RESEARCH ARTICLE

Usefulness of the outer membrane proteins of *Shigella sonnei* in developing an antibody-based immunoassay for the diagnosis of shigellosis

Mahadi, N.N.F.N.[1], Leow, C.Y.[2], Banga Singh, K.K.[1*]

[1]Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
[2]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia
*Corresponding author: kiren@usm.my

ABSTRACT

Conventional culture method and biochemical tests remain as the 'gold standard' method for the identification of *S. sonnei* which are time-consuming. We have discovered previously the potential of three OMPs of *S. sonnei* (33.3 kDa, 43.8 kDa and 100.3 kDa) as biomarkers in the diagnostic test for shigellosis. Here, we evaluated the performance of the outer membrane proteins of *S. sonnei* for the development of an antibody-based immunoassay for the detection of *S. sonnei* infections. All three-target proteins were specifically recognized when probed with *S. sonnei* sera. In addition, another two potential proteins of molecular weight 29.0 kDa and 88.2 kDa in size were also exclusively recognized by the IgA when probed with *S. sonnei* sera. The optimized ELISA demonstrated higher sensitivity and specificity which exceeded 86.0%. In conclusion, the identified target proteins showed great potential as diagnostic biomarkers for the detection of *S. sonnei* infections in patients.

Keywords: Biomarker; outer membrane proteins; immunoassay; diagnostics; *Shigella sonnei.*

INTRODUCTION

Shigellosis is an invasive infection of the human colon that is caused by *Shigella* species. Patients who are infected with shigellosis normally will develop diarrhoea, which is often consisting of blood and mucus, fever, pain, and abdominal cramps. It has become a global burden throughout the world especially in developing countries with approximately 164.7 million cases reported annually which leads to 1.2 million deaths (Kotloff *et al.*, 1999; Bardhan *et al.*, 2010). The rapid emergence of multidrug resistant strains of *Shigella* spp. only added to the burden of the disease. In Malaysia, *Shigella* has been reported to be one of the major causes of childhood diarrhoea with *S. sonnei* as the commonest isolated etiological agent of shigellosis (Lee & Puthucheary, 2002; Banga Singh *et al.*, 2011). The routine test for diagnosis of shigellosis is by conventional culture method and biochemical identification. However, these techniques are time-consuming which take a minimum of 2 days to obtain laboratory results. Delay in diagnosis increases the risk of the outcome such as morbidity and mortality especially among young children and the elderly (Afroze *et al.*, 2017). Accordingly, significant efforts have been exerted in developing alternative methods in the rapid identification of *Shigella*.

ELISA has been reported as a powerful technique mainly used to investigate the presence of specific antibodies against certain antigens (Lee *et al.*, 1993, Uzal *et al.*, 1995). It provides high specificity and sensitivity when tested on clinical samples and reported to be more rapid and economical compared to the culture methods. The key advantages of ELISA assay are that it shows high sensitivity which allows quantitative and semi-quantitative antibody measurements, automatically adopted and is inexpensive (Hosseini *et al.*, 2018). In addition, this assay is simple and easy to carry out, as well as can be used to investigate a large number of serum samples in a short period of time (Sudan & Shanker, 2013). Three potential proteins of outer membrane proteins (OMPs) of *S. sonnei* (33.3 kDa, 43.8 kDa and 100.3 kDa), were reported as promising biomarker candidates to be used in the development of a rapid and reliable protein-based diagnostics for the identification of *S. sonnei* (Harikrishnan *et al.*, 2014, 2017). Thus, this study aims to further evaluate the potential of these protein candidates as diagnostic biomarkers in the development of an antibody-based immunoassay for the detection of *S. sonnei* in human.

MATERIALS AND METHODS

Growth and maintenance of bacterial strains
Shigella sonnei SH080 clinical strain was collected from the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia. The clinical strain was maintained in glycerol stock and kept at -80°C. Prior to use, the clinical isolate was allowed to grow in nutrient both at 37°C for overnight. To check for the purity of the strain, the organism was sub-cultured on blood agar followed by MacConkey agar for identification and confirmation of the strain. Colonies morphology resembling of *Shigella* spp. were confirmed by biochemical tests such as triple sugar iron agar, sulphide-indole motility (SIM) medium, urease, methylene red, and citrate.

Ethics approval and consent
Samples were collected from patients admitted in the ward or patients attending the outpatient clinics at the Hospital Universiti Sains Malaysia, Kelantan, immediately after the laboratory culture results were confirmed. All samples were collected in compliance with the Universiti Sains Malaysia Human Ethical Committee (Approval reference number: USMKK/PPP/JEPeM/[248.3(10)]. Written informed consent was obtained from all subjects before participation in the study.

Serum samples
A total of 130 human serum samples were collected from patients who developed diarrhoea in Hospital Universiti Sains Malaysia (Hospital USM). Bacterial infections were confirmed by conventional culture method and biochemical tests of the isolated colonies from patients' stool. Thirty (30) cultured confirmed *S. sonnei* infected sera and 100 non-*Shigella* sera were obtained from patients (Table 1).

OMP extraction and immunogenicity determination by Western blot
Extraction of the OMPs of *S. sonnei* incubated at 37°C was performed according to the protocol described by Harikrishnan *et al.* (2013). Briefly, bacteria were grown in 1 L nutrient broth overnight at 37°C (Forma Orbital Shaker, Model-420, USA) and centrifuged at 15 900 x g for 12 minutes. The pellet was re-suspended in 3400 µl HEPES buffer (pH 7.4), 16 µl RNase (Sigma Chemical Company, USA), 16 µl DNase (Sigma Chemical Company, USA) and 1600 µl PMSF (Calbiochem, USA). The bacterial cells were disrupted until achieved at least 95% of cell lysis via vortexing for 2 hours with one-minute alternate on ice with the sterilized glass beads of size approximately 0.2 mm, (BDH chemical Ltd., England). The cell lysate was collected by washing the glass beads with HEPES buffer (pH 7.4), followed by removing of whole cells and insoluble debris using a compact high speed refrigerated centrifuge (Kubota 6930, Japan) at 7,800 x g, 4°C for 15 minutes. Supernatants containing cell envelope were collected and subjected to centrifugation with an ultracentrifuge (Hitachi CP 80MX, Japan) at 146,161 x g, 4°C for 1 hour (using Sorvall TH-660 rotor). The pellet (crude cell envelops) was collected and re-suspended in 15 ml of HEPES buffer (pH 7.4). For OMP extraction, 15 mL of Triton X-100 (Bio-Rad, USA) was added and incubated for 10 minutes at room temperature. The suspension was centrifuged using ultracentrifuge (Hitachi CP 80MX, Japan) at 183,345 x g, 4°C for 1 hour. The pellets were re-suspended with 8 ml of 30 mM Tris HCl, pH 8.0.

Immunogenicity determination by Western blot
The OMP was separated using 10% SDS-PAGE gel. The OMP samples were prepared by mixing with 0.1% SDS and 2-β-mercaptoethanol (10%). Pre-stained broad range standards (Amersham, UK) was used to determine the location of the target proteins. Electrophoresis was performed at a constant voltage of 25 mA for 45 minutes and then was transferred onto PVDF membrane using a semi-dry electrophoretic transfer cell (Towbin *et al.*, 1979) with some modifications. The electrophoretic transfer was set at 10 V for 30 minutes according to the manufacturer's instruction. The membrane was blocked with 5% non-fat skimmed milk for 30 minutes and then washed with PBS-T (0.05% Tween 20). Then, the membrane strips were probed with human sera in 1:100 dilutions. After overnight incubation, the strips were washed and followed by incubation with 1:400 dilutions of secondary antibody (goat anti-human IgA conjugated with alkaline phosphatase; Abcam, UK) for 2 hours at room temperature. The reaction was developed with alkaline phosphatase conjugated substrate (Bio-Rad, USA) and the reaction was stopped by washing with distilled water.

Purification of OMPs target proteins
Based on the immunoblot analysis, proteins that are specific and highly antigenic against *S. sonnei* were identified. Protein elution was performed to isolate the protein of interest from the complex mixture. The protein bands that have been excised from the polymerized gel were washed with distilled water and minced before being loaded into the eluter tube. The elution was performed at 10 mA/glass tube for 4 hours. The eluted proteins were mixed with ice-cold absolute ethanol to extract the purified protein through precipitation and left at -20°C for overnight. The suspension was then centrifuged three times at 12,000 x g for 15 min (4°C). The pellet was left to dry at room temperature. Prior to use, the purified protein powder was diluted with an appropriate amount of PBS and kept at 4°C.

Optimization of ELISA for the detection of *S. sonnei* infections
ELISA parameters were optimized in order to develop a rapid and reliable enzyme immunoassay test for the detection of shigellosis. Determinations of the optimal parameters were made by using checkerboard assays. Parameter that gave the highest positive to negative (P/N) ratio of known positive and negative control samples as well as obtaining a reasonable OD value in the positive sample was considered to be the optimal condition. The optimization procedure was applied for each individual targeted protein. All samples and controls were diluted in PBS (pH 7.4).

First, each target protein was diluted to their individually optimal concentrations (1.25 – 5.0 µg/ml) and 100 µl of each target protein was coated into separate wells (NuncMaxisorp® flat bottom 96-well plate). Wells were washed with PBS-T before blocked with blocking buffer for 1 hour. Next, 100 µl of diluted primary antibody (1:50, 1:100 and 1:200) were added and incubated for 1 hour. This was followed by the addition of diluted (1:800, 1:1000 and 1:1200) HRP-conjugated anti-human IgA (Abcam, UK). After 1 hour incubation, 100 µl of ABTS substrate was added. The optical density (OD) value was immediately recorded using VersaMax™ Microplate Reader at wavelength 405 nm.

Diagnostic performance of the optimized ELISA
A panel of patients' sera compromising of 30 *S. sonnei* infections and 100 other related bacterial infections were collected and tested with the optimized ELISA (Table 1). Conventional culture methods and biochemical tests were the gold standard in confirming the infections. The diagnostic performance for the target proteins was interpreted separately based on the established cut-off value for each protein following the formula described by Ferrandiz *et al*. (2004). The cut-off values were calculated as the mean OD_{405} value of 30 negative control sera samples with the addition of three standard deviations of the mean value. A test value that exceeds the cut-off value is considered as a positive test result and a lower value is considered as negative test result. The sensitivity and specificity of the optimized ELISA were calculated based on the formula described by Crowther (2009).

Table 1. Panels of patients' sera used in this study

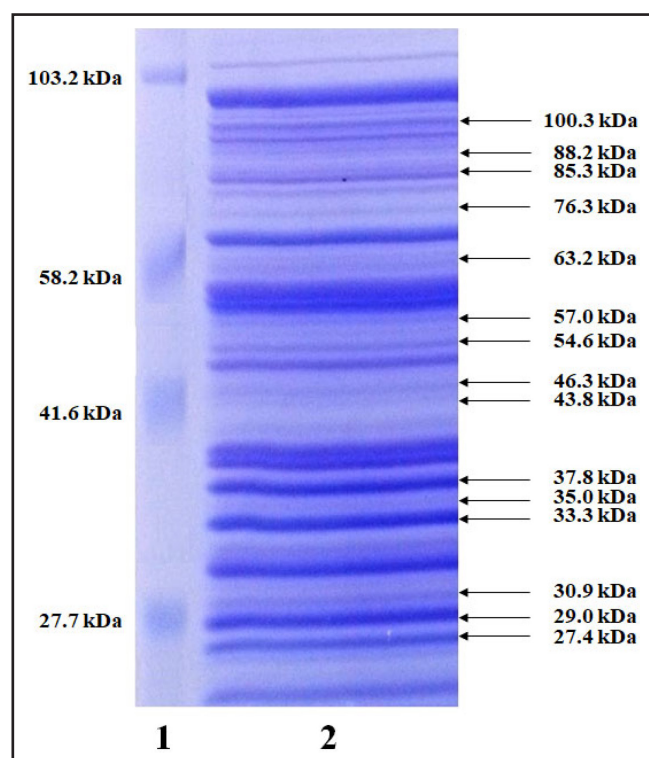| Bacterial strains	Quantity	Source
Shigella sonnei	30	
Salmonella spp.	51	
Enteropathogenic *Escherichia coli*	21	Department
Aeromonas hydrophila	10	of Medical
Campylobacter jejuni	8	Microbiology
Salmonella Typhi	7	& Parasitology,
Vibrio cholerae	2	USM
Salmonella Paratyphi C	1	
Total	130	

Figure 1. SDS-PAGE of the OMPs of *S. sonnei* clinical isolates SH080 expressed at 37°C (10% gel). Lane 1: molecular weight marker, Lane 2: OMPs of *S. sonnei*.
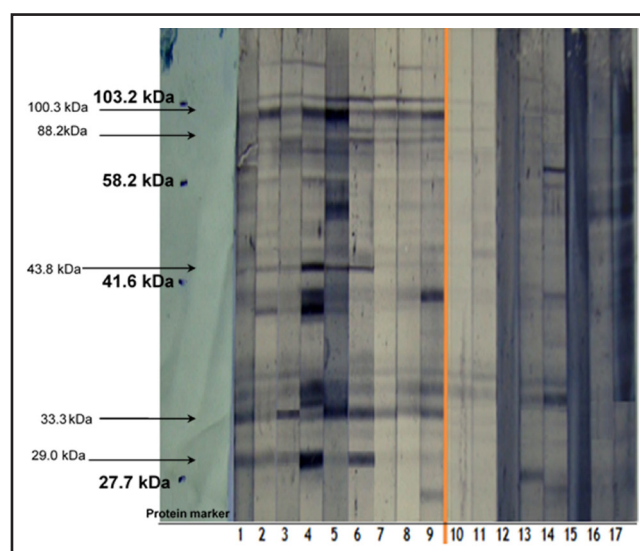

Figure 2. Western blot analysis showing the immunogenicity of OMPs of *S. sonnei* probed with anti-human IgA. The strips were probed with the following patient's sera: Strip 1: *S. sonnei* (SS01), Strip 2: *S. sonnei* (SS02), Strip 3: *S. sonnei* (SS03), Strip 4: *S. sonnei* (SS04), Strip 5: *S. sonnei* (SS05), Strip 6: *S. sonnei* (SS06), Strip 7: *S. flexneri* (SF01), Strip 8: *S. flexneri* (SF02), Strip 9: *S. flexneri* (SF03), Strip 10: Enteropathogenic *Escherichia coli* (EPEC01), Strip 11: Enteropathogenic *Escherichia coli* (EPEC02), Strip 12: *Campylobacter jejuni* (CAMPY01), Strip 13: *Aeromonas hydrophila* (AEHY01), Strip 14: *Aeromonas hydrophila* (AEHY02), Strip 15: *Aeromonas hydrophila* (AEHY03), Strip 16: *Aeromonas hydrophila* (AEHY04), Strip 17: *Salmonella* spp. (SASP01).

RESULTS

Protein profile and immunogenicity determination of OMP of *S. sonnei*

The OMPs profile of *S. sonnei* clinical isolate SH080 is shown in Figure 1. In total, fifteen OMPs protein were expressed with all target proteins were presented (shown in Figure 1).

In the Western blot analysis, a total of 17 sera were used including *S. sonnei* (n=6), *S. flexneri* (n=3) and other related infections sera (Figure 2). All the three target protein bands (33.3 kDa, 43.8 kDa and 100.3 kDa) were exclusively recognized against anti-human IgA when probed with all six *S. sonnei* sera and did not cross-reacted with sera from patients with other related infections. This result verifies the immunoreactivity of the target proteins against *S. sonnei* positive patients' sera. When probed with *S. sonnei* sera, the IgA also specifically recognized additional two protein bands, with 29.0 kDa and 88.2 kDa in sizes. Cross-reactivity analysis showed no cross-reaction with other non-shigellosis sera. Other antigenic bands were cross-reacting with most of the non-shigellosis sera tested. Based on the findings a total of five protein candidates (molecular weight 29.0 kDa, 33.3 kDa, 43.8 kDa, 88.2 kDa and 100.3 kDa) showed great potential to be used as diagnostic biomarker for early detection of *S. sonnei* infections. These five protein candidates were subjected to protein purification for diagnostics evaluation.

Optimization and diagnostic evaluation of ELISA using the purified target proteins of *S. sonnei*.

We performed the checkerboard assay to determine the optimal parameters for ELISA based on the highest OD_{405} ratio between positive to negative samples. The optimum antigen concentration for 29.0 kDa, 88.2 kDa and 100.3 kDa was 1.25 μg/ml. For protein 33.3 kDa and 43.8 kDa, the optimal antigen dilution was 2.5 μg/ml and 5.0 μg/ml, respectively. A dilution of 1:50 for primary human sera was used followed by 1:800 dilutions for secondary antibody.

Table 2. Diagnostic performances of the optimized ELISA using five target proteins for diagnosis of *S. sonnei* infections

Protein biomarker	Positive $n=30$	Negative $n=100$	Sensitivity (%)	Specificity (%)
29.0 kDa	26	96	86.7	96.0
33.3 kDa	26	96	86.7	96.0
43.8 kDa	27	96	90.0	96.0
88.2 kDa	26	96	86.7	96.0
100.3 kDa	27	98	90.0	98.0

A total of 130 patients' sera (30 culture-confirmed positive sera and 100 culture-confirmed negative sera) were screened using the optimized ELISA to evaluate the diagnostic performance of the new developed ELISA using the purified target proteins. The overall diagnostic evaluations is shown in Table 2. The optimized ELISA using protein 29.0 kDa. 33.3 kDa and 88.2 kDa as captured antigens exhibited 86.7% sensitivity (26/30) and 96% specificity (96/100). While, for protein 43.8 kDa, the optimized ELISA demonstrated 90% sensitivity (27/30) and 96% specificity (96/100). The diagnostic performance for protein 100.3 kDa was calculated 90% for sensitivity (27/30), 98% for specificity (98/100). The reduced in specificity were contributed by cross-reactions that were observed when probed with sera from patients infected with enteropathogenic *Escherichia coli* and *Aeromonas hydrophila*.

DISCUSSION

Current routine diagnostic methods for detection of *Shigella* are time-consuming and laborious which takes 2 to 3 days to obtain the results. It also has significant limitations such as low sensitivity and requires well-trained laboratory personnel. Delayed in

detection of this bacterium in patients caused problems in disease control and result in severe complications in patient care and management (Duran *et al.*, 2013). Hence, significant efforts have been exerted in developing alternative methods in identification of *Shigella*. Detection of anti-*S. sonnei* antibodies in patients serum has been reported as a promising tool in screening/identification of *Shigella sonnei* by immunoassay techniques such as latex agglutination, immunodiffusion, radioimmunoassay, enzyme immunoassay, enzyme-linked immunosorbent assay (ELISA) and immunochromatography test (Harikrishnan *et al.*, 2017; McPherson & Pincus, 2021). Development of such test would require the finding of specific antigenic proteins of *Shigella* to be used as biomarkers in the assay. Criteria for a good biomarker candidate is that it does not cross-react with other bacteria and specific only to the bacterial species to be identify.

Previously, our group identified three proteins candidates (33.3 kDa, 43.8 kDa and 100.3 kDa) as potential biomarkers in the diagnosis of *S. sonnei* infections (Harikrishnan, 2014). Current study was embarked using these target proteins as capture antigens to develop an ELISA assay for the diagnosis of *S. sonnei* infections. Protein expressed at temperature 37°C was selected in this study because it mimics the human normal body temperature, which is also the optimal growth temperature for *Shigella*. A study by Maurelli *et al.* (1984) suggested that at 37°C, *S. sonnei* were found to be fully virulent and able to invade intestinal epithelial cell.

Immunoblot analysis has confirmed that the target proteins (33.3 kDa, 43.8 kDa and 100.3 kDa) were immunogenic with all six *S. sonnei* sera tested and no cross-reaction occurred when probed with sera of other enteric-related infections. In addition to the previously identified target proteins, we also discovered two additional proteins of molecular weight 29.0 kDa and 88.2 kDa as potential biomarker candidates, since they were also specifically recognized by anti-human IgA probed with *S. sonnei* sera and did not show any cross reactivity when tested with other enteric-related diseases. Hence, this study demonstrated that a total of five protein candidates (molecular weight 29.0 kDa, 33.3 kDa, 43.8 kDa, 88.2 kDa and 100.3 kDa) showed great potential to be used as diagnostic biomarker for early detection of *S. sonnei* infections.

Accordingly, the potential uses of the purified targeted proteins as diagnostic biomarkers were evaluated through the development of an ELISA for quantitative analysis of the specific IgA in patients' serum. This ELISA assay uses components of the immune system and chemicals to detect immune responses in the body. Numerous studies have reported that ELISA gave high specificity and accuracy when tested on clinical samples and it is more rapid and economical compared to the culture methods (Gan & Patel, 2013, Dogruman-Al *et al.*, 2015). Furthermore, this technique required minimum number of equipment which is suitable to be used in low resources set-ups and also during the outbreak of the disease. A successful test must be simple to perform, highly sensitive, specific for *Shigella* spp. and are able to detect any of a large number of *Shigella* serotypes (Rigby, 1984).

Determining the optimal conditions for the ELISA is mandatory to achieve accurate results while avoiding any discrepancy that may appear because of variable titers of the anti-*S. sonnei* antibody presence in serum. After the successful development and optimization of ELISA for the detection of *S. sonnei* infections, it is crucial to assess the developed ELISA whether it has achieving proper standard on its quality and performance. In this study, the overall diagnostic performances of the developed ELISA assay was achieved at the maximum values of 90% and 98% for diagnostic sensitivity and specificity, respectively. The diagnostic performance of the developed ELISA in this study were found to be equivalent to an evaluation study conducted by Selseleh *et al.* (2012), who investigated the potential use of recombinant *Toxoplasma gondii* surface antigen 1 for the diagnosis of toxoplasmosis using ELISA technique. High sensitivity and specificity (93% and 95%

respectively) were reported by the authors using the newly developed ELISA for diagnosis of toxoplasmosis. Many other studies have been performed to detect pathogens causing gastrointestinal infections using the ELISA method. A study reported by Kumar *et al.* (2011) successfully developed sandwich ELISA for detection of pathogenic *Vibrio parahaemolyticus* in seafood using monoclonal antibodies against TDH-related hemolysin (TRH) of pathogenic *Vibrio parahaemolyticus*. Another study described the use of recombinant antigens of cholera toxin B (CtxB) and outer membrane protein W (OmpW) for the simultaneous detection of toxigenic and non-toxigenic *Vibrio cholerae* from rectal swabs and environmental samples by sandwich ELISA (Tuteja *et al.*, 2007). In this study, we successfully developed the ELISA for the rapid identification of *S. sonnei* infections that can be completed within three hours. This duration is similar to that reported by Shylaja and Batra (2014), in which a simple dot-ELISA was developed using three generated monoclonal antibodies against the outer membrane protein of *S. sonnei*.

Despite of all the advantages that have been highlighted in this newly developed ELISA, there are some limitations of using IgA as a biomarker for diagnosis of *S. sonnei* infection. Study on the kinetics of the anti-*Shigella* IgA antibodies showed that their positive detection often occurs one day after the onset of fever and reaching their highest level around day eight following onset of the fever (Sonjai *et al.*, 2001). Further work is needed to elucidate the identities of these proteins biomarkers by determining the sequence of the proteins or its corresponding gene. The synergistic effect of the target proteins can be further studied by validating their combination usefulness as diagnostic markers for early detection of *S. sonnei*.

In conclusion, this study highlighted the usefulness of the ELISA in the serological screening of *S. sonnei* infections which will be useful in facilitating earlier detection of the pathogen causing this infection. Rapid and reliable diagnostic test is crucial in the early detection of the disease since it can control the spread of the disease and complications including mortality in patients especially among young children and elderly patients.

ACKNOWLEDGEMENTS

Conflict of interest
The authors declare that they have no conflict of interest.

REFERENCES

Afroze, F., Ahmed, T., Sarmin, M., Smsb Shahid, A., Shahunja, K.M., Shahrin, L. & Chisti, M.J. (2017). Risk factors and outcome of *Shigella* encephalopathy in Bangladeshi children. *PLoS Neglected Tropical Diseases* **11**: e0005561. https://doi.org/10.1371/journal.pntd.0005561

Banga Singh, K.K., Ojha, S.C., Deris, Z.Z. & Rahman, R.A. (2011). A 9-year study of shigellosis in Northeast Malaysia: Antimicrobial susceptibility and shifting species dominance. *Journal of Public Health* **19**: 231-236. https://doi.org/10.1007/s10389-010-0384-0

Bardhan, P., Faruque, A.S.G., Naheed, A. & Sack, D.A. (2010). Decreasing shigellosis-related deaths without *Shigella* spp specific interventions, Asia. *Emerging Infectious Diseases* **16**: 1718-1723. https://doi.org/10.3201/eid1611.090934

Crowther, J.R. (2009). The ELISA Guidebook. In: Methods in Molecular Biology, Walker, J.M. (Series Editor) 2nd edition. Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-60327-254-4

Dogruman-Al, F., Turk, S., Adiyaman-Korkmaz, G., Hananel, A., Levi, L., Kopelowitz, J., Babai, O., Gross, S., Greenberg, Z., Herschkovitz, Y. *et al*. (2015). A novel ELISA test for laboratory diagnosis of Blastocystis spp. in human stool specimens. *Parasitology Research* **114**: 495-500. https://doi.org/10.1007/s00436-014-4208-y

Duran, C., Nato, F., Dartevelle, S., Thi Phuong, L.N., Taneja, N., Ungeheuer, M.N., Soza, G., Anderson, L., Benadof, D., Zamorano, A. *et al*. (2013). Rapid diagnosis of diarrhea caused by *Shigella sonnei* using dipsticks; comparison of rectal swabs, direct stool and stool culture. *PLoS One* **8**: e80267. https://doi.org/10.1371/journal.pone.0080267

Ferrandiz, J., Mercier, C., Wallon, M., Picot, S., Cesbron-Delauw, M. & Peyron, F. (2004). Limited value of assays using detection of immunoglobulin G antibodies to the two recombinant dense granule antigens, GRA1 and GRA6 Nt of *Toxoplasma gondii*, for distinguishing between acute and chronic infections in pregnant women. *Clinical and Vaccine Immunology* **11**: 1016-1021. https://doi.org/10.1128/CDLI.11.6.1016-1021.2004

Gan, S.D. & Patel, K.R. (2013). Enzyme immunoassay and enzyme-linked immunosorbent assay. *Journal of Investigative Dermatology* **133**: e12. https://doi.org/10.1038/jid.2013.287

Harikrishnan, H., Ismail, A. & Banga Singh, K.K. (2013). Temperature-regulated expression of outer membrane proteins in *Shigella flexneri*. *Gut Pathogens* **5**: 38. https://doi.org/10.1186/1757-4749-5-38

Harikrishnan, H. (2014). Dissertation: Demonstration of antigenic and specific heat shock protein(s) of *Shigella flexneri* and *Shigella sonnei*. Universiti Sains Malaysia.

Harikrishnan, H., Ismail, A. & Banga Singh, K.K. (2017). Outer membrane proteins analysis of *Shigella sonnei* and evaluation of their antigenicity in *Shigella* infected individuals. *PLoS One* **12**: e0182878. https://doi.org/10.1371/journal.pone.0182878

Hosseini, S., Vázquez-Villegas, P., Rito-Palomares, M. & Martínez-Chapa, S.O. (2018). Advantages, disadvantages and modifications of conventional ELISA. In: Enzyme-linked Immunosorbent Assay (ELISA). SpringerBriefs in Applied Sciences and Technology. Springer, Singapore. https://doi.org/10.1007/978-981-10-6766-2_5

Kotloff, K.L., Winickoff, J.P., Ivanoff, B., Clemens, J.D., Swerdlow, D.L., Sansonetti, P.J., Adak, G.K. & Levine, M.M. (1999). Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bulletin of the World Health Organization* **77**: 651-666.

Kumar, B.K., Raghunath, P., Devegowda, D., Deekshit, V.K., Venugopal, M.N., Karunasagar, I. & Karunasagar, I. (2011). Development of monoclonal antibody based sandwich ELISA for the rapid detection of pathogenic *Vibrio parahaemolyticus* in seafood. *International Journal of Food Microbiology* **145**: 244-249. https://doi.org/10.1016/j.ijfoodmicro.2010.12.030

Lee, B.W., Bey, R.F., Baarsch, M.J. & Simonson, R.R. (1993). ELISA method for detection of influenza A infection in swine. *Journal of Veterinary Diagnostic Investigation* **5**: 510-515. https://doi.org/10.1177/104063879300500402

Lee, W.S. & Puthucheary, S.D. (2002). Bacterial enteropathogens isolated in childhood diarrhoea in Kuala Lumpur – the changing trend. *Medical Journal Malaysia* **57**: 24-30.

Maurelli, A.T., Blackmon, B. & Curtiss, R. (1984). Temperature-dependent expression of virulence genes in Shigella species. *Infection and Immunity* **43**: 195-201.

Mcpherson, R.A. & Pincus, M.R. (2021). Henry's clinical diagnosis and management by laboratory methods. 24th edition, E-book. Elsevier Health Sciences.

Rigby, C.E. (1984). Enzyme-linked immunosorbent assay for detection of *Salmonella* lipopolysaccharide in poultry specimens. *Applied and Environmental Microbiology* **47**: 1327-30. https://doi.org/10.1128/aem.47.6.1327-1330.1984

Selseleh, M.M., Keshavarz, H., Mohebali, M., Shojaee, S., Modarressi, M., Eshragian, M. & Selseleh, M.M. (2012). Production and evaluation of *Toxoplasma gondii* recombinant surface antigen 1 (SAG1) for serodiagnosis of acute and chronic Toxoplasma infection in human sera. *Iranian Journal Parasitology* **7**: 1-9.

Shylaja, R.R.M. & Batra, H.V. (2014). Rapid identification of *Shigella sonnei* by the use of specific monoclonal antibody based dot ELISA. *International Journal of Advanced Research* **2**: 897-906.

Sonjai, K., Soisangwan, R., Sakolvaree, Y., Kurazono, H., Chongsa-Nguan, M., Tapchaisr, P. & Chaicumpa, W. (2001). Validation of salmonellosis and shigellosis diagnostic test kits at a provincial hospital in Thailand. *Asian Pacific Journal of Allergy and Immunology* **19**: 115.

Sudan, V.J.A. & Shanker D. (2013). Recent trends in the diagnosis of toxoplasmosis. *Clinical Reviews and Opinions* **5**: 11.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**: 4350-4354. https://doi.org/10.1073/pnas.76.9.4350

Tuteja, U., Kumar, S., Shukla, J., Kingston, J. & Batra, H.V. (2007). Simultaneous direct detection of toxigenic and non-toxigenic *Vibrio cholerae* from rectal swabs and environmental samples by sandwich ELISA. *Journal of Medical Microbiology* **56**: 1340-1345. https://doi.org/10.1099/jmm.0.47166-0

Uzal, F.A., Carrasco, A.E., Echaide, S., Nielsen, K. & Robles, C.A. (1995). Evaluation of an indirect ELISA for the diagnosis of bovine brucellosis. *Journal of Veterinary Diagnostic Investigation* **7**: 473-475. https://doi.org/10.1177/104063879500700408