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Cloning, sequencing and phylogenetic analysis of *omp25* gene from *Brucella abortus* field strain

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ABSTRACT

The present study was carried out to clone, sequence and analyze complete *omp25* gene of *Brucella abortus* field strain. The primers for *omp25* gene of *Brucella abortus* were designed and sites for *NcoI* and *XhoI* were inserted in the forward and reverse primers, respectively. PCR amplification resulted in an amplicon of ~693bp. The amplified product was cloned in TA cloning vector. One positive clone named *Brucella omp25*/3 was subjected to sequencing and the data obtained were analyzed. Sequencing data exhibited an *orf* of 693bp. The deduced *orf* of *omp25* gene was submitted to NCBI (Accession No. JQ839278.1). The cloned *omp25* gene sequence shows high degree of homology with subject sequences available in the NCBI database. Codon based test of neutrality indicated that natural selection or purifying selection was operating on the *omp25* sequences and test of homogeneity indicated that the evolution of sequences through same pattern was homogeneous except for *Brucella* species CCM 4916 (AM712384). Evolutionary divergence of nucleotide sequence indicated that *omp25* gene sequence has less genetic variability among divergent species, which signifies that *omp25* is highly conserved among different species of *Brucella.* Therefore *omp25* gene can be used for development of recombinant antigen based immunological assays, subunit or DNA vaccine as well as for detection and differentiation of the pathogen by polymerase chain reaction.

Key words: *Brucella,* Cloning *omp25*, Phylogenetic analysis, Sequencing

Brucellosis is a re-emerging zoonoses. Protein antigens from *Brucella abortus* may be of potential value as a vaccine and as a diagnostic reagent for the prevention and diagnosis of bovine brucellosis. Omp25 is an outer membrane protein which has been reported to be immunodominant and conserved in pathogenic *Brucella* species (Cloeckaert *et al.* 2002). The major difference was seen in *omp25* gene of *B. ovis* and *B. melitensis.* There was a short deletion of 36 nucleotides at the 3' end of gene in *B. ovis* and absences of *EcoRV* site in *B. melitensis* (Cloeckaert *et al.* 1996). Studies are now focused on the characterization of *omp25* deletion mutant of *B. abortus, B. melitensis* and *B. ovis* have revealed that Omp25 protein is a virulence factor. The *omp25* gene mutant strain is not expressed and such mutant could be possibly used as new live attenuated vaccine strains (Cloeckaert *et al.* 2002). Conventionally, for sero-diagnostic assays, purified native antigens were used, which required handling of live organism and therefore, was hazardous. Modern molecular tools, viz. cloning and expression provide purified antigen in bulk at a lower cost and with no need to handle live pathogen. The objective of the study was to clone, sequence and analyse complete *omp25* gene of *Brucella abortus* field strain.

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MATERIALS AND METHODS

In the present study, *B. abortus* field strain was isolated from aborted buffalo foetus and suspected isolates were confirmed by PCR using *Brucella* genus specific B4/B5 (Baily *et al.* 1992) and F4/R2 primers (Romero *et al.* 1995). Species specific identification was done by rapid slide agglutination test with *B. abortus* positive serum (procured from IVRI, Izatnagar). The *B. abortus* strain was grown in brain heart infusion broth at 37°C for 3–5 days under microaerophilic conditions. Genomic DNA from bacterial strains extracted as per the standard chromosomal DNA extraction protocol of Sambrook and Russell (2001). For cloning of *omp25* gene, primers were designed on the basis of available *omp25* gene sequences on NCBI database after thorough clustal analysis and were custom synthesized. *NcoI* and *XhoI* restriction endonuclease sites were included in the forward and reverse primers, respectively. The sequence of the primers designed and used were *Brucella omp25* F: 5' cgc*ccatgg*ccatgacgttcaaaaatctacttggcgc 3' and *Brucella omp25* R: 5' cgc*ctcgag*tcagaacttataggcaacgccgag 3'.

The PCR assay was optimized using 25µl reaction mixture. Standardized PCR reaction was containing 1X PCR buffer, 2.5mM $MgCl_2$, 200µM dNTP mix, 20pM of each of forward and reverse primers, 1.5U of *Taq* DNA polymerase and approximately, 100ng of template DNA. Cycling conditions standardized for PCR amplification included one cycle of initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. The PCR product was analyzed by running on 1% agarose gel prepared in 1X TBE buffer and visualized on chemidoc XRS gel documentation system.

Gel extraction of the *omp25* amplicon was done using gel extraction kit. Gel purified PCR product was ligated with pGEMTEasy™ cloning vector in 10 µl reaction mixture. Ligation mixture was incubated at 4°C for overnight for optimum ligation. Competent *E. coli* (DH5α) cells prepared by calcium chloride ($CaCl_2$) method and transformation of ligated product into freshly prepared DH5α competent cells was done by heat shock at 42°C for 2.0 min followed by immediate chilling of the tubes on ice for 10 min. After brief incubation (~45 min) of the transformed cells mixed with SOC broth at 37°C under shaking, plating was done on LB agar plates containing ampicillin (100µg/ml), X- Gal (40mg/ml) and IPTG (100mM) with the help of sterile L-spreader. Plates were incubated at 37°C for 12–15 h (overnight). Randomly, 6 white colonies were picked and inoculated in LB broth containing ampicillin (100µg/ml) and kept overnight at 37°C under shaking at 200rpm. Plasmids were isolated by alkaline lysis method then confirmation of the clones was done by PCR and restriction double digestion of the isolated plasmids using *NcoI* and *XhoI*.

One positive clone confirmed by PCR and restriction double digestion was selected and named *BrucOmp25*/3. *BrucOmp25*/3 was inoculated in semisolid agar and sent for commercial sequencing to University of Delhi South Campus, New Delhi. The obtained nucleotide sequences (forward and reverse) of *omp25* gene sequence were displayed and analyzed using BioEdit sequence alignment editor, Version 7.0.9.0 (Hall 1999). Vector sequences at both 5'- and 3'-termini of sense and antisense strands were trimmed. The open reading frame was deduced and the sequence was submitted to NCBI GenBank (accession no. JQ839278.1). The nucleotide sequence was subjected to BLASTn (Altschul *et al.* 1997) to compare for sequence similarities with other sequences available in the NCBI database. The related sequences from other *Brucella* species were retrieved from GenBank and the equivalent sequences were selected and saved in FASTA format and aligned using alignment explorer/Clustal of MEGA 4.0 software (Tamura *et al.* 2007). Nucleotide sequences of *omp25* gene of *Brucella* species were used to construct the phylogenetic tree using maximum parsimony (MEGA 5.2) method with bootstrap re-sampling based on 500 bootstrapped replications. The MEGA 5.2 software package was used for analysis of codon based test of neutrality by Nei-Gojobori method. There were 227 positions in the final dataset. For estimation of base substitution per site for evolutionary divergence between sequences composite maximum likelihood method was used. The analysis involved 8 nucleotide sequences namely *B. abortus* strain A9 (JQ839278), *B. melitensis* biovar *Ovis* 63/290 (AY484547), *B. cetaceae* B202R (AY484553), *B. pennipediae* B2/94 (AY484550), *Brucella* sp. CCM 4916 (AM712384), *B. suis* 1330 (AE014291), *B. canis* HSK A52141 (CP003174), *B. abortus* S19 (CP000887). Codon positions included were 1st+2nd+3rd+noncoding sequences. All positions containing gaps and missing data were eliminated. There were a total of 684 positions in the final dataset and codon based evolutionary divergence was studied for synonymous and non synonymous differences per synonymous and non synonymous sites, respectively, by Nei-Gojobori method for the selected nucleotide sequences. The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (disparity index test). A Monte Carlo test (500 replicates) was used to estimate the P-values.

RESULTS AND DISCUSSION

Brucella culture showed pinpoint colonies after 7–10 days of inoculation in *Brucella* selective medium. Genomic DNA extracted using standard protocol showed an $OD_{260/280}$ ~1.8 indicating the purity of the chromosomal DNA. PCR amplification of *omp25* gene resulted in ~693bp amplicon on 1% agarose gel (Fig. 1). Gel purified PCR product was successfully ligated with linearized pGEMTEasy cloning vector as transformation in DH5α competent cells resulted in development of mostly white colonies on the selective medium (LB agar plates with ampicillin, X- Gal and IPTG) with few blue colonies. Results also indicated optimum transformation efficiency as large number of colonies appeared on the selective medium. Six white colonies picked and processed for plasmid isolation, all 6 resulted in specific amplicon of 693bp by PCR and also resulted in the release of specific size insert by restriction double digestion by *NcoI* and *XhoI* enzymes as seen on 1% agarose gel (Fig. 2). One positive clone named *BrucOmp25*/3 was selected and sequenced. Sequencing data obtained was analyzed using various bioinformatics softwares and *orf* was deduced and submitted to NCBI (Accession No. JQ839278.1).

The BLASTn search result using the *omp25* coding sequence (cds) revealed a high degree of homology between the query sequence and other subject sequences available in the NCBI database (99% with *B. pinnipedialis* B2/94, *B. microti* CCM 4915, *B. melitensis* bv. 1, *B. cetaceae* and *B. melitensis* biovar *Ovis*). The predicted Omp25 amino acid sequence showed high degree of similarity when subjected to multiple sequence alignment (Fig. 3). The phylogenetic

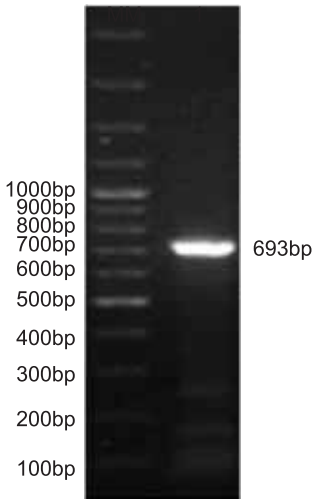

Fig. 1. PCR amplification of *omp25* complete gene from *Brucella abortus* field strain; Lane 1: PCR amplicon of *omp25* gene; MM: GeneRulerTM 100 bp plus DNA ladder.

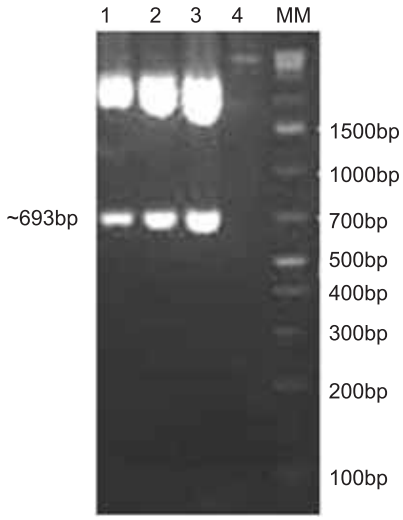

Fig. 2. Confirmation of the clone *omp25* carrying insert by restriction digestion; Lane 1-4 : Plasmids from selected clones digested by *NcoI* and *XhoI;* MM:GeneRulerTM 1kb plus DNA ladder.

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#JQ839278.1_Babortus_strain_A9           ---------M TFKNLLGASL VAVITSTSAY AADAIVAQEP APIAIAPSFS WAGAYFGGQV GYGWGRAKLE NRTNGGTSEF
#AY484547.1_Bmelitensis_biovar_Ovis_63... ---------. KL.A..F..T .VLVAA.G.N ....VIE... ..VVV..T.T .N...L...I ..A..KSHFS YDPT-DLGDI
#AY484553.1_Bcetaceae_B202R              ---------. KL.A..F..T .VLVAA.G.N ....VIE... ..VVV..T.T .N...L...I ..A..KSHFS YDST-.LGDI
#AY484550.1_Bpinnipediae_B2/94           ---------. KL.A..F..T .VLVAA.G.N ....VIE... ..VVV..T.T .N...L...I ..A..KSHFS YDST-.LGDI
#AM712384.1_Brucella_sp._CCM_4916        MNIGCLEGTV NI.S..L..T .VLVAA.G.K ....VIE... ..VVV..T.T .N...L...I ......ST.S DEDT--SIRV
#AE014291.1_Bsuis_1330                   ---------. .......... .......... .......... .......... .......... .......... ..........
#CP003174.1_Bcanis_HSK_A52141            ---------. .......... .......... .......... .......... .......... .......... ..........
#CP000887.1_Babortus_S19                 ---------. .......... .......... .......... .......... .......... .......... ..........

#JQ839278.1_Babortus_strain_A9           KPNGFIGGLY TGYNFDTGNN FILGLDANVD YNNLKKSRDF ITSGNPVQTT GETQLRWSGA VRARAGYAID RFMPYIAGGV
#AY484547.1_Bmelitensis_biovar_Ovis_63... ..D..L.... A.....L..S VV..I.GD.T ..DVS.NI.. LDEN.AAA.F -.NR..... ........V. ..L.......
#AY484553.1_Bcetaceae_B202R              ..D..L.... A.....L..S VV..I.GD.T ..DVS.NI.. LDEN.AAA.F -.NR..... ........V. ..L.......
#AY484550.1_Bpinnipediae_B2/94           ..D..L.... A.....L..S VV..I.GD.T ..DVS.NI.. LDEN.AAA.F -.NR..... ........V. ..L.......
#AM712384.1_Brucella_sp._CCM_4916        ..D..L.... A.....M... .V..V.GDIT ..D.DA.HSD TDPDLDLT.G VDSK..... ....M.V.M. .W........
#AE014291.1_Bsuis_1330                   .......... .......... .......... .......... .......... .......... .......... ..........
#CP003174.1_Bcanis_HSK_A52141            .......... .......... .......... .......... .......... .......... .......... ..........
#CP000887.1_Babortus_S19                 .......... .......... .......... .......... .......... .......... .......... ..........

#JQ839278.1_Babortus_strain_A9           AFGGIKNSLR ----IGGEES SKSKTQTGWT VGAGIEYAAT DNVLLRLEYR YTDYGKKNFG LND----LDT RGSFKTNDIR
#AY484547.1_Bmelitensis_biovar_Ovis_63... ...SLR.RGE ----FE.AGF .Q...L.... I...MD.... ...I...... .....N.DY. FD.----AAV TNN.......
#AY484553.1_Bcetaceae_B202R              ...SLR.RGE ----FE.TGF .Q...L.... I...MD.... ...I...... .....N.DY. FD.----AAV TNN.......
#AY484550.1_Bpinnipediae_B2/94           ...SLR.RGE ----FE.TGF .Q...L.... I...MD.... ...I...... .....N.DY. FD.----AAV TNN.......
#AM712384.1_Brucella_sp._CCM_4916        ...NV...VS LTDG.ESIGV .Q...M...A A...VD.... ...IV..... .....H.D.S VV.GDLSVEA .NK...H...
#AE014291.1_Bsuis_1330                   .......... ----...... .......... .....D.... .......... .......... ...----... ..........
#CP003174.1_Bcanis_HSK_A52141            .......... ----...... .......... .....D.... .......... .......... ...----... ..........
#CP000887.1_Babortus_S19                 .......... ----...... .......... .....D.... .......... .......... ...----... ..........

#JQ839278.1_Babortus_strain_A9           LGVAYKF*
#AY484547.1_Bmelitensis_biovar_Ovis_63... F.......
#AY484553.1_Bcetaceae_B202R              F.......
#AY484550.1_Bpinnipediae_B2/94           F.......
#AM712384.1_Brucella_sp._CCM_4916        .......
#AE014291.1_Bsuis_1330                   .......
#CP003174.1_Bcanis_HSK_A52141            .......
#CP000887.1_Babortus_S19                 .......
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Fig. 3. Multiple sequence alignment of predicted amino acid sequences of Omp25 protein of *Brucella abortus* field strain.

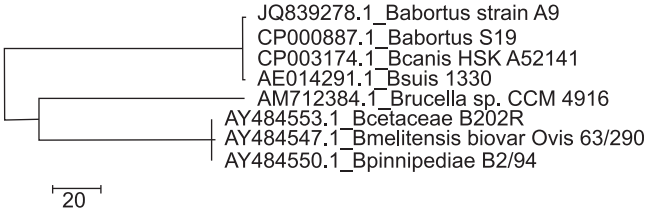

Fig. 4. Phylogenetic tree obtained from maximum parsimony method using nucleotide sequences of *Brucella* species.

tree revealed that the *omp25* cds of *B. abortus* is highly conserved gene (Fig. 4).

Similar results were reported and suggested *omp25* gene as marker for *Brucella* typing and diagnostic as well by Cloeckaert *et al.* (1995). In the present study it was observed that null hypothesis (dN=dS) has been rejected for sequences namely *B. melitensis* biovar *Ovis* 63/290 (AY484547), *B. cetaceae* B202R (AY484553), *B. pinnipediae* B2/94 (AY484550) and *Brucella* species CCM 4916 (AM712784)

Table 1. Codon- based test of neutrality (dN=dS) of nucleotide sequences for the numbers of synonymous (dS) and nonsynonymous (dN) substitutions per site

Sequence Acc. No.	Test statistic (dN-dS)	Probability (P)
AY484547	–7.498	0.000***
AY484553	–7.463	0.000***
AY484550	–7.463	0.000***
AM712384	–9.240	0.000***
AE014291	–0.658	0.512
CP003174	1.042	0.229
CP000887	1.042	0.229

******* P<0.001.

with respect to *B. abortus omp25* sequence (JQ839278.1) by using codon based test of neutrality (Table 1).

It indicated that natural selection or purifying selection was operating on the *omp25* sequences. Test of homogeneity indicated that the evolution of the sequences was homogenous except for *Brucella* species CCM 4916 (AM712384) (Table 2).

Table 2. Test of the homogeneity of substitution patterns between sequences

Sequence Acc. No.	Disparity index per site	Probability (P)
AY484547	0.000	1.000
AY484553	0.000	1.000
AY484550	0.000	1.000
AM712384	0.969	0.008**
AE014291	0.001	0.426
CP003174	0.000	1.000
CP000887	0.000	1.000

**P<0.01.

Codon based test of neutrality (dN=dS) also indicated that the sequences have evolved through same pattern of substitution however the test of homogeneity of substitution pattern between sequences also reflect the significance of test only for the sequence of *Brucella* species CCM 4916 (AM712384). Multiple sequence alignment shows that amino acid sequence of *Brucella* species CCM 4916 (AM712384) has got insertion of 4 extra amino acid in 2 different locations of the translated peptides of different species. Existing less genetic variability for *omp25* gene sequence among divergent species which signifies that *omp25* is highly conserved among different species of *Brucella* was confirmed by estimating no. of base substitution per site in evolutionary divergence of sequence analysis (Table 3).

Table 3. Evolutionary divergence of nucleotide sequence for estimating the number of base substitutions per site with respect to the *B. abortus* (Accession No JQ839278.1) nucleotide sequence obtained from the present study

Sequence Acc. No.	No. of base substitution/site ± SE
AY484547	0.484
AY484553	0.487
AY484550	0.487
AM712384	0.547
AE014291	0.003
CP003174	0.001
CP000887	0.001

Therefore *omp25* gene can be used to evaluate the immunogenicity in experimental animals as well as to test its potential for development of recombinant antigen based immunological assays (e.g. ELISA) as well as for development of subunit or DNA vaccine. Since the *omp25* gene is known to be present only in pathogenic strains and highly conserved, the same can also be attempted for detection and differentiation of the pathogen by PCR method.

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