Mass Spectrometric Analysis and Database Search

The MS and tandem-MS (MS/MS) spectra of tryptic peptides (Fig. 1E) were obtained using the NanoFrontier nLC and NanoFrontier eLD Liquid Chromatography Mass Spectrometer (Hitachi High-technologies, Tokyo, Japan). The nanoLiquid Chromatography/ ElectroSpray Ionization/ Linear Ion Trap/ Time of Flight (nLC-ESI/LIT/TOF) and collision induced dissociation (CID) modes were used for MS detection and peptide fragmentation as described [32]. In the NanoFrontier nLC, the trypsinized peptides (1–10 μL) suspended in 0.3% formic acid were trapped on monolith trap column [C18-50-150 column, (0.05 mm I.D. × 150 mm L). Hitachi High-technologies] and separated on a packed nano-capillary column [NTCC-360/75-3-123, (0.075 mm I.D. × 100 mm L, particle diameter 3 μm), Nikkyo Technos Co., Ltd, Tokyo, Japan] at a flow rate of 200 nL/min. The peptides in the column were eluted using a stepwise ACN gradient (mobile phase A: 2% ACN, 0.1% formic acid; mobile phase B: 98% ACN, 0.1% formic acid, The A:B concentration gradient was 0.0 min: (A:B = 100:0%) → 60 min (0:100%). In the NanoFrontier eLD spectrometer, the eluted peptides were ionized with a capillary voltage of 1700 V and detected in a detector potential TOF range of 2050–2150 V.

Acquired MS and MS/MS spectra were converted into Mascot generic format (mgf) using a Data Processing software 2008 (Hitachi High-technologies) and subsequently searched in a locally established database for *S. japonicum* sequences downloaded from Chinese National Human Genome Centre at Shanghai (http://www.chgc.sh.cn/japonicum/Resources.html) (containing 12,657 predicted proteins) [33] using the MS/MS Ion Search provided by MASCOT Sequence Query sever version 2.3 (http://www.matrixscience.com). With the MASCOT search, the following search parameters were used, enzyme: trypsin, variable modifications: carbamidomethylation, carboxymethyl (C) and oxidation (M), mass values: monoisotopic, protein mass: unrestricted, peptide mass tolerance: ± 0.5 Da, fragment mass tolerance: ± 0.2 Da (CID data), maximum missed cleavages: 1 and Instrument type: ESI-TRAP. For MASCOT output, significant peptides were determined by the peptides score from the probability-based molecular weight search (MOWSE) which identifies proteins from the molecular weight of peptides created by the proteolytic digestion [34]. Peptide score > 27 indicate identity or extensive homology ($p<0.05$). Further stringency was added by eliminating any single peptide that could be assigned to more than one protein. To ensure a non-redundancy, the protein identifications were examined manually in the database for possible redundancies including multiple names and homologies.

Characterization of Identified Proteins

Identified *S. japonicum* proteins were characterized

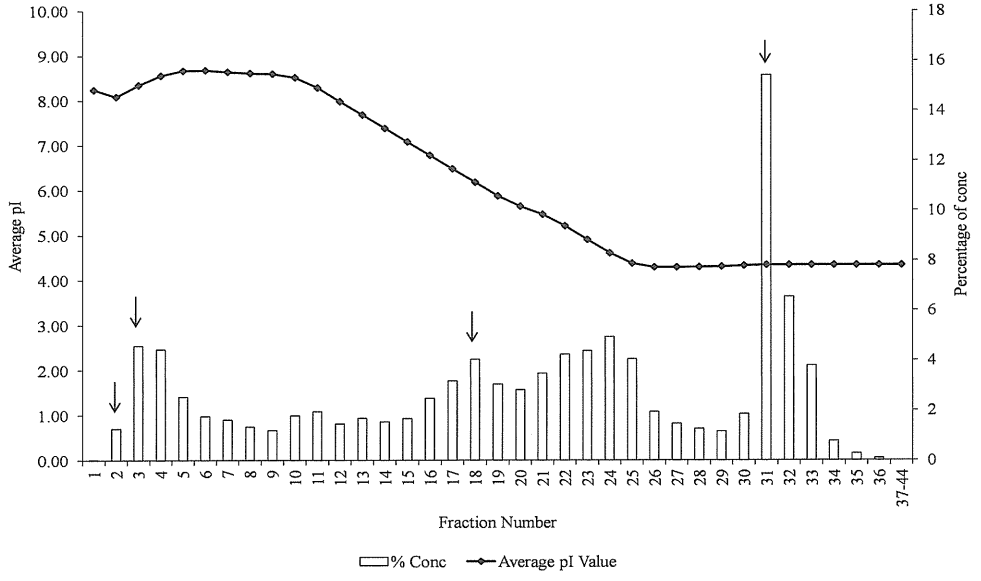

Fig. 2. Elution profile and percentage concentration of SWA into various fractions during chromatofocusing. Chromatofocusing was performed using the ProteomeLab PF 2D protein separation system. The pH gradient was formed using two proprietary buffers: "ProteoSep Start" buffer at a pH 8.5 and "ProteoSep Elution" buffer at a pH 4.0. As shown, the SWA was well fractionated with fraction 31 containing most of the void proteins. Proteins identified were obtained from fractions numbers indicated by arrows (2, 3, 18 and 3).

according to antigenic propensity, hydrophobicity, antigenic determinant, domains and gene ontology (GO) types with respect to their antibody isotype recognition. An estimate of hydrophobicity was determined from the grand average of hydropathicity index (GRAVY) [35] using ProtParam from *ExPASy* [36]. The GRAVY index is the average hydropathy score for all the amino acids in the protein. The positive GRAVY index indicates hydrophobic protein and negative, hydrophilic protein. The immune peptides or antigenic determinants and antigenic propensity were also estimated [37]. With reference to conserved domain sequences (CDS) that were similar to known genes and domains, the proteins were further characterized into GO terms and annotations (biological processes, molecular functions and cellular components) using UniProt-GOA server (ver. 106) (http://www.ebi.ac.uk/QuickGO/).

Statistical Analysis

The data generated were analyzed by Microsoft Excel and GraphPad Prism ver. 5. All the data were expressed as the mean ± SD. Differences between groups were analyzed for statistical significance by one-way analysis of variance and the *t*-test (Unpaired t test) using the GraphPad Prism. A *p*-value of <0.05 was considered as statistically significant.

RESULTS

Fractions and Dot-blot Screening

Protein fractionation by chromatofocusing is used to enrich proteins with similar isoelectric point (IEP or pI) and collected in one fraction [38]. Liquid fractionation of crude SWA into 1- and 2-D was achieved using the ProeteomLab PF 2D instrument. In the chromatofocusing, SWA proteins were separated and eluted into 44 fractions. Fig. 3 shows the fraction number against the elution profile (average pI) and percentage concentrations while Fig. 3, 2-D elution profiles of fractions 2, 3, 18 and 31. As expected, the basic proteins started to elute (fractions 1 to 17). The elution continued with the neutral proteins (18 to 20) followed by the weak and strong acidic proteins (21 to 44). Most of the proteins were eluted in fraction 31 (15.42%) with mainly acidic proteins. Fractions 37 to 44 had undetectable level of percentage concentration. The pIs of almost all the proteins identified in the higher pH gradient were consistent with the expected pI (Table 1). However, lower pH gradient showed a weak correlation to the expected pI range even though proteins with the expected pI were also found in these fractions (F31-H7). Such unexpected phenomenon is possible because the last few fractions can be enriched with proteins that carry post-translational modifications (PTM) such as phosphorylation which can cause a shift in their pI. Usually, this phenomenon is observed in 2-D electrophoresis and is useful for identifying proteins with different modification condition [38].

Four human circulating antibody isotypes (IgE, IgG1, IgG3 and IgG4) recognition by the 1-D fractions were evaluated using dot-blot ELISA. The reactivity intensity of each fraction was transformed into pixel unit and then expressed as relative reactivity intensity. The IgE was bound by 19 fractions (43.18%) with mean intensity of 0.22 ± 0.18 while IgG1 had 33 (75.00%) with mean intensity, 0.24 ± 0.23. For IgG3 and IgG4 there were 35 (79.55%) and 27 (61.36%) fractions detected with mean intensity of 0.28 ± 0.26 and 0.36 ± 0.30, respectively. The IgE isotype was detected by fewer fractions as compared to the remaining isotypes. The most detected isotype was IgG3 followed by IgG1 and IgG4 accordingly. It was observed that fractions 2 to 6 reacted with all the isotypes with varying intensity (Fig. 3). Likewise, the 2-D fractions (2, 3, 18 and 31) were further screened by dot-blot analysis to identify wells containing proteins that were IgE or IgG4 preferred (Fig. 3). The positive dot-blot fractions of such 2-D wells were selected from basic fractions (F2-C5, F2-C7, F2-C3; F3-E1, F3-H7), neutral fractions (F18-B1, F18-C3) and acidic fractions (F31-C3, F31-E1, F31-H7) which were processed for LC-MS/MS. Fig. 4A shows a representative 2-D dot-blot reactivity.

ESI-MS/MS Analysis

A total of 25 non-redundant proteins were identified by two-dimensional fractionation through ESI MS/MS analysis of 10 fractions (Table 1). A representative tandem mass spectrum is presented in Fig. 4B. Proteins identified with peptides obtained from the MS/MS mostly had a single confirmed peptide matches with a minimum Mascot score of 27. The 2-D wells (F2-C3, F2-C5 and F2-C7) originating from 1-D F2; 1-D F3 (F3-E1 and F3-H7); 1-D F18 (F18-B1 and F18-C3) and 1-D F31 (F31-C3, F31-E1 and F31-H7), yielded 3, 14, 4, 4 proteins respectively. Two proteins (chromatin licensing and DNA replication factor 1 and zinc finger, RanBP2-type, domain-containing) were sequenced from two sequential fractions (F2 and F3) and at the same time one of them (chromatin licensing and DNA replication factor 1), in other two non-sequential fractions (F18 and F31). Proteins with a wide range of molecular weight were identified from less than 20 kDa to more than 300 kDa (Table 1). The pI and the molecular weight profiles therefore indicate the benefit of protein fractionation which is an important aspect of protein profiling in identifying proteins with different biochemical properties. The majority of the *S. japonicum* proteins were recognized by IgE, IgG3 and IgG4 but not IgG1.

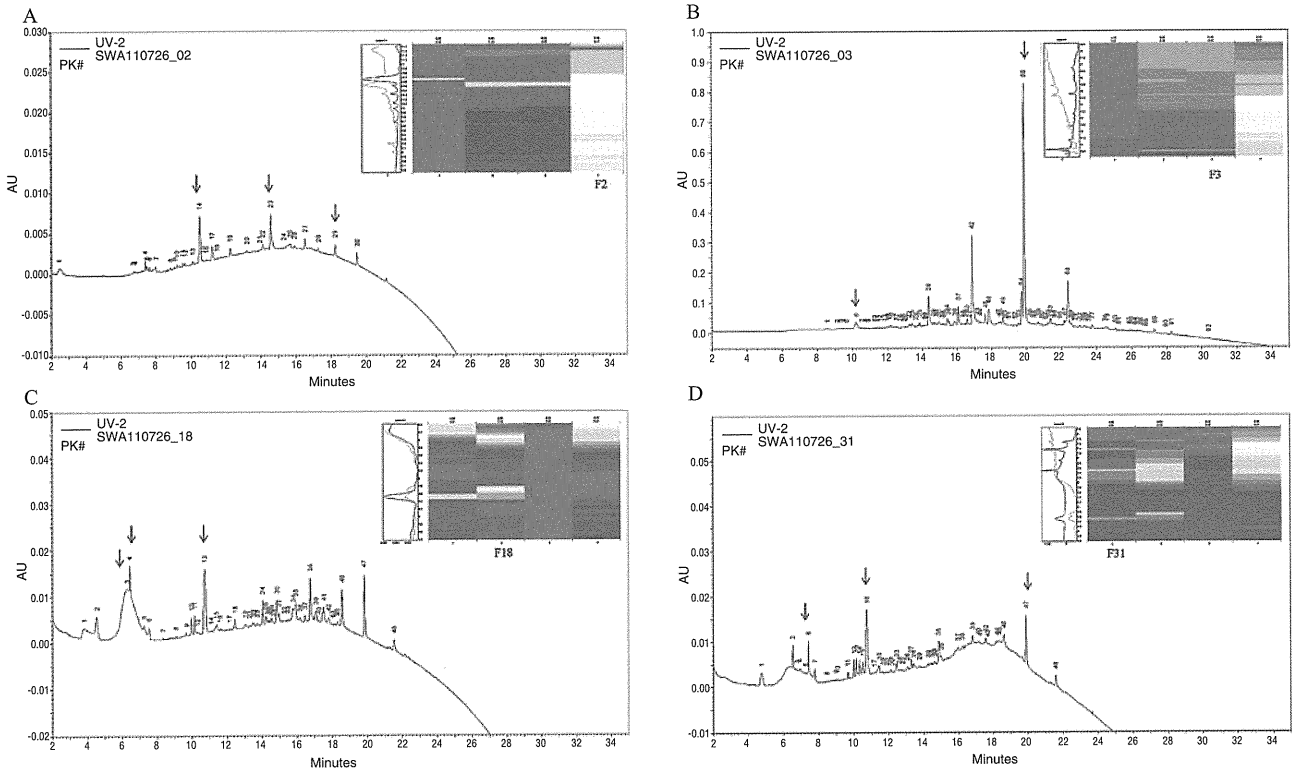

Fig. 3. Second dimensional elution profile of 1-D fractions 2 (A), 3 (B), 18 (C) and 31 (D). 1-D fractions were run in the 2-D reversed phase chromatography using the ProteomeLab PF 2D. As shown is the elution profile with respect to time and absorbance units (AU). The elution gradient was achieved using two solvents, 0.1% TFA in water (A) and 0.08% TFA in ACN (B). The 2-D fractionation was run at column temperature of 50°C and a buffer flow rate of 0.75 ml/min with the absorption monitored at 214 nm. The column was eluted with a 0–100% linear gradient of solvent A and B for 35 min. Insert, 2-D UV difference maps obtained by ProteoVue of the fractions and the pH range; 31 (pH 4.29–4.34), 18 (pH 6.03–6.33), 3 (pH 8.22–8.45) and 2 (pH 8.45–8.47). Arrows indicate fractions from which proteins were sequenced. F2 P# = 14, 23, 29. F3 P# = 14, 23, 29. F18 P# = 3/4, 13. F31 P# = 6, 16, 47. P#, peak numbers.

Characterization

It is worth noting that all the proteins identified have a GRAVY index within negative range (Table 2). Meaning the proteins are hydrophilic in consistent with 2-D reversed phase elution profile (Fig. 3). Meiosis-specific nuclear structural protein 1 (MNS1) was found to be the most hydrophilic (–1.306) and BRO1 domain-containing protein BROX, less hydrophilic (–0.177) protein. With respect to the number of possible epitope each protein have, ubiquitin-conjugating enzyme E2 N had 8 antigenic determinants, being the least yet with average antigenic propensity of 1.0386.

The GO terms for the annotated proteins in terms of biological processes, molecular function and cellular components were identified for almost all the proteins (Table S1). In terms of biological processes, some are associated with DNA metabolic and physiological processes including nucleotide metabolic process (GO:0009117), cell division (GO:0051301), mitotic cell cycle (GO:0000278), RNA interference (GO:0016246) and protein physiological processes such as potassium ion transport (GO:0006813), ATP binding (GO:0005524) and protein modification process (GO:0006464). Others were associated with regulation, development or stress response including cell differentiation (GO:0030154), reproduction (GO:0000003) and response to oxidative stress (GO:0006979) and so on. In terms of cellular components, the proteins were found to be associated with cytoplasm (GO:0005737), intracellular (GO:0005622), nucleus (GO:0005634), plasma membrane (GO:0005886) and extracellular region (GO:0005576). The PDZ and LIM domain protein 3 expressed in the cytoplasm is associated with response to oxidative stress (GO:0006979). In addition, a membrane protein, Small conductance calcium-activated potassium channel (SK) protein 2 with Calmodulin binding domain (CaMBD) (GO:0015269), has been found to be a secreted protein [39–42].

Table 1. Fractions and identified proteins by ESI MS/MS. *S. japonicum* adult worms extract was liquid fractionated using ProteomeLab PF 2D system into 1-D by chromatofocusing and reversed phase chromatography followed which were screened by dot-blot. Immunoreactive 2-D fractions were subjected to ESI MS/MS and peptide identification using MASCOT server with a locally established *S. japonicum* protein sequences downloaded from *S. japonicum* database at http://www.chgc.sh.cn/japonicum/Resources.html. MP, MASCOT peptides score. *Total MASCOT Peptide Score.

Fraction Well	Protein ID	Match to	Unique peptide	m/z	z	error (Da)	Peptide	MP Score	pI/Mw
F2-C3	Sjc_0203170	Chromatin licensing and DNA replication factor 1	1	530.81	2	0.0092	DVIDLVKMK	56	9.74/64520
F2-C5	Sjc_0213700	Zinc finger, RanBP2-type, domain-containing	1	450.29	2	0.0517	INLSSLPR	27	9.34/57532
F2-C6	Sjc_0213700	Zinc finger, RanBP2-type, domain-containing	1	450.29	2	0.0503	INLSSLPR	30	9.34/57532
F3-E1	Sjc_0034740	Meiosis-specific, nuclear structural protein 1	1	689.85	2	–0.0309	RELEAINAYTAK	40	6.79/45874
	Sjc_0203170	Chromatin licensing and DNA replication factor 1	1	530.8	2	–0.008	DVIDLVKMK	36	9.74/64520
	Sjc_0058190	NACHT and WD repeat domain-containing protein 1	1	590.33	2	0.0613	MCEQLLKTR	34	5.28/228171
	Sjc_0037420	Triple functional domain protein	1	606.37	2	0.0134	QFLAK	30	6.13/173854
	Sjc_0083690	RNA Helicase	1	606.37	2	0.0465	MQLAK	30	9.07/86337
	Sjc_0040110	Protein kinase	1	699.86	2	0.0254	ENFVLYDEIEK	29	8.86/209937
	Sjc_0302250	BRO1 domain-containing protein BROX	1	564.33	2	0.0046	EKAGQAIAALR	31	8.28/47921
	Sjc_0111110	Cell division control protein CDC7	2	464.79	2	0.1187	LLEPCPEK	32*	9.5/36413
				716.92	2	0.0794	TTDLNISENNRR		
	Sjc_0063180	Centriolin	3	572.88	2	0.1375	GELEQIKAEK	51*	7.04/278991
				723.43	2	0.0615	KISDQSELKLER		
				730.45	2	0.2049	RDYSLMRSCVR		
	Sjc_0046400	Conductance calcium-activated potassium channel protein 2	2	481.32	2	0.1387	FISLCNHK	31*	7.09/169207
				899.53	2	0.1672	NLVTSVMGVLSDYMPR		
	Sjc_0009730	CREB-binding protein	2	679.52	2	0.153	MILMR	32*	8.00/113822
				414.23	2	0.0905	YTVCER		
	Sjc_0106490	PDZ and LIM domain protein 3	3	564.31	2	0.0968	VPMHPECLK	30*	9.08/35351
				506.97	2	0.2293	VPMHPECLKCCK		
				534.22	2	0.0493	CCKCGIGLR		
	Sjc_0054640	Prolyl-tRNA synthetase	3	488.93	2	0.0718	KGTQQGLRCCVR	31*	7.5/128901
				699.86	2	0.0117	CKVEPHVRTGSK		
				621.88	2	0.0013	LALQNTVLSKR		
F3-H6	Sjc_0213700	Zinc finger, RanBP2-type, domain-containing	1	450.29	2	0.0415	INLSSLPR	30	9.34/57532
F18-B1	Sjc_0045150	E3 ubiquitin-protein ligase HUWE1	1	466.78	2	0.0381	RWTNLSR	29	5.25/301151
	Sjp_0042440	5'-nucleotidase, cytosolic II	1	384.8	2	0.1307	VTSVHLL	27	7.17/55038
F18-C3	Sjc_0203170	Chromatin licensing and DNA replication factor 1	1	530.83	2	0.0376	DVIDLVKMK	40	9.74/64520
	Sjc_0042100	Ubiquitin-conjugating enzyme E2 N	2	487.32	2	0.0935	QNEAEALAK	28*	5.42/19728
				601.4	2	0.1123	LGRICLDILK		
F31-C3	Sjc_0203170	Chromatin licensing and DNA replication factor 1	1	530.8	2	0.0104	DVIDLVKMK	36	9.74/64520
F31-E1	Sjc_0001260	Putative Serine/threonine-protein kinase C05D10.2	2	683.38	2	0.0558	LCDFGLARSLK	34*	9.18/120799
				590.36	2	0.1803	CQNGNKINCK		
F31-H7	Sjc_0203170	Chromatin licensing and DNA replication factor 1	1	530.81	2	0.0056	DVIDLVKMK	36	9.74/64520
	Sjc_0058190	NACHT and WD repeat domain-containing protein 1	1	590.33	2	0.0763	MCEQLLKTR	31	5.28/228171

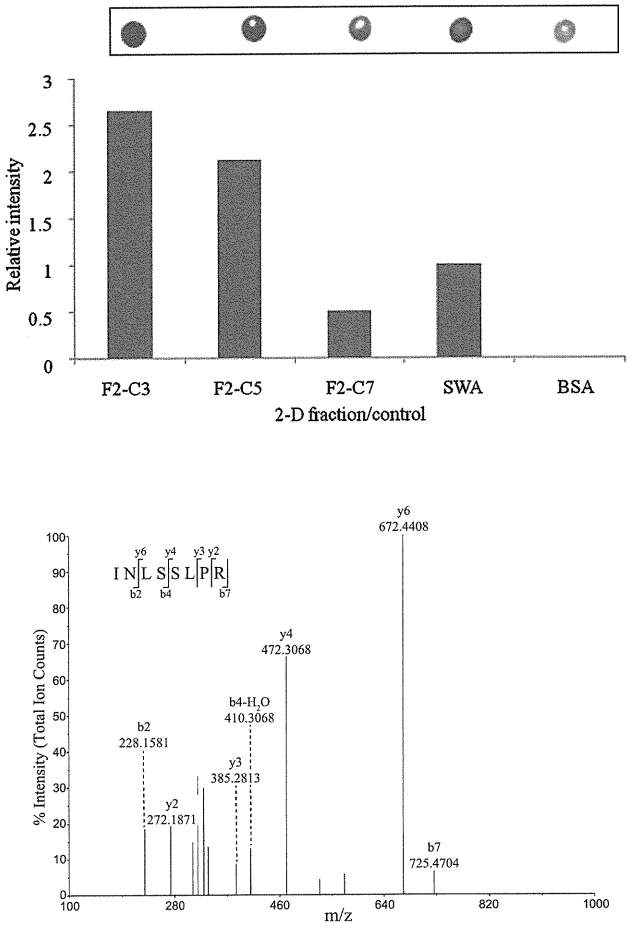

Fig. 4. Representative 2-D fraction dot-blot reactivity and tandem mass spectrum. Represented by A, IgE reactivity intensity of F2-C3, F2-C5 and F2-C7 with crude SWA and BSA controls. B, a tryptic INLSSLPR peptide of Zinc finger, RanBP2-type, domain-containing protein, Sjc_0213700 (GenBank: CAX74641.1). The precursor ion was *m/z* 450.30(2+). Sjc_0213700 was sequenced from F2-C5 and F2-C7 with strong preference for IgE.

DISCUSSION

Following skin penetration by cercariae, *S. japonicum* adult worms migrate to the hepatic portal system, where they mature and survive for many years where the female occasionally migrating to the smallest venules to lay eggs [43]. The adult schistosomes are constantly exposed to the host immune system with antibodies produced against fractions of the worms. These antibodies are often used as potential diagnostic tools. Several immunoepidemiological studies have examined antibody isotype responses to schistosomal protein extracts in the form of isolated proteins, recombinant proteins or crude antigen [44, 45]. Many studies have also presented a global proteomics approach using 2-D gels to identify major *S. japonicum* excretory and secretory proteins as well as adult worm and egg extracts [46–50]. In this study, for the first time, proteomics approach was extended to identifying *S. japonicum* proteins in ProteomeLab FP 2D derived liquid fractions reactive to antibody isotypes in plasma samples from *S. japonicum*-infected population. ESI MS/MS was applied to sequence fractions containing immunreactive proteins. In all, 18 proteins were identified; characterized and GO categories determined to enhance understanding of the immunological significance of these proteins.

In IgE, IgG1, IgG3 and IgG4 antibody isotype recognition of 1-D fractions, it has been shown that some fractions from the adult *S. japonicum* proteome are preferentially recognized by certain isotypes. For instance, IgE isotype was detected by fewer antigenic fractions (43.2%). The most recognized isotype was IgG3 (79.5%) followed by IgG1 (75.0%) and IgG4 (61.4%) accordingly. The IgG3 response was directed against a larger repertoire of antigens in the fractions. This suggests that there were fewer dominant antigens stimulating IgE response. Earlier report [51, 52] showed where IgE reactivity to glycolipids extracted from schistosome eggs (SEA) or adult worms (SWA) was more than IgG4 and that proteins alone do not constitute the major binding targets of IgE, and that this isotype is substantially directed towards carbohydrate moieties portion of glycolipids on proteins present in SEA or AWA. Weiss *et al.* [53] showed that a carbohydrate epitope recognized by a monoclonal antibody that was raised against the cercarial glycocalyx was present on glycoproteins and glycolipids of various schistosomes' life cycle stages. This might explain why IgG4 recognized more proteins than IgE in the 1-D dot-blot.

Furthermore, zinc finger, RanBP 2-type, domain-containing protein was strongly recognized by IgE but moderately by IgG3 and IgG4 and weakly by IgG1 indicating that it might play less role in IgG subclasses directed immune response. The antigens recognized strongly by IgE are of interest as such antigens could be associated with development of resistance to schistosomiasis [2–4, 6]. The E3 ubiquitin-protein ligase, ubiquitin-conjugating enzyme E2 N and 5'-nucleotidase, cytosolic II were strongly recognizing by IgG subclasses (IgG1, IgG3 and IgG4) but not IgE suggesting these enzymes might be of IgG subclasses preferred. The serine/threonine-protein kinase with a relatively higher antigenic propensity of 1.0118 was sequenced from a single 2-D well strongly reactive with all the four isotypes indicating strong preference for IgE, IgG1, IgG3 as well as IgG4 suggesting shared antigenic determinates or multiple epitopes. This highlights the importance of vaccine research

Table 2. Biochemical properties and immunoreactivity pattern of the proteins. Dot-blot assay was performed for each second dimension fraction containing the proteins identified. The reactivity was quantified into pixels unit and graded according to reactivity intensity with respect to that of the crude parasite antigen to obtain relative reactive intensity. Using the relative reactive intensity, each reactive spot was scored as 'weak reactivity' (–), 'moderate reactivity' (±) or 'strong reactivity' (+). In addition to reactive pattern, proteins were characterized using GRAVY index (ProtParam tool), number of antigenic determinant contained in the full amino acid length and the antigenic propensity where >1 indicates high antigenic character. In this Table antibody reactivity pattern is not repeated for proteins from the same fraction well.

Fraction Well	Protein identified	GRAVY index	Amino acids	Antigenic determinants	Antigenic propensity	Antibody reactivity pattern			
						IgE	IgG1	IgG3	IgG4
F2-C3	Chromatin licensing/DNA replication factor 1	–0.348	578	20	1.0414	+	–	–	+
F2-C5	Zinc finger, RanBP2-type, domain-containing	–0.986	513	21	1.0108	+	–	–	±
F2-C7	Zinc finger, RanBP2-type, domain-containing	–0.986	513	21	1.0108	±	–	–	±
F3-E1	Meiosis-specific, nuclear structural protein 1	–1.306	376	11	0.9936	+	±	+	+
	Chromatin licensing/DNA replication factor 1	–0.348	578	20	1.0414	+	±	+	+
	NACHT/WD repeat domain-containing protein 1	–0.282	2005	81	1.0271				
	Triple functional domain protein	–0.414	1554	68	1.0336				
	RNA Helicase [EC:3.6.1.-]	–0.409	762	25	1.0308				
	Protein kinase [EC:2.7.1.-]	–0.22	1921	63	1.0397				
	BRO1 domain-containing protein BROX	–0.177	426	16	1.0459				
	Cell division control protein CDC7	–0.333	330	13	1.0363				
	Centriolin	–0.847	2444	92	1.0139				
	Small conductance calcium-activated potassium channel protein 2, putative	–0.393	1536	58	1.0294				
	CREB-binding protein	–0.708	986	29	1.0269				
	PDZ and LIM domain protein 3	–0.674	317	13	1.0199				
	Prolyl-tRNA synthetase [EC6.1.1.15]	–0.473	1135	41	1.0295				
F3-H7	Zinc finger, RanBP2-type, domain-containing	–0.986	513	21	1.0108	–	–	±	–
F18-B1	E3 ubiquitin-protein ligase HUWE1	–0.423	2720	105	1.0231	–	+	+	+
	5'-nucleotidase, cytosolic II	–0.2	476	21	1.0368				
F18-C3	Chromatin licensing/DNA replication factor 1	–0.348	578	20	1.0414	–	+	+	+
	Ubiquitin-conjugating enzyme E2 N	–0.228	173	8	1.0386				
F31-C3	Chromatin licensing/DNA replication factor 1	–0.348	578	20	1.0414	–	+	+	+
F31-E1	Putative serine/threonine-protein kinase C05D10.2	–0.774	1061	39	1.0118	+	+	+	+
F31-H7	Chromatin licensing/DNA replication factor 1	–0.348	578	20	1.0414	–	–	–	–
	NACHT/WD repeat domain-containing protein 1	–0.282	2005	81	1.0271				

focusing on induction of protective isotype-specific antibody response to specific peptides since a single protein from the parasite might possess undetermined antigenic determinants capable of stimulating various antibody productions. Therefore, further investigations employing peptide mapping techniques will be essential in determining specific antigenic determinants for the isotypes.

There were also proteins found with strong immunogenic activity to IgE, IgG3 and IgG4 but not IgG1. Some of these proteins were the Small Conductance calcium-activated potassium channel protein 2, BRO1 domain-containing protein BROX both of which are membrane associated proteins and PDZ/LIM domain containing protein 3. Notwithstanding, it is possible that such proteins might have multiple isotype-specific or shared antigenic determinants within each protein. The PDZ/LIM domain containing proteins are known to be both interaction modules associated with proteins of diverse functions [20, 54].

Proteins associated with nucleus, or cytosolic component are not immediately and directly exposed to the immunity of the definitive host and hence might not evoke immune response. However, Harn, *et al.* [55], identified glycolytic enzymes, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase to be expressed on the surface of larval schistosomes and it appears very unlikely that these fundamentally cytosolic proteins are exposed at the external surface of the parasites. However, evidence from other cell systems showed that both glycolytic enzymes and various heat shock proteins can bind to cytoskeletal structures such as actin, microfilaments and microtubules [56, 57] where they can evoke immune response from the host. In addition, small conductance calcium-activated potassium channel (SK) protein 2 with calmodulin binding domain (CaMBD), a membrane associate protein that can also be secreted has been reported to function in host-parasite interaction [39, 41, 42].

In this study, attempt was made to identify and characterize potential IgE and IgG4 immunoreactive proteins employing immunoproteomics approach and related identified proteins with their biochemical properties and gene ontology types. A number of proteins were sequenced from immunoreactive fractions. It is anticipated that, immunoreactive proteins identified herein will stimulate further studies to evaluate their immunogenicity through recombinant protein expression, immunomoderation properties in terms of protective potentials and novel compounds that have therapeutic importance.

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Performance of Thirteen Clinical Rules to Distinguish Bacterial and Presumed Viral Meningitis in Vietnamese Children

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Abstract

Background and Purpose: Successful outcomes from bacterial meningitis require rapid antibiotic treatment; however, unnecessary treatment of viral meningitis may lead to increased toxicities and expense. Thus, improved diagnostics are required to maximize treatment and minimize side effects and cost. Thirteen clinical decision rules have been reported to identify bacterial from viral meningitis. However, few rules have been tested and compared in a single study, while several rules are yet to be tested by independent researchers or in pediatric populations. Thus, simultaneous test and comparison of these rules are required to enable clinicians to select an optimal diagnostic rule for bacterial meningitis in settings and populations similar to ours.

Methods: A retrospective cross-sectional study was conducted at the Infectious Department of Pediatric Hospital Number 1, Ho Chi Minh City, Vietnam. The performance of the clinical rules was evaluated by area under a receiver operating characteristic curve (ROC-AUC) using the method of DeLong and McNemar test for specificity comparison.

Results: Our study included 129 patients, of whom 80 had bacterial meningitis and 49 had presumed viral meningitis. Spanos's rule had the highest AUC at 0.938 but was not significantly greater than other rules. No rule provided 100% sensitivity with a specificity higher than 50%. Based on our calculation of theoretical sensitivity and specificity, we suggest that a perfect rule requires at least four independent variables that posses both sensitivity and specificity higher than 85–90%.

Conclusions: No clinical decision rules provided an acceptable specificity (>50%) with 100% sensitivity when applying our data set in children. More studies in Vietnam and developing countries are required to develop and/or validate clinical rules and more very good biomarkers are required to develop such a perfect rule.

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Introduction

Accurate and rapid diagnosis of acute bacterial meningitis (ABM) is essential as successful disease outcome is dependent on immediate initiation of appropriate antibiotic therapy [1,2]. Differentiating ABM from presumed acute viral meningitis (pAVM) often proves challenging for clinicians as their symptoms and laboratory tests are often similar and overlapping. Classical clinical manifestations of ABM in infants and children are usually difficult to recognize given the absence of meningeal irritation signs and delayed elevation of intracranial pressure. In addition, the various parameters examined in the cerebral spinal fluid (CSF) are less discriminative in children than in adults, especially in enterovirus meningitis where the CSF parameters may be similar to bacterial meningitis values. The vast majority of patients with acute meningitis are administered broad-spectral antibiotics targeting ABM while awaiting results of definitive CSF bacterial cultures. In the absence of ABM, this practice may enhance the local frequency of antibiotic resistance [3], cause adverse antibiotic effects [4], and high medical costs [5]. Thus, it is not only important to recognize ABM patients who promptly require antimicrobial therapy, but also pAVM patients who do not need antibiotics or hospital admission at all. An ideal diagnostic rule

should demonstrate 100% sensitivity in detecting bacterial meningitis [6], while retaining a high specificity.

Unfortunately, no single clinical symptom or laboratory test has differentiated ABM from pAVM with 100% sensitivity and high specificity [7,8]. More recently, numerous researchers have investigated potential clinical decision rules that recognize ABM from pAVM including: Thome [9], Spanos [10], Hoen [11] (also called Jaeger et al [12]), Freedman [13], Nigrovic [14], Oostenbrink [15], Bonsu 2004 [16], Brivet [17], Schmidt [18], De Cauwer [19], Chavanet [20], Dubos [21], Bonsu 2008[22], Tokuda [23], and Lussiana [24]. A few rules have included complicated multivariate models that require the use of a computer [10,11], while others have used scoring systems [9,15], tree model decisions [23], or a simple list of items [13,14,17,18,19,20,21,22]. These clinical decision rules require extensive test prior to their use in hospitals [25] and have rarely been compared in a single study. In addition, several rules are yet to be tested by independent researchers [17,21,22,23,24] or tested in children [17,23]. The Nigrovic's rule, also called Bacterial Meningitis Score (BMS) [14], performed perfectly in several studies [8,26,27,28,29,30], but failed to provide 100% sensitivity in other independent data sets [7,19,20,31]. Simultaneous test and comparison of these rules is required to enable clinicians to select an optimal rule to limit the number of patients being unnecessarily treated with antibiotics, and to guarantee that patients with bacterial meningitis receive appropriate antibiotics.

Materials and Methods

Identification of clinical rules

Two electronic databases including PubMed and Scopus were searched for suitable clinical rules. The search terms used were as follows: "dengue AND (rule OR score)". We supplemented these searches with a manual search of articles that developed and/or compared clinical rules. Since we aimed to find the clinical rule that could be applied in our hospital and test the generalizability of clinical rules [32], no restrictions were applied with respect to country, year, and language of studies that developed clinical rules. A total of 15 clinical rules were identified. Among them the Bonsu 2008 [22] and Dubos rules [21] were not tested as band leukocytes and procalcitonin were not available in our hospital.

Study design

The current study was performed at the Infectious Department of Pediatric Hospital Number 1, Ho Chi Minh City, Vietnam. The hospital is a tertiary pediatric hospital in southern Vietnam with 1200 beds. It was a retrospective cross-sectional analysis of the clinical signs and laboratory tests obtained from previously healthy children (≤15 years) that were diagnosed with acute meningitis. Discharge diagnosis was reviewed to identify meningitis patients based on the International Classification of Diseases, 10th Revision (ICD-10) with the following codes: G00, G00.x, G01*, G02.0*, G03, and G03.x. The study was approved in advance by the Ethical Review Committee of the Pediatric Hospital Number 1, Ho Chi Minh City, Vietnam. Written informed consent from the patients or their parents was waived by the Committee, because all data were retrospectively collected after the discharge of patients and numerically coded to ensure patient anonymity.

The entry criteria were as follows: children with proven acute bacterial meningitis (ABM) or presumed acute viral meningitis (PAVM), who had received a lumbar puncture between December 2003 and December 2008. Patients exhibiting blood-contaminated CSF (CSF erythrocyte count >10,000 cells/μL) [33], tuberculous meningitis, HIV infection, immune depression, and those found to have histories of pulmonary tuberculosis, liver diseases such as autoimmune disease, alcoholic liver disease and metabolic disease, kidney disease, neurosurgical disease or had undergone recent neurosurgery were excluded from the study. Neonates (less than 28 days old) and patients with missing laboratory variables listed in Table 1 were also excluded.

Proven ABM was diagnosed if the patient demonstrated CSF pleocytosis (CSF leukocyte count >7 cells/μL) [34,35] in addition to one of the following test results: (1) positive CSF culture for bacterial pathogens, (2) positive CSF latex agglutination test, or (3) positive blood culture. PAVM was defined as patients with a pleocytosis in the CSF (CSF leukocyte count >7 cells/μL) in addition to positive culture for viral pathogens or rapid remission without extensive antibiotic therapy combined with an absence of any four criteria of proven ABM [10,14,20,26,36,37].

Blood cultures were performed using 5% sheep blood agar before 2005 and a BACTEC 9240 system instrument (BD Biosciences, China) from 2005. CSF culture was done on 7% horse blood agar and 5% chocolate blood agar plates and incubated at 36°C for 24 h. Observed colonies were further identified by standard microbiological methods. Viral culture was not routinely performed, only five CSF samples were sent to Pasteur Institute (Ho Chi Minh City, Vietnam) for virus isolation.

At the time of admission, the relevant patient history regarding clinical symptoms and signs, and laboratory parameters listed in the Table 1 was collected. Clinical signs and symptoms that were not noted in the patient medical record were coded as normal.

Data analysis

All information was entered into a Microsoft Office Excel 2007 computerized database. Missing clinical signs and symptoms were not included and the number of patients per group was also adjusted before analysis. Our analysis showed that there were no significant differences in selected variables between patients with and without missing data." into the data analysis (page 6).

A score, judge, or probability of ABM (pABM) was calculated from each patient for each of the clinical decision rules according to the authors of the rules (Method S1). The overall accuracy of these rules represented by area under a receiver operating characteristic curve (ROC-AUC) was compared by the method of DeLong [38] using MedCalc statistical software (11.0, MedCalc Software bvba, Belgium). AUC values ≥0.5, 0.75, 0.93, or 0.97 were considered as fair, good, very good, or excellent accuracy [39]. The sensitivity and specificity of each rule was then calculated using our patient data set. To do so, we applied the thresholds indicated by the authors of the rules and by our own ROC analyses. The rules demonstrating 100% sensitivity were further analyzed to compare their specificity using the McNemar test [8].

The minimal required sample size and power of comparison were calculated using the MedCalc statistical software based on 5% type I error rate and 20% type II error rate. Assuming that ROC-AUCs of all clinical rules are at least 90% compared to the null hypothesis value 70% [22] , the required sample size was 48 subjects per group in this case.

In order to explain the limitation of Nigrovic's rule, we calculated the theoretical sensitivity and specificity of simple list of items rule with cut-off value at one item. Since selected variable demonstrated an independent predictor of ABM [14], the theoretical sensitivities and specificities of the simple list of items rule with cut-off value ≥1 can be derived from individual sensitivity and specificity of each variable as presented by equation 1 and 2, respectively (Figure 1). The individual sensitivity and specificity of each variable were derived from the current study

Table 1. Characteristic of variables used in the clinical decision rules to distinguish ABM from pAVM.

Variables	Scores using equation			List of items					Classified scores				Tree model
	Spanos (1989)	Hoen (1995)	Bonsu (2004)	Freedman (2001)	Nigrovic (2002)	Brivet (2005)	Schmidt (2006)	De Cauwer (2007)	Thome (1980)	Oostenbrink (2004)	Chavanet (2007)	Lussiana (2011)	Tokuda (2009)
Clinical variables													
Age	⊕		⊕	⊕									
Admission month	⊕												
Symtoms duration										⊕			
Seizure					⊕	⊕			⊕				
Vomit										⊕			
Body temperature									⊕				
Disturbed consciousness						⊕			⊕	⊕			⊕
Focal neurological						⊕							
Shock						⊕							
Meningeal irritation										⊕			
Cyanosis										⊕			
Purpura or petechiae									⊕	⊕			
Blood variables													
WBC		⊕							⊕				
Neutrophils %													
Neutrophil count				⊕	⊕								
Neutrophil band count													
Glucose		⊕											
CRP								⊕		⊕			
CSF variables													
Gram stain	⊕			⊕	⊕								⊕
WBC				⊕			⊕		⊕		⊕	⊕	
Neutrophils %								⊕	⊕		⊕		⊕
Neutrophil count	⊕	⊕	⊕		⊕								⊕
Protein		⊕	⊕	⊕	⊕		⊕	⊕	⊕		⊕	⊕	
Glucose				⊕				⊕	⊕		⊕	⊕	
CSF/blood glucose ratio	⊕			⊕									
Lactate							⊕						
Threshold	pABM* ≥0.1			≥1 item					Complex judge				

*Probability of ABM (pABM).

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- Equation 1: Theoretical sensitivity of rule = 1- $\prod_{i=1}^{n}(1 - Sens_i)$
- Equation 2: Theoretical specificity of rule = $\prod_{i=1}^{n}(Spec_i)$

Where *n*, Sens*i*, and Spec*i* are number of variables, sensitivity, and specificity of variable *i*, respectively.

Figure 1. Equation for calculation of theoretical sensitivity and specificity of simple list of items rule with cut-off value at one item.
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unless otherwise stated. The method calculation was described in the (Figure 2).

Results

Characteristic of patient population

Between December 2003 and December 2008, 192 patients met our inclusion criteria. A total of 63 patients were excluded from the final analysis due to the following reasons: (1) age of 0–28 days (n = 34), (2) traumatic lumbar puncture (n = 14), (3) recent neurosurgery or head injury (n = 12), or (4) HIV infection (n = 3). The high number of excluded patients could be explained by the characteristics of the tertiary hospital. A total of 129 patients including 80 ABM (62%) and 49 PAVM (38%) patients were selected for the final analysis (Table 2). Among the 80 patients with proven ABM, death occurred in 6.3% (n = 5), and neurological sequelae was observed in 25% (n = 15, Table 2). Of the 80 ABM cases, bacterial pathogen was identified in the CSF Gram-stain of 34 cases (43%), in the CSF culture of 39 cases (49%), blood culture of 18 patients (23%), in the blood culture alone of one patient (1.2%), and by latex agglutination in 65 patients (81%). Bacterial infections were caused by *Haemophilus influenzae* (n = 49, 61.3%), *Streptococcus pneumoniae* (n = 26, 32.5%), *Streptococcus agalactiae* (n = 1, 1.3%), *Neisseria meningitides* (n = 1, 1.3%), *Escherichia coli* (n = 2, 2.5%) and *Morganella morganii* (n = 1, 1.3%). Of the 49 PAVM cases, Herpes simplex virus 1 was the only viral pathogen isolated (n = 2).

Comparison of clinical rules

The overall accuracy of the rules was explored by calculation of the ROC-AUCs. All 13 clinical rules possessed AUC values between 0.75 and 0.94, indicating good accuracy (Table 3 and Figure S1) [39]. The Spanos rule had the highest AUC at 0.938. However, when comparing with the other four best rules (De Cauwer, Freedman, Nigrovic, and Thome), the Spanos rule was not significantly better by Delong method [38] (P>0.05, Figure 3).

When applying the thresholds indicated by the authors of the rules, no rule demonstrated 100% sensitivity, as prediction rules failed to identify six ABM patients by Thome, one ABM patient by Spanos, 19 ABM patients by Hoen, one ABM patients by Freedman, three ABM patients by Nigrovic, 18 ABM patients by Oostenbrink, seven ABM patients by Bonsu, 15 by Brivet, 33 ABM patients by Schmidt, one ABM patient by De Cauwer, 18 ABM patients by Chavanet, ten by Tokuda, and eight by Lussiana's rule. When applying the thresholds computed by our ROC analysis to achieve 100% sensitivity, all rules showed low specificity (< 25%). The Spanos's rule demonstrated the highest specificity at 24%, followed by Oostenbrink (8%), Bonsu (8%),

1. ***Theoretical sensitivity of a clinical rule that combines n varialbes:***

- Probability of a ABM patient is missed by test *i* = (FN*i*)/(TP*i* + FN*i*) = 1- (TP*i*)/ TP*i* + FN*i*) = 1- Sens*i*

Where *n*, TP*i*, FN*i*, and Sens*i* are number of tests (variables), true positive, false negative, and sensitivity of test *i*, respectively.

- Probability of a ABM patient is missed by all *n* test (test*1, 2, ..i,..n*) = $\prod_{i=1}^{n}(1 - Sens_i)$
- Sensitivity of simple list of items rule with cut-off value ≥ 1 is equal to probability of a ABM patient met by any test =1- $\prod_{i=1}^{n}(1 - Sens_i)$

2. ***Specificity of simple list of items rule:***

- Probability of a pAVM patient is missed (diagnosed as pAVM) by test *i* = (TN*i*)/(FP*i* + TN*i*) = Spec*i*

Where TN*i*, FP*i*, and Spec*i* are true negative, false positive and specificity of test *i*, respectively.

- Specificivity of simple list of items rule with cut-off value ≥ 1 is equal to probability of a pAVM patient that is missed by all n test (test*1, 2, ..i,..n*) = $\prod_{i=1}^{n}(Spec_i)$

Figure 2. Explanation for calculation of theoretical sensitivity and specificity. The theoretical sensitivity is the likelihood of sensitivity of the clinical rule after combining *n* tests, thus its values is depend on the individual sensitivity of each test. For example, a clinical rule combining two tests with sensitivities at 90% and 80%, respectively, the likelihood of the combined sensitivity (of the clinical rule of two tests) is calculated as 1–(1–0.90)×(1–0.80) = 0.98 or 98%. Therefore, combination of several tests will enhance the rule's sensitivity. In contrast, a clinical rule combining two tests with specificities at 80% and 70%, the likelihood of the combined specificity (of the clinical rule of two tests) will be decreased as the follow calculation: 0.80×0.70 = 0.56 or 56%.
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Table 2. Characteristics of the 129 patients in this study.

Characteristic	ABM n (%) or mean ± SD	pAVM n (%) or mean ± SD
Number of patients	80	49
Age ≤ 12 month	49 (62)	13 (28)
> 12 month	31 (38)	36 (72)
Sex: male	50 (63)	33 (67)
Duration of illness (days, median, 95% CI for the median)	3 (3–5)	2 (2–3)
Hospitalization days	16.3±8.8	5.3±2.6
Nausea	3 (4)	4 (8)
Vomiting	47 (60)	31 (62)
Fever	78 (98)	48 (96)
Purpura	0 (0)	0 (0)
Cyanosis	7 (9)	1 (2)
Seizure	54 (68)	11(22)
Fever	59 (74)	36 (72)
Cyanosis	13 (16)	1 (2)
Purpura or petechiae	4(5)	0 (0)
Meningeal signs	28 (35)	20 (40)
Bulging **fontanelle**	37 (46)	11 (22)
Altered mental status	35 (44)	4 (8)
Focal neurological deficits	23 (29)	5 (10)
Shock	3 (4)	0 (0)
Blood WBC	15,398±9,033	13.420±4,989
Blood neutrophil %	58.5±18.1	53.0±21.4
Blood neutrophil count	9,776±8,224	7.298±4,709
Blood glucose (mg%)	84.5±30.4	89.8±18.2
Blood CRP	136.7±97.5	25.0±47.9
CSF WBC	2,946±5,809	136±215
CSF neutrophils %	71±21	36±23
CSF neutrophil count	2,469±4,920	36±48
CSF protein (g/L)	1.13±0.70	0.39±0.31
CSF glucose (mg%)	26.1±19.6	56.9±12.9
CSF/blood glucose ratio	0.34±0.26	0.65±0.19
CSF lactate (mmol/L)	7.0±4.3	2.1±0.7
Blood culture (+)	18 (23)	
Gram-stain (+)	34 (43)	
CSF culture (+)	39 (49)	
Latex (+)	65 (82)	
Death	5 (6)	0 (0)
Neurological sequelae or death	20 (25)	0 (0)

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Hoen's rules (4%), while the Freedman, Nigrovic, Thome, Brivet, Schmidt, De Cauwer, Chavanet, Tokuda, and Lussiana's rules could not achieve 100% sensitivity.

Our calculation showed that the theoretical sensitivity of Nigrovic's rule was 96.6% when computing the variables' sensitivity values observed in our study. The strength of the theoretical sensitivities was in the following order: Freedman = De Cauwer > Nigrovic > Schmidt > Brivet > Chavanet, which was almost identical to the order of real sensitivities performed in our data set (Table S1). The theoretical sensitivity of Nigrovic's rule was just slightly increased (98.1%) upon computing the variables' sensitivity values observed in Nigrovic's studies [14], further supporting that the rule is not perfect. Similarly, the strength of theoretical specificities was in the following order: Chavanet > Schmidt > Brivet > Nigrovic > De Cauwer > Freedman. These findings were similar to the order of real specificities in the data set. Furthermore, the correlation between the theoretical and real accuracy was analyzed by a Spearman rank test. Our results demonstrated that the theoretical sensitivity and specificity were highly correlated with real sensitivity and

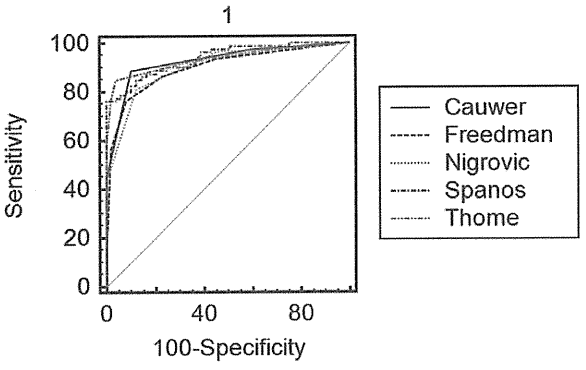

Figure 3. ROC curves of five best clinical rules for differential diagnosis of ABM from PAVM. The AUCs of ROC curves were 0.927 for De Cauwer rule, 0.900 for Freedman, 0.907 for Nigrovic, 0.938 for Spanos, and 0.935 for Thome. Pairwise comparison of all ROC-AUCs showed no significant difference of the five selected rules.
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specificity, respectively (Figure S2). Overall, there was no statistical difference between theoretical calculations and real values in data sets in regards to sensitivity and specificity, suggesting that our calculation was correct.

Discussion

To our knowledge, this is the first study that simultaneously tested more than ten prediction rules for clinical practice in meningitis. No clinical rule had superior overall accuracy compared to other rules. In addition, no rule provided 100% sensitivity with acceptable specificity (>50%). The overall accuracy of the two earliest rules (Spanos and Thomas rules) was not outperformed by recent developed rules, probably due to the similar epidemiology to the pre-vaccination era [10]. The high frequency of *H. influenzae* in our study could be explained by the lack of conjugate Hib vaccine in the Vietnamese national vaccination policy, and only a small number of children (0.5%) reportedly received conjugate Hib vaccine [40].

Among reported clinical decision models, the Nigrovic's rule [14] is the only rule that has been tested by more than three independent groups, and performed perfectly in several studies [8,26,27,28,29,30]. However, it only provided 96.3% sensitivity in our study, which is also in the range of other independent data sets [7,19,20,31] and well agreed with the theoretical sensitivity (96.64%) and specificity values (53.35%), explaining that the Nigrovic's rule could not identify all ABM patients in several data sets.

Based on these evidences and our equations, an ideal simple list of items clinical rule with theoretical sensitivity >99.99% and theoretical specificity >50% should include at least four independent variables that posses both sensitivity and specificity >85–90%. In addition, to improve the rule sensitivity without significantly reducing its specificity, we recommend adding additional variables with extremely high specificity (approximately 100%). We are not aware of more than three such conventional parameters to derive such an ideal rule. However, recent studies have proposed that blood procalcitonin [21,41], CSF lactate [42,43,44], and blood C-reactive protein (CRP) [45] are very good biomarkers for bacterial meningitis. Upon addition of procalcitonin test (99% sensitivity and 83% specificity [37]), the theoretical sensitivity of Nigrovic's rule would be significantly increased from 96.64% to 99.77% (Calculation: $1-(1-0.9664)\times(1-0.99)=0.9997$), while the theoretical specificity value would be dropped

Table 3. Accuracy comparison of clinical rules.

Rule	AUC	Cut-off values	Sensitivity % (95% CI)	Number of ABM patients missed by the rule	Specificity % (95% CI)
Thome	0.935	≥2#	92.5 (84.4–97.2)	6	65.3 (51.2–78.8)
Spanos	0.938	**pABM>0.04***	**100 (96.3–100)**	**0**	**[a]24 (13.1–38.2)**
		pABM≥0.10#	98.7 (93.2–99.9)	1	34 (21.2–48.8)
Hoen	0.883	pABM>0.0026	**100 (96.3–100)**	0	[a]4 (0.5–13.7)
		pABM≥0.10#	77.2 (65.4–85.1)	19	80 (67.7–89.2)
Freedman	0.900	≥1#	98.7 (93.2–99.9)	1	12.2 (5.8–26.7)
Nigrovic	0.907	≥1#	96.3 (91.2–98.7)	3	55.1 (46.9–59.0)
Oostenbrink	0.758	≥2⊕	100 (96.3–100)	0	[a]8 (2.2–19.2)
		≥8.5#	78.5 (66.8–86.1)	18	50 (35.5–64.5)
Bonsu	0.812	**pABM≥0.014**	**100 (96.3–100)**	0	**[a]8 (2.2–19.2)**
		pABM≥0.10#	92.4 (82.8–96.4)	7	28 (16.2–42.5)
Brivet	0.790	≥1#	81.3(71.0–89.1)	15	70 (55.4–82.1)
Schmidt	0.880	≥2#	58.8 (47.2–69.7)	33	100 (92.9–100)
De Cauwer	0.927	≥1#	98.7 (93.2–99.9)	1	40.8 (33.3–43.7)
Chavanet	0.878	≥2#	78.5 (66.8–86.1)	18	96 (86.3–99.5)
Tokuda	0.876	High risk	87.5 (78.2–93.8)	10	88 (75.7–95.5)
Lussiana	0.868	High risk	90.0 (81.2–95.6)	8	75.5 (61.1–86.7)

#Thresholds indicated by the authors of the rules.
⊕Thresholds computed by ROC analysis to achieve 100% sensitivity.
*Probability of ABM (pABM).
Numbers in boldface indicate rule with 100% sensitivity.
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from 53.35% to 44.28% (Calculation: $0.5335 \times 0.83 = 0.4428$). However, these three parameters have rarely been measured in the same study and their usefulness and independent contribution in the differential diagnosis of ABM from pAVM are rarely evaluated [46,47]. Thus, further studies are required to evaluate the contribution of these variables in the performance of clinical rules.

There were several limitations in our study. The first limitation was that the design was retrospective. Secondly, we only analyzed data from only one hospital. Therefore our results would be different from other hospitals, particularly in high-resources countries, where the epidemiology, clinical characteristics and outcome are different. Thirdly, our study focused on hospitalized patients in a big city. Therefore, further studies recruiting patients in clinics or local hospitals are required to further test these clinical rules. Fourthly, we could not confirm all pAVM patients as aseptic meningitis due to limited diagnosis in our hospital, which may affect the result. Another limitation is that the number of pAVM patients was much smaller than that of ABM, because several patients with extensive antibiotic therapy were excluded from criteria of pAVM. Finally, we were unable to include band leukocytes and blood procalcitonin, thus we could not test two promising Bonsu 2008 [22] and Dubos's [21] rules in the current study.

In conclusion, accurate bacterial meningitis is serious and the outcome is dependent on immediate initiation of appropriate antibiotic therapy. The best method for differentiating accurate bacterial meningitis from viral meningitis remains unclear. Several clinical decision rules have been derived to assist clinicians to distinguish between bacterial meningitis and viral meningitis, but barely tested and compared by independent studies. When applying our data set, no clinical rule provided an acceptable specificity (>50%) with 100% sensitivity. More studies in developing countries are required to confirm due to several limitations related to population and more accurate biomarkers are required to develop such a perfect rule.

Supporting Information

Figure S1 ROC curves of 13 clinical rules for differential diagnosis of ABM from PAVM when applying our data set. The AUCs of ROC curves were 0.812 for Bonsu 2004, 0.790 for Brivet, 0.927 for De Cauwer, 0.878 for Chavanet, 0.900 for Freedman (upper panel), 0.883 for Hoen, 0.868 for Lussiana, 0.907 for Nigrovic, 0.758 for Oostenbrink (middle panel), 0.880 for Schmidt, 0.938 for Spanos, 0.935 for Thome, and 0.876 for Tokuda rule (lower panel). Pairwise comparison of all ROC-AUCs was shown as the follow: -Spanos rule was significantly better than Schmidt, Chavanet, Tokuda, Lussiana, Bonsu, Brivet, and Oostenbrink rule. -Thome rule was significantly better than Hoen, Chavanet, Tokuda, Lussiana, Bonsu, Brivet, and Oostenbrink rule. -De Cauwer rule was significantly better than Bonsu 2004, Brivet, and Oostenbrink rule. -Nigrovic rule was significantly better than Brivet and Oostenbrink rule. -Freedman rule was significantly better than Bonsu 2004, Brivet, and Oostenbrink rule. -Hoen rule was significantly better than Bonsu 2004, Brivet, and Oostenbrink rule. -Schmidt rule was significantly better than Bonsu 2004, Brivet, and Oostenbrink rule. -Chavanet rule was significantly better than Brivet and Oostenbrink rule. -Tokuda rule was significantly better than Bonsu 2004, Brivet, and Oostenbrink rule. -Lussiana rule was significantly better than Oostenbrink rule. -Other pairwise comparison showed no significant difference.
(TIF)

Figure S2 Correlation between real and theoretical accuracy of six simple list of items rules. The Spearman correlation showed an r value of 0.971, $P = 0.001$, $n = 6$ for sensitivity correlation, and r value of 1.0, $P < 0.001$, $n = 6$.
(TIF)

Method S1 Description and calculation of clinical rules. The rules were derived from original studies.
(DOC)

Table S1 Theoretical sensitivities and specificities of simple list of items rule calculated and compared using our data set.
(DOC)

Author Contributions

Conceived and designed the experiments: NTH NTHT DTND KH. Performed the experiments: NTHT NTH NTK DTND. Analyzed the data: NTH NTHT NAT CCM DTND KH. Wrote the paper: NTH NTHT DTND CCM KH.

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RESEARCH LETTER

Development of a single-tube loop-mediated isothermal amplification assay for detection of four pathogens of bacterial meningitis

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Abstract

Several loop-mediated isothermal amplification (LAMP) assays have been developed to detect common causative pathogens of bacterial meningitis (BM). However, no LAMP assay is reported to detect *Streptococcus agalactiae* and *Streptococcus suis*, which are also among common pathogens of BM. Moreover, it is laborious and expensive by performing multiple reactions for each sample to detect bacterial pathogen. Thus, we aimed to design and develop a single-tube LAMP assay capable of detecting multiple bacterial species, based on the nucleotide sequences of the 16S rRNA genes of the bacteria. The nucleotide sequences of the 16S rRNA genes of main pathogens involved in BM were aligned to identify conserved regions, which were further used to design broad range specific LAMP assay primers. We successfully designed a set of broad range specific LAMP assay primers for simultaneous detection of four species including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *S. suis* and *S. agalactiae*. The broad range LAMP assay was highly specific without cross-reactivity with other bacteria including *Haemophilus influenzae*, *Neisseria meningitidis* and *Escherichia coli*. The sensitivity of our LAMP assay was 100–1000 times higher compared with the conventional PCR assay. The bacterial species could be identified after digestion of the LAMP products with restriction endonuclease DdeI and HaeIII.

Introduction

Rapid diagnosis of bacterial meningitis (BM) is essential as successful disease outcome is dependent on immediate antibiotic therapy (Saez-Llorens & McCracken, 2003; Zimmerli, 2005). However, accurate and rapid identification of BM is challenging for clinicians as its symptom and laboratory test are often similar and overlapping with those of aseptic meningitis. Conventional diagnosis of BM relies on the detection of bacteria in cerebrospinal fluid and/or blood by Gram staining, latex agglutination and culturing. However, Gram staining and latex agglutination tests are low in sensitivity (Kennedy *et al.*, 2007), while culturing takes few days. Furthermore, antimicrobial therapy prior to lumbar puncture often reduces the frequency of positive cultures from the CSF and blood (Pandit *et al.*, 2005).

PCR assays have recently been developed to detect several bacterial pathogens of BM. These assays have been widely used in clinical practice and proved to have both high sensitivity and specificity. However, the PCR method requires expensive instrument, experienced technician and few-hour performance. To overcome the limitations of current PCR, the loop-mediated isothermal amplification (LAMP) assay has been invented as an accurate, rapid and cost-effective method, which amplifies the target

FEMS MICROBIOLOGY LETTERS

nucleic acid under isothermal conditions, usually between 56 and 65 °C (Notomi *et al.*, 2000). The amplified product of LAMP assay can be detected in < 1 h and identified by agarose gel electrophoresis, simple visual inspection, real-time monitoring of turbidity or visual colour change using fluorescent dye. Importantly, the assay can be performed at bedside and in rural areas using only a water bath (Tomita *et al.*, 2008). Several LAMP assays have been developed to detect common causative pathogens of BM such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli* and *Staphylococcus aureus* (Seki *et al.*, 2005; Yamazaki *et al.*, 2008; Hanaki *et al.*, 2011; Kim *et al.* 2011; McKenna *et al.* 2011). However, no LAMP assay has been reported to detect *Streptococcus agalactiae* and *Streptococcus suis*, which are two of the most common pathogens of BM in some countries (Mai *et al.*, 2008; Chiba *et al.*, 2009). Moreover, it is laborious and expensive by performing multiple reactions for each sample to detect bacterial pathogen. Thus, we aimed to design and develop a LAMP assay capable of detecting multiple bacterial species based on the nucleotide sequences of the 16S rRNA genes of the bacteria.

Materials and methods

Design of LAMP assay primers for seven common bacteria in BM

The broad range LAMP primers were designed to be specific for eubacterial 16S rRNA-specific gene. This gene was chosen because of its highly conserved regions among species and has been widely used as a target for broad range PCR method (Gray *et al.*, 1984; Lane *et al.*, 1985). The partial nucleotide sequences of the 16S rRNA genes of *S. aureus* (GenBank FJ907240.1), *S. pneumoniae* (Z22807), *S. suis* (Z22776.1), *S. agalactiae* (Z22808), *N. meningitidis* (Z22806), *H. influenzae* (Z22809.1) and *E. coli* (AY513502.1) were retrieved from the GenBank database and were aligned to identify potential target regions using MULTIALIN software (Corpet, 1988). Several conserved regions were chosen for designing of LAMP primer set using the LAMP primer design software Primer Explorer version 4 (Eiken Chemical Co., Ltd, Tokyo, Japan). A set of four primers including two outer primers (forward primer F3 and backward primer B3) and two inner primers [forward inner primer (FIP) and backward inner primer (BIP)] that identified six distinct regions on the potential target sequence was designed. This study was approved by the institutional ethical review committees of the Institute of Tropical Medicine, Nagasaki University.

Bacterial strains and samples preparation

Serotypes 3 and 10 of *S. pneumoniae* were isolated from upper respiratory tract in Vietnamese patients. Two strains (8-01 and 8-02) of *S. suis* serotype 2, *E. coli*, *S. aureus* and *S. agalactiae* were also isolated from Vietnamese patients. In addition, *H. influenzae* and *N. meningitidis* were isolated from Japanese patients. The *S. pneumoniae* was cultured on rabbit blood Muller Hinton agar, while other bacteria were grown on rabbit blood brain heart infusion agar. Grown bacteria were harvested and suspended in normal saline. The cells were pelleted, suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and serially diluted with TE buffer ranging from 10^8 down to 10^0 colonies of bacteria mL^{-1} (CFU mL^{-1}). DNA was released from the bacteria by boiling for 20 min followed by centrifugation at 10 000 *g* for 10 min. The supernatant was used as the DNA template.

Optimization of LAMP reaction

The LAMP reaction was carried out in a 25-μL reaction mixture with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd) as described in our previous work (Kubo *et al.*, 2010). The reaction mixture contained 40 pmol (1 μL) each of FIP and BIP, 5 pmol (1 μL) each of F3 and B3 and 20 pmol (1 μL) each of Loop F and Loop B. LAMP reaction was performed at several different temperatures ranging from 55 to 68 °C in 90 min using LA-320C Loopamp real-time turbidimeter (Teramecs, Japan). The best condition for LAMP procedure was at 63 °C and in 60 min. Therefore, all of mixtures were incubated at 63 °C for 90 min, followed by heating at 80 °C for 5 min to inactivate the reaction. Two microlitre of the extracted DNA was used as the template in each reaction mixture. A negative control (a reaction mixture with distilled water instead of DNA template) and a positive control (a confirmed positive sample) were included in each run. Precautions were taken to prevent cross-contaminations.

Analysis of LAMP product

The LAMP product was analysed by three methods including a real-time turbidimeter, agarose gel analysis and naked eye visualization. The LA-320C Loopamp real-time turbidimeter (Teramecs) was used to monitor the LAMP reaction based on the turbidity of magnesium pyrophosphate at 405 nm, a byproduct of the reaction. The turbidity threshold value for a positive sample was fixed at 0.1, and samples above this threshold value were considered as positive. After amplification, 2 μL of the

LAMP product was further separated by 2% agarose gel electrophoresis, which was stained with ethidium bromide and visualized under UV light. In addition, 1 μL of SYBR Green I (Invitrogen) was added to the remained LAMP product, a change from orange to fluorescent green colour was considered as positive. To further distinguish bacterial species, 2 μL of the LAMP product was digested with 10 U of DdeI or HaeIII at 37 °C for 90 min. The digested LAMP product was analysed by 2% agarose gel electrophoresis as described above.

Conventional PCR assay

A conventional PCR was also carried out with the universal primer set targeting 16S rRNA genes to compare the sensitivity of the LAMP assay. The paired primers were 5′-CCAGCAGCCGCGGTAATACG-3′ and 5′-ATCGG(C/T)TACCTTGTTACGACTTC-3′ (Lu *et al.*, 2000). Twenty-five microlitre of PCR assay contained 2 μL of DNA template, 1 μL of each primer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 μL of 10 × buffer and 1.25 U *Taq* HS DNA polymerase (Takara Bio, Shiga, Japan). The reactions were amplified as follows: initial activation of one cycle at temperature 94 °C for 10 min and then followed by 35 cycles at 94 °C for 30 s, 55 °C for 50 s and 72 °C for 2 min. The final extension step was carried out at 72 °C for 10 min. Amplified products were then detected by ethidium bromide staining after 2% agarose gel electrophoresis.

Results and discussion

Design of broad range LAMP assay primers

We aimed to develop a LAMP assay capable of detecting many bacterial species (multispecies LAMP assay) for diagnosis of BM. The bacterial species used in this study were *S. aureus*, *S. pneumoniae*, *S. suis*, *S. agalactiae*, *N. meningitidis*, *H. influenzae* and *E. coli*. The nucleotide sequences of the 16S rRNA genes of these bacteria were retrieved and aligned to design broad range specific LAMP assay primers using EXPLORER VERSION 4 (Eiken Chemical Co., Ltd). We could not design any broad range specific LAMP assay primers for all the seven bacteria due to high level of variation in the target 16S rRNA gene among species (Fig. 1a). Next, we repeatedly aligned the target gene and designed broad range specific LAMP assay primers each time removing each species. However, no broad range specific LAMP assay primers were found for the detection of any set of more than four bacterial species. Finally, we successfully designed a set of broad range specific LAMP assay primers for the detection of four species including *S. aureus*, *S. pneumoniae*, *S. suis* and

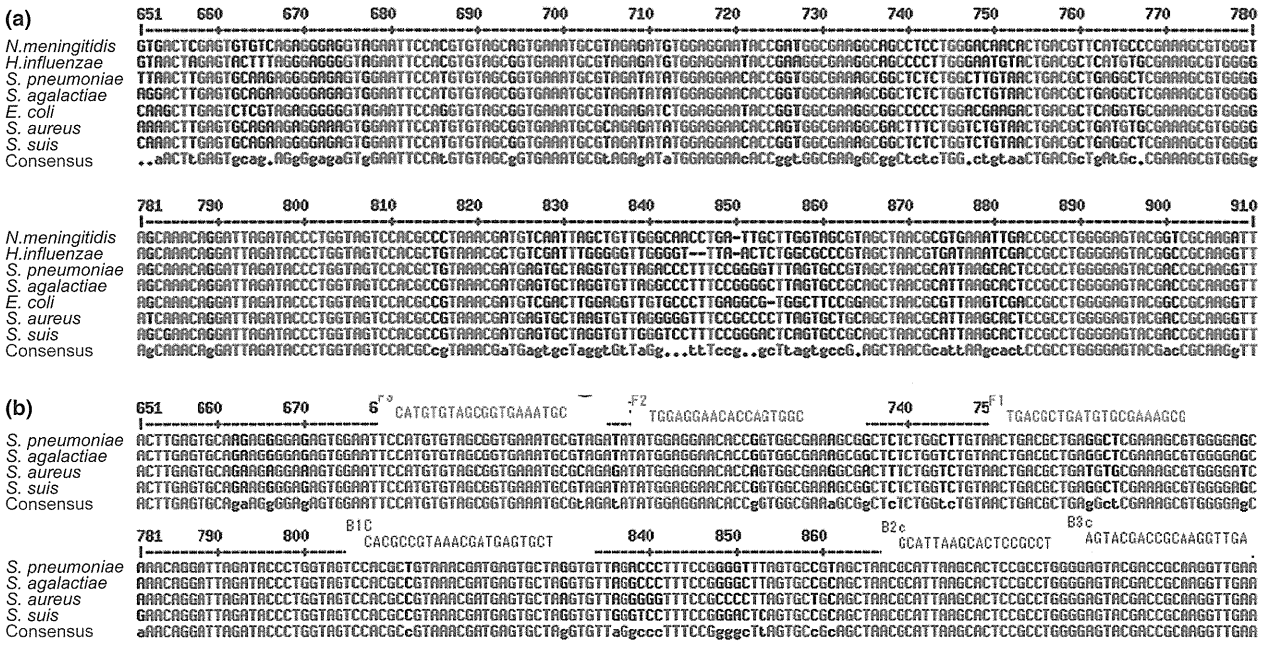

Fig. 1. Alignment of nucleotide sequences of the 16S rRNA genes of bacteria. The target gene of seven common bacteria of BM including *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus suis* (a) and four bacteria including *S. pneumoniae*, *S. agalactiae*, *S. aureus* and *S. suis* (b) were aligned to identify the highly conserved regions, which were used for LAMP primers design. Consensus shown similar nucleotides in red colour was used to design a universal set of LAMP primers for simultaneous detection of multiple bacteria.