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Two dimensional gel electrophoresis (2-DE) for high-throughput proteome analyses of *Mycoplasma bovis*

Farhan Anwar Khan[1,2,3]✉, Xi Chen[2,3], Muhammad Shoaib[1], Muqader Shah[1], Faisal Ahmad[1], Hayatullah Khan[1], Faiz ur Rehman[1], Muhammad Saeed[1], Ijaz Ahmad[5], Irshad Ahmad[4] and Aizhen Guo[2,3]✉

[1]Department of Animal Health, The University of Agriculture Peshawar, Khyber Pakhtunkhwa, 25120, Pakistan; [2]The State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China; [3]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China; [4]Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan; [5]Department of Livestock Management, Breeding and Genetics, The University of Agriculture Peshawar, Khyber Pakhtunkhwa, 25120, Pakistan

Expression proteomics approaches do not only directly confirm protein coding genes of sequenced genomes but also facilitate resolution of minute qualitative protein differences and improve the quality of genome annotation. Despite development of many tools, use of 2-DE coupled with MS in proteomics is not uncommon. With an accelerated trend of genome sequencing of microorganisms, proteome analysis of animal pathogens with 2-DE has gained more attention in the last decade. Therefore, in this study primarily the protein extraction, sample preparation and loading, IPG strip rehydration, IEF, and SDS-PAGE conditions were improved for high throughput resolution and reproducible 2-DE map of proteins of *Mycoplasma bovis* HB0801 (*M. bovis* HB0801-Chinese Strain), a pneumonia pathogen in feedlot cattle, and its attenuated strains. Literally, higher amount of proteins was extracted exploiting the French pressure cell coupled with TCA precipitation when compared to the sonication method. Total protein concentration was determined using a 2D quant Kit. About 330–380 µg TCA treated protein sample, solubilized in calibrated rehydration solution, loaded on 17 cm IPG gel strip (pH 3–10 NL) followed by active rehydration at 50V and isoelectric focusing at final 10 000 Volt (33 uA/gel strip) for 80kVh had revealed well resolved proteins spots on 10% gel stained by CBB R250 (0.15%), representing 83–89% of the total protein coding genes of *M. bovis* HB0801, estimated by PD Quest (Bio-Rad, USA). Conclusively, this effort attempted to provide more precise 2-DE platform and suitable conditions, after extensive calibration, for future comprehensive proteome and immunoproteome analyses and future research on the elucidation of factors related to pathogenesis of *M. bovis* in cattle.

Key words: cattle; *M. bovis* HB0801; mass spectrometry; 2-DE; proteomics

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✉e-mail: farhan82@aup.edu.pk (FAK); aizhen@mail.hzau.edu.cn (AG)

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Abbreviations: 2-DE, two dimensional gel electrophoresis; CBB, Coomassie Brilliant Blue; ELISA, enzyme linked immune-sorbent assay; iELISA, Indirect Enzyme linked immune-sorbent assay; IPG, Immobilized pH gradient; IEF, Isoelectric focusing; MS, mass spectrometry; *M. bovis* HB0801-*Mycoplasma bovis* HB0801; PPLO, Pleuropneumonia like organism; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; VSP, variable surface proteins

INTRODUCTION

Mycoplasma bovis (*M. bovis*), a causative agent of mastitis, was documented as a pathogen of cattle pneumonia in 1976 (Caswell & Archambault, 2007). It is still a serious threat to the beef and dairy industry, due to its evasion from the host immunity, variability in the surface antigen, resistance to antibiotics (Robino *et al.,* 2005) and invasion of peripheral-blood mononuclear cells and erythrocytes (Van der Merwe *et al.,* 2010).

Although *M. bovis* has been discovered nearly six decades ago, its pathogenesis remains obscure. Accessible information regarding its proteome is very scant for inferring its pathogenesis. Certain proteins, such as p48 (Sachse *et al.,* 1993), pMB67 (Behrens *et al.,* 1996), variable surface proteins (VSPs) (Scherm *et al.,* 2002), p26 (Thomas *et al.,* 2003), GAPDH (Prysliak *et al.,* 2013), TrmFO (Guo *et al.,* 2017), NADH oxidase (Gang *et al.,* 2017) and P27 (Xi *et al.,* 2018) have been reported to be involved in its adhesion to the host cells and are immunogenic. However, the immune response to these antigens proved to be non-protective. Fluctuations in the expression, conformation, and antigenicity of the VSPs (Nicholas & Ayling, 2003) and pMB67 proteins might be one way in which *M. bovis* continuously evades the host immune response (Caswell & Archambault, 2007).

Comprehensive investigation of pathogenesis of *M. bovis* was sought and began in 2008 in China with the identification and isolation of *M. bovis* strain HB0801 from calf lung lesions in the Hubei province (Lei *et al.,* 2008). Consequently, complete genome sequencing of the HB0801 strain and its comparison with the genomes of other strains and *Mycoplasmas* species laid down a foundation for the clarification of pathogenesis of *M. bovis* (Qi *et al.,* 2012; Rasheed *et al.,* 2017).

Full genome sequence of the HB0801 strain basically paved the way for proteomics in order to analyse protein coding genes for understanding of the biological value of the sequenced genome. Proteomics based on MS represent a potentially important tool for incorporating protein-level information into the genome

annotation process. Combination of 2-DE with MS is currently the workhorse for proteomics (Jungblut *et al.,* 2008; Khan *et al.,* 2017), and particularly for immunoproteomics (Thomas *et al.,* 2005; Zhang *et al.,* 2008; Jores *et al.,* 2009; Sun *et al.,* 2012; Zhao *et al.,* 2012; Khan *et al.,* 2016; Khan *et al.,* 2017).

M. bovis proteins were usually analyzed employing conventional SDS-PAGE (1D), ELISA and immunoblotting. Utilization of 2-DE for the first time in the comparative proteomic analysis with detergent phase proteins of virulent and non-virulent strains of *M. bovis* identified a novel 24 kDa membrane associated protein (Thomas *et al.,* 2005). More recently, 2-DE and immunoblotting were performed with whole-cell proteins of *M. bovis* PD revealing 19 immunogenic proteins. Moreover, an iELISA was established for diagnosis exploiting the E1 beta subunit of the pyruvate dehydrogenase complex (Sun *et al.,* 2014). Although infrequent, successful application of 2-DE revealed important proteins of *M. bovis* as mentioned. These findings provide an insight for further improvement and application of 2-DE for comprehensive analyses of total proteins, membrane proteins, and immunogenic proteins of *M. bovis*.

The study presented here had efficiently calibrated each technical link of 2-DE for the analyses of total proteome, and more importantly immunoproteome of *M. bovis*. The 2-DE conditions standardized in this study would be likely very helpful in the future research on the elucidation of important factors of *M. bovis* related to its virulence in cattle.

MATERIALS AND METHODS

Strain and culture conditions

M. bovis strain HB0801 (CCTCC # M2010040), isolated from pneumonia calf lung lesions (Lei *et al.,* 2008), was utilized in the subsequent analysis. This strain was grown in a stationary CO_2 incubator as reported previously (Han *et al.,* 2015), with minor modification, at 37°C in the presence of 5% CO_2 in PPLO (Pleuropneumonia like organism) broth for 36 hrs, supplemented with 0.1% (w/v) sodium pyruvate and 0.5% (w/v) yeast extract, 0.001% (w/v) phenol red (pH indicator of the medium, color of PPLO broth changes with the growth of *M. bovis* due to acidic pH), 20% (v/v) heat inactivated (56°C, 30 min) donor equine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 40 000 U of penicillin G/100ml. Stock of this isolate was prepared by freezing broth culture in aliquots (1 ml and 5 ml) with 15% (v/v) glycerol as preservative at –80°C. For further analysis, the frozen stock was thawed and inoculated at a ratio of 1:100 in PPLO broth, in triplicate.

Confirmation of *M. bovis* HB0801

The bacterial strain used was confirmed to be *M. bovis* HB0801 by physical examination of biofilm formation over the PPLO broth (characteristic of *M. bovis* growth) and by PCR after 36 hrs of incubation in a log phase with 10^9 CFU, as described elsewhere (Qi *et al.,* 2012). Briefly, two sets of primers were used for targeting the *uvrC* gene and Mbov-0732 gene for confirmation and differentiation from attenuated strains of HB0801. PCR was conducted using 2 μL of genomic DNA as a template in a 25 μL reaction mixture with 1 U of LA Taq DNA polymerase (TaKaRa, Tokyo, Japan) in 1X buffer supplied by the manufacturer, 200 μM dNTPs and 50 pmol of each primer. The amplification was programmed over 30 cycles, each consisting of 35 s at 95ºC, 40 s at 55ºC, and 42s at 72ºC. Initial denaturation step was programmed at 95ºC for 4 min, and final extension at 72ºC for 5 min.

Bacterial cell lysis for the extraction of proteins

Two methods, i.e. sonication and high pressure homogenization, commonly used for bacterial cell lysis and extraction of whole cell proteins were employed and their extraction efficiency and quality was compared.

Sonication. Total proteins of HB0801 were extracted after 36 hrs of incubation in log phase with 10^9 CFU as reported previously (Jungblut *et al*., 2008), with slight alteration. Briefly, PPLO broth cultured cells were centrifuged at 15400 × *g*, for 20 min, at 4°C. Cell pellets (100–200 mg), in triplicate, were washed three times with cold PBS (150 mM NaCl, 1.5 mM KH_2PO_4, 9 mM Na_2HPO_4–$12H_2O$, 2.5 mM KCl, pH 7.4) to remove interfering substances and then resuspended in the lysis buffer (Table 1) containing a protease inhibitor (Sigma-Aldrich, USA). *M. bovis* cells were lysed by sonication with a 3 mm diameter probe, at 60–80% amplitude, 60% cycle duty (0.6 sec pulse rate) for 30 min at 4°C in ice-water, incubated for 3 h at room temperature and centrifuged 30 min at 3000 × *g* to remove debris and unlysed cells. Protein concentration was estimated with 2D Quant Kit (GE healthcare, Fisher Scientific, Thermo Fisher Scientific, USA). Solubilized proteins were immediately used or stored in aliquots at –80°C until further use.

High pressure homogenization

Slightly modified high pressure lysis conditions were used as reported previously (Parraga-nino *et al*., 2012; Zhang *et al.,* 2008). In brief, cells grown in PPLO broth

Table 1. list of evaluated lysis buffers

No.	Lysis buffers	Reference
1	7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer 4–7, and 65 mM DTT	Zhang *et al.,* 2008
2	30 mM Tris–HCl (pH 8.0), 7 M urea (Roth, Karlsruhe, Germany), 2 M Thiourea (Sigma), and 4% (w/v) CHAPS (Roth)	Jores *et al.,* 2009
3	7 M Urea, 2 M Thiourea, 4% CHAPS, 2% IPG Buffer, 40 mM DTT, 20 mM Tris-cl (pH 7.5)	Chen *et al.,* 2012
4	8 M urea, 2 M thiourea, 2.5% CHAPS, 2% ASB-14, 60 mM DTT, 40 mM Tris–HCl (pH 8.8) and protease inhibitor cocktail	Parraga-Nino *et al.,* 2012
5	7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 1% (v/v) cocktail, 0.5% (v/v) IPG buffer and 40 mM Tris-base (pH 9.6)	Sun *et al.*, 2014
6	8 M urea (Sigma), 2 M thiourea (Sigma), 4 % CHAPS (Sigma), 2% ASB-14 (Sigma), 60 mM DTT (Sigma), 40 mM Tris–HCl (pH 8.8) (Sigma) and protease inhibitor cocktail (Roche)	Optimized in this study

Table 2. List of evaluated rehydration solutions

No.	Rehydration solutions	Reference
1	7 M Urea, 2 M Thiourea, 2% w/v CHAPS, 1% w/v DTT, 0.5% v/v IPG buffer (pH 4–7), 0.002% w/v bromophenol blue	Zhang *et al*., 2008
2	30 mM Tris–HCl (pH 8.0), 7 M urea (Roth, Karlsruhe, Germany), 2 M Thiourea (Sigma), and 4% (w/v) CHAPS (Roth)	Jores *et al*., 2009
3	7 M Urea, 2 M Thiourea, 2% CHAPS, 0.002% bromophenol blue, 0.5% IPG Buffer, 40 mM DTT	Wei *et al*., 2012
4	7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 2% ASB-14 w/v, 0.5% pharmalytes (pH 3–10), 100 mM DeStreak reagent	Parraganino *et al*., 2012
5	6 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.5% (v/v) IPG, 0.04% (w/v) bromophenol blue and 40 mM Tris-base (pH 9.6)	Sun *et al*., 2014
6	7 M Urea (Sigma), 2M Thiourea (Sigma), 2.5% w/v CHAPS (Sigma), 2% ASB-14 w/v (Sigma) , 40 mM Tris–HCl (pH 8.8) (Sigma), 65 mM DTT (Sigma), 0.5% IPG buffer (pH 3–10) (Biorad)	Optimized in this study

were centrifuged at 15400×*g*, for 20 min, at 4°C. Pellet (100–200 mg), in triplicate, was washed with cold PBS (1X) three times. Cells were resuspended in 30 ml PBS (1X) containing a protease inhibitor (Sigma-Aldrich, USA) and lysed by three passages through a French pressure cell (Thermo, USA) at 20000 lbf/in. (pound-force per square inch); unlysed cells were removed by centrifugation at 15400×*g*, for 20 min, at 4°C. Supernatant was collected in another tube and treated with 15% TCA (Sigma). After 1h incubation at –20°C, the sample was centrifuged at 15400×*g*, for 20 min, at 4°C. Protein pellet was then washed three times with ice-cold acetone. After drying, protein pellet was resuspended in the lysis buffer (Table 2) and incubated for 3h at room temperature. Protein concentration was estimated with 2D Quant Kit (GE healthcare, Fisher Scientific, Thermo Fisher Scientific, USA).

Two dimensional gel electrophoresis (2-DE)

Various technical steps of 2-DE given below were compared and analyzed.

Immobilized pH gradient (IPG) gel strips. IPG gel strips (Bio-Rad, USA) of various pH ranges and lengths (Table 3), in triplicate, were thoroughly and separately evaluated in this study to generate a high-throughput resolution and reproducible proteome map of *M. bovis* HB0801.

Rehydration of IPG strips. To figure out promising rehydration conditions for IPG strips, each strip (in triplicate) of different length (7 cm/17 cm) and pH range (pH 3–10 NL/pH 4–7) was rehydrated passively (Sun *et al.,* 2014) for 1 h without overlaying mineral oil (Bio-Rad IPG strip manual). This was followed by active rehydration (50 V) of each 7 cm strip for 12 h and 17 cm for 16 h in IEF tray (Bio-Rad) at 20°C, layered with mineral oil (Parraga-Nino *et al*., 2012). Moreover, the effect of passive rehydration in disposable rehydration tray for 12 h (7 cm strips) and 16 h (17 cm strips) was also examined. For re-swelling of dry IPG strips, the rehydration solutions, as described elsewhere (Thomas *et al.,* 2005; Zhang *et al.,* 2008; Jores *et al.,* 2009; Sun *et al.,* 2012; Zhao *et al.,* 2012) were compared, evaluated, and standardized (Table 3).

Table 3. Various Immobilized pH gradient strips

IPG strip (cm)	pH range				
7	3–10 L*	3–10 NL**	4–7	5–8	–
17	3–10 L	3–10 NL	4–7	5–8	7–10

*Linear, **Non linear

First dimension – iso-electric focusing (1D-IEF). Using the Protean IEF cell (Bio-Rad), IEF was carried out on 7 cm IPG strips (Table 1) according to a program reported elsewhere (Zhang *et al.,* 2008) under slightly modified conditions, i.e. 200 V for 30 min, 300 V for 30 min, 1000 V for 3 h, and 4000 V for 10000 vhr. Whereas the IEF program, as described previously (Sun *et al.,* 2014), for 17 cm was modified as followed: 150 V for 3 h, 300 V for 3 h, 1000 V for 6 h, 10000 V for 3 h, and 10000 V for 60000 vhr. IEF on each strip (7 cm/17 cm) was performed in triplicate.

Equilibration of IPG strips. IPG gel strips were equilibrated as described previously (Zhang *et al.,* 2008), with modification. In brief, reduction of focused proteins on each strip (7 cm/17 cm) was carried out in (5 ml/10 ml) reducing solution (6 M urea, Sigma, 50 mM Tris-HCl, pH 8.8, Sigma, 30%, v/v glycerol, Sigma, 2% w/v SDS, Sigma, 2% dithiothreitol, Sigma), twice for 10 min each in a glass test tube, followed by alkylation in another solution (6 M urea, Sigma, 50 mM Tris–HCl, pH 8.8, Sigma, 30%, v/v glycerol, Sigma, 2%, w/v SDS, Sigma, 4% iodoacetamide, Sigma), twice on a rocking platform. Equilibration of IPG strips was carried out in triplicate.

Second dimension SDS-PAGE (2D-SDS PAGE). The SDS-PAGE was carried out under modified conditions, as previously reported (Jores *et al.,* 2009; Sun *et al.,* 2012; Zhao *et al.,* 2012). Briefly, IPG strips (7 cm, 17 cm) were subjected to electrophoresis after equilibration, by laying on acrylamide gels with various percentages (8%, 10%, and 12%), cast in glass plates (7×8.5 cm, 18.5×19 cm). Strips (17 cm) of different pH ranges (Table 1) were run, each in triplicate, on 8%, 10%, and 12% gels at 12°C at 50 V for 3 h followed by 100 V for 12, 15, and 18 h, respectively, in Protean II xi Multi-Cell with 2-D conversion kit (Bio-Rad), and evaluated for standardized conditions. Similarly, IPG strips (7 cm) were run at room temperature at 50 V for 2 h in a Mini Protean Tetra Gel apparatus (Bio-Rad).

Gel staining. All 2D gels of each strip (Table 1) were stained with 0.15% CBB R-250 for about 7 h with freshly prepared stain and overnight with a used stain. Furthermore, 2-DE gels were twice de-stained with de-staining solution (40% ethanol, 10% acetic acid) for 30 min each, as previously reported (Sun *et al.,* 2014). Then, the gels were washed three times with distilled water and

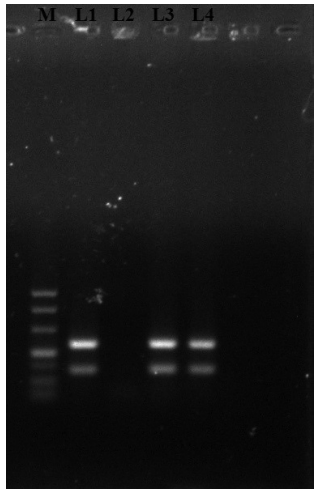

Figure 1. *M. bovis* HB0801 cultured on both, solid and liquid media, were subjected to PCR for confirmation and differentiation from its attenuated strains respectively.
Obtaining the 238 bp (*uvrC* gene) and 146 bp (absent in attenuated strains) bands confirmed *M. bovis* HB0801. Lane 1, positive control; Lane 2, Negative control; Lane 3, Agar grown *M. bovis*; Lane 4, Broth grown *M. bovis*

scanned by GS-800 Calibrated Densitometer (Bio-Rad) and analyzed by PD Quest Basic 8.0 program (Bio-Rad). Gel staining was performed in triplicate for each strip.

RESULTS

Confirmation of Strain

The strain under investigation was confirmed to be *M. bovis* strain both, physically by biofilm formation and color change of broth, and by molecular methods by obtaining a 238 bp amplicon of the targeted *uvrC* gene and 146 bp of Mbov-0732 gene with PCR (Fig. 1). Presence of Mbov-0732 gene differentiated the virulent HB0801 from its attenuated strains (not shown).

Total protein concentration of *M. bovis* HB0801

Two methods widely used for the extraction of total bacterial proteins (sonication and high pressure lysis) were compared for the whole cell protein extract of *M. bovis* HB0801 and were subsequently evaluated. Sonication at 4°C extracted a measurable protein concentration of about 6.2 mg/ml, whereas a higher protein concentration (8.3 mg/ml) with high purity was successfully acquired with French Pressure Cell coupled with TCA precipitation and acetone washing. Moreover, quality of the resultant 2-DE map of protein sample extracted by sonication was found to be lower than that of the high pressure lysis and TCA treated sample (Fig. 2 and 3).

IPG Strips, Sample loading and rehydration

Various IPG strips (Table 1) and different rehydration solutions (Table 3) were analyzed in a quest of finding a relatively reasonable strip's length and suitable rehydration solutions for the whole cell proteins of *M. bovis* HB0801. In this analysis, 17 cm IPG strip having nonlinear pH 3–10 (Fig. 2) was found to provide a relatively better separation distance and pH range for resolution than 7 cm (Fig. 4) and 17 cm (pH 3–10 linear, 4–7, and 5–8) strips (Fig. 5). Active rehydration, when compared to passive rehydration, at 50 V for 16 h with protein sample (330–380 µg) in a gel rehydration solution (7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 2% ASB-14 w/v, 40 mM Tris-HCl, pH 8.8, 65 mM DTT, 0.5% IPG buffer, pH 3–10) was found to generate a relatively better 2D image than previously reported (Thomas *et al.,* 2005; Chen *et al.,* 2012; Sun *et al.,* 2014).

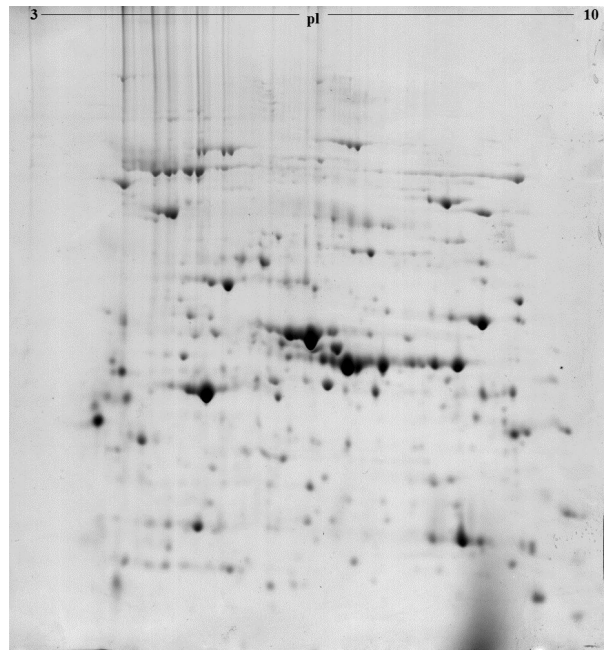

Figure 2. Two-dimensional gel electrophoresis (2-DE) of the whole-cell proteins of *M. bovis* HB0801 extracted by a high pressure homogenizer and precipitated with 15% TCA.
First, 330–380 µg of proteins were separated by IEF using a 17 cm, pH 3–10 NL IPG strip, followed by SDS-PAGE on 10% gels and stained with fresh CBB R-250. Protein samples of different batches were run in triplicates. High resolution and reproducible 2-DE image was obtained with the pH 3–10 NL strip. pI values are shown on top.

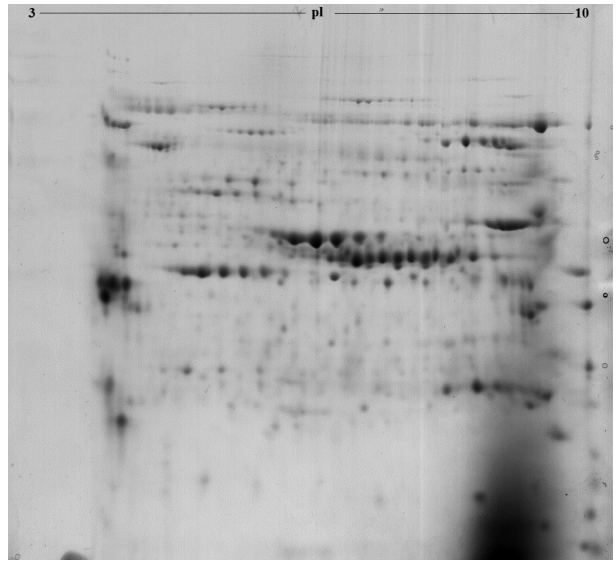

Figure 3. 2-DE map of *M. bovis* HB0801 proteins extracted by sonication.
First, 330–380 µg of proteins were separated on 17 cm, pH 3–10 NL IPG strip, and 10% SDS-PAGE, and stained with CBB R-250. Protein samples of different batches were run in replicates.

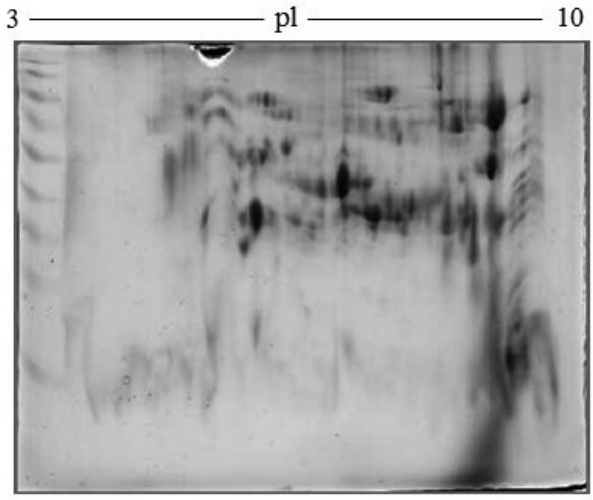

Figure 4. 2-DE map of *M. bovis* HB0801 proteins extracted by a high pressure homogenizer and precipitated with 15% TCA. Proteins were separated on a 7 cm, pH 3–10 NL IPG strip and 10% SDS-PAGE, and stained with CBB R-250. Protein samples of different batches were run in triplicates. Quality 2-DE image was obtained, but certain proteins at the basic end were missing as compared to Fig. 1. pI values are shown on top.

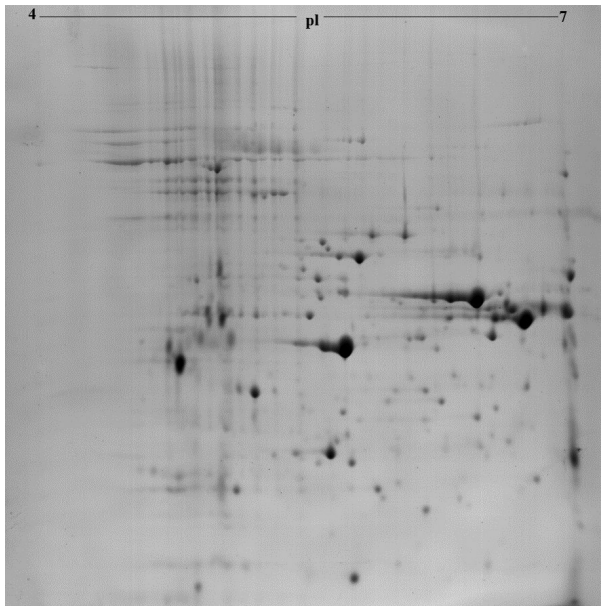

Figure 5. 2-DE map of *M. bovis* HB0801 proteins extracted by a high pressure homogenizer and precipitated with 15% TCA. Proteins were separated on 17 cm, pH 4–7 IPG strip and 10% SDS-PAGE, and stained with CBB R-250. Protein samples of different batches were run in triplicates. Quality 2-DE image was obtained, but certain proteins at the basic end were missing as compared to Fig. 1. pI values are shown on top.

IEF, Equilibration, and SDS PAGE

Two-dimensional electrophoresis was carried out analyzing different conditions. After extensive assessments, electrophoresis with IEF of proteins on 17 cm IPG strip compared to 7 cm IPG strips, gel side down, pH 3–10 NL using program: 150 V for 3 h, 300 V for 3 h, 1000 V for 6 h, 10 000 V for 3 h, and 10000 V for 60 000 vh, followed by equilibration as mentioned above were determined as good conditions, when compared to those reported elsewhere (Chen *et al.,* 2012; Sun *et al.,* 2014).

Gel running conditions, staining, and detection of protein spots

Focused IPG strip (17 cm), in triplicate, run simultaneously on freshly prepared gel (10%) for 3 h at 50 V and at 100 V for 15 h, in the same fresh electrode buffer and stained with fresh CBB-R250, were found to generate relatively better 2-DE images than 8% and 12% gels ran for 12 h and 18 h respectively. About 639–680 highly resolved protein spots were detected (Fig. 2), representing 83.85–89.23% of the total coding sequences of *M. bovis* HB0801. This 2DE process was found to be reproducible by running different batches of protein samples in triplicate.

DISCUSSION

M. bovis is one of the leading threats to the dairy and beef industry. It has been identified nearly six decades ago. However, its presence in China has been reported since 2008. Availability of very little information regarding virulence factors and pathogenesis makes its control very challenging. Recent complete genome sequences of *M. bovis* typed strain PG45 (Wise *et al.*, 2011), *M. bovis* Hubei-1 (Li *et al.,* 2011) and *M. bovis* HB0801 (Sun *et al.,* 2012) paved the way for elucidation of its pathogenesis exploiting proteomics approaches. Comprehensive proteomics approaches enable us to understand vital processes of any organism (Le Grand *et al.,* 1996). Proteomic studies are developing quickly and use of 2-DE is not uncommon for proteome analysis. Hence, obtaining high resolution 2DE maps with good reproducibility is the bottleneck of proteomics. Although the advent of IPG gel strips has enhanced the reproducibility of 2-DE results (Gorg *et al.,* 2007), there are several other factors which have to be streamlined in ordered to establish a 2DE for each particular subject, as documented previously (Ansong *et al.,* 2008). Furthermore, 2-DE with high resolution power has an ability to separate proteins with distinct post-translational modifications, which makes it an invaluable method for proteomic analyses.

Existences of significant evolutionary changes in the molecular machinery of *M. bovis* (Qi *et al.,* 2012) urge optimization of 2-DE for the in-depth exploration of common virulence factors in various strains of *M. bovis*. This may lead to the development of potentially more comprehensive control strategy. Although 2-DE used over the past decade for the proteome and immunoproteome analysis of *M. bovis* (Thomas *et al.,* 2005; Chen *et al.*, 2012; Sun *et al.,* 2014), the study presented here is presenting more efficient 2-DE conditions for the detail proteome analysis of *M. bovis* strain HB0801, after extensive calibration of each technical step of 2-DE.

Extraction and preparation of the protein sample are the benchmarks in the 2-DE. Their quality may directly affect the process of 2-DE and determine the accuracy of final results. An appropriate method and buffer have to be utilized to delicately remove and solubilize proteins with minimal losses. In this study, the HB0801 strain cells were lysed by sonication and high pressure homogenization. The same lysis buffer as described previously (Parraga-Nino *et al.,* 2012) was used for solubilization of proteins extracted with both methods. However, 2-DE images obtained with French pressure cell and TCA treated sample were of good quality (Fig. 2), which might be due to TCA, as described previously (Gorg *et al.,* 2007), which is effective in the removal of interfering substances (salts, nucleic acids, fats, and carbohydrates)

and eventually prevents vertical and horizontal streaking in 2-DE gels.

Reproducibility of 2-DE map has increased enormously with the advent of immobilized pH gradient strips (Gorg *et al.,* 2007). Still, certain 2-DE conditions still need extensive work to optimize for specific protein sample. Therefore, various IPG strips were evaluated in this project. Strip (17 cm) of pH ranging from 3–10 NL was proved to provide good separation distance for the proteins of HB0801. Additionally, narrow pH range strips (Table 1) were also evaluated, as described elsewhere (Gorg *et al.,* 2007), since they provide a large separation distance to proteins. Although 17 cm strips of pH ranges 4–7 and 5–8 (Fig. 4), were found good in resolution of respective proteins but some basic proteins were found missing in the gel strip of pH 4–7 when compared to pH 3–10 NL (Fig. 6). Moreover, 7 cm strips (pH 3–10 NL, 4–7, 5–8) were found not to be feasible in resolving *M. bovis* proteins, which might be due to their short length.

Appropriate rehydration solution is also very important for re-swelling of a dry IPG strip to their inherent size of 0.5 mm, according to manufacturer's instructions (Bio-Rad). Therefore, rehydration was performed both, actively at 50 V and passively for 16 h, respectively, as described elsewhere (Gorg *et al.,* 2007). Active rehydration with optimized rehydration solution (Table 3) excluding destreak reagents (Parraga-Nino *et al.,* 2012), and addition of 65 mM DTT (Li *et al.*, 2009), was observed to work well for resolution. DTT is known to be a good reducing agent, and in combination with other reagents in a given buffer it helps in solubilization of proteins by reducing the disulfide bonds of proteins. Furthermore, protein loading concentration also affects the quality of subsequent 2-DE map (Biorad Bulletin 3110). In this study, various protein loading concentrations were analyzed. Proteins concentration range of 330–380 µg for 17 cm strip, as reported (Li *et al.,* 2009), was found to be more promising than 1–4 mg reported elsewhere (Zhao *et al.,* 2012), which might be due to the differences in behavior of various microbial proteins in different 2DE environments.

Isoelectric focusing was performed with a slightly modified setup (Jores *et al.,* 2009; Pang *et al.,* 2010; Sun *et al.,* 2014), using Protean IEF cell at 50 µA/strip. Samples that were extracted by sonication were found to have ionic contaminants, which was initially indicated in the run by very early achievement of the target current for IEF at a very low voltage, and later on by poor quality 2DE images. On the other hand, the TCA/acetone treated samples (Zhang *et al.,* 2008), were found to be almost free of contaminants, which was indicated by 6 µA/strip at low voltage (150 V, 300 V) and 33 µA/strip at high voltage (10 000 V) till the end of IEF, and later on by an effective 2DE map. Equilibration of strips needs to be done delicately for better transfer of focused protein and movement during SDS-PAGE. Equilibration of the focused proteins was performed in a reducing and alkylating solution (Zhang *et al.,* 2008) for a bit longer (20 min each).

Furthermore, SDS-PAGE was performed using both, a relatively old and fresh 30% acrylamide. Fresh 30% acrylamide (Bio-Rad) with C=3.3 (29:1) was found to be relatively better in separation of proteins according to their respective molecular mass (M_r) than an old one (more than 6 months old).

Previously, certain efforts recognized some proteins of *M. bovis* by SDS-PAGE (1D), and 2-DE. However; potentially important virulent factors for understanding the pathogenesis of *M. bovis* are yet to be explored. 2-DE was utilized infrequently in the past decade for getting high resolution proteome map of *M. bovis*. Therefore, the effort presented here had standardized more precise 2-DE conditions, after extensive calibration of each step in the proteomics procedure, for a self-explanatory 2-DE map (Fig. 2). Exploiting these 2-DE conditions would be possibly useful in future efforts of elucidation of virulent factors and pathogenesis of *M. bovis* HB0801 (Chinese strain).

Conflict of interest statement:

The authors have no conflicts of interest to disclose.

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