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Whole cell protein profiling of *Brucella abortus* strain 19 through SDS-Page technique

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Abstract

Brucellosis is an important zoonotic disease which cause direct and indirect economic losses such as reduction in meat and milk production and abortion in mammals. *Brucella abortus* is the causative agent of bovine brucellosis. Usually, B. abortus vaccines; Strain 19 and RB51 are used as part of an eradication program or can be used to control the disease in endemic areas. Routine vaccination is often done in calves to minimize the production of persistent antibodies that can interfere with serological tests. Therefore, the present study was designed to determine the whole cell protein profiling of *B. abortus* strain 19 and to compare the vaccinal strain and field isolates on the basis of protein patterns to observe the extent of homology between them through SDS-PAGE. Pure culture of *B. abortus* was subjected to centrifugation followed by sonication and washing by normal saline. The proteins released after sonication were separated out by centrifugation. The sonicated supernatant was used for SDS-PAGE analysis. Our findings revealed that, a single vaccine against all the strains can be used to overcome brucellosis in cattle. However, for the development of specific vaccine against specific isolate needs further studies by using SDS-PAGE.

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Introduction

Brucellosis is an infectious as well as contagious disease and has significant zoonotic impact on public and animal health worldwide. The present study indicates that a single vaccine against all the strains can be used to overcome brucellosis in cattle. It is transmissible to humans and *causes* acute illness [1]. Brucellosis is one of the deadly infections by Brucella spp. leads to important economic losses [2]. *Brucella* is facultative intracellular Gram-negative bacteria and an important etiological agent that affects a variety of hosts like mammals that include man, cattle, sheep, goats, swine, rodents and even marine mammals. *Brucella* spp. has ability to invade the host defensive mechanism as it replicates as intracellular organisms before activation of antimicrobial mechanism of adaptive immunity, hence responsible for delayed morbidity [3, 4].

Brucella abortus mainly affecting cattle and buffalos *Brucella suis* affects swine, reindeer, cattle and bison and *Brucella melitensis* principally affecting goats. *Brucella abortus* has at least nine biotypes, all members of genus Brucella are closely related as per genetic and immunological evidence. Approximately 5% infections are from biotype 1 which is a major cause of abortion in large ruminants [5]. Brucellosis is characterized by abortion in late pregnancy, retained placenta arthritis, orchitis, epididymitis, excretion of causative organism in the uterine discharges and milk [6]. The organism causes infertility in both sexes. Brucellosis causes heavy economic losses in the form of low reproduction rate, reduced milk and meat yield and impaired quality of milk products. Routine serological tests like Rose Bengal Test (RBT), Serum Agglutination Test (SAT), 2-Mercaptoethanol Test (2ME), Rivanol Test (RIV) and Enzyme Linked immunosorbent Assay (ELISA) are used for the diagnosis of *Brucella* infection and to check the efficacy of vaccines by monitoring humoral immune response. The true diagnosis of the disease can be difficult and challenging, is frequently delayed because the clinical picture may mimic other infectious and noninfectious conditions [7].

The effective control and eradication measures against brucellosis are quick and accurate diagnosis, segregation and vaccination. In order to control brucellosis comprehensive surveillance before and after import testing is of paramount importance [8]. *Brucella abortus* strain 19 and RB 51 are used as vaccinal strains. The most efficient measure to control and reduce the incident rate and prevalence of brucellosis in cattle is vaccination. The success story of disease control is mainly due to of vaccination [9]. The potency of Brucella vaccine can be evaluated by different approaches like testing in laboratory animals, testing on natural hosts that are experimentally challenged or testing under natural conditions [10].

Vaccination is one of the most effective measures to reduce the prevalence of bovine brucellosis and has largely contributed to the success of many control programs [11]. The S19 and RB51 are the two B. abortus vaccines more broadly used in the control of brucellosis in cattle, being effective in the prevention of abortion and infection, besides offering long lasting protection. Prevention and control of Brucella infection needs such practical solutions that can be easily applied to the field [12]. The first vaccine against *B. abortus* is Strain 19 that is live attenuated vaccine which is being extensively used to control brucellosis in bovines [13]. *B. abortus* S19 has low pathogenicity, relatively high immunogenicity and moderate antigenicity [14]. The antibody response induces by attenuated Strain 19 of *B. abortus* biovar 1 cannot be differentiated from antibody response produced by field strain infection [4,15]. The majority of antibodies resulting from S19 immunization or natural infection are directed to the lipopolysaccharide (LPS) O-side chain of immunodominant antigen [16]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell protein is extensively used for identification, have a good level of taxonomic classification at species and subspecies. SDS-PAGE can be helpful in the separation of protein, to calculate the dispersal of proteins among fractions, to determine relative molecular mass and also check the relative abundance of proteins in test sample [17]. The protein profile of Brucella looks to be a reflection of the genome of the strain, so the whole protein profiling has a key role in classification, identification and comparative studies [18]. A recent study reveals that SDS-PAGE is an important technique for identification and comparative studies of bacterial protein profile. [19]. The present study was designed to perform SDS-PAGE to determine the whole cell protein profiling of *B. abortus* strain 19 and to compare vaccinal strain and field isolates on the basis of protein patterns to observe the extent of homology between them. The ultimate goal was to provide a base for selection of a better vaccinal strain and indigenous control of Brucellosis in Pakistan.

Materials and Methods

Bacterial strain

Brucella abortus strain 19 was recovered from stocks, kept at -20 ℃ in lyophilized condition at Bacteriology Laboratory of Animal Health Programme, National Agricultural Research Centre, (NARC) Islamabad.

Bacterial revival and culturing

The lyophilized field isolate was reconstituted in 1ml normal saline (pH 7.0) and cultured in Petri plate having Tryptone Soya Agar (TSA). The plate was incubated at 37ºC for 48 hrs. Isolation and identification methods were done according to WHO methods.

Isolation & Harvesting of cultures

Based on morphological purity, one pure colony was used to sub-culture and propagate in 30 TSA plates to get growth in bulk. Plates were incubated at 37ºC for 48hrs. Colonies obtained were observed morphologically by Gram's staining and confirmed bio-chemically. The tests for production of urease, catalase, oxidase, H_2S and indole; sensitivity to dyes (thionin and basic fuchsin) were carried out as described by Alton and co-workers. The Pure culture was centrifuged in 10 ml falcon tubes at 10,000 rpm at 4ºC for 30 minutes. This step was repeated till the whole culture was centrifuged. Supernatant was discarded and pellet of bacterial cells was suspended in normal saline. The suspended pellet was kept overnight at 4ºC to dissolve. Next day pellet was washed by centrifugation at 10,000 rpm at 4 ºC for 30 minutes. The pellet obtained was re-suspended in normal saline. Step was repeated twice [20].

Sonication of cells & Protein estimation

Bacterial suspension was sonicated in a sonicator (Branson Sonifier 450) at Soil Mineralogy Laboratory NARC, Islamabad, for 14 minutes (2 minutes for 7 times with 1-minute interval) to release inner protein. This material was centrifuged at 4,000 rpm for 30 minutes at 4ºC to remove the intact cells and debris. The supernatant thus obtained was collected containing whole cell protein (WCP) [21].

The WCP was quantified by Lowry's method. The reagents were prepared, and 20 test tubes (t.t) were arranged after labeling in the test tube rack. The sequence of test tubes was; 1 blank in the start and in the end, 5 standards, 1 t.t for original sample and 2 for dilutions; each in duplicate. Linear dilutions of Bovine serum albumin (BSA 0.1-0.5 mg/ml) were made along with 1:2 and 1:4 dilutions of the sonicated supernatant in normal saline. Lowry's method was performed and the optical densities of standards (BSA), sonicated supernatant and their respective dilutions were taken at 750 nm in a spectrophotometer. A standard graph was plotted between linear BSA dilutions and their respective optical densities and protein was estimated from this graph [21].

Preparation of samples for SDS-PAGE

Working sample buffer was made by mixing stock sample buffer, mercaptoethanol and distilled water in the Eppendorf tubes. Sample was mixed with working sample buffer in equal quantity (1:1). This solution was then heated in water for 5 minutes in boiling water. Before loading sample was mixed with loading dye bromophenol blue. One µl loading dye was added in 100 µl sample. Molecular weight standard used comprised of β- galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 k.Da), REase Bsp 981 (25 kDa), β- lactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa) [22].

SDS-PAGE analysis of whole cell proteins

After preparation of gel tank components of separating gel (pH 8.8) were mixed in a 100 ml beaker TEMED was added at the end, it initiates polymerization. After pouring TEMED, gel solution was immediately transferred to the gel cassette after mixing until it reaches 1 cm away from the bottom of the comb. To ensure the smoothness of gel surface, a drop of n-butanol was added at the top of separating gel. It took almost 30 minutes for polymerization of gel.

When the separating gel was set, poured off the overlying water. Mixed components of stacking gel (pH 6.6), added TEMED in the end. After mixing transferred this solution on the separating gel till solution had reached the cut way edge of the plate. Comb was placed in to this solution and left to set. This took about 20 minutes. Comb was carefully removed from the stacking gel. Wells formed were washed with distilled water to rinse out unpolymerized gel. Rubber was removed from the gel plates and the cassette was assembled in the electrophoretic tank. The top reservoir and the bottom tank were filled with electrophoretic buffer and was checked for any leaks, tilt the apparatus to remove any bubbles caught under the gel.

Prior to loading samples, loading dye bromophenol was mixed with each sample. 10 µl samples were loaded by micropipette just above the bottom of the gel in each well. Molecular weight markers were

loaded in the extreme well. The whole apparatus was covered by the lid and was then connected to power supply. Gel was run at 30 mA constant current and 250V voltage. Electrophoresis was continued until bromophenol had reached the bottom of the gel; it took 50 minutes for smaller gels to run. The gel apparatus was then dismantled and the gel cassette was opened by inserting a strip in the middle of the plates. The gel was removed and after discarding the stacking gel, the separating gel was placed in staining solution and left overnight. Next day, staining solution was replaced with de-staining solution and was left for 4 hours with constant shaking in shaking incubator. The gel was rinsed with distilled water and photographed under white light [23].

Results and Discussion

Morphological characteristics

Brucella abortus strain 19 was found to be pink i.e. Gram's negative coco-bacilli after Gram's staining in the microscope at 10x and 100x (**Table 1 and Figure 1**).

Table 1: Biochemical characterization of B. abortus strain 19

Sr. No.	Biochemical Tests	Reactions	Results
1	Oxidase Test	Cytochrome oxidase	+
2	Indole Test	Trytophan reduction	-
3	Urease Test	Urease	+
4	H2S Production	Hydrogen sulphide	+

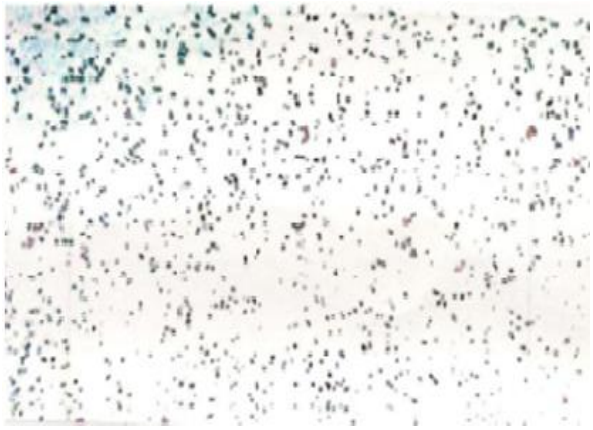

Figure 1: Gram-stained smear from a culture of *Brucella abortus*

Protein estimation

The whole cell protein was quantified by Lowry's method. The optical densities of standards (BSA), sonicated supernatant and their respective dilutions were taken at 750 nm in a spectrophotometer. The minimum protein concentration was 0.1 mg/ml whereas the maximum protein concentration was 0.5 mg/ml. A standard graph was plotted between linear BSA dilutions and their respective optical densities and protein was estimated from this graph as shown in **Figure 2**.

Protein profiling of Brucella abortus Strain 19 using SDS-PAGE

Proteins were extracted by sonication of bacterial cells. The supernatant obtained after centrifugation of this mixture was used in SDS-PAGE. Original samples were very concentrated, so samples were diluted to 1:2 and 1:4 times in normal saline. Both low and high molecular weight markers were used in the study to compare with the proteins and to assess their molecular weights. The electrophoretic profile of B. abortus was shown on 12 percent polyacrylamide gel (**Figure 3**). Using SDS-PAGE, a total of 11 polypeptides ranging from 100 kDa to 15.4 kDa were deduced in the sonicated solution (**Figure 4**). A graph was plotted between R_f value and log of molecular weight (**Figure 5**). From this graph the approximate weight of proteins was calculated (**Table 2**).

Table 2: Calculation of approximate molecular weight from log of molecular weight and R_f value (from graph)

Bands Distance (cm)	R_f Value	Log of mol weight (from graph)	Molecular weight (approximate)
0.4	0.07	5.0	100000
0.7	0.12	4.82	66200
1.2	0.21	4.72	53000
1.7	0.30	4.65	45000
1.9	0.35	4.54	35000
2.1	0.38	4.52	33000
2.6	0.47	4.48	30000
2.8	0.51	4.45	28000
3.6	0.65	4.32	21000
4.0	0.73	4.26	18400
4.2	0.76	4.19	15400

$$R_f = \frac{Distance\ covered\ by\ the\ specific\ band}{Distance\ covered\ by\ the\ solvent\ front}$$

Distance covered by the solvent front – 5.5 cm

The electropherogram evidenced the presence of 11 protein fractions of about 15.4 kDa to 100 kDa. Fractions with approximate molecular weight of s100, 66.2, 53, 45, 35, 33, 30, 28, 21, 18.4 and 15.4 kDa were found.

Onate and Folch [24] analyzed the protein constituents of *Brucella abortus*, *Brucella melitensis* and *Brucella*

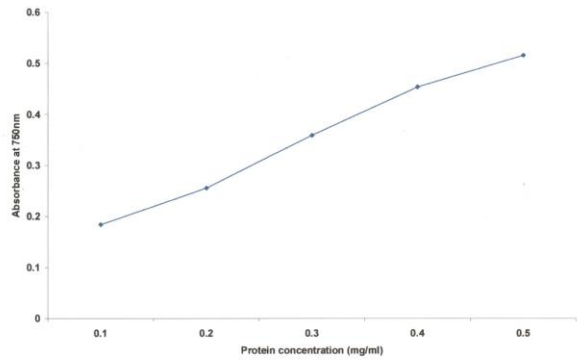

Figure 2: Protein concentration of *Brucella abortus* strain 19 by Lowry's method

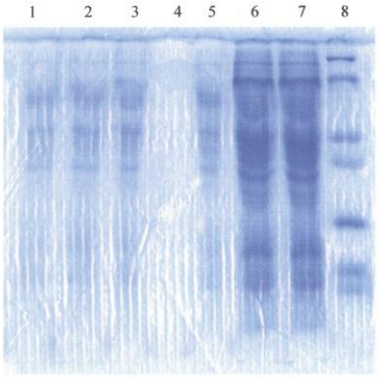

Figure3: Electrophoretic profiles of the WCP of *B.abortus* strain 19.
Molecular weight Marker (8); *B. abortus,* Original sonicated sample (6, 7); *B. abortus* 1:2dil (5, 3) *B. abortus* 1:4 dil (1, 2).

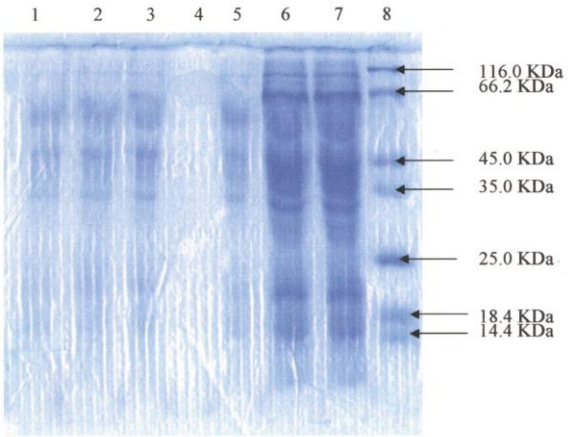

Figure4: Electrophoretic profiles of the WCP of *B.abortus* strain 19.
Molecular weight Marker (8); *B.abortus,* Original sample (6, 7) *B.abortus* 1:2dil (5, 3) *B.abortus* 1:4 dil (1, 2).

ovis. From the comparison appears that the three species of *Brucella* studied showed a different electrophoretic pattern especially at the level of small peptides. On the contrary when two strains of *B. abortus* were analyzed no differences were noticed.

Conclusion

In conclusion, the characterization of Brucella abortus Strain 19 through SDS-PAGE leads us to believe that a single vaccine against all strains can be used to overcome brucellosis in cattle. However, for the development of specific vaccine against specific isolates needs further studies.

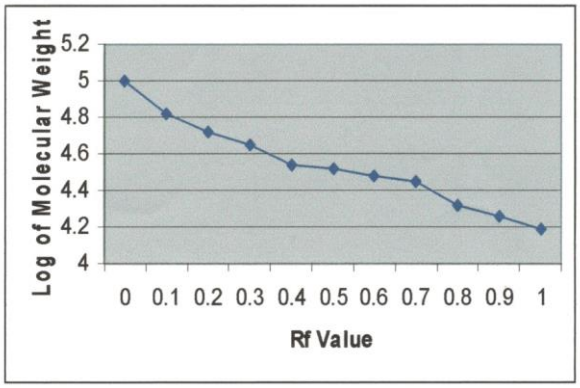

Figure 5: Graph between R_f values and log of molecular weight of total protein of B. abortus separated on the SDS-Page.

Conflict of Interest

Authors have no conflict of interest. No financial interest/relationships relating to the topic of this article have been declared.

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