ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Comparative proteome analysis of *Brucella abortus* under different growth conditions by two dimensional electrophoresis

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Manuscript Info

Manuscript History:

Received: 11 December 2014
Final Accepted: 26 January 2015
Published Online: February 2015

Key words:

Proteome; *Brucella abortus*; Two dimensional electrophoresis; Zoonosis; temperature; 2,2'-Bipyridyl.

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Abstract

Brucellosis is one of the most common zoonotic diseases in the world. Bovine brucellosis is majorly caused by *Brucella abortus* and is widespread in India and appears to be on the increase in recent times. In the present study, proteome of *B. abortus* was compared under different growth conditions i.e. temperature variations and iron limited conditions by two dimensional electrophoresis. *B. abortus* was cultured at different temperatures (30^{o}C, 37^{o}C and 42^{o}C) and iron limited condition by using different concentrations of iron chelator 2,2'-Bipyridyl (i.e. 100 µm and 200 µm) in Mueller Hinton agar. The cells were lysed in lysis buffer. Protein fraction was purified by 2DE cleanup kit (GE Healthcare, USA) and subjected to iso-electric focusing. Later, the strips were subjected to 2nd dimension SDS PAGE. The gels were stained with coomassie brilliant blue. Spots were scanned and analysed using Image master platinum 7. A total of 375, 382 and 363 protein spots were detected for *B. abortus* at 30^{0}C, 37^{0}C and 42^{0}C respectively. Under heat stress bacterial protein synthesis was altered as it synthesized 25 new proteins (30 - 70 kDa MW range) in response. A total of 380 spots were detected under normal growth conditions, while 356 and 368 spots were detected in bacteria supplemented with 100 µm and 200 µm of 2,2'-Bipyridyl containing media respectively. Around 39 were over expressed and 41 proteins were down regulated at higher iron chelator concentrations.

INTRODUCTION

Brucellosis is an important re-emerging infectious and zoonotic disease of worldwide concern. However, *Brucella* eradication has been held up since wild animals are its natural reservoirs and because brucellosis is still highly prevalent in less developed countries where livestock vaccination programmes are inadequate (1). *B. abortus* is a facultative intracellular bacterial pathogen which causes disease in both animals and human beings.

Survival within macrophage is apparently also associated with the production of various other proteins. These proteins tend to be stress - induced proteins such as heat shock proteins. They include 17, 24, 28, 60, and 62 kDa proteins. Two of them, 17 and 28 kDa proteins seem to be induced only during intracellular cohabitation of *Brucella* with in macrophages. The 24 kDa protein induce acid environment that is responsible for limiting antibiotic action and explains the discrepancy between *in vitro* and *in vivo* events (2).

Heat shock proteins are synthesized when cells are exposed to elevated temperatures or to a variety of other stresses (3). The heat shock response is highly conserved and presumably allows organisms to adapt to stressful environments. Intracellular pathogens by their very nature are exposed to a number of inhospitable environments, including extremes of pH, oxidative and nutritional stress. A stress response would presumably increase the chances of pathogen survival and dissemination.

B. abortus encounters extreme iron deprivation in their mammalian hosts. Two siderophores have been described for these bacteria, the simple catechol 2, 3-dihydroxybenzoic acid (2, 3-DHBA) and the more complex 2, 3-DHBA-based siderophore, brucebactin. The accumulation of excess intracellular iron can enhance oxidative damage to bacterial cells. Iron toxicity could be particularly problematic for the brucellae, as oxidative killing appears to be one of the primary mechanisms employed by host phagocytes to control the intracellular replication of these bacteria.

Rafie-Kolpin et al. (4) studied the *B. abortus* proteome in different *in-vitro* environmental conditions and demonstrated that different proteins were expressed when this organism was grown under acid heat stress, 35 new proteins were synthesized after exposure of *Brucella* cells to a higher temperature (42^0C). The expression of about 70 proteins increased after exposure to increased temperature. The major proteins expressed during temperature upshift in *Brucella* cells fall into groups with approximate molecular masses of about 20, 40, 60, and 70 kDa. The molecular masses of the major proteins induced in response to reduced pH were in the range of 15 to 20 and 60 kDa. Finally, expression of more than 100 proteins was found to be repressed in *B. abortus* as a response to pH 5.5.

Materials and methods

Maintenence of cultures

B. abortus was procured from Department of Veterinary Microbiology, GADVASU, India. They were maintained on Mueller Hinton Agar (Beef infusion from 300 g, casein acid hydrolysate 17.50 g, starch 1.50 g, agar 17.00 g per litre of distilled water) supplemented with 5-10% horse serum and *Brucella* selective supplement (HiMedia, Mumbai) under environment of 5-10% CO_2. The bacteria were grown at three different temperatures viz. 30^0C, 37^0C and 42^0C. They were also cultured under different iron restricted conditions viz. 100 µM and 200 µM 2,2'-Bipyridyl.

Whole protein extraction

Bacterial cells were harvested in the stationary growth phase by scraping (48–72 h) and washed with 1X phosphate buffer saline (PBS) for 2-3 times. Bacterial cells were lysed by adding lysis buffer (8 M urea, 4% w/v 3-cholamidopropyl-dimethylamminio-1-propane sulphonate (CHAPS), 2% Immobiline pH gradient (IPG) buffer (3-10 pH range), 10 mM Dithiothreitol (DTT), and cocktail protease inhibitor) and sonicated after 1 hour incubation at room temperature using microtip (Misonix, USA). The lysed cells were centrifuged at 12000 rpm for 8 min, and the supernatant containing protein was collected and purified using 2D-clean up kit (GE Health care, Sweden). Finally proteins were quantitated using nanodrop spectrophotometer (Thermo Scientific, USA).

Two-dimensional electrophoresis

First dimension - isoelectric focusing (IEF)

IEF was performed using the Ettan IPG phor Isoelectric Focusing Unit (GE Healthcare, Sweden) according to the manufacturer's recommendations. Seven centimetre immobilized pH gradient (IPG) strips (Immobiline Dry Strip, pH 3–10, GE Healthcare, Sweden) were rehydrated overnight at room temperature with the protein samples reconstituted in rehydration solution (8 M urea, 2% CHAPS, 0.2% DTT (dithiothreitol), 0.5% IPG Buffer pH 3–10, 0.002% bromphenol blue).About 200 µg of total protein in a volume of 125 µl/strip was used. To prevent dehydration, strips were overlaid with Dry strip cover fluid (GE Healthcare, Sweden). IEF was carried out at 20^0C for 8.5 h (maximum voltage of 5000 V, maximum current of 50 µA/IPG strip). Moist paper wicks were placed at each end of the strips, ensuring thorough contact between the gel and the electrodes. IPG strips were stored at -80^0C before second dimensional electrophoresis.

Second dimension - sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to SDS-PAGE, each IPG strip were washed in 2.5 ml of equilibration buffer 1 (50 mM Tris–HCl pH 8.8, 6 M urea, 1% DTT, 30% glycerol, 2% SDS, 0.002 % bromophenol blue) for 20 min followed by 2.5 ml of equilibration buffer 2 (50 mM Tris–HCl pH 8.8, 6 M urea, 2.5% IAA (iodoacetamide), 30% glycerol, 2% SDS, 0.002% bromophenol blue) for an additional 20 min. IPG strips were loaded onto 10% SDS- polyacrylamide gels and sealed with melted 1% agarose solution. Tris–glycine buffer system was used for electrophoresis (5). Electrophoresis was performed in Bio-Rad Separation Unit (Bio-Rad Laboratories, USA) at 25^0C supplying constant power in two steps: 10 mA/gel for 15 min and 20 mA/gel until the tracking dye reached the bottom of the gels (3–5 h). Gels were stained with Coomassie Brilliant Blue R-250 (CBB R-250).

Image analysis

After staining 2-D gels were documented by an Image Scanner III (GE Healthcare, Sweden). Images were processed on a PC workstation using Image Master Platinum Software (Version 7.0) (GE Healthcare, Sweden). Each sample was run in triplicates. An average gel was generated out of these gels including the protein spots presented in all of them. The synthetic average gels were compared to each other. The spots were quantified using the % of spot volume criterion, which is automatically calculated by the ImageMaster software. The match analysis

was performed in an automatic mode and further manual editing was performed to correct mismatched and unmatched spots. A criterion of $P < 0.05$ was used to define the significant difference when analyzing the paired spots between the three groups ($n = 3$) according to ANOVA.

Results and Discussion

Variation in Proteomic profile of *B. abortus* grown at different temperatures (30^0C, 37^0C and 42^0C).

Proteome maps of *B. abortus* grown at different temperatures i.e 30^0C, 37^0C and 42^0C in laboratory conditions, obtained using single type of IPG strips (7cm, pH range, 3.0 to 10.0) are presented in Fig. 1. A total of 375, 382 and 363 protein spots were detected for *B. abortus* at 30^0C, 37^0C and 42^0C respectively. An upshift of growing temperature from 37^0C to 42^0C resulted in variation of proteome profile. Under this stressful condition bacterial protein synthesis was altered as it synthesized 25 new proteins (30 - 70 kDa MW range) in response to heat stress, these may be heat shock or stress induced proteins. Heat stress also increased expression levels of 56 proteins. An appreciable reduction in synthesis of about 35 protein spots was observed in response to heat shock (Table 1a &1b).

Growth at sub-optimum temperature (30^0C) led to few variations in protein expression in *B. abortus*. At this temperature, 5 unique proteins were expressed and 28 proteins were down regulated. Previously, Rafie-kolpin et al. (4) found that 35 new proteins were synthesized in *Brucella* when the cells were exposed to a higher temperature (42^0C). The expression of about 70 proteins increased when exposed to higher temperature. The major proteins expressed during temperature upshift in *Brucella* cells fall into groups with approximate molecular weights of about 20, 40, 60, and 70 kDa. Teixeira-gomes et al. (6), studied proteome of *B.melitensis* under heat stress and revealed that 13 new proteins were synthesized in response to heat stress. They observed an appreciable reduction in synthesis of about 40 proteins in response to heat shock. About 17 of them were completely repressed. Amirmozafari et al. (7) studied comparison of heat shock response in *B.abortus* and *B. melitensis* and found that most significant protein groups of these isolates were in the range of 45-75 kDa in heat shocked bacteria at 42°C, not only a few new proteins spots appeared, but also there was a generalized increase in the level of most proteins and the amount of a 60 kDa protein (hsp 60) was significantly enhanced following heat shocks compared to controls. During host infection these heat shock proteins are required for intracellular survival of the bacteria (8). They are immunogenic, eliciting both cellular and humoral immune responses in infected hosts (9). Thus, analysis of *B. abortus* HSPs is worthwhile to explore their immunogenicity and their suitability as components of subunit vaccines (10).

Comparison of proteome profile of *B. abortus* under iron restricted conditions.

Proteome maps of *B. abortus* grown under normal and iron restricted conditions by using an iron chelator (2,2'-Bipyridyl) at different concentrations i.e. 100 µm and 200 µm in media are presented in Fig. 2. A total of 380 spots were detected under normal growth conditions, while 356 and 368 spots were detected in bacteria supplemented with 100 µm and 200 µm of 2,2'-Bipyridyl containing media respectively. The expression levels of proteins in the bacteria altered as the chelator concentration increased. This finding could be attributed to the fact that iron availability deceases in media as the concentration of the chelator increases which in turn effects protein expression in the bacteria. Whether this variation is a consequence of transcriptional, post-transcriptional, translational, or post-translational modifications cannot be stated. Protein expression levels may reflect differences in metabolic properties that may have resulted due to growth conditions. Around 17 proteins were uniquely expressed under iron limited condition. Out of 368 proteins, 39 were over expressed and 41 proteins were under expressed at higher iron restricted condition (Table 2a &2b).Eschenbrenner et al. (11) compared the proteomes of *B. melitensis* vaccine strain Rev 1 and a virulent strain, 16M. This vaccine strain was developed by attenuation of virulent strain by repeated passages on iron limited media, so the proteome profile of this vaccine strain resembles bacteria grown on iron limited conditions. About 9 proteins were uniquely expressed, around 45 proteins were under expressed and 40 proteins were over expressed in Rev I compared with virulent strain 16M.

Iron plays a salient role in the survival of pathogens once inside host cells (12). This element is essential in the synthesis of iron- and heme-containing enzymes. During infection, macrophages reduce iron availability by producing chelating agents and actively exporting iron from the phagosome, where the pathogen multiplies (13). In response, the pathogen synthesizes a battery of proteins designed to compete with the host for iron, such as bacterioferritin, outer membrane protein and an iron (III) binding periplasmic protein (11).

This laboratory induced stress condition resembles natural *in vivo* condition during macrophage infection in the host. Many bacterial stress proteins are targets of the host immune response in a broad spectrum of infections (9, 14 & 15). It has been reported that their immuno-dominance is probably related to their abundance within antigen-presenting cells under conditions of stress (16).

Figures

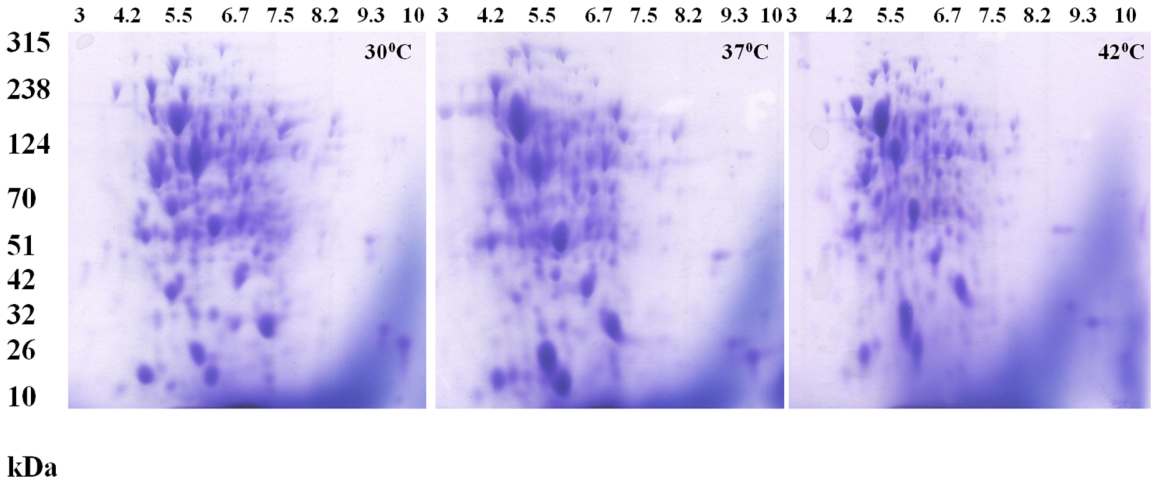

Fig. 1: Two dimensional electrophoresis gels showing proteomes of *B. abortus* grown at different temperatures i.e. 30^0C, 37^0C and 42^0C

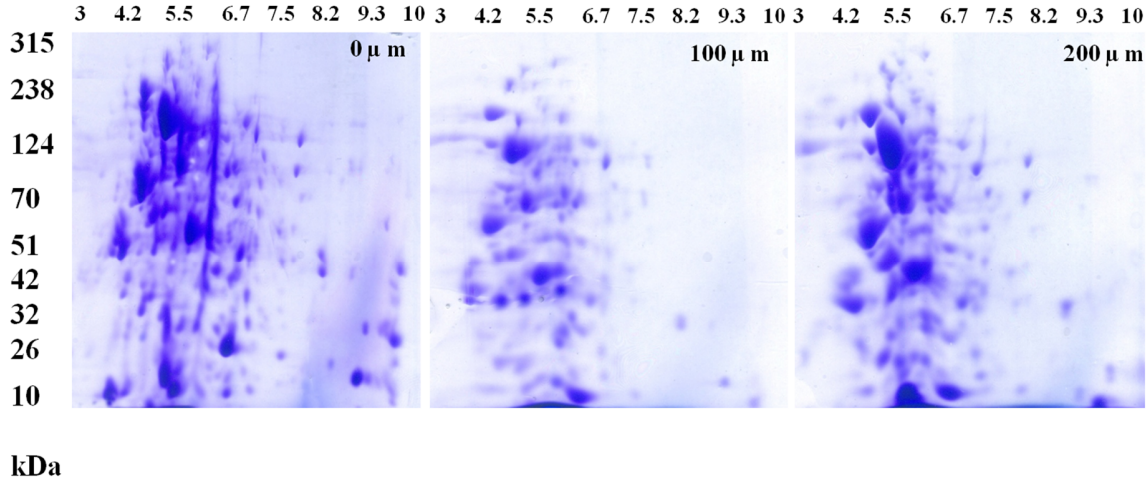

Fig. 2: Two Dimensional Electrophoresis gels showing proteomes of *B. abortus* grown under normal and iron restricted conditions using 100 µM and 200 µM 2,2'-Bipyridyl

Table 1a: Proteins present in 2-D gels of *B. abortus* grown at different temperatures (30^0 C, 37^0 C & 42^0 C)

Temperature	Total no. of protein spots	Uniquely expressed proteins
30^0C	375	05
37^0C	382	--
42^0C	363	25

Table 1b: Table showing expression levels of proteins at different temperatures (30^0C & 42^0C) as compared to normal temperature (37^0C)

Temperature	Over expressed	Under expressed
30^0C	03	13
42^0C	56	21

Table 2a: Proteins present in 2-D gels of *B. abortus* grown under normal and iron limited conditions

Iron chelator concentration	Total no. of protein spots	Uniquely expressed proeins
Normal	380	--
100 µM	356	06
200 µM	368	11

Table 2b: Expression levels of proteins under iron limited conditions

Iron chelator concentration	Over expressed	Under expressed
100 µM	21	30
200 µM	39	41

Conclusion:

Heat shock and iron chelation led to alteration of protein expression profile in *Brucella abortus*.

Acknowledgement:

The authors are thankful to the Director, School of animal biotechnology and DBT-HRD for providing necessary facilities tocarry out this work.None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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