, Mashhad, IRAN). Five percent stacking and 10% resolving SDS-PAGE gels were used. Proteins were stained with Coomassie blue. Protein ladder was utilized to confirm the molecular weight of the antibody.

Results and Discussion

Laboratory diagnosis of brucellosis is usually performed by cultivation and serological tests. Bacterial culture is the gold standard of brucellosis diagnostic methods (Akhtar et al., 2010). However, since culture is time-consuming and labor-intensive, serological tests based on agglutination are utilized as a conventional method in Iran. Wright diagnostic kits are produced in the Pasteur Institute of Iran – Karaj complex. The positive control used in the kit is prepared by rabbit immunization against *Brucella abortus*. In this procedure, a significant number of animals are needed, and the amount of blood collection is limited. Thus, in this study, we used goats as the host to produce the anti-serum. The main aim of antibody production in laboratory animals is to obtain high-titer and high-affinity anti-serum to be used in diagnostic tests such as agglutination, ELISA, immunofluorescence, and Western blot.

Antibody production and collection

Polyclonal antibodies are relatively inexpensive (Pihl, et al., 2015) and can be produced in large quantities in different animals like rabbits, goats, chickens, and horses. Chickens have several advantages for the production of polyclonal antibodies and mammalian proteins. These advantages include i) no blood sampling is required since engineered antibodies are secreted in egg yolk, ii) low quantity of antigen is required for immunization, iii) ethical considerations are some of its benefits. However, the production of antibodies in chickens is not yet widely accepted (Hanly et al., 1995). Rabbits are more commonly used than other animals (Pihl, et al., 2015). The appropriate size of the animal, easy handling, and antigenic precipitation properties of the antibodies are some of the important advantages of rabbits. In addition, rabbits are a suitable host for the production of antibodies against rodent and human proteins and have only one IgG class (Hanly et al., 1995).

The produced anti-serum in goats had several advantages compared to rabbits including i) higher quantity of serum, ii) higher titers, and iii) anti-complementary activity (Xavier et al., 2010). In each blood collection from the goats, approximately 50-60 mL of the blood was obtained, while in the case of the rabbits, this amount was about 13-15 mL, and led to death of the rabbits after a few times. The final volume of the serum produced in the goats was 60 mL (two-blood sampling), but in the rabbits, it was totally 27 mL (13 mL/rabbit), which is insignificant compared to the goats. In goats, the injection was performed intramuscularly. Intramuscular injections release the antigen to the bloodstream gradually, preventing anaphylactic shocks (Mirnejad et al., 2017), while for the safety of the rabbits, the lateral vein of the ear was used for injection.

Blood collection and serum separation were performed following the goat and rabbits immunization. Table 1 shows the volume of blood and serum of each host.

Marlies et al. (2005) and Carey Hanly et al. (1995) examined and compared the production of monoclonal and polyclonal antibodies in different animals. In goat, the amount of blood collection (150-400 mL) was suitable when large volumes of anti-serum were needed (Hanly et al., 1995; Leenaars and Hendriksen, 2005). Their studies overlapped and were comparable to ours in that all of them compared anti-serum production in rabbits and goats. Thus, the goat was considered as an appropriate host for the production of polyclonal antibodies in industrial scale.

Table 1. The amount of the blood collected each time (4 times) from the rabbits and goats and also the final volume of the blood and isolated serum.

	Goat (mL)	Rabbits (mL)
Blood vol. in each step	40-50	10-15 (one rabbit)
Final vol. of blood	120	52 (two rabbits)
Final vol. of serum	60	27

Rose-Bengal Test (RBT)

One drop (30µL) of the serum was mixed with an equal volume of RBT kit antigen. Agglutination was observed with naked eyes after one minute. The results showed 4+ agglutination using the goat anti-serum and 3+ agglutination using rabbit anti-serum (Fig. 1).

Figure 1. Rose Bengal test with serum control prepared from rabbits, goats, and negative control serum. The antiserum produced in goats has a higher intensity of agglutination than in rabbits and also no agglutination is observed in negative control serum.

Rapid slide agglutination test

Different dilutions of serum (1/100 to 1/6400) were mixed with an equal volume of the Wright Rapid antigen kit. Agglutination was observed after one minute. The results showed 4+ agglutination using goat anti-serum at 1/6400 dilution, compared to +1 agglutination rabbit anti-serum at 1/800 dilution (Fig. 2).

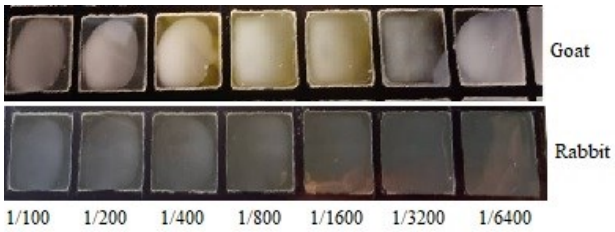

Figure 2. Wright rapid test on a slide for antiserum produced in the goats and rabbits. Dilutions of 1.100 to 1.6400 were prepared from the produced antiserum. The results showed that the titer was 1.6400 positive for antiserum produced in the goats, and the titer was 1.800 positive for antiserum, produced in rabbits.

Brucella tube agglutination test

The tube agglutination test was performed using various serial dilutions of anti-serum produced in goat and rabbit. Pancake-like agglutination in the bottom of the tube with a clear supernatant shows a positive result (3+) before shaking. Healthy serum was used as the negative control. Negative results (-) are indicated by the absence of agglutination and formation of button at the bottom of the tube (Fig. 3a). Using goat anti-serum, the positive results were displayed at 1/1280 (1+) dilution (Fig. 3b). However, agglutination was observed at 1/320 (1+) dilution of rabbit anti-serum (Fig. 3c).

The produced anti-serum was used in the agglutination test. A positive result was observed at 1/6400 dilution with goat anti-serum (4+) and 1/1600 dilution with rabbit antiserum (+). Therefore, the goat antibody is preferred to the rabbit antibody in terms of quantity (60 mL compared to 27 mL) and quality (high titer).

Keshavarz et al. (2008) used goats to prepare anti-serum against measles for quality control of vaccines, and they produced high-titer anti-serum (1/1024) in a short time and with appropriate stability (three years as a lyophilized) (Keshavarz et al., 2008). The results of our study are comparable to Keshavarz's and the anti-serum produced against *Brucella abortus* in goats has an adequate titer (1/6400).

Figure 3. Wright tube test for antiserum produced in rabbits and goats. The formation of a button at the bottom of the tube is a negative result and the formation of a network at the bottom of the tube indicates that the Wright tube test is positive. The result of Wright tube antigen titration showed that titer 1.1280 is positive for antiserum produced in goat (Figure 3a) and titer 1.320 is positive for antiserum produced in rabbit (Figure 3b), negative control serum (Figure 3c).

Protein assay

Optical density and Bradford assays were used to quantify protein concentration. The results are displayed in Table 2.

Table 2. The concentration of the purified antibodies in the ammonium sulfate precipitation step, the TFF dialysis step, and finally after ion exchange chromatography with two methods, optical density at 280 nm and Bradford.

Steps	O.D	Bradford assay (mg/mL)
Ammonium Sulphate precipitation	17.121	15.91
Dialysis	13.11	12.19
Ion exchange chromatography	9.11	8.29

SDS-PAGE

The purity and quality of the antibody were checked by SDS-PAGE after purification steps. A protein band with 150 KD appeared after staining (Fig. 4).

The results of quantitative (protein concentration) and qualitative (SDS-PAGE) studies indicate the appropriate concentration and high purity of the antibody. Therefore, it can be used in tests such as ELISA and immunofluorescence for *brucella* identification.

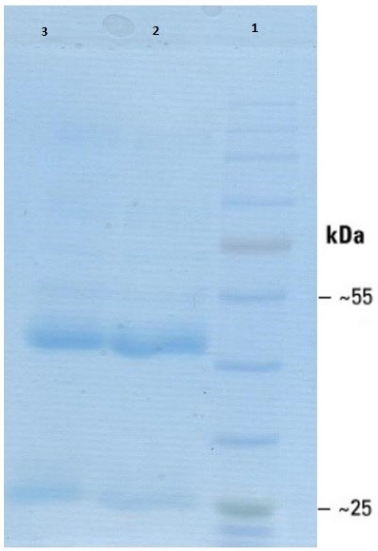

Figure 4. Analysis of purified antibodies on 10% resolving SDS PAGE. Lane 1: pre-stained molecular ladder (15-170kDa), Lane 2: Goat antibody and Lane 3: Rabbit antibody. The single and sharp bands observed in the 150 kDa zone indicate good antibody purification.

RBT after antibody purification

The slide agglutination test was performed using the purified antibody and RBT kit antigen. Agglutination was observed after one minute. The results showed agglutination as 4+ using purified goat anti-serum (Fig. 5).

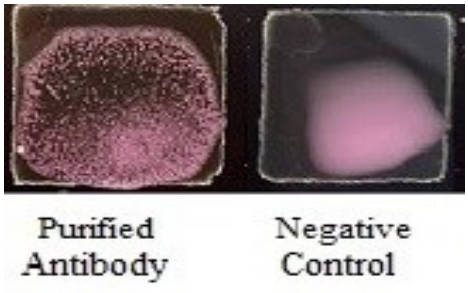

Figure 5. Rose Bengal test of the purified antibody. Left: the Purified Antibody produced in goat. Right: Negative Control.

The antibody purification was performed to use the produced goat antibody in ELISA, immunofluorescence, and western blot. In 2012, Alizadeh et al. prepared and purified polyclonal antibodies against *Mycobacterium avium paratuberclosis* antigens in rabbits (Alizadeh and Babaie, 2012). Their methods included Sedimentation with 35% ammonium sulfate, dialysis using 10 kDa dialysis bag, and ion-exchange chromatography (DEAE-Cellulose). The present study is similar and comparable in terms of the purification methods. In this study, we used the precipitation method using saturated ammonium sulfate and dialysis was performed using TFF mounted with 30 kDa filter to remove ammonium sulfate. This method has several advantages over dialysis bags, for example, a large amount of protein (antibody) could be dialyzed within 3-4 hours using TFF compared to dialysis bags, which is performed overnight to completely remove ammonium sulfate. Using TFF devices in antibody purification is a new method, and leads to the preparation of high purity antibodies in a few hours. Additionally, to obtain higher purity, ion-exchange chromatography (DEAE Sepharose FF) was also used.

Conclusion

The present study suggests that goats are ideal hosts for antibody production applicable in commercial kits. In fact, using goats as the host, we achieved a high titer of the antibody as well as high quality and quantity of anti-serum. In addition, it is time-saving and cost-effective. This is the first time in Iran that large scale anti-Brucella anti-serum is produced using goats. Instead of using conventional methods, we used specific purification steps and tangential flow filtration to obtain high quality and quantity of anti-Brucella anti-serum.

Ethical statement

All the procedures of the study have been approved by the Ethics Committee of Pasteur Institute of Iran (Code 1683), (Tehran) and confirmed by the provisions of Declaration of Helsinki.

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Competing Interests

The authors declare that there is no conflict of interest.

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Authors' Contribution

M. Paryan and R. Shokri performed the study concept and design, and also administrative, technical, and material support. Data were acquired by V. Kia. R. Shokri performed analysis and interpretation of data. S.

Mirzaei Samavat prepared the draft of the manuscript. Critical revision of the manuscript for important intellectual content was performed by V. Kia and D. Doroud. Statistical analysis was carried out by M. Paryan.

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