Original Article

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Salivary Anti-50 kDa Antibodies as a Useful Biomarker for Diagnosis of Typhoid Fever

Microbiology Section

NUR ELIYANA MOHD REDHUAN[1], KAI LING CHIN[2], AZREEN SYAZRIL ADNAN[3], ASMA ISMAIL[4], PRABHA BALARAM[5], KIA KIEN PHUA[6]

ABSTRACT

Introduction: Typhoid fever remains a scourge of humanity, especially in developing and under-developed countries due to poor sanitation and food hygiene. Diagnostic methods available for detection of this disease are not satisfactory due to a lack of sensitive, specific, rapid and convenient diagnostic test kits available in the market.

Aim: To evaluate the feasibility of a Dot-EIA method for Ig-class specific salivary antibody detection for diagnosis of typhoid fever.

Materials and Methods: Paired saliva and serum samples were collected in the year 2010 from patients and normal volunteers in Hospital Universiti Sains Malaysia, Kelantan, Malaysia, which is endemic for typhoid fever. A total of 11 culture-confirmed typhoid fever patients, 43 non-typhoid fever patients and 53 normal human control subjects were evaluated for antibodies against a 50 kDa antigen specific for *Salmonella* Typhi using Dot-EIA.

Results: Ig class-specific screening of the test samples showed a higher sensitivity for IgA (90.9%) compared to either IgG (72.7%) or IgM (72.7%) antibodies in saliva, but for serum, IgG (90.9%) had a higher degree of sensitivity compared to IgA (36.4%) and IgM (63.6%). Combining all isotypes (IgA, IgG or IgM), serum showed a higher sensitivity (100.0%) compared to saliva (90.9%). Also, the specificity for serum (100.0%) was much higher than saliva (85.4%).

Conclusion: Salivary IgA anti-50kDa antibody was found to be more suitable biomarker for routine screening, whereas serum IgG was more suitable for confirmatory test as it has higher specificity. Nevertheless, salivary IgA Dot-EIA is a convenient method for rapid testing, such as for Point-of-Care Diagnostics (POCD) and field epidemiological studies, due to its non-invasive nature and ease of use.

Keywords: Dot-EIA, Saliva, *Salmonella* Typhi, Widal

INTRODUCTION

Typhoid fever, grouped among the enteric fevers, is caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). This is a disease of the less developed countries including countries in Southeast Asia and Africa [1]. It is estimated that the global incidence of this disease to be 26.9 million cases per year [2]. Ochiai RL et al., reported an annual incidence as high as 24-500/100,000 persons per year in five Asian countries studied [3]. However, the actual incidence could be higher. This is because proper diagnosis of typhoid fever is handicapped by the lack of affordable diagnostic tests which are sensitive and specific. Current laboratory methods rely mainly on identifying the bacteria in blood or stool cultures, followed by serotyping which is tedious (taking 4-7 day's) and infamous for indeterminate results (low sensitivity, i.e., 45%-70%) [4-6]. This method has been in practice for over a century and has not seen a major improvement in its detection rate, probably due to the limitations involved with the test per se, such as false negatives in cases with prior antibiotic therapy, non-availability of culture facilities, expenses involved and the time required for the whole process. Furthermore, this test is mainly effective in the early acute phase (first week) of the disease where the live bacterial load is high enough to be detected [7]. In addition, blood sampling among the paediatric and geriatric population is often problematic [8-10]. The request for stool samples or rectal swabs in field studies for bacteria cultures are also not well accepted, and the source of the stool is often dubious amongst incarcerated subjects or food handlers.

Taking into consideration the above factors, a number of other methods have been developed using typhoid specific serological and DNA amplification tests. However, scientists have yet to succeed in a satisfactorily specific, rapid test that can be used for diagnosing typhoid fever in the less-developed world, where the incidence of typhoid is highest and where laboratory facilities may not be adequate. Serological tests using agglutination methods (Widal test) and rapid diagnostic test kits are being used widely [11,12]. ELISA and immunoblotting using specific antigens against flagellar or membrane antigens have been shown to be more sensitive and specific in comparison to the Widal test [13-15].

Blood is the most popular bio-fluid that is used for laboratory diagnosis [16]. Studies on the saliva proteome have revealed a plethora of putative proteins of importance in detection of infections. Saliva has also been used for detection of antibodies and drugs in various diseases suggesting that saliva can be successfully used as an alternative to serum for detection of infections including typhoid fever [17-21]. According to Greabu M et al., saliva is the mirror image of the body that could give information on the systemic status of various metabolites [22]. Tests using saliva as the diagnostic sample have reported it to be a promising bio-fluid as a non-invasive, faster, cheaper and potentially safer diagnostic method than blood sampling and to have the potential to be used as a POCD in laboratories with minimal infrastructure [23]. Compared to venipuncture, the ease of collection, the ease of conducting serial qualitative tests in the rural clinics or doctor's clinic itself adds to its potential use as a diagnostic fluid. Furthermore, in some countries it is recommended and used for rapid screening of several infectious diseases [24].

Immunoglobulins form a major proportion of the proteins in saliva and contain mainly secretory IgA (s-IgA) [25]. Elements specific to mucosal immunity predominate as observed by the high levels of s-IgA antibodies even in healthy subjects [26]. During infection, especially with enteric pathogens including *S.* Typhi, there is an increase in salivary IgA antibodies which enable its detection using moderately sensitive methods such as Dot-EIA [17]. In addition to

IgA antibodies, about 5%-10% of the remaining antibodies are made up of IgG and IgM derived by transduction through the crevicular fluid or leakage from plasma [27,28]. Therefore, IgG and IgM are also important candidates for diagnosis of infectious diseases.

The gold standard method for protein biomarker detection relies mainly on ELISA methods which require expensive reagents and equipment [29]. There is a continuing interest to develop new diagnostics that are cheaper, more rapid, sensitive and carries a smaller foot-print to enable applications in POCD. Of the serodiagnostic tests available for detection of typhoid fever, Typhidot® which is a commercially available diagnostic kit detects the presence of serum IgM and IgG antibodies against an outer membrane protein, 50 kDa antigen, specific for *S.* Typhi, is reported to have high sensitivity (78%-100%) and specificity (80%-100%) to diagnose typhoid fever [10,13,30]. Taking into consideration the above limitations, this preliminary study was carried out to evaluate the potential of salivary antibodies against the specific 50 kDa antigen of *S.* Typhi to diagnose typhoid fever. Such an assay would provide us with a non-invasive, easy to use and cost-effective method for diagnosis of typhoid fever in both symptomatic and asymptomatic individuals (typhoid carriers). Since typhoid fever is restricted to humans, human beings are the only carriers of the disease. Detection of these carriers must be an integral part of any programme to eradicate the disease.

MATERIALS AND METHODS

A prospective study was conducted in the year 2010. Samples were collected from patient age between seven to 70 years old and without history of typhoid fever. Sera and saliva samples were collected from Hospital Universiti Sains Malaysia (HUSM) after approval by the Ethical Committee (Human) of Universiti Sains Malaysia (USM) accordance with Declaration of Helsinki. Eleven patients grouped under 'typhoid fever cases' constituted of patients with prolonged fever (fever more than three days) and were positive for *S*. Typhi by blood culture and Typhidot® test. All 43 cases grouped under 'non-typhoid fever cases' were randomly selected cases of fever of more than three days duration, negative for *S*. Typhi culture and for Typhidot® test. This group included cases of dengue, leptospirosis, infective diarrhea, urinary tract infection, and fever of unknown origin. All samples were collected before start of any therapy at the hospital within one to three days of registration. 'Normal human control cases' consisted of randomly selected subjects who did not have any history of typhoid fever or typhoid vaccination during their life time, and had no clinically detectable signs of any infection at the time of sample collection, and were negative for *S.* Typhi culture and Typhidot® test. Informed consent and information of the duration of fever, antibiotic usage and previous history of typhoid fever or vaccination were obtained from the specimen donors. All participants' blood were collected by routine venipuncture and saliva were collected as described by Nurkka A et al*.,* [31]. The participants were asked to refrain from any food or liquid intake 30 minutes to an hour prior to collection of saliva to avoid the effects of food. About 3-5 ml of saliva was collected in sterile containers by drooling method over a period of 10-15 minutes after washing of the mouth by gargling at least three times with sterile distilled water. The collected samples were centrifuged at 2,000 rpm and the supernatant were stored in aliquots at -20ºC until ready for used.

50 kDa S. Typhi antigen

The antigen was isolated from a clinical strain of *S.* Typhi (S1188/03) from the stool of a typhoid fever patient during an outbreak in year 2003 in Kota Bharu, Kelantan, Malaysia and stored in the bacterial bank of the Institute for Research in Molecular Medicine (INFORMM), USM, Kelantan, Malaysia. The growth and maintenance of bacteria was done as described by World Health Organization (WHO) [32]. The antigen was prepared as described by Choo KE et al., as used in the Typhidot® assay with all the quality controls in place [13].

Dot Enzyme Immunoassay

Test sera (n=107) were screened using Typhidot® assays for both IgM and IgG antibodies and graded as per the instructions given in the test kit brochure. An in-house Dot-EIA method was used for detection of serum IgA antibodies. Paired saliva samples from all the three groups were tested for presence of IgA, IgM and IgG antibodies to the 50 kDa antigens. Initially, the concentration of antigen, saliva sample volume, dilution of secondary HRP-conjugated antibodies to be used and time of incubation were optimized by checkerboard titration. The optimum conditions as shown in [Table/Fig-1] were used for testing the test samples. Briefly, nitrocellulose strips dotted with the 0.03 µg/µl 50 kDa antigens, blocked with 1% bovine serum albumin overnight and air-dried at room temperature were incubated with 1 ml of neat saliva sample in sample troughs for one hour at room temperature on an orbital shaker (Heidolph Polymax 2040, Australia), followed by three times washing with 1xPhosphate-Buffered Saline (PBS), pH 7.4 for 15 minutes each prior to incubation with 1 ml of secondary antibody for one hour. The strips were separated into three troughs for incubation with HRP-conjugated rabbit anti-human IgA and IgM (Dako, Denmark) at 1:200 dilutions, and HRP-conjugated rabbit anti-human IgG (Dako, Denmark) at 1:400 dilution. On completion of the incubation period, the strips were washed three times with 1xPBS, pH 7.4 for 15 minutes each. The reaction was visualized by incubating with 1 ml HRP-substrate consisting of 0.01% hydrogen peroxide and chromogen (0.003% 4-chloro-1-naphthol in methanol) for 15 minutes at room temperature, and stoppage of the reaction by washing with distilled water. The colour intensity of the dots were recorded and graded on a scale from '-' (negative-no staining), '1+' (positive-weak staining), '2+', '3+' to '4+' (positive-moderate to strong staining) by comparing the reaction colour given by a reference positive saliva. Each sample was tested in triplicates. The results were tabulated and evaluated for sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) using the criteria described by NCSSM Statistics Leadership Institute for Categorical Data Analysis in July 1999 [33].

Parameter	Optimum condition
Antigen	0.3 µg/µl
Saliva	Neat (undiluted)
Primary antibody (saliva) incubation time	1 hour
Secondary antibody incubation time	1 hour
Secondary antibody dilutions:	
• HRP-conjugated rabbit anti-human IgM	1:200
• HRP-conjugated rabbit anti-human IgA	1:200
• HRP-conjugated rabbit anti-human IgG	1:400

[Table/Fig-1]: Optimized conditions used for salivary antibody detection.

RESULTS

The results of the Ig-class specific screening showed that number of paired samples positive for salivary anti-50 kDa IgA and IgM antibodies were higher compared to those in serum, suggesting that salivary IgA and IgM were more sensitive compared to its counterpart (serum) [Table/Fig-2]. Out of 11 typhoid fever samples, 10 samples (90.9%) were positive for salivary IgA anti-50 kDa antibody, whereas only four samples were positive for serum IgA (36.4%). For salivary IgM antibody, 8 samples (72.7%) were positive compared to 7 samples (63.6%) for serum IgM [Table/Fig-2]. In contrast, salivary IgG anti-50 kDa antibody was found in 8 samples (72.7%) compared to 10 serum samples (90.9%) [Table/Fig-2].

Subject Groups	Number of samples positive for anti-50 kDa antibodies			
	Any isotypes (IgA, IgG or IgM)	IgA	IgG	IgM
Culture-positive typhoid fever (n=11)				
Serum	11 (100.0%)	4 (36.4%)	10 (90.9%)	7 (63.6%)
Saliva	10 (90.9%)	10 (90.9%)	8 (72.7%)	8 (72.7%)
Non-typhoid fever (n=43)				
Serum	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Saliva	8 (18.6%)	5 (11.6%)	0 (0.0%)	4 (9.3%)
Normal controls (n=53)				
Serum	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Saliva	6 (11.3%)	5 (9.4%)	0 (0.0%)	1 (1.9%)

[Table/Fig-2]: Number of samples positive for anti-50 kDa antibodies in different groups of samples evaluated.

False-positive results were observed in the non-typhoid fever and normal control groups [Table/Fig-2]. For salivary IgA, 5 out of 43 non-typhoid fever (11.6%) and 5 out of 53 normal control (9.4%) samples showed false-positivity, whereas for salivary IgM, 4 out of 43 non-typhoid fever (9.3%) and 1 out of 53 normal control (1.9%) samples showed false-positivity. No false negative results (0.0%) were observed using serum and salivary IgG for diagnosis of typhoid fever [Table/Fig-2].

By combining all isotypes (IgA, IgG or IgM), serum showed higher sensitivity (100.0%), specificity (100.0%), PPV (100.0%) and NPV (100.0%) compared to saliva (90.9%, 85.4%, 41.7% and 98.8%, respectively) [Table/Fig-3]. Overall, this study showed that salivary IgA was found to be more suitable for routine screening test due to its rapidness and high sensitivity (90.9%), whereas serum IgG was more suitable for confirmation test as it has higher specificity.

Antibody positivity	Sample	Sensitivity (%) (n=11)	Specificity (%) (n=96)	PPV (%)	NPV (%)
Any isotypes (IgA, IgG or IgM)	Serum	100.0	100.0	100.0	100.0
	Saliva	90.9	85.4	41.7	98.8
IgA	Serum	36.4	100.0	100.0	93.2
	Saliva	90.9	89.6	50.0	98.9
IgG	Serum	90.9	100.0	100.0	99.0
	Saliva	72.7	100.0	100.0	97.0
IgM	Serum	63.6	100.0	100.0	96.0
	Saliva	72.7	94.8	61.5	96.8

[Table/Fig-3]: Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NVP) of anti-50 kDa antibodies in serum and saliva using Dot-EIA.

DISCUSSION

Saliva has been used by a number of investigators as a diagnostic fluid for various diseases [17-21]. However, very few studies have been carried out on typhoid fever patients using saliva. Herath et al., evaluated salivary IgA antibodies against Lipopolysaccharides (LPS) of *S.* Typhi with assay sensitivity of 83% and specificity of 97%, respectively [17]. A more recent study by Zaka-ur-Rab et al., found that *S.* Typhi LPS to have a sensitivity and specificity of 71.4% and 100%, respectively [34]. Also, a study by Chin KL et al., showed that salivary IgA against *S.* Typhi Haemolysin E protein can be used as a biomarker to differentiate acute typhoid, convalescent typhoid and other febrile diseases [35]. In this study, the potential of saliva as a diagnostic fluid and antibody against 50 kDa antigen were evaluated. Salivary IgA anti-50 kDa was more sensitive (90.9%) to diagnose typhoid fever than using salivary IgG (72.7%) and IgM (72.7%). This could be due to high concentration of IgA antibody in saliva. A similar observation was made by Romero MR et al., whereby a high concentration of IgA antibodies with traces of IgG and IgM present in parotid saliva. The mean concentration of salivary IgA in healthy subjects (81.0 µg/ml) was 400 times higher than salivary IgM (0.2 µg/ml) and five times higher than salivary IgG (14.8 µg/ml) [36]. This finding of an increased level of specific IgA in saliva in comparison to that in serum was also found in other studies [17,20,23]. This is consistent with the normal physiological occurrence of a higher IgA in saliva compared to serum. As observed in this study, the diagnosis of typhoid fever using levels of salivary IgG antibody as a diagnostic marker was found to be lower than that of serum. Even though, serum had high titres of IgG antibodies, the lower sensitivity of salivary IgG could be hypothesized to be due to a possible threshold level of antibodies are being required in the serum before they leak across capillaries and appear in the saliva as suggested by Nurkka A et al., [37].

The salivary antibodies showed false positivity in patients with two cases of leptospirosis, two cases of unknown fevers, one case of abdominal pain, one case of urinary tract infection and two cases of dengue among the non-typhoid fevers subjects, and six of 53 normal human controls. Thus, in this current study, the false positive rate for salivary antibodies against *S.* Typhi 50 kDa antigen in non-typhoid fever patients and normal individuals was 14.58% (14/96). Of these, the intensity of the reaction was high (4+) only in two cases of dengue fever and one case of leptospirosis. All the normal cases showed low intensity positivity (1+). The false negative result for salivary antibody detection of typhoid fever in this study was 9% (1/11 culture positive cases). The false positive and negative rates observed for saliva in this study were comparable to those in other studies. Herath HM et al., reported a false positive rate of 7.8% (4/51) and a false negative rate of 17% (4/29) [17], while Zaka-ur-Rab Z et al., reported a false positive rate of 10.8% (4/37) and false negative rate of 0% (0/60) in the evaluation of saliva for typhoid fever detection using anti-LPS antibody as the biomarker [34].

The occurrence of high IgA positivity in the saliva of culture negative non-typhoid fever cases could not be simply explained at this juncture. These results could not be simply dismissed as 'false positive cases' because they were *S.* Typhi culture-negative. Since the test subjects were from a typhoid endemic region, the frequency and level of anti-*S.* Typhi antibodies in this population could be higher. Also, non-specific binding may arise due to 3 factors: 1) HRP-conjugated anti-human IgA antibody binding to excess IgA found in saliva; 2) presence of cross-reactive antibodies in the test specimens which bind to both the 50 kDa antigen and other outer membrane proteins in *Salmonella enterica* species and; 3) low sensitivity of the test for detection of serum IgA antibodies which made it difficult to differentiate between antibody positive and negative samples. This study also found a high discrepancy in IgA results of the paired serum and saliva. This is due to the absent of a gold standard for assessing the predictive cut-off value for antibody level.

LIMITATION

Using the salivary anti-50 kDa antibody detection assay developed in this study as biomarker for diagnosis of typhoid fever, approximately 9% of the results were false negative and 15% were false positive. As the sample size used in this study was small due to the low number of cases of typhoid fever cases reported in Kelantan in the year 2010 [38], a larger sample size is needed to increase the accuracy (sensitivity and specificity) of the study. This antigen also showed cross-reactivity with dengue and leptospirosis infections, and thus differential diagnosis should be performed to rule out the possibilities of co-infections. Also, serial samples should be obtained to test its accuracy to diagnose typhoid fever.

CONCLUSION

Overall, this study showed that saliva has the potential to be an alternative fluid for detection of typhoid fever due to its characteristics of: a) greater acceptability (non-invasive and painless); b) greater

convenience (simple, rapid, and inexpensive collection with no requirement of specialized training); c) less hazardous (to subjects as well as to investigators, e.g., needle pricks); and d) better access and easy availability (in epidemic outbreaks, children, large populations and hard-to-reach high risk groups). The testing does not require sophisticated equipment and can be done in a rural clinic or even in the doctor's consultation room for POCD. It is very much suitable in children, they being the highest infected group for this disease. Despite the modest PPV of the test, the salivary antibodies could be a good biomarker to detect immune responses to typhoid infection. When the positivity to any class of antibody against 50 kDa antigen was considered as the marker, the detection by salivary antibodies was found to have a high sensitivity (90.9%) and specificity (85.4%) for typhoid fever.

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PARTICULARS OF CONTRIBUTORS:
1. PhD Student, School of Veterinary Medicine, Universiti Malaysia Kelantan, Kelantan, Malaysia.
2. PhD Student, Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia.
3. Lecturer, Chronic Kidney Disease Resource Center, Universiti Sains Malaysia, Kelantan, Malaysia.
4. Former Professor, Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia.
5. Former Professor, Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia.
6. Professor, Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Kia Kien Phua,
Professor, Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Minden-11800, Penang, Malaysia.
E-mail: kkphua7@gmail.com

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