Original Article

Optimization and Comparison of Different Methods and Factors for Efficient Transformation of *Brucella abortus* RB51strain

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Abstract

Background: The development of protective vaccines for *Brucella* spp. has been hampered by the difficulty in transformation of *Brucella* cells with foreign DNA for genetic manipulation. It seems that the formation of *Brucella* spheroplasts would increase the efficiency of transformation. The aim of this study was to devise an efficient method for the transformation of *Brucella* spp.
Materials and Methods: At first, spheroplast of *Brucella* was prepared by glycine and ampicillin induction and transformed using optimized protocols of $CaCl_2$, electroporation, and lipofection methods. Then, the efficacy of transformation was compared between the three-mentioned methods.
Results: Ampicillin-induced spheroplasts from early-log phase culture of *brucella* when incubated in a medium-containing 0.2 M sucrose during cell recovery had higher transformation efficiency in three different methods. Comparison of the transformation efficiency of *Brucella abortus* RB51 using the $CaCl_2$, lipofection, and electroporation methods revealed that the transformation efficiency with the lipofection method was significantly higher than with other two methods ($P < 0.05$).
Conclusions: Lipofection method by lipofectamine 2000 on ampicillin-induced spheroplasts can be a suitable approach for *Brucella* transformation.

Keywords: *Ampicillin, Brucella, lipofectamine 2000, lipofection, spheroplasts, sucrose*

Introduction

Brucella genus is Gram-negative intracellular microbes that cause brucellosis, which can result in abortion in domestic animals and undulant fever in human.[1] Brucellosis is the most common zoonotic disease and causes considerable economic losses in the world livestock industry; a potential concern also exists about the use of *Brucella* as bioterrorism weapons.[2,3] Hence, the development of sensitive and specific molecular diagnostic methods and effective vaccines to eradicate brucellosis are required. There are some limitations to develop recombinant *Brucella*, because of the difficulties associated through gene delivery and targeted gene deletion, insertion, or genetic manipulation of *Brucella*.[4,5]

No evidence has yet been presented for the existence of a natural genetic transformation system in *Brucella* spp.; hence, the transformation of these cells has to be induced by artificial methods.[6] An electroporation protocol has been used for many years as a current approach to transform *Brucella* spp. Nevertheless, transformation yield of these methods is insufficient, especially when homologous or nonhomologous recombination of DNA is desired and selected by antibiotics.[7-9] Optimization of a high-efficiency gene delivery system or method for *Brucella* spp. would facilitate genetic manipulation of these microbes.

The cell wall and outer membrane of *bacteria* comprise the main barriers for DNA uptake and transformation.[10] Using *Brucella* spheroplasts, which lack the cell wall and most of the outer membrane,[11,12] might be the best solution for the described transformation problems. At present, several cell-wall-weakening agents exerting

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different effects on the cell wall are widely used to enhance the transformation efficiency of bacteria.[13-15]

Cationic liposomes are being widely used to transform *eukaryotic cells* (lipofection); this method is characterized by high efficiency and safety.[16,17] Cationic lipid subunits through liposome formation entrap the negatively charged DNA molecules. The DNA-lipid complex overcomes the electrostatic repulsion of the cell membrane, and the cationic lipid fuses with the cell membrane lipids, and finally, the DNA molecule enters to the cell.[18,19]

We hypothesized that cationic liposomes could be used for the transformation of *Brucella* spheroplasts, that is why the inner membrane is exposed and might promote transformation efficiency. To test this, *Brucella* spheroplasts were formed and transformed with foreign DNA using three different methods, including chemical ($CaCl_2$), physical (electroporation), and lipofection method. In an effort to increase the transformation efficiency, the impact of some parameters on the efficiency of the three methods was examined.

Materials and Methods

Bacterial strains, growth conditions, and plasmids

Escherichia coli TOP10F' strain was grown at 37°C in Luria-Bertani (LB) broth or agar (Merck, Germany) containing 80 μg/mL of tetracycline. *Brucella abortus* RB51 strain was grown at 37°C in trypticase soy agar (TSA) or trypticase soy broth (TSB) (Quelab, Canada) supplemented with 50 μg/mL of rifampin and kanamycin or 50–100 μg/mL of ampicillin Plasmids used in this study are listed in Table 1. Plasmid pUC57/pBBR1ori (about 4 kb, bearing an ampicillin resistance gene) containing the origin of replication of pBBR1 plasmid and is able to replicate in *Brucella* spp.,[20,21] pBBR1ori fragment (1.7 kb) was designed and ordered to synthetize to Gene cust company (Gene cust, Luxembourg) and cloned in pUC57 using *Eco*RI and *Nde*I restriction enzymes. Furthermore, from plasmid pBGGT/ΔureC/kana/LLO which was prepared in another study (unpublished data), *Eco*RI and *Nde*I digested fragment-containing ΔureC/kana/LLO (5.3 kb) was separated and ligated to PBBR1ori fragment and pBBR1ori/ΔureC/kana/LLO (7 kb bearing a kanamycin resistance gene) was constructed.

Table 1: Plasmids used in this study

Plasmids	Characteristics	Reference
pBGGT	Cloning vector, Ap^r	Laboratory stock
pUC57/pBBR1ori	pMB1 and pBBR1 ori, Ap^r	Gene cust, luxembourg
pBGGT/ΔureC	pBGGT with *ureC Brucella* fragment	This study
pBGGT/ΔureC/kana	Km^r, Ap^r	This study
pBGGT/ΔureC/kana/LLO	Km^r, Ap^r, LLO from *Listeria monocytogenes*	This study
pBBR1ori/ΔureC/kana/LLO	pBBR1 ori, Km^r	This study

The plasmids were extracted by Miniprep extraction kit (SolGent, Korea) and dissolved in ddH_2O and quantified by nanodrop spectrophotometer (Nanolytik, Germany).

Spheroplast formation

Some factors such as the concentrations of ampicillin, glycine, and sucrose solutions used for spheroplast induction, culture growth phase, and the time of spheroplast formation were optimized.

A single colony of *B. abortus* RB51 was grown in 5 mL of TSB supplemented with rifampin at 37°C and shaking at 200 rpm until the optical density at 600 nm (OD_{600}) reached 0.2–0.4. Then, 200–300 mL of fresh TSB-containing rifampin was added, and the culture was incubated at 37°C with shaking for 24 h until OD_{600} intended for each transformation method was reached. The bacteria were pelleted by centrifugation, and the cells were added to TSB-containing ampicillin (10 μg/mL) with 0.2 M sucrose or TSB-containing 2% glycine and 0.2 M sucrose. Ampicillin and glycine were used as spheroplast induction agents. Glycine or ampicillin-containing media without sucrose were prepared and used as negative control treatments of the osmotic protection step. The treated cultures were incubated at 37°C with gentle rotation (80 rpm) for 24 h. Spheroplast formation was observed using phase contrast microscope at the end of the incubation period.[22]

Transformation with cationic lipid method

Once the glycine- and ampicillin-induced spheroplasts (OD_{600} = 0.2–0.4) were prepared, the cells (50 mL) were chilled on ice for 30 min, centrifuged at 1000 × *g* for 10 min, and washed 3–5 times with ice-cold DDW-containing 0.2 M sucrose. The spheroplasts were then suspended in 10 mL of ice-cold ddW-containing 0.2 M sucrose. Nearly 2 μL of each plasmid DNA (pUC57/pBBR1ori and pBBR1ori/ΔureC/kana/LLO, 1 μg/mL) and an optimized volume of 5 μL lipofectamine 2000 reagent (Invitrogen, USA) were mixed and incubated for 20 min at room temperature. A solution of each plasmid (1 μg/μg) without the cationic lipid was also prepared as a control. The prepared spheroplasts (100 μL) were added to the DNA-cationic lipid complex solutions and mixed by gently pipetting. The mixtures were then kept on ice for 1 h. Next, each mixture was split into two aliquots; one portion was transferred to 1 mL of TSB-containing 0.2 M sucrose (to continue the osmotic protection); the other portion was transferred to 1 mL TSB without sucrose. All tubes were incubated at 37°C for 6 h without shaking for cell recovery. Ampicillin or kanamycin (the related resistance genes were encoded by plasmids used in the study) were added to all tubes, to select transformants, and the incubation continued

at 37°C for 24 h with gentle shaking. Finally, all cells were transferred to TSA plates-containing antibiotics, incubated at 37°C for 48 h, and the resulted colonies were counted. Transformation of nonspheroplast *B. abortus* cells was performed similarly.

The $CaCl_2$ method for transformation

At first optimization of the culture growth phase, $CaCl_2$ concentration, heat-shock duration, and competent cell induction were performed. Once glycine- and ampicillin-induced spheroplast (50 mL) was formed (OD_{600} = 0.2–0.4), the bacteria were chilled on ice for 30 min, centrifuged at 1000 × *g* for 10 min, and washed five times with ice-cold DDW-containing 0.2 M sucrose. The pelleted spheroplasts were resuspended in 5 mL of 0.1 M $CaCl_2$ or 0.2 M $CaCl_2$ supplemented with 0.2 M sucrose and incubated on ice for 30 min. The spheroplasts were centrifuged at 1000 × *g* for 10 min, resuspended in 2 mL of 0.1 and 0.2 M $CaCl_2$-containing 0.2 M sucrose, and incubated on ice for 30 min; the cells were pelleted again and gently resuspended in 1 mL of 0.1 or 0.2 M $CaCl_2$ solution with 0.2 M sucrose. The competent spheroplasts (100 µL) were gently mixed with 1 µg/µg of each plasmid DNA solution (pUC57/ori or pBBR1ori/ΔureC/kana/LLO) and incubated on ice for 30 min. This was followed by a heat-shock, 2 min at 42°C; the cells were immediately incubated on ice for 2 min. Next, each batch of transformed competent cells was split into two portions. One portion was added to 1 mL of TSB-containing 0.2 M sucrose (to continue the osmotic protection), the other portion was added to 1 mL of TSB without sucrose. All tubes were incubated at 37°C for 6 h without shaking to allow the cells to recover. Ampicillin or kanamycin was added depending on the plasmid used for transformation; the incubation was continued at 37°C for 24 h with gentle shaking. Finally, all transformed cells were spread on TSA plates containing the appropriate antibiotics, incubated at 37°C for 48 h, and the transformants were counted. Spheroplasts that had not been treated by $CaCl_2$ were used as a negative control. Transformation of nonspheroplast *B. abortus* cells was performed in the same condition.

Electroporation

Three 250 mL cell culture samples were grown to different OD_{600} values, i.e. early-log phase (OD_{600} = 0.1–0.4), mid-log phase (OD_{600} = 0.4–0.7), and late-log phase (OD_{600} = 0.7–1.0). The cultures were treated by glycine or ampicillin, with or without sucrose, to induce spheroplast formation as described above. To prepare electrocompetent cells, spheroplast and nonspheroplast cells were chilled on ice for 30 min. The spheroplasts were centrifuged at 1000 × *g* for 10 min, washed three times with ice-cold ddH_2O-containing 0.2 M sucrose, and two times with an ice-cold solution containing 10% glycerol 0.2 M sucrose. Finally, the cells were resuspended in 1 mL of 10% glycerol supplemented with 0.2 M sucrose. Electro-competent cells were prepared from nonspheroplast cells as above, except that sucrose was excluded from washing solutions. Immediately before electroporation, 100 µL of competent cells were mixed with 2 µL of two different plasmid solutions (pUC57/pBBR1ori or pBBR1ori/ΔureC/kana/LLO, 1 µg/mL). The mixtures were loaded into prechilled 2-mm gap width cuvettes, and electroporation was performed using a Gene Pulser (Bio-Rad, USA) set at 2.5 kV, 25 µF, and 600 Ω. After a single electrical pulse, the cells were immediately diluted with 1 mL of TSB with or without 0.2 M sucrose and incubated at 37°C for 24 h without shaking to allow cell recovery. The cells were then transferred to 1 mL of TSB (with or without sucrose, with an appropriate antibiotic) and incubated at 37°C by shaking for 24 h. Finally, the cells were harvested and plated on TSA plates supplemented with ampicillin or kanamycin. The transformants were counted following 48–72 h of incubation at 37°C.

Statistical analysis

The data were analyzed using the SPSS software (version 17.0, Chicago, IL, USA). The experiments were repeated five times, and the means and standard deviations of transformation efficiencies were calculated for each method. Independent *t*-test and one-way ANOVA were used for the comparison of transformation efficiencies with different treatments within and between the three different transformation methods. A value of $P < 0.05$ was considered as statistically significant.

Results

Spheroplast formation

Spheroplast formation was induced in a 24 h culture of *B. abortus* RB51 grown in TSB with shaking at 37°C, with either glycine or ampicillin and sucrose, as inducing agents. Microscopic observations revealed spherical morphology of >95% of cells treated with ampicillin and about 75% of cells treated with glycine were converted into spheroplasts. Complete lysis of bacterial cells was observed in the control groups, in the absence of sucrose.

The result of cationic lipid method for bacterial transformation

The following parameters were optimized for this transformation method: mixing temperature and incubation time of the spheroplast and DNA-cationic lipid complex; the concentration of the cationic lipid used during transformation; the number of spheroplast washes before transformation; and the recovery time after transformation.

In the current study, nonspheroplast and glycine- and ampicillin-induced spheroplasts of *B. abortus* RB51 were transformed with two different plasmid-cationic liposome complexes. We attempted at least ten times transformations of nonspheroplast *B. abortus* cells by the lipofection method, but no transformants were obtained. Moreover, no

transformants were observed when DNA was mixed with spheroplast cells without the cationic lipid as a control. In Figure 1, the details of the transformation efficiency (cfu/μg of DNA) of spheroplasts transformed using the lipofection method in different treatment conditions were shown. All spheroplast groups were successfully transformed with a reasonable efficiency compared with control and nonspheroplast cells ($P < 0.05$ for all treated groups). The transformation efficiency of ampicillin-induced spheroplasts transformed with 4- or 7-kb plasmid-liposome complexes and incubated during cell recovery in a medium-containing 0.2 M sucrose were significantly higher than that of glycine-induced spheroplasts treated similarly ($P < 0.001$). The transformation efficiency of ampicillin-induced spheroplasts treated with a 4-kb plasmid-liposome complex and incubated in a medium without sucrose was significantly higher than glycine-induced spheroplasts that underwent the same treatment ($P = 0.035$). The transformation efficiency of ampicillin-induced spheroplasts transformed with a 7-kb DNA-liposome complex and incubated in the absence of sucrose was significantly higher than glycine-induced spheroplasts treated in the same manner ($P < 0.001$). The transformation efficiency of ampicillin-induced spheroplasts transformed by a 4- or 7-kb plasmid-liposome complex was significantly higher when sucrose was included in the recovery medium than in the absence of sucrose ($P < 0.001$). The same was true for glycine-induced spheroplasts ($P = 0.008$ for the 4-kb plasmid-liposome complex, and $P = 0.001$ for the 7-kb plasmid-liposome complex). For both ampicillin- and glycine-induced spheroplasts, transformation with a 4-kb plasmid-liposome complex showed higher efficiency than transformation with a 7-kb plasmid-liposome complex, regardless of the presence of sucrose in the recovery medium ($P < 0.001$). These data clearly indicated that spheroplast formation is prerequisite for the transformation of *B. abortus* RB51 by this method.

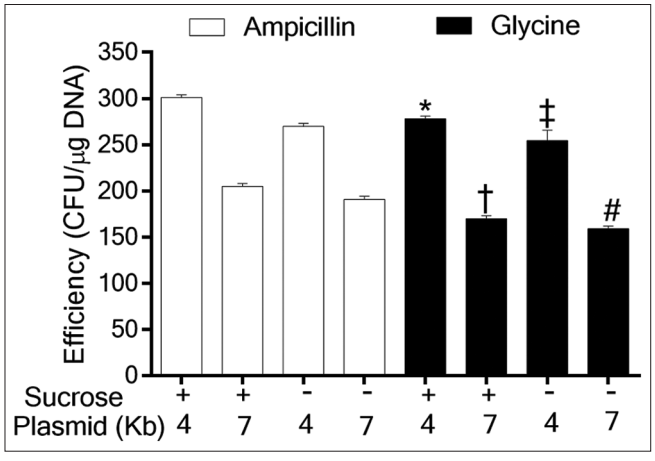

Figure 1: The effect of different treatments of *Brucella abortus* on transformation efficiency using the lipofection method. Results are mean ± standard error of the mean. *$P < 0.05$ compared to the ampicillin/sucrose (+)/plasmid 4 kb, †$P < 0.05$ compared to the ampicillin/sucrose (+)/plasmid 7 kb, ‡$P < 0.05$ compared to the ampicillin/sucrose (−)/plasmid 4 kb, #$P < 0.05$ compared to the ampicillin/sucrose (−)/plasmid 7 kb. Plasmids: 4 kb, pUC57/pBBR1ori; 7 kb, pBBR1ori/ΔureC/kana/LLO

The result of $CaCl_2$ method

Transformation results of spheroplasts that had not been treated by $CaCl_2$ as a control and ampicillin or glycine-induced spheroplasts of *B. abortus* transformed by 0.1 M $CaCl_2$ were negative. The transformation efficiency of *B. abortus* RB51 spheroplasts (by both ampicillin and glycine methods) using the 0.2 M $CaCl_2$ method is presented in Figure 2. There were no significant differences between the performances of ampicillin- or glycine-induced spheroplasts in the 0.2 M $CaCl_2$ transformation method, even though the average efficiency of transformation was somewhat higher for ampicillin-induced spheroplasts ($P > 0.05$). The transformation efficiency of ampicillin-induced spheroplasts treated with 0.2 M $CaCl_2$ transformed by a 4-kb plasmid and recovered in a medium-containing sucrose was not significantly different than that for glycine-induced spheroplasts treated in an analogous manner ($P = 0.17$). The transformation efficiency, of ampicillin-induced spheroplast transformed by a 4-kb plasmid incubated in a medium without sucrose, was not significantly different than that for glycine-induced spheroplasts that were submitted to the same treatment ($P = 0.17$). The transformation efficiencies, of both ampicillin- and glycine-induced spheroplasts transformed by the 4-kb plasmid, were significantly higher in sucrose supplemented recovery medium than in sucrose devoid recovery medium ($P = 0.037$). The transformation efficiencies of both ampicillin- and glycine-induced spheroplasts transformed by the 4-kb plasmid were higher for transformation with the 7-kb plasmid, regardless of the presence of sucrose in the recovery medium ($P < 0.001$). Transformation efficacy of both ampicillin and glycine spheroplasts treated 0.2 M $CaCl_2$ and 7-kb plasmid was negative.

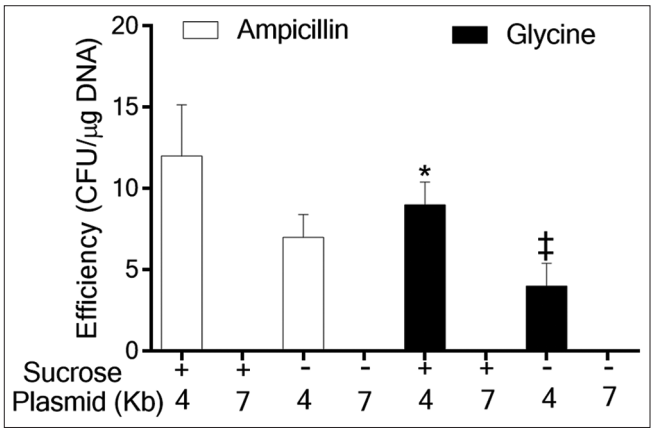

Figure 2: The effect of different treatments of *Brucella abortus* on transformation efficiency using the 0.2 M CaCl2 method. Results are mean ± standard error of the mean. *$P < 0.05$ compared to the ampicillin/sucrose (+)/plasmid 4 kb, ‡$P < 0.05$ compared to the ampicillin/sucrose (−)/plasmid 4 kb. Plasmids: 4 kb, pUC57/pBBR1ori; 7 kb, pBBR1ori/ΔureC/kana/LLO

The result of electroporation

Nonspheroplast cells and glycine- and ampicillin-induced spheroplast of *B. abortus* RB51 prepared from three cultures with different OD_{600} values were electroporated in the presence of two different plasmids. The results are shown in Figure 3 indicate that the electroporation of spheroplasts was more efficient than electroporation of nonspheroplast cells ($P < 0.05$). The electroporation efficiencies of ampicillin- and glycine-induced spheroplasts prepared from cultures at OD_{600} of 0.1–0.4 or 0.4–0.7 were significantly higher than those of nonspheroplast cells ($P < 0.001$). The electroporation efficiency of ampicillin- and glycine-induced spheroplasts induced from cultures at OD_{600} of 0.7–1.0 was significantly higher than those of nonspheroplast cells ($P = 0.004$). In addition, the use of early-log phase cultures ($OD_{600} = 0.1–0.4$) resulted in higher electroporation efficiency than when mid- and late-log phase cultures were used, for both spheroplast and nonspheroplast cells ($P < 0.001$ for ampicillin- or glycine-induced spheroplasts, and $P = 0.008$ for nonspheroplast cells). The independent *t*-test revealed no significant differences between the use of mid- and late-log phase cultures for nonspheroplasts and glycine-induced spheroplasts despite a high average electroporation efficiency when mid-log phase cultures were used ($P = 0.24$ for glycine spheroplast, and $P = 0.056$ for nonspheroplast cells). On the other hand, the electroporation efficiency of ampicillin-induced spheroplasts from mid-log phase cultures was significantly higher than for late-log phase culture spheroplasts ($P = 0.044$).

As shown in Figure 4, ampicillin induction resulted in higher electroporation efficiency than glycine treatment ($P < 0.05$); the only exception was when ampicillin-induced spheroplasts were electroporated with a 7-kb plasmid and incubated in a medium without sucrose: compared with glycine-induced spheroplasts that were submitted to the same treatment, no statistically significant differences were observed, despite a higher average electroporation efficiency of ampicillin-induced spheroplasts ($P = 0.35$). The electroporation efficiency of ampicillin-induced spheroplasts electroporated with a 4-kb plasmid and incubated in a medium-containing sucrose was enhanced compared with glycine-induced spheroplasts that underwent the same treatment ($P < 0.001$); the same was observed when no sucrose was present in the recovery medium ($P = 0.002$). The electroporation efficiency of ampicillin-induced spheroplasts electroporated with a 7-kb plasmid and incubated in a medium-containing sucrose was significantly higher than for glycine-induced spheroplasts treated in an analogous manner ($P = 0.001$). In all treatment groups, the inclusion of sucrose in the recovery medium after electroporation significantly increased the efficiency of electroporation compared with medium lacking sucrose ($P < 0.001$). Furthermore, in all treatment groups, the electroporation efficiency was also significantly increased when the 4-kb plasmid was used rather than the 7-kb plasmid ($P < 0.001$).

Comparison of the different cell treatments in the three optimized transformation methods revealed that the lipofectamine method resulted in the highest transformation efficiency of all treatment groups ($P < 0.001$) [Figure 5].

Discussion

Because of the difficulty in transforming *Brucella* spp. with foreign DNA, optimization of an efficient DNA transfer method for the introduction of exogenous genes into *Brucella* cells is the first step for carrying out genetic manipulation.[5] Hence, we optimized and compared three different transformation methods and the effect of

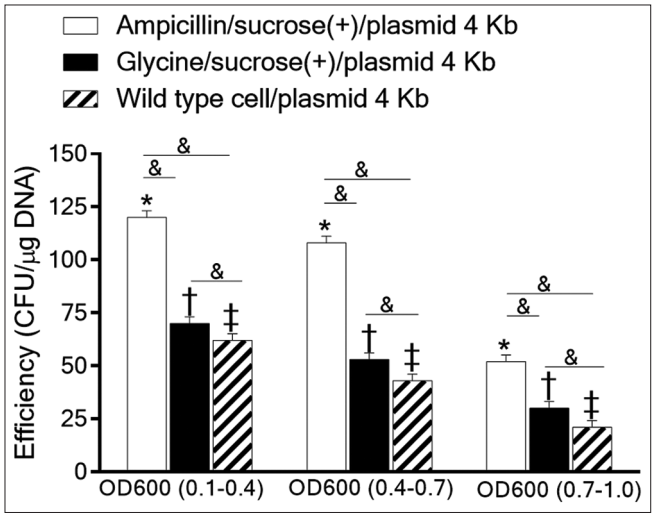

Figure 3: Comparison of electroporation efficiencies of nonspheroplast and spheroplasts of *Brucella abortus* prepared from cultures grown to three different OD600 values. Nonspheroplast and glycine and ampicillin-induced spheroplasts were prepared and electroporated. The results are mean ± standard error of the mean and $^{\&}P < 0.05$. The same symbols indicate statistically significant differences ($P < 0.05$) between the groups

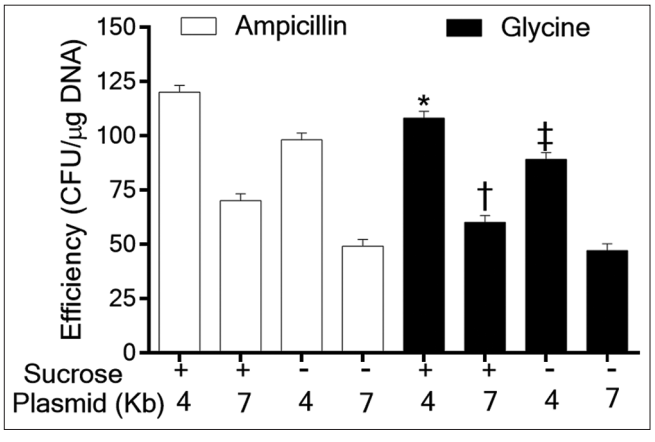

Figure 4: The effect of different treatments of *Brucella abortus* on the electroporation efficiency. Results are mean ± standard error of the mean. $^{*}P < 0.05$ compared to the ampicillin/sucrose (+)/plasmid 4 kb, $^{\dagger}P < 0.05$ compared to the ampicillin/sucrose (+)/plasmid 7 kb, $^{\ddagger}P < 0.05$ compared to the ampicillin/sucrose (−)/plasmid 4 kb. Plasmids: 4 kb, pUC57/pBBR1ori; 7 kb, pBBR1ori/ΔureC/kana/LLO

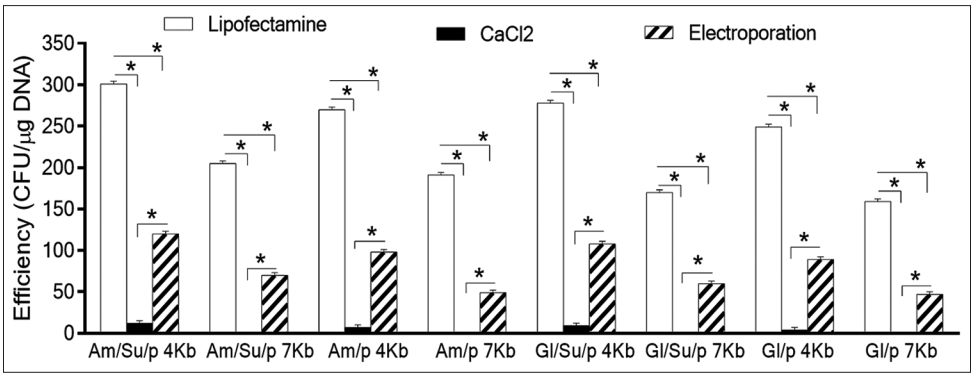

Figure 5: Comparison of the efficiency mean value of 10-time transformation of *Brucella abortus* RB51 using the CaCl2, lipofectamine, and electroporation methods. Results are mean ± standard error of the mean and **P* < 0.05. Am: Ampicillin, Su: Sucrose, P: Plasmid

several parameters that may have potentially improved the transformation. In the current study, the induction of spheroplasts was achieved by cell-wall-weakening and osmotic protection agent. The transformation efficiencies of the three-mentioned methods were significantly higher with spheroplasts than with nonspheroplast cells. The obtained results supported our initial hypothesis that the formation of spheroplasts as an approach to overcome the cell wall as a barrier would improve the exogenous DNA uptake by *B. abortus*. Similar results have been obtained by Liu *et al*. in a study of the transformation of *E. coli* spheroplasts through the chemical $CaCl_2$ method[23] and in several other studies with different bacteria.[14,24-28] However, some researchers reported that cell-wall-weakening either had no effect on the transformation frequency.[29] It seems that type of bacteria, cell-wall-weakening agents for spheroplast formation, and the transformation method used should be affected on the result. Based on our findings, the use of ampicillin for spheroplast induction resulted in a higher percentage of *Brucella* cells converting to spheroplasts than glycine induction; further, the efficiency of transformation of ampicillin-induced spheroplasts was significantly higher than for glycine-induced spheroplast after lipofection or electroporation. The same was observed by Zhang *et al*. who investigated the transformation of *Bacillus subtilis* ZK.[25] Ampicillin induces *Brucella* spheroplast formation via the degradation of the peptidoglycan structure; while glycine by replacing alanine in the peptidoglycan of cell wall causes peptidoglycan cross-linking and susceptibility to spheroplast formation.[15,30]

Another important finding of the current study is the notion that the cationic liposome method resulted in the highest transformation efficiency of *Brucella* spheroplasts while no effect was observed for the nonspheroplast *Brucella* cells. Transformation of *E. coli*[31] and the archaeon *Methanosarcina*[32] was also successfully achieved with this method. In the current study, the transformation efficiency of ampicillin-induced spheroplasts transformed by the lipofection method was significantly higher than that of glycine-induced spheroplasts.

In contrast with the lipofection method, both nonspheroplast and spheroplast forms of *B. abortus* could be transformed by electroporation. However, the efficiency of electroporation was higher for spheroplasts than for nonspheroplast cells. Several previous studies documented that the use of a cell-wall weakening agent in culture before electroporation improves the transformation efficiency of cells.[24,25,33] The growth phase of the bacteria was another factor examined in the current study; the most electroporation efficiency was observed in the early-log phase and the least one in the late-log phase. It seems that during the early-log phase, the cells are growing most rapidly, with the most dynamic cell wall and poorer cell-wall intensity that render them competent for a physical or chemical transformation. Previous studies have reported conflicting observations for different bacteria.[26,34] In *Bacillus thuringiensis* and *Bacillus cereus*, similarly to our result, the use of early-log phase (OD_{600} = 0.1–0.4) cultures resulted in the highest electroporation efficiency;[35,36] in *Bacillus amyloliquefaciens* and *B. subtilis* IH6140, the late-log phase (OD_{600} = 0.7–1.0) culture cells had the highest electroporation efficiency.[25,26] In the current study, comparison of the electroporation and lipofection revealed that the efficiency of lipofection was higher than electroporation. In addition, the incubation and recovery time of cells after transformation using the optimized electroporation method were longer than in the cationic lipid method. A probable explanation for these results may be the electrical shock of cells submitted in electroporation which cause more injury in the cell wall.

We also observed that using a high concentration of $CaCl_2$ had no significant effect on transformation efficiency of *B. abortus*. In a report by Li *et al*., transformation efficiency of *E. coli* DH5α significantly decreased when the concentration of $CaCl_2$ exceeded 200 mM.[37]

Another surprising observation was that repeated washing and addition of 0.2 M sucrose to the washing solution and recovery medium significantly enhanced the transformation efficiency in the three transformation methods. One possible explanation is that slow-growing *Brucella* cells, especially in spheroplast form, require a long time to grow

and convert to the normal, nonspheroplast form. Osmotic protection afforded by sucrose, therefore, aids this process. Two previous studies examined the effect of osmotic protection of sucrose in the culture medium and treatment solutions before transformation and evidenced increased transformation efficiency in the presence of sucrose.[24,29] The effect of sucrose in media after the transformation has not been examined. Finally, we observed that the transformation efficiency significantly decreased with increased plasmid size. A similar observation was reported by other studies.[31]

Conclusions

The current study showed that employment of optimized lipofectamine protocol using ampicillin-induced spheroplasts form of *Brucella* could be the best alternative for the electroporation method in *Brucella* transformation.

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Conflicts of interest

There are no conflicts of interest.

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