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Comparative Widal Reaction for IgG/IgM Complement C3, lymphocyte and Neutrophils Assay in Patients with Suspected Typhoid Fever in Selected Hospitals in Kaduna State, Nigeria

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ABSTRACT

Typhoid also known as enteric fever is endemic in Nigeria most often diagnosed by the widal reaction though the results of this test are being questioned in many quarters. This has necessitated the search for other methods for analysis. This work is aimed at comparing the widal reaction with the rapid immunochromatographic assay the complement C3 and selected haematological indices. Two ml of blood was collected from 350 patients with suspected typhoid infection and analysed by the widal slide agglutination reaction and the IgG/IgM Immunochromatographic assay. Complement C3 was assayed by ELISA while neutrophils and Lymphocyte counts were also performed. The finding showed that 41(11.7%) and 29 (8.3%) were positive for *S. typhi* IgG and IgM respectively out of 350 patients. It was found that 207 patients had O-antigen widal reaction titres 1/80 and above for *S. typhi* out of which, 30 (14.5%) and 22(22.6%) were IgG and IgM positive respectively. Those with reactions 1/80 and above to the H-antigen were 118 with 24(20.3%) cases of IgG and 13(11.0%) of IgM. The mean neutrophil and lymphocyte count in the IgM positive were 48.90 ± 20.060 and 60.28 ± 17.64 as compared to the negatives (48.46 ± 18.95 and 55.02 ± 19.19 respectively). The mean neutrophil and lymphocyte count of IgG positive were 50.68 ± 19.65 and 57.22 ± 19.72) while the negatives were 48.20 ± 1.08 and 55.23 ± 19.04 . the mean plasma levels of complement factors C3 in the IgG positive was 630.70 ± 327.41 as compared to those that are negative (626.97 ± 247.72). The complement C3 levels was significantly higher ($P = 0.000$) in the IgM positive (816.45 ± 406) as compared to the IgM negatives (610.33 ± 233.40). No significant association was observed between the clinical features and the typhoid positives.

Keywords: Typhoid Fever, Widal test, Immunochromatographic test, Complement 3, IgG, IgM

Introduction

Typhoid also known as enteric fever is a caused by a highly virulent *Salmonella typhi* which is also very invasive. The illness is frequently encountered in tropical countries like Nigeria where it constitutes a serious source of morbidities and mortalities (Ibekwe, *et al.*, 2008). Typhoid

carriers are of special concern from a public health point of view because they are sources for the spread of diseases (WHO, 2003). Studies to immune response against these microorganisms are still evolving (Mashi, 2001). However, it has previously been shown that circulating

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immunoglobulins secreted by lymphocytes, are substantially sought to understand the host immune response elicited in *Salmonella* infection, however, events related elevated in the peripheral blood of adults with typhoid and paratyphoid fever and this increase can be used as both sensitive and specific diagnostic assay to identify patients with enteric fever (Sheikh, *et al.*, 2009). These activated lymphocytes may represent cells activated early in infection either at mucosal surface or systematic sites.

Diagnosis of typhoid fever is essentially based on Widal Agglutination reaction (Elderman and Levine, 1986; Onuigbo, 1990; Mbuh, *et al.*, 2003). However, this test has numerous setbacks such as poor specificity (Passey, 1995; Wain, *et al.*, 2008). This necessitates the importance of a reliable, rapid diagnostic procedure for typhoid fever. New typhoid rapid antibody tests have been developed and evaluated in Asia and other developing countries, where typhoid is highly endemic and these assay kit are now commercially available Crump, *et al.*, 2004; Ochiai, *et al.*, 2008).

However, rapid typhoid tests are yet received enough attention to make them commercially available in Nigeria. To a large extent, studies on typhoid infection and the role of complement activation been exploited.

Also, biochemical and haematological changes have been associated with typhoid fever. Olubuyide *et al.* (1989) reported an elevation of C-reactive proteins thus suggesting that the increase could be used as a diagnostic parameter for typhoid fever. The aim of this research is therefore to determine the importance of some specific and non-specific immune responses in patients presenting with symptoms characteristic of typhoid fever recruited from selected hospitals within Zaria metropolis, Kaduna, Nigeria.

Materials and Methods

Study Area and Population

The study recruited patients from selected hospital in Zaria, a city in Kaduna State in Northern Nigeria

covering 300km³. It is made up of two local Government Area, Zaria city and Sabon Gari local Government with 12 districts and a population of 408, 198 people with an annual growth rate of 3.3% (NPC, 2013).

The selected hospitals used were Hajia Gambo Sawaba Hospital located in Zaria city area, Major Ibrahim Memorial Hospital located in Sabon gari local Government and Ahmadu Bello University Health Services, Sickbay, Samaru. The patients who attended the hospitals with symptoms characteristic of typhoid fever and who consented to the study were recruited.

Study design, Inclusion and Exclusion Criteria

The study was hospital based, cross sectional and experimental study carried out over a period of six months. Clinical samples were collected with the assistance of a trained medical personnel. Similarly, a structured questionnaire was administered of the patients that fulfilled the criteria for typhoid fever to obtain socio-demographic data before collection of samples. Ethical approval (MOH/ADM /744/VOL.1) was obtained from the ethical committee of the Kaduna State Ministry of Health prior to sample collection and commencement of the Research. A written consent was solicited and obtained from patients and those who gave their approval were included in the studies.

Sample Size determination and collection

The sample size was calculated using the formula by Naing *et al.* (2006) and a prevalence of 46% (Adabara *et al.*, 2012).

$$N = Z^2 pq / d^2$$

$$N = (1.96)^2 \times 46 \times 1 - 0.05 / 0.05^2$$

$$= 3.841 \times 0.45 \times 0.5 / 0.0025 = 345.744.$$

Therefore, 350 samples were collected, for the sake of this study. A total of 3mls of blood samples was collected by the laboratory personnel using sterile five milliliters syringe aseptically by venous

puncture. The blood was dispensed into EDTA containers and transported on ice in an insulated cold box to the Bacteriology Laboratory of Microbiology Department, ABU, where they were centrifuged at 1500rpm. The plasma was transferred into sterile screwed cap bottles and stored at -20°C until analysis.

Detection of typhoid fever using the widal agglutination reaction

Samples were screened for typhoid fever using the Widal kit (Lab care diagnostic, India) as instructed in the Manufacturers specifications. One drop (0.1ml) of undiluted plasma was added to circles on clean glass slides using a Pasteur pipette. Then, a drop *S. typhi* and *S. paratyphi* H and O antigens, the positive as well as the negative control were dispensed to their respective cycles. The antigen was mixed together with the plasma using separate Pasteur pipettes to fill whole of the individual circle. Then the slides were observed for agglutination.

Detection of the Typhoid IgG and IgM using Immunochromatographic Assay Kit

All the samples were tested for the typhoid IgM and IgG using Sensitive *Salmonella typhi* IgG/IgM Rapid test kit (Ubio Biotechnology, India) based on the Manufacturer's instruction. The test card was taken out from the foil porch and was placed on a horizontal surface, 5ul of plasma of patients was added to the sample well labeled S, when the sample was fully absorbed, 2 drops of the diluents provided with the assay was added to the sample hole, after 15mins, the results was read.

Detection of the Complement C3 levels using ELISA

Typhoid positive samples were screened for C3 using Human complement C3 ELISA based on Manufacturer's (Assaypro Human Complement C3 ELISA) Instruction. All samples and reagents were removed from the refrigerator and allowed to come to room temperature (25°C). The coated strips were

placed in a holder and labeled (one blank well, seven controls, two calibrators and 91 wells for sample specimens). About 3ul of the test samples, seven control and calibrators were added to 240ul of the serum diluents and mixed well to make 1 in 80 dilutions. Hundred microlitre (100ul) each of the diluted samples was dispensed into appropriate wells, ensuring that there were no air bubbles. Air bubbles present in the liquid were removed by tapping the holder. Hundred microlitre (100ul) of the serum diluents was added into the reagent blank well. The wells were incubated at room temperature 25°C for 30 minutes. After incubation, liquid from all wells was removed by washing three times with 300ul of the wash buffer. Hundred microlitre (100ul) of enzyme conjugate was added into each well and incubation was repeated for another 30 mins at room temperature (25°C). Excess enzyme conjugate was removed by washing three times with the wash buffer. Hundred microlitre (100ul) of chromogen/substrate solution (TMB) was dispensed into each well and incubated again as before for 15 mins. Finally, 100ul of stop solution (1M H₂SO₄) was added and the plate was tapped gently to mix contents of the wells. The reading was done using ELISA microplate reader (GF-M300 Microplate reader, B BRAN Scientific \$ Instrument, Company England) at 450nm.

Determination of Percentage neutrophils and lymphocyte population

Typhoid positives samples were determined for the neutrophils/lymphocyte's levels using staining technique (Cheesbrough 2006). A drop of blood was placed on the end of a clean dry slide, was smeared to give a thin film and allowed to air-dry. The film was fixed in methanol for 2 mins, then the slide was placed on a staining jar, 10% of the Giemsa stain was added, the slide was allowed to stay for 45mins, then the stain was washed away rapidly with water. When dried, oil immersion was added and it was viewed under microscope using x 100 objective lens.

Data Analysis

The data obtained were analysed with SPSS (statistical package for social sciences) version 20.0 software program. Pearson's chi square χ^2 was employed to determine association of variables set at a significance of at 95% confidence interval and $P < 0.05$ was considered significant. Means of parametric variables were analyzed using Student *t* tests.

Results

The finding showed that 207 (59.1%) and 118 (31.7%) patients had titres above 80 to the O and H antigens of *S. typhi* be the widal reaction. Similarly, 153 (43.7%) and 124 (38.3%) reacted to the O and H antigens of the combined *S. paratyphi* group (Table 1). The distribution of the *S. typhi* O antigen titre among the IgG and IgM positives is presented in table 2. Out of the 40 samples with 1/160 agglutination titre, 5 were positive for IgG while 4 where IgM positive. Similarly, of the 167 demonstrating 1/80 titre, 21 were IgG positive and 18 were IgM positive. On the other hand, 9 out of the 27 tested patients demonstrated 1/60 titre were IgG Typhoid positive and only 4 where IgM positives, and 15 were positive for IgG out of 91 demonstrating the 1/80 titre while 9 were IgM positive. A significant association was established between the H antigen and the IgG but not with the IgM positives (table 3).

The mean neutrophil count in the IgM typhoid positives (48.90 ± 20.060) was slightly higher than those that are negative (48.46 ± 18.95) while the mean lymphocyte count was significantly higher in typhoid patients (60.28 ± 17.64) than the IgM negatives (55.02 ± 19.19). The mean neutrophils of the IgG typhoid positive (50.68 ± 19.65) was higher than the IgG negatives (48.20 ± 1.08) and a higher mean lymphocytes counts was also observed in the IgG positives (57.22 ± 19.72) as compared with the negatives (55.23 ± 19.04). No significant association was observed between the mean neutrophils/lymphocytes count and IgM levels of Typhoid patients (Table 4).

The Complement C3 levels was significantly higher ($P = 0.000$) in the IgM positive (816.45 ± 406) as compared to the IgM negatives (610.33 ± 233.40). There was no significant association ($P > 0.05$) between the complement factor C3 and the Immunoglobulin IgG. There was an increase in the Complement C3 levels of the IgM positives. (816.45 ± 406) as compared to the IgM negatives (610.33 ± 233.40), a significant association ($P < 0.05$), was observed between the IgM positives and the complement C3 levels (Tables 5). The relationship between clinical features associated with typhoid fever and the corresponding IgG and IgM positives is presented in Table 6. No significant association was observed between the clinical features and the typhoid positives.

Table 1: Distribution of the Individual Widal Titre against the Serotypes of *Salmonella*

Serotype	Antigen	Agglutination Titre / Number positive (%)					
		1/20	1/40	≤ 40	1/80	1/160	≥ 80
<i>S. typhi</i>	O	79	64	143	167	40	207
		(22.6)	(18.3)	(40.9)	(47.7)	(11.4)	(59.1)
<i>S. typhi</i>	H	83	149	232	91	27	118
		(23.7)	(42.6)	(66.3)	(26.0)	(7.7)	(33.7)
<i>S. paratyphi</i>	O	109	88	197	142	11	153
		(31.1)	(25.1)	(56.2)	(40.6)	(3.1)	(43.7)
<i>S. paratyphi</i>	H	123	93	216	117	17	124
		(35.1)	(26.6)	(61.7)	(33.4)	(4.9)	(38.3)

O= Somatic antigen, H= Flagella antigen

Table 2: Distribution of *S. typhi* O antigen and the corresponding IgG and IgM Positive Samples

O-antigen	No examined	IgG			IgM		
		No positive (%)	χ^2	P-value	No positive (%)	χ^2	P-value
1/20	79	5(6.33)	7.173	0.067	4(5.1)	3.690	0.297
1/40	64	6(9.38)			3(4.7)		
Sub total	143	11(7.69)			7(4.9)		
1/60	40	5(12.5)			4(10.0)		
1/80	167	21(12.57)			18(10.8)		
Sub total	207	30 (14.5)			22(10.6)		
TOTAL	350	41(11.7)			29(8.3)		

χ^2 =Chi square, P >0.05, No significant association.

Table 3: Distribution of the *S. typhi* H antigen and the corresponding IgG and IgM positive Samples

H-antigen	No examined	IgG			IgM		
		No positive (%)	χ^2	P-value	No positive (%)	χ^2	P-value
1/20	83	5(6.0)	18.1732	0.000*	5(6.0)	2.541	0.468
1/40	149	12(8.1)			11(7.4)		
Sub total	232	17(7.3)			16(6.9)		
1/60	27	9(33.3)			4(14.8)		
1/80	91	15(16.5)			9(9.9)		
Sub total	118	24(20.3)			13 (11.0)		
TOTAL	350	41			29		

χ^2 =Chi square, P = 0.000* Significant association (P<0.05)

Table 4: The mean neutrophils and lymphocyte count in relation to the IgG and IgM status of the study population

	IgG			IgM		
	N	Neutrophils	Lymphocytes	N	Neutrophils	Lymphocytes
Positive	41	50.68 ±19.65	57.22± 19.72	21	49.90± 20.6	60.28 ±17.64
Negative	309	48.20± 1.08	55.23± 19.04	329	48.46 ±17.64	55.02± 19.19
	350			350		

Table 5: The mean C3 levels (ug/ml) in relation to IgG and IgM status of the study population

	IgG	C3	IgM	C3
Positive	41	630.70± 327.41	29	816.45± 406.0
Negative	309	626.97 ±247.72	329	610.33 ±233.40
P-value		0.931		0.000*

(SD±t-test)=Mean Standard Deviation, t-test, χ^2 = Chi square, P =0.000* Significant association (P<0.05)

Table 6: Clinical manifestation in relation to IgM and IgG status

Clinical Manifestation	Patient's Response	IgG			IgM		
		No Positive (%)	χ^2	P-value	No Positive (%)	χ^2	P-value
Headache							
Yes	119	39(32.8)	0.156	0.693	28(23.5)	0.138	0.710
No	231	2(0.9)			1(0.4)		
Fever							
Yes	328	37(11.3)	0.172	0.458	25(7.6)	0.132	0.716
No	22	4(18.2)			4(18.2)		
Weakness							
Yes	309	20(6.5)	0.032	0.854	11(3.6)	1.144	0.285
No	41	21(51.2)			18(43.9)		
Nausea							
Yes	166	16(9.6)	0.307	0.580	9(5.4)	0.234	0.628
No	184	25(13.6)			20(10.9)		
Diarrhoea							
Yes	119	5(4.2)	0.710	0.710	10(8.4)	0.003	0.954
No	231	26(11.3)			19(8.2)		

χ^2 =Chi square, P>0.05 No significant Association

Discussion

The most frequently recorded significant titre for the O antigen of *S. typhi* is 1/80 and 1/140 for the H antigen (Ohanu *et al.*, 2019) and this was adopted as the cut-off value for the interpretation of the result obtained. This agrees with a result from India (Aruni *et al.* (2014), which showed the significant titres above 1/80 for O antigen and 1/60 for H antigen for a diagnosis of Typhoid fever. However, it was in contrast with the study by Oyeyinka and Salimonu (1999) who recorded a titre of < 1/20 for *S. typhi* O and H agglutination at Ibadan city. The variation in the widal reaction depends on the level to which Typhoid is endemic in a particular area, a fact which may change overtime (Punia *et al.*, 2003) and the sanitary condition of that area. Other possible contributory factors include to the sharing of antigens by other serotypes in the Salmonellae family (Olopenia and King, 2000). Early use of antibiotics which can lead to low antibody titre and technical differences in the performance of the test.

The present study also found that titres of agglutinins to *Salmonella typhi* is higher than that of *S. Paratyphi* which is similar to other studies (Jeyakumari, *et al.*, 2014). This indicated exposure to *S. typhi* is more among the population in this area. The rapid antibody assay used in this study is one out of several kits that have been modified to replace the widal reaction in the diagnosis of Typhoid fever. This is because the Widal test, which detects agglutinating antibodies in Patient serum against O and H antigen of *S. typhi* has been faced with a lot of controversy (Olopenia and King, 2000). In developed countries, diagnosis of typhoid fever during the acute phase of the illness by the Widal agglutination has largely been discontinued, especially in view of the low prevalence of the disease.

The Rapid kit used in this study was able to detect IgG/IgM antibodies elicited in the patients in response to the antigens against of

Salmonella typhi in human blood/serum during Typhoid infection. The present study also revealed an increase in complement factor C₃ levels in Typhoid positive patients as against those that were negative, this was in agreement with the work of Ghassan, (2006) who observed an increase in the levels of complement factors C₃ and C₄ among patients in Iraq. However, it was in contrast with the work of Kumar *et al* (1974) who reported the Complement C3 levels in Typhoid patients to be within normal range. The increase observed in this study is consistent with the fact that complement rises with most inflammatory response. The innate immune system plays an essential role in the early response to pathogenic bacteria and may be enough to control progression to disease in most subclinical infections. Also complement fixation on the bacterial surface could promote complement-receptor-facilitated uptake by phagocytes and activation of the complement cascade via the classical and alternative pathways which may eventually play a major role in resistance to many Gram-negative bacteria (Brown, 1991). Also, the significant association observed between the complement C3 and IgM reflects ongoing infections, since the IgM rises early in the acute phase of the disease (Boes, 2000).

The increase in the mean neutrophil count of Typhoid patients observed in this study was in agreement with the reports of Sarkinfada and Abubakar (2001) who reported an elevated neutrophil count in typhoid patients than healthy controls in Kano, but was in contrast with the findings of Emenuga *et al.* (2014) who observed a lower mean Neutrophil count in Typhoid patients than controls among Igbos in Enugu, Nigeria.

The elevated mean lymphocyte counts obtained in the Typhoid patient in this study was in agreement with the report of Emenuga *et al.* (2014) and Obeaghu, (2017) who also recorded a higher lymphocyte count in Typhoid patients than healthy individuals in Abia state, Nigeria.

However, the elevated lymphocyte was it was in contrast with a lower lymphocyte count observed in typhoid patients by Abdool-Gaffar *et al.* (1992) in South Africa. In this study, typhoid resulted in neutrophilia) and a corresponding increase in lymphocytes (Lymphocytosis). Neutrophils play significant roles in the innate response and are crucial acute infection such as typhoid fever. It has also been reported that certain infectious agents may impair the bone marrow leading to its suppression or activation and are considered important mechanism in producing hematological changes (Khosla *et al.*, 1995). Typhoid fever is known to be a multisystem disease that affects most organs including the bone marrow which leads to changes in PCV, neutrophils and lymphocytes.

Although a high number of the patients were positives for the agglutination titre in the Widal test, a few of them were detected positive for IgG and IgM, This further support the reports by various authors that the Widal test is subjected to many faults such as reaction between antigens of other serotypes in the Salmonellae family, Furthermore patients may not demonstrate rise in antibody due to pre-exposure to TAB vaccines.

Conclusions

A total of 41(11.7%) and 29 (8.3%) tested positive for IgG and IgM to *S. typhi* respectively of the 350 patients. It was found that 207 patients had O-antigen widal reaction titres 1/80 and above for *S. typhi* out of which, 30 (14.5%) and 22(22.6%) were IgG and IgM positive respectively. The low positive recorded by the rapid assay confirm the report that the widal reaction results should by subjected to other confirmatory tests before a diagnosis is made. The neutrophil and lymphocyte counts were higher in the IgG and IgM positive patients as compared with those that were negative. These could serve as complimentary tools for diagnosis of typhoid disease. The mean complement C3 level was significantly elevated in the patients that were positive for typhoid IgM

and IgG as compared with those that tested negative (P =0.000). None of the clinical features showed association with the disease indication that these are not sufficient and must be accompanied with laboratory analysis for conclusive diagnosis of typhoid fever.

Conflict of Interest:

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