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Original Article

Identification of a proteomic biomarker associated with invasive ST1, serotype VI Group B *Streptococcus* by MALDI-TOF MS

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Sequence type

Abstract *Background:* Group B *Streptococcus* (GBS) is an important invasive pathogen in neonates, pregnant women and the elderly. Serotype VI GBS, which has been rarely reported globally, has emerged as a significant pathogen in Asia. However, traditional serologic latex agglutination (LA) methods may fail to type isolates that lack of or low expression of CPS.
Methods: A total of 104 GBS strains were analyzed by MALDI-TOF MS. Multiplex PCR and multilocus sequence typing (MLST) were also performed to confirm their strains. The protein markers were purified with gel electrophoresis and LC-column, followed by identification with nanoLC–MS/MS analysis.
Results: Protein peak of 6251-Da was appeared in most (20/24, 92%) serotypes VI (94% ST-1 or single locus variant of ST-1), and protein peak of 6891-Da was appeared in most serotypes III (15/18, 83%) and Ib (19/23, 83%) strains. The protein peak of 6251-Da and 6891-Da were identified as CsbD family protein and UPF0337 protein gbs0600, respectively.

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Conclusions: The protein peak of 6251 Da may play a role of emergence of ST-1 clone, serotype VI GBS in central Taiwan and could be useful in rapid identifying invasive serotype VI from III isolates, which is hardly achieved by LA.

Introduction

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is an important invasive pathogen in neonates, pregnant women, and immunocompromised adults.[1–3] Based on surface capsular polysaccharide (CPS), GBS isolates are conventionally classified into ten serotypes: Ia, Ib, II, III–VIII, and provisional IX.[4] Common serotypes in infants are III, Ia, V, Ib and II,[1,5] while serotypes V, Ia, II and III are prevalent in non-pregnant adults.[1] However, serotype VI, which has been rarely isolated (around 1%) worldwide,[5] has recently emerged as a significant pathogen in Japan,[6] Taiwan,[7] and other Asian countries.[8]

Regarding GBS serotyping, the most common serologic test is commercial latex agglutination (LA) methods on the bases of antibodies specific for CPS. However, LA assay may fail to type isolates that lack of or low expression of CPS.[9] Therefore, PCR-based capsular gene typing methods have been developed to overcome the above limitations.[10,11] A multilocus sequence-typing (MLST) method, according to fragments of seven housekeeping genes, is a powerful tool to study the genetic lineages of GBS strains.[12] However, these methods are time-consuming, high cost, or labor-intensive. Recently, because of its high throughput, sensitivity and specificity, MALDI-TOF MS has been widely used to identify bacteria based on their unique protein profiles.[13–15] For example, Lartigue et al.[16] have identified a 6250-Da protein specific to ST-1 strains (most were serotype V) and a 7525-Da protein specific to ST-17 strains (most were serotype III) for GBS recognition. Later, applying SELDI and LC–MS/MS, Lanotte et al.[17] detected a 6258-Da biomarker overexpressed in ST-1 (serotype V), which was further identified as CsbD-like protein. Therefore, MALDI-TOF MS could be a rapid and valuable method in identifying virulent ST-17 or ST-1 GBS clones. However, there is still lacking of reports for rapid identification of serotype IV based on MALDI-TOF.

Previous studies indicate the emerging of invasive ST-1, serotype VI GBS in Taiwan.[7,18] Thus far, the cause of dissemination of serotype VI, ST-1 GBS in Taiwan is still unknown. Therefore, in this study, we aimed to use MALDI-TOF MS to identify potential protein markers for rapid identifying invasive serotype VI, ST-1 GBS clone and try to find clues to explain the spreading of serotype VI.

Methods

***S. agalactiae* isolates.** 104 GBS strains from strain library of China Medical University Hospital (CMUH), Taiwan, were cultured on 5% sheep blood plate for 24 h at 37 °C in 5% CO_2 atmosphere, and were identified according to the colony morphology, β-hemolysis, Gram staining, and Lancefield grouping with type B antisera.

Serotyping by multiplex PCR and latex agglutination. Serotyping was performed in a total of 104 GBS isolates by a multiplex PCR assay as previously described by Imperi et al.[11] A total of 67 GBS isolates were also serotyped with the Group B Streptococcus Serotyping Kit (Essum AB, Umea, Sweden). The kit recognizes 9 serotypes (polysaccharide antigens): Ia, Ib, and II to VIII.

Multilocus sequence typing (MLST). MLST was performed by sequencing seven housekeeping genes, as originally described,[12] and sequence type (ST) determination was carried out using the GBS MLST databases (http://pubmlst.org/sagalactiae).

MALDI-TOF MS analysis

MS profiling spectra were performed on a Microflex and an Ultraflex III TOF/TOF machine (Bruker). For sample preparation, one GBS colony on agar was directly smeared on a MALDI target. A 1 μL aliquot of the α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution [saturated, 50% acetonitrile (ACN)/2.5% trifluoroacetic acid (TFA)] was overlaid on the sample spot of an MALDI-target or a PDMS-coated MALDI plate[19] and then air dried. The mass spectra were acquired in the mass range of 2000–20,000 m/z in linear mode.

SDS-gel electrophoresis

For sample preparation, one GBS colony was suspended in 500 μL of 70% ethanol, followed by centrifugation for 3 min at 12,000*g*. The supernatant was discarded, and the bacterial pellet was resuspended in 50 μL of 70% formic acid (FA) followed by addition of 50 μL of pure acetonitrile (ACN) (LC–MS grade, JT-Baker, Mallinckrodt Baker, Inc.). After vortex, the extracted protein sample was centrifuged for 3 min at 12,000*g*, and the supernatant was collected and dried. The extracted proteins were then solubilized with 10% SDS, and separated on 4%–12% Bis–Tris NuPAGE® gels (Invitrogen) at room temperature for 65 min and 135 V with constant current.[20]

Protein extraction from gel band

The A and B bands on NuPAGE gels (Fig. 2) were sliced into small pieces separately and each of them comprised of three sections to elute the intact protein. The gels were destained by washing with buffer (50% ACN/0.1% FA) and

dehydrated by vacuum concentrators (SpeedVac, Thermo SCIENTIFIC). Then, the gels were eluted by 50% ACN, 5% TFA solution with gentle agitation. For better protein recovery, the extraction procedure was repeated three times and the extracts were pooled together. The eluted protein was dried by SpeedVac and dissolved in 0.1% FA for MALDI-TOF analysis.

In-gel digestion

The excised gel bands were cut into small pieces and washed with 25 mM ammonium bicarbonate (ABC) (pH 8.2) containing 50% ACN for 15 min three times. The gel pieces were further reduced by 10 mM dithiothreitol (DTT) at 5 °C for 15 min and alkylated with 55 mM iodoacetic acid (IAA) in the dark for 20 min. After washing the gel by 25 mM ABC containing 50% ACN for 10 min, they were digested with trypsin (1:50 trypsin to protein ratio in weight) at 37 °C overnight. After digestion, the tryptic peptides were extracted from the gel using 0.1% TFA in 50% ACN.

LC protein fractionation and in-solution digestion

Protein purification[21] by LC separation was performed on a Dionex Ultimate 3000 HPLC system (Dionex, Germany). A linear gradient on a C4 LC column (Waters, XBridge, BEH 300, 3.5 μm, 2.1 × 250 mm) was used to separate the protein mixture with a flow rate of 0.25 ml/min. The mobile phase A consisted of water containing 0.1% (v/v) formic acid and mobile phase B containing 99.9% (v/v) acetonitrile and 0.1% (v/v) formic acid. A gradient elution was applied from 10% (v/v) B to 50% B in the first 16 min, and to 80% B over 2 min. It was then held at 80% B for another 5 min, which was followed by a return to the starting conditions and re-equilibration of the column for 2 min with 1% B (v/v) prior to the next injection. Protein fractions are collected at 1-min intervals and dried by using a centrifugal concentrator. The collected protein fraction was then rehydrated with 20 mM ABC solution, reduced with 10 mM DTT at 56 °C for 15 min and alkylated with 55 mM IAA in the dark at room temperature for 20 min. Trypsin was added to the protein solution at an enzyme-to-substrate ratio of 1:50 (wt/wt), and the mixture was incubated for 12 h at 37 °C for digestion.

Protein identification by nanoLC—MS/MS

LC—MS/MS system was performed using nanoESI Q-TOF mass spectrometer (maXis impact, Bruker, Germany) coupled on line with an Ultimate 3000 RSLCnano LC system (Dionex) consisted of a tunnel-frit trap column (C18, 5 μm, 150 μm × 20 mm)[22] and a reverse phase analytical column (Acclaim PepMap C18, 2 μm, 75 μm × 250 mm, Dionex). The peptides were eluted with H_2O/ACN gradient (300 nl/min) from 1 to 40% of solvent B (80% ACN, 0.1% FA) in 30 min, 50—80% of B in 10 min and 80% of B in 8 min, returning to 99% A (5% ACN, 0.1% FA) in 2 min. Peptide fragment mass spectra were acquired in data-dependent Auto-MS/MS mode with a scan range of 100—2000 *m/z*. The MS data was searched in the Swissport database (release 51.0) using an in-house MASCOT 2.2.04 server. Search parameters were selected as Taxonomy — bacterial; enzyme — trypsin; fixed modifications — carbamidomethyl (C); variable modifications — oxidation (M, H, W), deamidated (N, Q); Precursor peptide tolerance 50 ppm; MS/MS tolerance 0.05 Da; Peptide ion score ≧25 was accepted.

Statistical data analysis

Statistical analyses of the MS profiling spectra from GBS isolates were performed with the ClinPro Tools™ software (Bruker). All spectra are normalized to their own total ion count (TIC). The MS data from GBS isolates were presented in a 2-D cluster plot.

Results

Serotyping by multiplex PCR and latex agglutination

One hundred and four isolates were serotyped by a multiplex PCR assay. Among 104 isolates, 67 isolates from blood of children (n = 6) and non-pregnant adults (n = 53) and anorectal swab of pregnant women (n = 8) were also typed by latex agglutination (LA). All results are shown in Table 1. Serotypes VIII and IX were not detected. There are 75% of 67 isolates in agreement with typing by latex agglutination (LA) and multiplex PCR. However, there are some disagreement when typing to serotypes Ia, II, III, IV and V (concordance from 0 to 81.8%). Ten of 23 isolates originally typed as III by LA were further distinguished as VI (n = 7), Ia (n = 2) and Ib (n = 1) by multiplex PCR.

MALDI-TOF MS analysis

GBS strains (n = 104) were investigated with MALDI-TOF MS analysis, and two major protein profiles were found (Fig. 1a). Some strains have protein pattern with the presence peaks of 6251.0 *m/z* (Fig. 1a, top panel) and some have peaks of 6891.0 *m/z* (Fig. 1b, bottom panel). The MS spectra from 98 strains were converted to a pseudo-gel analysis to conveniently observe peak profiling in all strains (Fig. 1b). The protein peak of 6251 appeared in serotype Ia (4/11, 36%), Ib (4/23, 17%), II (9/12, 75%), III (2/18, 11%), V (11/16, 69%) and VI (22/24, 92%) strains. The protein peak of 6891 was found in serotype Ia (7/11, 64%), Ib (19/23, 83%), II (3/12, 25%), III (15/18, 83%), V (5/16, 31%) and VI (2/24, 8%) strains. Interestingly, the pseudo-gel showed that distribution of proteins 6251 Da and 6891 Da are mutually exclusive in all strains (Fig. 1b). The peak of 6251 Da has been reported with high appearance in serotype VI (92%) strains. Therefore, serotype VI strains were further typed with MLST, and the result showed that 81% of serotype VI strains with protein peak of 6251 Da were identified as ST-1 or single locus variant (SLV) of ST-1 (ST-679) (Table 2).

Protein purification and identification

To identify the protein peak of 6251 Da and 6891 Da, a gradient 4%—12% Bis—Tris gel with higher separation resolution was performed to purify these two proteins. As

Table 1 Comparison of typing results between agglutination test and multiplex PCR assay of 67 GBS clinical strains.

Serotype	Latex	Multiplex PCR											
		Ia	Ib	II	III	IV	V	VI	VII	VIII	IX	Total	Concd %[a]
Ia	8	6			1		1					8	75
Ib	15		15									15	100
II	5	1		3		1						5	60
III	23	2	1		13			7				23	56.5
IV	1						1					1	0
V	11	1					9		1			11	81.8
VI	4							4				4	100
VII													
VIII													
IX													
Total	67	10	16	2	14	1	11	11	1			67	

[a] Percentage of concordance.

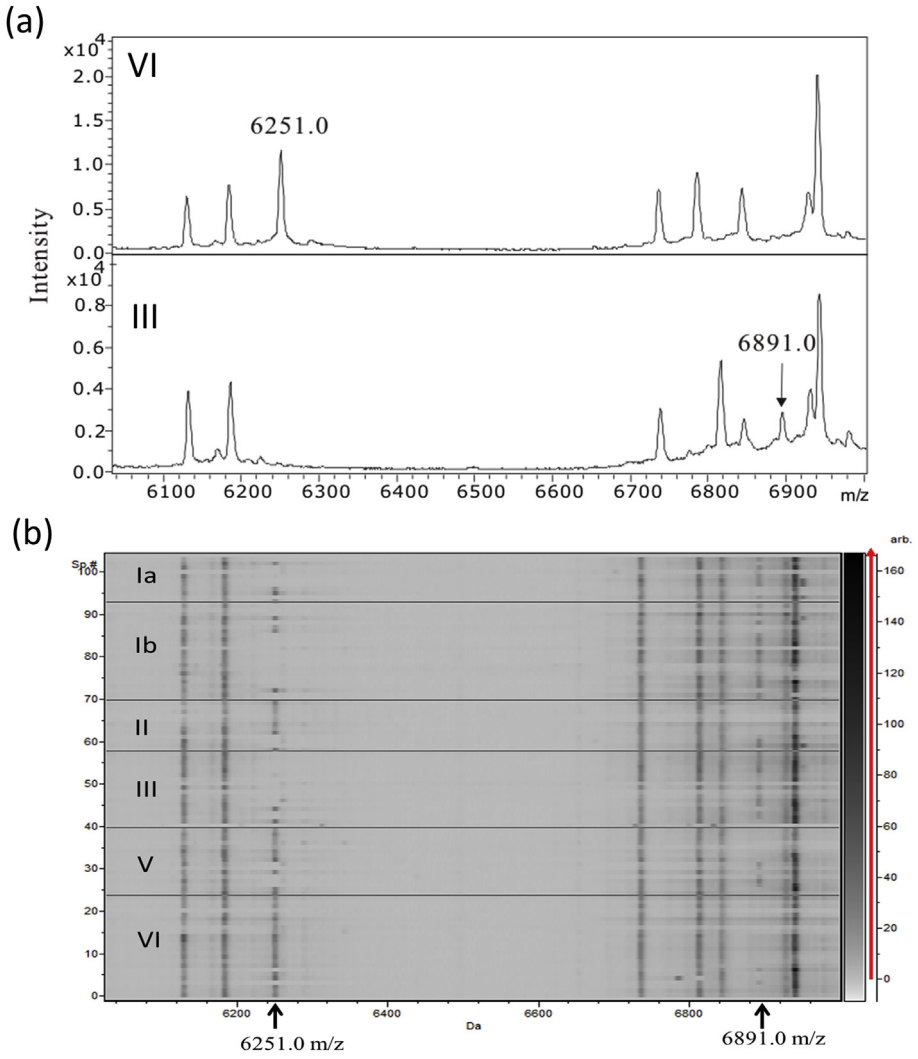

Figure 1. (a) MALDI-TOF mass spectra of GBS serotype VI and serotype III. Cell were grown on BHI agar plates and then extracted with 50 μL of 70% FA and 50 μL of ACN. (b) Pseudogel view of protein profiles of the isolates. The horizontal line in the figure indicates the separation between serotype Ia, Ib, II, III, V and VI groups. The differential density along the x axis represents the abundance of specific peptide in the samples.

Table 2 Distribution of the peak 6251 Da and 6891 Da in different serotypes of Group B *Streptococcus* isolates.

Serotype	No. of isolates	p6251 (+) no. of isolates (%)	p6891 (+) no. of isolate (%)
Ia	11	4 (36%)	7 (64%)
Ib	23	4 (17%)	19 (83%)
II	12	9 (75%)	3 (25%)
III	18	2 (11%)	15 (83%)
V	16	11 (69%)	5 (31%)
VI	24	22 (92%)	2 (8%)

shown in Fig. 2a, after gel electrophoresis and coomassie blue staining, a slightly stained gel bands of A and B bands below 10 kDa was excised and extracted. As shown in Fig. 2b, high purify of protein peaks 6251 and 6891 Da can be successfully extracted and detected on MALDI-TOF. This protein gel bands of 6251 Da and 6891 Da were separately subjected to in-gel digestion and nanoLC—MS/MS analysis. In the 6251-protein peak sample, three peptides fragmented ion spectra were identified as a CsbD-like family protein (gi: 445998854) (Fig. 3a), and the protein peak of 6251 could be the degraded form of the CsbD-like family protein (average neutral mass: 6379 Da) with

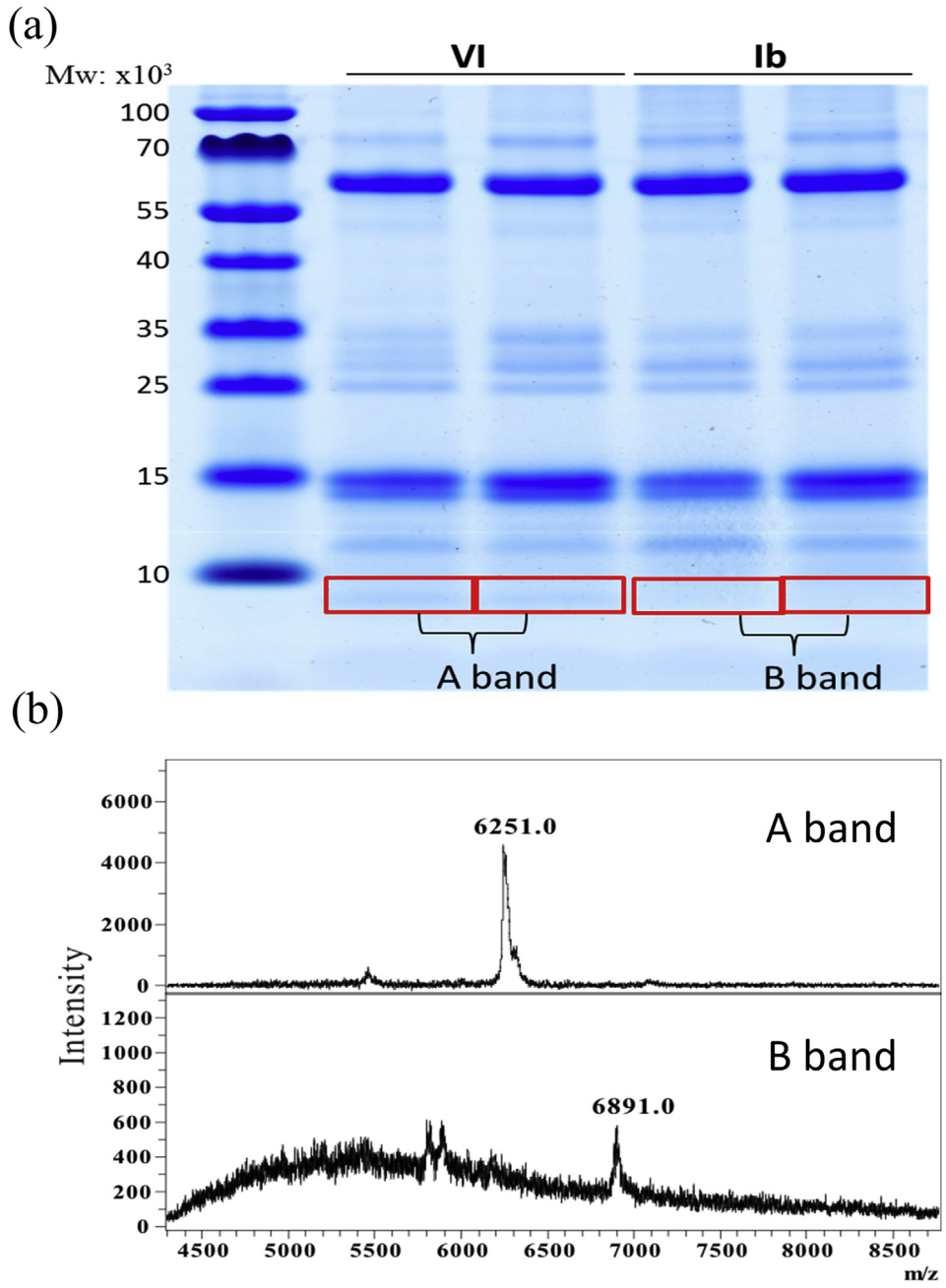

Figure 2. (a) Bis—Tris gel analysis of protein sample extracted from serotype VI and Ib strains. Resulting gels were stained with Coomassie blue G250. (b) MALDI-TOF analysis of intact protein extracted from A and B bands on Bis—Tris gradient gels.

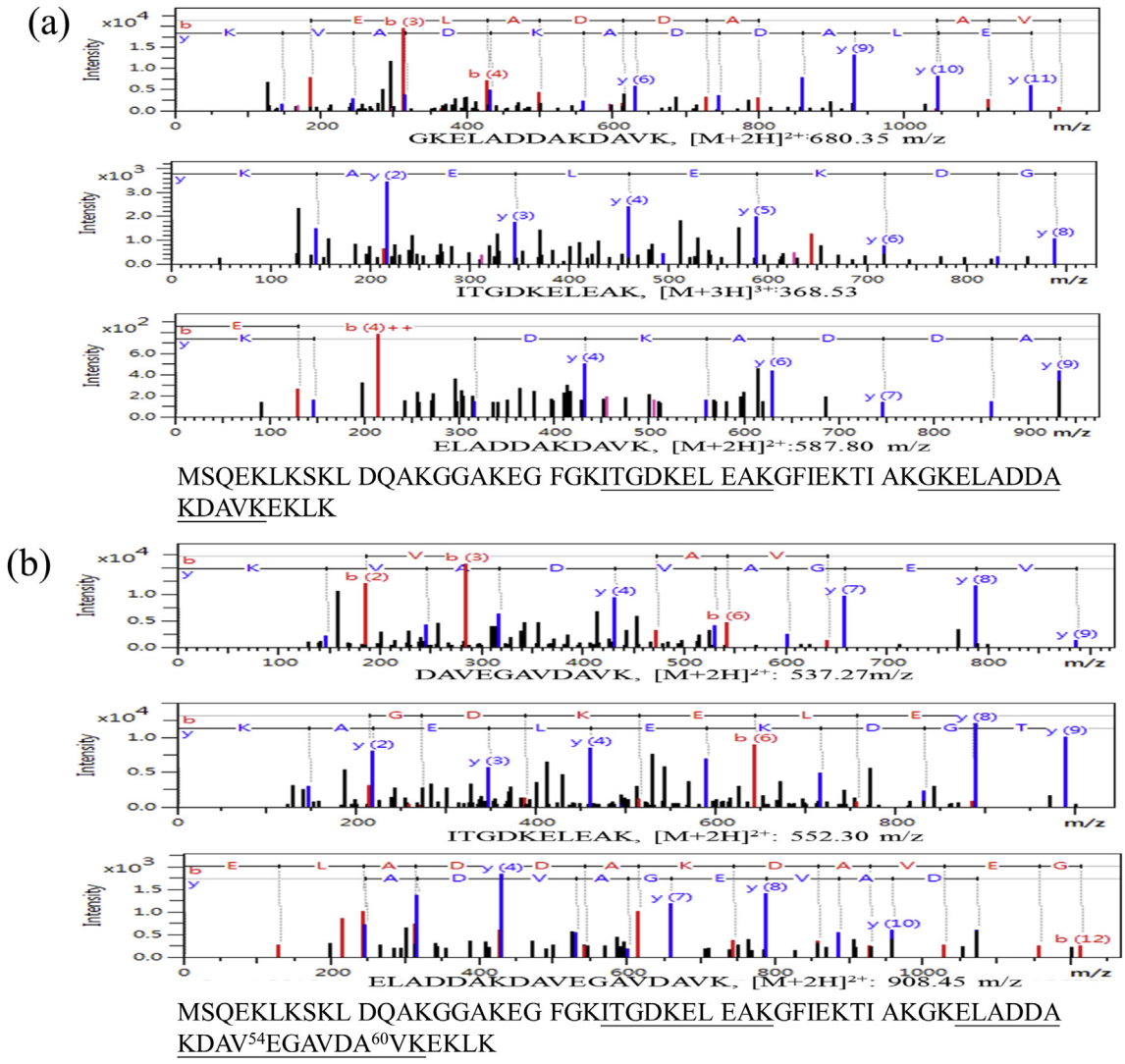

Figure 3. NanoLC—MS/MS spectra for identification of (a) CsbD-like family protein (gi:445998854) and (b) UPF0337 protein gbs0600 (gi:115311231). The identified protein sequence was underlined.

one-lysine losing at its C-terminal. In the 6891-protein peak sample, three peptides fragmented ion spectra were identified as a UPF0337 protein gbs0600 (gi: 115311231) (Fig. 3b), and the protein peak of 6891 Da could be the degraded form of UPF0337 protein (average neutral mass: 7021 Da), with one-lysine at its C-terminal. Because the CsbD-like family protein (6251 Da) and the UPF0337 protein (6891 Da) have the same protein sequences except that UPF0337 has the additional sequence of $E^{55}GAVDAV^{61}$, in order to further confirm the protein peak of 6891, LC separation was also used to purify protein peak of 6891 Da. As shown in Fig. 4a, the protein peak of 6891 Da was successfully purified in one of the LC fraction, and this LC fraction was subjected to in-solution digestion and nano-LC—MS/MS analysis. The protein with mass of 6891 Da was only identified as UPF0337 protein in the 6891-presented LC fractionated sample compared to the same time-eluted LC fraction from one of the ST-1 strain samples (without protein peak of 6891 Da) (Fig. 4b).

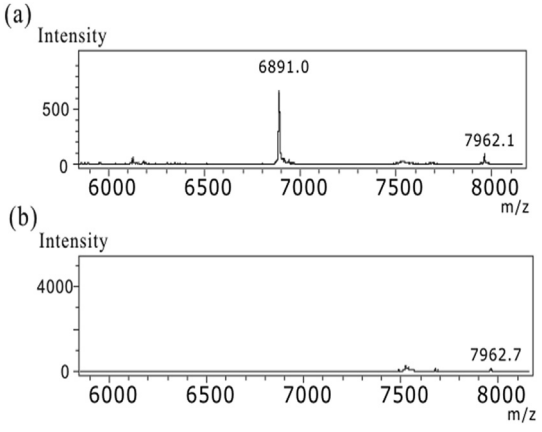

Figure 4. MALD-TOF analysis of (a) the LC subfraction at specific time containing purified protein peaks of 6891 Da from serotype Ib strain and (b) the LC subfraction at the same specific time from serotype VI strains.

Discussion

For typing GBS, LA is easy to implement,[9] less time-consuming than previously described serotyping methods, such as multiplex PCR,[10,11,23,24] PFGE[25] and MLST,[12] and appropriate for large-scale epidemiological survey. Nevertheless, in this study, LA is not able to accurately discriminate serotype VI from serotypes III. The reason might be due to cross-reactions between the latex solution of serotype III and serotype VI.[9] Moreover, serotype VI was rarely recognized in previous studies,[5] and LA data regarding serotype VI is limited. Besides, pan-genome analysis of eight pathogenic GBS isolates indicated that the classical and convenient typing method of bacteria based on their capsular polysaccharide compositions does not response the genetic diversity of the species.[26]

Considering the emergence of invasive serotype VI strains in Asia,[6,7] a rapid identification method is required. In this study we found that a protein peak of 6251-Da protein was significantly appeared in serotypes VI strains (most ST-1 clone), while a protein peak of 6891-Da protein was found in most serotype III strains. In addition, protein peaks of 6251 and 6891 appeared mutually exclusive in these strains (Fig. 1b) and are successfully purified and identified as a CsbD family protein (gi: 445998854) and UPF0337 protein gbs0600 (Gi:115311231), respectively. Therefore, the identification of 6251 protein peak on MALDI-TOF could be an attractive and practical strategy in differentiating serotypes III and VI. Although 6251 Da is also presented in other serotypes, 6251 is lowly presented in serotype III. Therefore, for unknown samples, latex assay can be used to identify most serotypes, and then MALDI-TOF could be complementary applied to accurately identify serotype VI from isolates, which were mis-identified as serotype III isolates by latex assay.

Although Lartigue et al. also found a similar protein mass of 6250-Da protein specific to ST-1 strains (serotype V), they did not purify or identify this protein peak.[16] Afterwards, Lanotte et al. successfully identified the protein marker of 6258 Da as a CsbD-like protein overexpressed in invasive ST-1 serotype V strains.[17] However, in our study, a 6251-Da protein was identified as a CsbD family protein (gi: 445998854) and specific to serotype VI (ST-1 clone), but the sequence was not the same as the one (gi:447210780) identified by Lanotte P et al.[17]

CsbD family protein was thought as a stress response protein.[27] The role of CsbD family protein expression in *Bacillus subtilis* is mediated by sigma factor, manipulating the expression of the general stress genes[28]; however, its role in stress response of bacteria is still uncertain. A recent study identified that *B. subtilis* CwlQ (previous YjbJ, CsbD family protein) is one of the supposed cell wall hydrolases and a bifunctional enzyme exhibiting muramidase and soluble-lytic transglycosylase activities.[29] CsbD family proteins may contribute to virulence in invasive GBS strains, whereas 6251 Da proteins maybe a potential spreading factor of recent emergence of ST-1 clone, serotype VI GBS in central Taiwan.

Interestingly, similar amino acid sequence of CsbD were identified in the lytic phage JX01 genome.[30,31] The sequence found in JX01 (gi:402760705) was similar to one of our protein marker peaks, 6891 Da. The protein peak of 6891 Da appeared in the majority of strains of serotypes III, Ib and Ia, and was identified as UPF0337 protein gbs0600, which belongs to the UPF0337 (CsbD) family. We found that the difference between peak of 6251-Da (CsbD) and peak of 6891-Da were only 7 amino acid residues (E^{55}GAVDAV^{61}). Since 6251-Da located nearby the Prophage Lambda SA03, it is possible that 6891-Da was modified through the phage infection process of 6251-Da (CsbD). Previous studies have indicated that unique prophage groups were belonged to specific serotype strains and they were responsible to the virulence distribution in *Streptococcus agalactiae*.[32–34] Furthermore, according to Domelier's research,[32] specific marker belonging to the prophage SA03 was also exist in other phages and was able to induce specific clonal lineages infection and lytic process. Therefore, it was reasonable to speculate that phage infection play critical role to transfer CsbD among different serotypes.

Recently the serotype VI, ST-1 GBS clone has emerged in Taiwan.[7,18] Because GBS is a normal flora component in the human gastrointestinal and genitourinary tracts, the possible spread pathway may include food consumption and sexual behavior. A recent study by Wang et al.[18] reported that serotype VI has increased adaptation compared to the conserved serotypes in serotype V, increasing its spread possibility.

A recent comparative genomics analysis[31] indicated that GBS isolates from cultured tilapia in China are closely related to the human strain A909 (serotype Ia, ST-7) which also carry a protein (gi:406649967)[34] identical to CsbD (gi:445998854) in human strain A909. Furthermore, an Ia ST-7 invasive human GBS could cause disease and death in Nile tilapia. Besides, consumption of tilapia has been associated with an increased risk of GBS serotypes Ia and Ib colonization in college women and men.[35] In addition to the piscine GBS, bovine GBS was possible to cause VI clone emerged, because bovine GBS serotype III were closely related to human GBS serotype III.[36] Recently, the comparative genomic analysis showed that bovine GBS were closely related to human and fish isolates.[31] Although there is no evidence showing that humans could be infected by piscine or bovine GBS through wounds or consumption, attention should be paid to its potential threat to public health security, because ST7 fish strains potentially bearing CsbD family protein might act as reservoirs of human pathogenic lineages with close genomic relationships.[26,31,37]

In this study, we found that the protein peak of 6251 Da was specifically appeared in ST-1 strains and identified as a CsbD-like superfamily protein. Because newly raised GBS serotype VI in Taiwan belongs to ST-1, combination of LA and MALDI-TOF MS could rapidly identify GBS and differentiate serotype VI from serotype III. The functions of 6251 Da CsbD-like superfamily in serotype VI strains is still not clear but it may play an important role in the spreading of serotype VI. Besides, preventing invasive GBS infections in infants, pregnant women and immunocompromised non-pregnant adults necessitates comprehensive epidemiology study and eventually an effective glycoconjugate vaccine incorporating common serotypes.

Conflict of interest disclosure

The authors declare no competing financial interests.

Acknowledgments

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