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Binding to CSA receptor is associated with asymptomatic and mild malaria: a preliminary study using *P.falciparum* field isolates from Sudan

Hassan DA[1*], Mohamed HS[2], El-Hussein AM[1], Ibrahim ME[2] and Abdulhadi NH[3]
[1] Central laboratory, Ministry of science and technology, Khartoum, Sudan.
[2] Institute of endemic diseases, University of Khartoum, Khartoum, Sudan.
[3] College of Pharmacy, The National Ribat University, Khartoum, Sudan.

***Correspondence: Dina A. Hassan, Central Laboratory, Ministry of science and technology, Khartoum, Sudan. E-mail: dinaibrahim6@gmail.com**

Abstract:
Malaria imposes great socio-economic burden on humanity, and afflicts approximately 90 countries and territories in the tropical and subtropical regions, almost one half of them are in Africa, South of Sahara. Sequestration of parasitized erythrocytes within the small vessels of vital organs is a key event in the pathogenesis of malaria and responsible of virulence of *Plasmodium falciparum* parasite. To find out whether the ability of infected red blood cells (IRBCs) to adhere to a specific receptor is a risk factor for developing severe clinical manifestation of the disease, *in-vitro* cytoadhesion and inhibition experiments were performed on field isolates obtained from five symptomatic and five asymptomatic patients inhabiting Gazira State, Central Sudan. The results showed significant lower levels ($p<0.02$) of cytoadhesion among asymptomatic compared to symptomatic patients. Percent inhibition by FA6-152, a monoclonal antibody for CD36, was comparable between the two study groups. However, the inhibition by CSA protein was less among symptomatic compared to asymptomatic patients. These results shed light on possible role of CSA receptors expressed on endothelial cells in ameliorating the events associated with the severe phenotype of the disease.

INTRODUCTION

Plasmodium falciparum malaria remains the major cause of death in Sub Saharan Africa, mostly among children below the age of five years. According to the mechanical hypothesis which was first proposed in 1894 by Marchiafava and Bignami [cited by 1], the pathogenicity of *P. falciparum* results from its unique ability to adhere to capillary and post-capillary venular endothelium during the second half of the 48-h erythrocytic life cycle, a process that is called cytoadherence. The resulting sequestration of infected red blood cells (IRBCs) leads to alterations in microcirculatory blood flow, metabolic dysfunction, and, as a consequence, many of the manifestations of severe falciparum malaria [2]. It has been reported that patients who die in the acute phase of falciparum malaria have intense sequestration of erythrocytes containing mature forms of the parasite in the microvasculature of vital organs [reviewed by 3].

In vitro models have shown that erythrocytes parasitized with mature stage of *P.falciparum* may adhere to receptors found on the endothelial cell surface. A key question is whether the ability of IRBCs to adhere to a specific receptor is a risk factor for developing severe clinical manifestation of the disease. Several studies among different populations were carried out to investigate the correlation between binding to specific host receptor and disease outcome. However, results obtained from these studies were considerably variable. A study among Malawian children [4] has investigated the binding of purified receptors CD36 (cluster of differentiation 36), ICAM-1 (intercellular adhesion molecule), CSA (chondroitin sulphate A) and thrombmodulin (TM) to fresh parasite isolates cultured to trophozoite stage from children with uncomplicated and severe malaria. Eighty five percent of all isolates bound to CD36, and 56% bound to ICAM-1. Fewer bound to CSA (27%) or TM (15%). However, after standardizing for parasitemia, parasite isolates from children with uncomplicated disease bound at higher levels to all receptors, significantly so for ICAM-1 and CD36. Conversely, a similar study at Kilifi on the Kenyan coast using cryopreserved and thawed isolates, has reported that the binding to ICAM-1 was highest among isolates from cerebral non-anaemic malaria patients whereas CD36 binding was slightly elevated among non-severe cases [5]. The study also reported that very few isolates could bind to VCAM-1 and none to E-selectin. Another study among Gambian children with falciparum malaria using monoclonal antibodies also showed that CD36 and ICAM-1 antigens were the major mediators of adhesion of IRBCs to vascular endothelium [6]. Cytoadherence characteristics were also studied among Thai patients [7] with complicated and non-complicated malaria using *in vitro* model of human lung endothelial cells (HLEC). According to this study, cytoadherence was significantly associated

with malaria severity and in consistence to previous studies CD36 was the main receptor in patients with uncomplicated malaria while ICAM-1 and CSA were major receptors among those with complicated malaria. Moreover, VCAM-1 and E-selectin played minor role in cytoadhesion among both groups. In a study conducted in non-immune travellers [8], it has been shown that IRBCs from severe malaria isolates appeared to adhere more frequently and/or strongly to ICAM-1 and CD36 in comparison with uncomplicated cases.

Recent attempts to explain the variable pathology seen in malaria stated that apposition of IRBCs activate signalling pathways. This is dependent, at least in part, on the cytoadherence characteristics of the invading isolate, such that the avidity of the IRBC for the receptor on host endothelium is proportional to the level of activation of the signalling pathways [9]. It has also been proposed that the balance between positive and negative regulation of genes involved in inflammation and cell death, by IRBCs and TNF will contribute to endothelial pathology during malarial infection [9].

The current study is a preliminary study intended to define host endothelial cell receptors favoured by the field isolates from Gazira state, Central Sudan and the impact of adherence to these receptors on the pathogenicity of malaria.

SUBJECTS AND METHODS

Subjects included in this study were inhabitants of Wadmadani city, Gazira State, central Sudan or its suburbs, an area characterized by seasonal malaria transmission. The experiment included five field isolates of *P.falciparum* from symptomatic children attending Wadmadani paediatric hospital and five isolates from asymptomatic children during screening for *P.falciparum* in a primary school in the area. Diagnosis of malaria was confirmed by immuonochromatographic rapid test (Hexagon Malaria CombiTM; Human, Wiesbaden, Germany) and Giemsa stained blood films. Classification of cases was based on clinical signs, and laboratory findings of malaria at the time of presentation.

One and half mililiters of venous blood were drown in citrate Vacutainer® tube (BD, Franklin Lakes, NJ) from each child enrolled after obtaining a signed consent form by parents or guardians. The study protocol was approved by the Ethical Committee of the institute of endemic diseases, University of Khartoum.

Human lung endothelial cells (HLEC) culture

HLECs are used in this experiment (gift from Laboratoire de Parasitologie Expe´rimentale, Faculte´ de Me´decine, Universite´ de la Me´diterrane´e, Marseille, France). The cells are characterized by expressing CSA, CD36 and ICAM-1 receptors. Cells were cultured in DME/F-12 media (Sigma, USA) at a density of 3,000 Cells/well in 12-well IFA slides for 24 to 48 hours under standard conditions to reach confluence as described by [10].

Maturation and enrichments of parasite isolates

Field isolates were cultured as previously described [11] in RPMI 1640 medium supplemented with 0.2% sodium bicarbonate, 0.25% AlbuMax, 0.6% hepes and 10 µg/ml gentamicin and incubated in a candle jar at 37°C. Cultures were harvested after 48 – 60 hours when parasites were mostly at the mature stage of schizonts and trophozoites. Erythrocytes Infected with mature stages were enriched by gelatin sedimentation technique described by [12] and the suspension of each isolate was used in duplicate static cytoadhesion and inhibition assays with HLECs. Parasitemia after gelatin sedimentation was adjusted to $1.5x10^{10}$ IRBCs/ml.

Static cytoadhesion assays

Static cytoadhesion assays were carried out in duplicate for each sample on 12-well IFA slides on which HLECs had been cultured to confluence [10] Briefly, the cell culture media was aspirated carefully from the edge of each well and 20 µl of (IRBCs) suspension prepared by gelatin sedimentation and 20 µl of RPMI 1640 media supplemented with 10 µg/ml gentamicin were added to each well. The IRBCs were allowed to cytoadhere to the confluent HLECs for 2 h at 37°C. Unattached IRBCs were removed by washing with RPMI 1640 media, and the cells were fixed for 1 hour with 2.5% glutaraldehyde (Sigma, USA). Number of IRBCs adhering to HLECs was counted under inverted microscope in four fields for each well (Fig 1). The result expressed as IRBCs/mm^2 was calculated as follows:

IRBCs/mm^2 = number of IRBCs in each field/area of field.

Average IRBCs/mm^2 = Sum IRBCs/mm^2 of the four fields/4.

Cytoadhesion inhibition assays

Soluble CSA at a concentration of 100 µg/ml (Fluka; 27042) and FA6-152 anti-CD36 monoclonal antibody at a concentration of 5 mg/ml were used to inhibit cytoadhesion to CSA and CD36 receptors respectively. Briefly, 20 µl IRBCs suspension from each isolate were incubated with HLECs in the presence of either 20 µl of soluble CSA or anti-CD36 mAb. IRBCs/mm^2 after inhibition were calculated by the same procedure used for cytoadhesion assays (Fig 2,3). The percent inhibition of cytoadhesion was calculated using the following equation:

% inhibition = 1-(average IRBCs/mm^2 after inhibition) / average IRBCs/mm^2 without inhibition) * 100.

Mann-Whitney tests were used to evaluate the statistical significance of differences.

RESULTS

Average cytoadherence counted as IRBCs/mm2 was significantly higher among symptomatic

patients compared to asymptomatics (p<0.02), (table 1). Inhibition of cytoadhesion using soluble CSA protein reduced the cytoadhesion by 5.04% and 40.8% (p<0.08) among symptomatic and asymptomatic patients respectively. On the other hand, % inhibition by FA6-152 anti-CD36 mAb was comparable between the two study groups (Fig 4).

TABLE 1: LEVELS OF CYTOADHESION AND INHIBITION OF CYTOADHESION BY CSA PROTEIN AND EA6-152 ANTI CD36 MAB AMONG THE STUDY GROUPS

Parameter (average±SD)	Symptomatic infection	Asymptomatic infection	p-value
Cytoadhesion (IRBC/mm2)	480.20±127.0	136.08±21.0	p<0.02*
% inhibition by CSA protein	5.04±2.3	40.80±16.6	p<0.08
%inhibition by FA6-152	72.60±6.8	72.50±9.6	p<0.7

*Statistically significant difference; p<0.05

FIGURE 1: CYTOADHESION OF AN ISOLATE FROM ASYMPTOMATIC PATIENT (A) AND SYMPTOMATIC PATIENT (B)

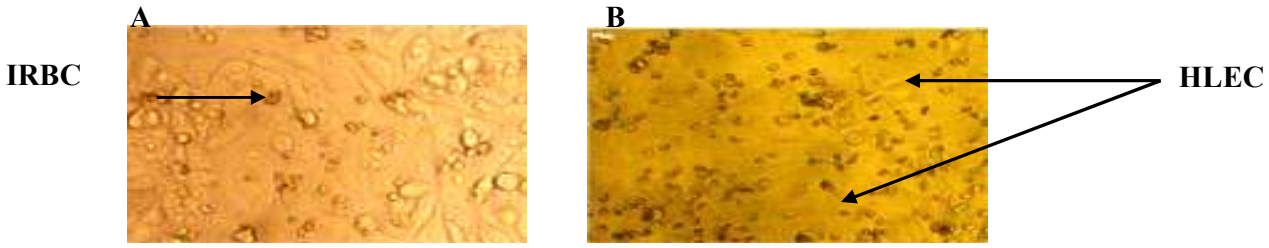


FIGURE 2: INHIBITION OF CYTOADHESION BY SOLUBLE CSA PROTIEN OF AN ISOLATE FROM ASYMPTOMATIC PATIENT (A) AND SYMPTOMATIC PATIENT (B)

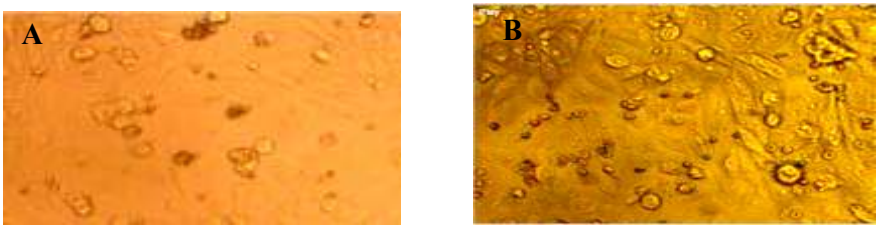
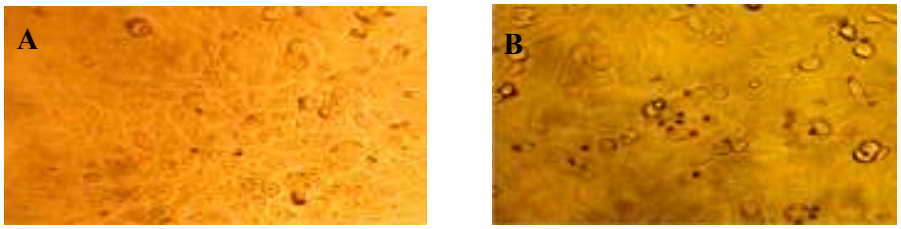
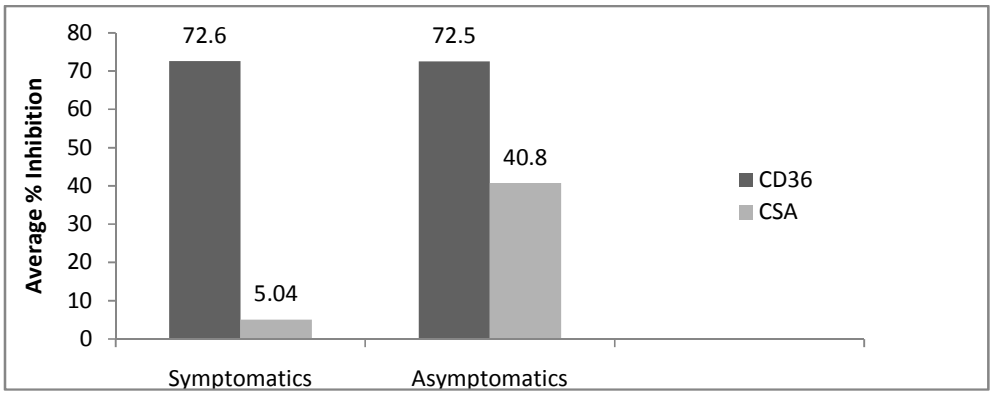


FIGURE 3: INHIBITION OF CYTOADHESION BY FA6-152 ANTI CD36 MAB OF AN ISOLATE FROM ASYMPTOMATIC PATIENT (A) AND SYMPTOMATIC PATIENT (B)



HLEC monolayer

FIG 4: PERCENTAGE INHIBITION OF CYTOADHESION BY FA6-152 AND CSA PROTEINS IN SYMPTOMATIC AND ASYMPTOMATIC PATIENTS.



DISCUSSION

In human *P. falciparum* malaria infection, IRBCs either sequester or are removed from the circulation primarily by the spleen. The balance between splenic clearance and sequestration, which allows the parasite to evade the immune system, is a major determinant of the rate of increase and magnitude of the infecting parasite burden. Within this paradigm, pathogenicity is proportional to the size of the sequestered parasite burden and the pattern of vital organ sequestration [reviewed by 3]. A key question is whether the ability of IRBCs to adhere to a specific receptor is a risk factor for developing severe clinical manifestation of the disease. In an attempt to answer this question and to determine the relative importance of CD36 and CSA receptor molecules for wild-type parasites among Sudanese, we have used static binding assays of IRBCs taken directly from the peripheral blood of patients with falciparum malaria to HLECs.

Our results confirmed the correlation between cytoadhesion and disease severity. Indeed, the level of cytoadhesion increased by 3.5 folds among symptomatic compared to asymptomatic patients. Inhibitions assays were carried out to evaluate the contribution of each of the selected receptors to the level of cytoadherence. Our results showed that (91.7%) of all tested isolates bound to CD36 receptor. This is consistent with previous studies using wild-type isolates among Thai [7], Malawian [4] and Kenyan [5] patients with severe and uncomplicated malaria. All isolates from Thai and Kenyan patients and 85% of Malawian patients bound to purified CD36 receptor. Similar results were also obtained with lab adapted strains [12]. However, the absence of significant difference in the percent cytoadhesion inhibition between the two study groups (p<0.7) indicates lack of correlation between CD36 binding and clinical severity of the disease. This is in agreement with previous studies reported among Kenyan children [5]. In contrast to our results, CD36 was found to be associated with uncomplicated malaria among Thai patients [7] and Malawian children [4]. It was suggested that this negative association between CD36 binding and disease severity might be due to the sequestration of parasites in less harmful parts of the body. Interestingly, a study among non-immune travelers reported that IRBCs from severe malaria appeared to adhere more frequently and/or strongly to ICAM-1 and CD36 in comparison with uncomplicated cases [8]. The contradicting reports on the association between CD36 binding and severity of malaria among the different populations might be attributed, at least partially, to polymorphism in the gene encoding CD36 protein. It has been reported that African populations contain an exceptionally high frequency of mutations in CD36 [13]. To our knowledge no studies were conducted so far on CD36 polymorphism among Sudanese population and its relation to malaria severity.

On the other hand, the binding of IRBCs to CSA receptors was quite variable among the different isolates. Although CSA receptor is closely associated with placental malaria where it is expressed on syncytiotrophoblasts that line the placental intervillous space, some reports pointed to the occurrence of CSA binding variants in non-pregnant individuals [14].The restriction of the CSA specific phenotype to the placenta is ruled out by the desequestration of IRBCs obtained when *P. falciparum* infected non-pregnant *Saimari sciureus* monkeys are injected with CSA. The specificity of this desequestration demonstrates that IRBCs are able to cytoadhere to CSA in the microvasculature of non-pregnant animals of this primate species, as well as in the placenta [10].

According to our data, only 58.3% of all study isolates adhere to CSA compared to 91.7% adherence to CD36 receptors. The reduced adherence to CSA receptors compared to CD36 is in agreement with previous studies among Malawian children [4] and Thai patients [7]. Our results

showed that the average percent cytoadhesion inhibition by CSA protein was higher among the asymptomatic group. However, the difference was not statistically significant probably due to the small sample size. In agreement with our results, a non-significant increase in CSA binding among Malawian children with uncomplicated malaria was reported [4]. In contrast, a higher percent of cytoadhesion inhibition by CSA antibodies among complicated malaria cases compared to uncomplicated ones was reported among Thai patients [7].

On conclusion, this study shed light on the importance of CSA as a putative receptor for uncomplicated or asymptomatic malaria among Sudanese in the study area and may protect against developing severe symptoms. It is possible that binding to CSA receptors in the study population does not provoke harmful immune response. Moreover, the high percentage of cytoadhesion inhibition by CD36 mAb may reflect a strong selective pressure exerted by CD36 on the parasite isolates in this region. It is possible that *P.falciparum* parasites may use this receptor in vivo to promote parasite survival and immune evasion.

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LABORATORY DIAGNOSIS OF MALARIA IN CHILDREN UNDER FIVE YEARS IN A RURAL COMMUNITY: MICROSCOPY VERSUS MALARIA PF TEST

ENWURU[1], C.P., UMEH[2], S.I., AGBASI,[3] U.M., and EGBUOBI,[4] R.C.
1. St Johns Lab Limited, Owerri, 2. Department of Microbiology, Federal University of Technology, Owerri, 3. Department of Science Lab Technology, Imo State Polytechnic, Umuagwo, 4. Department of Medical Laboratory Science, Imo State University, Owerri, Nigeria.

CORRESPONDENCE: C. P. ENWURU,P.O. BOX 38, URUALLA, IDEATO NORTH, IMO STATE. e-mail: globachik@yahoo.com

ABSTRACT
The morbidity and mortality associated with malaria in children below 5 years is really worrisome especially in the rural communities with little or no laboratory diagnostic facilities. This study was carried out to compare microscopy with Malaria Pf test for the diagnosis of malaria in a rural community in Ideato North Local Government Area of Imo State. Two hundred and fifty blood smears of children below 5 years were stained with Giemsa and examined microscopically for malaria parasites. Also the Malaria Pf rapid diagnostic test was used to test the same blood samples for malaria antigens. Thirty two per cent of the blood samples were positive for malaria parasite. Compared with microscopy, the sensitivity of the Malaria Pf test was 90.0%, the specificity was 98.2%. The positive predictive value was 96.0% and negative predictive value was 95.4%. The Malaria Pf test is reliable in the parasite based diagnosis of malaria in children under 5 years. We recommend the application of this test for parasitological confirmation of malaria in all places where it is not possible to provide facilities for good quality microscopy especially in the rural communities.

KEY WORDS: MALARIA, DIAGNOSIS, CHILDREN, MICROSCOPY, MALARIA PF

INTRODUCTION

Ever since the demonstration by Ronald Ross of the transmission of the parasite causing malaria from humans to mosquito and vice versa (1), malaria remains a scourge the world over especially in the sub-Saharan Africa, parts of Asia and the Americas (1, 2).Each year, 350-500 million cases of malaria occur world-wide (1). The World Health Organization had estimated 881000 deaths resulting from malaria in 2006, 91% of which occurred in Africa (3). Eighty five per cent of these deaths were children under 5 years of age. Another WHO report says 1 out of every 5 children die in Nigeria, 25% of which is caused by malaria (1). The malaria burden is indeed a threat to life and a drain in the economy of the already impoverished people of the sub-Saharan Africa (4, 5).
Early diagnosis, prompt and effective therapy are the pivots of the global malaria control strategy aimed at reducing unnecessary use of antimalarials and also reducing the mortality and morbidity associated with malaria (6). Treatment of malaria based on clinical diagnosis leads to unnecessary use of antimalarials (7, 8, 9, 10) with the attendant economic and health consequences. This is because clinical signs and symptoms associated with malaria are not specific (1, 5, 6). Malaria can be suspected presumptively from the signs and symptoms, but for a definitive diagnosis to be made, laboratory tests must demonstrate the parasite or its components (1). WHO (6) recommends that parasitological confirmation should be part of good clinical practice in order that the quality of care is improved.

Blood film stained with a Romanowsky stain (eg: Giemsa, Leishman and Fields stain) and examined by an experienced laboratory personnel remains the gold standard for laboratory confirmation of malaria diagnosis as this provides more detailed information such as parasite density, the Plasmodium species identification and different stages of the parasite (11). But this method depends on the quality of reagents, of the microscope, and on the experience of the laboratory personnel (1). Furthermore, blood smear may also not yield a reliable result if the slide is scratched and unclean, and if the pH of the buffer is not correct, if the stain contains debris (not filtered) or if the blood has been stored for some time in anticoagulant (12).
There are other methods of testing which include immunological methods like the antigen detection tests (1) as rapid diagnostic tests that produce results within 5-15 minutes, antibody detection tests example: enzyme linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT). The last 2 are less sensitive and unsuitable for routine diagnosis of malaria (1, 2, 12). Molecular method employing polymerase chain reaction (PCR) is highly sensitive but expensive, requiring greater sophistication in materials and labour and therefore not suitable for routine diagnosis (12).

The World Health Organisation (6) had stated that rapid diagnostic tests make it possible to provide accurate parasite based diagnosis for remote populations, reaching those who lack access to good quality microscopy services.
Furthermore, it has been reported that Plasmodium falciparum is the most common cause of severe and potentially fatal malaria, causing an estimated 700000 to 2.7 million deaths annually, most of them in young African children (1). Because of this it has been recommended that rapid diagnostic tests in Africa need high sensitivity for *Plasmodium falciparum*, and specificity to avoid over estimation of the malaria burden, false perception of therapeutic failure when fever is due to other illnesses, and unnecessary drug pressure (5).
This study was therefore carried out to assess the effectiveness of the malaria Pf rapid diagnostic test in detecting active malaria infection in children under 5 years of age in a rural community.

MATERIALS AND METHODS

Subjects
The subjects comprise children under 5 years who attend Osina Community Hospital, Osina, Ideato North, and Chika Medical Centre, Osina, Ideato North, both in Imo State from May to August, 2009

Procedures for testing
Consent of the parents/ guardians of the children were obtained prior to sample taking for the tests, and venous blood of the patients were collected.

Microscopy
Thick and thin blood smears were prepared according to standard techniques (13), allowed to air-dry and stained with 10% Giemsa solution for 30 minutes. The thin blood smear was fixed in absolute methanol for about 2 minutes before staining. Afterwards, the stain was washed in running tap water, allowed to air-dry in a slanting position and examined under oil immersion for malaria parasites by experienced Medical Laboratory personnel blinded to the result of the rapid diagnostic test. At least 200 high power fields were examined before a patient test is recorded as negative. Both thick and thin blood films were examined for each patient.

Malaria Pf Test
The kit comprises:

- Cassette contained in a sealed foil (pouch).
- Assay diluent (or buffer) in a dropper plastic bottle.
- Disposable pipettes.

The test was performed strictly according to manufacturer's instructions. The test kit was allowed to equilibrate at room temperature before testing. The cassette was removed from the pouch and placed on a clean and level surface. Using the dropper provided, the blood sample was drawn up to the fill line (about 10 µl) and transferred to the sample well (S) on the test cassette.

Three full drops (about 120 µl) of the sample diluent or buffer were added on the sample well.
The cassette was then examined for the appearance of coloured lines on the result window within (but not beyond) 20 minutes.

Interpretation of results
Positive test is indicated by the appearance of 2 coloured lines, 1 on the control (C) region and the other on the test (T) region. A negative test is indicated by the appearance of only 1 coloured line on the control (C) region and none on the test region. An invalid test is indicated by the non-appearance of coloured line on the control region with or without a coloured line on the test region.

RESULTS
A total of 250 children under the age of 5 were tested with both Giemsa stained blood smears and Malaria Pf rapid diagnostic test for malaria parasite. Eighty patients (32%) were positive by microscopy and 75 patients (30%) were positive by Malaria Pf test. Ten per cent of those positive by microscopy (n=8) were negative by the rapid diagnostic test (RDT) while 4% of those positive by RDT (n=3) were negative by microscopy (table 1). Using the microscopy as gold standard, the sensitivity (14) of the Malaria Pf test was 90.0% and the specificity was 98.2%. The positive predictive value (15) was 96.0% and negative predictive value was 95.4%.

TABLE 1 STATISTICAL VALUES OF MALARIA PF TEST

SENS	SPEC	PPV	NPV	FPR	FNR	LR+	LR-	F-MEASURE
90.0%	98.2%	96.0%	95.4%	1.8%	10.0%	5.0	0.1	93.9%

KEY: SENS=Sensitivity, SPEC= Specificity, PPV= Positive predictive value, NPV= Negative predictive value, FPR=False positive rate, FNR= False negative rate, LR+ = Likelihood ratio for positive tests, LR- = Likelihood ratio for negative tests.

DISCUSSION
The prevalence of malaria parasite among children under 5 years in this community is 32%. The sensitivity of the Malaria Pf test is 90.0% and the specificity is 98.2%. This means that this kit is capable of detecting 90 out of every 100 children with malaria and is also capable of giving a clean bill of health to 98 out of every

100 children who do not have malaria. In other words the kit is good at confirming the presence of malaria with a precision (positive predictive value) of 96.0%, thus enabling prompt and accurate treatment of a child with malaria. It also shows that the kit is good enough for ruling out the presence of malaria (negative predictive value = 95.4%) thus prompting search for other possible causes of febrile conditions in a child. Other studies comparing microscopy with RDTs especially in pregnant women have given equally good results (2, 16). The false positive rate of 1.8% is low and acceptable. Although the false negative rate of 10% seems to be on the high side, other factors that may give rise to false negative and false positive malaria tests need to be considered. Factors such as low parasite density (17, 18), sequestration of parasite in tissue capillaries (5, 19), Mutation of parasites (20), cross-reactivity with rheumatoid factor (5) etc. have variously been reported. Nonetheless, the result of this study gives a reasonable confidence in the diagnosis of malaria in small children. This is considered when weighed with the risk of unnecessarily exposing every child with fever to antimalarials, given the fact that children in the sub-Saharan Africa are also prone to other conditions such as respiratory tract infections (1), septicaemia etc. that usually give rise to febrile conditions. Furthermore, the use of this RDT in the diagnosis of malaria will go a long way to reduce over diagnosis and miss-diagnosis of malaria which give false impression of therapeutic failure and antimalarial drug resistance. This view is re-enforced by the discovery during the course of this study that laboratories that claim to diagnose malaria by microscopy use very low quality microscopes and some lack electricity and use reflected light from the sun using a mirror. This will obviously produce miss-leading results. Therefore, in any situation where it is not possible to provide good quality microscope and well experienced laboratory personnel, then the rapid diagnostic test is highly recommended. Moreover, the need to insist on parasitological confirmation of malaria before treatment has variously been emphasised (6). However, there is this suggestion that parasitological confirmation of diagnosis of malaria was recommended in all cases except for children under 5 years of age residing in areas of high prevalence of *Plasmodium falciparum* (5). This suggestion was in consideration of the risk of not treating false negative children. This argument sounds plausible but however, we are of the opinion that parasitological confirmation of diagnosis is necessary even in children under 5 years given the fact that should it turn out that the child does not have malaria after all, it will take days of worsening condition for
that to become obvious and a wasted opportunity for earlier search and adequate treatment for the real cause of the ailment. Worse still, some of such cases could unfortunately be misconstrued as antimalarial drug resistance with further administration of more expensive and complex antimalarials posing a further risk to the child and financial loss to the parents. This will also increase drug pressure on malaria parasite due to sub therapeutic dose encountered by newly acquired parasites, thus helping the parasite to develop resistance to the drug (2, 4, 5).

CONCLUSION AND RECOMMENDATION

We conclude that the Malaria Pf rapid diagnostic test is comparatively good for the parasite based diagnosis of malaria in children under 5 years. It is therefore recommended that the kit be provided in all places where facilities for microscopic diagnosis of malaria could not be provided and where laboratory personnel were under trained for recognition of malaria parasites in stained blood films. This will go a long way to reduce the miss-diagnosis and over diagnosis of malaria in our environment. Selection of drug resistant malaria due to drug pressure will be reduced if the policy of parasite based diagnosis prior to treatment is adopted even in children under 5 years of age. We also recommend that strict quality assurance measures be adopted in the use of the rapid diagnostic tests, a well trained and regularly retrained medical laboratory personnel designated to monitor the use within a given area and the results regularly compared with microscopy. Finally, it is necessary that laboratory personnel in both public and private health institutions be retrained in microscopic identification of malaria parasite if the war against malaria is to be won.

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MULTIDRUG RESISTANT *SALMONELLAE* ISOLATED FROM BLOOD CULTURE SAMPLES OF SUSPECTED TYPHOID PATIENTS IN WARRI, NIGERIA.

Ehwarieme,D.A. *Department of Microbiology, Delta State University, Abraka. Abraka, Delta State, Nigeria.*
ayoboladaniel@yahoo.com.

ABSTRACT
This study investigates the prevalence of R-plasmids in *Salmonella sp.* isolated from blood samples of suspected typhoid patients in Warri, Nigeria. A total of 136 blood samples were collected between May and December,2009 and screened for the presence of *Salmonellae* using standard blood culture techniques of which 20(14.7%) was positive for the pathogen. The multidrug resistant (MDR) isolates obtained (n=16; 80.0%), exhibiting the Ampicillin, Chloramphenicol, Cotrimoxazole and Tetracyclin (ACCoT) resistance profile, were subjected to plasmid curing. All (100%) of these MDR isolates bore their resistance markers on plasmids, as they lost their resistance sequel to the curing experiment. The low prevalence (14.7%) of the pathogen in the blood samples indicate that a good number of the suspected typhoid cases may not be incidences of the disease afterall. Furthermore, the high prevalence of MDR and plasmid-mediated MDR (80.0% and 100% respectively) isolates, suggest that treatment failures may be rampant if precise susceptibility test is not conducted prior to prescription.
Key words: Multidrug resistant, blood culture, typhoid fever.

INTRODUCTION

Cases of typhoid fever has become an endemic public health menace especially in developing countries. It is reported that yearly, there are about 16.6million cases of the infection with about 600,000 cases resulting in death (1,2). With the near absence of properly treated public water supply, these alarming figures are expected since typhoid fever is largely spread by the consumption of contaminated water (3). The etiological agent, *Salmonella typhi* is an enteric pathogen and so, the fecal-oral route of transmission is highly significant.

Symptoms of typhoid fever resembles those of some other infections, making diagnosis very paramount in the management of the disease. Routinely, the screening test for typhoid is the Widal serological technique (4) but, the reliability of this test is doubtful. False positive test is commonly encountered (5). Culture therefore remains a more definite diagnostic tool for confirmation of typhoid cases. Stool culture is preferable at about the third week of disease onset, a time when the patient may have been exposed to antibiotic therapy, interfering with chances of recovering the pathogen in the sample. Since there is a 70-90% chances of finding the organism in blood during the first ten days of infection (6,7), the blood culture technique was adopted in this study.

The first line of drugs for treatment of typhoid fever is Chloramphenicol, Amoxicillin and Trimethoprim-Sulfamethosazole (WHO,2003). However, there has been growing concern about the prevalence of Multidrug Resistant (MDR) *S.typhi* in developing nations, Nigeria inclusive (8,9,10). Most of the MDR *S.typhi* indicates plasmid mediation to be of importance. Resistance to the routinely prescribed antibiotics: ampicillin(A), chloramphenicol(C), cotrimoxazole(Co) and tetracycline(T) is usually plasmid- mediated and has been reported widespread from most parts of the world (11,12,13,4).

The oil city of Warri is a classical case of urbanization without development(14). Numerous factors still predispose individuals to typhoid fever infection. This study was designed to obtain the etiological agent of typhoid fever from blood of suspected typhoid patients, determine their pattern of resistance to the commonly prescribed drugs and, make a preliminary enquiry into the involvement of plasmids in the resistance attributes of *S.typhi* isolated from patients in Warri, Nigeria.

METHODS

Collection Of Samples

A total of 136 blood samples were obtained between May and December, 2009 from adults attending various private clinics in Warri, Nigeria, suspected of having typhoid fever, and processed according to standard procedures(7). These were patients with Physician's request for Widal test. Consent of the patients were sought prior to collection of samples. Using aseptic techniques, a sterile syringe and needle was used to withdraw 10mL of venous blood from the patients. Samples were collected twice and inoculated in duplicate into 50mL of Glucose and Sodium taurocholate broth. Incubation was at 37^0C for up till 7 days. Subculture was made onto

MacConkey and Blood agar plates, from broth showing signs of bacterial growth. Initial bacteriological analyses were undertaken at the Microbiology Laboratory of the Delta State University, Abraka. Subsequent plasmid analysis was carried out at the Biotechnology Laboratory of the Lahor Research Labs., Benin-City Nigeria.

Bacterial Identification And Standardization

Bacteria isolates were characterized and identified using standard techniques(15) as previously described (16,17). Stock of isolates were prepared by suspending a loop full of each microbial growth in about 10mL Nutrient Broth(NB). After incubation at 37^0C for 12H, the turbidity was adjusted to be visually comparable with a 0.5 McFarland's standard.

Antibiotic Sensitivity Testing

Sensitivity of the pure culture of bacteria isolates to different antibiotics, was determined using the Kirby-Bauer disc diffusion technique and interpreted based on the guidelines of the Clinical and Laboratory Standards (18). Discs used contained the following antibacterial agents: Ampicillin A(10µg), Chloramphenicol C(30µg), Cotrimoxazole Co(25µg), Tetracyclin T(30µg), Cpx(10µg).

Muella-Hinton (MH) agar plates were swabbed with cells from the bacteria stock solution, already adjusted to the 0.5 MacFarland's turbidity standard. The discs were thereafter, carefully layered on the agar and incubated at 37^0C for 24H.

Plasmid Curing Experiment

All bacteria isolates exhibiting the ACCoT antibiotics resistance pattern, were subjected to plasmid curing experiment (19). 10^3-10^4 cells was inoculated into a series of tubes containing acridine orange at graded concentrations. Incubation was at 37^0C for 24-48H. Subcultures were made from the sub-lethal concentration tube and, again screened for antibiotics resistance. Organisms that lost their resistance after curing were considered to bear the specific resistance marker on plasmids.

RESULTS

A total of 136 blood samples were collected and screened for bacteremia, from suspected typhoid fever patients. Of this number, 111(81.62%) were negative, as no growth was observed in the blood culture after a 7day incubation. However, positive blood culture revealed the occurrence of 20(14.7%), 4(2.94%) and 1(0.74%) respectively for *Salmonella sp., Klebsiella pneumonia* and *Staphylococcus aureus* (Table1).

The resistance profile of *Salmonella* (n=20) isolated, was tested to the antibiotics ampicillin(A=10µg), chloramphenicol(C=30µg), cotrimoxazole(Co=25µg), tetracycline(T=30µg), Nalidixic acid(Na=30µg), gentamycin(G=10µg), amoxicillin(Ax=30µg) and ciprofloxacin(Cpx=10µg). The least resistance was to Cpx,1 (5.0%) and Na,2 (10.0%) while the most of *Salmonella sp.* isolated was resistant to C,18 (90.0%) (Table 2).

Moreover, 16(80.0%) of the 20 isolates exhibited the ACCoT resistance profile. All but CS-003, 004, 006, 009, 010, 014 and 018, were equally resistant to at least one other antibiotic. (Table3).

TABLE1: PERCENTAGE PREVALENCE OF ORGANISMS IN BLOOD OF SUSPECTED TYPHOID PATIENTS.

Organisms	n(%) N=136
Salmonella sp	20(14.7)
Klebsiella pneumonia	4(2.9)
Staphylococcus aureus	1(0.7)

TABLE2: PERCENTAGE RESISTANCE OF *SALMONELLA* TO TEST ANTIBIOTICS.

Drug(concentration)	Resistance n(%) N=20
Ampicillin(10µg)	17(85.0)
Chloramphenicol(30µg)	18(90.0)
Cotrimoxazole(25µg)	16(80.0)
Tetracyclin(30µg)	17(85.0)
Nalidixic acid(30µg)	2(10.0)
Gentamicin(10µg)	10(50.0)
Amoxicillin(30µg)	2(10.0)
Ciprofloxacin(10µg)	1(5.0)

TABLE3: RESISTANCE PROFILE OF *SALMONELLA* ISOLATES BEFORE AND AFTER PLASMID CURING.

Isolates Code number	Before Curing	After Curing
CS-002	ACCoTG	G
CS-003	ACCoT	-
CS-004	ACCoT	-
CS-006	ACCoT	-
CS-007	ACCoTG	G
CS-008	ACCoTG	G
CS-009	ACCoT	-
CS-010	ACCoT	-
CS-011	ACCoTG	-
CS-013	ACCoTG	G
CS-014	ACCoT	-
CS-015	ACCoTG	-
CS-016	ACCoTAx	-
CS-017	ACCoTNaCpx	-
CS-018	ACCoT	-
CS-020	ACCoTNa	Na

DISCUSSION

The percentage of bacterial isolation among patients with typhoid fever vary enormously (20,21) Detection of bacteria by blood culture may be influenced by the culture medium employed, the number of circulating bacteria, the time of blood collection, the volume of blood employed for the culture, the host's immune response system as well as the intracellular character of the bacteria (20,21,19). In the present study, we obtained positive blood culture results from 25(18..38%) of the patients under study.

The low prevalence (14.7%) of the pathogen *Salmonella*, coupled with the presence of other bacteria- *Klebsiella pneumonia* 4(2.94%) and *Staphylococcus aureus* 1(0.74%) in the blood of suspected typhoid fever patients, indicate that some of the patients were not actually suffering from typhoid fever. A good number of infections may present symptoms which are similar to those of typhoid fever. Hence, these patients may not respond to treatment if they are placed on a regime strictly for typhoid fever.

The high prevalence of MDR *Salmonellae* is remarkable. Report from various parts of the world also tend to correlate present findings (22). This goes to suggest that most of the patients will not respond to treatment if placed on these first-line drugs. Equally remarkable, is the fact that all MDR isolates exhibiting the ACCoT resistance pattern lost their resistance sequel to plasmid curing.(Table 3). This is a major clinical challenge since the ease with which resistance markers are transferred, increases with the resistance being plasmid-borne (23).

Of particular interest is isolate CS-017 (Table 3) that equally lost its resistance to the fluoroquinolones(FQs) Na and Cpx after acridine orange treatment. This is a case of an emerging Plasmid Mediated Quinolone Resistance (PMQR). Further research into this isolate is ongoing, to establish whether its FQ resistance is due to a qnr, aac(6')Ib-cr or qep protein which has been established to confer PMQR to certain Enterobacteriaceae (23).

With the rising incidence of typhoid fever infection in our locality, the scenario cannot be any worse, if treatment failure are increasingly encountered. It is thus recommended that proper diagnosis be carried out before establishing cases of typhoid infection. Furthermore, sensitivity tests should inform the choice of drug to be administered.

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DIAGNOSTIC ACCURACY OF RAPID UREASE TEST FOR THE DIAGNOSIS OF *HELICOBACTER PYLORI* IN GASTRIC BIOPSIES IN NIGERIANS WITH DYSPEPSIA

Jemilohun[1], A.C., Otegbayo[2], J.A ., Ola[2], S.O., Oluwasola[3], A.O. and Akere[2] ,A.
[1]Department of Medicine, Federal Medical Centre, Iddo- Ekiti, Nigeria, [2]Department of Medicine, College of Medicine, University of Ibadan and University College Hospital, Ibadan, Nigeria, [3]Department of Morbid Anatomy and Histopathology, College of Medicine, University of Ibadan and University College Hospital, Ibadan, Nigeria.

Correspondence: Dr A.C. Jemilohun, Federal Medical Centre, Iddo- Ekiti, Nigeria, P.M.B. 201, Iddo-Ekiti, Ekiti State, Nigeria.Email:chrislohun2010@hotmail.com. Tel: 08038674623

Running title: **DIAGNOSTIC ACCURACY OF RAPID UREASE TEST**

ABSTRACT
Background: The strong association of *Helicobacter pylori (H. pylori)* with dyspepsia has caused a major paradigm shift in patients' management. It has been observed that histology is usually employed as the routine test for the diagnosing *H. pylori* in centres where Oesophagogastroduodenoscopy (OGD) is available in Nigeria. Because of the drawbacks attendant to the use of histology, in terms of cost effectiveness and technical expertise, it is necessary to evaluate the diagnostic accuracy of a simpler alternative for ease of management of patients with dyspepsia.
Objective: This study evaluated the diagnostic accuracy of rapid urease test (RUT) in the diagnosis of *Helicobacter pylori* (*H. pylori*) in patients with dyspepsia.
Methods: Eighty-six consecutive adult patients with dyspeptic symptoms presenting for endoscopy were recruited after giving informed consent. Gastric antral biopsies were collected at endoscopy for RUT and histology. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of RUT was calculated using histology as the reference standard.
Results: Of the 86 subjects, there were 39 (45.3%) males and 47(54.7%) females. The mean age was 49.19±13.75 years. The age range was 23 to 85 years. The sensitivity, specificity, PPV, NPV of RUT was 93.33%, 75.6 %, 80.76 %, and 91.17 % respectively.
Conclusion: RUT is accurate for the diagnosis of *H. pylori* infection. Its use will serve as a good alternative to histology in management of patients with dyspepsia in resource poor environments, except in patients who need histology for reasons other than *H. Pylori* diagnosis.

Key words: ***Dyspepsia, Helicobacter pylori, Rapid Urease test, Histology.***

LIST OF ABBREVIATIONS

H&E	Haematoxylin and Eosin
H. pylori	*Helicobacter pyl*ori
IgG	Immunoglobulin G
NPV	Negative predictive value
OGD	Oesophagogastroduodenoscopy
PPV	Positive predictive value
RUT	Rapid urease test

INTRODUCTION

Helicobacter pylori is a gram negative, spiral, flagellated bacterium with the capability of abundant urease production. *H. pylori* bacterium is usually found under the mucus layer in the gastric pits and in close apposition to gastric epithelial cells (1). Since the discovery of *H. pylori* by Warren and Marshall (2), it has been evidently demonstrated that the organism plays a major role in several upper gastrointestinal diseases which present as dyspepsia (2- 4). *Helicobacter pylori* infection causes chronic active gastritis in the antrum (antral gastritis), the corpus (corpus gastritis) or in both (pangastritis). It is a major aetiological factor in peptic ulcer disease, gastric carcinoma, and gastric mucosal associated lymphoid tissue (MALT) lymphoma (2, 5, 6). Haemorrhage and perforation are the most frequent complications of peptic ulcer disease and are associated with substantial morbidity, mortality and health care costs (6). Peptic ulcer disease can be cured by eradicating *H. pylori* so that complications no longer occur (1).

There are various diagnostic tests for *H. pylori* which can be broadly classified into invasive and non-invasive tests (7). Invasive tests utilise endoscopic gastroduodenal biopsy samples for histology, culture, rapid urease test (RUT), polymerase chain reaction and fluorescent in-situ hybridization. The non-invasive tests do not require endoscopy; they include urea breath test, immunoglobulin G, A and M serology, stool antigen test, saliva antibody test (8, 9) and urinary antibody test (10). In Nigeria, the non-invasive tests are not generally available with

the exception of IgG serology. Serological screening is of limited value, especially in a hyper-endemic area like Nigeria, because it has low discriminatory the routine test for diagnosing *H. pylori*. There are relatively few histopathologists in Nigeria, and they are usually concentrated in government owned Teaching Hospitals located in urban centres. Histology report on gastric mucosal biopsies for *H. pylori* usually takes two weeks or more to be available while RUT results for *H. pylori* could be read within 5 minutes to 24 hours of test (11). The RUT is also much cheaper compared to histology for *H. pylori* diagnosis, as the test is carried out in the endoscopy suite by the Endoscopist or an assistant. There has been a recent modest increase in the availability of gastrointestinal endoscopic facilities and it is envisaged that more centres with no histopathology services will begin to have such facilities as expertise increases in Nigeria. In view of the foregoing advantages of the RUT, and the generally low socioeconomic status of majority of the Nigerian populace, it is desirable to evaluate RUT diagnostic accuracy using histology as the reference standard.

MATERIALS AND METHODS

The study was carried out at the Endoscopy sub-unit of the Gastrointestinal & Liver Unit, Department of Medicine, University College Hospital, Ibadan, Nigeria. Ethical approval was sought and obtained from the Joint University of Ibadan/ University College Hospital Institutional Review Committee. Eighty- six consecutive adult patients with dyspeptic symptoms presenting for OGD were recruited after giving informed consent. Diagnosis of dyspepsia was made clinically according to the Rome working teams' definition (12). Patients' symptoms had persisted for a minimum of 3 months or recurrent in nature for the same period. Those who were previously treated for *H. pylori* infection or who had received antibiotics, proton pump inhibitors or bismuth compounds in the preceding 4 weeks were excluded. Base line bio-data were obtained.

OGD was performed on all the participants using Olympus (GFI-XQ20) or Pentax (FG29W) forward-viewing oesophagogastroduodenoscopes. All patients had pharyngeal spray with 2% xylocaine. Most of the patients had conscious sedation with intravenous diazepam and pethidine or pentazocine. All were monitored with multiparameter pulse oximeter(EDANinstruments). power between previous and current infection. Centres that have OGD usually employ histology as

Two gastric antral mucosal biopsies were taken for each of RUT and histology.

Rapid urease test (RUT)

Two of the four antral biopsies taken from each patient were used immediately for RUT. The RUT consisted of two dry filter paper containing urea, phenol red (a *pH* indicator) in a sealed plastic slide. If the urease enzyme of *H. pylori was* present in an inserted tissue sample, the resulting decomposition of urea to CO_2 and NH_3 caused the pH to rise and the colour of the dot turned from yellow to a bright magenta. Results were read within 3 hours after sampling according to the manufacturer's specification. The colour change from yellow to magenta was considered a positive result while no colour change was regarded as negative.

Histology

The other two antral biopsies were fixed in 10% formaldehyde and transferred to the histopathology laboratory for processing. Four micron thick paraffin sections were stained with routine Haematoxylin and Eosin (H&E) for detection of *H. pylori* and gastritis. Giemsa stain which is a special histochemical stain for *H. pylori* was also used for better yield. Slides were examined microscopically for *H. pylori* by the help of a Gastrointestinal Pathologist. The presence of submucosal helical (*Helicobacter-like)* organisms was regarded as positive while its absence was regarded as negative.

Data analysis

Data was analyzed using Statistical Package for Social Sciences, version16.0 (SPSS Inc. Chicago Illinois). Results were presented as means ± standard deviation for quantitative variables and number (percentages) for qualitative variables. Sensitivity, specificity, positive and negative predictive values of RUT was calculated by two by two standard method.

RESULTS

- A total of 86 adult patients with dyspepsia participated in the study. The mean age was 49.19 (±13.75) years. There were 39 (45.3%) males and 47(54.7%) females. The minimum age was 23 years and the maximum was 85 years. The results of the diagnostic tests are shown in table 1. The RUT was positive in 52 (60.5 %), while histology was positive in 45(52.35%).

TABLE1: RESULTS OF RAPID UREASE TEST AND HISTOLOGY IN SUBJECTS

Test	Frequency(n=86) Positive	Negative
Rapid urease test	**52 (60.5 %)**	**34(39.5 %)**
Histology	**45(52.3%)**	**41(47.7%)**

TABLE 2: COMPARISON BETWEEN RUT AND HISTOLOGY

	Histology (n=86)		
	Positive	Negative	Total
Rapid urease test			
+ve	**42**	**10**	**52**
-ve	**3**	**31**	**34**
Total	**45**	**41**	**86**

TABLE 3: DIAGNOSTIC ACCURACY OF RUT

VARIABLE	VALUE %	95% C. I.*
Sensitivity	93.33	81-98
Specificity	75.6	59-87
P PV	80.76	67-90
NP V	91.17	76-98

*C. I. = Confidence Interval

Table 2 shows the comparison between results of RUT and histology. The total number of those who were both positive for RUT and histology (true positive) was 42(48.84%), those who were positive for RUT but negative for histology (false positive) were 10(11.62%), those that were both negative for RUT and histology (true negative) were 31(36.04%), while those that were positive for histology but negative for RUT (false negative) were 3 (3.49%).
The sensitivity, specificity, PPV and NPV of RUT were 93.33%, 75.6 %, 80.76 %, and 91.17 % respectively (table 3).

DISCUSSION

The diagnosis of *H. pylori* by culture, gram stain and histology, which are biopsy based methods, is well established. However, several drawbacks attend to them. Firstly, the delay in the availability of results, and secondly the rarity of good microbiology and histopathology laboratory support especially in developing countries like Nigeria. Other problems associated with histological diagnosis of *H. pylori* arise because the result depend on the competence of the pathologist, the time spent to examine the slides (inter-observer variability) and the variability of staining techniques (11, 13). Special stains for biopsy specimens improve visual detection of the bacteria. To mitigate these problems in our study, the service of a Gastrointestinal Pathologist was employed and Giesma stain was used in addition to routine H&E. Giemsa stain is the most frequently used stain for *H. pylori* diagnosis in routine clinical practice, because of its diagnostic performance and lack of technical difficulty in preparation in comparison with the other stains (13, 14). A major advantage of histological examination over other biopsy based methods is that it also provides information about gastric mucosal pathology.

The RUT practically overcomes these drawbacks since it is not dependent on the experience and accuracy of individual laboratories as in the case of other biopsy based methods. As usefully and attractive as RUT is, it has its own draw backs. In theory, patients that salivate or have reflux alkaline bile into the stomach could have a weak positive reaction because the liquid may contaminate a small gastric biopsy specimen such that the resulting surface pH is >6.0 (6). Similarly, chronic use of proton pump inhibitor may lead to achlorhydria and subsequent superficial colonisation of the gastric mucus layer with urease-producing organisms e.g. *Klebsiella* or *Proteus mirabilis* (1, 6).These organisms can give a false-positive urease test after 24 hours of inoculation but generally negative tests remain so when read within the specified time by the manufacturer (6, 15).

It has been shown that the use of proton pump inhibitors increases the numbers of false-negative tests (16). Two possible mechanisms by which this is done have been identified. Firstly, the medication may directly inhibit *H. pylori* urease activity. Secondly, the changing patterns of *H. pylori* colonisation after acid suppression may delay the positivity of RUT. *Helicobacter pylori* often only resides in the corpus during long-term use of proton pump inhibitors and can therefore not be detected in antral biopsies (16). The problem of chronic proton pump inhibitor use was avoided in our study by excluding patients who were on the drug in the preceding four weeks to the test.
The presence of blood may also adversely affect the performance of RUT leading to a false negative result. This is due to the buffering effect of serum albumin on the pH indicator, rather than by a direct inhibition of the urease activity.

Rapid urease tests have specificity and sensitivity of greater than 90%, but false-positive results do occur (17). The RUT had a sensitivity and specificity of 97.4 and 96.1% respectively in an earlier study conducted by van Keeken *et al.* (6) in the Netherlands. The relatively lower values obtained in our study compared to that of van

Keeken *et al*. and other previous similar studies (13,18, 19, 20) could be explained by the fact that they all used more than one diagnostic method as reference standard, as no single presently available test provides the definitive diagnosis of *H. pylori* by itself (13, 21). For instance, van Keeken *et al*. used a combination of histology and culture as the reference standard in their study. The implication of this is that any infection missed by one test due to the patchy distribution of the infection and consequent sampling error could be easily picked by the other tests, thereby increasing the number of positive results by the reference standard. This is further buttressed by the fact that there were less positive results by histology (52.3%) as compared to RUT (60.5 %) in our study. It is concluded from our study that the RUT with two gastric biopsies is accurate for the diagnosis of *H. pylori* nfection. Compared with histology, RUT is simpler, it gives more rapid test result and it is much cheaper. It will serve as a good alternative to histology in managing patients with dyspepsia in resource poor environments like Nigeria, except in patients who require histology for reasons other than *H. Pylori* diagnosis.

CONFLICT OF INTEREST

We declare no conflict of interest

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HEPATITIS B IMMUNIZATION AT THE UNIVERSITY COLLEGE HOSPITAL, IBADAN: AN EIGHT YEAR REVIEW OF VACCINE ADMINISTRATION RECORDS.

Ola SO, Akere A, Otegbayo JA, @Omokhodion F, ^Olofin AA, #Bamgboye EA.

Departments of Medicine, @Community Medicine and #Epidemiology, Medical Statistics & Environmental, Health, College of Medicine, University of Ibadan and ^Staff Medical Services Department, University College Hospital, Ibadan, Nigeria.

Correspondence : Dr. S.O. Ola, Departments of Medicine, College of Medicine, University of Ibadan, University College Hospital, Ibadan; Nigeria. Email Address: <soola@comui.edu.ng>, soola2001@yahoo.com Tel.00234 2 2410088 Ext 2676 Fax 00234 2 2413545.Mobile Tel 002348076727548.

Summary

Vaccination of health care workers (HCWs) against hepatitis (HBV) infection is highly necessary in Nigeria where the infection occurs in hperendemic proportions. We hereby determine the trends in the administration of HBV vaccine at the University College Hospital (UCH), Ibadan, Nigeria. The study reviewed the records for the administration of vaccine against HBV at the Staff Medical Services Department of UCH, Ibadan, Nigeria, from 1994 to 2001. A total of 1,437 subjects consisting of 686 (47.7%) males and 751 (52.3 %) females were vaccinated against HBV from 1994 to 2001. They were aged 16 to 64 years and consisted of 356 students (24.8%) and 1081 healthcare workers (HCWs) (75.2%) which comprised Doctors (30.9%), Dentists (1.9%), Paramedics (19.6%), Non-medics (14.6%) and subjects with undisclosed occupational category ((10.7%). About 11% to 100% of the subjects had annual prescreening for HBsAg sero-negativity from 1996 to 2001 but none had post vaccination assay of anti-HBs titre. All the subjects received 1st dose of 0, 1, 2 accelerated HBV vaccination schedule while on annual basis, 16.7% to 91.8% of the subjects received the 3rd dose of the vaccine. Despite the proportional participation of the different occupational groups was highest among the doctors and dentists but lowest among the paramedics, only 59.7% of all the vaccinees had three dose(s) of the vaccine during the 8 year period. In conclusion, although the HBV vaccination programme had shortcomings, it is an established practice at UCH, Ibadan and efforts should be made to improve on its prevailing standard.

Keywords: Immunization, HBV, Healthcare Workers, Nigerians, UCH, Ibadan.

Introduction

Although, hepatitis B virus (HBV) infection occurs in hyperendemic proportions in sub-Saharan Africa including Nigeria [1]; the introduction of vaccination against the infection has curtailed its chronic sequelae in countries where there have been strict and proper implementations of the immunization strategy [2]. The scheme has also been incorporated into the national immunization programme of the different countries [3]. Also, protection of at-risk groups such as the health care workers (HCWs) is particularly important in order to reduce the spread of the virus as well as the prevalence of its attendant sequalae in the community [1, 4].

The prevalence of HBV infection among Nigerian HCWs is high (38.7%) [5]. A high prevalence rate of the infection also exists among Nigerian students [6, 7]. These reports support the need to vaccinate HCWs against HBV especially with use of the recombinant DNA technology type of HBV vaccines. The Rapid Immunisation Schedule consisting of series of three intramuscular injections given at 0, 1, 2 months and administration of a booster dose of vaccine at the 12th month after the first dose will confer protection against HBV infection for further 5 to 8 years when the next booster dose will be due [8-10]. Completion rates of the vaccination has been reported to be as high as 71% for the receipt of 3 or more doses of hepatitis B vaccine among HCWs in United States between 2002 and 2003 [4].

The question arises if the programme is being pursued in accordance with the international recommendation. This has led to our study on the determination of the trends in the vaccination of HCWs against HBV at the University College Hospital, Ibadan (UCH), Nigeria.

Materials and Methods

This retrospective study reviewed the records for the administration of vaccination against HBV at the

Immunization Unit of the Staff Medical Services Department, UCH from 1994 to 2001. Data on sex, age, occupational distribution of all the vaccinees were extracted. Information on the prescreening of the vaccinee for Hepatitis B surface antigenaemia (HBsAg) and anti-bodies to Hepatitis B core antigen (anti-HBc) were also obtained. Similarly, the number of dosages of the vaccines received as well as the type of vaccines administered, were collected. The study was approved by the Joint UCH/UI Ethical Review Board prior to its execution. The data obtained were handled with strict confidence and expressed in tables and graphs. The significance of any hypothesis was investigated using appropriate statistical test at 5% probability level.

Results

A total of 1,437 of the HCWs at UCH, Ibadan were involved in the study and they comprised 686 males (47.7%) and 751 females (52.3%) with a sex ratio of1:1.6. The HCWs were vaccinated against HBV from 1994 to 2001, Table 1. Their ages ranged from 16 to 64 years but the subjects were below 40 years until 1999 when those aged up to 64 years participated in the vaccination programme. The year 1999 had the highest participation while 1995 had the least. The participation in 1994 was twice that of 1995. Thereafter, it rose to a peak in 1999 and further declined progressively for the subsequent two years. The schedule for 1999 was principally carried out by the hospital consultants as an effort at immunizing all HCWs against HBV.

Table 2 shows that the subjects consisted of 1081 HCWs (75.2%) and 356 students (24.8%). Only 1283 (89.3%) of the HCWs disclosed the type of their occupation. The HCWs who received first dose of HBV vaccine included doctors comprising Physicians (14.1%), Paediatricians (2.7%), Surgeons (3.8%), Obstretricians and Gynaecologists (2%), and House Officers (8.3%); Dentists (1.9%), Paramedics (Nurses, Nursing Assistants, Laboratory Technologists, Pharmacists, Radiographers, Medical and Social Workers, Physiotherapists, Medical Record Staff, Occupational Therapists, Medical Illustration Unit Staff) (19.6%) and Non-medics (administrative, secretariat, engineering, maintenance and other hospital support staff), 14.6%.

Prescreening for HBsAg prior to vaccination was not carried out for the initial period of two years and was commenced in 1996, Table 3. Only 10.5% of the participants had prescreening in 1996. The figure rose to 70.1% in 1997 and 100% from 1999 to 2001. The exercise in 1999 involved prescreening of all workers for not only HBsAg but also anti-HBc. All the prescreened subjects were HBsAg sero-ngative before they administered HBV vaccine.

Table 1: Age and annual distribution of healthcare workers vaccinated against HBV at UCH, Ibadan, 1994-2001

Year	No	Mean Age ± SD years	No > 40 years (%)	Male	Female
1994	20	27.7± 7.2	2(10)	11	9
1995	10	28.7 ± 9.3	1(10)	7	3
1996	38	26. 0± 4.2	0(0)	27	11
1997	41	25.7± 7.7	2(4.9)	23	18
1998	88	23.2 ± 6.7	2(2.3)	34	54
1999	546	49.0 ± 14.3	244(44.7)	275	271
2000	359	25.3 ± 8.5	26(7.2)	134	225
2001	335	25.8 ± 8.5	19(5.7)	175	160

Two available types of recombinant vaccines - Genevac B (5.3%) and Engerix B (94.7%) were administered. Also, three doses of vaccines at 0, 1, and 2 or 0, 2and 6 months were scheduled per participant every year from 1994 to 2001. Although, all the participants received the first dose of the

Table 2: Departmental distribution of vaccinees and yearly pattern of the receipt of the first dose of vaccine against Hepatitis B virus

Group	Number (%)	Years							
		1994	1995	1996	1997	1998	1999	2000	2001
Doctors	443 (30.9)	17	5	35	23	26	115	148	74
Physicians	202 (14.1)	8	1	12	11	6	56	64	44
Paediatricians	39 (2.7)	4	-	3	2	2	19	5	4
Surgeons	54 (3.8)	3	-	8	4	5	24	3	7
Obstetrics & Gynaecologists	29 (2)	2	1	7	4	2	5	2	6
House Officers	119 (8.3)	-	3	5	2	11	11	74	13
Dentistry	28 (1.9)	2	2	2	-	1	6	4	11
Paramedics	282 (19.6)	-	-	-	3	5	206	47	21
Non- medicals	174 (12.1)	-	1	-	-	-	164	5	4
Students	356 (24.8)	1	2	-	15	31	36	121	150
Sub-total	1283(89.3)	20	10	37	41	63	527	325	260
No record	154 (10.7)	-	-	1(2.6)	-	25(28.4)	19(3.4)	34(9.5)	75(22.4)
Total	1437(100)	20(1.4)	10(0.7)	38(2.6)	41(2.9)	88(6.1)	546(38)	359(25)	335(23.3)

Parenthesis - percentages
Paramedics - Nurses, Nursing Assistants, Laboratory Technologists, Pharmacists, Radiographers, Medical and Social Workers, Physiotherapists, Medical Record Staff, Occupational Therapists, Medical Illustration Unit Staff
Non-medics – Administrative, Secretariat, Engineering, Maintenance and Other hospital support staff

Table 3: Percentage of healthcare workers prescreened for HBV infection and their vaccination status at UCH, Ibadan, Nigeria - 1994 to 2001.

Year	Number of HCWs vaccinated	Percentage of HCWs Prescreened	Percentage of HCWs vaccinated with different doses			P-values	
			One	Two	Three	p1	p2
1994	20	0	100	85.0	60.0	NS	NS
1995	10	0	100	90.0	60.0	NS	NS
1996	38	10.5	100	86.8	34.2	NS	0.0003
1997	41	70.1	100	95.1	56.1	NS	0.02
1998	88	92.0	100	81.8	58.0	NS	0.0004
1999	546	100.0	100	96.2	91.8	NS	NS
2000	359	100.0	100	80.2	54.9	0.003	0.0000
2001	335	100.0	100	58.2	16.7	0.0000	0.0000
Total	**1437**	**94.1**	**100**	**81.9**	**59.7**	**0.0000**	**0.0000**

p1 - between attendances of 1st and 2nd dose NS – Not significant p2 – between attendance of 1st and 3rd dose

vaccine with information on the scheduled dates for the two subsequent doses, the percentage attendances for the second dose were between 80.2% to 96.2% for the initial seven years and it dropped to 58.2% at the 8th year, $p<0.05$. There were no significant differences between the annual participations for the first dose and those for the second dose of vaccination except in 2000-1, $p<0.05$ while the attendances for the third dose was significantly lower than those of the 1st dose in all the years except in 1994-5 and 1999, Table 3. No vaccinee had assay of its post vaccination anti-HBs titre.

Only 1437 (35.9%) and 859 (21.5%) subjects had received one and three dose(s) of the vaccine respectively by 2001 when compared with the population of HCWs (4,001) at UCH, Ibadan in that same year. Participation in the vaccination programme was highest among the doctors and lowest among the paramedics even though the doctors and the paramedics accounted for less than a sixth and about half percentile of the 2001 population of HCWs respectively at the institution, Table 4.

Table 4: Population of the different occupational groups of healthcare workers at UCH, Ibadan, Nigeria; 2001

Occupational groups	Population of HCWs	
	Number	%
Paramedics	1938	48.4
Students	930	23.3
Doctors & Dentists	572	14.3
Non- medics	561	14.0
Total	4001	100

Discussion

Although vaccination against HBV began in 1984 first with a plasma-derived vaccine among non-immune children under the age of 5 years in the Gambian villages of Keneba and Manduar, and later a recombinant DNA-derived vaccine [11], active programme for the vaccination of HCWs against HBV commenced at UCH, Ibadan in 1994. This study showed that the programme was not only commenced but has been sustained and has evolved from few numbers of participants at onset to sixty fold by the sixth year. It showed a commendable effort being made by the hospital at controlling HBV infection primarily among the staff and secondarily in the community. This is particularly important because Nigeria is a hyperendemic zone for the infection with high proportions of the consequences of the infection present among its population [12]. The gender distribution of the participants being predominantly males in the initial three years of the programme is probably secondary to the presence of highest vaccination rate among the doctors who also are predominantly males although the cultural belief that the health of the man takes precedence over those of the other members of the family could not be ruled out despite the fact that the utilization of health facilities should be without consideration of status of any member of the family. The improvement in the gender utilization of the service is in consonance with increasing education of the females since they formed the bulk of HCWs right from foundation of the health care institution.

The general low number of participants in 1994 to 1997 could be due to poor knowledge about HBV among HCWs in 1994 as well as the non-availability of the protective vaccine. With increasing knowledge of the usefulness of the vaccine vis a vis the consequences of lack of protection, acceptability of the vaccination programme by the staff and the determination of the hospital administration to protect its staff against the virus could be responsible for the rise in the number of the participants over the study period. The downward trend in the number of the participants from 1999 to 2001 could be secondary to the coverage of greater proportion of the staff particularly in 1999 with only new entries being added thereafter although there may be low drive of the HCWs towards their being vaccinated against HBV. Furthermore, the low participation of subjects above 40 years of age could be due to the presence of more staff in the younger age groups however the knowledge that the sequalae of the infection is predominantly in the 2nd to 5th decades of life[12] among Nigerians could be the motivating force inspiring the greater participation of the younger adult population in comparison to the older adults.

The distribution of the different proportions of the HCWs involved the programme is not unexpected as it follows previous report [4]. It shows the awareness the HCWs of their risk status for the infection [1, 6-10]. The participation of student HCWs in the programme supports the need for their immunization against HBV since they are also at high risk of contacting the infection [6, 13]. The presence of incomplete data on a significant proportion of the workers is unacceptable and it suggests an improvement is needed in the collection of data from the vaccinees. Furthermore, the coverage of the immunization programme is very low among the total population of the HCWs at UCH, Ibadan, hence there need for ensuring 100% coverage of the workers.

The completion rate of a three-dose accelerated HBV vaccine schedule among the HCWs studied, falls below the report among similar groups of subjects in the USA [4, 15]. Efforts should be made to improve compliance to the vaccination schedule since receipt of only one dose confers no protection. The receipt of two doses may offer only some protection (61.4%) [16] but it is lesser than what occurs with the receipt of three doses (80-95%) [8]. The supervision of the programme in 1999 was associated with the high participatory rate of the workers for the receipt of three doses of vaccine while the converse could be responsible for the poor compliance recorded in the other years. Hence, this study has demonstrated the positive role of a supervisory vaccination prograrmme [15] actively financed and purposely carried out by the management of a tertiary Nigerian heath institution at ensuring a high compliance rate from the vaccinees with consequent development of high protection rate against the infection.

However, a fourth dose at 6th or 12 month post 1st dose will confer higher anti-HBs titre with protection beyond 12 months (offered by 3 dose regimen) to 5-8 years when a booster dose will be required [8, 17]. It is obvious from this study that no worker had a fourth or booster dose post the 1st dose. The booster dose at 5 year is advocated for HCWs in order to

ensure their safety for patient care [8]. It is advocated that all workers should be educated on the importance of the dosing schedule as well as their compliance to completion of the vaccination programme. Furthermore, efforts should be made to immunize all the workers as this ensures their protection against the virus than when not immunized [18]. The prescreening rate of the workers for HBV prior vaccination from 0% in 1994 and 1995 to 100% in 1999 to 2001 is commendable because the assay is necessary in Nigeria, a hyper endemic area for HBV [6,13] and particularly among HCWs who have high rate of HBV [5,18,19]. However, the prescreening for HBV prior to vaccination is a good course of action which needs to be maintained as it helps to determine the status of the worker in an area of HBV hyper-endemicity and thus prevents a false protection for an already infected worker.

The prescreening for both HBsAg and anti-HBc carried out in 1999 follows acceptable norm [2, 18, 19] especially in HBV hyper-endemic areas [1]. The use of either Engerix – or Genevac –B vaccine at any of the dose of the HBV vaccination schedule observed from the study follows previous reports [8-10, 20]; hence any available recombinant DNA technology type of HBV vaccines could be administered. This will enhance compliance of the subjects to the vaccination schedule.

Inspite of the efforts made at vaccinating the HCWs against HBV, the absence of assay of anti-HBs titre after the receipt of third dose of the vaccine left the vaccination programme uncompleted since the responses of the vaccinated subjects were undetermined and thus their immuned status are uncertain.

In conclusion, vaccination of HCWs has been implemented in a Nigerian tertiary hospital and efforts should be made and sustained at overcoming the shortcomings and obstacles at ensuring a full completion rate of a four-dose accelerated HBV vaccine schedule regimen (0, 1, 2 and 12 months), 100% prescreening of workers for HBV infection, vaccination of all non-immuned and HBsAg sero-negative workers as well as proper collection of data from all vaccinees. In addition, all categories of workers should be offered facilities for another form of accelerated HBV vaccine schedule (0, 1, 3 weeks 12 months) if they have to travel away within one month of being vaccinated and post vaccination anti-HBs titre for the determination of the protection level as well as administration of booster dose.

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ORIGINAL ARTICLE

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EVALUATION OF IMMUNITY AGAINST POLIOVIRUS SEROTYPES AMONG CHILDREN IN RIVERINE AREAS OF DELTA STATE, NIGERIA

Donbraye, E., Adewumi, M.O., Odaibo, G.N., Bakarey, A.S., Opaleye, O. O. and Olaleye, D.O.

Department of Virology, College of Medicine,

University of Ibadan, University College Hospital

Ibadan, Nigeria.

Correspondence: Professor D. O. Olaleye, Department of Virology, University College Hospital, Ibadan, Nigeria. Email: ibvirology@yahoo.com, foreodaibo@hotmail.com

ABSTRACT

Nigeria remains one of the major reservoirs for wild poliovirus transmission despite the reported success in National Immunization Days and acute flaccid paralysis surveillance. Two hundred children aged ≤ 10 years, were enrolled following parental consent from hard-to-reach riverine areas of Delta state of Nigeria to assess the level of protective immunity to poliovirus. Neutralizing antibodies to the three poliovirus serotypes in the serum samples of the children were determined by the beta method of neutralization.

Eight (4%) of the children had no detectable antibody, 178 (89%), 180 (90%) and 181 (90.5%) were positive for antibodies to poliovirus types 1, 2 and 3, respectively. Overall, 162 (81%) of the children had antibodies to the three poliovirus serotypes at a titre of at least 1:8. The study shows the need for proper monitoring of vaccination coverage in such hard-to-reach riverine areas to achieve the objective of the global eradication of poliovirus.

Keywords: Neutralizing, antibody, poliovirus, serotypes, riverine, Nigeria

INTRODUCTION

In May 1988, the World Health Assembly (WHA) resolved to eradicate Poliomyelitis globally by the year 2000 (1). The global eradication of polio involves both halting the incidence of the disease and the worldwide eradication of the virus. In the African Region (AFRO) of the World Health Organization (WHO), eradication strategies were accelerated following supporting resolutions by WHO's Regional Committee for Africa in 1995 and the Organization for African Unity in 1996. Remarkable progress has been made since the initiative began in 1988 (2).

However, there have been reports of less-than-optimal responses to trivalent OPV in developing countries. Patriarca *et al.* (3) reviewed data that accumulated in developing countries over 25 years and found 32 studies from 15 developing countries on the response of children to three doses of trivalent Sabin-derived OPV. After three doses of trivalent OPV, there was wide variation in the proportion of children seroconverting with rates of 73% for type 1, 90% for type 2, and 70% for type 3. Also, a large-scale randomized trial in Brazil and the Gambia has confirmed these findings. Even after eight OPV doses delivered during mass campaigns, gaps in immunity as defined by serum antibody levels, persist in some countries, especially for type 3 (4).

Although there has been improvement in the quality of surveillance for acute flaccid paralysis (AFP) and synchronized house-to-house NIDs conducted since late 2000 in Nigeria, the country still remains one of the most significant poliovirus reservoirs with 998 reported cases of acute flaccid paralysis out of which 101 were due to poliovirus infection as of early August, 2002. Consequently, children can develop poliomyelitis when infected by circulating wild polioviruses except they have sufficient levels of neutralizing antibodies against polioviruses to block the infection.

This study was therefore conducted to determine the proportion of infants with protective levels of serum neutralizing antibodies after at least two doses of OPV among children within the age at greatest risk of poliomyelitis in the riverine areas (known as hard-to-reach areas because of the difficult terrain) as a true biological marker of protection against the virus rather than using solely the vaccination coverage rate of the National Immunization Campaigns in Nigeria.

MATERIALS AND METHODS

SAMPLING METHOD

Random sampling method was used for subject selection in this study. Blood samples were collected from children aged one week to 10 years after due parental consent to participate in the study. Healthy vaccinated children were selected at random from designated health centres on

immunization days, schools and households. Subjects selected were from the riverine communities in two local government areas of Delta state of Nigeria (5).

Bomadi and Kpakiama communities in Bomadi; and Burutu, Ogulagha and Odimodi communities in Burutu local government area of the state (Figure1).

COLLECTION, TRANSPORT, PREPARATION AND STORAGE OF SAMPLES

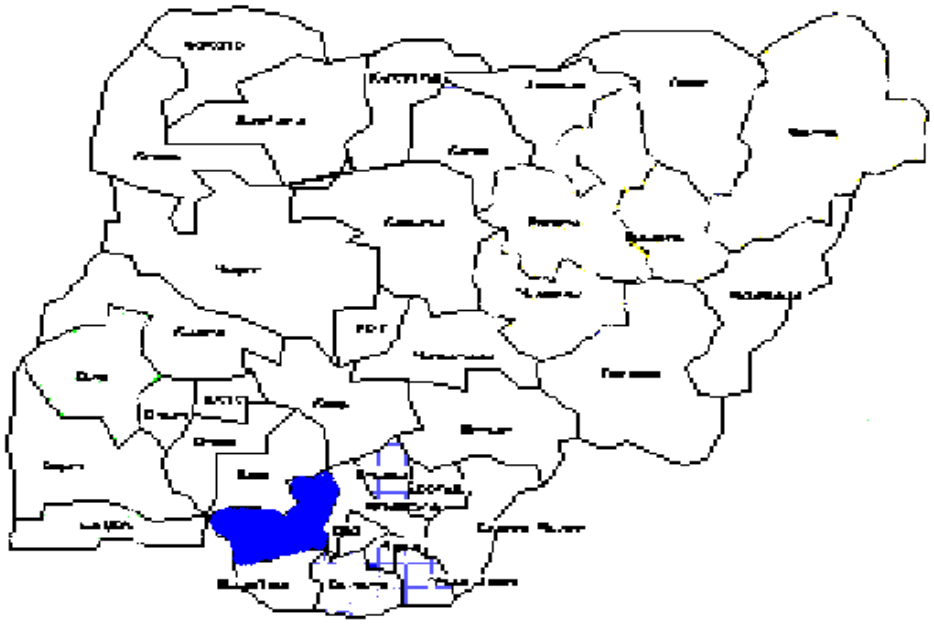
About 2ml of blood sample was collected by venepuncture from each child into a labelled sterile container free of anticoagulants or preservatives. Samples were transported to the laboratory immediately in a cold box with frozen ice packs to achieve a condition of about 4 to 8^{o}C. Serum samples were separated by low-speed centrifugation at 500g for 5 minutes, or direct

SITES AND SAMPLE SIZE

A total of 200 blood samples were collected from children aged one week to 10 years from two local government areas in Delta state, Nigeria (Bomadi = 80, Burutu =120). The samples were collected from removal of the serum using a sterile disposable pipette after retraction of the clot. The serum was transferred to two labelled sterile cryovials per sample and stored at -20^{o}C until ready for analysis.

The samples were inactivated at 56^{o}C in water bath prior to use for neutralization assay. Virus suspensions of the laboratory strain of the three poliovirus serotypes (Sabin strains) were prepared in L20B cell line. Challenge dose of 100 $TCID_{50}$ of poliovirus serotypes 1, 2 and 3 was determined and used for the neutralization test by the standard method of constant virus, varying serum dilutions microneutralization test as described by WHO (5).

FIGURE 1: MAP OF NIGERIA SHOWING THE STATE USED FOR THE STUDY



Delta State

RESULTS

All serum samples were collected from children 10 years of age and below. There were 97 males and 103 females. Overall, 178 (89%), 180 (90%) and 181 (90.5%) of the 200 blood samples tested had antibody titres of 8 or higher against poliovirus types 1, 2 and 3, respectively (Table 1). Also, 167 (83.5%), 160 (80%) and 158 (79%) had antibody titres of 32 or higher against poliovirus types 1, 2 and 3, respectively (Table 1). Moreover, 162 (81%) had antibody titres of 8 or higher against all three polioviruses, while 8 (4%) were completely seronegative to the three poliovirus serotypes, 2 (1%) had antibody titre of 8 or higher against P1 only, 2 (1%) had antibody titre of 8 or higher against P2 only and 1 (0.5%) had antibody titre of 8 against P3 only. The parents claimed to have received two or more doses of OPV for their children.

TABLE 1: NEUTRALIZING ANTIBODIES TO POLIOVIRUS SEROTYPES

ANTI BODY TITRE	P1	P2	P3
	NO. (%)	NO. (%)	NO. (%)
≥8	178(89.0)	180(90.0)	181(90.5)
≥32	167(83.5)	160(80.0)	158(79.0)

For the male children, 81 (83.5%), 82 (84.5%) and 83 (85.6%) had antibody titres of 8 or higher against poliovirus types 1, 2 and 3 respectively. However, among the female children, 97 (94.2%), 98 (95.1%) and 98 (95.1%) had antibody titres of 8 or higher against poliovirus types 1, 2 and 3 respectively (Figure 2).

DISCUSSION

Tests for serum neutralizing antibodies are considered to be the most specific for evaluation of the protective antibody response to poliovirus infections. Persons are presumed to be protected against disease caused by a particular type of poliovirus if they develop type-specific serum neutralizing antibodies to the virus. Antibody surveys are a more reliable method of assessing immunity than either statistics of vaccine uptake (or coverage) or sporadic cases or outbreaks of paralytic poliomyelitis which signals a dangerous decline of immunity due to complacent under vaccination or to technical flaws in the vaccination procedure.

This study showed the presence of neutralizing antibodies against poliovirus types 1, 2 and 3 in children from five riverine communities in two local government areas of Delta state, Nigeria. After claims to have received at least two doses of oral polio vaccine (OPV), by their parents, there was variation in the percentage of children seroconverting. Overall, 89%, 90% and 90.5% had antibody titre of 8 or higher against poliovirus types 1, 2 and 3, respectively. These results agree with reports from other countries where there have also been less than optimal responses to trivalent OPV (3, 6).

Eighty-one percent (81%) of the children had antibody to poliovirus types 1, 2 and 3 at a titre of 8 or higher. These leave non-immunized pockets of children who were seronegative to one or more poliovirus types. These non-immunized children, as against NPI reports of 108% immunization coverage in the second round of the 1999 NIDs, favour continued spread and outbreaks of poliovirus infection (2, 7). These gaps in immunity levels raise concerns of either primary vaccine failure, that is, lack of initial antibody responses where potent vaccines are used or, failure of the cold chain and the subsequent use of non-potent vaccines in the field.

Furthermore, the children had 83.5%, 80% and 70% seropositivity for poliovirus types 1, 2 and 3, respectively, for neutralizing antibody titre of 32 or above. Although the level of serum neutralizing antibody which protects against clinical illness has not been determined, studies have indicated that persons with low serum neutralizing antibody titre post immunization can be reinfected by wild virus or when challenged with vaccine virus (8, 9, 10). In animal experiments, passively administered antibody that provides moderate serum antibody levels (titre of 20 or higher) is likely to protect against clinical illness, but this cannot be compared with the natural situation where challenge with wild or vaccine strains occurs (11). These studies suggest that persons with low but detectable serum antibody levels are probably not in danger of developing clinical poliomyelitis. However, they may be reinfected with poliovirus and possibly become a source of infection for others who have not been vaccinated or have been vaccinated but seronegative.

In addition, it was observed that a higher percentage of the female children, compared to the males, were seropositive to poliovirus types 1, 2 and 3. On the other hand it was also noticed that seropositivity for neutralizing antibodies against poliovirus types 1, 2 and 3 is not age dependent. Seropositivity did not decrease with increasing age of children. This can be explained to be a consequence of antibody boosting resulting from continuous exposure to the virus in an endemic area (9).

The areas where the samples were collected from Delta state (Bomadi and Burutu) of Nigeria are riverine often described as hard-to-reach. In these

areas, households are crowded and mostly with poor hygiene. Members of a household defecate; take their baths, swim and sometime drink from the same water source (streams and rivers). Faeces of infected children serve as source of contamination of water and virus dissemination is facilitated by poor sanitation and overcrowding. Ashley *et al.* (12), in a survey in Jamaica obtained sera from more than 2500 children and adolescents in a population based study involving children aged up to 19 years and showed that among children 1-4 years that had never received vaccine 53%, 77% and 55% were seropositive for P1, P2 and P3 respectively, probably reflecting circulation of both wild and secondary vaccine virus. Circulation of poliovirus due to poor sanitation and existence of unhygienic environment conditions could be contributing factors to the spread of the virus (13).

CONCLUSION

The present study demonstrates 81% herd immunity for the three-poliovirus serotypes among children in five riverine communities in two local government areas of Delta state of Nigeria. Identification of some children without detectable antibodies to one or more poliovirus serotypes, in their sera, show that pockets of susceptible individuals are still present within the population. Such individuals favour the continued spread and circulation of the virus in the population. Therefore, increased vaccination coverage as a result of more frequent NIDs, and increased coverage by routine immunization in Nigeria, may reduce the number of such children and go a long way to ensure the eventual elimination of poliomyelitis and wild poliovirus from the country.

There is the need to re-evaluate the vaccination programmes and implementation of the global polio eradication policy in Nigeria to improve effectiveness and eventual eradication of polio from the country. Also there is the need for monitoring of the various stages of the programme and include vaccine potency testing and protective polio antibody testing to assess the level of immunity to poliovirus serotypes among children at risk of the virus infection.

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REVIEW ARTICLE

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MARBURG HAEMORRHAGIC FEVER: RECENT ADVANCES

Adegboro[1] B. and Adeola[2] O. A.

[1]Department of Medical Microbiology & Parasitology, Bingham University Teaching Hospital (ECWA Evangel Hospital), Jos and Bingham University, Karu, Nigeria
[2]Department of Virology, College of Medicine, University of Ibadan, Ibadan, Nigeria
[1]Correspondence: Boaz Adegboro.

ABSTRACT
Viral hemorrhagic fevers (VHFs) are a group of etiologically diverse viral diseases unified by common underlying pathophysiology. These febrile diseases result from infection by viruses from four viral families: Arenaviridae, Bunyaviridae, Filoviridae, and Flaviviridae. The viruses in the four families are all RNA viruses. All share the feature of having a lipid envelope. Survival and perpetuation of the viruses is dependent on an animal host known as a natural reservoir, but humans are not the natural reservoir. With the exception of a vaccine for yellow fever and ribavirin, which is used for treatment of some arenaviral infections, no specific chemotherapy for viral hemorrhagic fever exists. Only supportive treatment is possible The filoviruses, Marburg virus (MARV) and Ebola virus (EBOV), have been associated with hemorrhagic fever (HF) that produce severe disease and high mortality rates among infected humans and non-human primates. MARV and EBOV are also considered potential biological weapons. Although much progress has been made in developing preventive vaccines and postexposure interventions that can protect laboratory animals and nonhuman primates against lethal challenge with MARV, none of these has been approved for humans. Because MARV haemorrhagic fever, when it occurs, has the potential to spread to other people especially health care staff and family members who care for the patient, there is need for periodic review of recent developments relating especially to its diagnosis and treatment. This would help to increase awareness among health-care providers and limit the spread of the disease during outbreaks.

Keywords: Marburg virus, viral haemorrhagic fever, recent advances

Background

Viral hemorrhagic fevers (VHFs) are a group of etiologically diverse viral diseases unified by common underlying pathophysiology. These febrile diseases result from infection by viruses from four viral families: Arenaviridae, Bunyaviridae, Filoviridae, and Flaviviridae. The viruses in the four families are all RNA viruses. All share the feature of having a lipid envelope. Survival and perpetuation of the viruses is dependent on an animal host known as a natural reservoir, but humans are not the natural reservoir. With the exception of a vaccine for yellow fever and ribavirin, which is used for treatment of some arenaviral infections, no specific chemotherapy for viral hemorrhagic fever exists. Only supportive treatment is possible (1).

The filoviruses, Marburg virus (MARV) and Ebola virus (EBOV), have been associated with hemorrhagic fever (HF) that produce severe disease and high mortality rates among infected humans and non-human primates (2). MARV and EBOV are also considered potential biological weapons. Although much progress has been made in developing preventive vaccines and postexposure interventions that can protect laboratory animals and nonhuman

primates against lethal challenge with MARV, none of these has been approved for humans (3).

History

MARV was first identified during simultaneous outbreaks in 1967 when infected monkeys, imported from the Lake Kyoga region of Uganda, transmitted the virus to laboratory workers and scientists at facilities in Marburg and Frankfurt, Germany and Belgrade in the former Yugoslavia (3,4). The persons affected had contact with the blood or tissues of monkeys or with other infected persons. Other Marburg haemorrhagic fever epidemics which have occurred since then include one from October 1998 through September 2000 in Durba, Democratic Republic of the Congo (5). The outbreak involved 154 patients (48 confirmed and 106 suspected cases); the case fatality ratio was 83% (6).

In March 2005, the Centers for Disease Control and Prevention (CDC) investigated a large HF outbreak in Uige Province in northern Angola, West Africa. In total, 15 initial specimens were sent to CDC, Atlanta, for testing for viruses associated with viral HFs known to be present in West Africa, including Ebola virus. Marburg virus was also included despite the fact that the origins of all earlier outbreaks were linked directly to East Africa. Surprisingly, Marburg virus was confirmed (12 of 15 specimens) as the cause of the outbreak. The outbreak likely began in October 2004 and ended in July 2005, and it included 252 cases and 227 (90%)

fatalities (report from the Ministry of Health, Republic of Angola, 2005), making it the largest Marburg HF outbreak on record (7). Two smaller outbreaks occurred in 2007 and 2008 in Uganda and the Netherlands respectively. The outbreak in Uganda involved 2 cases, one fatal, in young males working in Lead and gold mine in Kamwenge District, Uganda. The latter case, which was fatal, involved a 40-year-old Dutch woman in the Netherlands with a recent history of travel to the Python Cave, Uganda (3). On February 9, 2009, it was reported that in January 2008, a US Citizen from Colorado was the first patient treated in the United States for Marburg. The patient had contracted the virus while overseas in Uganda and traveled back to the USA, where she was later treated successfully for the infection (8).

Aetiology

Marburg virus, or simply Marburg, is the common name for the genus Marburgvirus which contains one species: Lake Victoria marburgvirus. The virus causes the disease Marburg Hemorrhagic Fever (MHF), also referred to as Marburg Virus Disease, and previously also known as Green Monkey Disease due to its primate origin. Marburg originated in Central and East Africa, and infects both human and nonhuman primates. The Marburg Virus is in the same taxonomic family as Ebola, and both are identical structurally although they elicit different antibodies (8). The two viruses comprise the family Filoviridae, order Mononegavirales (Peters and Khan, 1999 9). MARV is a single species consisting of viruses differing from one another by up to 21% at the nucleotide level. For instance, during the epidemics which occurred from 1998 to 2000 in the Democratic Republic of Congo, at least nine genetically distinct lineages of the virus were in circulation (6).

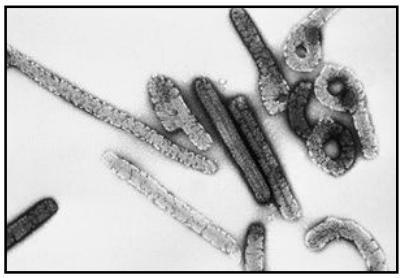

Plate 1: Marburg virus particles (Appro ximately 100,000x magnification). Adapted from species.wikimedia.org/wikifiloviridae

In contrast, four distinct species of ebolavirus (Zaire, Sudan, Reston, and Ivory Coast) have been defined, which differ genetically from one another by approximately 37 to 41% (9). The structure of MARV is typical of filoviruses, with long threadlike particles which have a consistent diameter but vary greatly in length from an average of 800 to 14,000 nanometers (nm), with peak infectious activity at about 790 nm (Plate 1). Marburg virus contains a single molecule of linear negative-sense, 19.1 kb single-stranded RNA whose seven gene products are, in order, nucleoprotein (NP), VP35, VP40, glycoprotein (GP), VP30, VP24, and the polymerase (L) (10).

Epidemiology and Ecology of Marburg Virus Haemorrhagic Fever

Outbreaks of Marburg are centered in Africa, where the natural reservoir is believed to be located. Historically, sources of MARV were confined to East Africa. They had been centered almost exclusively within 500 miles of Lake Victoria, with the exception of a single case in Zimbabwe in 1975, when a traveler became infected and seeking medical treatment, subsequently transmitted the virus to a health care worker in South Africa. This previous close association of MARV with East Africa contrasts with the observed distribution of EBOV,which has caused human HF outbreaks throughout tropical Africa, ranging from Coted'Ivoire to Uganda. However, a large MARV HF outbreak occurred in Uige Province in northern Angola, West Africa in 2005 (7).

MARV and EBOV HF outbreaks are generally thought to involve the relatively rare introduction of the virus into the human population followed by waves of human-to-human transmission (usually through close contact with infected individuals or their body fluids) (31). Although the environmental reservoir of MARV was previously unknown (CDC, 2005 11), in a study carried out to determine reservoir hosts for MARV in Democratic Republic of the Congo, the fauna of a mine which was associated with a protracted outbreak of Marburg hemorrhagic fever during 1998 to 2000 were examined and MARV nucleic acid was found in 12 bats, comprising of two species of insectivorous bat and one species of fruit bat. Antibodies to the virus were also detected in the serum of some of the insectivorous and fruit bat species, but attempts to isolate virus were unsuccessful (17). Pourrut *et al*.,2009 (30) also suggested, based on results of their studies, that the bat species *Rousettus aegyptiacus* may be involved in the natural cycle of both Marburg and Ebola viruses.

Transmission and Pathogenesis of Marburg Haemorrhagic Fever

Marburg virus is transmitted by direct contact with the blood, body fluids and tissues of infected persons. Transmission of the Marburg virus also occurred by handling ill or dead infected wild animals (monkeys, fruit bats) (3). After gaining access to the body, filoviruses initially infect monocytes, macrophages and other cells of the mononuclear phagocytic system (MPS), probably in regional lymph nodes. Some infected MPS cells migrate to other tissues, while virions released into

the lymph or bloodstream infect fixed and mobile macrophages in the liver, spleen and other tissues throughout the body. Virions released from these MPS cells proceed to infect neighboring cells, including hepatocytes, adrenal cortical cells and fibroblasts (12).
Infected MPS cells become activated and release large quantities of cytokines and chemokines, including TNF-, which increases the permeability of the endothelial lining of blood vessels. Endothelial cells apparently become infected by virus only in the later stages of disease. Circulating cytokines contribute to the development of disseminated intravascular coagulation (DIC) by inducing expression of endothelial cell-surface adhesion and procoagulant molecules and tissue destruction results in the exposure of collagen in the lining of blood vessels and the release of tissue factor (12). Massive lysis of lymphocytes occurs in the spleen, thymus and lymph nodes in the late stages of filovirus infection. There is no sign that the lymphocytes themselves are infected, rather they die through apoptosis, perhaps induced by cell-surface binding of chemical mediators released by MPS cells or by a viral protein. Massive cytolysis, immune dysfunction, fluid shifts, microvascular coagulation and interstitial hemorrhage all play a role in the development of shock and death (12).

Clinical Signs and Prognosis of Marburg Haemorrhagic Fever

Filovirus infections, in general, are the most severe of the viral hemorrhagic fevers. After an incubation period of 4 to 10 days, to a maximum of three weeks (13Jeffs, 2006), infected individuals abruptly develop flu-like symptoms characterized by fever, chills, malaise, and myalgia. Approximately the fifth day after onset of symptoms, a maculopapular rash might occur, after which patients usually develop other signs and symptoms that indicate systemic involvement, such as prostration and gastrointestinal (anorexia, nausea, vomiting, abdominal pain, and diarrhea), respiratory (chest pain, shortness of breath, and cough), vascular (conjunctival injection, postural hypotension, and edema), and neurological (headache, confusion, delirium and coma) manifestations (11).

The target organ in the VHF syndrome is the vascular bed; correspondingly, the dominant clinical features are usually a consequence of microvascular damage and changes in vascular permeability. Bleeding is manifested as petechiae, ecchymosis, uncontrolled oozing from venipuncture sites and gingiva, mucosal hemorrhages, and bloody diarrhea. In later stages, the general condition of patients deteriorates due to multiorgan failure, including disseminated intravascular coagulopathy, resulting in death (11,14). If a patient survives, recovery is usually prompt and complete, though it may be prolonged in some cases, with inflammation or secondary infection of various organs, including: orchitis, hepatitis, transverse myelitis, uveitis, and parotitis. Recovered patients often have little or no memory of being sick, though only 10-40% survive (16). Case fatality rates of Marburg haemorrhagic fever have varied greatly, from 25% in the initial laboratory-associated outbreak in 1967, to more than 80% in the Democratic Republic of Congo from 1998-2000, to even higher in the outbreak that began in Angola in late 2004 (3,11).

Prevention of Marburg Virus Infection

MARV is a biosafety level-four agent (BSL-4), and thus requires the highest level of precautions (18). While over-reaction on the part of medical personnel is inappropriate and detrimental to both patient and staff, it is prudent to provide isolation measures as rigorous as feasible. At a minimum, these should include the following: stringent barrier nursing; mask, gown, glove, and needle precautions; hazard-labeling of specimens submitted to the clinical laboratory; restricted access to the patient; and autoclaving or liberal disinfection of contaminated materials, using hypochlorite or phenolic disinfectants (MARV is susceptible to 1% sodium hypochlorite, 2% gluteraldehyde or formaldehyde, ultraviolet light and heat). For more intensive care, however, increased precautions are advisable. Members of the patient care team should be limited to a small number of selected, trained individuals, and special care should be directed toward eliminating all parenteral exposures. Use of endoscopy, respirators, arterial catheters, routine blood sampling, and extensive laboratory analysis increase opportunities for aerosol dissemination of infectious blood and body fluids.

A few research groups are working on vaccines to fight the virus. In 1998, a group at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) published the first peer reviewed article detailing the development of the first experimental Marburg virus vaccine demonstrated to completely protect animals from lethal Marburg virus infection (19Hevey *et al.*, 1998). Following this, in 2002, Genphar, a company doing research for the United States Army's biodefense program, announced that an experimental vaccine protected animals from a high dose of Marburg virus. The tests were conducted by USAMRIID. According to the company, all animals in the control group died within days whereas all animals that received the regular dosage of the vaccine were fully protected (8).

Post-exposure Treatment of Marburg Virus Infection

There is no specific antiviral therapy indicated for treating Marburg, and hospital care is usually supportive in nature. Hypotension and shock may

require early administration of vasopressors and haemodynamic monitoring with attention to fluid and electrolyte balance, circulatory volume, and blood pressure. Viral haemorrhagic fever (VHF) patients tend to respond poorly to fluid infusions and may develop pulmonary edema. (3,8). However, several attempts have been made to develop postexposure interventions against the filoviruses. Some degree of success has been achieved by using strategies that mitigate the coagulation abnormalities characterizing filoviral infection (20,28). Also, new postexposure treatment approaches, based on small interfering RNA (21) and antisense oligomers (22), have shown promising results in rodent models, but no reports have been published of evaluations of either strategy in the more stringent macaque models.

In 2006, the first complete postexposure protection of nonhuman primates against a filovirus was reported. This was done by administering a live-attenuated recombinant vesicular stomatitis virus (rVSV) vaccine vector expressing the MBGV glycoprotein (GP) (VSVΔG MBGV GP) shortly after a high-dose MBGV challenge (27). In a follow-up study to the one above, rhesus monkeys were protected from MARV disease when a recombinant vesicular stomatitis virus–based vaccine was administered 20 to 30 minutes after infection with Marburg virus. Five out of six (5/6) monkeys were protected when this vaccine was given 24 h after challenge, while 2/6 animals were protected when the vaccine was administered 48 h postinfection (22).

More recently, results obtained from studies conducted by the U.S. Army Medical Research Institute of Infectious Diseases in collaboration with AVI BioPharma, a Washington-based biotechnology firm, have remained very promising for post-exposure treatment of MARV infection. Their studies show that novel antisense therapies targeting specific viral genes protected monkeys infected with deadly Ebola or Marburg viruses, even when therapeutics were administered one hour after exposure—suggesting the approach holds promise for treating accidental infections in laboratory or hospital settings (23).

Diagnosis of Marburg Virus Infection

It should be kept in mind that the diagnosis of MARV infections will initially have to be based on clinical assessment (24). Clinicians should consider the diagnosis of Marburg VHF among febrile patients who, within 10 days before onset of fever, have either 1) traveled in northern Angola; 2) had direct contact with blood, other body fluids, secretions, or excretions of a person or animal suspected of having VHF; or 3) worked in a laboratory or animal facility that handles hemorrhagic fever viruses. The likelihood of acquiring VHF is considered extremely low in persons who do not meet any of these criteria. The cause of fever in persons who have traveled to areas where VHF is endemic is more likely to be a different infectious disease (11). When the identity of a VHF agent is totally unknown, isolation in cell culture and direct visualization by electron microscopy, followed by immunological identification by immunohistochemical techniques is often successful. Immunohistochemical techniques are also useful for retrospective diagnosis using formalin-fixed tissues, where viral antigens can be detected and identified using batteries of specific immune sera and monoclonal antibodies (24).

Formal laboratory diagnosis requires a laboratory with special containment facilities (BL-4 containment) (24). Antigen-capture enzyme-linked immunosorbent assay (ELISA) testing, IgM-capture ELISA, polymerase chain reaction (PCR), and virus isolation can be used to confirm a case of Marburg hemorrhagic fever within a few days of the onset of symptoms. The IgG-capture ELISA is appropriate for testing persons later in the course of disease or after recovery. The disease is readily diagnosed by immunohistochemistry, virus isolation, or PCR of blood or tissue specimens from deceased patients (13, 14). MARV grows well in a large variety of cell lines, although Vero or Vero E6 cells have been most used. The virus is relatively stable and may survive unfavorable handling and shipping (15 Sanchez *et al.*, 2001). Diagnosis by viral cultivation and identification requires 3 to 10 days. However, viral isolation should not be attempted without BL-4 containment (24).

Weaponization and Bioterrorism

The viral hemorrhagic fever (VHF) agents, including MARV, are all highly infectious via the aerosol route, and most are quite stable as respirable aerosols. This means that they satisfy at least one criterion for being weaponized, and some clearly have the potential to be biological warfare threats (24). The former Soviet Union reportedly had a large biological weapons program involving Marburg. They developed a new strain, called "Variant U," which was successfully weaponized and approved by Soviet Ministry of Defense in 1990 (25). Bioterrorism grants in the United States are funding research to develop a vaccine for Marburg virus (8).

Conclusion

Marburg hemorrhagic fever is a very rare human disease. However, when it occurs, it has the potential to spread to other people, especially health care staff and family members who care for the patient. Increasing awareness, among health-care providers, of clinical symptoms in patients that suggest Marburg hemorrhagic fever is therefore

critical for limiting the spread of the disease during outbreaks.

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DIPHTHERIA MORTALITY IN NIGERIA: THE NEED TO STOCK DIPHTHERIA ANTITOXIN

***SADOH, A. E. & Sadoh, W. E. Department of Child Health, University of Benin Teaching Hospital, Benin City, Nigeria.**

***Correspondence: Phone no: +2348033435312 Email: ayebosadoh@yahoo.com**

ABSTRACT
INTRODUCTION: Diphtheria had been a major of cause of childhood mortality until the advent of an effective vaccine. Even in Nigeria with low to moderate coverage with the third dose of DPT the number of reported cases of diphtheria had been reducing. However, in a recent report we noted an increase in the incidence of diphtheria. The mainstay of management of diphtheria is the Diphtheria antitoxin. Diphtheria antitoxin is not available in Nigeria. We present the mortalities from diphtheria to highlight the need to stock the diphtheria antitoxin.
METHODOLOGY: A review of the case notes of patients managed for diphtheria between August 2008 and 2010 was done and relevant data extracted.
RESULTS: Nine cases of diphtheria were seen and three mortalities were recorded giving a mortality rate of 33.3%. One of the deaths was from myocardial involvement and acute renal failure while another was from possible septicaemia. The third mortality occurred at home after discharge from hospital
DISCUSSION: Most of the symptoms of diphtheria are due to the effects of the diphtheria exotoxin. The non availability of the antitoxin for the management of these children contributed to their mortality.
CONCLUSION: As long as diphtheria remains uneradicated the need for the antitoxin is imperative.

INTRODUCTION

Diphtheria, a disease caused by *Corynebacterium diphtheria* and its exotoxin is characterised by high case fatality (1-3). Diphtheria commonly affects the tonsils, pharynx and larynx. The C. diphtheria remains in the superficial mucosa or skin and elaborates its exotoxin. The diphtheria exotoxin, a potent 62 kd polypeptide inhibits protein synthesis leading to local tissue necrosis (2). The exotoxin is absorbed into the mucous membranes and causes destruction of epithelium and a superficial inflammatory response (3). The necrotic epithelium becomes embedded in exuding fibrin and red and white cells, resulting in a dense necrotic coagulum of organisms, epithelial cells, fibrin, leukocytes, and erythrocytes (3,4). This advances – commonly over the tonsils, pharynx, or larynx, and becomes a gray-brown, leather-like adherent **pseudomembrane** (*Diphtheria* is Greek for leather). With increase in the concentration of toxin, it is spread to other tissues through haematogenous dissemination (2).

Laryngeal involvement, which may occur on its own or as a result of membrane extension from the nasopharynx, presents as hoarseness, stridor, croupy cough and dyspnea (3). These patients are at significant risk for suffocation because of local soft tissue edema and airway obstruction by the diphtheritic membrane (3).
There may be toxin-mediated paralysis of soft palate, posterior oropharynx and hypopharynx (4). Although the toxin has no target organs the myocardium and peripheral nerves are most affected.[2]Other toxin mediated complications of diphtheria are toxic cardiomyopathy which occurs in 10–25% of patients with respiratory diphtheria and is responsible for 50–60% of deaths (3). Neurotoxicity and renal damage can also occur. Some of these features may present up to six weeks after the onset of the illness suggesting an immunological basis for the pathophysiologic mechanism for these delayed features of diphtheria (2).

The mainstay of management of diphtheria is the antitoxin. WHO (5) recommends immediate administration of diphtheria antitoxin and antibiotics following clinical diagnosis. The diphtheria vaccine is one of the major approaches for the control and prevention of diphtheria. In the pre-vaccine era diphtheria was common place with annual reported cases of 125,000 and 10,000 annual deaths from diphtheria being reported in the United States of America (3). With the advent of an effective vaccine against diphtheria and following the introduction of mass immunization the incidence of diphtheria fell to such levels that at the beginning of the 1980s many countries in the world were progressing toward the elimination of diphtheria (6). It in fact became a medical rarity (3). This was until the striking resurgence of diphtheria in the Newly Independent States (6).

The numbers of reported cases of diphtheria in Nigeria have been declining. Reported cases from Nigeria were 5,039 in 1989, 3,995 in 2000, 2,468 in 2001, 790 in 2002 and 312 in 2006 (7). Immunization coverage with three doses of DPT in Nigeria has been inconsistent reaching an all time low in 2003 (8). Current DPT 3 coverage in Nigeria is 72% (7). There are few reports on clinical diphtheria in Nigeria but most of these are old reports corroborating the possible declining prevalence of diphtheria in Nigeria. However, we reported 5 cases over a one year period in 2009 with 40% mortality and speculated on the possible resurgence of the disease (9).

In this report we note the continued presence of cases suggesting the possible veracity of resurgence of the disease in Nigeria. The focus of this report however, are the mortalities recorded among the cases of diphtheria between 2008 and 2010 with a discussion on the need to stock the diphtheria antitoxin which is the mainstay of management of diphtheria.

MATERIALS AND METHODS

Records of patients managed for diphtheria over the period 2008 and 2010 in the University of Benin Teaching Hospital, Benin City were reviewed. In all cases diagnosis was clinical and confirmed microbiologically. The relevant clinical data were extracted. Each child received intravenous crystalline penicillin once the diagnosis was made or strongly suspected. Resection of the pseudomembrane was done in two children both of whom eventually required tracheostomy. No child received the diphtheria antitoxin as this is not available in Nigeria. The public health unit investigated each case and reported the cases to the appropriate authorities. Family and close contacts were treated with erythromycin while unimmunized children were immunized

RESULTS

Nine patients were admitted over the two year period. Their ages ranged 11 months to 10 years with a mean of 5.8±3.5 years. There were 4(44.4%) males and 5(55.6%) females. Three mortalities were recorded giving a mortality rate of 33.3%. The three deaths were one male infant (11months old) and two females aged 8 and 10 years. All three mortalities presented with fever, dysphagia/drooling of saliva and enlarged cervical lymph nodes. Other clinical features are as shown in table 1.

Case A in addition to the features in table 1 had had measles two weeks prior to the onset of pharyngotonsillar symptoms. In fact he did not recover from the measles as he continued to have fever before the onset of the pharyngotonsillar symptoms. He also had visited a traditional healer who had made scarifications on his abdomen. Although he had been seen earlier in the General Practice Clinic of the hospital diphtheria was not suspected until he presented in the children's emergency room with symptoms of pharyngotonsillar disease.

TABLE 1: CLINICAL FEATURES OF CHILDREN WHO DIED FROM DIPHTHERIA

Clinical features	Individual cases of diphtheria		
	a	b	c
age	11mth	8yrs	10yrs
sex	male	female	female
fever	+	+	+
cough	+	-	+
nasal discharge	+	+	-
nausea/vomiting	+	-	-
diarrhea	+	-	-
dysphagia/drooling/ inability to suck	+	+	+
*duration of symptoms (in days)	2	3	4
neck swelling/enlarged lymph nodes	+	+	+
enlarged tonsils	+	+	-
membrane on tonsils	-	+	+
im status	dpt2	?complete	complete
contact**	-	-	-
mother's loe	pry	?	pry

+ attribute present - attribute absent
im – immunization status * symptoms of pharyngtonsilar disease
****contact with a case of diphtheria loe – level of education**

pry – primary dpt – diphtheria, petussis, tetanus vaccine

Even so the initial diagnosis was septicaemia. He developed severe anaemia for which he was transfused. Thereafter he started bleeding from the nostrils and rapidly deteriorated and died.

Case B improved following treatment and was discharged home after a week on admission. She developed nasal speech and regurgitation of water one week after discharge and died at home four days later

Case C in addition to the features in table 1 developed bradycardia and hypotension two days after admission. Electrocardiogram showed abnormalities of the ST segment. She was adjudged to have toxic cardiomyopathy and managed with fluids (including blood transfusion) and dopamine infusion. She subsequently developed acute renal failure with pulmonary oedema and died

DISCUSSION

The major features of morbidity resulting from diphtheria are from the exotoxin and once bound is not amenable to treatment. It is thus imperative that antitoxin is available for the management of cases of diphtheria. In fact, it is recommended that antitoxin be administered once diphtheria is suspected(3, 10). Worsening outcomes have been associated with

delays in administration of the antitoxin (2). The antitoxin has been recommended to be given at an empirical dose based on the degree of toxicity, site and size of the membrane, and duration of illness (2). Similar doses are given to adults and children (3). The intravenous route is preferred (2).

Diphtheria is associated with high mortality and one of the prognostic factors is the speed with which the antitoxin is administered (2). For those in whom the disease is recognized on the first day and appropriate treatment instituted mortality is 1% but those in whom such treatment is delayed till the fourth day mortality rises to 20%.[2]The high mortality rate recorded in this series may have been due to the lack of antitoxin. The high mortality recorded in this series is similar to that reported from India in which a case fatality rate of 30.8% was recorded during an outbreak of diphtheria in which none of the patients benefited from antitoxin since none was available (11).
The role of antimicrobial therapy in diphtheria is to halt toxin production, treat localized infection and prevent transmission of the organism to contacts (2, 3). Delay in making the diagnosis occasioned by late presentation of patients and a low index of suspicion in the first mortality in this series may also have contributed to the high mortality recorded. Delays allow time for multiplication of the organism and elaboration of toxins before the administration of effective antibiotics. Bound toxin is not amenable to treatment and in this series even the unbound toxin could not have been treated as there was no antitoxin. In a series reported from Thailand, early recognition and prompt treatment reportedly decreased complications and mortality (12).

Early recognition of diphtheria is dependent on a high index of suspicion of the attending physician as there are other common causes of pharyngotonsillar disease such as exudative pharyngitis due to streptococcus pyogenes and Epstein-Barr virus, Vincent's angina, bacterial epiglottitis, severe laryngotracheobronchitis, staphylococcal or streptococcal tracheitis.[3] Since diphtheria had hitherto become uncommon it is necessary for practicing medical personnel to be reminded of diphtheria through continuing medical education programmes. Caregivers also need to be sensitized concerning diphtheria to improve care seeking behaviour.

Rarely diphtheria may result in septicaemia with uniformly fatal outcome (2). While this is a possibility in the first mortality it was not confirmed by a positive blood culture. The possibility of septicaemia in this child is also likely given the fact that he had suffered from measles which is known to impair immunity and the causative organism may not necessarily be Corynebacterium diphtheria since he had scarification marks which may have served as portal of entry for other organisms.

Some of the complications of diphtheria can occur as late as six weeks after the illness (2, 3). Thus in the second case death occurred after the child had been discharged home in the third week of the illness. Although the history suggested that the child had neurological complications as evidenced by the nasal speech and the regurgitation of water, it is possible that the child may have had other more life threatening complications such as myocarditis. Complete recovery is often the expected outcome for neurological complications of diphtheria however in a series from India fatal aspiration led to mortality in a minority of cases with palatal paralysis (13). In the same series poor outcome was associated with delay in instituting diphtheria antitoxin. Since a post mortem examination was not done in the current case it is not possible to state the definitive cause of death. It can however be speculated that if the child had received antitoxin the outcome may have been different.

Early complication of the illness with myocardial disease has been associated with high mortality as was the case with the third mortality (3). The additional complication of acute renal failure may have been due to the effect of the toxin. It could also have resulted from the repeated episodes of cardiogenic shock with resultant hypoperfusion of the kidneys.

In developed countries such as the United States where diphtheria is all but a medical rarity the antitoxin is still stocked at Centre for Disease Control (CDC) and can be obtained within 24hours. While prevention by effective vaccination remains the best option, until the disease is eradicated the antitoxin should be available.

Two of the three mortalities occurred in older children one of whom is said to have had complete immunization in infancy. It has been documented that the level of immunity wanes with time such that booster doses of DPT are required (14). This becomes more pertinent in the face of improving social environments with fewer opportunities for cutaneous diphtheria which is believed to serve as boosters in developing countries. Research is needed to determine the levels of immunity in Nigerian children to guide policy decisions on the need for booster doses for diphtheria.Of importance is the need to strengthen the immunization programme. One of the mortalities had received only two doses of DPT and had not received measles vaccine despite being 11months old. He suffered measles prior to developing diphtheria a classic case of "double jeopardy"

In conclusion Diphtheria would seem to be increasing in incidence in Nigeria. There is therefore a need to strengthen the immunization services as a means of prevention. However, as long as Diphtheria is not eradicated Diphtheria antitoxin should be available to treat clinical cases of diphtheria so as to reduce case fatality rates. The presence of diphtheria in any given community remains a potential risk for the rest of the world since the world has become a global village.

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ORIGINAL ARTICLE

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BACTERIOLOGICAL ANALYSIS OF WELL WATER SAMPLES IN SAGAMU.

A.O Idowu*, B.B Oluremi, K.M Odubawo

Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Olabisi Onabanjo University, Ago-Iwoye, Nigeria.
***Correspondence. E-mail: Solaid2002@yahoo.com. Tel: 08027348864, 07042261824**

ABSTRACTS:
Majority of the population in semi-urban and urban areas of Nigeria depend on wells as their source of water supply. Due to increasing cases of water-borne diseases in recent times, this study was carried out to examine the microbial quality of well water in Sagamu, Nigeria as a way of safeguarding public health against water borne diseases. Water samples from a total of forty wells, covered and uncovered from four different locations of varying socio economic and demographic status were assessed for their bacteriological quality using serial dilution to obtain total bacteria count and the multiple tube fermentation technique to determine the coliform count using the most probable number method. Positive tubes of the presumptive test were further cultured on appropriate solid media. The organisms isolated were further characterized using standard procedures. The result of the study revealed that all the wells were grossly contaminated with bacteria pathogens such as *Klebsiella spp* (95%), *Escherichia coli* (72%) and *Salmonella typhi* (32.5%). Comparatively, the uncovered wells were more highly contaminated with bacteria pathogens than the covered well especially in the highly populated areas. All the water samples exceeded the standard limit of the most probable number (MPN) per 100ml set for untreated drinking water. This result highlight the fact that most well water in Sagamu metropolis are not safe microbiologically for drinking without additional treatment such as boiling or disinfection and this could lead to outbreak of water borne diseases. Good and proper environmental and personal hygiene must be maintained especially by the users of those wells to prevent their contamination with bacterial pathogens.

Keywords: Well water, bacteriological analysis, bacterial pathogens, water borne, diseases

INTRODUCTION

Water of good drinking quality is of basic importance to human physiology as well as indispensable to man's continued existence [1]. Its role as a medium of water borne disease which constitutes a significant percentage of the diseases that affect human and animals cannot be underestimated. This is the most important concern about the quality of water. Guideline for bacteriological water differs from country to country but they all conform to WHO recommendation. The standards for drinking water are more stringent than those for recreational waters. Availability of facilities and financial constraints are the major obstacles in the provision of water of good quality in developing countries and rural areas. In Nigeria, treated pipe borne water is limited to urban areas and the quantity provided inadequate and the frequency of supply epileptic. Such services may not even be available in certain areas within the metropolis. Due to this scenario, an increasing no of people in semi urban and urban areas in Nigeria including Sagamu depends on wells as their source of water supply. There has been increasing cases of food and water borne diseases in many parts of the country particularly typhoid fever and Cholera. There is a need therefore to have water supply surveillance as a way of keeping a careful watch at all times from the public health point of view, over the safety and acceptability of drinking water supplies [2]. In line with this, this study was conducted to investigate the bacterial content of well water that serve as the major source of drinking water in Sagamu, a semi urban area in Nigeria.

MATERIALS AND METHODS

(A) STUDY AREA AND SAMPLING

Water samples from forty wells from four different locations of varying socioeconomic and demographic status were randomly collected for bacteriological analysis. The areas are Isale Oko (Location W) Hospital Road (Location X), Station/GRA (Location Y) and Sabo (Location Z). Well water constitutes the major source of drinking water in these areas. Most of the wells under study were privately owned and are usually open to general public. Half of the numbers of the studied wells were covered while the others were not. Drawing of water from these wells was done by the use of 5-7 litre containers, which is tied directly to the well cover. In certain cases where this is not possible, individual fetcher usually comes with small bucket to draw water. The wells are not less than 10years old;

some were constructed with concrete and are on average five meters deep.

(B) COLLECTION OF WATER SAMPLES

Water samples were collected in sterile bottle tied with a strong string to a piece of metal (about 500g) as the weight. The bottle cap was aseptically removed and the weighted bottle lowered into the well to a depth of about 1-2 meters. The bottle was brought up to a surface and covered with a screw cap when no air bubbles were seen inside.

(C) BACTERIOLOGY

Total bacterial count:- For each water sample, 1ml of a (1:100) dilution was inoculated into 19ml of molten nutrient agar, properly mixed and poured into a sterile Petri dish. The agar was allowed to set, and then incubated at 37^0C for 24hours. The formed colonies were counted and result expressed as cfu/ml.

PRESUMPTIVE COLIFORM TEST

The multiple tube fermentation technique as described by Collins and Lynne [3] and Mackie and Mc cartney [4] was used. In this method, varying amounts of water sample were added to double and single strength Mac Conkey broth in bottles containing inverted sterile Durham tubes as follows.

- 1 x 50ml of water to 50ml double strength medium
- 5 x 10ml of water to 10ml double strength medium
- 5 x 1ml of water to 5ml single strength medium

The bottles were incubated aerobically at 37^0C for 18-24hours after which they were examined for production of acid and gas. Sterile distilled water was used as a control for each test batch.

Presumptive coliform count was obtained by the most probable number (MPN) of coliform per 100ml of water sample by making reference to the Mc Crady's probability table after combination of various positive and negative results

(D) IDENTIFICATION OF ISOLATES

Positive tubes of the presumptive test were subcultured on Mc Conkey agar for the enumeration of *Escherichia coli* and other enteric coliforms. *Salmonella typhi* and *Shigella spp* were enumerated using *Salmonella Shigella* agar and Triple Sugar Iron agar (TSI). All the inoculated media were incubated aerobically at 37^0C for 24 hours, after which the isolates were further characterized by a combination of colonial and morphological characteristic on solid media as well as standard biochemical tests as described by Cowan and Steel [5]

RESULTS:

The total viable bacterial counts obtained are shown in Table 1. The value obtained from the well water samples ranged from 0.1 – 6.5 x 10^4 cfu/ml. The total viable bacterial counts obtained from uncovered wells 1.1 – 6.5 x 10^4 cfu/ml were higher than the values obtained from covered well (0.1 – 2.8 x 10^4 cfu/ml). The values of the total viable bacterial count were lower in the 2 categories of wells studied in location Y than in the other three locations.

Table 1: TOTAL BACTERIA VIABLE COUNT FROM WELL WATER SAMPLES IN SAGAMU

	TOTAL BACTERIAL VIABLE COUNTS CFU/MLX10^4			
SAMPLES	**W**	**X**	**Y**	**Z**
C1	**2.0**	**1.2**	**0.4**	**1.6**
C2	**1.9**	**0.9**	**0.2**	**0.8**
C3	**2.4**	**1.6**	**0.6**	**1.3**
C4	**1.7**	**1.9**	**0.1**	**1.2**
C5	**2.8**	**1.4**	**0.3**	**2.1**
U1	**3.5**	**2.8**	**2.1**	**5.2**
U2	**2.9**	**2.1**	**1.9**	**2.5**
U3	**4.3**	**3.3**	**1.3**	**4.7**
U4	**5.2**	**2.2**	**1.6**	**4.2**
U5	**3.2**	**4.2**	**1.1**	**6.5**

Table 2: PRESUMPTIVE COLIFORM COUNT FROM WELL WATER SAMPLE IN SAGAMU

	Location	W				X				Y				Z			
	Quantity of water	50ml	10ml	1ml	MPN	50ml	10ml	1ml	MPN	50ml	10ml	1ml	MPN	50ml	10ml	1ml	MPN
Samples	Numbers of sample of each quantity tested	1	5	5		1	5	5		1	5	5		1	5	5	
C1	NUMBER GIVING POSITIVE REACTION (ACID AND GAS)	1	5	4	160	1	5	4	160	1	4	3	30	1	5	4	160
C2		1	5	3	90	1	5	2	50	1	4	5	40	1	5	3	90
C3		1	5	2	50	1	5	3	90	1	5	2	50	1	5	4	160
C4		1	5	4	160	1	4	5	40	1	4	4	35	1	5	4	160
C5		1	5	3	90	1	5	3	90	1	5	3	90	1	5	3	90
U1		1	5	5	180^{+}	1	5	5	180^{+}	1	5	4	160	1	5	5	180^{+}
U2		1	5	5	180^{+}	1	5	4	160	1	5	4	160	1	5	5	180^{+}
U3		1	5	5	180^{+}	1	5	5	180^{+}	1	5	4	160	1	5	5	180^{+}
U4		1	5	5	180^{+}	1	5	4	160	1	5	5	180^{+}	1	5	5	180^{+}
U5		1	5	5	180^{+}	1	5	5	180^{+}	1	5	4	160	1	5	5	180^{+}

Table 3: DISTRIBUTION OF BACTERIAL PATHOGEN ISOLATED FROM WATER SAMPLES IN SAGAMU

		IDENTIFIED ISOLATES			
SAMPLES	LOCATION	W	X	Y	Z
C1		*Escherichia coli, Klebsiella spp*	*Klebsiella spp*	*Klebsiella spp*	*Escherichia coli, Klebsiella spp*
C2		*Escherichia coli, Klebsiella spp*	*Klebsiella spp*	*Klebsiella spp*	*Escherichia coli, Klebsiella spp*
C3		*Escherichia coli, Klebsiella spp*	*Escherichia coli, Klebsiella spp*	*Klebsiella spp*	*Escherichia coli, Klebsiella spp*
C4		*Escherichia coli, Klebsiella spp*	*Escherichia coli, Klebsiella spp*	*Klebsiella spp*	*Escherichia coli, Klebsiella spp*
C5		*Escherichia coli, Klebsiella spp*	*Escherichia coli, Klebsiella spp*	*Klebsiella spp*	*Escherichia coli, Klebsiella spp*
U1		*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Escherichia coli, Klebsiella spp*	*Escherichia coli, Klebsiella spp, Salmonella typhi*
U2		*Escherichia coli, Klebsiella spp*	*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Escherichia coli*	*Escherichia coli, Klebsiella spp, Salmonella typhi*
U3		*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Klebsiella spp*	*Escherichia coli, Klebsiella spp, Salmonella typhi*
U4		*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Escherichia coli*	*Escherichia coli, Klebsiella spp, Salmonella typhi*
U5		*Escherichia coli, Klebsiella spp*	*Escherichia coli, Klebsiella spp, Salmonella typhi*	*Klebsiella spp*	*Escherichia coli, Klebsiella spp, Salmonella typhi*

For tables 1-3
Key: C1 – C5 are water samples from covered wells
U1 – U5 are water samples from uncovered wells
Location W- Isale Oko area
Location X – Hospital road area
Location Y – Station/GRA area
Location Z – Sabo area

The presumptive coliform count measured by the most probable no per 100ml in the multiple tube fermentation technique of bacterial enumeration from the covered and uncovered well water samples from the different locations are indicated in Table 2. The most probable number MPN per 100ml for the well water sample ranged between 20 and 180^{+} which clearly exceeded the standard limit set by the World Health Organization (WHO). Table 3 showed bacterial pathogens isolated from well water from different locations. Thirty five (87.5%) well water samples from the tested locations were with two or more species of bacterial pathogen.

FIGURE 1: BAR CHART SHOWING THE DEGREE OF CONTAMINATION OF WATER SAMPLES.

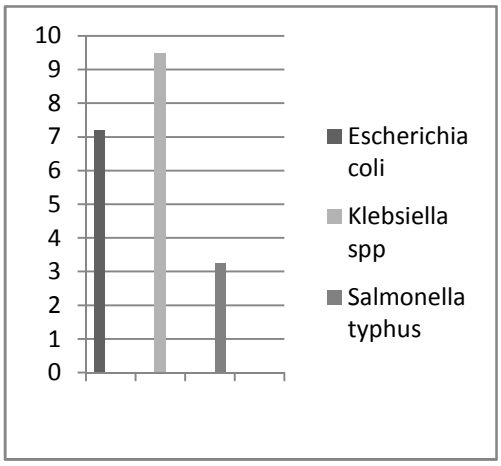


FIGURE 2: BAR CHART SHOWING DISTRIBUTION OF ORGANISMS AT VARIOUS LOCATIONS

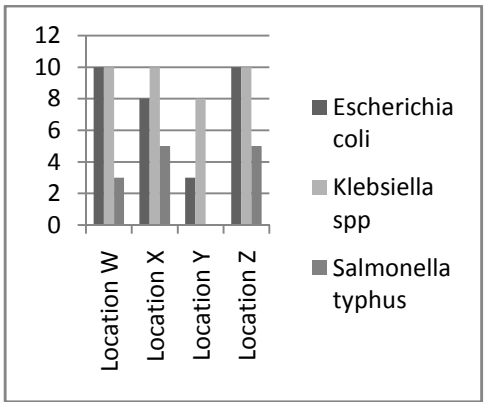


FIGURE 3: BAR CHART SHOWING DISTRIBUTION OF ORGANISMS FROM COVERED WELL AT DIFFERENT LOCATIONS

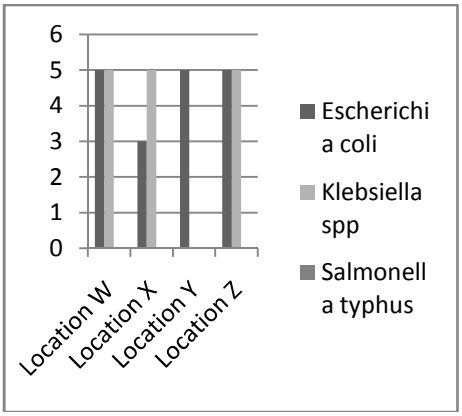
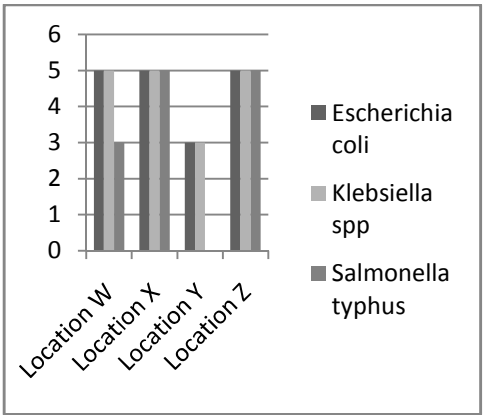


FIGURE 4: BAR CHART SHOWING DISTRIBUTION OF ORGANISMS FROM UNCOVERED WELLS AT DIFFERENT LOCATIONS



Klebsiella spp was the most predominant (95%) in the well water samples, followed by *Escherichia coli* (72%) and *Salmonella typhi* (32.5%) fig 1. The distribution of organism at the different locations varies as shown in Fig 2. *Salmonella typhi* was absent in water samples from location Y. The distribution of organisms in covered and uncovered wells samples (Fig 3 and Fig 4) showed that *Salmonella typhi* was only encountered in samples from uncovered wells.

DISCUSSION

The spread of diseases through faecal contamination of water sources particularly in developing and underdeveloped countries are a common phenomenon that has been well reported [2, 6, 8, 9]. In Ogun state as well as most part of Nigeria, availability of treated pipe-borne water is rare and restricted where it exist to urban and semi-urban areas. Even in these areas, public water supply is quite irregular. Consequently rich individuals dig boreholes as alternative water sources. The poor or average classes which constitute more than 80% of the population which cannot afford the high cost of borehole drilling are forced to dig wells as alternative source of water supply for drinking and sanitary purpose.

The viable bacterial count which is a measure of the microbial load of the water sample obtained in this study (0.1 – 6.5 x 10^4 cru/ml) exceeded the recommended limit. This shows that the wells contain too high level of microbial contaminant that make water obtained from them a threat to public health.

The values obtained for the samples from the uncovered well were higher than the ones from

the covered well. This is expected possibly due to the openness of the wells which permit unhindered access of particles from the surroundings compared to the covered wells.

It was observed that location Y is a low population density area inhabited by people of high socioeconomic status where there might be better sanitation practices. Both categories of wells from this location recorded a lower microbial count than the other three locations. This indicates that better environmental condition as well as reduce pressure of use may reduce the degree of contamination that may be encountered in well water samples. However, the sanitary quality of potable water is determined primarily by the kinds of micro-organisms present rather than by the microbial count per see [10]

The most probable number (MPN) per 100ml obtained for the well water samples ($20\text{-}180^{+}$) clearly exceeded standard limit set by WHO. This suggest that the well water samples have been contaminated by potentially dangerous microorganism and is therefore not fit for drinking purposes. This was confirmed by the characterization of the isolates from the well water samples from the locations under study which were highly contaminated with one or more bacterial pathogens namely *Escherichia coli*, *Klebsiella spp* and *Salmonella typhi*. The most predominant is the enteric coli form *Klebsiella spp* (95%) followed by *Escherichia coli (*87.5%) and *Salmonella typhi* (32.5%).

These are pathogenic organisms mainly of faecal origin. Any water source used for drinking or cleaning purpose should not contain any organism of faecal origin [10,12]Presence of enteric coliforms especially *Escherichia coli* makes the water samples unsuitable for human consumption according to the guidelines set by WHO for the evaluation of bacteriological quality of drinking water [2]

Curiously, *Salmonella typhi* which was encountered only in the uncovered wells was not isolated from any of the water samples in location Y. This may not be unconnected with good and proper household hygiene envisaged in this area because of the calibre of people living in this area as well as absence of overcrowding which is the case in other locations under study. Apart from environmental hygiene and population density, the presence of *Salmonella typhi* in some of the uncovered wells in those areas may also be attributed to drainage and flooding from contaminated surface water into unprotected well shafts. Findings from this study clearly highlight the non conformity of well water samples studied with the WHO standard recommendation for safe potable water [8], a guideline being adopted by the NAFDAC.

This study corroborates earlier studies in Lagos and Ibadan where it was discovered that well water used as source of water for drinking and cleaning purposes were grossly contaminated with pathogenic organisms [13, 14] A situation where enteric pathogens are grossly isolated from sources of water consumed by humans is a serious problem which calls for vigilance on the part of the authorities as it signals possible future outbreak of water borne diseases. Such disease outbreak may spread widely within the country and even possibly extend to neighboring countries since Nigeria shares boundaries with many West African states.

The reason for the gross contamination of well waters by pathogens as observed in this study may be due to openness and shallowness of the wells which allows easy entrance of particles from the surroundings. It may also be due to poor sanitary condition around the areas where such wells are located or drawing water from the wells with contaminated containers, a practice that is common among the users since individuals bring along their own water containers.

The high morbidity that is recorded from enteric diseases such as diarrhea, dysentery and typhoid fever in the country may be due to widespread consumption of contaminated well water.

CONCLUSION

This study has shown that there is a high incidence of contamination of well waters by pathogenic organisms. To reduce the widespread incidence of contamination of well water, it is advocated that wells dug must be deep and covered adequately. Also good and proper personal and environmental sanitary practices must be maintained in and around the wells. Boiling well water before being used for drinking purposes would also go a long way to prevent incidence of waterborne diseases.

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