

Using bacteriophages in the extraction of *Brucella* DNA from milk and culture samples and its evaluation by multiplex PCR

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Summary

Molecular methods have been progressing at a noticeably rapid rate recently. Obtaining the DNA required for most of these methods is also trying to catch up with this progress. Although many different kits and processes are utilized for DNA extraction, bacterial isolation is still accepted as a gold standard in the diagnosis of brucellosis. This study aims to create a novel way that can be used as an extraction method including bacteriophages. Bacteriophages that are specific to *Brucella* (*B.*) genus and species are used commonly in conventional identification and biotyping. However, using bacteriophages in the extraction of the DNA of the target bacteria has not been tried so far. In this study, DNA was extracted from *Brucella* culture and milk samples through the use of Izat Nagar (Iz) phage with 10^2 - 10^4 routine test dilution. These milk samples were artificially infected by *Brucella suis* bacteria with the amounts changing between 3.68×10^9 and 0.44×10^7 cfu/ml. Positive results were obtained from multiplex PCR by observing the *B. suis* profile with 7 bands. The methodology of the study consisted of 4 main phases; namely, first centrifugation, phage addition, incubation and the second centrifugation. When the effects of phages on viable bacteria are considered, it might be said that a method, which could be named 'Phage-Exsen', and which supports the gold standard, has been suggested. This method combines the advantages of the conventional method and molecular ones and forms a basis for prospective studies. Moreover, it illustrates the use of bacteriophages for DNA extraction in an unusual way, which might form a new perspective.

Keywords: bacteriophage, *Brucella*, extraction, milk, polymerase chain reaction

Brucellosis is one of the most common zoonotic diseases caused by *Brucella* (*B.*) spp. Due to the transmission of *Brucella* species via aerosol, it is classified as a potential bioterror agent, as well as belonging to risk group 3 (20, 23, 25). Diagnosis depends on isolation and identification of *Brucella* from abortion materials, udder secretions or post-mortem tissues (24). Isolation of bacteria and the bacteriological culture method is considered as the gold standard in the diagnosis of brucellosis (3, 14, 25). In this gold standard, focusing on viable bacteria is very important. However, the decline of isolation sensitivity is a possible result because of the contaminant organisms in the samples (11). In such samples, the rate of the diagnosis of infected animals can be increased by PCR techniques through detecting the DNA from dead bacteria cells (8).

In this sense, molecular methods seem to be appropriate alternatives to classical bacteriology, which has

limited diagnostic ability because of the contaminated samples. Moreover, PCR-based methods are valuable tools and they are more advantageous compared to conventional methods (6). However, there is a disadvantage of the PCR method; it can detect the viable and dead bacteria (1), but cannot discriminate them (18). Even for the cases in which there is no bacteria isolation, identification can be achieved through PCR (6). Thus, it is obvious that the procedures composed of 2 different types of diagnosis (molecular and bacteriological) may present more reliable and rapid results about the existence of an active infection.

Brucella organisms are identified with the help of the simultaneous use of several tests (25). Lysis activity by phages among these tests provides significant data. Lysis activity with Tbilisi (Tb), Weybridge (Wb), Iz, and Rough/Canis (R/C) phages are among the distinctive features of the *Brucella* species. In particular,

lysis activity with Iz has genus-specific characteristics since it shows activity with all the classical *Brucella* species except for *B. ovis* and *B. canis*. The interaction between *Brucella* culture and lysis by phage is very specific. *Brucella* phages cannot replicate themselves in other bacteria and *Brucella* strains cannot be lysed by other phages (10).

The use of bacteriophages for the detection of *B. abortus*, *Listeria monocytogenes*, *Yersinia (Y.) pestis* and *Bacillus anthracis* or some other bacteria out of the scope of conventional phage typing was investigated in several studies (4, 9, 12, 16, 18, 19). These studies including phage-based detection tests followed different methodologies while providing the detection of the pathogens through a rapid, simple and efficient way (4, 9, 18, 19). The release of ATP or Adenylate kinase (AK) due the phage mediated lysis of host bacteria (4, 9) or measuring the bioluminescent signal resulting from the luciferase reporter phages (12, 19) were used as the indicators of the host bacteria for the diagnostic applications. Moreover, detecting the amplification of phage DNA due to the replication in the live hosts bacteria like *B. abortus* or *Y. pestis* was considered as another novel way for the molecular diagnosis of the pathogen through real-time PCR (18, 19).

Similar to the novelty and benefits of these studies, this study also presents a new DNA extraction method combined with PCR. In this study, testing the DNA extracted by *Brucella* bacteriophages via PCR is a significant progress because it enriches the PCR method through showing the viable bacteria presence. Using phages for DNA extraction is an unusual way and it has the necessary potential of enlarging the area of its use as well as supporting PCR results. PCR, which analyses the DNA extracted by phages, and bacterial isolation as the gold standard have something in common, which is the presence of viable bacteria. The present study, which has attempted to benefit from this common point, investigated the potential value of this new method.

Material and methods

B. suis biovar 1, as a reference strain, and Iz phage and pasteurized milk were utilized in this study. The reference strains and *Brucella* bacteriophages were provided by the culture collection of the National Laboratory for Brucellosis of Pendik Veterinary Control Institute, Istanbul, Turkey. The determination of Routine Test Dilution (RTD) of the phages was conducted with the help of classical methods (3, 7).

In the first stage of the study, the lyophilized reference strain was reconstituted and the *Brucella* strain was inoculated to a solid medium and a suspension (300 µl) was prepared from cultivated bacteria at the end of the incubation period. 100 µl Iz phage (10^4 RTD) was added to this suspension. This suspension with phage addition was incubated for 24 hours, after this it was centrifuged at 8490 relative centrifugal force (rcf) for 3 minutes. 1 µl of supernatant was analyzed by multiplex PCR as a template DNA sample.

Multiplex PCR was performed according to the method described by Mayer Scholl et al. (13). To this end, 25 µl reaction mixture containing 12.5 µl Qiagen Multiplex Master Mix (Cat No./ID: 206143, Qiagen, Germany), 2.5 µl primer mix composed of 9 primer pairs (each primer 2 pmol/µl) and 1 µl template DNA were used. An initial denaturation step at 95°C for 15 minutes was followed by template denaturation at 94°C for 30 seconds, primer annealing at 58°C for 90 seconds, a 3-minute-primer extension at 72°C for a total of 25 cycles, with a subsequent final extension phase of 10 minutes at 72°C. The PCR products were analyzed with a 1.5% agarose gel. Afterwards, the DNA fragments were observed as bands under UV light. The same method was followed while utilizing the milk samples that were artificially infected by *Brucella*.

In the second stage of the study including the milk samples, 1 ml suspension of *brucella* bacteria was inoculated to each milk sample (9 ml pasteurized milk). Milk was used in this study because it is considered to be an important material for the diagnosis of brucellosis in animals. Consumption of milk including *Brucella* spp. may lead to infection in humans; therefore, milk has significance within the scope of the zoonotic nature of the disease (2, 6). Serial dilution method was preferred to be able to reveal the viable *Brucella* bacteria count in the suspension of *B. suis* strain (3). At the end of the 24-hour-incubation period, each sample was centrifuged (5000 g) for 15 minutes and supernatant was discarded. The precipitant was diluted with 500 µl PBS (phosphate buffer solution). Next, 350 µl Iz phage was added. After another 24-hour-incubation period, the suspension was centrifuged for 3 minutes at 8490 rcf. In the end, 1 µl of supernatant was analyzed by multiplex PCR as a template DNA sample. In addition to the aforementioned way that was followed, many different trials were conducted simultaneously by using the cream layer, supernatant or precipitate after the first or second centrifugation.

Results and discussion

Both in the culture and in some of the milk samples *B. suis* profile was observed through gel electrophoresis of PCR amplified products. Extracted DNA displayed the correct band profile of *B. suis*, which is composed of 7 bands. However, DNA bands were either faint or non-existent for some of the milk samples. Before the first 24-hour-incubation period, 1 ml *B. suis* suspension with 3.68×10^9 cfu/ml was added to each milk sample. 350 µl Iz $\times 10^4$ RTD was added to the samples before the second 24-hour-incubation period. The results of the samples varying according to the collecting point in the sample tube after the first or the second centrifugation are listed in Table 1.

The DNA band views of these PCR results observed under the UV light on gel are shown in Figure 1.

Sample 6 was analyzed by following the procedure described in the Method part of this study. The best band view was obtained from sample 6, which is why the same procedure was followed in the following trials as well as while reducing the bacteria count and phage

titer. The results of the sample 6, 7 and 8 were particularly compared and contrasted. After the first centrifugation of the milk samples, no PCR band was observed when the cream layer accumulated at the top of the tube that was used as in sample 7 instead of the precipitant. However, when the supernatant located below the cream layer was used as in sample 8, PCR bands were not as clear as in sample 6 in which the precipitant was used during the same procedure.

Repeat testing was performed by using the precipitant for this reason. The amount of bacteria (cfu/ml) and the titer of the phage were reduced in the following trials. The amount of bacteria and phage titer used in the following trials were listed in Table 2. Table 2 also shows the PCR results of these samples. In this trial, 350 µl phage was also added to the milk samples although the phage titer was reduced. When the results of the samples in Table 1 and sample 6 in Table 2 were compared and contrasted, the *brucella* bacteria count and phage titer were reduced 830 and 100 times respectively in sample 6 as listed in Table 2.

The DNA band views of these results observed under the UV light on gel are shown in Figure 2.

Despite these reductions, the *B. suis* profile was maintained at variable visibility as seen through PCR bands. In Figure 2, the top bands of the samples might look a bit faint but it was observed more clearly on the gel with naked eyes right after the electrophoresis.

It has been suggested that the bacteriophage can be used as a targeted reagent for the release of intracellular ATP from *Listeria* and it was observed that bacteriophage lysis combined with ATP detection enabled the identification of 2.5×10^5 cells (4, 9). Taking the above mentioned suggestion into consideration, phage was added to samples from uterus discharge and an ATP determination kit was used. During the test, it was observed by a luminator that there was an increase in the luminescence due to the luciferin-luciferase reaction. It was enabled by the ATP which was released from bacteria because of phage lysis (9). In another study with the same method, the *Esherichia coli* and *Salmonella newport* were analysed. However, they focused on the bacteria's AK production instead of ATP as the key point of the process (4).

In this study, obtaining DNA from viable bacteria through use of phages for PCR increased the significance of this novel approach. More than 85% of the infected pregnant ruminants have a high *Brucella* bacteria burden up to 1×10^{10} cfu/ml in allantoinic, amniotic fluids and placenta. This bacteria load up to 1×10^{13} cfu/g can be present in cotyledon tissues (14, 15, 27). In this sense, the bacteria count for this test

Tab. 1. PCR results of the samples prepared in various ways prior to the PCR

Sample no	After the 1 st centrifugation	After the 2 nd centrifugation	PCR band profile
1	not done	cream layer	none
2	not done	supernatant (below cream)	<i>B. suis</i>
3	cream layer	cream layer	none
4	cream layer	supernatant (below cream)	<i>B. suis</i>
5	precipitate	supernatant (no cream layer existent)	<i>B. suis</i> (faint bands)
6	precipitate	supernatant	<i>B. suis</i>
7	supernatant	cream layer	none (very faint bands)
8	supernatant	supernatant	<i>B. suis</i>



Fig. 1. DNA band views of the samples on gel
 Explanations: lane 1-8 – sample 1-8; lane 9 – negative control; lane 10 – *B. melitensis*; lane 11 – *B. ovis*; lane 12 – *B. canis*; lane 13 – *B. suis*; lane 14 – 2000 bp ladder.



Fig. 2. DNA band views of the samples on gel
 Explanations: lane 1-6 – sample 1-6; lane 7 – negative control; lane 8 – *B. melitensis*; lane 9 – *B. ovis*; lane 10 – *B. canis*; lane 11 – *B. suis*; lane 12 – 2000 bp ladder.

Tab. 2. PCR results of the samples regarding bacteria count and phage titer

Sample no	Suis (cfu/ml)	Iz	PCR results
1	0.44×10^9	10^4 RTD	<i>B. suis</i>
2	0.44×10^8	10^4 RTD	<i>B. suis</i>
3	0.44×10^8	10^3 RTD	<i>B. suis</i>
4	0.44×10^7	10^4 RTD	<i>B. suis</i>
5	0.44×10^7	10^3 RTD	<i>B. suis</i>
6	0.44×10^7	10^2 RTD	<i>B. suis</i>

is lower than the bacteria burden mentioned in those studies. Prospective studies with lower bacterial count might be analysed by modifying the method.

It is also important to state that the study was carried out with a conventional multiplex PCR and only observing the *B. suis* profile with 7 bands was regarded as a positive result. The 7 bands were compared and contrasted with the results of the previously mentioned study (13). If real time PCR is preferred instead of conventional multiplex PCR, it might be possible to achieve a lower detection limit in future studies.

In the first stage of the study, successful results were obtained while working with culture samples. However, under normal circumstances it is possible to get false positive results by PCR from culture samples mixed with microorganisms having a close genetic relationship with the target bacteria. Therefore, the use of phages can increase the reliability of the positive results by leading to the lysis of only the target bacteria even if the sample is a mixed culture. In a study by Sergueev et al. (18), a similar confusion was mentioned. They showed the presence of *B. abortus* in the mixed culture indirectly by detecting the increase of phage DNA through real time quantitative PCR. This mixed culture was composed of *B. abortus*, *Ochrobactrum anthropi* or *Afpia felis*, which belong to alpha-proteobacteria.

The members of the genus *Brucella* are fastidious microorganisms and need a longer incubation period than the contaminants in the samples that grow quickly (3, 21). In their study, Stack et al. (21) could not recognize the growth of *Brucella* bacteria in some of the infected milk samples with *Brucella* spp. In order to overcome the difficulties of the *Brucella* isolation from milk, the use of more than one medium has been recommended (2). What is more, keeping the incubation period up to 6 weeks by using enrichment broth is also recommended for *Brucella* isolation from milk samples (25). In this study, the results were obtained in 2 days from artificially infected milk samples. Therefore, this method has the potential advantage of saving time.

Better results were obtained from milk samples when the test was conducted with a precipitant after centrifugation. Researchers compared the count of viable *Brucella* bacteria in the milk samples regarding the sampling point on a tube including milk. They discovered that it is possible to get higher counting results from the bottom than the top of the centrifuged tube (26). Similarly, we might get a productive DNA extraction by using the precipitant in which bacteria accumulation occurred. This possibility might also affect the visibility of the band in gel.

The master mix used includes 3 mM MgCl₂. It is known that the components of milk samples such as milk fat and Ca have inhibitor effects on PCR. For this reason, more visible bands were observed by increasing the MgCl up to 4 mM to avoid the inhibition by

Ca (26). It might be possible to obtain clear bands by making this manner of changes.

Agents of brucellosis are capable of being well adapted to an intracellular environment. The isolation of strains requires specific medium, a well-equipped laboratory and qualified staff trained to work with infectious materials (22). Moreover, in the OIE manual the necessity of working at appropriate biosafety levels by conducting a biorisk analysis at the laboratories working with viable *Brucella* spp. culture or suspected infectious material has been stated (24). In addition, it is stated that the practices related to brucellosis must be classified and regarding this classification BSL-3 or higher biosafety level conditions have to be provided (25). Furthermore, *Brucella* species are considered to be the most common pathogens related to laboratory acquired infection (17, 25). A PCR conducted with Phage-Exsen can both decrease risky practices and can detect the presence of viable bacteria, which is a requirement for the gold standard in the diagnosis of brucellosis. It is also pointed out that the phage-based detection assays like the one in this study have applicability and the potential to analyse different environmental and clinical samples (9, 16, 18).

It is contended that due to its highly specific lysis ability, phage is a good way of accurate identification of bacteria and it might also be possible to benefit from it for diagnosis (22). There are also some studies which focused on phagotyping and phage susceptibility of *Brucella* strains associated with human and animal cases (5, 10). In this study, which created the 'Phage-Exsen' method, using phages in the extraction of DNA thanks to their lysis capability was considered as a new way apart from using phages in typing bacteria. The findings of this study are promising in that it will be possible to do a similar study with raw milk and organ samples in the future.

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