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Rapid determination of AB_5 enterotoxins using membrane electrospray ionization mass spectrometry

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ABSTRACT

Toxigenic bacterial pathogens play important role in enteric infections which remain a major cause of morbidity around the world. Although many methods have been reported for detection of enterotoxins, rapid analyses of AB_5 enterotoxins on protein-level in real samples is still challenging. A rapid quantitative method was developed in this study to determine bacterial AB_5 enterotoxins targeting at their unique peptides using membrane electrospray ionization mass spectrometry (MESI-MS). Precursor/production pairs of peptides HDDGYVSTSISLR (HR), TPNSIAAISMEN (TN), MASDEFPSMCPADGR (MR) and AVNEESQPECQITGDRPVIK (AK) unique to enterotoxins Ctx, LT, Stx1 and Stx2 respectively were used for both toxin identification and quantitative analysis. The methodologies were validated including sensitivity, accuracy, precision and recovery in detection of simulated real samples. Larger scaled real sample detections with statistical analysis demonstrated that target peptides in developed enterotoxins analyses method are reliable.

1. Introduction

Enteric infections remain a major cause of morbidity around the world, which represent a notable economic burden due to medical expense, reduce of productivity and decrease in quality of life. Bacterial pathogens, which produce the AB_5 toxins, occupy an important position in enteric infections [1]. AB_5 toxins are so termed owning to that they comprise a catalytic A-subunit accounting for interference in essential host functions, and a pentameric B-subunit that mediates the receptor recognition [2].

AB_5 toxins are deployed by different categories of diarrheogenic organism, each of which has their own specific AB_5 toxin, such as Cholera toxin (Ctx) produced from *Vibrio cholera*, classic Shiga toxin (Stx) produced from *Shigella dysenteriae*, heat labile enterotoxin (LT) produced from enterotoxigenic *Escherichia coli* (ETEC) and (Stx1 and Stx2) produced from Shigatoxigenic *E. coli* (STEC) [3]. Although AB_5 toxins share the homology structural architecture, they recognize distinct glycan receptors located on the cell surface and differ with cellular catalytic activities. Ctx and LT specifically bind the ganglioside GM1 expressed mainly on the epithelial cell membrane of the small intestine and causing ion dysregulation of infected cells, which could induce severe copious watery diarrhea resulting in life-threatening dehydration and electrolyte imbalance [4,5]. Stx, including Stx1 and Stx2, show affinity with glycosphingolipid Gb_3 expressed on the surface of the microvascular or kidney endothelium and causing cytotoxicity for endothelial cells, which also elicit serious gastrointestinal diseases involving diarrhea and hemorrhagic colitis, along with life-threatening sequelae such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura [6]. Every year, AB_5 toxins related infections affect millions of people and cause more than a million deaths [7]. For these reasons, there is an increasing demand for detection of AB_5 toxins in clinical samples.

Current clinical diagnostic methods mainly focused on determination of AB_5 enterotoxins' coding genes. For instance, the polymerase chain reaction (PCR) ranging from multiplex PCR to real-time quantitative PCR [8,9]; and the DNA hybridization assays [10]. Moreover, there are also a large number of methods target-

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ing AB_5 toxin protein. Some are based on immunoassays, such as enzyme-linked immunosorbent assay (ELISA) [11], microfluidic biosensor [12] and liposome PCR (LPCR) immunoassay [13]; the other are novel technologies under development such as localized surface plasmon resonance (LSPR) [14] and mass spectrometry (MS) [15] and so on. MS is a powerful tool with multiple advantages, such as high sensitivity, high resolution and small sample consumption. The chromatography–mass spectrometry method allows qualitative and quantitative analyses of trace amount analytes, which has been widely used in many ways [16,17].

Many methods have been reported to quantitatively detect enterotoxins on protein level by liquid chromatography tandem mass spectrometry, such as *Staphylococcal* enterotoxins A, *Staphylococcal* enterotoxins B and toxic shock syndrome toxin-1 in biological, food and water samples [18–23]. A comparative study of UPLC-MS/MS and ELISA methods for identification and quantification of *Staphylococcal* enterotoxins in milk and shrimp samples confirmed that the two methods showed good coherence [21]. However, it is time-consuming in both sample preparation and chromatography separation processes. With the developments of ambient ionization technologies, direct sample analysis in an open environment is allowed for MS analysis. Techniques, such as direct analysis in real-time (DART) [24], desorption electrospray ionization (DESI) [25], paper spray ionization (PS) [26], etc. [27–31], could achieve direct analyte detections in complex matrices with minimized sample handling process.

Previously, membrane electrospray ionization (MESI) was developed in our group [31], in which membrane was applied for three-dimensional molecule separation in real-time. As an ambient ionization technique, MESI-MS could not only achieve sensitive biomolecule quantitation, but also minimize the sample handling process. In this study, an analytical method was developed to detect Ctx, LT, and Stx (Stx1 and Stx2) in human faecal and serum samples using MESI-MS. This rapid method can be potentially coupled with portable MS and used in field investigation of outbreaks in the future.

2. Materials and methods

2.1. Bacterial strains and chemical reagents

Bacteria strains were all provided by Chinese CDC (Beijing, China), and detailed information on strains and primers were given in Tables 1 and 2. Shigatoxins-1 and 2 were purchased from Toxin Technology, Inc. (FL, USA); urea, dithiothreitol (DTT), iodoacetamide (IAA), NH_4HCO_3, KH_2PO_4, K_2HPO_4 and NaCl were purchased from Sigma-Aldrich (MO, USA); sequencing-grade modified trypsin was purchased from Promega (WI, USA); tryptone, yeast extract, HPLC-grade formic acid (FA) and methanol (MeOH) were purchased from Fisher Scientific (NJ, USA); Amicon Ultra Centrifugal Filter (3 kDa) were purchased from Merck Millipore (Darmstadt, Germany); Quick Start™ Bradford Protein Assay Kits (#500-0204) were ordered from Bio-Rad Laboratories (Shanghai, China); pure water was purchased from Wahaha (Hangzhou, China); filter papers were purchased from Beijing Chemical Company (Beijing, China); dialysis membrane (molecular weight cut-off (MWCO) 3.5 and 8–14 kDa) was purchased from Ebioeasy Co., Ltd. (Shanghai, China). Human faeces and serum were provided by healthy volunteers in accordance with the requirements of medical ethics. Four synthesized peptides (HR, HDDGYVSTSISLR; TN, TPNSIAAISMEN; MR, MASDEFPSMCPADGR; AK, AVNEESQPECQITGDRPVIK) with purity >95%, assessed by MALDI-TOF MS and HPLC, were obtained from Pepmic Co., Ltd. (Suzhou, China).

Table 1
Strains used in this study.

Bacterial origin	Strain No.	Toxin detected	Source
Toxigenic *V. cholerae*	VC995	Ctx	China CDC, Beijing, China
	VC1119	Ctx	China CDC, Beijing, China
	VC1283	Ctx	China CDC, Beijing, China
	VC2448	Ctx	China CDC, Beijing, China
	VC4480	Ctx	China CDC, Beijing, China
	VC7258	Ctx	China CDC, Beijing, China
Non-toxigenic *V. cholerae*	VUN54	ND[a]	China CDC, Beijing, China
	VC235	ND	China CDC, Beijing, China
	VC2113	ND	China CDC, Beijing, China
	VC5524	ND	China CDC, Beijing, China
	VC5528	ND	China CDC, Beijing, China
	VC197	ND	China CDC, Beijing, China
ETEC	ET002	LT	China CDC, Beijing, China
	ET007	LT	China CDC, Beijing, China
	ET011	LT	China CDC, Beijing, China
	ET019	LT	China CDC, Beijing, China
	ET036	LT	China CDC, Beijing, China
	10407	LT	ATCC
STEC	ST011	Stx1	China CDC, Beijing, China
	ST012	Stx1	China CDC, Beijing, China
	ST033	Stx1	China CDC, Beijing, China
	ST039	Stx1	China CDC, Beijing, China
	ST046	Stx1	China CDC, Beijing, China
	ST004	Stx2	China CDC, Beijing, China
	ST005	Stx2	China CDC, Beijing, China
	ST017	Stx2	China CDC, Beijing, China
	ST049	Stx2	China CDC, Beijing, China
	ST056	Stx2	China CDC, Beijing, China
	EDL933	Stx1 & Stz2	ATCC
Non-toxigenic *E. coli*	A127	ND	China CDC, Beijing, China
	A137	ND	China CDC, Beijing, China
	A144	ND	China CDC, Beijing, China
	JM109	ND	Takara Biotech Co. Ltd., Dalian, China
	BL21	ND	Takara Biotech Co. Ltd., Dalian, China

[a] ND, not detected.

2.2. Bacterial culture and protein digestion

Bacteria strains used in this study, including toxigenic *Vibrio cholerae*, non-toxigenic *V. cholerae*, enterotoxigenic *E. coli* (ETEC), Shigatoxigenic *E. coli* (STEC) and non-toxigenic *E. coli*, were cultured in Luria-Bertani (LB) medium at 37 °C with constant shaking. Fresh LB medium was inoculated with each kind of bacteria from the culture in the exponential phase (OD_{600} = 0.6) of growth.

After the overnight cultivation, the bacterial cultures were centrifuged at 3000 × *g* for 10 min at 4 °C, and the cell free supernatants were concentrated by ultrafiltration with ultra-centrifugal filters at 5000 × *g* for 20 min at 4 °C. Protein concentrations of the condensed supernatants were then determined by the Bradford assay. Microwave-assisted protein digestion was carried out [32]. Briefly, the protein samples were dissolved in 50 μL of 50 mM NH_4HCO_3 and reduced for 5 min at 95 °C in 50 mM NH_4HCO_3 containing 5 mM DTT and then alkylated for 30 min in the dark by adding 15 mM IAA. Sequencing-grade modified trypsin was added at an enzyme/substrate ratio of 1:50 (w/w) and allowed to digest the proteins in a domestic microwave for 15 min at 700 W.

2.3. PCR detection of the enterotoxins' coding gene

Bacteria strains were confirmed for *ctx*, *lt stx*1 and *stx*2 genes carrying by the PCR assay, and primers (Table 2) were selected according to published methods [33,34]. The PCR process was carried out as follows: pre-denaturation at 94 °C for 5 min, 30 cycles