Indian Journal of Experimental Biology
Vol. 46, September 2008, pp. 621-626

Comparative analysis of protein profiles of wild virulent (E156) and *aro*A-*htr*A double deletion mutant vaccine strain (S30) of *Salmonella enterica* subsp. *enterica* serovar Abortusequi under *in vivo* and *in vitro* growth conditions

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Received 2 April 2008; revised 19 June 2008

In the present study, cell lysate and cell supernatant of the both strains i.e., virulent wild type (E156) and mutant (S30) vaccine strains of *Salmonella enterica subspecies enterica* serovar Abortusequi (*S*. Abortusequi), grown under varied *in vivo* and *in vitro* conditions were subjected to SDS PAGE and western blotting (using rabbit hyperimmune serum). Variation in growth conditions did not have any significant effect on expression of different proteins. SDS PAGE of E156 and S30 cell lysate (CL) revealed 26 and 28 bands, respectively with 3 prominent proteins of 71, 46 and 42 kDa in cell lysate of E 156 and 4 prominent proteins 71, 65, 46 and 40 kDa in S30 strain. The cell supernatant (CS) from both the strains, subjected to SDS PAGE, exhibited similarity in protein profile among these strains, however three bands of 65, 53 and 40 kDa were more prominent in CS preparation of S30, whereas a 56 kDa protein was prominent in CS of E156. Western blotting of E156 and S30 revealed 3 unique proteins of 65, 53 and 40 kDa present in CS preparation of S30 strains which could be used for differentiation of mutant and wild strains and also in development of test for differentiating vaccinated animals from naturally infected.

Keywords: Mutant, Protein profile, SDS PAGE, *Salmonella*, Western blot

Salmonella enterica subspecies *enterica* serovar Abortusequi (*S*. Abortusequi) is an important host specific *Salmonella* primarily infecting equids. It has been graded as the most probable single pathogen responsible for infectious abortions in mares (at 7-8th month of gestation) all over the world[1-3]. Besides abortion, it leads to mortality in foals and infertility in mares. Many types of bacterins (killed vaccines) have been tried and used in past to control the disease, but due to certain limitations those vaccines could not confer long lasting immunity and multiple vaccination (3 to 5) during one gestation[4-6].

With the advancing molecular biology the construction of genetically defined stable attenuated strains of pathogens harboring mutations in specific genes have shown a great potential for future needs. At the National *Salmonella* Centre (Vet.), Indian Veterinary Research Institute (I.V.R.I.), a double deletion mutant having deletion at *htr*A and *aro*A genes has been developed as a novel oral vaccine candidate[4,5], which promises stable attenuation and good immunogenic potential against equine salmonellosis[6]. The vaccine strain has been used to vaccinate more than 700 mares as well as dozens of foals in several equine breeding units including experimental farms at IVRI, Izatnagar and Mukteshwar, Equine Breeding Stud of Indian Army at Babugarh, Equine breeding Farm of Uttranchal government at Pashulok, Rishikesh, Haryana Government Livestock Farms at Hissar, farmers mares in Moradabad, Bijnor and Jyotirbafule district of Uttar Pradesh, and in thoroughbred horse breeding farms at Sohna and Tohana in Haryana. The vaccine proved it safe and conferred immunity for about a year i.e., single vaccination for full term of gestation was sufficient[7].

Being live vaccine candidate (S 30) it is liable to be excreted in environment and might confuse bacteriologists while investigating outbreak of disease. Although the vaccine strain can be identified using *aro*A+*htr*A gene amplification PCR as well as growth characteristic in the laboratory (inability to

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grow in defined M_9 broth and also at 42°C) additional immunological differentiation characteristics are always desirable to improve their rapidity and simplicity. Thus, the protein profiles of wild type and mutant strains grown under *in vitro* and *in vivo* conditions were studied for identification of immunodominant proteins. Immunodominant proteins can be exploited for diagnosis and differentiation of wild from the mutant *Salmonella* strains and also to differentiate between vaccinated and non vaccinated immunopositive animals to give the vaccine strain a DIVA (Differentiation between infected and vaccinated animals).

Materials and Methods

Bacterial strains—The reference wild type *S.* Abortusequi (E156) strain and *aro*A+*htr*A double deletion vaccine strain (S30) available at the National *Salmonella* Centre (Vet.), IVRI, Izatnagar[4, 5], were revived from the preserved stocks and utilized for the study.

Maintenance of Bacterial Strains—The wild type (E156) and mutant strain (S30) of *S.* Abortusequi were maintained throughout the study in 30% glycerinated nutrient broth (Hi-media, Mumbai) stocks in several aliquots at –70°C and on nutrient agar slants at 4°C.

Sterilization of dialysis bags—A total of 24 dialysis tubings (Sigma, USA), 15 cm long tied at one end by linen thread, were sterilized by boiling for 10 minutes in carbonate buffer (*p*H, 9.2, 0.2m*M*).

Preparation of culture—Ten ml overnight growth in trypticase soy broth (TSB, Difco, USA) of E156 and S30 were centrifuged at 8000*g* for 30 min and the respective pellet was suspended in 1ml of PBS (*p*H 7.4). Each of the above bacterial suspension (10µl) was re-suspended in Luria Bertani (LB) broth to make a final volume of 1 ml.

Preparation of dialysis bags—A total of 12 (Sigma, USA) sterilized dialysis bags (Sigma, USA) were filled with 1ml of E156 culture as prepared above and an equal number of bags were filled with S30 and were tied at the other end by linen thread. Later, 6 bags of each culture were boiled in water for 10 minutes to kill bacteria and were maintained at room temperature in normal saline solution (NSS), while 6 bags containing live culture of each strains were stored at 4°C till used for the study on the same day.

Procurement of guinea pigs—Healthy, adult female guinea pigs (12) were procured from Laboratory Animal Facility of the institute and tested for *Salmonella* infection *via* serology and faecal culture methods[8]. Animals free of anti-*Salmonella* antibodies and without any excretion of *Salmonella* for three consecutive weeks were selected for the study. Guinea pigs were maintained in groups of 3 animals as per guidelines of the institute animal ethic committee (IAEC) in separate trays.

In vivo (guinea pigs) experiment—With due approval from the IAEC, 12 guinea pigs were divided into 4 groups of 3 animals each (A to D). For inserting dialysis bags containing E156 and S30 cultures. Animals were anaesthetized[9] using 30mg/kg of ketamine (Sigma, USA) and 5mg/kg of xylazine (Sigma, USA), aseptic incision was made in right flank and dialysis bags containing *Salmonella* cultures were inserted in the peritoneal cavity with all aseptic precautions, keeping both ends stuck on the skin and sutured. Three animals were used each for E156 live, E156 killed, S30 live and S30 killed culture preparations. The animals were euthanized after 24 hr to remove bags. The contents from the dialysis bags were collected and pooled group wise. Contents were centrifuged at 8000*g* for 30 min to collect supernatant and pellets which were stored at -20°C till further use for SDS PAGE and western blotting, separately.

In vitro experiment—The live and killed cultures of E156 and S30 filled in the dialysis bags as described above, were immersed in 200 ml of LB broth and incubated at 37°C for 24 hr. The contents from the dialysis bags were collected, pooled and centrifuged at 8000*g* for 30 min to separate supernatant and stored at -20°C as above.

In vitro experiment with addition of polymixin B—E156 and S30 were grown individually for 12 hr at 37°C in 100ml LB broth and later in the same broth polymixin B sulphate (20 µg/ml; Sigma, USA) was added. The cultures were further incubated for 4 hr at 37°C. Then cultures were centrifuged at 8000*g* for 30 min to separate supernatant and cell pellet were stored at -20°C till further use[10].

Ammonium sulphate precipitation—Ammonium sulphate (Sigma, USA) was added to the supernatant up to 80% saturation and kept at 4°C overnight and was centrifuged at 8000*g* for 30 minutes. The pellet was dissolved in 1 ml 0.15*M* (PBS) *p*H 7.4 and dialyzed thrice against the same buffer during 24hr intervals. The products so obtained were stored at –20°C till further use[11].

Estimation of protein concentration—The pellets obtained in the above experiments were resuspended individually in 1ml of PBS (*p*H 7.4) and the protein concentration in supernatant and pellet was calculated using modified Lowry's method [12] and concentration was adjusted to equality in each preparation.

Salmonella Abortusequi anti-serum—Standard anti-*Salmonella* Abortusequi (E156) serum was induced in rabbits at N.S.C. (Vet), IVRI, Izatnagar and used with high antibody titre (1:20,000).

Protein profiling—SDS PAGE was performed using a uniform stacking (4%) and separating gel (12%)[13]. Pellets and supernatants were separately mixed with sample loading dye (5x) for loading in electrophoresis gel. Gels run for 4 hr at 25 m*A*/slab were removed and stained with 0.25% of Coomassie brilliant blue (R-250) for 4 hrfollowed by destaining and stored in glacial acetic acid (7%).

Western blotting—Western blot was performed using Transblot apparatus (Atto, Japan) for electrophoretic transfer of polypeptides from SDS gel to nitrocellulose membrane (NCM; Sigma, USA)[14]. After transfer of protein on NCM, remaining sites were blocked by incubating membrane overnight at 4°C in 5% (W/V) skimmed milk (Difco, USA) and 0.2% tween-20 (Difco, USA) to block all remaining hydrophobic binding sites and washed thrice for 5 min each in PBST (0.15*M* PBS of *p*H 7.4 + 0.2%, Tween 20). The blot was probed with primary antibodies (1:500) diluted in skimmed milk (3% in PBST) and was incubated for 2 hr at room temperature with continuous agitation and was subsequently washed thrice for 5 min each in PBST. Further, it was incubated with goat anti rabbit immunoglobulin-G, horse radish peroxidase (IgG-HRPO) conjugate (Genie, Bangalore) (1:1000) diluted in skim milk (3% in PBST) for 1 hr at room temperature with agitation. After three more washings with PBST, the blot was incubated in substrate buffer containing 3,-3' diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2) in dark at room temperature with agitation for 20 min. Finally, the blot was rinsed with PBS (*p*H 7.2) to remove H_2O_2 and to stop reaction. All the chemicals used in the study unless specified were from Sigma, USA.

Results

Protein profile of cell lysate (CL)—SDS PAGE profiles of cell lysates of wild (E156) and vaccine (S30) strains of *S.* Abortusequi grown under *in vivo* and *in vitro* conditions (Fig. 1) had a total of 26 and

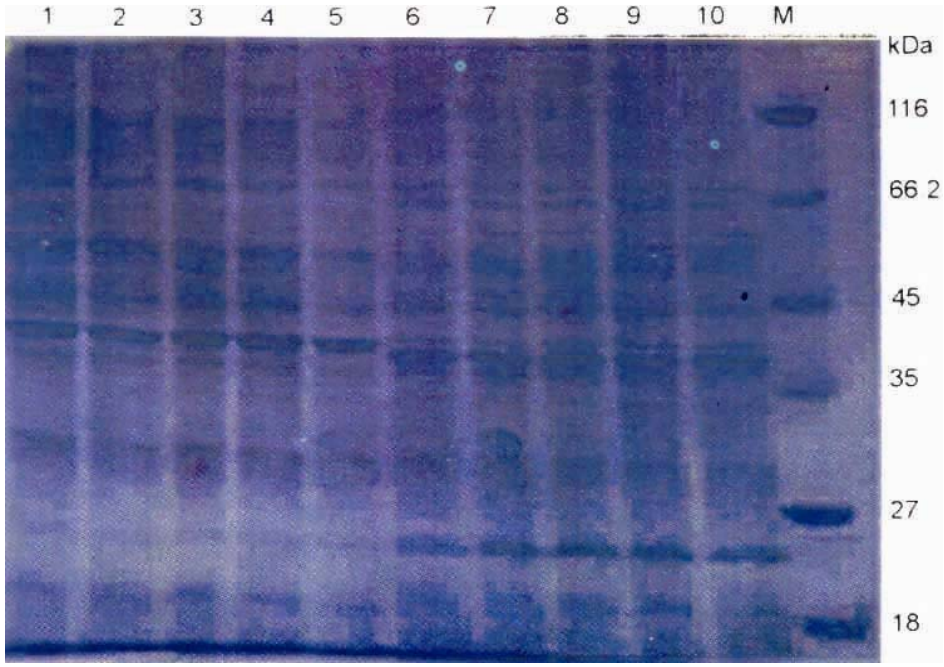

Fig. 1—SDS-PAGE protein profile of cell lysate of *Salmonella* Abortusequi strains grown *in vivo* and *in vitro* [Lane (M) marker, (1) E156 grown *in vitro,* (2) E156 (killed) *in vitro,* (3) E156 grown *in vivo*, (4) E156 (killed) *in vivo*, (5) E156 grown with polymixin B sulphate, (6) S30 grown *in vitro*, (7) S30 (killed) *in vitro*, (8) S30 grown *in vivo*, (9) S30 (killed) *in vivo*, (10) S30 grown with polymixin B sulphate]

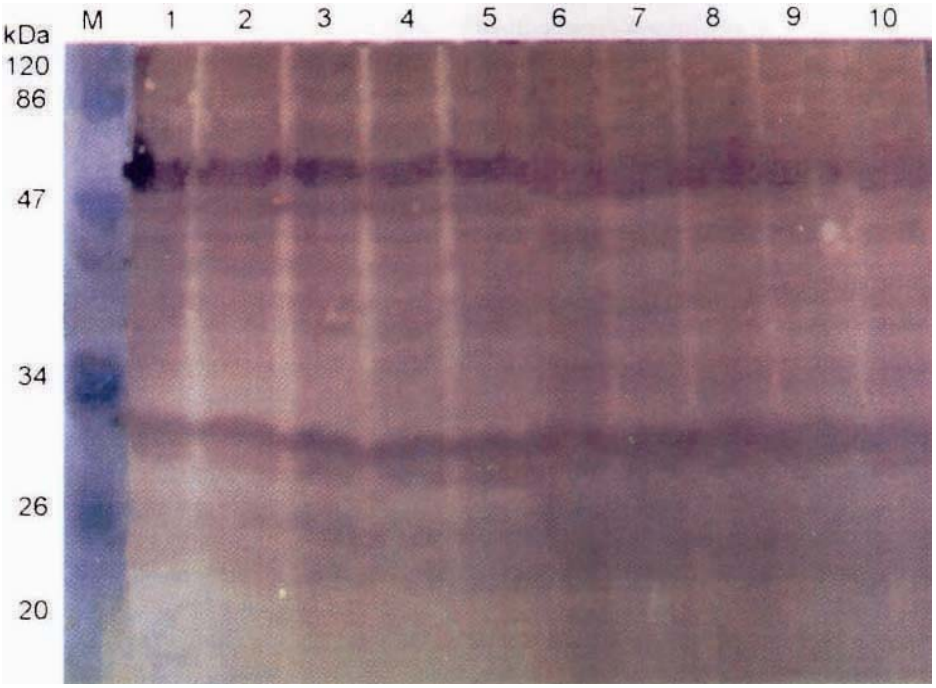

Fig. 2—SDS-PAGE protein profile of cell supernatant of *Salmonella* Abortusequi strains grown *in vivo* and *in vitro* [Lane (M) marker, (1) E156 grown *in vitro,* (2) E156 (killed) *in vitro,* (3) E156 grown *in vivo*, (4) E156 (killed) *in vivo*, (5) E156 grown with polymixin B sulphate, (6) S30 grown *in vitro*, (7) S30 (killed) *in vitro*, (8) S30 grown *in vivo*, (9) S30 (killed) *in vivo*, (10) S30 grown with polymixin B sulphate]

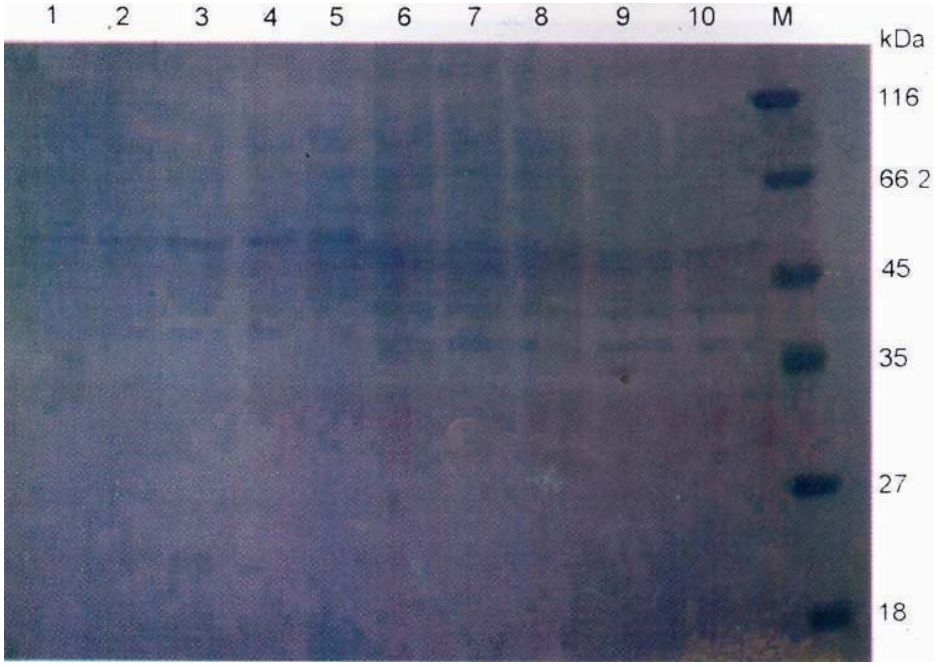

Fig. 3—Western blot of proteins present in cell lysate of *Salmonella* Abortusequi strains grown *in vivo* and *in vitro* [Lane (M) marker, (1) E156 grown *in vitro,* (2) E156 (killed) *in vitro,* (3) E156 grown *in vivo*, (4) E156 (killed) *in vivo*, (5) E156 grown with polymixin B sulphate, (6) S30 grown *in vitro*, (7) S30 (killed) *in vitro*, (8) S30 grown *in vivo*, (9) S30 (killed) *in vivo*, (10) S30 grown with polymixin B sulphate]

28 protein bands, respectively; irrespective of growth conditions. In CL of E156, 3 protein bands of molecular weight 71, 46 and 42 kDa were prominent, whereas, in the cell lysate of S30, 4 major protein bands of 71, 65, 46 and 40 kDa were prominent. Of the most prominent protein expressed in wild strain, 42 kDa band appeared as a minor band in preparations of S30 strain.

Protein profile of cell supernatant (CS)—SDS PAGE profile of wild (E 156) and vaccine (S 30) strains revealed similarity in protein profile (Fig. 2). In the supernatant of E156 there were 10 bands whereas, in the supernatant of S30, 12 bands were visible. Besides, a few faint bands were also visible. The proteins of 65, 53 and 40kDa were visible in CS from S30 strain only.

Western blotting of CL—Western blotting, CL of E156 and S30 revealed many immunodominant proteins (Fig. 3). Two important proteins of 56 and 30 kDa were visible in CL of both the strains and there was no effect of varied environmental conditions on proteins expressed.

Western blotting of CS—On western blotting, CS of E156 and S30 also revealed many immuno reactive proteins which were 16 and 18 in number in E156 and S30 supernatants, respectively (Fig. 4). Western blotting of CS of E156 and S30 also revealed 3 unique proteins of 65, 53 and 40 kDa present only in CS preparation of S30 strain whereas, in the CS of E156 a very prominent band (56 kDa) was present.

Discussion

Studies on protein profile of *S.* Abortusequi parent and mutant culture preparations undertaken under *in vivo* and *in vitro* conditions to find out differences with respect to immunodominant proteins among these strains to identify unique protein(s) capable of differentiating immunized from non immunized animals and also between wild and mutant strains revealed three mutant specific epitopes in CS preparations. The study is in concurrence of earlier reports indicating that the role of different proteins under *in vivo* growth conditions does not seem to be associated with numbers but with their sequence and extent of excretion or translocation to different effecter cells[15, 16]. The concerted events of synthesis, secretion and translocation, in response to environmental stresses, decide the fate of the pathogen in host and amount of effectors to be secreted[15]. No significant difference between protein profiles of *in vivo* and *in vitro* growth though in

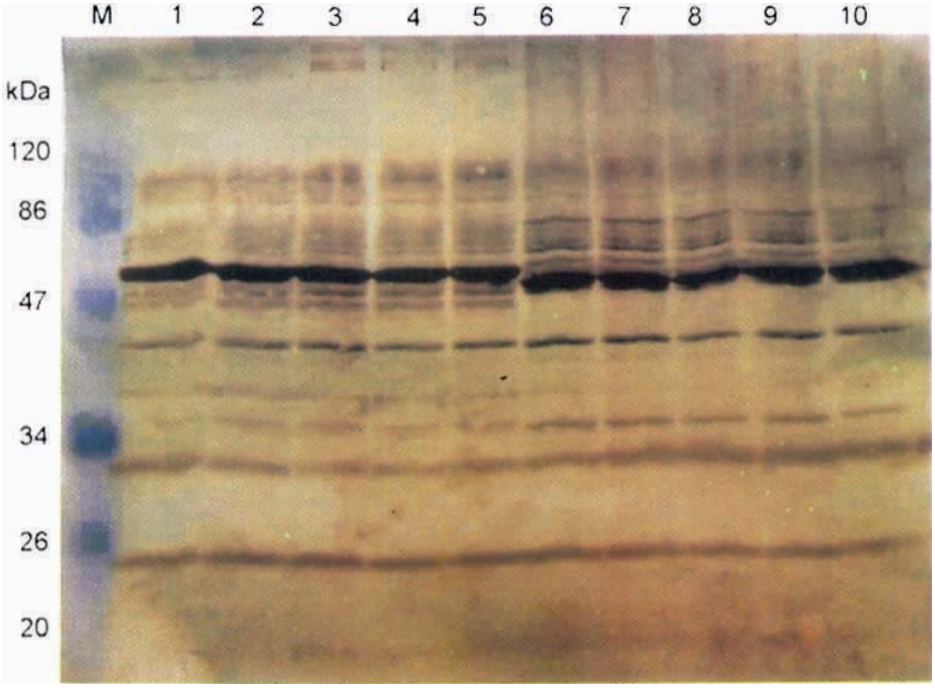

Fig. 4—Western blot of proteins present in cell supernatant of *Salmonella* Abortusequi strains grown *in vivo* and *in vitro* [Lane (M) marker, (1) E156 grown *in vitro,* (2) E156 (killed) *in vitro,* (3) E156 grown *in vivo*, (4) E156 (killed) *in vivo*, (5) E156 grown with polymixin B sulphate, (6) S30 grown *in vitro*, (7) S30 (killed) *in vitro*, (8) S30 grown *in vivo*, (9) S30 (killed) *in vivo*, (10) S30 grown with polymixin B sulphate]

contrast to earlier observation[17], might be either due to sensitivity of the SDS PAGE and Western blotting[11,16] in which very minor proteins though might be crucial in pathogenesis could not be detected or might be due to absence of true infection in the experiment as the infective agent was restricted to an enclosed bag during *in vivo* growth. In earlier studies the bacteria was inoculated into the host while in our study bacteria was in host environment but not in direct contact with the host. The host factor might not have come into contact of bacteria due to dialysis membrane barrier, moreover there is every possibility that low molecular weight proteins might have leached out of dialysis bags.

Both the mutant and wild virulent reference strain had almost similar protein profile which was on the expected line because both of them were isogenic. However, identification of few unique proteins (65, 53 and 40 kDa) in CS preparations of S30 strain only, might be because of truncated products of partially deleted *aro*A and *htr*A genes of *S.* Abortusequi and more amount of truncated protein might have been produced due to continuous stimulation of the gene in absence of active product. The other reason might be expression of some alternate proteins in absence of usual *aro*A and *htr*A gene products to meet the need of the bacterium. However, all the 3 unique protein of S30 reacting with hyperimmune serum of *S.* Abortusequi raised using parent strain proves that these unique proteins could be normally expressed minor proteins of *S.* Abortusequi which might have got expressed in large quantities in vaccine strain for normal growth of the pathogen to compensate the deficiency due to mutation. The compensatory mechanism is common even in higher life and might be very probable in *Salmonella* too. It can be concluded from the study that vaccine strain can be immunologically differentiated for wild bacterium and differential diagnostics can be evolved.

Acknowledgement

The authors acknowledge Director and Joint Director (Academic), I.V.R.I., Izatnagar, for providing the necessary facilities to carry out the research work.

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