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COMPARATIVE PROTEIN PROFILE ANALYSIS OF DIFFERENTIALLY EXTRACTED WHOLE CELL BACTERIAL PROTEIN DERIVED FROM *SALMONELLA* TYPHI AND INVASIVE NON-TYPHOIDAL *SALMONELLA*

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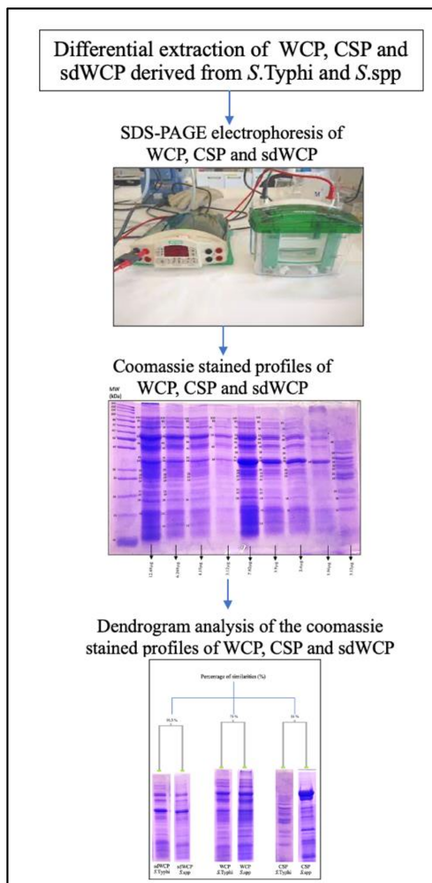
Graphical abstract



Abstract

Typhoid fever is an endemic disease that has been acknowledged as a major global health burden. Typhoid fever is a neglected re-emerging infectious disease that is transmitted through fecal oral route. Nevertheless, non-typhoidal *Salmonella* outbreak has been increasing globally with large number of cases involving immunocompromised individuals. The clinical diagnosis of typhoid is difficult due to the overlapping symptoms of typhoid fever, non-typhoidal *Salmonella*, and other associated febrile diseases, which causes to delayed treatment. Herein, this study aims to provide a reproducible, discriminative protein fingerprint of two different Salmonella serovars using a differential extraction procedure comprising of whole cell protein (WCP), cell surface protein (CSP) and surface-depleted whole cell protein (sdWCP) derived from whole cell bacterial protein of *Salmonella* Typhi (*S.*Typhi) and *Salmonella* spp (*S.*spp). In the present research, we perform comparative analysis to characterize protein profiles of two differentially extracted *Salmonella* serovars by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dendrogram analysis. The findings showed that the proteins were reproducible in optimized concentration. Separation of protein into three different extractions revealed discriminative protein profiles with major and micro-heterogenicity. We observed the diverseness of differentially extracted proteins between two strains which provided an effective adjunct that can be used in identification of *Salmonella* strains. The results achieved when differential extraction procedure was applied, provides a promising, future opportunity for further immunoproteomic classifications.

Keywords: *Salmonella* Typhi, *Salmonella* spp, Differential Extraction, SDS-PAGE, typhoid fever

Abstrak

Penyakit demam tifoid adalah penyakit endemik yang diakui sebagai beban kesihatan global yang besar. Demam tifoid adalah penyakit infeksi terselindung yang semakin berkembang dan dipindahkan melalui laluan feses-oral. Walau bagaimanapun, wabak *Salmonella* bukan tifoid telah meningkat secara global dengan bilangan kes yang melibatkan individu yang kekurangan daya tahan. Diagnosis klinikal demam tifoid sukar disebabkan oleh simptom-simptom demam tifoid yang mirip dengan *Salmonella* bukan tifoid, dan penyakit-penyakit yang lain. Ini menyebabkan kelewatan dalam mendapatkan rawatan yang betul. Oleh itu, kajian ini bertujuan untuk memberikan sidik jari protein yang boleh diterbitkan dan diskriminatif bagi dua serovar *Salmonella* yang berbeza dengan menggunakan prosedur ekstraksi yang berbeza yang melibatkan protein sel keseluruhan (WCP), protein permukaan sel (CSP) dan protein sel keseluruhan

tanpa permukaan (sdWCP) yang diperoleh daripada protein bakteria sel keseluruhan *S.* Typhi dan *S.* spp. Dalam kajian ini, kami melakukan analisis perbandingan untuk mencirikan profil protein dua serovar *Salmonella* yang diekstrak secara berbeza dengan elektroforesis poliakrilamid-sodium dodekil sulfat (SDS-PAGE) dan analisis dendrogram. Temuan menunjukkan bahawa protein itu boleh diterbitkan dengan konsentrasi yang disesuaikan. Pengekstratan protein ke dalam tiga ekstrak yang berbeza mendedahkan profil protein yang dapat membezakan antara kedua-dua strain dan boleh digunakan untuk mengenal pasti serotip *Salmonella*. Keputusan yang dicapai apabila prosedur ekstraksi berbeza diterapkan, memberikan peluang masa depan yang menjanjikan untuk klasifikasi imunoproteomik yang lebih lanjut.

Kata kunci: *Salmonella* Typhi, *Salmonella* spp, ekstrasi berbeza, SDS-PAGE, demam tifoid

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1.0 INTRODUCTION

Typhoid fever is a potentially fatal, neglected re-emerging global infectious disease. The disease is acquired through faecal oral route by consumption of contaminated food and water [1], [2]. The clinical presentation of typhoid fever is unpredictable and mimics many other acute undifferentiated febrile illnesses (AUFI). Therefore, clinical based diagnosis can be challenging and complicated in typhoid cases. Typhoid fever is a common infection in immunocompetent individuals. However, communities with poor sanitation, children and immunocompromised individuals due to primary and secondary immunodeficiency or malnutrition are more susceptible to typhoid fever with severe outcome [3], [4]. Therefore, timely treatment is essential to prevent complications and fatal outcomes. If untreated the disease can progress to severe complications involving multi organ systems and lead to death within one month of infection. The survivor may be left with long term neurological comorbidities[5]. The standard antimicrobial drug of choice for treatment of typhoid fever are fluoroquinolones, azithromycin and cephalosporin depending on the severity of disease [4], [5]. However, treatment for typhoid fever remain challenging due to the global widespread of the extensively drug resistance stains (XDR). The global spread of XDR strains limiting the choice of antibiotic treatment and may lead to scenario of untreatable typhoid fever[6], [7].

On the other hand, invasive non-typhoidal *Salmonella* (iNTS) further complicate the clinical and laboratory diagnosis of typhoid fever. iNTS causes frequent localized and systemic infection. iNTS disease is a global burden in the community whereby children and immunocompromised individuals are more susceptible to this disease. The increasing population of immunocompromised individuals globally as reported in literature led to upsurged of iNTS cases which caused the emergence of drug resistance strains [9], [10]. These extensive antimicrobial resistance among iNTS isolates makes treatment more challenging [11]. The clinical presentations of iNTS also mimic typhoid fever, therefore differential and definitive laboratory diagnosis of typhoid fever against iNTS infection is required [12].

Identification and classification of clinical isolate is important in routine diagnosis and epidemiological investigation of typhoid fever. The routine biological classification of clinical isolates of *Salmonella* Typhi are based on serotyping and molecular methods. Kauffman White is a conventional serotyping method used for classification of *Salmonella* based on somatic (O) antigens and flagella (H) antigens. Currently, there are more than 2600 serovars that have been classified based on this method [13]. Bacterial characterization by molecular methods are random amplification polymorphic DNA analysis (RADP-PCR), DNA amplification fingerprinting (DAF), restriction fragment length polymorphism (RFLP), plasmid profiling, pulsed field gel electrophoresis (PFGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [14]. Alternative serotyping tool include SeqSero genome based serotyping focusing on O and H antigens loci using next generation sequencing (NGS) data based analysis [15], [16]. Limitation of conventional and molecular serotyping include requirement of expensive antisera, trained personnel to perform the test, may produce inconclusive data, unable to predict serotype and incorrect identification [17], [18].

Several reports in literature showed the potential use of protein profiles for cost effective typing of bacteria [19]–[21]. Protein profiling by SDS-PAGE is an effective analytical technique used to produce high resolution separation of proteins based on their molecular weights [22]. This method has been widely used for strain and species identification and classification [23]. Report in literature has also described the correlation in SDS-PAGE profiling with DNA hybridization. SDS-PAGE has been proven to be a simple, quick and low-cost technique for bacterial identification. It is also an excellent method to be applied in resource-constraint environment [19].

Previous study has substantiated the characterization of common immunodominant

surface protein of different *Salmonella* serovars which contributed to protective measures for Salmonellosis [24]. Hence, further characterization and comparison between different serovars of *Salmonella* is necessary. Therefore, in this study, the protein profiling of differentially extracted whole cell bacterial proteins which includes whole cell proteins (WCP), cell surface proteins (CSP) and surface depleted-whole cell proteins (sdWCP) derived from *Salmonella* Typhi (*S*.Typhi) and invasive non-typhoidal *Salmonella* (*S*.spp) were analyzed by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Comparative analysis have been carried out to characterize and classify the two different serovars and develop an alternative protein serotyping tool for *Salmonella* species.

2.0 METHODOLOGY

2.1 Bacteria Culture Preparation and Maintenance

Two clinical isolates from blood cultures were obtained from Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan. Purity of the culture were verified by bacterial serotyping. The stock cultures from the hospital were collected and maintained at Proteomic Laboratory, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM), Kepala Batas, Pulau Pinang. The cultures were maintained in Luria broth with 20% glycerol in -80°C. Ethical approval was granted for the use of the stock cultures (Protocol code: USM/JEPeM/21050354 and date of approval: 11 August 2021).

2.2 Whole Cell Protein Extraction

Single colony of each bacterial strains were picked from Blood sheep agar, inoculated into 10ml Luria broth and incubated overnight at 37°C. After three consecutive overnight sub-culture, the broth was centrifuged (10000 rpm, 4°C, 10 minutes) and the pellet was resuspended in 1X sample preparation buffer (SPB) (pH 6.8). The suspension was boiled for 5 minutes and centrifuged (10000 rpm, 4°C, 10 minutes). The suspension was precipitated with ethanol and incubated overnight at -20°C. Subsequently, the suspension was centrifuged (10000 rpm, 4°C, 10 minutes). The protein pellet was dissolved in Tris with pH 7.4 containing phenylmethanesulfonylfluoride (PMSF) and stored at -20°C until further use.

2.3 Cell Surface Protein Extraction

Briefly, each bacterial strain was grown in 10ml of Luria broth at 37°C for 24 hours with constant shaking and sub-cultured three times consecutively in 1:100 ration in Luria broth. The overnight broth culture was harvested (10000 rpm, 4°C, 10 minutes) and the pellet was resuspended in glycine-HCL (pH 2.2). The suspension was incubated for 15 minutes at room temperature and centrifuged (10000 rpm, 4°C, 10 minutes). The pH of supernatant was adjusted to 7.4 and soluble protein was precipitated with ice cold ethanol at -20OC overnight. The cell surface protein was extracted by centrifugation (10000 rpm, 4°C, 10 minutes), dissolved in Tris-PMSF and stored at -20°C for future use.

2.4 Surface Depleted-Whole Cell Protein Extraction

Briefly, 10ml of Luria broth was inoculated with one colony of each bacterial strain and incubated (37°C, 24h) with constant shaking. The overnight broth was sub-cultured three times in 1:100 ratio. The culture was harvested (10000 rpm, 4°C, 10 minutes) and the pellet was resuspended in glycine-HCL (pH 2.2). The suspension was incubated for 15 minutes at room temperature and centrifuged (10000 rpm, 4°C, 10 minutes). The pellet was resuspended in 1X SPB (pH 6.8), boiled for 5 minutes and centrifuged (10000 rpm, 4°C, 10 minutes). The suspension was precipitated with ice cold ethanol and incubated overnight at -20°C. Subsequently, the protein was extracted by centrifugation (10000 rpm, 4°C, 10 minutes), dissolved in Tris-PMSF and stored at -20°C until further use.

2.5 Protein Assay

Total protein concentration for each extract of WCP, CSP and sdWCP of both strains was determined with Bradford Bio-Rad protein assay kit which is based on dye binding assay (Bio-Rad, CA,USA). The microassay procedure was performed using bovine serum albumin (BSA) as standard. Working standards with a concentration of 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml were prepared. 800 µl of each BSA standard and sample solution were mixed with 200µl of dye reagent concentrate in a separate test tube. The mixtures were incubated at room temperature for 5 minutes and the optical density (OD) were determined using a spectrophotometer (Cary 60 UV-Vis, Agilent) at wavelength of 595 nm using Bio-Rad's disposable polystyrene cuvettes.

2.6 SDS-PAGE Analysis

SDS-PAGE separation was done to analyze the profiles of each differential extracted protein[25]. A 10% resolving gel was prepared by mixing 5ml acrylamide, 3.75ml resolving buffer, 6ml deionized water, 200µl 20%AP and 20µl TEMED . The solution was mixed well and poured into Protean II slab gel mold and immediately overlaid with deionized water and allowed to polymerize at room temperature for 1 hour.

Staking gel was prepared by adding 0.65ml acrylamide, 1.25ml stacking buffer, 3.05ml deionized water, 100µl of 20% AP and 10µl TEMED. The solution was mixed well and poured into the glass slab mold inserted with 10 wells preparative comb and allowed to polymerize for 45 minutes.

The polymerized gel was removed from the gel casting apparatus and clamped to the cast before transferring them into electrophoresis tank. The tank

was filled with 1X running buffer that was made up of Tris, glycine and SDS. The protein samples were prepared by adding an equal volume of sample (6µl) to equal volume of SPB (6µl) dye. The protein samples were denatured by boiling the specimens for 5 minutes followed by centrifugation for 90 seconds in 10 000 rpm at 4°C. SDS-PAGE was then carried out at room temperature with current set of 25mA for one gel . After completion, the SDS-PAGE gel was incubated with Coomassie brilliant blue (CBB) staining solution for one hour followed by destaining with destaining solution (10% Methanol, 10% Acetic acid) until protein band could be seen clearly.

2.7 Protein Profile Analysis

The SDS-PAGE gel was analyzed by gel doc analysis software. The presence of clear distinct bands were labelled according to their molecular weight and tabulated by using Microsoft Excel software. The scanned gel was analyzed by using Unweighted Pair Group Method of Analysis (UPGMA) in CLIQS software. The relationships and similarities in all of the protein profiles were presented as dendrogram.

3.0 RESULTS

3.1 SDS-PAGE Protein Profiles of Differentially Extracted WCP of S.Typhi

Protein profiles of WCP,CSP and sdWCP derived from *S.*Typhi were determined by SDS-PAGE separation. Figure 1 shows SDS-PAGE gel of *S.*Typhi stained with CBB which demonstrated the protein profiles of WCP, CSP and sdWCP. Titration was done for WCP and sdWCP derived from *S.*Typhi and *S.*spp to obtain the optimum standardized concentration where each protein band are expressed. Four different concentrations were loaded in each lane of WCP and sdWCP of *S.*Typhi. From the result of the WCP from *S.*Typhi observed in lane 2 to lane 5 in Figure 1, up to 22 distinct, clear bands ranging from 13-100 kDa were observed. Common heavy protein bands were identified on lane 2 between 25-70 kDa while well separated bands were identified as the concentration went down in lane 3, lane 4 and lane 5.

Lane 6 to lane 9 of the sdWCP produced up to 24 bands ranging from 13-100 kDa. Common heavy protein bands were recorded on lane 6 between 25-70 kDa while well separated bands were recorded in lane 7 lane 8 and lane 9. On the other hand, CSP in lane 10 produced 12 distinct, clear bands ranging from 15-46 kDa. Table 1 shows the summary of WCP, sdWCP and CSP protein profiles derived from *S.*Typhi.

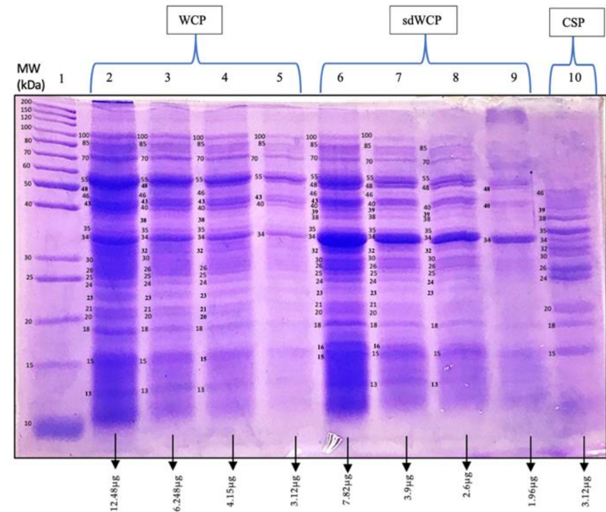

Figure 1 Protein profile of *S.*Typhi at different concentrations. Lane 1 = Molecular weight marker, Lane 2, Lane 3, Lane 4, Lane 5 = WCP, Lane 6, Lane 7, Lane 8, Lane 9 = sdWCP, Lane 10 = CSP. Numbers on the left side of each lane represents molecular weight of each band. Arrows below each lane indicates concentration of protein

Table 1 Summary of WCP, CSP and sdWCP coomassie stained protein profiles of *S.*Typhi in different concentrations

Bands molecular weight (kDa)	Lane								
	2	3	4	5	6	7	8	9	10
	WCP	WCP	WCP	WCP	sdWCP	sdWCP	sdWCP	sdWCP	CSP
100	+	+	+	+	+	+	-	-	-
85	+	+	+	+	+	+	+	-	-
70	+	+	+	+	+	+	+	-	-
55	+	+	+	+	+	+	+	-	-
48	+	+	-	-	+	+	+	+	-
46	+	+	+	-	+	+	-	-	+
43	+	+	+	+	+	+	+	+	-
40	-	+	+	+	+	+	+	+	-
39	-	-	-	-	+	+	+	-	+
38	-	+	+	-	+	+	+	-	+
35	+	+	+	-	+	+	+	-	+
34	+	+	+	+	+	+	+	+	+
32	-	+	+	-	+	+	+	-	+
30	+	+	-	-	+	+	+	-	+
26	+	+	+	-	+	+	+	-	+
25	+	+	+	-	+	+	+	-	-
24	+	+	+	-	+	+	-	-	+
23	+	+	+	-	+	+	+	-	-
21	+	+	+	-	+	+	-	-	-
20	+	+	+	-	+	+	-	-	+
18	+	+	+	-	+	+	+	-	+
16	-	-	-	-	+	+	-	-	-
15	+	+	+	-	+	+	+	-	+
13	+	+	+	-	-	+	+	-	-

3.2 Protein Profiles of *S.*spp in Different Concentrations

Figure 2 shows SDS-PAGE gel stained with CBB which demonstrated the protein profile of WCP, CSP and sdWCP derived from *S.*spp. The WCP and sdWCP derived from *S.*spp were loaded at three different concentrations. The SDS-PAGE separation of extracted WCP in lane 2 to lane 4 produced up to 21

distinct, clear bands ranging from 13-100 kDa. Common heavy protein bands were recorded between 25-60 kDa in lane 2 while well separated bands were recorded on lane 3 and lane 4 from Figure 2.

For sdWCP, up to 19 clear distinct bands ranging from 13-100 kDa were observed in lane 5 and lane 6 while only 12 clear bands were spotted in lane 7. As for the separation of bands, lane 5 has common heavy protein bands in the region below 18 kDa while lane 6 and lane 7 has well separated protein bands. The SDS-PAGE separation of extracted CSP in lane 8 produced 18 distinct, clear bands ranging from 11-71 kDa with common heavy protein band between 35-60 kDa. Table 2 shows the summary of WCP, sdWCP and CSP protein profiles derived from *S.*spp.

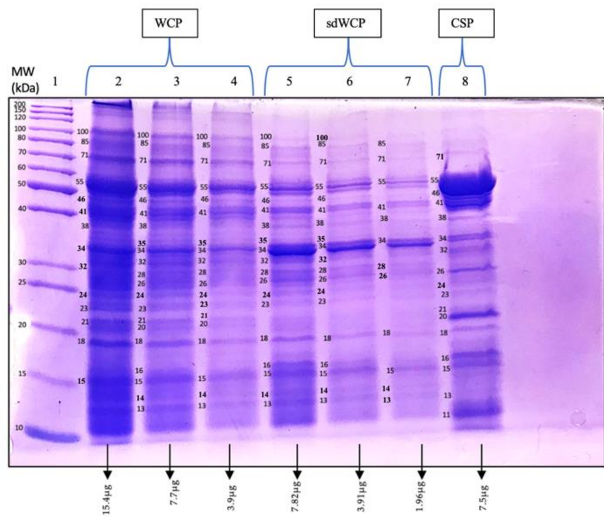

Figure 2 Protein profile of *S.*spp at different concentrations. Lane 1 = Molecular weight marker, Lane 2, Lane 3, Lane 4 = WCP, Lane 5, Lane 6, Lane 7 = sdWCP, Lane 8 = CSP. Numbers on the left side of each lane represents molecular weight of each band. Arrows below each lane indicates concentration of protein

Table 2 Summary of WCP,CSP and sdWCP coomassie stained protein profiles of S.spp in different concentrations

Bands molecular weight (kDa)	Lane						
	2	3	4	5	6	7	8
	WCP	WCP	WCP	sdWCP	sdWCP	sdWCP	CSP
100	+	+	+	+	+	-	-
85	+	+	+	+	+	+	-
71	+	+	+	+	+	+	+
55	+	+	+	+	+	+	+
46	+	+	+	+	+	-	+
41	+	+	+	+	+	+	+
38	+	+	-	+	+	+	+
35	-	+	+	+	+	-	-
34	+	+	+	+	+	+	+
32	+	+	+	+	+	-	+
28	-	+	+	+	+	+	-
26	-	+	+	+	+	+	+
24	+	+	+	+	+	-	+
23	+	+	+	+	+	-	+
21	+	+	+	-	-	-	+
20	-	+	+	-	-	-	+
18	+	+	+	+	+	+	+
16	-	+	-	+	+	-	+
15	+	+	+	+	+	+	+
14	-	+	+	+	+	+	-
13	-	+	+	+	+	-	+
11	-	-	-	-	-	-	+

3.3 Protein Summary on SDS-PAGE Protein Profiles of Whole Cell Protein Derived from *S.*Typhi and *S.*spp

From the protein profiles titration gel of *S.*Typhi and *S.*spp, the concentration of 6.24 µg is chosen for WCP of *S.*Typhi and 7.7 µg for WCP for *S.*spp as the optimum concentration for further analysis. For sdWCP, the concentration of 3.91 µg for *S.*Typhi and *S.*spp is chosen. The optimum concentration selected for CSP of *S.*Typhi is 3.12 µg and 7.5 µg for *S.*spp.

Table 3 shows the molecular weight of protein present in WCP, CSP and sdWCP derived from *S.*Typhi and *S.*spp at their optimized concentration. Table 4 shows the total number of protein bands present in WCP, CSP and sdWCP derived from *S.*Typhi and *S.*spp.

Table 3 Molecular weight of protein bands present in WCP, CSP and sdWCP of S.Typhi and S.spp

	Molecular weight (kDa)	
	*S.*Typhi	*S.*spp
Protein present in whole cell protein (WCP)	100, 85, 70, 55, 48, 46, 43, 40, 38, 35, 34, 32, 30, 26, 25, 24, 23, 21, 20, 18, 15, 13	100, 85, 71, 55, 46, 41, 38, 35, 34, 32, 28, 26, 24, 23, 21, 20,18, 16, 15, 14, 13
Protein present in bacterial surface (CSP)	46, 39, 38, 35, 34, 32, 30, 26, 24, 20, 18, 15	71, 55, 46, 41, 38, 35, 34, 32, 26, 23, 24, 21, 20, 18, 16, 15, 13, 11
Protein present in surface de-pleted-whole cell protein (sdWCP)	100, 85, 70, 55, 48, 46, 43, 40, 39, 38, 35, 34, 32, 30, 26, 25, 24, 23, 21, 20, 18, 16, 15, 13	100, 85, 71, 55, 46, 41, 38, 35, 34, 32, 28, 26, 24, 23, 18, 16, 15, 14, 13

Table 4. Total number of protein bands present in WCP, CSP and sdWCP of S.Typhi and S.spp

	Total number of bands	
	*S.*Typhi	*S.*spp
WCP	22	21
CSP	12	18
sdWCP	24	19

3.4 Dendrogram analysis of SDS-PAGE protein profiles of differentially extracted whole cell protein derived from *S.*Typhi and *S.*spp

Figure 3 shows the dendrogram analysis of WCP, CSP and sdWCP derived from *S.*Typhi and *S.*spp at the optimized concentrations. This dendrogram is developed by using numerical analysis unweighted pair group method of analysis (UPGMA) based on the banding pattern. The percentage of similarity between each differentially extracted protein is shown in the dendrogram. The numerical analysis showed that WCP, CSP and sdWCP derived from *S.*Typhi and *S.*spp has distinct similarity to each other. The highest percentage of similarity was observed between sdWCP of *S.*Typhi and *S.*spp at 91.3 %. The lowest similarity of 55 % was observed between CSP cluster derived from *S.*Typhi and *S.*spp. The

percentage similarity of WCP coomassie stained profiles derived from *S.*Typhi and *S.*spp was 75%.

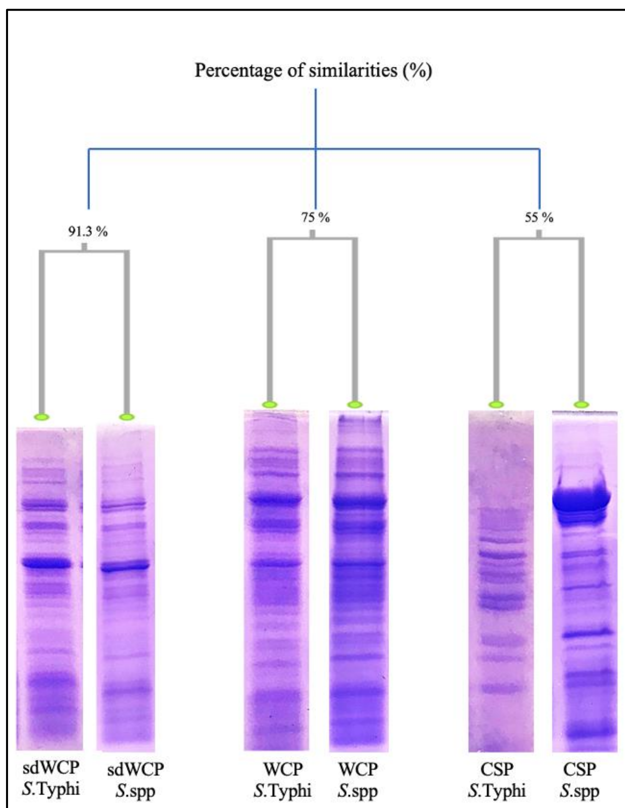

Figure 3 Dendrogram analysis on the coomassie stained protein profiled of differentially extracted proteins derived from *S.*Typhi and *S.*spp separated by SDS-PAGE. The analysis shows the percentage of similarity of the SDS-PAGE profiles of WCP, CSP and sdWCP of *S.*Typhi and *S.*spp by the unweighted pair group method of analysis (UPGMA). The Pearson coefficient has been used to compute the similarity matrix between each lane

4.0 DISCUSSION

Identification and differentiation of clinical pathogens are critical in monitoring infection and source of outbreak especially during an epidemic. The characterization of somatic (O) and flagella (H) antigens has been the primary choice in *Salmonella* serotyping[26]. However, emerging data have evidenced the issue of the over expression of flagella masking the O antigen which led to misidentification of clinical pathogens[27]. Ideally, a robust, cost effective and highly discriminative protein fingerprint that goes beyond serotype is needed in routine laboratory.

A high resolution SDS-PAGE has been described as an important tool of protein typing with higher discrimination power[28]. WCP profiling by SDS-PAGE has been reported as an effective means of identification and differentiation of clinical pathogens[29]. However, bacterial characterization solely by WCP is not the optimal approach since *Salmonella* WCP profiles are very homogeneous, making distinction of various *Salmonella* strains difficult[14]. The method of differential extraction followed by protein profiling has been described to produce striking differences in the protein profile of different strains of the same species[30]. The concept or theory on protein profiling is that the bacteria will demonstrate or convey the same set of proteins that are greatly reproducible under a standard optimized growth condition. This pattern of protein profile will represent each specific bacterial strain as their very own "protein fingerprint"[31]. This study focuses on protein profiling of two different *Salmonella* strains by three different extractions.

When screening differentially extracted proteins using SDS-PAGE, an optimal concentration is important in exposing the bands that cannot be seen at high concentration or low concentration. The low and high abundant protein are naturally distributed across the SDS-PAGE profile in various concentrations. Titration was performed in this study to recover as many proteins as possible and to optimize concentration at a maximum protein profile that includes low and high abundant protein for analysis.

Therefore, these results revealed heterogenicity of bands in various concentrations of protein. Based on the results in Figures 1 and 2, the appearance of clear and visible bands can be seen across different concentrations of protein. Concurrently, zone of smearing bands can be observed in lane 2 in both Figures 1 and 2, whereby some of the bands are masked with the high concentration of protein. However, several of the bands were either lost or unable to be detected when the protein concentration was too low as observed on lane 5 in Figure 1 and lane 7 in Figure 2. The SDS-PAGE protein profiles of the differentially extracted protein derived from *S.*Typhi and *S.*spp in various concentrations proved the feasibility to conclude the number of protein bands present in each strain without missing some of the proteins and therefore suggesting the added value of this approach for efficient protein-based typing.

Based on Table 3, the differentially extracted protein derived from *S.*Typhi and *S.*spp identified relatively homogenous and distinctive bands. WCP of *S.*Typhi and *S.*spp both yielded some major bands within 100 kDa to 13 kDa with the similarity percentage of 75%. Referring to Tables 3 and 4, four additional bands on the region of 48, 43, 30 and 25 kDa were detected in WCP of *S.*Typhi compared to WCP of *S.*spp. Concurrently, two additional bands with the molecular weight of 16 and 14 kDa were identified specifically in WCP of *S.*spp. The relatively minor differences in WCP derived from *S.*Typhi and *S.*spp indicated that WCP alone was insufficient for serotyping. Similarly, many reports have also claimed that whole cell profiles between closely related strains were relatively similar and is not sufficient enough for protein typing[14], [24], [32], [33]. These reports correlate with the results in this study when comparing WCP of both strains.

The difference in the protein profiles were more discrete in CSP derived from *S*.Typhi and *S*.spp. Based on Tables 3 and 4, CSP of *S*.spp recognized up to six additional protein bands compared to CSP of *S*.Typhi. Parallelly, two bands at 30 and 39 kDa present in CSP of *S*.Typhi was not detected in CSP of *S*.spp. This distinctive pattern of bands observed in the SDS-PAGE protein profile of CSP derived from *S*.Typhi and *S*.spp can be used as protein marker for strain typing.

The sdWCP from both *S*.Typhi and *S*.spp recognized a limited number of distinctive protein bands. As observed in Figure 1 and Figure 2, the sdWCP profiles of both strains recognized largely identical bands in the range of 100 kDa to 13 kDa with the similarity percentage of 91.3 %. Looking at the overall results, protein profiles of WCP and sdWCP exhibit considerable similarity in both strains. These results are in agreement with Berber, Cokmus and Atalan in 2003, who reported on the insufficient protein pattern difference of whole-cell and extracellular protein profiles Staphylococcus species[34]. In contrast to the report, a clear, distinct protein profile pattern was observed between CSP of both strains in this study. These prominent findings were supported and strengthen by the numerical analysis from the dendrogram (UPGMA) clustering in Figure 3 which indicated the percentage of similarity between the differentially extracted proteins derived from *S*.Typhi and *S*.spp. Much more complex protein in sdWCP and WCP in both strains explained the high similarity value based on dendrogram analysis. The CSP derived from *S*.Typhi and *S*.spp showed highest discrimination with a similarity of 55% based on the dendrogram analysis correlated with the pattern of protein profiles observed. To date, no previous study has been found in the comparison analysis between WCP, CSP and sdWCP of *Salmonella*. The differential extraction of both strains revealed unique, enhanced, distinctive proteins that could play a role in definitive characterization of *Salmonella*.

It is lucid from the present results that differential extraction and analysis of protein profiles by SDS-PAGE displayed heterogenous number of protein bands. This observation has been clearly delineated by Duncan *et al.* (1997), where certain degree of heterogenicity was observed in differentially extracted proteins of different species. Differential extraction results in distinguishable patterns of protein between two species that may assist in characterization for taxonomic of bacteria[30]. The present findings extent and confirmed this analysis, demonstrating that protein profiling of differentially extracted bacterial proteins derived from *S*.Typhi and *S*.spp is an effective approach to define and characterize at the strain level.

Serotyping has been the initial gold standard for characterization of *Salmonella*[15]. However, there has been major drawbacks in the classic phenotypic method. The prevalence of serotypes varies according on geographical location and the emergence of XDR strains further complicate the characterization of *Salmonella.* This study indicated a promising alternative solution in characterization of various *Salmonella* strains including the XDR strains.

The limitation of this study is the exclusion of paratyphoid fever as this study primarily focused on typhoid fever. Generally, infection by *S*.Paratyphi A, *S*.Paratyphi B and *S*.Paratyphi C are mild compared to typhoid fever. However, given the relatively similar clinical presentations of paratyphoid with typhoid fever, there is a diagnostic gap in early health intervention of paratyphoid[35]. Therefore, in the future study, *S*.Paratyphi A, *S*.Paratyphi B and *S*.Paratyphi C can be included in the investigation to conclude appropriate reference protein profiles for each species of *Salmonella* for routine laboratory application. Further follow-up investigation suggests the inclusion of all the other different *Salmonella* serovars present and came out with a profile that changes the whole scenario of typing based on O and H antigens.

5.0 CONCLUSION

The comparative analysis of protein profiles of S. Typhi and iNTS, this study has demonstrated the feasibility of using SDS-PAGE as an alternative protein serotyping tool for Salmonella species. This method can be a useful tool for quick and cost-effective identification and differentiation of clinical isolates, particularly in resource-limited settings. However, further studies with larger sample sizes are needed to validate the use of this technique and to assess its sensitivity and specificity. Overall, the findings of this study have the potential to contribute to the development of effective surveillance and control strategies for typhoid fever and iNTS, particularly in regions where these diseases are endemic. Further follow-up study in identifying the antigenicity of the protein bands has been carried out by western blot analysis and enzyme immunoassay. Further research in developing a rapid, highly sensitive diagnostic kit model based on the differential extraction approach has also been developed and filed for patent.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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