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Development of an indirect ELISA based on whole cell *Brucella abortus S99* lysates for detection of IgM anti-*Brucella* antibodies in human serum

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ABSTRACT

Background: Brucellosis is the most common zoonotic diseases worldwide. The aim of this study was to develop and evaluate the diagnostic performance of an indirect-ELISA (I-ELISA) method based on whole cell *Brucella abortus* S99 lysates for detection of IgM anti-*Brucella* antibodies in a human serum.

Materials and methods: The study was conducted in two species-rich endemic areas of Iran (Tehran and Lorestan provinces). Serum samples of 102 patients and 150 healthy individuals were tested by the new kit and the commercial Vircell kit for the presence of anti-*Brucella* IgM antibodies. The disease status was confirmed by Wright agglutination test. The difference in the mean optical densities (OD) recorded by the new and the Vircell kits for patients and healthy individuals were tested using Two-tailed Student *t*-test. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the new kit were informed using Receiver operating curve analysis. The results were used to guide the choice of cutoff. Agreements in ODs recorded by the new and the Vircell kit was visually inspected using Bland-Altman plot.

Results: The new I-ELISA showed excellent diagnostic performance (sensitivity and PPV = 95.7%, specificity and NPV = 97.8%) for the diagnosis of brucellosis. The cut-off area for the antibody index (AI) was determined to be 8–10, where AIs less than 8 and greater than 10 were considered *Brucella*-negative and -positive, respectively. AIs of 8–10 show equivocal results, requiring re-testing. The Vircell kit showed low (36.8%) sensitivity and perfect (100%) specificity on the same samples. The Bland-Altman plot showed low agreement between both tests in recording the OD values for the same individuals.

Conclusion: The new I-ELISA based on whole cell *Brucella abortus* S99 showed a good performance for the detection of *Brucella* spp. Lack of agreement between the new and the Vircell kit suggest that the performance of ELISA kits might be dependent on the geographical area under study. Hence, validation of the new and the Vircell kits is recommended prior to their implementation in other geographical areas.

1. Introduction

Brucellosis is a worldwide re-emerging zoonosis, which mainly infects domestic animals including cattle, swine, goats, sheep, and dogs [1]. Humans generally acquire the disease through eating or drinking contaminated animal products (e.g., milk and meat), or by inhaling airborne agents. Animal infection imposes great direct and indirect economic burden on the livestock industry through reduction of milk and meat and increases in the abortion rate of animals [2–4]. Among humans, brucellosis also remains a major public health problem in many parts of the worlds, including the Mediterranean region, western Asia, Africa, and Latin America [5].

The causing pathogen of brucellosis is a gram-negative, motionless, intracellular and acapsular coccobacilli belonging to the *Brucella* genus [6]. Four types of *Brucella* bacteria cause the majority of brucellosis infections in humans including *Brucella* melitensis, *Brucella* suis, *Brucella abortus* and *Brucella* canis. *Brucella* melitensis found in sheep and goats is the main pathogen responsible for human brucellosis, followed by *Brucella abortus* and *Brucella* suis [2].

The gold standard for brucellosis diagnosis is the isolation of

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bacterium from body fluids such as blood, bone marrow and CSF [7]. Bone marrow cultures may provide higher sensitivity while yielding faster culture times and may be superior to blood culture techniques in the detection of *Brucella* spp. [8]. However, bacterial culture requires strict biosafety measures (biosafety level 3 facilities) and skilled laboratory personnel. Therefore, alternative methods such as serological and molecular tests should be developed for routine clinical diagnosis and surveillance programs [8].

Indirect enzyme-linked immunosorbent assay (I-ELISA) has been acknowledged for its better performance in *Brucella* diagnosis over other methods such as rose bengal plate test (RBPT), microagglutination test, and polymerase chain reaction (PCR) [9,10]. The reason is believed to be the sensitivity of the primary binding assays which is higher for I-ELISA rather than RBPT and the complement fixation test (CFT) [11].

Brucella has a complex antigenic structure. The outer membrane of the cytoplasm contains two main classes of antigens: outer membrane proteins (Omps) and lipopolysaccharide (LPS) [12]. Omps include Omp28 [13], Omp22 [14] and Omp16 [15]. Structurally, the LPS can be either smooth (S) or rough (R) depending on the *Brucella* spp. The S-LPS is found in wild-type strains of all classical *Brucella* spp. but not in *B. ovis* and *B. canis*, which carry an R-LPS. O-polysaccharide (OPS) is an epitope shared among major *Brucella* spp, including *B. abortus*, B. suis and B. melitensis. Therefore, it can be used for diagnostic purposes [15]. In addition, almost all common epitopes of *Brucella* spp. are present in *B. abortus*, which makes this species a good candidate for extraction of OPS antigens [16]. Moreover, due to various yet complex antigenic nature of *Brucella* spp., serological tests based on whole cell antigens might have the potential to detect a wider range of *Brucella* biovars. However, to the best of our knowledge, few attempts have been done in this regard.

Keeping this view in mind, the present work is undertaken to develop and evaluate the diagnostic performance of an Indirect ELISA (I-ELISA) system based on whole cell extracts of *B. abortus* S99 for detection of IgM anti-*Brucella* antibodies in human serum.

2. Materials and methods

2.1. Selection of a suitable strain

According to other studies, a lyophilized strain of *B. abortus* S-99 (biovar 1) was purchased from the bacteria collection of Pasteur Institute of Iran and considered for the preparation of antigen [6,17].

2.2. Antigen preparation procedure

For the preparation of whole cell lysate antigens, we modified the procedure described by Manley, et al. [18]. To do this, bacteria were grown overnight in *Brucella broth* (Sigma-Aldrich, B3051) supplemented with 5% defibrinated bovine blood (SBA) at 37 °C with 5% CO2. More than 10 McFarland bacteria were obtained prior to heating at 90 °C. Pellets from centrifuges (at 4000 rpm for 10 min) were stored in the phosphate-buffered saline (PBS buffer). After measuring the antigen concentration by spectroscopy, serial dilutions were made, and concentrations were assessed by Bradford protein assay (Bio-Rad, Hercules, California). The best amount of antigenic dilution for coating process in microplates (polystyrene) was identified with 7 µg/ml. One hundred µl of the 7 µg/ml dilution was placed in wells and incubated for 24 h at 4 °C and then, contents of the well were removed and washed with the PBS 1X buffer. To facilitate bonding process of the antigen to flat-bottom 96-well immunoplates (Maxisorp; Nunc, Roskilde, Denmark), the coating buffer (Bicarbonate/carbonate coating buffer, pH 9.6 at 25 °C) was used (Passive adsorption). Moreover, to prevent non-specific binding between antigen and antibody, microplates were washed twice with PBS followed by incubation for 2 h at 37 °C with blocking buffer (3% bovine serum albumin (BSA), 0.1% fish skin gelatin, 0.1% Triton X-100, 0.05% Tween-20) with 2% goat serum (Sigma, St. Louis, MO). Then, the buffer was removed and wells were freeze-dried in upside-down form for 6 to 7 h at 4 °C and stored in sealed bags with a silica gel packet.

2.3. Serum sample collection

A total of 252 sera samples originating from different medical centers and hospitals in Tehran and Lorestan provinces of Iran were collected over a period of 16 months for the validation of this assay (102 and 150 random serum samples with positive and negative Wright tube tests, respectively). Clinical diagnosis was made by the physicians based on the presence of compatible signs and symptoms with the demonstration of specific antibodies at significant titer (Wright test titer > 1:160). A questionnaire was completed for each subject at the time of sampling to record relevant information such as age, sex, and contact history with animals. Minimum sample size in *Brucella*-positive and *Brucella*-negative groups was allocated considering a minimum sensitivity of 80%, a minimum prevalence of 20%, and a type I error of 0.05, using the sample size table recommended by Bujang and Adnan [19]. All serum samples were evaluated in terms of the potential and common pathogens including HBsAg, anti − HCV, anti-HTLV-1/2 and anti-HIV-1/2.

2.4. Increasing antibody stability in control samples

BioStab antibody stabilizer reagent (Sigma-55514, St. Louis, MO, USA) was used to prevent the reduction of antibody titers in control samples. The best ratio to increase the longevity and stability of the antibody, as well as prevent the reduction of antibody dilution was evaluated, and a volume of antibody stabilizing reagent and three volumes of control were mixed.

2.5. Eliminating interference factors

The presence of Rheumatoid Factor (RF) in serum causes a false positive result by forming a complex with IgG. On the other hand, IgG can degrade IgM and causes a false negative response. So, to remove these two interacting factors, the Goat Anti-Human IgG-Fc Fragment (Bethyl, Montgomery, TX, US) was purchased and the ratio of 1/5000 was used.

2.6. Individual components

A Secondary antibody: Goat anti-Human IgM Antibody HRP Conjugated (Bethyl Co, Catalog No. A80-100 P) diluted 1/100,000 in a red-colored Proclin-containing buffer (antibody stabilizer).

B Substrate/chromogen: 15 ml of substrate solution containing tetramethylbenzidine (TMB) (BioLegend Co, Catalog No. 421,101) stored in an amber vial.

C Stop solution: 15 ml of stopping solution (0.5 M or 1 N H2SO4) was made.

D Sodium azide: NaN3 (Merck Co, Germany, catalog No. 822,335) was purchased as a preservative and antifungal compound and added to the antigenic suspensions, controls, washing solution and serum diluents solutions in the standard proportions.

E Washing solution 20x and Serum Diluent 1x: were made based on existing protocols [20] and evaluated. 50 ml of 20x washing solution: a phosphate buffer containing TweenR-20 and Proclin per the Vircell standard kit protocol was used for all wash steps and as the sample diluent; 25 ml of serum dilution solution: a blue colored phosphate buffer containing protein stabilizers and Proclin.

F Serum controls: 500 µl of positive, cut off and negative control serums were lyophilized at 1:100 containing proclin.

2.7. Cross-reactivity with samples from other infections

Freshly prepared kits were assayed for cross-reactivity to IgM from Brucellosis patients using eight known high Brucellosis IgM-positive sera from primary infections. In addition, eight *Yersinia enterocolitica* IgM-positive, eight salmonella typhi IgM-positive and eight malaria-positive samples were assayed for cross-reactivity in the new kit.

2.8. Determination of assay cut off and accuracy

For the accuracy and precision studies, Wright agglutination test was used as the reference standard. Individuals, whose Wright test results were positive, were considered as *Brucella* infected, while those with negative Wright test were considered uninfected. Positive and negative samples were tested with the new kit as well as commercial *Brucella* ELISA IgM assays (Vircell SL, Spain) to check for its performance measures. The recorded optical densities (OD) were summarized with descriptive statistics, including OD values' mean, standard deviation, median, and interquartile range. To assess if the new kit can properly discriminate infected from uninfected individuals, the difference in the mean OD values recorded for the two groups was tested using the Two-tailed Student *t*-test. Performance measures (i.e., sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy) of the new kit were also informed using ROC curve analysis, given Wright test as the reference standard. Estimates of sensitivity and specificity computed by the ROC curve were used to guide the choice of cutoff. Ninety-five percent confidence intervals (CI) were calculated using exact score interval for proportions.

For interpretation of the test results, antibody indexes were calculated from the OD values, using the formula described below:

$$\text{Antibody index} = (\text{Sample OD}) / (\text{Cut-off serum mean OD}) \times 10$$

Antibody indexes (AI) were then summarized with descriptive statistics. Calculating 5th to 95th percentiles of antibody indexes for infected and uninfected individuals, the area with overlapping values among both groups was identified and considered as the equivocal area. Individuals whose AI value falls within this area should be re-tested for brucellosis.

2.9. Performance of the Vircell kit for the diagnosis of Brucellosis on study samples

Besides the new kit, we also measured the sensitivity and specificity values for the Vircell kit, given the Wright test as the reference standard. Confidence intervals were also calculated using the exact score interval for proportions. To measure the degree of agreement between the new and the Vircell kit, the distributional parameters and curves of the OD values recorded by the two tests were compared. Paired *t*-test was used to determine whether there was a significant difference in the mean OD values recorded by the two tests. Bland-Altman plot was also created to display the scatter of the mean difference of the OD values for the new and the Vircell kits (vertical axis) to the OD values of the Vircell kit (horizontal axis), corresponding to the level of agreement between the OD values of the two tests. Data were analyzed using Stata software (v. 11). Statistical tests were considered significant at 0.05 levels.

3. Results

3.1. Status background of brucellosis patients

Serum samples from 102 brucellosis patients (68 male and 34 female) were collected from community and laboratory hospitals in urban and rural areas of central (Tehran province = 45 samples) and western (Lorestan province = 57 samples) Iran. The two provinces are among endemic areas for brucellosis in Iran. Male and female participants' mean (SD) age was 37.3 (13.9) and 36.2 (10.7) years, respectively. The highest proportion of positive samples (58.8%) belonged to the age group of 31–40 years, followed by the age group of 41–50 years (18.62%). Moreover, most patients (66.6%) had a history of contact with live animals, among which 24.5% were also keeping livestock within their residential property.

3.2. Stability

To determine shelf life, the designed components of the kit were assayed over a period of one year after production. When the kit was compared to the comparative Vircell kit, a downward trend of the P/N values was observed over time from month 0 to month 12 (mean P/N reductions of 32% (RT) and 19% (4 °C). No obvious decrease in the P/N's, however, was seen due to the three-month storage at 4 °C. The positive control exhibited an OD of approximately 35% of the month 0 value when it was stored for 12 months at RT, whereas no deterioration was seen for the positive control when the kit was stored at 4 °C. After storing the kit at 4 °C and testing it at month 12 the P/N and OD values remained consistent with those measured at month six. By contrast, the kit that was stored at RT for 12 months showed a slow downward trend in overall OD's in the test serum panel, although the resulting P/N values were not adversely affected.

3.3. Cross-reactivity

Of the eight *Brucella* sera with high P/N values, seven yielded positive IgM results when the new kit was used. However, the P/N values were in average 5-fold higher for the *Yersinia enterocolitica* sera compared to the P/N values for *Brucella* IgM. Using the new kit, we found two malaria-positive and one typhoid fever-positive samples.

3.4. Determination of cut-off, accuracy, and precision

The mean OD recorded by the new kit for *Brucella*-infected individuals (those with positive Wright agglutination test results) was significantly higher than non-infected individuals (those with negative Wright test results; independent *t*-test < 0.0001, Table 1). In the same way, the distribution curves of infected and uninfected individuals' OD values had a small overlapping region, suggesting that the new kit recorded different values for infected and uninfected individuals and possesses the ability to properly discriminate these groups (Fig. 1, Panel a). The area under the ROC curve was 0.97 (95% CI = 0.94–0.99), suggesting the high accuracy of the new kit in discriminating infected and uninfected individuals (Fig. 1, Panel b) (Table 2–4).

The 5th to 95th percentile limits of the OD values recorded for infected and uninfected individuals were 0.09–0.73 and 0.60–1.72, respectively. The 0.6–0.8 interval overlapped between both groups, and was considered as the cut-off point area. OD values above 0.8 were considered as a positive test result (infected with *Brucella* spp.), while OD values below 0.6 were considered as negative results. In the ROC curve analysis, these cut-points yielded high sensitivity, specificity, PPV and NPV for the new kit, all of which were above 95%.

Table 1
Distribution of the new kit's OD results in Wright-negative and Wright-positive individuals.

OD of New kit	Wright-negative	Wright-positive	*P* value*
Mean ± SD	0.31 ± 0.22	1.17 ± 0.35	
Median (IQR)	0.27 (0.19–0.38)	1.26 (0.91–1.41)	< 0.0001
Percentile 5–95	0.09–0.73	0.60–1.72	
Minimum–Maximum	0.06–1.55	0.30–1.90	

SD: Standard Deviation; **IQR**: Inter quartile range.
* *P* value is generated using Two-tailed Students *t*-test.

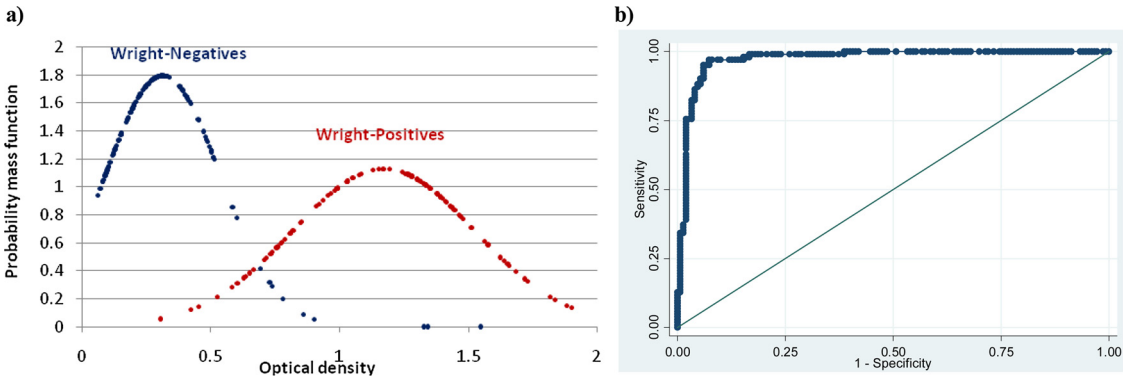


Fig. 1. Distribution of the new kit's OD results (Panel a) and accuracy (Panel b) given Wright test as the reference standard.

The 5th to 95th percentile limits of the antibody indexes calculated for infected and uninfected individuals (based on the formula described under the heading VII), was 0.26–9.26 and 8.14–26.54, respectively, with the 8.14–9.26 interval overlapping between both groups. Therefore, the AI interval of 8–10 was considered as the equivocal area, where the test should be repeated. Test results with antibody indexes over 10 were considered positive (infected with *Brucella* spp), while test results with antibody indexes less than 8 were considered negative.

3.5. Comparison of the Vircell and the new kits for diagnosing brucellosis

Testing study samples with the Vircell kit and comparing their results to the Wright test as reference standard revealed a low sensitivity (36.8%) for this kit on study samples (95% CI = 27.2–47.4%) in detecting Iranian brucellosis cases. The NPV of the Vircell kit was also 71.3% (95% CI = 64.6–77.3%), much lower than the new kit (97.8%). The specificity and PPV of the Vircell kit, however, was perfect (100%), showing its ability to rule out all false negative cases. The area under the ROC curve for the Vircell kit was slightly smaller than the new kit (0.95 vs. 97.0, respectively).

We also compared the OD values of the Vircell and the new kits. The distribution curves of OD values recorded by the two kits was not the same, either in general or across uninfected and infected groups. The results showed an overall mean difference of 0.13 between the OD values of the two tests (Fig. 2, Panel a), which was also highly statistically significant (Paired *t*-test < 0.0001). Among uninfected individuals, the distribution of OD values was more peaked for the Vircell kit than the new kit (Fig. 2, Panel b). While for infected individuals, the distribution was more flat for this kit (Fig. 2, Panel c). The mean of OD values recorded by the new kit for uninfected individuals was about 0.16 units greater than that of the Vircell kit (0.15 vs. 0.31, respectively; Fig. 2, Panel b), showing that the new kit usually records greater OD values for uninfected individuals than the Vircell kit. For infected individuals, the new kit also recorded greater OD values, but here with smaller standard deviations (higher precision) than the Vircell kit, suggesting the outperformance of the new kit for accurate detection of positive cases compared to the Vircell kit.

The distribution of the two tests' OD values can also be seen in the Bland-Altman plot (Fig. 3). The plot shows that the scatter of the difference between the OD values of the Vircell and the new kit decreases as the OD values for the Vircell kit increases (Fig. 3), all in all, suggesting lack of proper agreement between both tests in recording the OD values of the same samples.

Table 3
Distribution of the new kit's antibody index values for Wright-negative and Wright-positive individuals.

Antibody index	Wright-negative	Wright-positive
Mean ± SD	3.77 ± 3.27	16.47 ± 5.41
Median (IQR)	3.25 (1.31–4.99)	16.89 (12.50–20.08)
Percentiles 5% - 95%	0.26–9.26	8.14–26.54
Minimum–Maximum	0.13–19.04	4.10–30.56

SD: Standard Deviation; **IQR**: Inter quartile range.

Table 4
Distribution of the OD results read by the Vircell and the new kit.

Optical density	New kit	Vircell kit
Total		
Mean ± SD	0.66 ± 0.51	0.53 ± 0.63
Minimum–Maximum	0.06–1.90	0.001–2.53
Median (IQR)	0.42 (0.25–1.10)	0.21 (0.11–0.77)

4. Discussion

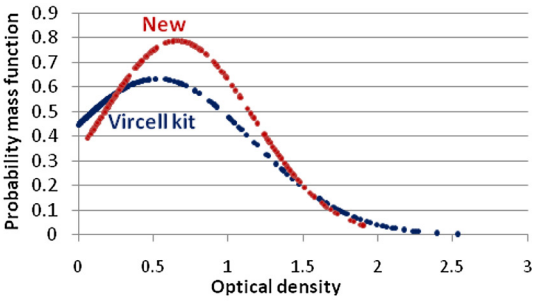
In this study, the new kit designated based on whole cell antigen was tested on samples from case-patients diagnosed through the Wright test to evaluate its ability to diagnose brucellosis among human subjects. The new kit proved to be sensitive, specific, and precise, while this was less evident from the results obtained for the Vircell kit. The Bland-Altman plot suggested that the ODs of the new and the Vircell kits do not agree to a great extent. ROC curve analyses indicated that the new kit was capable of greater sensitivity and accuracy than the Vircell kit in diagnosing *Brucella*, while the Vircell kit had a perfect specificity and PPV.

Our results showed that the new kit has been able to discriminate

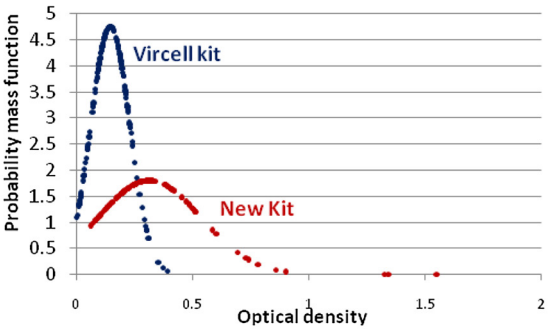
Table 2
Selected cut-offs and performance measures* of the new kit.

	OD	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Negative (Healthy)	< 0.6	95.7 (88.0–99.1)	97.8 (93.6–99.5)	95.7 (88.0–99.1)	97.8 (93.6–99.5)
Positive (Diseased)	> 0.8				
Cut off point	0.6–0.8	–	–	–	–
Maximum Plausible Value	2	–	–	–	–

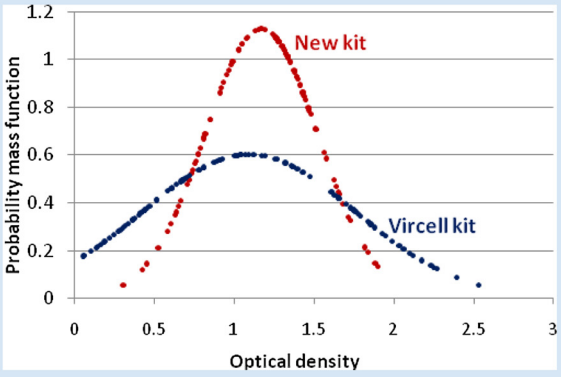
PPV: positive predictive value, **NPV**: negative predictive value.
* Performance measures are derived from ROC curve analysis, given Wright test as reference standard.



OD	New kit	Vircel
	a) Total	
Mean ± SD	0.66 ± 0.51	0.53 ± 0.63
Min – Max	0.06 – 1.90	0.001 – 2.53
Median (IQR)	0.42 (0.25 – 1.10)	0.21 (0.11 – 0.77)



	b) Wright-negative individuals	
Mean ± SD	0.31 (0.22)	0.15 ± 0.08
Min – Max	0.06 - 1.55	0.001 - 0.39
Median (IQR)	0.27 (0.19 - 0.38)	0.13 (0.09 - 0.21)



	c) Wright-positive individuals	
Mean ± SD	1.17 (0.35)	1.09 ± 0.66
Min – Max	0.30 - 1.90	0.06 - 2.53
Median (IQR)	1.26 (0.91 - 1.41)	1.03 (0.47 - 1.70)

Fig. 2. Distribution of the OD results read by the Vircell and the new kit. a) Total; b) distribution among Wright-negative individuals; c) distribution among Wright-positive individuals.

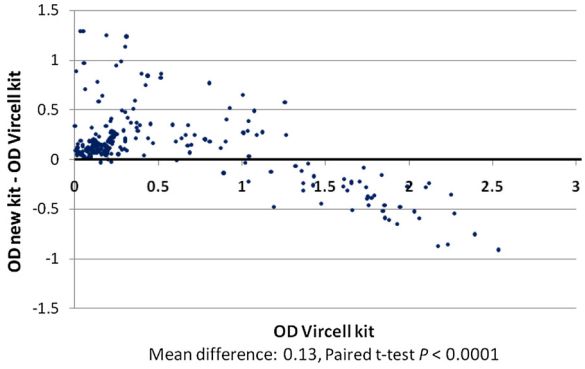


Fig. 3. Bland-Altman plot of the differences between the new kit and the Vircell kit in the OD values compared to the OD values of the Vircell.

between brucellosis patients and healthy individuals. In fact, mean OD values measured by the new kit differed significantly between both groups. Estein, et al. [21] have noted that incorporating hot saline extract (HS), vesicles, proteins, rough LPS, and internal antigens into six serological tests (i.e., agar gel immunodiffusion test, and complement fixation test, both using HS, and four ELISA using HS, R-LPS, cytosolic proteins, and 18-kDa cytosolic protein) is an effective method for the detection *B.ovis* species in folks. Comparing five serological tests (i.e., the buffered plate antigen test, the standard tube agglutination test, the complement fixation test, the hemolysis-in-gel test, and I-ELISA), Dohoo, et al. [22] reported the outperformance of the ELISA for diagnosis of brucellosis. The test showed a sensitivity and specificity of over 90% in detecting *Brucella* antibodies. The same result was reported later in various settings and among different living species [23–26]. More specifically, the use of unpurified SLPS, along with other *B. melitensis* antigens incorporated into an ELISA kit has been reported as a good candidate for diagnosis of brucellosis [27].

The Bland-Altman plot showed that there is a low agreement between ODs of the Vircell and the new kit, with the former reading OD values in a lower range and having lower sensitivity and specificity. This difference might be a result of different antigenic compositions used by the Vircell and the new kit, with the former using a purified *Brucella* antigen that is able to detect a narrow range of *Brucella* spp. while the latter is a test based on whole cell lysate. Both types of assays are useful depending on the objective and the socio-economic and

geographical settings. Purified *Brucella* antigens have the advantage of being more specific, while assays based on whole cell lysates antigens provide a more comprehensive assessment, increasing the probability of detecting various *Brucella* spp. The latter seems more promising choice for resource-limited high-burden countries.

Brucella samples used in this study were from endemic regions of Iran, a country with a high diversity of *Brucella* spp. circulating among human and animal populations [28]. This might be the reason behind observing a lower diagnostic ability for the Vircell kit, which is developed to detect a narrow range of *Brucella* spp. This implies that assays with a wider antigenic range, such as assays based on whole cell lysates antigens, might be better diagnostic tools for areas where different strains of *Brucella* spp. are circulating within human and animal populations.

This can also be the reason for observing heterogeneity in the Vircell kit's diagnostic performance across various geographical areas [29–32]. Fadeel, et al. [30], compared the performance of four commercial kits for the diagnosis of brucellosis in Egypt and the USA. Although the Vircell kit showed good correlation with the microplate serum agglutination test (MAT), it had low sensitivity in detecting *Brucella* infection comparing to the three other commercial kits tested. Performance of the Vircell kit has been also various in other populations, including Egyptians [30,33], Americans [30], Danes [30], and Iranians [31].

Variations in the results of the Vircell kit might be also attributed to differences in the immune system profile of human populations. As human genetic structure varies across populations, their antibody composition and protection levels against *Brucella* infection may also vary accordingly, leading to different response patterns to a particular pathogenic agent.

Variation of the performance measures is not unique to the Vircell kit, but it is commonly reported by other commercial kits due to similar reasons mentioned above [34]. This highlights the need for the development of population-specific diagnostic assays. External validation of already available assays prior to its large-scale implementation on the target population may also reveal the extent of the assays misclassification.

There are notable limitations to this study. First, this study has been conducted on samples collected from brucellosis patients in Iran. Although Iran is a country with a high degree of *Brucella* spp. variation, there is still a need for validation of the new kit's diagnostic performance in other geographical areas and across different populations. While the newly developed kit is not currently recommendable for use in routine practice, we believe that these preliminary results provide promising evidence for out-performance of the whole cell *B. abortus S99* lysates comparing to the standard Vircell kit. So, further investments to validate the new kit against culture method and in different populations seems to be reasonable.

Second, despite high coverage of this new whole-cell antigen-based kit, it still fails to detect *B. canis* and *B. ovis* species, as these species lack the OPS antigen. To diagnose brucellosis caused from *B. canis* and *B. ovis* it is necessary to use other kind of kits.

This study has also important strengths. The development and evaluation of the new kit have been performed in a controlled laboratory environment using samples of known *Brucella* status. As mentioned previously, the new kit also shows the appropriate ability for the diagnosis of Brucellosis patients that are derived from species-rich areas of Iran. Methodological details of the kit development, provided in this manuscript will hopefully be of help to other researchers in the field, as this type of information is usually lacking in the literature because of the commercial nature of most kits.

5. Conclusions

This study introduces a new indirect ELISA system for the detection of anti- *Brucella* IgM antibodies. Using samples from two species-rich endemic areas of Iran, the newly developed immunoassay showed appropriate diagnostic performance and outperformed the commercial Vircell kit. The OD values of the Vircell kit had a low agreement with the newly developed kit. These findings suggest that the performance of ELISA kits might be dependent on the geographical area under study, and highlight the need for their validation in new geographical areas and populations. Therefore, validation of the new kit's diagnostic performance in other parts of the country is recommended prior to its commercialization. It is also recommended to evaluate the diagnostic performance of the new (and the Vircell) kits before their implementation in other parts of the world.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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References

[1] M.N. Seleem, S.M. Boyle, N. Sriranganathan, Brucellosis: a re-emerging zoonosis, Vet. Microbiol. 140 (2010) 392–398.
[2] K. Franc, R. Krecek, B. Häsler, A. Arenas-Gamboa, Brucellosis remains a neglected disease in the developing world: a call for interdisciplinary action, BMC Public Health 18 (2018) 125.
[3] T. Lytras, K. Danis, G. Dounias, Incidence patterns and occupational risk factors of human brucellosis in Greece, 2004-2015, Int. J. Occup. Med. Environ. Health 7 (2016) 221–226.
[4] J.B. Muma, K.L. Samui, V.M. Siamudaala, J. Oloya, G. Matope, M.K. Omer, M. Munyeme, C. Mubita, E. Skjerve, Prevalence of antibodies to Brucella spp. and individual risk factors of infection in traditional cattle, goats and sheep reared in livestock–wildlife interface areas of Zambia, Trop. Anim. Health Prod. 38 (2006) 195–206.
[5] G. Pappas, P. Papadimitriou, N. Akritidis, L. Christou, E.V. Tsianos, The new global map of human brucellosis, Lancet Infect. Dis. 6 (2006) 91–99.
[6] K. Nielsen, W. Yu, Serological diagnosis of brucellosis, Prilozi 31 (2010) 65–89.
[7] M.J. Ducrotoy, P.M. Muñoz, R. Conde-Álvarez, J.M. Blasco, I. Moriyón, A systematic review of current immunological tests for the diagnosis of cattle brucellosis, Prev. Vet. Med. 151 (2018) 57–72.
[8] S. Christopher, B. Umapathy, K. Ravikumar, Brucellosis: review of the recent trends in pathogenicity and laboratory diagnosis, J. Lab. Phys. 2 (2010) 55.
[9] M.R.U. Islam, M.P. Gupta, P.K. Sidhu, G. Filia, H.M. Saxena, T.A. Shafi, Comparative evaluation of indirect enzyme-linked immunosorbent assay, rose Bengal plate test, microagglutination test, and polymerase chain reaction for diagnosis of brucellosis in buffaloes, Turk. J. Vet. Anim. Sci. 37 (2013) 306–310.
[10] K. Nielsen, Diagnosis of brucellosis by serology, Vet. Microbiol. 90 (2002) 447–459.
[11] I. Jacques, V. Olivier-Bernardin, G. Dubray, Efficacy of ELISA compared to conventional tests (RBPT and CFT) for the diagnosis of *Brucella* melitensis infection in sheep, Vet. Microbiol. 64 (1998) 61–73.
[12] M.J. Kuehn, N.C. Kesty, Bacterial outer membrane vesicles and the host-pathogen interaction, Genes Dev. 19 (2005) 2645–2655.
[13] P. Chaudhuri, R. Prasad, V. Kumar, A. Gangaplara, Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis, Mol. Cell. Probes 24 (2010) 142–145.
[14] P. Minhas, H.N. Pawar, D. Kaur, D. Deka, R.K. Agrawal, Development and evaluation of PCR assay based on outer membrane protein 22 gene for genus-specific diagnosis of *Brucella* spp, Proc. Natl. Acad. Sci. India Sect. B Biol. Sci. 83 (2013) 615–619.
[15] K.A. Pasquevich, S.M. Estein, C.G. Sammartino, A. Zwerdling, L.M. Coria, P. Barrionuevo, et al., Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well as systemic and oral protection against *Brucella abortus* infection, Infect. Immun. 77 (2009) 436–445.
[16] E.A. Murphy, J. Sathiyaseelan, M.A. Parent, B. Zou, C.L. Baldwin, Interferon-γ is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice, Immunology 103 (2001) 511–518.
[17] F.F. Tuon, R.B. Gondolfo, N. Cerchiari, Human-to-human transmission of Brucella – a systematic review, Trop. Med. Int. Health 22 (2017) 539–546.
[18] J.L. Manley, A. Fire, M. Samuels, P.A. Sharp, In vitro transcription: whole-cell extract, Recombinant DNA Methodology, Elsevier, 1989, pp. 653–666.
[19] M.A. Bujang, T.H. Adnan, Requirements for minimum sample size for sensitivity and specificity analysis, J. Clin. Diagn. Res. 10 (2016) YE01.

[20] K. Kiianitsa, N. Maizels, Ultrasensitive isolation, identification and quantification of DNA–protein adducts by ELISA-based RADAR assay, Nucleic Acids Res. 42 (2014) 108-e.
[21] S.M. Estein, P.C. Baldi, R.A. Bowden, Comparison of serological tests based on the outer membrane or internal antigens for detecting antibodies to Brucella ovis in infected flocks, J. Vet. Diagn. Invest. 14 (2002) 407–411.
[22] I.R. Dohoo, P. Wright, G. Ruckerbauer, B. Samagh, F. Robertson, L. Forbes, A comparison of five serological tests for bovine brucellosis, Can. J. Vet. Res. 50 (1986) 485.
[23] M. Sanogo, E. Thys, Y.L. Achi, D. Fretin, P. Michel, E. Abatih, et al., Bayesian estimation of the true prevalence, sensitivity and specificity of the Rose Bengal and indirect ELISA tests in the diagnosis of bovine brucellosis, Vet. J. 195 (2013) 114–120.
[24] G. Matope, J. Muma, N. Toft, E. Gori, A. Lund, K. Nielsen, et al., Evaluation of sensitivity and specificity of RBT, c-ELISA, and fluorescence polarisation assay for diagnosis of brucellosis in cattle using latent class analysis, Vet. Immunol. Immunopathol. 141 (2011) 58–63.
[25] D. O'Grady, W. Byrne, P. Kelleher, H. O'Callaghan, K. Kenny, T. Heneghan, et al., A comparative assessment of culture and serology in the diagnosis of brucellosis in dairy cattle, Vet. J. 199 (2014) 370–375.
[26] S.L. Chisi, Y. Marageni, P. Naidoo, G. Zulu, G.W. Akol, H. Van Heerden, An evaluation of serological tests in the diagnosis of bovine brucellosis in naturally infected cattle in KwaZulu-Natal province in South Africa, J. S. Afr. Vet. Assoc. 88 (2017) 1–7.
[27] M.J. De Bagüés, C. Marin, J. Blasco, I. Moriyon, C. Gamazo, An ELISA with *Brucella* lipopolysaccharide antigen for the diagnosis of *B. Melitensis* infection in sheep and for the evaluation of serological responses following subcutaneous or conjunctival *B. Melitensis* strain Rev 1 vaccination, Vet. Microbiol. 30 (1992) 233–241.
[28] M. Golshani, S. Buozari, A review of brucellosis in Iran: epidemiology, risk factors, diagnosis, control, and prevention, Iran. Biomed. J. 21 (2017) 349.
[29] J. Ruiz-Mesa, J. Sanchez-Gonzalez, J. Reguera, L. Martin, S. Lopez-Palmero, J. Colmenero, Rose Bengal test: diagnostic yield and use for the rapid diagnosis of human brucellosis in emergency departments in endemic areas, Clin. Microbiol. Infect. 11 (2005) 221–225.
[30] M.A. Fadeel, A.R. Hoffmaster, J. Shi, G. Pimentel, R.A. Stoddard, Comparison of four commercial IgM and IgG ELISA kits for diagnosing brucellosis, J. Med. Microbiol. 60 (2011) 1767–1773.
[31] H. Peeridogaheh, M.G. Golmohammadi, F. Pourfarzi, Evaluation of ELISA and *Brucella* capt tests for diagnosis of human Brucellosis, Iran. J. Microbiol. 5 (2013) 14.
[32] S. Al Dahouk, L. Sprague, H. Neubauer, New developments in the diagnostic procedures for zoonotic brucellosis in humans, Rev. Sci. Technol. 32 (2013) 177–188.
[33] T.F. Ismail, H. Smits, M.O. Wasfy, J.L. Malone, M.A. Fadeel, F. Mahoney, Evaluation of dipstick serologic tests for diagnosis of brucellosis and typhoid fever in Egypt, J. Clin. Microbiol. 40 (2002) 3509–3511.
[34] A.F. Longosz, D. Serwadda, F. Nalugoda, G. Kigozi, V. Franco, R.H. Gray, et al., Impact of HIV subtype on the performance of the limiting antigen-avidity enzyme immunoassay, the bio-rad avidity assay, and the BED capture immunoassay in Rakai, Uganda, AIDS. Res. Hum. Retrovir. 30 (2014) 339–344.