Construction of a recombinant intergenus multidomain chimeric protein for simultaneous expression of haemolysin BL of *Bacillus cereus*, listeriolysin O of *Listeria monocytogenes* and enterotoxin B of *Staphylococcus aureus*

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Haemolysin BL (HBL) of *Bacillus cereus*, listeriolysin O (LLO) of *Listeria monocytogenes* and enterotoxin B (SEB) of *Staphylococcus aureus* are among the major toxin components contributing to the pathogenicity of these organisms in foodborne illnesses. In this study, an intergenus non-toxic multidomain fusion protein (r-HLE) was generated with specificity for HBL, LLO and SEB. The fusion gene (*r-hle*) comprising the conserved regions of *hblD* and the *hly* and *entB* genes was codon-optimized for expression in *Escherichia coli* and encoded a 50 kDa recombinant multidomain chimeric protein (r-HLE). Hyperimmune antiserum raised against r-HLE specifically reacted with the L₁ (38 kDa) component of the HBL complex of *B. cereus*, LLO (58 kDa) of *L. monocytogenes* and SEB (28 kDa) of *S. aureus* during Western blot analysis when tested on standard strains. During testing on isolates, the antiserum again identified the appropriate toxin molecules and was highly specific to the relevant bacterial species. The antigenicity of the SEB component of the r-HLE protein was also confirmed using a commercially available TECRA kit. The described procedure of creating a single antigenic molecule carrying components of three different toxins whilst still retaining the original antigenic determinants of individual toxins will be highly advantageous in the development of rapid, reliable and cost-effective immunoassays.

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INTRODUCTION

Bacillus cereus, *Listeria monocytogenes* and *Staphylococcus aureus* are closely related bacterial pathogens with the potential to cause clinical infections and foodborne infections by secreting a variety of extracellular toxins (Fsihi *et al.*, 2001; Becker *et al.*, 2003; Schoeni & Wong, 2005; Ler *et al.*, 2006). Among the array of toxin components possessed by these pathogens, haemolysin BL (HBL) of *B. cereus*, listeriolysin O (LLO) of *L. monocytogenes* and enterotoxin B (SEB) of *S. aureus* are important virulence factors that play vital roles in the pathogenicity of these organisms. HBL is a unique membrane-lytic toxin and has a variety of toxic activities including haemolysis, cytotoxicity, vascular permeability, dermonecrosis, enterotoxicity and ocular toxicity (Beecher & Macmillan, 1991; Prüss *et al.*, 1999; Gaur *et al.*, 2001; Callegan *et al.*, 2002). SEB is the primary cause of

staphylococcal food poisoning and is a potent mitogen that elicits life-threatening polyclonal T-cell proliferation and cytokine production at very low concentrations (Fraser, 1989; Rajagopalan *et al.*, 2006). At low serum concentrations, SEB can trigger toxic shock, profound hypotension and multiorgan failure, and is a recognized biowarfare molecule (Anderson & Tary-Lehman, 2001; Ulrich *et al.*, 2001). *L. monocytogenes*, the aetiological agent of listeriosis, possesses a bacterial pore-forming toxin, LLO, generally believed to be the central molecule in host-pathogen interactions, responsible for lysing the vacuolar membrane and allowing the organism to escape into the cytoplasm of the cell (Gholizadeh *et al.*, 1997; Slutsker & Schuchat, 1999; Dramsi & Cossart, 2003).

Numerous immunoassays have been reported for the detection of these toxins individually among pathogenic strains of the organisms, but, owing to their ubiquitous presence in nature, the co-existence of these organisms during foodborne illnesses or clinical infections may cause confusion and possible misinterpretation during the examination of clinical/food samples and could lead to delay in the initiation of treatment or control procedures.

Abbreviations: HBL, haemolysin BL; LLO, listeriolysin O; SEB, enterotoxin B.

The GenBank/EMBL/DDBJ accession number for the sequence of the r-HLE protein constructed in this study is EU912538.

We recently reported a novel approach using a chimeric protein that entailed joining together truncated portions of the HBL toxin, i.e. the L₁ and L₂ components, for their simultaneous detection in virulent *B. cereus* strains (Kumar *et al.*, 2008). This approach permits the production of the desired multicomponent antigens as a single chimeric fusion protein for expression in an *Escherichia coli* host to obtain high-epitope-density antigens with enhanced detection sensitivity and to serve as a cost-effective alternative to the preparation of whole toxin antigen. We used a similar approach in the present study to develop a recombinant intergenus multidomain protein with truncated portions of HBL, LLO and SEB, termed r-HLE, for possible utilization in detection/diagnosis.

METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 3. All bacteriological media used in this study were from Hi-Media Laboratories. Reference strains were obtained from the American Type Culture Collection (ATCC), the National Collection of Industrial Microorganisms (NCIM) and the Defence Research and Development Establishment (DRDE). Isolation of these three important foodborne micro-organisms was carried out by standard conventional isolation and biochemical identification procedures. All of the standard strains and isolates were checked for the presence of the genes under study (*entB* in *S. aureus*, *hly* in *Listeria* and *hblD* in *B. cereus*) using PCR primers developed in house (Table 1).

Plasmid construction. Conserved regions of the *hblD*, *hly* and *entB* genes were amplified by PCR using *B. cereus* ATCC 14579, *L. monocytogenes* ATCC 15313 and *S. aureus* ATCC 51740 whole genomic DNA as template (Sambrook *et al.*, 1989). PCR was performed in a 25 µl reaction volume containing 400 nM each primer, 200 µM each dNTP, 1.0 U *Pfu* polymerase (MBI Fermentas), 1.5 mM MgSO₄ in 1 × PCR buffer (MBI Fermentas) and 50 ng template DNA. The amplified PCR products were separated on 1% agarose gel (Sigma) and subjected to restriction digestion with their respective enzymes (MBI Fermentas). The fusion gene was constructed as shown in Fig. 1. The resultant 1.37 kb gene (*r-hle*) was ligated into the *Bam*HI and *Hind*III sites of the bacterial expression vector pRSET A, which was also cut by both enzymes to generate the plasmid pR-r-HLE. In this plasmid, the *r-hle* gene was inserted in frame with the ATG codon and a His₆-encoding sequence (at the 5' end) provided by the pRSET A vector to produce a protein with the expected size of 50 kDa. The ligated product was transformed into *E. coli* BL21(DE3)pLysS cells and selected by growth on ampicillin plates. Recombinant clones were subjected to direct colony PCR

screening, using the L₁ forward primer and EntB reverse primer to identify recombinants harbouring the synthetic *r-hle* gene (Table 2).

Expression and purification of the r-HLE chimeric protein. *E. coli* cells carrying the recombinant plasmid were grown at 37 °C in Luria-Bertani broth (100 ml) with 50 µg ampicillin µl⁻¹ at 200 r.p.m. in a 500 ml flask. When the culture OD₆₀₀ reached 0.6, IPTG (Sigma) was added to a final concentration of 1 mM. After 5 h of induction, cells were harvested by centrifugation at 4000 g for 20 min. All samples were examined by 12% SDS-PAGE.

As the r-HLE protein was mainly localized as inclusion bodies, the protein was purified under denaturing conditions. The pellet from 200 ml culture was resuspended in 5 ml buffer containing 6 M urea and 0.1 M sodium phosphate (pH 8.0). Cells were stirred at room temperature for 1 h. The lysate was centrifuged at 10 000 g for 30 min at 4 °C. The resulting supernatant was mixed with 1 ml Ni-NTA Superflow resin (Qiagen) that had been pre-equilibrated with the lysis buffer. This suspension was gently mixed for 1 h at room temperature and then packed into a column. After collecting the flowthrough, the column was washed with buffer containing 8 M urea, 0.1 M sodium phosphate and 0.01 mM Tris/HCl (pH 6.3) to facilitate the removal of contaminants. The recombinant protein was eluted with buffer containing 8 M urea, 0.1 M sodium phosphate and 0.01 mM Tris/HCl (pH 4.5). Refolding of r-HLE in 8 M urea in buffer A (20 mM Tris/HCl, pH 8.0) was achieved by sequential dialysis with 4 M urea and 2 M urea in buffer A, and finally with buffer A only. The peak fractions containing r-HLE were combined and dialysed against 7 vols 4 M urea in buffer A for 30 min at room temperature followed by one change of the dialysis solution, and then dialysed for an additional 30 min. The same procedure was repeated with 2 M urea in buffer A. The final dialysis was against buffer A with two changes of buffer for 30 min each and finally overnight dialysis at 4 °C.

Preparation of hyperimmune polyclonal antiserum.

Hyperimmune anti-r-HLE polyclonal antiserum was generated by intraperitoneal immunization of male BALB/c mice (6 weeks old) with affinity-purified r-HLE protein. Primary immunization of each animal was carried out with 50 µg purified r-HLE in Freund's complete adjuvant (Sigma). Two boosts of 10 µg each of r-HLE in Freund's incomplete adjuvant (Sigma) followed on days 14 and 28. The antibody reactivity of the immunized mice sera was measured using a plate ELISA (Engwell and Perlman). Hyperimmune serum obtained 14 days after the last boost was used for Western blot analysis as described below.

Exoprotein preparations. Exoprotein preparations were prepared from all three strains by 80% ammonium sulphate precipitation, and protein concentrations were determined using the Lowry method.

Western blot analysis. The affinity-purified r-HLE protein and toxin preparations from standard strains as well as isolates were

Table 1. Primers for detection of the *hblD*, *hly* and *entB* toxin genes

Primer*	Gene	Toxin	Sequence (5'→3')	Product size (bp)	GenBank accession no.
Hbl (F)	<i>hblD</i>	Haemolysin BL L ₁ component	CGCAAAAACAATGATTAAGC	256	AJ237785.1
Hbl (R)			CTCTTTTAGACCTGCCTTATCT		
Hly (F)	<i>hly</i>	Listeriolysin O	TGCAAGTCCTAAGACGCCAA	325	AY750900
Hly (R)			CCACACTTGAGATATATGCAGGA		
EntB (F)	<i>entB</i>	Enterotoxin B	CCAGATCCTAAACCAGATGAGTT	753	AY856382
EntB (R)			GTTTTTCGTTTGTCAGTTTGATG		

*F, Forward; R, reverse.

Fig. 1. Construction of the plasmid used for expression of the HBL fusion protein. Left panel: plasmid pRSET A, an expression vector containing the T7 promoter (P), a synthetic ribosome-binding site (R), a His₆ affinity tag sequence (H), an express epitope (EX), a multiple cloning site (MCS), a transcriptional terminator (T), an origin of replication (O) and an ampicillin-resistance gene (*amp*^r). BXSBBPPKNEBH in the MCS represents the sites for the restriction endonucleases *Bam*HI, *Xho*I, *Sac*I, *Bgl*II, *Pst*I, *Pvu*II, *Kpn*I, *Nco*I, *Eco*RI, *Bst*BI and *Hind*III, respectively. Right panel: *hblD* (encoding the L₁ component of HBL), *hly* (encoding LLO) and *entB* (encoding SEB) were amplified by PCR with new restriction sites added at the ends of the primers and cloned into the *Bam*HI and *Hind*III sites of pRSET A to generate the fusion gene construct *r-hle*.

separated by 12 % PAGE, transferred to a nitrocellulose sheet (Millipore) and immersed in TBS [150 mM NaCl, 50 mM Tris/HCl (pH 7.4)] containing 0.05 % Tween 20 and 5 % skimmed milk at 4 °C overnight to saturate the non-specific protein-binding sites. The antigen electroblotted onto the nitrocellulose membranes was incubated with the hyperimmune mouse serum at a 1 : 1000 dilution followed by horseradish peroxidase-conjugated anti-mouse IgG (Sigma) at a 1 : 1000 dilution. The positive bands were detected by immersing the sheet in a developing solution (70 mM sodium acetate, pH 6.2) containing 0.3 % diaminobenzidine tetrahydrochloride (Sigma) and 0.03 % H₂O₂ at room temperature for 5 min. The enzyme reaction was terminated by washing the sheet in 0.1 M H₂SO₄.

SEB analysis. The r-HLE protein was evaluated for the presence of native antigenic determinants of SEB using a TECRA VIA *Staphylococcus* enterotoxin kit (TECRA International). The enterotoxin assay was carried out as recommended by the manufacturer. Colour reactions developed by the TECRA kit were determined by measuring the *A*₄₁₄.

ELISA. To determine whether the anti-r-HLE antibody also bound native toxins, an antibody ELISA was performed according to the

method of Harlow & Lane (1988) using plastic 96-well microtitre plates (PolySorb; Nunc) as the solid phase. The plates were coated with 100 µl diluted antigen and incubated at room temperature for 2 h. Each well was washed twice in PBS, and the remaining wall area was blocked with 3 % BSA in PBS for 2 h at room temperature. A dilution of the anti-r-HLE antibody in 1 % BSA in PBS/0.05 % Tween was added and incubated for 2 h. Each well was washed three times with PBS/0.05 % Tween. Anti-sheep IgG conjugated to horseradish peroxidase was added and incubated for 1 h, washed three times and developed using *O*-phenylenediamine dihydrochloride (Sigma) and H₂O₂ in 0.15 M phosphate citrate buffer (pH 5.0), and the *A*₄₅₀ was measured.

Initial assays were performed to determine the antigen concentration that best discriminated between ELISA reactions of culture supernatants from confirmed toxin-producing organisms and non-toxin-producing organisms. Chequerboard titrations were performed with 25-, 50- or 200-fold serum dilutions and antigen concentrations per well of 5, 25, 50 and 100 ng. In subsequent assays to determine sensitivity and specificity, plates were coated with the predetermined antigen concentrations. Incubations were performed with 50- and 20 000-fold dilutions of primary sera and secondary antibody

Table 2. Primers for cloning the L₁ component of HBL and the LLO and SEB toxin genes

Primer*	Gene	Toxin	Sequence (5'→3')	Product size (bp)	GenBank accession no.
L ₁ (F)	<i>hblD</i>	Haemolysin BL L ₁ component	ACTACCGGATCCACTATTTCATG	575	AJ237785.1
L ₁ (R)			TGAAGTTGTGGTACCGTTGC		
LLO (F)	<i>hly</i>	Listeriolysin O	ATGGGTACCCTTCGCGCAATCAGTG	462	AY750900
LLO (R)			TCTGAGCTCATGGGAACCTCTGGTGTT		
Ent (F)	<i>entB</i>	Enterotoxin B	ATAGAGCTCACCAGATCCTAAACCAGATG	327	AY856382
Ent B (R)			TAGAAGCTTGTTTGTCAGTTTGATGCG		

*F, Forward; R, reverse.

Fig. 2. Electrophoretic analysis of r-HLE protein expression in *E. coli*. All samples were separated by 12 % SDS-PAGE. The gel was stained with Coomassie blue to visualize the protein components. Lanes: M, molecular mass standards; 1, BL21(DE3)pLysS cells; 2, uninduced *E. coli* BL21(DE3)pLysS cells with the vector containing the fusion gene; 3, cells expressing the HBL chimeric protein after 5 h; 4–7, HBL chimeric protein during the first, second, third and fourth elutions of Ni-NTA affinity chromatography, respectively.

conjugate, respectively. Individual culture supernatant samples were tested in duplicate and the means of the two measurements were calculated for analysis. Paired measurements that differed by greater than 10 % were retested. One positive control serum sample that reacted with all recombinant antigens and one negative control serum sample were included, in duplicate, on each plate as a quality control measure.

RESULTS

Design of a novel intergenus multidomain protein (r-HLE)

In order to create a multidomain protein that could be useful in simultaneous detection of HBL, LLO and SEB toxins, we focused on the conserved domains of the HBL, LLO and SEB components when selecting truncated regions. Care was taken during design of the primers for amplification of the *hblD* (L_1 component of the HBL complex), *hly* (LLO) and *entB* (SEB) genes to accommodate the open reading frame of each gene with regard to the

open reading frame of the preceding gene. The fusion gene, designated *r-hle*, containing the conserved domains of *hblD*, *hly* and *entB*, was designed to be in frame with the initiator codon and N-terminal His₆ tag-encoding sequence provided by the vector. The *r-hle* gene was predicted to encode a 50 kDa recombinant multidomain chimeric protein, r-HLE (Fig. 1). Restriction enzyme digestion of the recombinant plasmid revealed a 2.9 kb linearized vector and 1.37 kb insert as predicted. DNA sequence analysis of the plasmid indicated that the open reading frame of the insert was correct. *E. coli* BL21(DE3)pLysS cells were transformed with the recombinant plasmid.

Expression and purification of the r-HLE chimeric protein

The r-HLE chimeric protein was purified from inclusion bodies under denaturing conditions. Following affinity chromatography, the protein was found to be about 90 % pure with small amounts of degraded products and other impurities (Fig. 2). Peak fractions containing r-HLE in 8 M urea were pooled and dialysed sequentially against 4 and 2 M urea in buffer A and then with buffer alone. r-HLE appeared to be properly folded by this process as it remained soluble when no urea was present in the final dialysis.

Western blot analysis

Western blot analysis showed that mouse anti-r-HLE serum reacted strongly with all three native toxins (Fig. 3). Hyperimmune antiserum against r-HLE did not exhibit any cross-reactions with other organisms tested (Table 3).

SEB analysis

The r-HLE protein sample tested positive for SEB moieties using a TECRA VIA *Staphylococcus* enterotoxin kit (TECRA International).

Development of an ELISA

Although Western blot analysis, which separates out proteins, could clearly detect toxin-producing strains, it

Fig. 3. Western blot analysis of culture-free supernatants of *B. cereus* group, *Listeria* spp. and *S. aureus* standards and isolates. Lanes: 1, prestained molecular mass standards; 2, recombinant HLE chimeric protein; 3, *S. aureus* ATCC 159051; 4, *L. monocytogenes* ATCC 13932; 5, *B. cereus* ATCC 14579; 6, mixture of culture supernatants of *S. aureus*, *L. monocytogenes* and *B. cereus*; 7, *B. cereus* ATCC 10876; 8, *S. aureus* NCIM 2654; 9, *S. aureus* NCIM 2120; 10, *Listeria ivanovii*.

Table 3. ELISA, Western blot and PCR analysis of culture-free supernatants of *B. cereus*, *S. aureus* and *Listeria* standards and isolates

Organism	Source	ELISA	Western blot			PCR		
			HBL (L ₁)	LLO	EntB	hblD	hly	entB
<i>B. cereus</i> 14579	ATCC	+	+	—	—	+	—	—
<i>B. cereus</i> 10876	ATCC	+	+	—	—	+	—	—
<i>B. cereus</i> 11778	ATCC	—	—	—	—	—	—	—
<i>B. cereus</i> 2156	NCIM	+	+	—	—	+	—	—
<i>B. cereus</i> 2155	NCIM	—	—	—	—	+	—	—
<i>B. cereus</i> 2703	NCIM	—	—	—	—	+	—	—
<i>B. cereus</i> 2158	NCIM	—	—	—	—	—	—	—
<i>B. cereus</i> 2185	NCIM	+	+	—	—	+	—	—
<i>B. cereus</i> 2702	NCIM	—	—	—	—	—	—	—
<i>B. cereus</i>	DRDE	—	—	—	—	+	—	—
<i>B. cereus</i> 2322	NCIM	—	—	—	—	+	—	—
<i>B. cereus</i> 2701	NCIM	+	+	—	—	+	—	—
<i>Bacillus thuringiensis</i> 10792	ATCC	+	+	—	—	—	—	—
<i>B. thuringiensis</i> 5019	NCIM	+	+	—	—	+	—	—
<i>B. thuringiensis</i> 5117	NCIM	+	+	—	—	+	—	—
<i>B. thuringiensis</i> 5118	NCIM	—	—	—	—	+	—	—
<i>B. cereus</i> isolate 1	Milk	—	—	—	—	+	—	—
<i>B. cereus</i> isolate 2	Milk	+	+	—	—	—	—	—
<i>B. cereus</i> isolate 3	Soil	—	—	—	—	+	—	—
<i>B. cereus</i> isolate 7	Rice	—	—	—	—	+	—	—
<i>B. cereus</i> isolate 9	Soil	+	+	—	—	+	—	—
<i>B. cereus</i> isolate 11	Vegetable	—	—	—	—	+	—	—
<i>B. cereus</i> isolate 14	Meat	+	+	—	—	+	—	—
<i>B. cereus</i> isolate 17	Whey	+	+	—	—	+	—	—
<i>B. cereus</i> isolate 18	Whey	+	+	—	—	+	—	—
<i>S. aureus</i> 51740	ATCC	+	—	—	+	—	—	+
<i>S. aureus</i> 2654	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 2120	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 2120	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 2654	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 2492	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 2127	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 5021	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 2121	NCIM	+	—	—	+	—	—	+
<i>S. aureus</i> 2672	NCIM	—	—	—	—	—	—	—
<i>S. aureus</i> 2794	NCIM	—	—	—	—	—	—	—
<i>S. aureus</i> 2492	NCIM	—	—	—	—	—	—	—
<i>S. aureus</i> 2079	NCIM	—	—	—	—	—	—	+
<i>S. aureus</i> 2901	NCIM	—	—	—	—	—	—	—
<i>S. aureus</i> isolate 3	Clinical	+	—	—	+	—	—	+
<i>S. aureus</i> isolate 6	Clinical	+	—	—	+	—	—	+
<i>S. aureus</i> isolate 9	Food	+	—	—	+	—	—	+
<i>S. aureus</i> isolate M	Food	+	—	—	+	—	—	+
<i>S. aureus</i> isolate	Rabbit	+	—	—	+	—	—	+
<i>S. aureus</i> isolate	IVRI	+	—	—	+	—	—	+
<i>S. aureus</i> isolate TS	DRDE	+	—	—	+	—	—	+
<i>S. aureus</i> isolate	FRI	+	—	—	+	—	—	+
<i>L. monocytogenes</i> 13932	ATCC	+	—	+	—	—	+	—
<i>L. monocytogenes</i> 15313	ATCC	+	—	+	—	—	+	—
<i>L. monocytogenes</i> 835	MTCC	+	—	+	—	—	+	—
<i>L. ivanovii</i> 700402	ATCC	+	—	+	—	—	+	—
<i>L. monocytogenes</i> isolate	Milk	+	—	+	—	—	+	—
<i>L. monocytogenes</i> isolate	Milk	+	—	+	—	—	+	—
<i>L. monocytogenes</i> isolate	Rice	+	—	+	—	—	+	—

Table 3. cont.

Organism	Source	ELISA	Western blot			PCR		
			HBL (L ₁)	LLO	EntB	<i>hblD</i>	<i>hly</i>	<i>entB</i>
<i>L. monocytogenes</i> isolate	Rice	+	—	+	—	—	+	—
<i>L. monocytogenes</i> isolate	Clinical	+	—	+	—	—	+	—
<i>L. monocytogenes</i> isolate	Clinical	+	—	+	—	—	+	—
<i>Aeromonas hydrophila</i> 49140	ATCC	—	—	—	—	—	—	—
<i>Salmonella typhimurium</i> 2501	NCIM	—	—	—	—	—	—	—
<i>E. coli</i> 730	MTCC	—	—	—	—	—	—	—

was considered impractical for routine clinical use. An ELISA-based system was therefore developed that could be used in both clinical and food research laboratories. This would also have the advantage of quantifying the toxin (HBL, SEB and LLO) levels produced by different *B. cereus*, *S. aureus* and *L. monocytogenes* strains, which may be related to the varying severity of illness. Maximum absorbance values were observed at antigen concentrations of 30 ng ml⁻¹. The ratio of mean absorbance values between toxin-positive and control individual samples was not significantly different at 50-, 100- and 200-fold dilutions of the primary antiserum, and therefore a 50-fold dilution was chosen for subsequent assays.

Evaluation of the detection systems

The presence of toxin genes in all standard strains and isolates of *B. cereus*, *S. aureus* and *L. monocytogenes* was checked by PCR to compare the results of the Western blot and ELISA immunoassays. The results are summarized in Table 3. There was a 90% correlation between PCR and Western blot assay results.

DISCUSSION

Molecular detection of pathogenic micro-organisms from various environmental sources including food matrices/clinical specimens relies mainly on PCR amplification of specific target genes of interest. Recently, a large number of multiplex PCR formats have been reported, wherein simultaneous identification of two to ten different micro-organisms is technically possible. Their application for routine analysis is still under debate as they only identify potential toxin-producing bacteria, which may not always correlate with the expression of toxin components by the organism, and, in addition, a variety of interfering substances from the sample may compromise the sensitivity of the system. Moreover, because of the relatively high cost of PCR laboratory infrastructure and the cost of reagents, coupled with inherent problems with the over-sensitivity of the system, PCRs in either monoplex or multiplex format have yet to qualify as an ideal test for routine investigations.

Immunoassay-based detection systems have been reported and also commercialized (e.g. TECRA, TECRA International; BCET-RPLA, Oxoid). In the present work, we have reported a successful strategy to develop a recombinant intergenus multidomain protein, r-HLE, comprising truncated regions of HBL, LLO and SEB, for possible utilization in detection/diagnosis. This is supported by our earlier work, where polyclonal antiserum raised against a multidomain non-toxic chimeric protein (comprising truncated regions of the L₁, L₂ and B components of HBL) could effectively identify all three toxin components of HBL of *B. cereus* simultaneously in Western blot analysis (Kumar *et al.*, 2008). Here again, the approach of joining truncated proteins was followed to avoid toxicity, as in this form the toxins fail to achieve their proper native structure. The fusion gene in the current study, which was codon-optimized for *E. coli* expression, was first generated by ligation of PCR-amplified products encoding L₁ of the HBL toxin complex of *B. cereus*, LLO of *L. monocytogenes* and SEB of *S. aureus*. The resultant 1.37 kb fusion gene was sequenced and its expression resulted in a single recombinant multidomain chimeric protein, r-HLE, in the *E. coli* host (Fig. 1).

As one of the major objectives of this study was to investigate the potential of the r-HLE protein as a diagnostic antigen for simultaneous toxin detection, the r-HLE protein was evaluated using a commercial TECRA kit to identify the presence of the SEB antigenic determinants. The hyperimmune antiserum raised against the r-HLE protein specifically reacted with the L₁ (38 kDa) component of the HBL complex of *B. cereus*, LLO (58 kDa) of *L. monocytogenes* and SEB (28 kDa) of *S. aureus*. It also provided reaction bands at the expected regions when tested on the other standard strains and the isolates of *B. cereus*, *L. monocytogenes* and *S. aureus*, and appeared to be highly specific (Fig. 2, Table 3). Although no circular dichroism data are available for the r-HLE protein for comparison, the strong seroreactivity of r-HLE that we observed with mice sera suggests that it presents epitopes similar to those found in the full-length native protein. In our earlier work developing a chimeric protein of the HBL toxin components, we were able to observe the neutralizing ability of the recombinant fusion protein on the haemolytic

activity of the native HBL toxin of the organism, thereby providing some support that the molecule might have potential as a candidate vaccine. As the antigenicity of the recombinant chimeric protein in this study appeared to be retained similar to that of the native toxin, this may serve as a potential candidate vaccine, although it was not possible for us to assess the native structural integrity of the protein including protein folding. This aspect of the r-HLE protein needs to be explored. As we targeted truncated regions of the native toxin components rather than full-length toxins, the fusion protein is unlikely to exhibit any toxicity. This may avoid the requirement for detoxification procedures before evaluating the molecule for its vaccine potential.

In conclusion, this paper has described the successful construction and expression of a multidomain *r-hle* fusion gene with specificity tailored towards HBL of *B. cereus*, LLO of *L. monocytogenes* and SEB of *S. aureus* coupled to *E. coli*-based expression with a simple one-step purification procedure for cost-effective production. This protein may be useful in rapid and reliable immunoassays for proper surveillance and for initiating a timely response to clinical infections or foodborne outbreaks. The recombinant chimeric protein-based immunodetection approach may eventually provide advantages over PCR formats.

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