Brucella Melitensis invA Gene (BME_RS01060) Transcription Is Promoted Under Acidic Stress Conditions.

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***Brucella melitensis invA* gene (BME_RS01060) transcription is promoted under acidic stress conditions.**

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Running Title: *Brucella melitensis invA* gene transcription

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Abstract

The *invA* gene of *Brucella melitensis* codes for a NUDIX (nucleoside diphosphate linked to moiety X) hydrolase related to invasiveness. The objective of this work was to evaluate *invA* transcription under acidic conditions. The *invA* gene transcription was up regulated at pH 3 and pH 5 observed with semiquantitative real-time PCR in *B. melitensis* 133 strain. Results indicated that *invA* gene transcription at pH 3 showed a basal and decreased transcription compared to that of pH 5 incubation. Transcription levels of the *dnaK* gene were similar to those obtained with *invA* gene. The survival rates of wild type and *invA* mutant strains at pH 5 were above 90% in all post-incubation times. In contrast, at pH 3 there was a time-dependent reduction on both strains at 15 min ($P < 0.05$). These results suggest that *invA* gene transcription is promoted under acidic conditions in *Brucella melitensis*.

Keywords *Brucella melitensis*, transcription, acidic stress, NUDIX, bacterial adaptation.

Declarations

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Conflicts of interest/Competing interests.

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval

Not applicable

Consent to participate.

Not applicable

Consent for publication

Not applicable

Availability of data and material

Data on bacterial survival experiments are in the Additional File 1.xls.

Data on *invA* and *dnaK* gene transcriptions analysis are in the Additional File 2.xls

Code availability

Not applicable

Author´s contribution

RSB: carried out the experiments, interpreted and analyzed data and drafted the manuscript.

HBG: carried out the experiments, made substantial contributions to the work and revised the manuscript critically.

JMB: made substantial contributions to the work and revised the manuscript critically.

BAR: designed the experiment and revised the manuscript critically.

ABG: designed the experiment and revised the manuscript critically.

RHC: interpreted and analyzed the data and revised the manuscript critically.

JAP: funding obtaining, designed and carried out the experiments, interpreted and analyzed the data and drafted the manuscript.

All authors read and approved the final manuscript.

Introduction

Members of the genus *Brucella* are intracellular pathogens that infect animals and humans, producing reproductive disorders in domestic animals and flu-like symptoms with possible human complications (Olsen et al. 2010). In order to have a successful infection, bacterial pathogens have to overcome iron sequestration, oxidative stress, hyperosmotic conditions, among other stressors (Guan et al. 2017). Acid stress is originated as a defense mechanism against pathogens. Before reaching the intracellular niche, *Brucella* sp. encounters acid conditions in the host stomach (pH 3.0) if the infection is acquired through the oral mucosa (Sangari et al. 2007). During cell infection, *Brucella* bacteria are enclosed in a phagosome (called *Brucella* containing vacuole, BCV) that matures to phagolysosome, with a pH conditions in the range of 4.5 to 5.0 (Porte et al. 1999). Phagosome-lysosome fusion increases the vacuole proton concentration rendering low pH, thereby counteracting intracellular bacteria (Duclos and Desjardins 2000). Not only decreased pH is encountered inside of BCV, but also nutrient deprivation, oxidative environment and lytic enzymes. This harsh environment worsens until bacteria reach the endoplasmic reticulum (ER), where they became replicative. It is speculated that 90% of *Brucella* bacterial cells die in this phagosome maturation process, and only 10% of them survive to become replicative (Celli 2019). Mechanisms of low-pH resistance in *Brucella* are necessary for full virulence and replication. Different elements have been reported that are associated with the response to acid stress conditions. DnaK protein (Teixeira-Gomes et al. 2000), the response regulator OtpR (Liu et al. 2015), the glutamic acid decarboxylase system (*gad* operon) in *B. microti* (Occhialini et al. 2012), the HdeA acid-chaperone (Valderas et al. 2005) among others plays a decisive role in controlling acid stress. Of particular interest is the activation of the Type IV Secretion System (TIVSS), a principal virulence mechanism that is promoted under intracellular acidic conditions (Boschiroli et al. 2002; Celli et al. 2003; Sieira et al. 2010)

Under these considerations' possible unknown mechanisms of stress resistance or adaptation could interact for *Brucella* survival. In *Brucella melitensis* genetic sequence, a possible NUDIX hydrolase gene has been identified, named *invA* (BME_RS01060). *B. melitensis invA* mutant strain showed a lower invasion phenotype in non-professional phagocytes and attenuation in the murine model of infection (Alva-Pérez et al.

2014). In the present work, we demonstrate that the *invA* gene transcription is promoted under acidic conditions *in vitro*.

Materials and Methods

Bacterial strains

Brucella melitensis 133 (Bm 133) is a biotype 1 Mexican field strain, nalidixic acid-resistant (Hernández-Castro et al. 2000), and *B. melitensis invA*-km (Bm 133 *invA*-km) is *invA* gene mutant strain, kanamycin-resistant (Alva-Pérez et al. 2014). Both strains were maintained on Trypticase Soy Broth or Trypticase Soy Agar (TSA, BD Bacto^{TM}, Sparks Maryland, USA), at 37 ^{o}C. For mutant strain, kanamycin (200 µg/ml, Bio Basic Ontario, Canada) was added to TSB or TSA when needed. All bacterial manipulations were done on biosafety level III facilities.

Acid stress survival assay

Fresh frozen aliquots of Bm 133 and Bm 133 *invA*-km were thawed at room temperature and seeded in TSA. After 48 h of incubation at 37^{o}C, five colony forming units (CFU) were added in 100 ml of TSB and incubated in a shaker at 37^{o}C at 200 rpm for 26 h. Bacterial cell concentration (one milliliter of each bacterial strain, $10^{9.7} – 10^{10}$, late log phase) were harvested by 2, 000 x *g* centrifugation for 10 min at 4^{o}C. Bacterial cells were re-suspended in 1 ml of acidified medium (pH 5 or pH 3, TSB) and incubated 5, 10 or 15 min at room temperature (time post-incubation, i.e. pi). HCl 2N (Sigma-Aldrich, Missouri, USA) achieved the acidified medium added to the required pH. The control group was designed as 1 ml of each strain centrifuged and incubated at the same conditions, but TSB medium was at pH 7.2. After incubations in acidified medium or neutral pH medium bacterial numbers were calculated by diluting and plating on TSA. Bacterial concentration in neutral pH medium was determined as 100% per cent of survival. The experiment was carried out in triplicate, and every experiment had three replicates.

invA gene transcription under acid condition

The *invA* gene transcription assay was determined for Bm 133 incubated in acid conditions as described previously. After incubations, bacterial cells were harvested at 2, 000 x *g* for 1 min at 4ºC. RNA extraction was done with Tri Reagent (MRC Ohio, USA) following the manufacturer´s instructions. Isolated bacterial RNA was re-suspended in 30 µl of nuclease-free water (Sigma-Aldrich, Missouri, USA). DNAse I (Invitrogen California, USA) incubation was done following the manufacturer´s instructions for DNA elimination. The RNA concentration and purity were determined by spectrophotometer readings (JENWAY Genova, Staffordshire UK). Samples were kept at -20 ºC.

The *invA* transcription analysis was carried out using a semiquantitative real-time PCR (qPCR) assay. Primers and probes (Table 1) were designed for qPCR with on-line software PrimeQuest of Integrated DNA Technologies (https://www.idtdna.com/Primerquest/Home/Index). 16srRNA gene was used as a housekeeping gene and *dnaK* gene as acid stress positive control (Teixeira-Gomes et al. 2000). We use the single tube step format, where RNA is transformed into complementary DNA (cDNA), and then PCR is achieved in the reaction. Super Mix RT-PCR 2X (Biotecmol, Mexico City, Mexico) was used. The reaction was integrated of 5 µl of retrotranscription PCR buffer (2x), 0.5 µM of reverse and forward primer (1 µl each, table 1), 0.5 µM of *Taq-man* probe (1 µl, Table 1), 100 ng of RNA sample, and nuclease-free water (up to 10 µl). qPCR protocol was as follows: reverse transcription step at 42 ºC for 30 min, denaturation step at 95 ºC for 10 min, followed for 40 cycles of 95 ºC for 5 sec and 60 ºC for 30 sec. qPCR was achieved in Step One thermocycler (Applied Biosystems, California USA). *invA* gene transcription (relative expression) was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Experiments were done in triplicate and for every time evaluated, two independent experiments were done. In order to evaluate primer efficiency, different Bm 133 cDNA concentrations were tested for *dnaK* or *invA* qPCR amplifications (Livak and Schmittgen 2001). The *dnaK* gene specific primers resulted in efficient amplification and was similar to that of the amplification of endogenous gene (equation of *dnaK* gene: -3.993x + 14.556; R^2: 0.9899; equation of 16sr RNA gene: -4.0748x +12.677; R^2: 0.9897). Similarly, the *invA* gene specific primers resulted in efficient amplification and was similar to that of the amplification of endogenous gene (equation of *invA* gene: -3.4247x + 11.405; R^2: 0.9995; equation of 16sr RNA gene: -3.384x + 9.6809; R^2: 0.9993).

Table 1. Oligonucleotides used in this study

Gene	Primer	Sequence
invA	Foward	5´-GAGGGCGATGAAAGTGAAATTG-3´
	Probe	5´-ACTGCTGAGTTCGATTGCTGGGAA-3´
	Reverse	5´-CGCAACCACCTGTTCATAGA-3´
16sr RNA	Foward	5´-ACCTTCCTCTCGGCTTAT-3´
	Probe	5´-CCCTTAGAGGCCCAACTGAATGCT-3´
	Reverse	5´-GTCGTGAGATGTTGGGTTAAGT-3´
dnaK	Foward	5´-GACGTGTCGGTTCTGGAAAT-3´
	Probe	5´-TGAAGTGAAGTCCACCAATGGCGA-3´
	Reverse	5´-CTTGAACTCGGCAACCAGATA-3´

Statistical analysis

Survival bacterial results and qPCR results were statistical analyzed with two-way ANOVA and mean multiple comparisons were made with Tukey´s test. The significance level was set at $P < 0.05$ or $P < 0.001$. The GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA) was used for analysis and graphics construction.

Results

Bacterial survival rate under acid stress conditions

Survival assay results at pH 5 demonstrate no significant differences, except for mutant survival strain comparison, where Bm 133 *invA*-km survived almost 10% more at 10 min compared to that of 15 min ($P < 0.05$, Figure 1a). At 5- and 10-min pi, mutant and wild-type strains had more than 90% of survival rate, and at 15 min pi, a slight decrease in the *invA* mutant strain survival rate was observed, compared to that of wild type strain (84.6 ± 4.8 and 92.7 ± 4.6, respectively). Nevertheless, no significant difference was observed. In contrast, survival assay results at pH 3 demonstrate a reduced bacterial survival at 15 min pi (Figure 1b). Bm 133 *invA*-km survival at 15 min was less compared to that of the same strain at 5 min pi (62.9 ± 6.5 vs 93.8 ± 3.9, $P =$

0.009), in the same way, the mutant strain at 15 min pi. survived less than that of the parental strain at 10 min (62.9 ± 6.5 vs 87.4 ± 4.9, $P = 0.005$). Also, wild-type strain at 10 min survived less than that of the mutant strain at 5 min (87.4 ± 4.9 vs 93.8 ± 3.9, $P = 0.029$). Overall tendencies to better bacterial survival were observed at pH 5 than that of pH 3. However, bacterial survival did not decrease to 50% for both strains, indicating a good resistance to low pH. All the bacterial survival data are given in the Additional File 1.xls

Relative expression of *invA* gene

Relative transcription results demonstrate different patterns of *invA* mRNA concentration depending on pH and time incubation (Figure 2). Under pH 5 conditions maximum *invA* gene transcription was observed at 15 min pi and compared at 5- and 10-min pi, *invA* gene transcription was 25.4 and 15.4 times more up-regulated, respectively ($P < 0.0001$, Fig 2a). In addition, *dnaK* gene was significantly up-regulated since 10 min pi (above 2 Relative Transcription Units, RTU). In contrast, at pH 3, levels of *invA* mRNA transcripts were below 1 RTU (maximum level observed at 5 min pi, i.e. 0.79 ± 0.26, Fig 2b). Likewise, *dnaK* gene transcription was below 1 RTU and it was significantly lower than that of *invA* gene transcription at 5 min pi (0.46 ± 0.1 compared to 0.79 ± 0.26, $P < 0.05$). Levels of mRNA transcripts for both genes beyond 10 min pi were below 0.5 RTU, indicating a constant decrease in transcription. These results demonstrate that a pH 5 *invA* gene transcription is promoted and is time dependent. In contrast at pH 3 *invA* gene transcripts remained low at basal levels. All the transcription data and analysis are shown in the Additional File 2.xls

Discussion

Low pH reduced nutrient availability and oxidative environment are present during the invasion, at the initial stages of *Brucella* infection. There are multiple systems to sense and respond to environmental factors that could be detrimental to bacterial cells to survive and multiply. Acid stress is detrimental to bacterial protein structure and function, and cellular bioenergetics (Krulwich et al. 2011). Therefore, *Brucella melitensis* survival to acid stress is crucial for a successful intracellular infection (Roop et al. 2009). In the present work we demonstrated survival to two different low pH conditions. The oral route of *Brucella* infection is the most common route of infection. Live *Brucella* counteract the proteolytic and acid environment of the stomach of mammals (pH 1.0 to 3.0). Passage to this acid milieu is fast and transitional (in a calf-ligated ileal loop, *Brucella* bacteremia is detected in 30 min) (Rossetti et al. 2013). Urease activity of *Brucella* have been related as the

principal system for stomach low-pH resistance (Sangari et al. 2007). Subsequently, *Brucella* bacterial cells can disseminate systemically through transepithelial migration or invasion on M cells (Rossetti et al. 2013). Final intracellular infection in epithelial and monocyte-macrophage cells is determinant for the establishment of successful infection. In the initial steps of infection (5 to 15 minutes), traffic through endosome-phagosome with a pH to 4.0 to 5.0 determines the intracellular fate of *Brucella* (Celli 2019). *Brucella* transition to low-pH environment could be fast (no more than 30 minutes). Considering these two pH scenarios, we hypothesized that *invA* gene transcription could be necessary for the *Brucella melitensis* acid resistance mechanism. In pH 5, survival of both strains was higher up 80%, still at pH 3 bacterial survival was decreased according to time evaluated, with less survival at 15 min. No differences at the same time evaluated were observed between Bm 133 (wild-type) and Bm 133 *invA*-km (mutant) strains. Similar results were observed by Cui et al. (2013) at pH 3, where *B. melitensis* 16M strain survived less than 40%, and *hfq* mutant survived 20%. Gene transcription analysis showed that *invA* is progressively upregulated at pH 5. Conversely, at pH 3 *invA* transcription is basal; nevertheless, *invA* mRNA transcripts are present, suggesting a constitutive expression under acidic conditions. These results demonstrate that *invA* gene transcription is up-regulated under acidic stress. Other factors that are up-regulated under acidic conditions (pH 4.4) are two-component response regulator BMEI1329, *dnaK* and *dnaJ* gene (Liu et al. 2016), the two-component system OtpR (Occhialini et al 2012) and the *vjbR* gene at a pH of 5.5 (Arocena et al. 2012)

Ttranscriptomic analysis demonstrated that oxidation-reduction, catalytic, and bacterial transport processes are critical in a low pH environment in virulent *B. melitensis* (Salmon-Divon et al. 2019). Meanwhile, in *B. melitensis* Rev1 strain (vaccine strain) metabolism, DNA repair and stress adaptation (i.e., GroEL (BMEII1048) and cold shock proteins (BMEI0498 and BME_RS02600) expression) are essential processes for low pH adaptation (Salmon-Divon and Kornspan 2020). These studies demonstrate that regardless of attenuated or virulent strain studied, the bacterial metabolic process is determinant for acid stress adaptation. In this context, upregulation of the *invA* gene could promote *B. melitensis* low pH adaptation through oligophosphate nucleosides hydrolysis (catalysis of potentially toxic compounds). The amino acid analysis demonstrated that the *invA* gene of *B. melitensis* has nucleoside hydrolase characteristics. The *invA* gene preserves the NUDIX box and has 76% similarity with the *invA* gene of *Rickettsia prowazekii* and *nudH-ialB* locus of *Bartonella*

bacilliformis (Alva-Pérez et al. 2014). Further studies are required to demonstrate oligophosphate nucleoside hydrolysis by *invA* gene of *B. melitensis* and relevance.

NtrX is a response regulator protein implicated in low oxygen fitting of *B. abortus* (Carrica et al. 2013). Fernandez and coworkers, demonstrate that phosphorylation of the response regulator (RR) of the NtrX protein occurs only in the acidified medium (Fernández et al. 2018). Furthermore, the NtrY/X two-component system activates denitrification enzymes that consume protons, thereby counteracting low pH (Carrica et al. 2012). Another genetic factor that requires acidification is the *virB* operon, which encodes the TIVSS in *Brucella*, considered the principal virulence factor (Ke et al. 2015). These observations indicate that adaptation to low pH is not only necessary but also a signal for an overall adaptation to intracellular stress conditions (i.e. low oxygen tension). We hypothesize that InvA works together with other factors, as well, for adaptation. It would be of interest to specify how InvA protein contributes to the stress response network of *Brucella* and clarify the specific time InvA protein is contributing to intracellular pathogenesis. Current experiments are underway to decipher these questions.

Through this study we demonstrate that *invA* gene is up-regulated under low-pH conditions. At pH 5 *invA* gene up-regulation was observed according to time, nevertheless at pH 3 up-regulation was almost basal, probably indicating minimal *invA* gene intervention in low-pH adaptation. Acid stress adaptation for intracellular invasion in *Brucella* spp. are critical in order to have a successful infection. It has been demonstrated that an *invA* gene mutant strain was a lower invasion phenotype compared to parental strain. This observation indicates that, under *in vitro* intracellular infection, *invA* gene aids to *Brucella melitensis* invasion. In this work, we demonstrate that low-pH up-regulates *invA* transcription, probably indicating that the InvA protein is contributing to stress adaptation.

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Figures Titles and Legends

Fig 1. Bacterial survival percentage under acidic conditions of *B. melitensis* 133 and *B. melitensis invA*-km. Results are presented as the average of three independent experiments ± standard deviation. *: $p < 0.05$ **: $p < 0.001$. a) Bacterial survival at pH 5 and b) Bacterial survival at pH 3

Fig 2. Relative *invA* and *dnaK* transcription of *B. melitensis* 133 under acidic conditions. Results are presented as the average of two independent experiments ± standard deviation. RTU: Relative Transcription Units. Different letters are presented as different statistical significance between transcription analyzed. a) Transcription at pH 5 and b) Transcription at pH3

Supplementary material

Additional File 1.xls Spreadsheet of bacterial survival. It contains the three representative experiments. Data are in percentages by time, strain and pH (sheet 1: pH5, sheet 2: pH3)

Additional File 2.xls Spreadsheet of *invA* and *dnaK* transcription It contains the Ct (crossing threshold) values of the qPCR of *invA*, *dnaK* and *16srRNA* (endogenous control) genes. In addition, it contains the $2^{-\Delta\Delta Ct}$ analysis method. Results of every pH analyzed are in different sheets

Figures

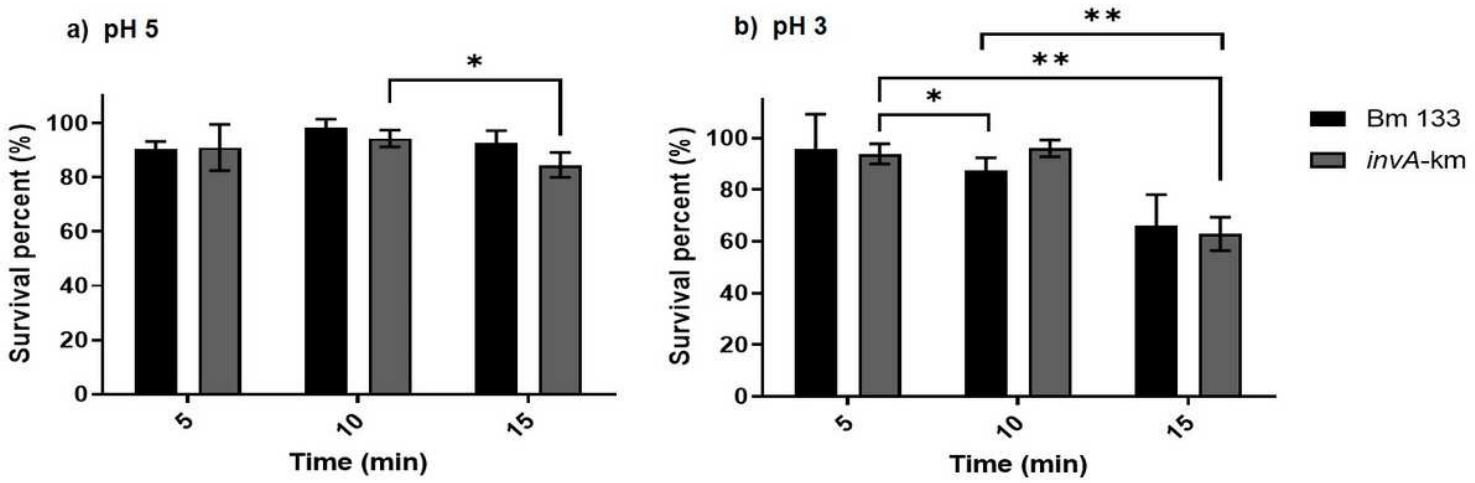

Figure 1

Bacterial survival percentage under acidic conditions of B. melitensis 133 and B. melitensis invA-km. Results are presented as the average of three independent experiments + standard deviation. *: $p < 0.05$ **: $p < 0.001$. a) Bacterial survival at pH 5 and b) Bacterial survival at pH 3

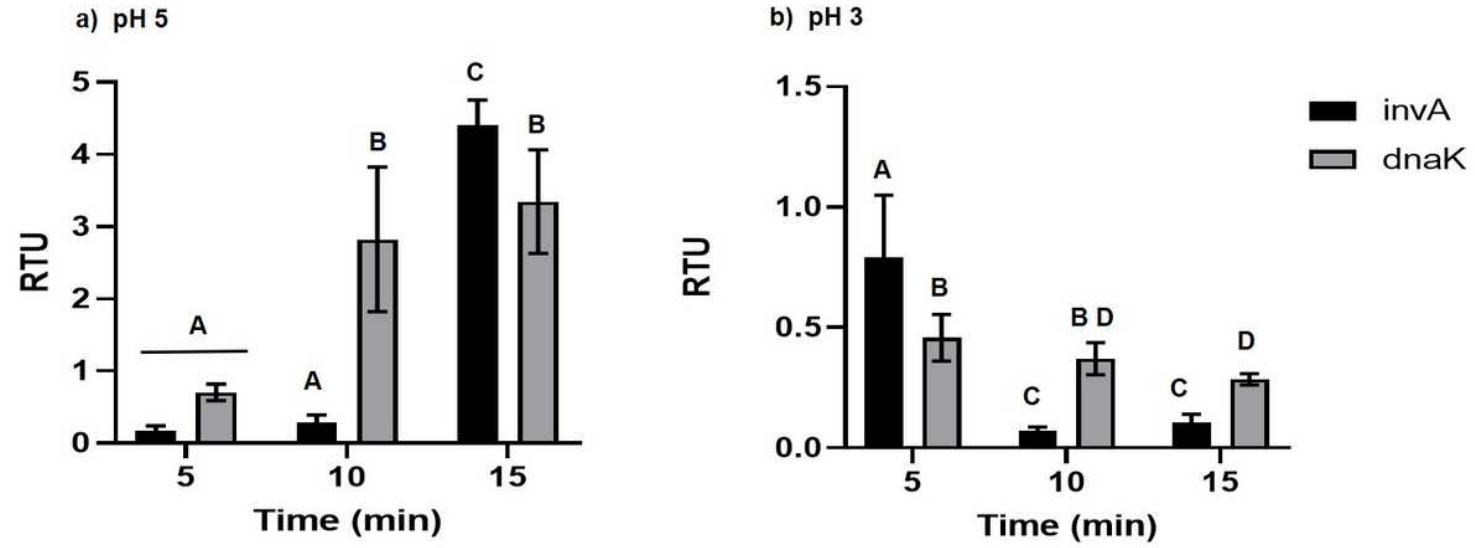

Figure 2

Relative invA and dnaK transcription of B. melitensis 133 under acidic conditions. Results are presented as the average of two independent experiments + standard deviation. RTU: Relative Transcription Units. Different letters are presented as different statistical significance between transcription analyzed. a) Transcription at pH 5 and b) Transcription at pH3

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1.xlsx
- AdditionalFile2.xlsx