Journal of Microbiological Methods 106 (2014) 40–46

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Antigenicity and immunogenicity of fused B-subunit of heat labile toxin of *Escherichia coli* and colonization factor antigen I polyepitopes

Nastaran Sadat Savar [a,*], Amir Dashti [a], Elham Darzi Eslam [a], Ali Jahanian-Najafabadi [b], Anis Jafari [a,*]

[a] Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran
[b] Department of Pharmaceutical Biotechnology, and Bioinformatics Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences and Health Services, Isfahan, Iran

ARTICLE INFO

Article history:
Received 16 May 2014
Received in revised form 27 July 2014
Accepted 28 July 2014
Available online 7 August 2014

Keywords:
Colonization factor antigen
Enterotoxigenic *Escherichia coli*
Fusion protein
Polyepitopes
Heat-labile enterotoxin B subunit

ABSTRACT

Linear B-cell epitopes (^{93}AKEFEAAAL^{101} and ^{66}PQLTDVLN^{73}) of CfaB were genetically fused to *ltb-(gly)$_5$-cfaB$_{(1-25)}$*. Sera of rabbits immunized with fusion proteins reacted strongly with solid-phase bound ETEC bacteria bearing CFA/I fimbriae. Sera failed to agglutinate or inhibit hemagglutination promoted by CFA/I-positive strain which may be due to solvent inaccessibility of epitope residues on intact fimbriae.

1. Introduction

Enterotoxigenic *Escherichia coli* is a major cause of infant diarrhea in developing countries and travelers to these regions. One of the main difficulties in developing a vaccine against enterotoxigenic *E. coli* (ETEC) is that it should provide protection against a wide range of virulent strains (Cegelski et al., 2008; Fleckenstein et al., 2013). ETEC causes diarrhea through the action of heat-labile (LT) and heat-stable (ST) enterotoxins, which are secreted either alone or together to intestinal epithelia after bacterial adherence through colonization factors (CFs) (Croxen and Finlay, 2010). The B subunit of heat labile enterotoxin (LTB) of ETEC elicits antitoxin response and could be considered as a potent immunogen in designing new vaccines (Turner et al., 2011).

Bacterial colonization of host tissues plays a crucial role in pathogenesis of every bacterial disease. Impairing this ability represents an ideal defense approach due to its importance in the early stages of the infectious process (Cegelski et al., 2008). ETEC expresses one or more of over 25 different CFs that mediate bacterial adhesion to the intestinal cells resulting in a large variation in virulence factor profiles in clinical isolates (Sjöling et al., 2007).

Epidemiological and human challenge studies have provided strong evidence showing that the antibody produced against CFs is protective and nearly all the candidate vaccines developed in the past three decades, have included a heat-labile enterotoxoid and one or more CFs, but none has performed well enough to be approved and released for use (Fleckenstein et al., 2013; Holmgren et al., 2013). Development of strains expressing more than one CF or using potentially protective epitopes that might elicit effective immunity has been proposed as a means of overcoming the shortcomings of the present candidate vaccines (Tobias et al., 2010). The use of selected epitopes instead of the complete antigen for generation of specific immune responses represents a new strategy for development of a potentially useful immunogen (Ruan et al., 2014; Zhou et al., 2009).

Colonization factor antigen I (CFA/I), the archetype of eight genetically related ETEC colonization factors, is the most epidemiologically relevant ETEC fimbrial adhesin and a 4-gene operon is required for its assembly. CFA/I consists of flexible rods 7–8 nm in diameter composed of >1000 copies of the major pilin subunit CfaB and 1 or a few copies of the tip-residing adhesive minor subunit CfaE (Jansson et al., 2006; Li et al., 2009; Mu et al., 2008).

Experimental and computational analyses have predicted several linear B-cell epitopes within the B subunit and the combined or partially overlapping B- and T-cell epitope domains which have been identified to include amino acids 3–45, 66–101 and 124–142 [Cassels et al.,

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; LTB, heat labile enterotoxin B subunit; CF, colonization factor; CFA/I, colonization factor antigen I.

* Corresponding authors at: Department of Molecular Biology, Pasteur Institute of Iran, Pasteur Ave., Tehran 13164, Iran. Tel.: +98 21 66 95 33 11 20.
E-mail addresses: nstrn_savar@yahoo.com (N.S. Savar), anisjafari@yahoo.com (A. Jafari).

http://dx.doi.org/10.1016/j.mimet.2014.07.035
0167-7012/

1992]. Antigenicity and immunogenicity of some of these epitopes have previously been evaluated (Bouzari et al., 2010; Cassels et al., 1997; Luna et al., 1997a, 1997b; Rudin and Svennerholm, 1996).

A major drawback of epitope-based vaccines is their inability to induce a strong immune response after immunization, requiring appropriate adjuvants/carriers to augment immunogenicity. The B subunit is the non-toxic binding domain of LT consisting of five identical 103 aa peptides forming a potent immunogenic donut-shaped pentamer which binds to GM_1 ganglioside receptors (Mudrak and Kuehn, 2010). Various studies have shown that LTB has adjuvant immunostimulatory activities which require the ability to bind to GM_1 gangliosides (Connell, 2007).

In this study experimentally identified linear B-cell epitope ^{93}AKEFEAAAL^{101} (within a 92–102 amino acid (aa) segment) was used singly and epitope ^{66}PQLTDVLN^{73} (in a 63–75 aa stretch) (Cassels et al., 1992) was utilized after being joined to aa 92–102. A chimeric gene containing the N-terminal first 25 amino acid-coding fragment and the B subunit of LT linked by a pentaglycine linker had been previously constructed (Bouzari et al., 2010) and the addition of the new epitopes to this ensemble gave rise to constructs expressing LTB-$(gly)_5$-$CfaB_{(1–25::92–102)}$ and LTB-$(gly)_5$-$CfaB_{(1–25::63–75::92–102)}$.

The antigenicity and immunogenicity of the new fusion proteins designated LTB-$CfaB_{2epi}$ and LTB-$CfaB_{3epi}$ respectively were evaluated. Considering the importance of CfaB polymerization on antigenicity and recognition of selected epitopes by antibodies as well as the ability of the antibody raised against the hybrid proteins to recognize the native form of fimbriae in agglutination reactions, in silico accessibility and flexibility of the amino acid residues in monomeric and polymeric phases were also investigated.

2. Materials and methods

2.1. Bacterial strain and culture condition

The wild-type CFA/I-positive ETEC strain used in this study for PCR amplification of epitopes, polyclonal antibody production, and as whole-cell bacteria in coating ELISA plates for evaluation of humoral antibody response had been isolated from a diarrheal patient during a previous study (Shahrokhi et al., 2011). *ltb* and *cfab* genes were detected by PCR and confirmed by sequencing and production of CFA/I fimbriae was established by slide agglutination using anti-CFA/I monoclonal antibody (kindly provided by Professor Svennerholm, Microbiology and Immunology Department, Göteborg University, Göteborg, Sweden).

For CFA/I expression ETEC strain was cultured on CFA agar containing 20 g/l agar, 10 g/l casamino acids (Difco, USA), 1.5 g/l yeast extract, 0.05 g/l $MgSO_4$ and 0.005 g/l $MnCl_2$ (Merck, Germany). To avoid plasmid loss, sub-culturing was limited to 3–4 passages on CFA agar and the isolate was stored at −70 °C.

2.2. Design, construction and expression of the fusion proteins

Antigenicity scores of individual amino acid residues of ^{66}PQLTDVLN^{73} and ^{93}AKEFEAAAL^{101} in CfaB, rLTB-$CfaB_{2epi}$ and rLTB-$CfaB_{3epi}$ were calculated using IBED analysis resource (Kolaskar and Tongaonkar, 1990).

Recombinant DNA manipulations were performed according to the standard protocols (Sambrook et al., 1989). Construction of *ltb-cfaB*$_{(1–25)}$ has been described previously (Bouzari et al., 2010). Primers for amplification and fusion of fragments containing^{92}TAKEFEAAALG^{102} and ^{63}ADTPQLTDVLNST^{75} linear B cell epitopes of CfaB subunit by overlap PCR, were designed based on the nucleotide sequence of strain MB/ETEC400-2 *cfaB* gene (GenBank accession no.; FJ215858) and synthesized (Metabion, Germany). *ltb*F and R_2 primers were used to amplify *ltb-cfaB*$_{(1–25)}$ fragment, while F1 and $R_{epi2/3}$ primers were utilized to amplify the region containing amino acids 92–102 (Table 1).

Each fragment was amplified separately in a 25 μl volume with a final concentration of PCR buffer (1×), $MgCl_2$ (1.5 mM), dNTPs (200 μM), primers (20 pmol) and *Pfu* DNA polymerase (2.5 U) in a 30 cycle program with 95 °C, 5 min (1 cycle), 95 °C, 45 s, 60 °C, 45 s and 72 °C, 30 s. Purified PCR products corresponding to *ltb*-$(gat)_5$-*cfaB*$_{(1–25)}$ and amino acids 92–102 were mixed (100 ng each) and used as template for overlap PCR using primers *ltb*F/$R_{epi2/3}$ (Table 1). The following program was used for fusion of these fragments; 95 °C, 5 min (1 cycle), 95 °C, 45 s, 45 °C, 45 s and 72 °C, 30 s (5 cycles), and a 30 cycle program as described above. For assembly of rLTB-$CfaB_{3epi}$ nucleotide sequence corresponding to aa 63–75 and 92–102 was amplified using primers F2/R3 and F4/$R_{epi2/3}$ respectively (Table 1) and tandemly fused using the same program and the same procedure as mentioned above. The assembled fragment was then joined to the C-terminal end of the gene expressing LTB-$CfaB_{(1–25)}$ using primers *ltb*-F/$R_{epi2/3}$ and the program outlined above. Restriction sites for *Nco*I and *Xba*I in primers *ltb*-F and $R_{epi2/3}$ are underlined (Table 1).

Table 1
Amino acid and oligonucleotide sequences of the two B-cell epitopes of CfaB and primers used for their amplification. Restriction sites for *Nco*I and *Xba*I enzymes are underlined.

Templates/primers	Sequence
ltb-$(gat)_5$-*cfaB*$_{(1–25)}$	
ltb-F	5′-gac cca tgg ctc ctc agt cta tta ca-3′
R2 (355–343/75–63)	5′-ttc ttt ggc agt cag agc att gcc-3′
Amino acids: 92–103	^{92}TAKEFEAAALG^{102}
Nucleotides: 343–376	5′-act gcc aaa gaa ttt gaa gct gct gct ttg ggt-3′
F1 (63–75/343–355)	5′-ggc aat gct ctg act gcc aaa gaa-3′
$R_{epi2/3}$ (376–360)	5′-ccg tct aga tcc caa agc agc agc t-3′
Amino acids: 63–75	^{63}ADTPQLTDVLNST^{75}
Nucleotides: 256–295	5′-gct gat aca cca cag ctt aca gat gtt ctg aat tca act-3′
F2 (63–75/256–268)	5′-ggc aat gct ctg gct gat aca cca-3′
R3 (355–343/295–283)	5′-ttc ttt ggc agt agt tga att cag-3′
F4 (283–295/343–354)	5′-ctg aat tca act act gcc aaa gaa-3′

The fused fragments were double digested with *Nco*I and *Xba*I (Fermentas, Lithuania), gel purified and ligated with similarly treated pBAD/gIII expression vector which contains an L-arabinose inducible promoter and C-terminal c-myc and histidine tags (Invitrogen, USA). The pBAD-*ltb-cfaB*$_{2epi}$ and pBAD-*ltb-cfaB*$_{3epi}$ constructs were transformed into competent *E. coli* Top10 (Invitrogen, USA), sequence verified (GATC, Germany) and were induced using L-arabinose (Merck, Germany). The expressed proteins were eluted from polyacrylamide gels using a mini-eluter (BioRad, USA) or purified by Ni-NTA magnetic beads according to manufacturer's instructions (Qiagen, Germany). SDS-PAGE was used to assess the purity and Bradford assay (Bradford, 1976) to quantify the purified proteins.

Eluted proteins were treated with Triton X-114 (Sigma, USA) to remove lipopolysaccharide (LPS) contamination (Aida and Pabst, 1990). Briefly purified proteins were mixed with 1% (v/v) Triton X-114 and chilled on ice for 10 min and vortexed. The mixture was then incubated at 37 °C for 15 min and centrifuged at 10,000 ×*g* for 2 min and the aqueous phase aspirated. The collected phase was passed through Econo-Pac columns according to manufacturer's instructions (BioRad, USA) and tested for LPS contamination by Limulus amoebocyte lysate (LAL) test (QC Department, Pasteur Institute of Iran).

Polyclonal antibodies against the fusion proteins were raised in white female New Zealand rabbits weighing approximately 2 kg (Pasteur Institute of Iran) as previously described (Bouzari et al., 2010) and against ETEC CFA/I-positive isolate as described by Favre et al. (2006).

2.3. Western blotting

Recombinant proteins (rLTB-$CfaB_{2epi}$, rLTB-$CfaB_{3epi}$, rLTB and rCfaB) were electrophoresed in 15% SDS-polyacrylamide gels, electrotransferred to nitrocellulose membrane (Schleicher and Schuell,