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Precise detection of *L. monocytogenes* hitting its highly conserved region possessing several specific antibody binding sites

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ABSTRACT

Listeria monocytogenes, a facultative intracellular fast-growing Gram-positive food-borne pathogen, can infect immunocompromised individuals leading to meningitis, meningoencephalitis and septicaemias. From the pool of virulence factors of the organism, ActA, a membrane protein, has a critical role in the life cycle of *L. monocytogenes*. High mortality rate of listeriosis necessitates a sensitive and rapid diagnostic test for precise identification of *L. monocytogenes*. We used bioinformatic tools to locate a specific conserved region of ActA for designing and developing an antibody–antigen based diagnostic test for the detection of *L. monocytogenes*. A number of databases were looked for ActA related sequences. Sequences were analyzed with several online software to find an appropriate region for our purpose. ActA protein was found specific to *Listeria* species with no homologs in other organisms. We finally introduced a highly conserved region within ActA sequence that possess several antibody binding sites specific to *L. monocytogenes*. This protein sequence can serve as an antigen for designing a relatively cheap, sensitive, and specific diagnostic test for detection of *L. monocytogenes*.

1. Introduction

Several outbreaks of listeriosis within the last decade have been reported (Graves et al., 2005). Ingestion of *Listeria monocytogenes* contaminated food is the route of infection in humans. Clinical symptoms are wide, ranging from gastroenteritis to sepsis or meningo-encephalitis. In pregnant women, listeriosis leads to abortion or neonatal sepsis (Vazquez-Boland et al., 2001b). The disease has a high lethality rate in immunocompromised individuals, neonates, and fetuses. The mortality rate of listeriosis is as high as 20–30% (Vazquez-Boland et al., 2001a; Watson, 2009). Moreover, *L. monocytogenes* causes spontaneous abortions in farm animals (Czuprynski, 2005). The pathogen is able to replicate within macrophages and a number of nonprofessional phagocytes such as hepatocytes and epithelial cells (Cossart and Portnoy, 2000; Vazquez-Boland et al., 2001a). A 9 kb region of the chromosome is responsible for virulence factors such as PrfA, LLO, and ActA (Vazquez-Boland et al., 2001a). The membrane protein ActA plays a major role in crossing of the fetoplacental barrier in murine model (Le Monnier et al., 2007) and is involved in recognition of heparan sulfate receptor (Alvarez-Dominguez et al., 1997) and entry into epithelial cells (Suárez et al., 2001). Moreover, intracellular motility of *L. monocytogenes* is related to the major virulence factor, ActA (Smith et al., 1996; Vazquez-Boland et al., 2001a). ActA polarization (Rafelski and Theriot, 2006) and phosphorylation take place on the surface of the pathogen (Brundage et al., 1993; Darji et al., 1998). This is followed by polymerization of host cell actin after LLO-mediated escape from phagosomal compartment of *L. monocytogenes*, providing the force of unidirectional actin-based movement within eukryotic infected cells (Portnoy et al., 2002). Polymerization of actins occurs even during and for some time after the bacterium entry (Poussin and Goldfine, 2009). There is no direct interaction between ActA and actin (Cossart and Lecuit, 1998). ActA mimics the activity of WASP/WAVE family members (Chong et al., 2009), recruits Ena/VASP and the Arp2/3 complex, and thereby polymerizes actin monomers (Romero and Van Nhieu, 2009). In epithelial cells, actin assembly near the phagosome suggests that monomers of actins can enter to the compartment through LLO-mediated pores and polymerize there (Poussin and Goldfine, 2009). Actin filaments and actin-binding proteins network referred to as an actin "cloud" that rearranges into actin "tail", moves the bacterium to the host cell periphery (Smith et al., 1996), pushes the cell membrane outward, and forms pseudopod-like structure that is taken up by

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the surrounding cells. Then, the pathogen escapes again from double-membrane phagosome and the cycle is repeated (Suárez et al., 2001). So, the pathogen is able to spread cell to cell without encountering antibodies. Since ActA mutants do not induce actin polymerization and are unable to spread cell to cell, they are avirulent and grow as microcolonies in infected cells (Smith et al., 1996; Vazquez-Boland et al., 2001a). This ability of the bacterium undermines the role of humoral immunity in *L. monocytogenes* infection (Cossart and Lecuit, 1998; Zenewicz and Shen, 2007). ActA is a key player in evading autophagic recognition. This protein helps pathogen camouflage itself with host proteins and thereby escapes recognition by autophagy (Yoshikawa et al., 2009a). Autophagy is an eliminating system of damaged organelles, misfolded protein aggregates, undesirable molecules and intracellular pathogens. However, these pathogens can hamper autophagy system by using different strategies (Yoshikawa et al., 2009a). In addition, the ActA is involved in phagosome disruption and escape of the bacterium from the phagosome. As many as 156 amino acids of N-terminal of the ActA are responsible for actin nucleation. Deletion of amino acids 31–58 decreases the phagosome disruption. Amino acids 32–42 are pivotal in stability of the ActA and phagosome disruption, but have no important role in actin polymerization (Poussin and Goldfine, 2009). Two sets of "proline-rich-repeats" and "long repeats" were located in ActA sequence. These tandem repeats are involved in rate of actin-based motility, the percentage of moving bacteria, and the localization of VASP and profilin (Leong et al., 2009).

In the present study we used bioinformatic tools to locate a conserved specific region of ActA to design and develop a specific and sensitive diagnostic test for the detection of *L. monocytogenes*. Such a test will improve rapid clinical decisions and provide a method to epidemiologically evaluate management and control the infections caused by *L. monocytogenes*.

2. Methods

2.1. Study design

The present study is divided into two phases. In the first phase, database search and analysis were conducted to find a conserved and specific region of ActA leading to selection of a proper region expressed in several species of *L. monocytogenes*. The second phase includes various analyses of the selected region such as specificity to *L. monocytogenes*, B-cell epitope prediction, antigenicity etc.

2.2. Sequence availability and similarity search

Sequences were obtained from NCBI Database at http://www.ncbi.nlm.nih.gov. In order to find out the presence or absence of ActA across species we used Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database of physical and functional interactions (Szklarczyk et al., 2011). Also BLAST on all the sequences from each species included in the ortholog databases including NCBI COG, NCBI KOG, OrthoMCL DB, and MultiParanoid was performed using BLASTO (Zhou and Landweber, 2007) at http://oxytricha.princeton.edu/BlastO/, the program was weighted at the highest or positive favor to search within ortholog groups that contain genes from more species. These ortholog groups are therefore likely to be more conserved.

2.3. Primary sequence analysis

Variety of sequence properties including pI were evaluated using ProtParam (Gasteiger et al., 2005) at http://expasy.org/tools/protparam.html. Dotlet (Junier and Pagni, 2000) online software from http://myhits.isb-sib.ch/cgi-bin/dotlet/ was employed to detect repeat modules in primary amino acid sequences.

2.4. Alignments

Multiple sequence alignments were generated by T-Cofee (Notredame et al., 2000) at http://www.ebi.ac.uk/Tools/msa/tcoffee/ using various sequences of ActA. Alignment file was then edited in CLC Protein Work Bench. Aligned sequences were shaded and prepared for presenting using BOXSHADE (Hofmann and Baron, 1996) at http://www.ch.embnet.org/software/BOX_form.html. Needle program at http://www.ebi.ac.uk/Tools/psa/emboss_needle/ was employed for pairwise comparison of sequences.

2.5. Topology prediction

Secondary structure prediction was performed using GOR software (Garnier et al., 1996) at http://npsa-pbil.ibcp.fr/cgi-bin/secpred_gor4.pl. Presence and location of signal peptide cleavage sites in amino acid sequences was predicted by SignalP 4.0 (Petersen et al., 2011) at http://www.cbs.dtu.dk/services/SignalP/. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks.

A consensus prediction from five different topology prediction algorithms: SCAMPI (single sequence mode), SCAMPI (multiple sequence mode), PRODIV-TMHMM (Viklund and Elofsson, 2004), PRO-TMHMM and OCTOPUS (Viklund and Elofsson, 2008) was performed for evaluation of specification of the membrane spanning segments of signal peptide cleaved off sequence of ActA and its IN/OUT orientation relative to the membrane at http://topcons.cbr.su.se/ server using TOPOCON software (Bernsel et al., 2009).

2.6. Immuno-informatic analyses

Properties important in predicting the B-Cell epitopes positions were determined at www.immuneepitope.org. The server uses following methods: Chou and Fasman method (Chou and Fasman, 1978) was used to predict Beta-Turns, Karplus and Schulz (Karplus and Schulz, 1985) for predicting the flexibility, Emini method (Emini et al., 1985) for surface accessibility prediction, and Parker method (Parker et al., 1986) for hydrophilicity evaluation.

2.6.1. Prediction of continuous B-cell epitopes

A combination of hidden Markov model (HMM) with two best propensity scale methods (Parker and Levitt) (Levitt, 1978; Parker et al., 1986) was employed for predicting linear B-cell epitopes using Bepipred (Larsen et al., 2006) at http://www.cbs.dtu.dk/services/Bepipred/ the Score threshold for epitope assignment was set at 0.35. Also ABCpred software (Saha and Raghava, 2006) at http://www.imtech.res.in/raghava/abcpred/ predicted B-cell epitopes The server is able to predict epitopes with 65.93% accuracy using recurrent neural network. The overlapped predicted epitopes were considered as potential B-Cell epitopes. Accuracy of these two software was confirmed using COBEpro (Sweredoski and Baldi, 2009) at http://scratch.proteomics.ics.uci.edu/ which is a novel two-step system for predicting continuous B-cell epitopes. The software first uses a support vector machine to make predictions on short peptide fragments within protein sequence and then calculates an epitopic propensity score for each residue based on the fragment predictions. The degree of conservancy of these potential linear epitopes was calculated within three sequence collections viz. ActA sequences of

L. mocytogens, *L. ivanovii*, and *L. seeligeri*. The degree of conservation is defined as the fraction of protein sequences containing the epitope at an 80% identity level. This approach was performed at www.immuneepitope.org.

2.7. Important properties of construct

Protein solubility was evaluated using recombinant protein solubility prediction at www.biotech.ou.edu (Davis et al., 1999; Harrison., 2000; Wilkinson and Harrison, 1991). Probability of antigenicity was estimated at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html website using VaxiJen software (Doytchinova and Flower, 2007). (The leave-one-out cross-validation (LOO-CV) of the software has 82% accuracy, 91% sensitivity and 72% specificity).

2.8. Validating the method

In the present study we have used The NCBI Protein database that is a collection of sequences from several sources, including translations from annotated coding regions in GenBank, RefSeq and TPA, as well as records from SwissProt, PIR, PRF, and PDB; Also we have used Version 9.0 of STRING with high confidence (0.700) which covers more than 1100 completely sequenced organisms (Szklarczyk et al., 2011). We also specified software parameters precisely to achieve the best and reliable results.

3. Results

3.1. Similarity searches and sequence availability

Several sequences of ActA are available in the current databases; these sequences can be found in *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*. We chose sequences of various lengths for further analyses and a 604 amino acid sequence of ActA in *L. monocytogenes* (Acc. No. YP_002756940.1) as reference sequence. Results of BLAST against non redundant protein database at www.ncbi.nlm.nih.org/BLAST for ActA shows no homology in other organisms when our reference sequence served as a query. Search against conserved domains database of NCBI referred the majority of proteins to ActA superfamily (pfam accession no. 05058, a member of the superfamily cl09356). Also there were no homologous sequences anywhere in String database or even in ortolog groups of proteins from several databases.

3.2. Primary sequence analyses and alignments

The theoretical isoelectric pH of ActA is approximately 5. Sequences of ActA are containing repetitive modules. The short length modules are proline rich showing low complexity. Repeat sequences are shown in Fig. 1a.

Several identical blocks were observable within the alignments with no major or significantly notable difference except, at places, for size and number of repeat units. Sample of shaded alignment file is presented in Fig. 2.

3.3. Region selection

Based on alignment and primary sequence analyses and distribution of antigenic determinants, an ideal region for our purpose was selected and named as construct. The corresponding position of this region to ActA reference sequences is shown in Fig. 1b. No significant similarity was observed within pairwise alignments between our selected region and ActA sequences in two other species of listeria (*L. ivanovii* and *L. seeligeri*), in contrast, the construct shares significant similarity with ActA sequences of *L. monocytogenes* ranging from 80% to 100% (data not shown).

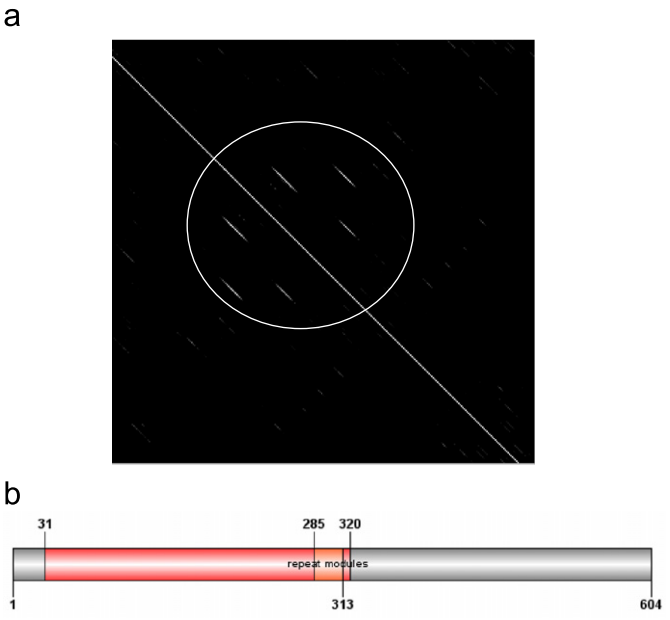

Fig. 1. Dotplot matrix of ActA. White circle shows the repeat containing region (a) horizontal and vertical axes are both ActA sequence. Graphical display of ActA reference sequence and the position of selected region. The selected region is presented in red and repeat modules are in orange (a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Topology prediction

The secondary structure constituents of ActA is random coil (59.93%), alpha helix (34.11%), and beta turn (4.14%) a shematic display of protein secondary structure is depicted in Fig. 3. Since cleavable signal peptides are easily confused with transmembrane segments, we firs applied SignalP software for prediction of signal peptide and cleavage site. For reference sequence of ActA submitted in SignalP, two different neural networks are used, one for predicting the actual signal peptide and one for predicting the position of the signal peptidase I (SPase I) cleavage site. High-peaking C-score referred to by a single peak at position 30 meaning that cleavage site between amino acid 29–30 corresponds to that the mature protein starts at (and include) position 30 (Fig. 4). The TOPCONS hidden Markov model (HMM), gave a consensus prediction for the mature (cleaved off) protein, together with a reliability score based on the agreement of the included methods across the sequence. In addition, ZPRED (Granseth et al., 2006) is used to predict the Z-coordinate (i.e. the distance to the membrane center) of each amino acid, and the ΔG-scale is used to predict the free energy of membrane insertion for a window of 21 amino acids centered around each position in the sequence; as a result majority of protein (except for a small segment of C-terminal) is outer membrane (Fig. 5).

3.5. Immunoinformatic assay

ABCpred result shows 62 hits of 16 meric peptide sequences as B-cell epitopes ranking based on scores for ActA reference sequence. 29 linear B-cell epitopes were potentially belongs to our selected region. 8 vast regions within protein reference sequence, sharing B cell epitope property, were predicted by

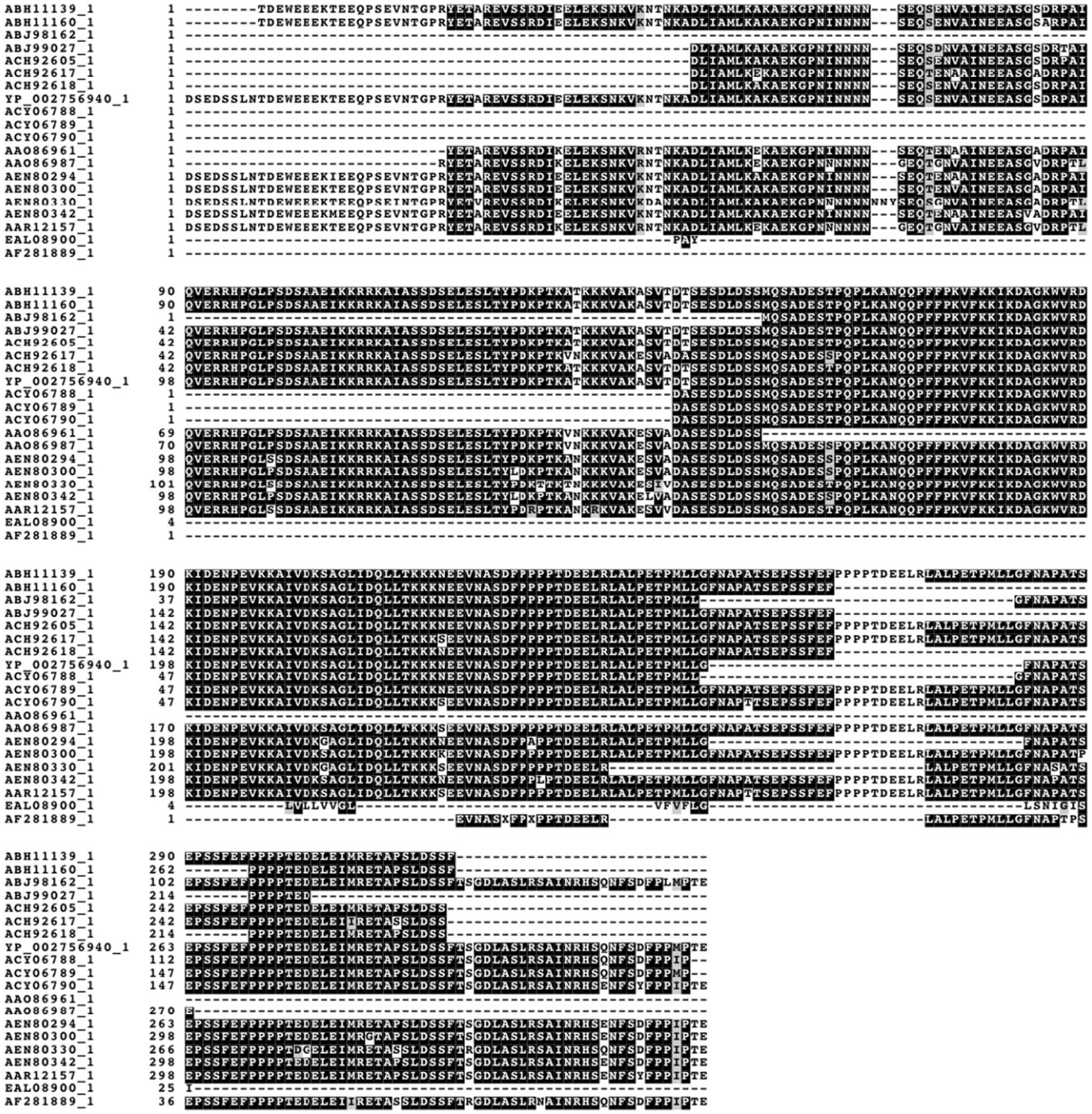

Fig. 2. Sample of alignment file. This file presents the aligned sequence of construct and ActA of various lengths from *L. monocytogenes*.

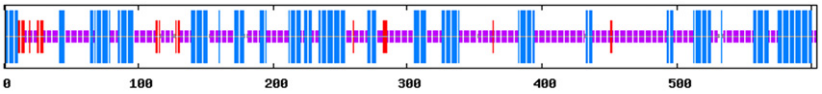

Fig. 3. Graphical results for secondary structure prediction of ActA reference sequence. Extended strand: purple, Coil: red, Helix: blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bepipred; the application of this method to a large number of proteins allowed an accuracy of 75% with Sensivity=0.49 and specificity=0.75. The subscription segments of these two softwares were considered as potential B cell epitopes (Table 1). The results derived therein were compared with those from COBEpro which confimed the accuracy of results. These linear epitopes were conserved within collection of ActA sequences and belong to *L. monocytogenes*. Antigenicity, Beta-turns, flexibility, surface accessibility, and hydrophilicity plots (Fig. 6) reveal the higher scores of these parameters for selected region (yellow regions above the threshold) and C and N –terminal of protein are significantly under the threshold. The degree of conservancy of

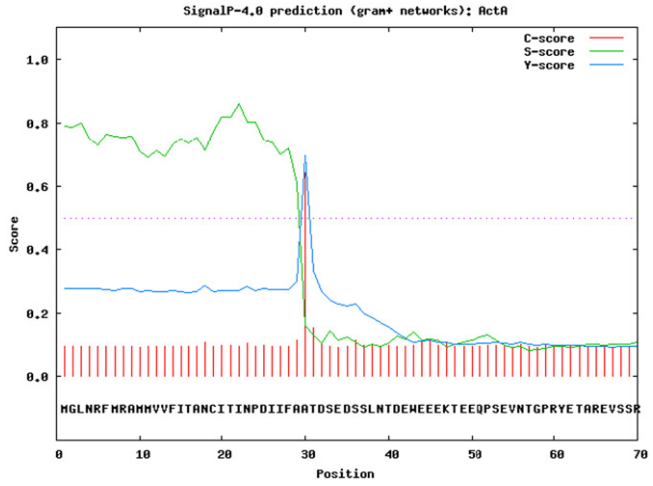

Fig. 4. The graphical output from SignalP (neural network). Description of the scores: The S-score is reported for every single amino acid position in the submitted sequence, with high scores indicating that the corresponding amino acid is part of a signal peptide. The C-score is the "cleavage site" score. C-score should be significantly high at the cleavage site. The cleavage site is assigned from the Y-score where the slope of the S-score is steep and a significant C-score is found. The signal peptide prediction is consistent with the database annotation.

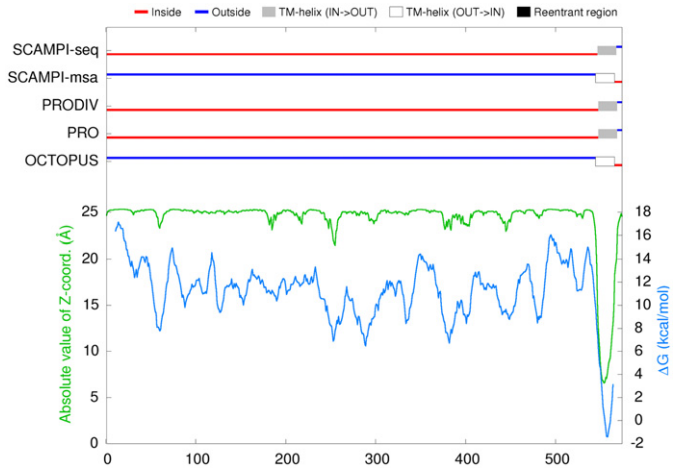

Fig. 5. Transmembrane topology results. Predictions are illustrated all in a single graph. TOPCONS graph is the consensus prediction. In addition, predicted Z-coordinates, predicted ΔG-values and reliability scores are given for each position in the sequence.

Table 1
Potential linear B-cell epitopes. Overlapped results of BepiPred and ABCpred.

Rank	Sequence	Start position	Score
1	SRDIEELEKSNKVKNT	38	0.88
2	LPETPMLLGFNAPATS	247	0.86
3	SEVNTGPRYETAREVS	22	0.84
3	GKWVRDKIDENPEVKK	192	0.84
3	DSELESLTYPDKPTKA	125	0.84
4	KKIKDAGKWVRDKIDE	186	0.83
5	KASVTDTSESDLDSSM	147	0.80
5	EWEEEKTEEQPSEVNT	11	0.80
5	DSAAEIKKRRKAIASS	109	0.80
6	PAIQVERRHPGLPSDS	95	0.79
6	PPTEDELEIMRETAPS	272	0.79
6	SADESTPQPLKANQQP	164	0.79
6	PTKATKKKVAKASVTD	137	0.79
7	AGLIDQLLTKKKNEEV	214	0.78
8	LKAKAEKGPNINNNNS	62	0.74
8	APATSEPSSFEFPPPP	258	0.74
8	PEVKKAIVDKSAGLID	203	0.74

these epitopes was calculated within collections of sequences of ActA in *L. monocytogenes* set at 80% of sequence identity (Table 2). Also these values were calculated within ActA sequences in two other species, *L. ivanovii* (Table 3) and *L. seeligeri* (Table 4) at 60% identity. All the linear epitopes were present in *L. monocytogenes* and none of them in *L. ivanovii* and *L. seeligeri* even at the level of 60% identity.

3.6. Other properties of construct

The construct sequence has a 54 percent chance of solubility when over expressed in *E. coli*. The overall prediction for the antigenicity of the construct was 0.60.

4. Discussion

Bioinformatics is one of the promising and standard approaches for selection of specific and immunogenic epitopes. A combination of *in silico* epitope mapping, in vitro, and in vivo verification, accelerates the detection process by approximately 10–20-fold (Amani et al., 2009). In recent decade, researchers take advantage of using in silico tools in clinical trials (Clermont et al., 2004), such as vaccine design (Davies and Flower, 2007; De Groot et al., 2001; Jahangiri et al., 2011; Korber et al., 2006) discovery of cancer or infectious diseases diagnostic agents (Aagaard et al., 2003; Bannantine et al., 2002; Leerkes et al., 2002; Roukos, 2009) identification and characterization of novel genes and proteins (Cai et al., 2011; Tomás-Roca et al., 2011), drug design or find targets for drugs (Shukla and Dixit, 2011). Validity of *in silico* approaches was confirmed experimentally by various authors. Zhang et al. (Zhang et al., 2002) identified several specific epitopes on a human adenovirus antigen using genomic alignment tools, antigenicity and 3-D structure prediction programs. Most of the predicted epitopes were originated in the prepared synthetic peptides and recombinant proteins. In order to find specific and immunogenic antigen for the serodiagnosis of *Chlamydia trachomatis* infections, Olfa et al. (Olfa et al.) used bioinformatics for analyzing the OmcB protein of *Clamydia tracomatis*. Further ELISA tests with several clinical specimens validated their *in silico* findings; their results confirmed the usefulness of bioinformatic tools for identifying species specific regions in an immunodominant antigen. We recently introduced an agent for precise identification of *Acinetobacter baumannii* using in silico approaches (Rahbar et al., 2011). Biofilm associated protein in *Acinetobacter baumannii* was bioinformatically analyzed in our laboratory and the antigenic regions of the protein was proposed as a vaccine candidate (Rahbar et al., 2010). This was followed by confirmation in the laboratory experiments (Fattahian et al., 2011). Current methods for identification of *L. monocytogenes* rely on physiological, biochemical, and molecular methods. The ACCUPROBE® LISTERIA MONOCYTOGENES CULTURE IDENTIFICATION TEST is a DNA probe test which utilizes the technique of nucleic acid hybridization for the identification of *L. monocytogenes* isolated from culture. A molecular method based on restriction fragment length polymorphism (RFLP) of PCR-amplified fragments of the 23 S rRNA gene was designed to identify *Listeria* strains to the species level (Paillard et al., 2003). Microarray-based assay, a general tool for identification and characterization of bacterial pathogens, was applied by Volokhov et al. (Volokhov et al., 2002) for detection and discrimination of six species of the *Listeria* genus: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. This method allowed unambiguous identification of all six *Listeria* species based on sequence differences in the *iap* gene. Two independent lines of monoclonal antibody which specifically

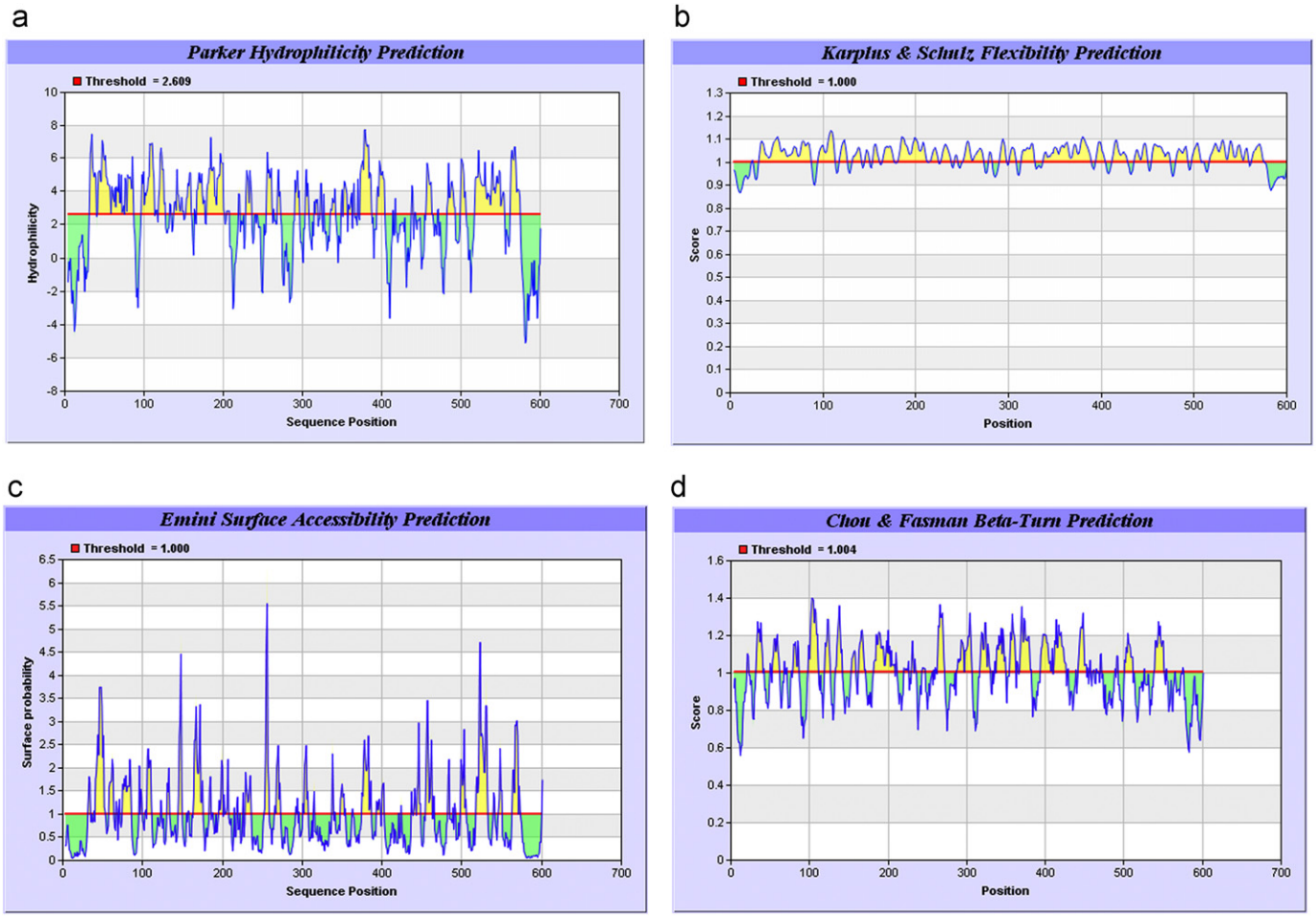

Fig. 6. Important properties for B cell epitope prediction. Hydrophilicity (a), Flexibility (b), Surface accessibility (c), Beta-turn (d) plots. Green color denotes unfavorable regions related to the properties of interest. Yellow colors are above the threshold sharing higher scores. Green and yellow colors indicate the below and above the threshold scores respectively. Horizontal red line is the threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Epitope conservancy among ActA sequences of *L. monocytogenes* at identity level of 80%.

No.	Epitope sequence	Epitope length	Percent of protein sequence matches at identity $\geq 80\%$	Minimum identity (%)	Maximum identity (%)
1	SRDIEELEKSNKVKNT	16	39.13% (9/23)	18.75	100.00
2	LPETPMLLGFNAPATS	16	82.61% (19/23)	18.75	100.00
3	SEVNTGPRYETAREVS	16	30.43% (7/23)	18.75	100.00
4	GKWVRDKIDENPEVKK	16	69.57% (16/23)	18.75	100.00
5	DSELESLTYPDKPTKA	16	56.52% (13/23)	25.00	100.00
6	KKIKDAGKWVRDKIDE	16	69.57% (16/23)	18.75	100.00
7	KASVTDTSESDLDSSM	16	47.83% (11/23)	25.00	100.00
8	EWEEEKTEEQPSEVNT	16	30.43% (7/23)	12.50	100.00
9	DSAAEIKKRRKAIASS	16	56.52% (13/23)	18.75	100.00
10	PAIQVERRHPGLPSDS	16	56.52% (13/23)	18.75	100.00
11	PPTEDELEIMRETAPS	16	73.91% (17/23)	12.50	100.00
12	SADESTPQPLKANQQP	16	69.57% (16/23)	18.75	100.00
13	PTKATKKKVAKASVTD	16	30.43% (7/23)	18.75	100.00
14	AGLIDQLLTKKKNEEV	16	69.57% (16/23)	18.75	100.00
15	LKAKAEKGPNINNNNS	16	56.52% (13/23)	18.75	100.00
16	APATSEPSSFEFPPPP	16	82.61% (19/23)	12.50	100.00
17	PEVKKAIVDKSAGLID	16	69.57% (16/23)	18.75	100.00

recognizes *L. monocytogenes* p60, and p6017 proteins was reported (Yu et al., 2004)., The same researchers developed efficient sandwich enzyme-linked immunosorbent assay (ELISA) systems which can specifically identify *L. monocytogenes* or generally detect *Listeria* species. ActA, in addition to its role in the bacterial entry (Suárez et al., 2001), is one of the virulence factors playing roles in the spread of the pathogen in host cells (Vazquez-Boland et al., 2001a), phagosome disruption (Poussin and Goldfine, 2009) and autophagy subversion (Yoshikawa et al., 2009b). Several potential linear B-cell epitopes were predicted by online softwares. Conservancy of predicted linear B-cell epitopes among several sequences is a support for specificity of these

Table 3
Epitope conservancy among ActA sequences of *L. ivanovii* at identity level of 60%.

No	Epitope sequence	Epitope length	Percent of protein sequence matches at identity ≥60%	Minimum identity (%)	Maximum identity (%)
1	SRDIEELEKSNKVKNT	16	25% (1/4)	25.00	62.50
2	LPETPMLLGFNAPATS	16	0.00% (0/4)	18.75	56.25
3	SEVNTGPRYETAREVS	16	0.00% (0/4)	18.75	31.25
4	GKWVRDKIDENPEVKK	16	0.00% (0/4)	25.00	31.25
5	DSELESLTYPDKPTKA	16	0.00% (0/4)	25.00	37.50
6	KKIKDAGKWVRDKIDE	16	0.00% (0/4)	25.00	37.50
7	KASVTDTSESDLDSSM	16	0.00% (0/4)	12.50	37.50
8	EWEEEKTEEQPSEVNT	16	0.00% (0/4)	18.75	37.50
9	DSAAEIKKRRKAIASS	16	0.00% (0/4)	25.00	56.25
10	PAIQVERRHPGLPSDS	16	0.00% (0/4)	25.00	31.25
11	PPTEDELEIMRETAPS	16	0.00% (0/4)	18.75	50.00
12	SADESTPQPLKANQQP	16	0.00% (0/4)	25.00	43.75
13	PTKATKKKVAKASVTD	16	0.00% (0/4)	31.25	37.50
14	AGLIDQLLTKKKNEEV	16	0.00% (0/4)	31.25	31.25
15	LKAKAEKGPNINNNNS	16	0.00% (0/4)	25.00	37.50
16	APATSEPSSFEFPPPP	16	0.00% (0/4)	25.00	50.00
17	PEVKKAIVDKSAGLID	16	0.00% (0/4)	18.75	37.50

Table 4
Epitope conservancy among ActA sequences of *L. seeligeri* at identity level of 60%.

No.	Epitope sequence	Epitope length	Percent of protein sequence matches at identity ≥60%	Minimum identity (%)	Maximum identity (%)
1	SRDIEELEKSNKVKNT	16	0.00% (0/5)	31.25	50.00
2	LPETPMLLGFNAPATS	16	0.00% (0/5)	25.00	37.50
3	SEVNTGPRYETAREVS	16	0.00% (0/5)	25.00	37.50
4	GKWVRDKIDENPEVKK	16	60.00% (3/5)	25.00	62.50
5	DSELESLTYPDKPTKA	16	0.00% (0/5)	25.00	37.50
6	KKIKDAGKWVRDKIDE	16	0.00% (0/5)	25.00	56.25
7	KASVTDTSESDLDSSM	16	0.00% (0/5)	31.25	37.50
8	EWEEEKTEEQPSEVNT	16	0.00% (0/5)	37.50	37.50
9	DSAAEIKKRRKAIASS	16	0.00% (0/5)	25.00	37.50
10	PAIQVERRHPGLPSDS	16	0.00% (0/5)	18.75	37.50
11	PPTEDELEIMRETAPS	16	0.00% (0/5)	25.00	43.75
12	SADESTPQPLKANQQP	16	0.00% (0/5)	25.00	31.25
13	PTKATKKKVAKASVTD	16	0.00% (0/5)	25.00	50.00
14	AGLIDQLLTKKKNEEV	16	60.00% (3/5)	25.00	68.75
15	LKAKAEKGPNINNNNS	16	0.00% (0/5)	25.00	37.50
16	APATSEPSSFEFPPPP	16	0.00% (0/5)	31.25	37.50
17	PEVKKAIVDKSAGLID	16	60.00% (3/5)	25.00	81.25

epitopes to the pathogen (Table 2). BLAST search revealed ActA specific to *L. monocytogenes*. Since this protein has no significant sequence similarity to other protein sequences in other bacteria, it could be assumed that probability of cross reactions of antibodies raise against the construct which may lower the specificity of serodiagnostic test is minimal to nil. Furthermore, importance of the virulence factor in pathogenicity of the bacterium is an evidence for existence of the protein in the all pathogen strains. Alignment results showing no significant differences among more than 220 protein sequences of ActA lead to this idea that all pathogenic strains could be detected by specific antibodies triggered against ActA. B cell epitopes share several properties such as hydrophilicity, accessibility, antigenicity and flexibility. Another important parameter is the secondary structure elements. ActA contains repeated modules (Fig. 1) and is an acidic protein with pI of about 5 attributable mostly to the abundance of Glu (10.9%)+Asp (5.6%). The acidic nature and repeated modules are attractive for B-cell responses (Kemp et al., 1987; Reeder and Brown, 1996; Reza Rahbar et al., 2010; Skeiky et al., 1994). The main difference found in the number of repeats can be indicative of involvement of these regions in speed of bacterial movement (Smith et al., 1996). Proline-rich repeats are accessible and flexible with Beta-turn secondary structure (Fig. 6). The highest score of B cell epitope prediction is related to the repeat region

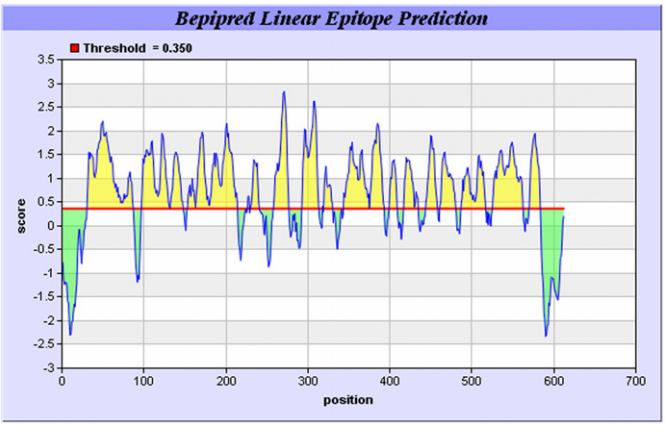

Fig. 7. Graphical result of Bepipred prediction for linear B cell epitopes. Yellow color denotes linear B cell epitopes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(amino acids 258–313) (Fig. 7) with predicted antigenicity of 0.5. Overall, this region could probably induce B cell responses efficiently to trigger antibodies. Flexiblity, beta turn and solvent accessibility analyses suggest that maximum of about 30 residues

from N and C terminals of the protein are not within the threshold and that all linear B Cell epitopes are located within 31–576 (Figs. 6 and 7). These suggest that none of the 30 residual regions of N and C terminals is favorable for selection. This selection is in agreement with other researchers (Cossart and Lecuit, 1998; Poussin and Goldfine, 2009; Vazquez-Boland et al., 2001a) who showed that N-terminal and central domains of ActA as functional and exposed to host cells. About 130 amino acids (aa) of N-terminal are involved in actin nucleation (Poussin and Goldfine, 2009) of which the first 28 (aa 31–58) are involved in phagosome disruption. The role of ActA in phagosome disruption suggests that amount of ActA expressed prior to disruption is sufficient for this purpose (Poussin and Goldfine, 2009). Involvement of ActA in epithelial cell invasion supports this issue (Poussin and Goldfine, 2009; Suárez et al., 2001). N-terminal amino acids of ActA are highly charged. Analysis of ActA (Fig. 6) from B cell epitope prediction point of view revealed that most of amino acids 31–320 were hydrophile, surface accessible and flexible most of which take part in Beta-turn structure. Theoretical pI and antigenicity of this region is 4.82 and 0.60 respectively. A BLAST search for this region as a query against non redundant protein data bank showed the region specific for ActA. So, antibodies raised against this region could also be used for detection of the antigen in addition to blockade of ActA functions such as phagosome disruption and decrease pathogenicity. Identifying clinical signs, and pathogens or factors (trauma, toxins, environment, etc.) that are known to produce these symptoms are golden rule of clinical disease diagnosis test. In everyday language, the best test will be the quickest, cheapest, and most reliable test available to meet these goals.

In conclusion N-terminal and central domains of the protein sequence including repeat modules play pivotal roles in functions of ActA and consequently the pathogen. These regions are also appropriate from B cell epitope points of view. Hence antibodies could be triggered against these regions. These properties make our construct (amino acids 31–320 of reference sequence of ActA) an ideal agent for development of an immunological detection system. ActA extracted sequence which introduced herein could offer a useful, rapid and cheap diagnostic test that can be introduced conveniently and immediately to the patient. This expedites immediate clinical management decisions on such an important issue.

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