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COMPARATIVE ANALYSIS OF ANTIBIOTIC RESISTANCE AND R-PLASMIDS OF STAPHYLOCOCCUS AUREUS ISOLATES FROM HUMAN AND DOG SAMPLES

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ABSTRACT

Bacterial resistance to antibiotics constitutes a major cause of failure in the treatment of bacterial infections. The genetic exchange of plasmids containing antibiotic resistant determinants between bacteria is believed to play a critical role in the evolution of antibiotics resistant bacteria and this has been shown in *S. aureus*. This study was therefore carried out to investigate the nature of plasmids that determine antibiotic resistance in *Staphylococcus aureus* isolates from man and animal.

Thirty multiply drug resistant *S. aureus* isolates from a total of 147 apparently healthy humans and dogs, as well as from clinical cases were determined by antibiotic susceptibility test using the standard disc agar diffusion method. Plasmid isolation was carried out by the alkaline lysis method of Birnboim and Dolly. Electrophoresis as well as the transformation experiment was done.

The result showed that no particular sensitivity pattern or plasmid profile can be ascribed to either human or animal sources of isolates. Two isolates from a domestic dog and its owner (human) were observed to have identical plasmid profile and almost the same antibiogram. 23.130 kbp and 25.119 kbp plasmids that were responsible for amoxycillin resistance were transferred.

In conclusion, the genetic basis of antibiotic resistance by *S. aureus* in our locality was found to be partly plasmid mediated. Plasmid analysis, in conjunction with the antibiogram is valuable in differentiating multiple resistant *S. aureus*.

Furthermore, domestic pet animals were found to be reservoirs and potential risk factors in the transfer of multiply antibiotic resistant *S. aureus* and their R-plasmids to antibiotic susceptible *S. aureus* and other bacteria.

Key words:- *Staphylococcus aureus*, antibiotic resistant plasmid, plasmid profile, resistance pattern, transformation.

INTRODUCTION

Bacterial resistance to antibiotics constitutes a major cause of failure in the treatment of infections by bacteria, and this is of concern, not only to clinicians, but also to medical scientists and the pharmaceutical industries (1). *S. aureus* is an important pathogen that is frequently found to be resistant to a large number of antibiotics (2, 3, 4). The genetic exchange of plasmids containing antibiotics resistant determinants between bacteria is believed to play a critical role in the evolution of antibiotic-resistant bacteria and this has been shown in *S. aureus* (1, 2, 5, 6).

A number of studies on the antibiotic resistance, carriage and infection of *Staphylococcus aureus* in both humans and animals have been conducted by researchers (2, 3, 7, 8, 9). However, only a limited number of studies have been conducted in Africa on the genetic basis of antibiotic resistance of bacteria, particularly on the animal strains of *Staphylococcus aureus*.

Molecular techniques are currently being used in differentiating strains of bacteria isolates and opinions vary as to the discriminatory power of the various types of these techniques (10, 11, 12,

13, 14, 15). Plasmid profile analysis has however been found by some authors as a molecular technique that is discriminatory enough in differentiating strains of *S. aureus* and other bacterial isolates (10, 13, 15, 16, 17, 22), while others have opined that no single technique was clearly superior to others for typing *S. aureus*, and that a combination of different techniques (both molecular and non-molecular) is sometimes necessary (7, 10, 14)

These observations necessitate and require further studies in our environment. This study was therefore conducted to analyze the antibiotic resistance and resistance plasmids of *Staphylococcus aureus* isolates from human and dog samples, in order to determine if there is any similarity or differences between animal and human strains of the isolates. Consequently the study will determine the discriminatory power of plasmid profile analysis in conjunction with antibiotic susceptibility pattern (antibiogram) in differentiating different strains of *Staphylococcus aureus*. The study will further demonstrate the potential transferability of resistance gene from *Staphylococcus aureus* to *Escherichia coli* with the aim of contributing to the knowledge of the genetic basis of antibiotic resistance by bacteria.

MATERIALS AND METHODS

Bacteria Isolates: A total of 30 multiple-resistant *Staphylococcus aureus* strains from human nasal colonisation (7 isolates), human ear colonisation (1 isolates), canine groin colonisation (4 isolates) and human clinical isolates (9) were subjected to plasmid extraction by modified alkaline lysis method of Birnboim and Dolly, 1979 (17). Multiple- resistance was defined as resistance to 4 or more antibiotics out of 8 different antibiotics the isolates were subjected to.

Colonisation isolates were obtained from samples collected between July and December, 2006, from humans and dogs in Agege and Alimosho Local Government Areas of Lagos State, Nigeria; and inserted into Stuart's Transport medium pending culture on appropriate culture media. Clinical isolates were obtained from clinical samples collected within the same period, from two University Teaching Hospitals in Lagos State and Ogun State of Nigeria.

All *Staphylococcus aureus* isolates were identified culturally, microscopically and biochemically by standard methods according to Jawetz *et al.* 2002 (21).

Culture Media and Growth Conditions

The culture media used for isolation and preliminary identification of *Staphylococcus aureus* strains were Mannitol Salt Agar (Britania) MacConkey agar (Biotec) and Blood Agar (Nutrient Agar, Biotec, supplemented with 5% expired human blood, HIV and Hepatitis B negative). Identified isolates were stored on Nutrient agar slopes at 4°C.

Antibiograms

The identified *S. aureus* isolates and *Escherichia coli* K-12 transformants were subjected to antimicrobial susceptibility test using the Disc diffusion technique as described by Bauer *et al.* (1966). Antibiotic multidisc (Abtec, U.K.) for Gram positive bacteria consisting of Augmentin (30ug); Amoxycilin (5ug) Erythromycin (5ug), Tetracycline (10ug); Gentamycin (10ug); cotrimoxazole (25ug); Chloramphenicol (30ug) as well as single disc of methicillin (10 units) were used.

The multidisc plates of Mueller-Hinton Agar were incubated at 37°C for 24 hours while the methicillin disc plates were incubated at 30°C for 24 hours and antibiogram were evaluated by comparing zones of growth inhibition of the test isolates with that of antibiotic sensitive oxford strains of *Staphylococcus aureus* NCTC 6571 as control.

Plasmid isolation

Plasmid extraction was carried out by the modified version of the alkaline lysis method of Birnboim and Dolly (1979) as described by Zuccarelli *et al.* (1989)

The multiply resistant organisms were grown on Luria-Bartani (LB) agar and incubated at 37°C overnight. Each organism was harvested into 1.0ml of TE buffer in Eppendorf tubes and washed by vortexing and centrifugation at 12000g for 10 minutes. The supernatant was discarded, the sediment was vortexed to mix and suspended in 2.0ml to TE buffer containing 50ug/ml lysostaphin. This was incubated at

37°C for 30mins and 0.4ml of 0.2M NaOH – 1% sodium dodecyl sulphate was added. The contents were mixed by inversion with hand and kept on ice for 10mins. After 10mins on ice 0.3ml of 3M potassium – 5M acetate was added and the lysate was cooled on ice for another 10mins. This mixture was centrifuged at 12,000 x g for 10mins and the supernatant transferred to another Eppendorf tube.

The supernatant was extracted once with buffered-saturated phenol and once with ether. The preparation was incubated with 2ul of heat-treated pancreatic RNase A (10mg/ml Worthington Diagnostics, Freehold, N.J.) at 37°C for 15mins. This was followed by extraction with 1.0ml of phenol-chloroform (1:1) through vortexing, centrifugation at 12,000 x g for 10mins and transfer of the upper layer into another eppendorf tube. The DNA content was precipitated with 1.0ml of absolute ethanol. The precipitate was collected by centrifugation, washed once with 1ml 70% ethanol by centrifugation. The supernatant ethanol was decanted and the DNA precipitate desiccated under vacuum. The dry precipitate was dissolved in 40ul of TE buffer, ready for agarose-gel electrophoresis.

Agarose Gel Electrophoresis

Electrophoresis of extracted plasmids was carried out in a horizontal agarose gel electrophoretic unit.

The agarose gel (0.8%) was prepared in Tris-Boric acid-EDTA (TBE) buffer, allowed to cool and 3 drops of ethidium bromide was added. The mixture was poured into the electrophoretic tank with the comb in place and allowed to set. The TBE buffer was poured into the tank until the appropriate mark was reached and the comb was removed to create the appropriate wells.

Using a micropipette, 20ul of each plasmid preparation, with added 0.2ul bromophenol blue dye (tracking dye) were applied into the horizontal well. Into the first well was added 20ul of DNA molecular Weight Marker(Roche Diagnostics, Germany; mol wt 0.12 – 23.1 kbp) to which 0.2ul of bromophenol blue had also been added as the standard. Electrophoresis was performed at 120v at room temperature for 2hrs. The electrophoretic bands were observed under a 302-nm transilluminator and then

photographed onto Polaroid Gel cam instant camera through orange filter (Peca Products Inc. U.S.A.)

b. Molecular Weight Estimation

The size of each plasmid was estimated by taking measurement of the mobility of the plasmids as well as that of the standard plasmid. Log₁₀ of molecular weight of the standard was plotted against the mobility of the standard. Molecular sizes of the *Staphylococcus aureus* plasmids were interpolated from the standard curve.

Gene Transfer Experiment

The method of Hanahan, 1983 (7) was used to transfer the resistant genes from *Staphylococcus aureus* isolates to strains of *Escherichia coli* K-12 devoid of plasmids

A single colony of recipient bacteria i.e. *Escherichia coli* k-12, from MacConkey agar plate was inoculated into fresh 5ml Mueller-Hinton broth and incubated at 37°C overnight. Into 20ml of fresh broth (Mueller-Hinton) was added 0.5ml of the overnight culture and the mixture was incubated on a rotating shaker at 37°C until cell density was 5 x 10⁷ cells per ml with an absorbance reading of 0.55 at 550nm.

The culture was chilled in ice for 10mins and centrifuged at 3,000 x g for 15mins at 4°C. The supernatant was decanted and the cell pellet re-suspended in 10ml of ice cold 0.1M MgCl. This was centrifuged as before at 3,000g for 15mins and at 4°C. The cell pellet was then suspended in 5ml of ice cold 0.1M CaCl, incubated on ice for 20mins and centrifuged as before. After centrifugation the cell were re-suspended in another 5ml ice cold 0.1M calcium chloride.

Two hundred microlitres (200ul) of competent cells were dispensed into labeled tubes. Into this was added 40ul of plasmid isolated from multiple resistant *Staphylococcus aureus* and the transformation mixture with experimental control (containing only competent cells) and sterility control (containing only plasmid DNA) were placed on ice for 30mins. The preparations were "heat shocked" by removing the tubes from the ice and placing them in a water bath, already set at 40°C for 20 mins. The tubes were removed and placed back on ice for 5mins. Into

each tube, was added 1ml of Mueller Hinton broth and incubated at 37°C for 1 hr.

After this incubation, 150ul of each of the tube content was plated into already prepared selective Luria-Bartani (LB) agar containing tetracycline (10ug/ml) and chloramphenicol (30ug/ml). The plate were incubated at 37°C overnight, and examined for growth the following day.

RESULTS

Resistance Pattern/Plasmid Profile Analysis

A total of 30 multiply resistant *Staphylococcus aureus* isolates were screened for possession of plasmids, out of which 16 (53%) were found to harbor plasmids (fig. 1). The profile of the plasmids in conjunction with the resistance pattern of the corresponding isolates is as shown in table 1.

A total of 8, representing 26% of the total number (30) of multiply antibiotics resistant *S. aureus*, whose plasmid content were determined, were found to possess 3 or more plasmids. Single plasmid isolates were 3 (10%) while isolates with 2 plasmids were 5 (17%). Fourteen isolates (47%) were found to have no plasmids (table 2.)

The major plasmid sizes, based on the frequency of occurrence in different isolates, are found in

human and canine isolates of different sources (table 3). This shows that the plasmid profile of isolates cuts across human and canine isolates of various sources, as no particular profile can be ascribed either to human or canine source alone. In the same vein, the resistance pattern of isolates cuts across human and canine sources as there were isolates from both sources having the same antibiotic resistance pattern (table 4). These groups of isolates were found to have different plasmid profile (table 4). On the other hand, 2 pairs isolates with same plasmid profile were found to have almost the same antibiotic resistance pattern, as there was only one antibiotic resistance difference in each of the 2 pairs of isolates. (table 5). One of the pair of isolates was a dog and its owner (human) cohabiting in the same residence while the other pair of isolates have no known relationship, and they were samples from human and nasal cavity (table 5).

Gene Transfer Result

The plasmid profile and the resistance pattern of donors and *Escherichia coli* k-12 transformant are shown in table 6 while fig. 2 is the electrophoretic mobility pattern of transformants.

Plasmid borne amoxycillin resistance of size 23.130 kbp and 25.1219 kbp was determined.

TABLE 1: PLASMID PROFILE AND RESISTANCE PATTERN OF PLASMID - CONTAINING ISOLATES.

| Serial No | LAB NO | RESISTANCE PATTERN | PLASMID PROFILE (kbp) |
|-----------|----------|--------------------------------|------------------------------------|
| 1. | UK 1 | Au, Am, Te, Me, Ge, Co | 2.322 |
| 2 | CN 'A' 2 | Er, Me, Co, CI | 0.282 |
| 3 | CN23 | Au, Am, Te, Me, CI | 1.259 |
| 4 | HN32 | Au, Am, Te, Me, CI | 25.119, 9.416 |
| 5 | CN26 | Au, Am, Te, Me, CI | 25.119, 9.416 |
| 6 | CN16 | Au, Am, Te, Me, CI | 23.130, 2.708 |
| 7 | EAS1 | Au, Am, Er, Te, Me, Ge, CI | 23.130, 6.557 |
| 8 | EK1 | Au, Am, Er, Te, Me, Co | 25.119, 1.259 |
| 9 | HN8 | Am, Te, Co, CI | 23.130, 1.259, 0.560 |
| 10 | HN27 | Au, Am, Te, CI | 23.130, 6.557, 4.361 |
| 11 | HNE4 | Au, Am, Er, Te, Me, Co | 23.130, 1.259, 0.708 |
| 12 | CNE2 | Au, Am, Te, Me, Co | 23.130, 1.259, 0.708 |
| 13 | HE36 | Au, Am, Er, Te, Me, Ge, Co, CI | 23.130, 1.259, 5.623 |
| 14 | CG22 | Au, Am, Er, Te, Me, Co, CI | 23.130, 1.259, 1.000 |
| 15 | WK1 | Au, Am, Te, Me, Co | 23.130, 6.557, 6.310, 3.54 |
| 16 | CG14 | Au, Am, Te, Me | 23.130, 6.557, 6.310, 2.322, 2.027 |

| | | | | | | | | |
|----|---|--------------|-----|---|-----------------|----|---|--------------|
| Au | = | Augmentin | Am | = | Amoxycillin | Er | = | Erythromycin |
| Te | = | Tetracycline | Me | = | Methicillin | Ge | = | Gentamycin |
| Co | = | Cotrimazole | Chl | = | Chloramphenicol | | | |

TABLE 2: PLASMID DNA CONTENT OF STAPHYLOCOCCUS AUREUS ISOLATES

| Number of plasmids | Molecular weight Range | Number of Isolates |
|--------------------|------------------------|--------------------|
| 0 | - | 14 (47%) |
| 1. | 0.282-1.259 | 3 (10%) |
| 2 | 0.708-25.119 | 5 (17%) |
| 3 | 0.546-23.130 | 6 (20%) |
| 4 | 3.548-23.130 | 1 (3%) |
| 5 | 2.027-23.130 | 1 (3%) |

TABLE 3: MAJOR PLASMID SIZES AND POSSESSING ISOLATE SOURCES.

| Plasmids Size | No (%) of Possessing Isolates | Isolate Sources |
|---------------|-------------------------------|-------------------------|
| 25.119 | 3 (19%) | HN, CN, EK |
| 23.130 | 10 (63%) | CN, EAS, WK, HN, HE, CG |
| 9.416 | 3 (19%) | HN, CN, HE |
| 6.557 | 4 (25%) | EAS, HN, WK, CG |
| 6.310 | 2 (12.5%) | HN, CN |
| 2.322 | 2 (12.5%) | CG, UK |
| 1.259 | 6 (38%) | EK, CN, HN, CG |
| 0.708 | 2 (12.5%) | HN, CN |

HN = Human Nose CN = Canine Nose EK/EAS/WK/UK = Clinical Samples
HE = Human Ear CG = Canine Groin

TABLE 4: ISOLATES WITH SAME RESISTANCE BUT DIFFERENT PLASMID PROFILE

| Isolates | Resistance Pattern | Plasmid Profile (kbp) | Host |
|---------------|------------------------|------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| CN23/EK1/HNE4 | Au, Am, Er, Te, Me, Co | i. 1.259 ii. 25.119, 1.259 iii. 23.130, 1.259, 0.708 | Canine (colonisation) Human (clinical) Human (colonisation) |
| HN32/CN16 | Au, Am, Te, Me, CI | i. 25.119, 9.416 ii. 23.130, 2.708 | Human (colonisation) Canine (colonisation) |
| CNE2/WK1 | Au, Am, Te, Me, Co | i. 23.130, 1.259, 0.708 ii. 23.130, 6.557, 6.310, 3.540 | Human (colonisation) Canine (colonisation) Canine (colonisation) Canine (clinical) |

TABLE 5: IDENTICAL ISOLATES WITH SAME PLASMID PROFILE AND SIMILAR RESISTANCE PATTERN

| Isolates | Plasmid Profile (kbp) | Resistance Pattern | Relationship | Basis for Identity |
|-----------|-----------------------|-----------------------------|--------------------------------------------------------------|--------------------------------------------------------------|
| HNE4/CNE2 | 23.130, 1.259, 0.708 | i. Au, Am, (Er), Te, Me, Co | Canine (Dog) and owner | Same plasmid profile and almost the same resistance pattern. |
| HN32/CN26 | 25.119, 9.416 | i. Au, Am, (Te), Me, Co | No known relationship. Human and dog nasal carriage samples. | Same plasmid profile and almost the same resistance pattern |

TABLE 6: PLASMID PROFILE AND RESISTANCE PATTERN OF DONORS & TRANSFORMANTS

| Lab no | Resistance pattern of Donor | Plasmid profile of Donor (kbp) | Plasmid Transferred (kbp) | Resistance Pattern of Transformant |
|-------------|-----------------------------|--------------------------------|---------------------------|------------------------------------|
| EAS1...(i) | Au, Am, Er, Te, Me, Ge, CI, | 23.130, 6.567 | 23.130 | Au, Am, Te, Er, CI, |
| ESA1...(ii) | Au, Am, Er, Te, Me, Ge, CI, | 23.130, 6.567 | 23.130 | Am, Te, Er, CI, |
| HN32...(i) | Au, Am, Te, Me, CI | 25.119, 9.416 | 25.119 | Au, Am, Te |
| HN32...(ii) | Au, Am, Te, Me, CI | 25.119, 9.416 | 25.119 | Au, Am, Te, CI |
| CN16...(i) | Au, Am, Te, Me, CI | 23.130, 2.708 | 23.130 | Au, Am, Te |
| CN16...(ii) | Au, Am, Te, Me, CI | 23.130, 2.708 | 2.708, 23.130 | Au, Am, Te, CI |
| HN8...(i) | Au, Am, Te, Co, CI | 23.130, 2.159, 0.564 | 23.130, 1.259 | Am, Te, CI |
| HN8...(ii) | Au, Am, Te, Co, CI | 23.130, 2.159, 0.564 | 23.130 | Am, Te, CI |
| CG22...(ii) | Am, Er, Te, Me, Co, CI | 23.130, 1.259, 1.000 | 23.130, 1.259, 1.000 | Am, Er, CI |

(i) And (ii) are antibiotic selective plates for tetracycline and chloramphenicol respectively.

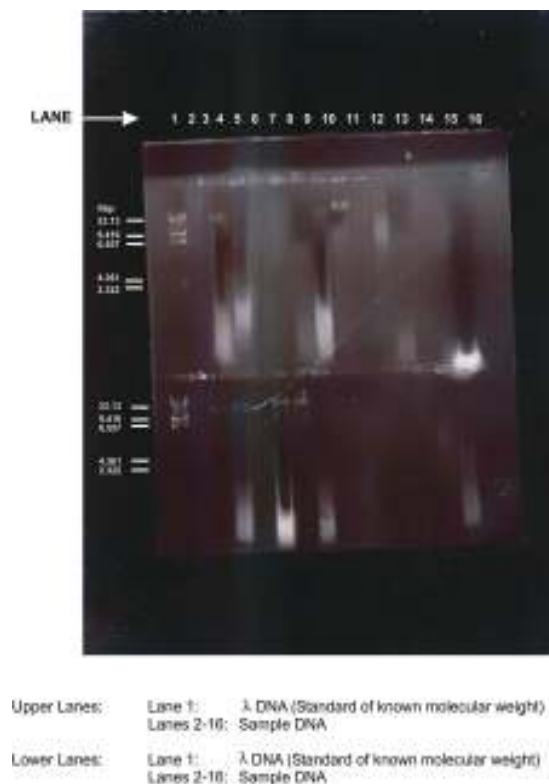


Fig. 1: Agarose Gel Electrophoresis Mobility Pattern of Plasmids

61(b)

Discussion

The resistance pattern and the plasmid profile of *S. aureus* isolates in this study revealed that the nature of resistance and the type of plasmids possessed cut across human and animal isolates of different sources. This observation is in conformity with findings of Whittam *et al.* 1989 (19) in their investigation of the genetic relationships among *Escherichia coli* isolates causing urinary tract infection in humans and animals. In the said work, they found that the independent isolates collected from humans and animals (dogs and cats) in separate geographical regions, share similar genotypes as identified by electrophoretic type; and they suggested that many cases of urogenital disease (in man and animal) may be caused by a small number of uropathogenic clones. In the same vein, the findings in this study, suggested that the various plasmid types (based on their sizes in terms of molecular weight) are widely distributed in



FIG. 2: Agarose Gel Electrophoresis Mobility Pattern of Transformants

humans and animals. This is also consistent with the findings that resistance pattern cuts across human and animal isolates of *S. aureus* in this study.

However, there was no consistency in relationship between resistance pattern and the corresponding plasmid profile of isolates. This findings also agrees with that of other workers who investigated the occurrences of multiple resistance and R-Plasmids in *Enterobacteriaceae* isolates from developing countries (11, 18, 19). Nevertheless, in this study while there existed isolates with the same resistance pattern but different plasmid profile, 2 pairs of isolates with the same plasmid profile also have similar (almost the same) resistance pattern.

One of these pairs of isolates were from nasal

swab of canine household pet (dog) and its owner (man) of the same residence, while the other is also from nasal cavity of man and animal, but of no known relationship. Hollis *et al.*, 1988 (7) has earlier suggested that family members may serve as reservoir for nosocomial infection with MRSA when a familiar carriage of MRSA and subsequent infection of a premature neonate was established. In their study, the *S. aureus* strains were characterized by antibiogram, plasmid analysis and genomic

DNA typing and the isolates were found to be identical by all 3 techniques (7). Other workers have also differently attested to the discriminatory power of plasmid profile analysis in conjunction with other non-molecular techniques like the antibiotic resistance pattern (antibiogram) in differentiating strains of *S. aureus* and other bacteria isolates.

The findings of this study support plasmid profile analysis and antibiogram as epidemiological investigating tool and extends the findings of Hollis *et al.*, (7) by suggesting, subject to further investigation (because of statistical insufficiency) that domestic pets are most probably a risk factor in transfer of both community acquired and nosocomial infections as well as R-plasmids.

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The result of transformation conducted identified a 23.130kbp and a 25.119 kbp plasmid as coding for amoxycillin resistance and this conforms with the findings of other workers (5, 10, 11). Although the result does not conclusively established the plasmid size coding for tetracycline or chloramphenicol resistance and further work is necessary to elucidate this, the existence of transformant in this study confirms the genetic basis of antibiotic resistance of *S. aureus* in our locality of being partly plasmid mediated and also probably chromosomal or transposon mediated, as no plasmid was detected in 47% of the multiple antibiotic resistance isolates whose plasmid content were investigated.

In conclusion, plasmid profile analysis in conjunction with the antibiogram is valuable in differentiating multiple resistant *S. aureus*. Furthermore, the combination of the findings of this study and other earlier studies suggested the transferability of multiple resistant *S. aureus* and their R-Plasmids, not only from man to man but also from domestic animal to man and vice versa.

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LASSA FEVER: ANOTHER INFECTIOUS MENACE

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Abstract

Nigeria is presently suffering from another Lassa fever epidemic. This was confirmed in the statement of the Minister of Health of the Federation in which he said, "There has been an upsurge in the reported cases of Lassa fever since the beginning of this year, especially in the Federal Capital Territory and its environs.

Within two weeks, 12 cases with five deaths due to the disease were recorded. 25 contacts are confirmed by laboratory investigations to have been infected, including 4 health staff working in the National Hospital, Abuja."¹

Lassa fever is an acute viral haemorrhagic fever first described in 1969 in the town of Lassa in Borno state, Nigeria.² It is endemic in West African countries, and causes 300,000 cases annually with 5000 deaths.³ Lassa fever epidemics occur in Nigeria, Liberia, Sierra Leone, Guinea and the Central African Republic.⁴ Lassa virus, the agent of the disease is a member of the Arenaviridae family. The virus is pleomorphic with single-stranded and bisegmented RNA genome.³ Its primary host is Natal Multimammate Mouse (*Mastomys natalensis*). Transmission to man occurs via exposure to the rat excrement through respiratory or gastrointestinal tracts⁵, exposure of broken skin or mucus membrane to infected material, direct contact, sexually and transplacentally.

The prevalence of antibodies to the virus is 8-22%⁹ in Sierra Leone, 4-55% in Guinea,¹² and 21% in Nigeria.¹³ The disease is mild or asymptomatic in 80% of infected people, but 20% have a severe multisystemic disease. Clinical features are difficult to differentiate from that of other viral haemorrhagic fevers and common febrile illness such as Malaria, Typhoid fever and so on. Definitive diagnosis is by viral isolation, Antigen and Antibody detection and Reverse Transcriptase PCR. Treatment is with Ribavirin, an antiviral agent. No vaccine is currently available. Prevention is by keeping rats away from homes.

Key Words: Lassa fever, Lassa Virus and Epidemic.

INTRODUCTION

Nigeria is presently suffering from another Lassa fever epidemic. The Minister Of Health of the country said in a statement on the recent Lassa fever outbreak that: " Since the beginning of this year, there has been an upsurge in the reported cases of Lassa fever especially in the Federal Capital Territory (FCT) and its environs. Within the last two weeks, we have recorded 12 cases with five deaths (41.7% case fatality). Much more worrisome is the danger that the outbreak poses to health workers. Four health staff working in the National Hospital, Abuja who were taking care of one of the Lassa fever

cases have also fallen ill and laboratory investigations have confirmed they are infected with the Lassa virus." "About 25 contacts in all were found to be positive with the Lassa virus from laboratory investigations but did not come down with the disease" (1).

Lassa fever is an acute viral haemorrhagic fever first described in 1969 in the town of Lassa in Borno state, Nigeria, located in the Yedseram river valley at the south end of Lake Chad (2). Clinical case of the disease had been known for

over a decade earlier but not connected with this viral

pathogen. The infection is endemic in West African countries, and causes 300,000-500,000 cases annually with approximately 5000 deaths (3). Outbreaks of the diseases occur in Nigeria, Liberia, Sierra Leone, Guinea and Central African Republic, but it is believed that human infections also exist in Democratic Republic of Congo, Mali and Senegal (4). Its primary animal host is the Natal Multimammate Mouse (*Mastomys Natalensis*), an animal indigenous to most of Sub-Saharan African (5). The virus is probably transmitted by the contact with the faeces and urine of animals accessing grain stores in residential areas (6).

CAUSATIVE AGENT OF LASSA FEVER

Lassa fever is caused by the Lassa virus, a member of the arena viridae; it is an enveloped, single-stranded, bisegmented RNA virus (3). The virions exhibit pleomorphic morphology when examined by cryoelectron microscopy. The surface of the virion envelope is studded with glycoprotein projections that consist of tetrameric complexes of the viral Glycoprotein GP1 and GP2 (6).

The genome of Lassa virus like other arena consists of two single-stranded RNA segments designated S (small) and L (large). In virions, the molar ratio of S to L RNAs is roughly 2:1. The 5' terminus of

each segment contains a tri-or diphosphate group and lacks a cap structure. The S RNA segment contains two genes that encode three final gene products- the nucleoprotein (NP or N) and the envelope glycoproteins GP1 and GP2 (also termed GP-1 and GP-2, or G1 and G2). GP1 and GP2 are first expressed as a precursor protein, GPC (or GP-C), which is cleaved post translationally (7). The L RNA segment contains two genes that encode two genes product, the viral polymerase (L protein) and Z protein, a small protein of undetermined function (7). On both segment, the genes are arranged in an ambisense orientation. The NP and polymerase genes reside at the 3' end of the S and L RNA segment, respectively, and are encoded in the conventional negative sense- that is, they are expressed through transcription of genome - complimentary mRNAs.

The genes located at the 5' end of the S and L RNA segment, GPC and Z, respectively, are encoded in mRNA sense but there is no evidence that they are translated directly from genomic RNA. These genes are expressed instead through transcription genomic-sense mRNAs from antigenomes, full length complimentary copies of genomic RNAs that function as replicative intermediates (6). Lassa virus will infect almost every tissue in the human body. It starts with the mucosa, intestine, lungs, and urinary system, and then progresses to the vascular system (4).

EPIDEMIOLOGY OF LASSA FEVER

Vectors: Lassa virus is zoonotic. The natural hosts for the virus are multimammate rats (*Mastomys natalensis*), which breed frequently throughout west, central, and east Africa⁸. They are probably the most common rodent in tropical Africa and are found predominantly in rural areas and in dwelling more often in surrounding country side (9); members of the genus are infected persistently and shed the virus in their excreta. Humans are infected by contact with rats or by eating them. Rats found in houses of infected people are seropositive for the virus ten times more often than those in control houses (10). Virus antibodies occur after a febrile illness in twice as many people who eat rats as in those who do not, and deafness (an effect of Lassa fever) occurs four times more frequently (11). Infection in humans typically occurs via exposure to animal excrement through the respiratory or gastrointestinal tract. Inhalation of tiny particles of infective materials (aerosol) is believed to be the most significant means of exposure (6). It is possible to acquire the infection through broken skin or mucous membranes that are directly exposed to infective materials. Transmission from person to person has also been established, presenting a challenge for health care workers. Sexual transmission and transplacental transmission of the virus have also been established (4). Transmission through breast milk has been observed (4).

PREVALENCE/INCIDENCE

Dissemination of the infection can be assessed by prevalence of antibodies to the virus in populations. The prevalence of antibodies to the virus is 8-52%⁹ in Sierra Leone, 4-55% in Guinea (12), and in Nigeria (13). Seropositivity has also been found in the Central African Republic Democratic of the Congo, Mali and Senegal (14). Sporadic cases have occurred in travellers returning to Britain, the Netherlands, and Germany from the endemic areas.

MORBIDITY AND MORTALITY

Lassa fever affects people of all ages. The disease is mild or has no observable symptoms in about 80% of people infected, but 20% have a severe multisystem disease. Incubation period is 6-21 days. The virus is excreted in urine for three to nine weeks from infection and in semen for three month (14). Sensorineural hearing deficit is a visual of the disease it was found in the 29% of confirmed cases compare with none of febrile controls in hospital in patients (15). In the general population, 81% of those who experienced sudden deafness had antibodies to Lassa virus as against 19% of matched controls (16). There is no apparent relationship between the severity of viral illness, initial hearing loss, or subsequent recovery(16).

Presentation of cases used to be highest during the dry season (January to March) and lowest during the wet season (May to November). However, recent data from Kenema, Sierra Leone show that admissions were highest during the change from the dry to the wet season (17).

Lassa fever was responsible for 10-16% of all adult medical admissions in 1987 into hospitals studies in Sierra Leone and for about 30% of adult deaths (18). The case fatality rate varied from 12% -23% for the period of 1997 -2002 (19). During pregnancy, high rate of maternal death (29%) and fatal and neonatal loss (87%) have been recorded, with 20% of all maternal deaths in Sierra Leone being due to Lassa fever (20). An estimate of the case fatality rate in the general population is 1-2%, must lower than in hospitalized cases, possibly as a consequence of differences in severity.

Using the figures for rural populations (available from United Nations Development Programme) and the epidemiology of the disease we estimate that the 'at risk' seronegative population (in Sierra Leone, Guinea, and Nigeria) may be as high as 59 million, with an annual incidence of illness of three million, fatalities up to 67,000 and up to three million reinfections (19).

CLINICAL FEATURES OF LASSA FEVER

Infection with Lassa virus leads to the gradual onset of fever and malaise after an incubation period of about 10 days (range, 5-21days), as the process develops, there is an increase in fever and myalgia, with severe prostration. Gastrointestinal manifestations such as abdominal pain, nausea, and vomiting, diarrhoea, or constipation are common. Sore throat occurs in two thirds of cases and is usually accompanied

by objective inflammatory or exudative pharyngitis. Retrosternal pain and cough are frequent, and pleural effusions may develop. Bleeding manifestations are seen in less than a third of patients but signal an unfavourable prognosis. Signs of increase vascular permeability such as facial oedema or pleural effusion are present in a minority of patient and also suggest a poor prognosis. Mortality in hospital patients is 15-20% (6).

A careful case-control study comparing Lassa fever to other febrile diseases seen in a West African hospital found features significantly associated with Lassa fever, including bleeding, oedema, exudative pharyngitis, conjunctivitis, and pharyngitis, but positive predictive values ranged between 0.61 to 0.74 (18). The same study also found vomiting, sore throat, tachypnoea, or bleeding to predict a 2.5-fold or higher increased risk of death. In spite of the relative non-specificity of the clinical findings, more than three fourths of patients thought to have severe Lassa fever are confirmed through viral assays. Lassa fever is a major paediatric problem as well (21, 22). Disease is more difficult to diagnose clinically. Occasionally, cases of infants developing anasarca have been described.

The course of fatal Lassa fever is relentless, with progression of signs and symptoms culminating in the onset of shock and death. In

Survivors symptoms and viremia persist until, 2 to 3 weeks after onset, there is defervescence

accompanied by the disappearance of virus from the blood. Pericarditis may occur in early convalescence, particularly in male patients. A case of polyserositis and recurrent pericarditis with constriction has been reported, which suggest that such complications should be sought more carefully (23).

Neurologic disease is not usually a dominant clinical manifestation in Lassa fever, but aseptic meningitis, encephalitis, global encephalopathy with seizures, and more subtle neurologic problems are well described (24-26). Cerebellar ataxia in convalescence is an uncommon but interesting occurrence. In convalescence, deafness is common; this is an important feature of Lassa fever, as it provides an important diagnostic clue (27). Late in course of the disease or early in convalescence, unilateral or bilateral hearing loss was noted in 29% of prospectively studied patients (15). No treatment is available and the effects may be transitory or often permanent. The auditory patterns and clinical course resemble idiopathic nerve deafness (16).

The clinical laboratory provides few clues to the diagnosis. The leucocytes count can be low, normal, or modestly elevated. Platelet counts are generally normal but may be modestly decreased (28). Albuminuria is common. AST is usually at least mildly elevated and the degree of elevation, which parallels the viremia, is useful predictor of mortality ²⁹. Patients with AST values in the hundreds or thousands are at

considerable risk of dying even with ribavirin treatment. Chest radiography may show infiltrates, pleural effusions, or, more commonly, no abnormalities. Electrocardiographic findings are often non-specifically abnormal (30).

Lassa virus also causes unusually high fetal mortality. Gravid women have been recognized to have an increased risk of death from Lassa fever, and prospective studies have shown that this is particularly pronounced 30% in the third trimester, compared to a 13% mortality in non pregnant women²⁰. Fetal loss was 87%, all infants infected in the last trimester died in-utero or during the neonatal period. Viremia, which is correlated with the risk of dying in Lassa fever patients, was higher in pregnant than non pregnant women. High concentrations of virus were found in fetal tissue as well as placenta (31). the biologic basis for these findings is unknown, but it seems likely that, once infected, the immature fetus is unable to mount an effective T-cell response to control the virus infection, maternal T cells would not be able to attack the placental infection because of the lack of MHC class I or II antigen expression on placental cells (32). Thus, the fetus and its supporting tissues would be a source of high level virus production.

LABORATORY DIAGNOSIS

There are a range of laboratory investigations that are performed to diagnose the disease and

assess its course and complications. Lassa virus is easily isolated from the blood or serum during the febrile phase of the disease up to 14 or more days after onset, even after the appearance of IFA antibody. Virus can also be detected in necropsy tissues (33, 24). Vero cell cultures examined by fluorescent antibody allow a diagnosis in 5 to 7 days or sooner. Lassa virus antigen can be detected by ELISA capture in serum within 4 hours of beginning testing and as it becomes negative, IgM antibodies appear (35). Antigen detection by ELISA is robust and reliable in rapidly fatal cases, even if the specimens are not handled properly for virus isolation (35). ELISA test for antigen and IgM antibodies give 88% sensitivity and 90% specificity for the presence of the infection (4). Reverse transcriptase (RT-PCR) is also a sensitive test for virus RNA, being positive in the blood of 23 of 29 patients at admission and 29 of 29 patients by the third day of hospitalization (36, 37).

Antibody can be detected by CF, IFA, or ELISA. IFA using lassa-infected vero cells as substrate is widely used (38) interpretation is subjective and discrepancies between laboratories are common. IFA IgG seroreversion has been reported and thought to represent loss of antibody by previously sero-positive individuals (9). Lassa IgG and IgM can also be detected by ELISA (35, 39, 40). ELISA IgM titres appear earlier and persist longer than IFA IgM titres. IgG ELISA antibody persists for long period, whereas IFA

antibody appears to wane below detectable limits within several years (6).

Other effects of illness include lymphocytopenia and a moderate thrombocytopenia, which are maximal 10-11 days after the onset of symptoms (19) the thrombocytopenia is associated with a serum inhibitor and with the occurrence of haemorrhage, depression of platelet aggregation, and the severity of Lassa fever (19).

TREATMENT

All persons suspected of Lassa fever infection should be admitted to isolation facilities and their body fluids and excrete properly disposed.

ANTIVIRAL DRUGS

Although several compounds have shown in vitro efficacy, only the guanosine analogue ribavirin has had practical application. The drug is efficacious in lassa fever and is the therapeutic agent of choice in the disease (6). Early and aggressive treatment of Lassa fever using ribavirin was pioneered by Joe McCormick in 1979. After extensive testing, it was determined that early administration is critical to success. Additionally,

ribavirin is almost twice as effective when given intravenously as when taken by mouth (41). ribavirin is a prodrug which appears to interfere with viral replication by inhibiting RNA dependent nucleic acid synthesis, although the precise mechanism of action is disputed (42). the drug is relatively inexpensive, but the cost of the drug is still very high for many of those in poverty stricken west African states.

When Lassa fever infects pregnant women late in their third trimester, it is necessary to abort the pregnancy for the mother to have a good chance of survival (20). This is because the virus has an affinity for the placenta and other highly vascular tissues. The fetus has only one in ten chance of survival no matter what course of action is taken, hence focus is always on saving the life of the mother (19). Following abortion, women should receive the same treatment as other Lassa fever patients.

Siga Technologies is developing an antiviral drug that has been shown to be effective in treating experimentally infected pigs. In a study conducted at the U.S Army research institute of infections. Disease (USAMRIID), treatment with ST-193 once a day for 14 days resulted in significant reduction in mortality (71% of the animals survived at the low dose), whereas all untreated animals and those treated with ribavirin died within 20 days of the infection (4). Intravenous interferon therapy has also been used in the management of Lassa fever infection (4).

SUPPORTIVE THERAPY

Supportive therapy is important in the management of patient with lassa fever (43, 44). Avoidance of travel and general trauma, gentle sedation and pain relief with conservative doses of opiates, the usual precautions of such patients with bleeding diatheses (such as avoiding intramuscular injections and acetylsalicylic acid), and careful maintenance of hydration are indicated. Bleeding should be managed by

platelet transfusions and factor replacement as indicated by clinical judgment and laboratory studies.

Management of shock is difficult. Vigorous infusion of crystalloid carries a high risk of pulmonary edema. Cautious administration of fluids and early use of pressors is indicated, but careful monitoring is important.

CONTAINMENT

The most dangerous exposure is parenteral and must be avoided through staff training. Thus, patients with these lassa fever should be treated in mask, gown, and glove isolation. Protection to caregivers and other patients should be enhanced by the addition of reparatory protection against small-particle aerosol (43, 45, 46). Close personal contacts should be monitored for fever for a period of 3 weeks. The patient may continue to excrete virus in urine or semen for weeks after recovery, so body fluids should be monitored for infectivity before the patient is released, meanwhile, a program of counselling emphasizing addition of disinfectant to toilets before use and protection of sexual partners should be followed. Special precautions are indicated when blood and other body fluids are handled in the clinical laboratory

PASSIVE ANTIBODY

Lassa virus infections are more difficult prospects for antibody therapy than other arenaviruses, because the volumes of plasma needed based on animal studies are large:

experimental studies of IgG for intravenous administration indicate that this could be a useful means of treatment only if selected, highly active preparations were available (48, 49). The future of antibody therapy in any of these diseases lies in development of standardized monoclonal antibody preparations of proven efficacy (50, 51).

PREVENTION AND CONTROL

Of all the arenaviridae, the Lassa fever virus has the greatest public health implication and control of the *Mastomys* rodent population is impractical, so measures are limited to keeping rodent out of homes and food supplies, as well as maintaining effective personal hygiene. Gloves, face masks, laboratory coats, and goggles are advised while in contact with an infected person.

Vaccine against Lassa fever is currently unavailable, though development is underway. The Mozambique virus closely resembles Lassa fever virus but lacks its deadly effects. This virus is being considered for possible use as vaccine.

Researchers at the USAMRIID facility have a promising vaccine against Lassa virus based on recombination vesicular stomatitis virus vectors expressing the Lassa virus glycoprotein. After a single intramuscular injection, test primates have survived lethal challenges, while showing no clinical symptoms (53).

CONCLUSION

Of all the Arenavirus diseases, Lassa fever has the greatest health impact and prospect for its

prevention through rodent control is least. No vaccine against the disease is currently available. The development of vaccines for Lassa fever carries several inherent problems. Clinically, Lassa fever infections are difficult to distinguish from other viral hemorrhagic fever and from more common febrile illness such as malaria, typhoid fever, shigellosis, leptospirosis, rickettsial disease and relapsing fever. Clinical laboratory provides little or no clue. RT-PCR tests which provide definitive diagnosis are not readily available in West African countries. Where, it is available it is too expensive and out of reach of the poor people living in the endemic areas. When the diagnosis is made, cost of Ribavirin and of barrier/isolated care is unaffordable. Furthermore, Lassa virus also causes usually high fetal mortality. Gravid women have been recognized to have an increased risk of death from Lassa fever, particularly pronounced in third trimester. All infants infected in the last trimester died in utero or during the neonatal period. The disease is thus contributing in no small way to the high maternal, neonatal, infant and under five mortality rates in West Africa. Failure to effectively control the disease makes the attainment of the millennium development goals of reducing maternal and infant mortality rates impossible.

The disease also has the potential of being used as biological weapon. It therefore constitutes an infectious menace that must be curbed.

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PREVALENCE OF BACTERIAL VAGINOSIS IN WOMEN WITH VAGINAL SYMPTOMS IN SOUTH PROVINCE, RWANDA

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Running title: Bacterial vaginosis and women with vaginal symptoms

Abstract

This is a prospective study of 297 Consecutive High Vaginal Swab (HVS) specimen from patients with vaginal symptoms at the laboratory of Butare University Teaching Hospital, South Province, Rwanda. The aim of the study was to evaluate the prevalence of bacterial vaginosis and the role of some micro-organisms and laboratory indices associated with it. The age range was 16-57 years with a mean of 30.8 years. The overall prevalence of bacterial vaginosis was 17.8% and the highest percentage of 52.8 % (28) found in the age group of 21-30 years compared with the lowest percentage of 1.9% (1) in the age group less than 20 years. Almost half of patients with trichomoniasis were found to have bacterial vaginosis ($P < 0.05$). The demonstration Clue cells in wet mount was found in significantly higher numbers (90.5%) in women with bacterial vaginosis ($P < 0.001$, positive predictive value 90.4%) while low sensitivity and positive predictive value were seen for vaginal discharge for detecting infection with bacterial vaginosis ($p > 0.05$, positive predictive value 26.0%). Bacterial vaginosis is common among women with vaginal symptoms in Rwanda as showed by gram stain examination. Further research into this pathology in other Rwandan women populations is needed.

Keywords: Bacterial vaginosis, laboratory based study, prevalence, Rwanda

Introduction

Vaginal symptoms are among the most common reasons of visits in clinical medicine, and occur frequently in women during their reproductive health, resulting very often in consulting an obstetrician or gynecologist or not (1). Bacterial vaginosis (BV) is the most common cause of vaginal discharge among women in reproductive age with a prevalence of 9-37%, depending on the population studied (2, 3). The

prevalence of vaginal infections, particularly BV, is high in many countries in Sub-Saharan Africa. For example, 20% to 50% of women of reproductive age are affected in Zimbabwe (4). It's characterized by a disorder of the vaginal ecosystem characterized by a change in the vaginal flora from the normally predominant lactobacillus to one dominated by sialidase-enzyme-producing organisms including *Gardnerella vaginalis*, *Mobiluncus spp.*, *Prevotella*

bifida, *bacteroides* spp., *Peptostreptococcus* spp., *Ureaplasma urealyticum*, and *Mycoplasma hominis* (5). All these organisms may have a synergistic role in producing the symptoms of BV. The condition is although common especially in low settings but under diagnosed. This may be due to confusion over its complex polymicrobial nature (6).

The two most widely accepted methods for the diagnosis of bacterial vaginosis, Amsel's composite criteria (7) and Nugent's Gram stain evaluation of bacterial morphotypes (8), are not used sufficiently in routine practice (9), mostly in low income countries. Although the diagnosis of BV by Amsel criteria is simple, it is relatively insensitive and not easily subjected to quality control, and the apparent complexity of the latter may have limited its routine application by clinical laboratories. As a consequence, surveillance studies on BV are mostly based on specialist clinic setting. Its prevalence in developed communities has been well documented as leading cause of vaginal discharge (10), whilst reports on the prevalence of BV from developing communities are scarce. Authors are not aware of other studies on this topic undertaken in Rwanda. The aim of our study was to evaluate the prevalence of BV among women who presented with vaginal symptoms at the Butare University Teaching Hospital, a tertiary health facility in South province, Rwanda, and to discuss the role of some micro-organisms and laboratory indices associated with BV.

Materials and methods

The study was conducted at the unit of microbiology (department of medical biology), Butare University Teaching Hospital, South Province, Rwanda, from March to December 2007. A total of 296 Consecutive High Vaginal Swab (HVS) specimen from patients (aged 16 to 57 years) with vaginal symptoms, who were referred to our laboratory, were included in the study. Almost all the patients were symptomatic and complained of one or more of the following symptoms: vaginal discharge, pruritus, and lower abdominal pain. Of 296 high vaginal swabs, clinical data on symptoms were available for 117 patients. All specimens were taken in patients who were already undergoing examination and the study was approved by the Butare University Teaching Hospital. The study was classified as human harm free since it is a laboratory-based study and all samples were analyzed anonymously to assure the patient confidentiality. A sterile cotton tipped swab was inserted in the posterior vaginal fornix at the time of speculum examination, then two swabs were taken, the first swab was rolled on a clean slide then a drop of isotonic saline was added and the slide was examined microscopically (X 400) for the presence of clue cells and *Trichomonas vaginalis*. A second swab of secretions was fixed by flame and Gram staining of the slide was performed for microscopic examination. The slide was examined under oil immersion microscopy (X 1000). Diagnosis of bacterial vaginosis was determined according to

a morphological scoring system(8), based on the proportions of lactobacilli relative to gram-negative anaerobes. Each slide was read by 2 independent technicians and was evaluated by another when discrepancies arose. The presence of yeast was done by slide smear examination of gram stain.

Data processing and statistical analysis were performed using SPSS software (version 15.0). The results were expressed as percentage, with significance at 5%

Results

A prospective study of consecutive 297 high vaginal swabs from women with vaginal symptoms was studied over the 10-month period for bacterial vaginosis; the age range was 16-57 years with a mean of 30.8 years. The overall prevalence of bacterial vaginosis was 17.8% and the highest percentage of 52.8 % (28) found in the age group of 21-30 years compared with the lowest percentage of 1.9% (1) in the age group less than 20 years (Table 1). Yeasts were present in samples from 83 (28%) patients and *T. vaginalis* was detected in samples from 11 (3.7%) patients.

Table 2 shows the distribution of *T. vaginalis* infection and the presence of yeast on gram stain over the categories of patients with and without

bacterial vaginosis. Almost half of patients with vaginal trichomoniasis were found to have bacterial vaginosis ($P<0.05$) while only few cases with yeasts were associated with bacterial vaginosis.

Of 296 high vaginal swabs, clinical data on symptoms reported were available and accurate for 117 patients, and only these data were analysed further. Most common symptoms identified was vaginal discharge (39.3%) followed by pruritus (31.6%), combination of vaginal discharge and pruritis (22.2%) and lower abdominal pain (6.8%). Table 3 shows a non-significant trend of a lower rate of symptoms in women with negative result compared to women with an infectious vaginal agent. A high rate of vaginal discharge was found in women with *Trichomonas vaginalis* than in bacterial vaginosis or in the absence of pathogen, although not to a significant extent. The demonstration Clue cells in wet mount was found in significantly higher numbers (90.5%) in women with bacterial vaginosis ($P<0.001$, positive predictive value 90.4%) while low sensitivity and positive predictive value were seen for vaginal discharge for detecting infection with bacterial vaginosis ($p> 0.05$, positive predictive value 26.0%).

TABLE 1: AGE DISTRIBUTION OF PATIENTS WITH BACTERIAL VAGINOSIS

| AGE GROUP (YEARS) | BV (%) | I (%) | N (%) |
|-------------------|-----------|-----------|-----------|
| 16-20 | 1 (1.9) | 4 (4.9) | 9 (5.6) |
| 21-30 | 28 (52.8) | 38 (46.3) | 94 (58.4) |
| 31-40 | 18 (34) | 28 (34.1) | 36 (22.4) |
| > 40 | 6 (11.3) | 12 (14.6) | 22 (13.7) |
| Total | 53 (100) | 82 (100) | 161 (100) |

BV, bacterial vaginosis: score of 7-10; I, intermediate: score of 4-6; N, normal: score of 0-3

TABLE 2: ASSOCIATIONS BETWEEN BACTERIAL VAGINOSIS AND PRESENCE OF OTHER MICRO-ORGANISMS

| CHARACTERISTIC | | VAGINAL FLORA MORPHOLOGY | | | |
|------------------------|----------|--------------------------|-----------|-----------|------------|
| | | n (%) | BV (%) | I (%) | N (%) |
| Trichomonas vaginalis* | Positive | 11 (3.7) | 5 (45.5) | 4 (36.4) | 2 (18.2) |
| | Negative | 285 (96.3) | 48 (16.8) | 78 (27.4) | 159 (55.8) |
| Yeasts | Positive | 83 (28) | 10 (12) | 20 (24.1) | 53 (63.9) |
| | Negative | 213 (72) | 43 (20.2) | 62 (29) | 108 (50.7) |

BV, bacterial vaginosis: score of 7-10; I, intermediate: score of 4-6; N, normal:score of 0-3.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ BV vs. I+N

TABLE 3: CORRELATIONS OF SYMPTOMS WITH LABORATORY DIAGNOSIS, NUMBERS IN PARENTHESES ARE PERCENTAGE OF CASES IN THE SAME ROW

| LABORATORY DIAGNOSIS | SYMPTOM N (%) | | | | |
|----------------------|-------------------|-----------|--------------------------------|----------------------|-------|
| | Vaginal discharge | Pruritis | Vaginal discharge and pruritis | lower abdominal pain | Total |
| Normal flora | 20 (33.3) | 22 (36.7) | 13 (21.7) | 5 (8.3) | 60 |
| Bacterial vaginosis | 6 (42.9) | 2 (14.3) | 5 (35.7) | 1 (7.1) | 14 |
| Yeats | 12 (36.4) | 12 (36.4) | 7 (21.2) | 2 (6.1) | 33 |
| Trichomona vaginalis | 2 (66.7) | 1 (33.3) | – | – | 3 |
| Mixed infection | 6 (85.7) | – | 1 (14.3) | – | 7 |
| Total | 46 (39.3) | 37 (31.6) | 26 (22.2) | 8 (6.8) | 117 |

TABLE 4: SENSITIVITY, SPECIFICITY AND POSITIVE PREDICTIVE VALUE OF SYMPTOMS AND SIGNS ASSOCIATED WITH BACTERIAL VAGINOSIS BY NUGENT SCORE.

| TEST | N OF THE RESULT WITH THE FOLLOWING RESULT (N=117) | | | | SENSITIVITY | SPECIFICITY | PPV | P VALUE |
|-------------------|---------------------------------------------------|----|----|----|-------------|-------------|------|---------|
| | TP | FP | TN | FN | (%) | (%) | (%) | |
| Vaginal discharge | 19 | 34 | 62 | 9 | 57.1 | 65.2 | 26 | NS |
| Clue cells > 20% | 12 | 2 | 94 | 2 | 90.4 | 97.7 | 90.4 | < 0.001 |

TP, true positive; FP, false positive; TN, true negative; FN, false negative; PPV, positive predictive value; NS, not significant

Discussion

Bacterial vaginosis is not a new syndrome but rather an infection that is being increasingly recognized in most of developed countries but still under-recognized condition in many low settings. There have been no reports of the prevalence of bacterial vaginosis in Rwanda. The present study, which is a cross-sectional laboratory-based study to define the presence of vaginal infections, provided unique information on the prevalence of bacterial vaginosis in the symptomatic women in the developing community.

In the present study our finding, with the overall prevalence of bacterial vaginosis of 17.8%, is in agreement with most of the studies that showed different rates ranging from 17% to 37% (11-13). However, higher rates of bacterial vaginosis have been reported in some studies carried out in developing countries. In a study from Haryana, India, bacterial vaginosis was diagnosed in a high percentage (48.5%) of rural women (14). These variations in the rate could be related to geographical distribution or systematic differences in the various population

samples. The contrasting prevalence figures may be because of various reasons such as differences in socio-economic status, educational level and method used for diagnosis of bacterial vaginosis.

Previous studies have found that bacterial vaginosis prevalence increased with age (15-17). In these studies, bacterial vaginosis prevalence was higher in women aged 25 years and older compared to women aged 14-24 years.

However, another study showed no association between bacterial vaginosis and age > 25 years (11). Although our findings showed that the prevalence of bacterial vaginosis is lower among women 16-20 years of age, there is no evidence to support this assertion as number of women in this group of age was very few.

In the present study, there were significant association of bacterial vaginosis with the presence of *Trichomonas vaginalis* in wet mount whereas no association was found with the presence of yeast on gram stain. The association of trichomoniasis with abnormal vaginal

ecology has been described by other authors (18, 19). The prevalence of this organism rise gradually with a decrease in the normal bacterial flora. This can be explained in 2 different ways: a normal vaginal ecology (score 0-3) may inhibit infection with *T. vaginalis* or trichomoniasis may change the vaginal ecology, causing it to resemble bacterial vaginosis. As found in a study (19) the presence of yeast on gram stain was inversely related to the level of ecological disturbance. This support the view that the bacterial vaginosis environment is not conducive to yeast multiplication, and yeast vaginitis therefore does not occur in the presence of bacterial vaginosis.

Out of 117 women with documented clinical information, the common symptom found was vaginal discharge in 39.3% followed by pruritis 31.6% and lower abdominal pain 6.8%. Clinical assessment is traditionally used for diagnosis. However, in the present study, none of the symptoms could significantly be attributed to a given infectious agent. A number of studies explored the association of vaginal discharge with vaginal infections (20, 21). With regard to clinical manifestations of vaginal infections among symptomatic women, all these studies found a variable degree of association between complaint of vaginal discharge and vaginal infections. Although these symptoms are not reliable, they are described in textbooks, as aids for the clinician to make a diagnosis of vaginal infection and to help choosing the proper medication. Even in our study, in more than

33.3% of cases with a laboratory diagnosis of normal flora, vaginal discharge was present.

Clue cells in wet mount were found to be significantly associated with bacterial vaginosis as shown by its sensitivity and positive predictive value. Our findings for positive clue cells also support previous studies that found it to have the highest sensitivity and specificity of the individual clinical criteria for diagnosing bacterial vaginosis (7, 22).

In conclusion, bacterial vaginosis is common among women with vaginal symptoms in Rwanda as showed by its diagnosis using gram stain. In fact, the prevalence of women with bacterial vaginosis in our study compared favourably with a lot of studies, but is different from some of the reports mostly from developing country where high rates prevail (12, 14). Clue cells in wet mount are valuable aid in bacterial vaginosis diagnosis. Although we did not evaluate the association of symptoms with vaginal infection in this study directly, our data suggest that the current approach to the diagnosis of vaginal infection should be reconsidered. Further research with larger sample size targeting both symptomatic and asymptomatic women in Rwanda is needed to study vaginal infections.

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PROFILE OF INSTITUTIONAL INFRASTRUCTURE FOR IMPLEMENTING UNIVERSAL PRECAUTIONS IN PRIMARY HEALTH CARE FACILITIES IN SOKOTO STATE, NIGERIA: IMPLICATION FOR OCCUPATIONAL SAFETY

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Abstract

Background: The adoption of primary health care in Nigeria has led to the expansion of health care delivery frontiers especially at the rural level. At this level is the most critical health services delivery point, with an attendant increase in contact between primary health care providers and patients. There is however also a simultaneous increased exposure to occupational and related health risks and hazards.

Methods: The objectives of this study were to assess the universal precaution profile of primary health care facilities and determine those factors that inform their prevailing safety status. Using a structured checklist, 23 representative primary health care facilities from the 23 local government areas in Sokoto State were randomly selected for the study, one from each of the local government areas.

Results:

The facilities were found to have poor universal precaution profile that could guarantee effective control of infection transmission and safety of their personnel. The facilities' mean score on measures and frameworks for ensuring the implementation of Universal Precautions was $53.12\% \pm 21.68\%$ with only 56.52% scoring above 50%.

Conclusion: Safety protocol and facilities for ensuring safe environment were inadequate and poorly developed. None of the facilities had full complement of facilities or resources for ensuring safety of working environment and for personnel's implementation of Universal Precautions. Policy for safety practice was poor, and post exposure intervention programmes for staff in event of accidental exposure grossly underdeveloped.

Interventions to improve safety environment and creation of safe climate are essential to protect primary health care workers against occupational hazards.

Key Words: Primary Health Care Centre, universal precautions, occupational safety

Introduction

Infections within the health care facilities are important in terms of the safety and wellbeing of patients and health care workers, and due to the enormous resources expended on avoidable infections. Their control thus remains a high priority globally. In the United Kingdom, they constitute a significant drain in terms of human and financial resources (1-3).

Interaction between patients and health care workers, therapeutic procedures, behaviour of

health care providers, state of a health facility environment, organizational profiles and estates and facilities have fostered the ease with which infections are transmitted within health care settings(1,4-6).

The characteristic organizational structure and functions of the health facility, which promote intimate interaction between the sick and health care workers, are capable of promoting the

transmission of infectious agents from sources to the susceptible within the health care environment. In the absence of, and unless proper precautions are taken, health care facilities can become sources of infection transmission.

Primary health care facilities in Nigeria are often faced with poor funding, inadequate facilities and poor environmental factors such as absent regular running water for safety practices. Services are often rendered in unpredictable environment, and in such situations the health care provider is unable to comply with universal precautions thereby increasing the potential of disease transmission. Inadequate infection control facilities and materials due to limited supplies and equipment are often characteristic of health care facilities in developing countries. These encourage infection transmission and militate against their effective control. Safety materials such as gloves, gowns and masks are often in short supply, and with these is the general scarcity of disinfectants and hand washing facilities (7, 8).

In developing countries, Nigeria inclusive, primary health care facilities constitute major sites for health service delivery to the vast majority of the population. This is due to the adoption of primary health care as the mainstay of health care delivery in Nigeria with a resultant expansion of health care delivery frontiers and interface of patient-health care worker interaction.

Primary health care services such as immunization, maternal and child health, family

planning, general medical and emergency, laboratory, school health, housekeeping, community-based, waste and instrument management are associated with infection transmission requiring adequate provision of enabling environment for effective control of infectious agents capable of been transmitted through them. In addressing the problem of infection transmission and its control within health care settings, WHO/CDC developed precautionary guidelines collectively known as Universal Precautions for implementation within health care settings (9 - 11). Its implementation was meant to reduce accidental exposure to blood and body fluids and the attendant infection that could result thereof. Working characteristics, organizational climate and administrative support are factors that have bearing in the profile of Universal Precautions in health facilities.

A growing body of research links working conditions such as working characteristics with safety for both patients and workers in health care settings. The provision of disposal facilities in the wards of a tertiary care centre in Vellore, India in 1998, saw to the reduction of percutaneous injury from 124 episodes in 1998 to 32 in 1999 (12). Vaughan *et al* showed that availability of infection control personnel and facilities were positive predictors for occupational safety (13). In a related study by Clarke, nurses working in hospitals with better practice environment were one-third less likely to be injured (14). Using data from 39 ICUs in 23 hospitals across the United States to examine the

impact of hospital structural characteristics and working conditions on occupational safety, the intensive care units with more positive organizational climates were found to have lower rates of occupational injuries and blood and body fluid exposures (15).

Thus organizational climate and administrative support are capable of influencing health care workers' likelihood of occupational injuries.

Where these are lacking, higher rates of occupational exposure have been observed. Aisen and Shobowale(16) showed that paucity of materials were responsible for 60% of health care workers' exposure to blood and body fluids. Atulomah and Oladepo(17) showed positive correlation between lack of institutional frameworks for safety measures and preponderance of related exposure to inherent risks in health facility environment. Clarke and colleagues buttressed this when, based on their study, they concluded that health care workers from poor organizational climates were generally twice as likely as those on better-organized units to report risk factors, needle stick injuries(18).

In countries of the developed world with strong administrative support and where government has made it compulsory that Universal Precautions be adhered to by both employers and employees, there is significantly high compliance and reduction in accidental exposures to hazards in health care settings, unlike what obtains in developing countries where Universal Precautions concept is not enforced or in existence. Thus administrative

responsibility is an important factor. The United States, United Kingdom, Canada, Hong Kong, Japan all have policies derived from the WHO/CDC Recommendations on Universal Precautions and to which strict adherence is a norm, requirement and practice (19-23).

Nigeria has its own national policy (23) which advocates for a nationwide adoption of Universal Precautions as developed by CDC. It outlines the minimum for the practice of universal precautions for the prevention of exposure to potentially infectious materials.

The policy has also established the Minimum Standard of Universal Safety Precautions to be observed by health workers in line with CDC Universal Precautions as well as guidelines for the implementation of post exposure management in event of accidental exposure to potential sources of infection with special reference to HIV.

In all, the policy is in line with international approach to prevention of blood borne pathogens especially blood borne viruses in event of accidental exposure in health care settings.

Developing world, characterised by lack or absence of these infrastructures, would parade higher rates of occupational exposure. This study was carried out to assess the status of safety measures in PHC facilities as well as their institutional framework for ensuring it and determine those factors that inform such prevailing profiles.

Methods

One primary health care centre was randomly selected from the PHC facilities in each of the 23 local government areas of Sokoto State. The study was conducted among these selected primary health care facilities using a set of checklist structured to ascertain the adequacy and appropriateness of measures and institutional framework for ensuring safe working condition and environment in each of the facilities.

Determination of appropriateness and adequacy of facility's measures and institutional framework for ensuring safe working environment was made through a scoring system. Assessment was done against eight (8) main items necessary for safety in health care setting. These consist of availability of universal precaution training and monitoring schedule; teaching, supporting and monitoring of appropriate hand washing; alternative arrangement in absence of water; teaching, supporting and monitoring of appropriate use of barriers; system for disposing equipment; appropriate system for processing instruments; waste disposal and housekeeping system. These were further broken down into 26 indicators for appropriate measurement.

For the purpose of determining the safety profile of these facilities, a scoring system based on the 26 indicators was designed. The percentage (%) scores on Checklist Assessment of Safe Working Characteristics of the Health Facilities is determined by the proportion of facility's total positive response to the total positive responses acceptable which is 26 overall. The result was

then used to grade the facility's profile with respect to its appropriateness and adequacy of its safety measures. A total score above 50%, indicating above average was accepted as satisfactory, while 50% and below was considered unsatisfactory.

Ethical approval was sought and obtained from the Ethical Committee of Usmanu Danfodiyo University Teaching Hospital, Sokoto, and permission obtained from the Sokoto State Government through the State Ministry of Local Government and Community Development. At the facility level, co-operation of the facilities' management team was obtained by explaining the purpose of the study.

Results

Teaching and supporting appropriate hand washing was the commonest safety measure in place, this being implemented in 65.23% of the primary health care facilities (Table 1). This is closely followed by the provision of alternative arrangement in absence of water and teaching, supporting and monitoring of appropriate use of barriers in 56.52% of the facilities. Among 47.83% of the facilities, there was appropriate waste disposal system, and established system for disposing used equipment and housekeeping in 39.13% that ensure safety for workers and the environment. Appropriate instrument processing system existed in 30.43% while safety training and monitoring schedule was available in only 8.70% of the primary health care centers. None of the facilities had the eight measures for safety completely available within it.

Resources for safety practice were similarly limited (Table 2). Equipment for processing instruments, as well as personnel for waste disposal was available in 82.61% of the primary health care centers surveyed. In 69.57% of these primary health care facilities, containers for sharps were available, while there was appropriate supply of disposable gloves as well as such gloves being readily available in treatment and laboratory areas as well as running water in 65.22% of the facilities.

Supply of disinfectants was appropriate in 56.52% and materials for cleaning and housekeeping adequate in 43.48% of the facilities. Functioning sink was found in treatment rooms of 34.78% and only 8.70% of the health centres had utility gloves for their cleaners for housekeeping. Water supply was from various sources among the facilities. As shown in Table 3, 47.83% of the health centres had both bore hole and well as sources of water, while 21.74% had either borehole or public water supply as source of water. Only 8.70%

was well as only source of water.

The commonest disinfectant in use among the facilities in the care of their instruments was Chloroxylonol 4.8% (52.86%). Methylated spirit was found as the main disinfectant in 46.43% of the facilities, while 25.24% of these centres used Chlorhexidine gluconate 1.5% and 14.29% commonly employed chlorine solution (bleaching solution).

Sources of gloves for patients' use were the health centre (43.48%) and patent medicine stores (56.52%). In both instances, the patients had to pay for them. All the facilities used boiling and disinfection in processing their instruments. Five (21.74%) of the centres however had functioning autoclaving machines for instrument care. None of the centres had any appropriate policy on post exposure management for staff in event of exposure to potential sources of infection. In all, the mean score of the facilities in ensuring safe environment was 53.21% (Table 4). Only 56.52% of the health centres had score above 50%.

TABLE 1: FACILITIES AND SAFETY MEASURES

| Safety Measures | Proportion of PHC Centres (%) |
|--------------------------------------------------------------------|-------------------------------|
| Availability of safety training and monitoring schedule | 2 (8.70) |
| Teaching and supporting appropriate hand washing | 15 (65.23) |
| Alternative arrangement in absence of water | 13 (56.52) |
| Teaching, supporting and monitoring of appropriate use of barriers | 13 (56.52) |
| System for disposing equipment | 9 (39.13) |
| Appropriate system for processing instrument | 7 (30.43) |
| Waste disposal | 11 (47.83) |
| Housekeeping | 9 (39.13) |

TABLE 2: RESOURCES FOR SAFETY IN THE FACILITIES

| Resources | PHC Facilities |
|---------------------------------------------------------------------|----------------|
| Running water | 15 (65.22) |
| Functioning sink in treatment room | 8 (34.78) |
| Appropriate supply of gloves | 15 (65.22) |
| Ready availability of gloves in treatment and laboratory areas | 15 (65.22) |
| Availability of gloves for cleaners | 2 (8.70) |
| Supply of disinfectants appropriate | 13 (56.52) |
| Containers for sharps available for use | 16 (69.57) |
| Equipment for steaming, boiling or chemical sterilization available | 19 (82.61) |
| Personnel for waste disposal available | 19 (82.61) |
| Cleaning supplies adequate | 10 (43.48) |

TABLE 3: SANITATION FACILITIES

| Description | % of Facilities |
|-----------------------------------------------|-----------------|
| Sources of water in the PHCs | 5 (21.74) |
| Tap water | 5 (21.74) |
| Bore hole | 11 (47.83) |
| Bore hole + Well | 2 (8.70) |
| Well alone | |
| *Commonly Used Disinfectants/Antiseptics | |
| Bleaching solution (Chlorine solution) | 14.29 |
| Chloroxylonol 4.8% | 52.86 |
| Hydrogen peroxide | 17.62 |
| Saponated cresol | 8.33 |
| Chlorhexidine gluconate 1.5% | 25.24 |
| Methylated Sprit | 46.43 |
| Others (Eusol etc) | 11.90 |
| Sources of Disposable Gloves for Patient Care | |
| Health Centre | 43.48 |
| Patent Medicine Store | 56.52 |
| *Instrument Processing | |
| Boiling and Disinfection | 100 |
| Autoclaving | 21.74 |
| Post Exposure Management Policy | 0.00 |

* Multiple responses allowed

TABLE 4: GRADING OF PHC FACILITIES' MEASURES AND INSTITUTIONAL FRAMEWORKS FOR SAFE ENVIRONMENT

| Score (%) | No. of PHC Centres |
|---------------------------------------------------------------------------|--------------------|
| 0 – 50 | 10 (43.48) |
| > 50 | 13 (56.52) |
| Overall Mean Score \pm SD = 53.21 \pm 21.68%; 95% CI = 34.43 – 66.98% | |

Discussion

The study showed that the facilities vary in the availability of measures and resources for implementing Universal Precautions concepts and guidelines that ensures safety of health care setting. This variation was found to be profound as indicated by the wide standard deviation and 95% confidence interval.

The mean score of 53.21% was marginally above average and can be considered not entirely satisfactory to guarantee a safe environment for health care workers in the rendering of their services. If considered as a system, the primary health care cannot be said to satisfactorily safe for primary health care workers in Sokoto State. Many of the measures and resources for implementing and practicing Universal Precautions were grossly lacking across the facilities. None of the facilities had entire measures and resources in place for Universal Precautions implementation. Only a few measures (teaching and supporting appropriate hand washing, alternative arrangement in absence of water, and teaching, supporting and

monitoring of appropriate use of barriers) and resources (equipment for instrument decontamination, waste disposal personnel, sharps containers, availability of gloves in treatment and laboratory, appropriate supply of gloves and running water) were available in more than 50% of the facilities.

Essential measures that improve knowledge and information dissemination and ensure safety of equipments in patient care were lacking in about 70% of the centres. With the absence of appropriate waste disposal system and poor housekeeping, health care workers as well as clients in quite a number of the primary health care centres are at risk of exposure to pathogenic micro-organisms. The lack of housekeeping personnel, inadequate cleaning materials and lack of functioning sinks in treatment rooms further reduce the capability of sustaining a safe working environment for staff or achieve an effective infection control.

The availability of water from diverse sources in all the facilities did not translate into availability of actual running water and functioning sinks in all the facilities. This may have affected the status of handwashing practice, a basic and cost effective safety procedure and other measures that requires the use of water. The alternative arrangement whereby water use is through scoops underlines the effect of non-functioning sinks. There were other gaps between resources available and their actual deployment in ensuring safety.

Various types of disinfectants were found to be in use in study setting. Chloroxylonol 4.8%, Methylated spirit and Chlorhexidine gluconate 1.5% are the commonly used antiseptics as against the more effective antiviral chlorine product (bleaching solution). Their use would likely create a false sense of protection especially among those who employ them in the care of accidental injured or exposed sites.

These have seeming implications for safe working environment in the primary health care centres. Such state as found from this study indicates low safety profiles of the centres, and which is unlikely to militate against infection control in the facility environment. The problem of conducive and enabling environment in the health institutions as observed in this study constitutes determining factors of ensuring the practice of the universal precaution concept. Lack of constant running water, shortage of personal protective equipment, paucity of institutional policy and frameworks would lead to poor compliance with Universal Precautions

by the various types of health care workers who make contact with patients with resultant increase in exposure of health care providers to infective agents.

Where these are thus lacking, higher rates of occupational exposure are resultant outcomes. This was the conclusion from the work of Aisen and Shobowale where paucity of materials was responsible for 60% of health care workers' exposure to blood and body fluids (16). In the study by Atulomah and Oladepo there was a positive correlation between lack of institutional frameworks for safety measures and preponderance of related exposure to inherent risks in health facility environment (17). Further credence to this was made through Clarke and colleagues in their study from which they showed that health care workers from poor organizational climates were generally twice as likely as those on better-organized units to report risk factors, needle stick injuries (18).

Where such measures and resources are available, accidental exposures are minimal. Availability of disposal facilities led to the reduction of percutaneous injury from 124 episodes in 1998 to 32 in 1999 in Vellore tertiary care centre in India, while infection control personnel were available as shown from the work of Vaughn and colleagues, occupational safety was enhanced (12,13).

The poor adherence to universal precautions among the respondents in the study by Aisien and Shobowale (16) was attributed not just to lack of knowledge (48%), but paucity of materials (60%) among the workers.

Administrative responsibility is an important factor in the overall process towards ensuring safe health facility environment as organizational support and administrative support are positive predictors of occupational safety in health care settings. Clarke demonstrated in health care settings with better practice environment, incidence of injuries would be less(14). Similarly, more positive organizational climate as shown from the data in 39 intensive care units in 23 hospitals across the United States was associated with lower rates of occupational injuries and blood and body fluid exposures (15).

None of the centres has any policy on post exposure management for staff in event of exposure to potential sources of infection. Policy guidelines and resources (human, material and monetary) are essential ingredients for an effective infection control within the health care facility and where absent or not implemented, workers are unlikely to know what to do or even comply. Such situations are common places in developing countries.

Developing world often characterised by lack or absence of these infrastructures would parade higher rates of occupational exposure.

Recommendations

Each primary health care centre should be encouraged to establish their respective safety training and monitoring schedules under the oversight of an organizational structure (a committee of some sort) within the facility for periodic updating and appraisal of staff and facility's safety profiles. Such a monitoring

system should strive to ensure the availability of necessary tools and supplies for safety practice, encourage and support behaviour change amongst the personnel.

Periodic review of primary health care centres' safety profile by the central supervisory body, the Ministry of Local Government and Community Development through its Directorate of Local Government Matters in collaboration with the State Ministry of Health through its Inspectorate Division in charge of ensuring standard or quality of health care delivery in all health institutions in the state. This is essential for the sustenance of safety practice and measures in the respective primary health care centers.

Policy on post exposure management for staff in event of exposure to potential sources of infection should be developed by the central supervisory organ for implementation at primary health care level.

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BRONCHOPULMONARY TUBERCULOSIS- LABORATORY DIAGNOSIS AND DOTS STRATEGY OUTCOME IN A RURAL COMMUNITY: A RETROSPECTIVE STUDY

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ABSTRACT

Pulmonary tuberculosis is still a global public health threat. Despite all efforts at its containment, the scourge is still menacing especially in the rural communities and among HIV infected patients. This retrospective study was carried out to determine the case detection rate of pulmonary tuberculosis in a rural community hospital in Nigeria from 2001-2006. A total of 1219 suspected patients were tested for pulmonary tuberculosis by sputum smear stained by Ziehl-Neelsen technique. Out of this number, 350 (28.7%) were positive for Acid-Fast Bacilli including 198 males and 152 females. Also 235 of the sputum-smear positive patients were tested for the human immunodeficiency virus (HIV) antibodies by Immunocomb 11 HIV 1 & 2 Bispot and confirmed by Immunocomb 11 HIV 1 & 2 Combfirm and HIV-1 Western Blot kit. Sixty three (26.8%) of the sputum-smear positive patients were co-infected with HIV.

Two hundred and seventy (77.1%) of the AFB positive patients were treated under the Directly Observed Therapy-Short course, 201 of them (74.4%) completed the treatment, 39 (14.4%) defaulted, 30 (11.1%) died before the completion of the treatment, 195 of the patients were declared cured and 6 were declared failed. Case detection rates could be improved upon by providing culture facilities at the DOTS centers. Also efforts should be made to ensure that all positive cases are followed to a logical conclusion and that anti-retroviral drugs are provided for patients co-infected with HIV to reduce the mortality rate of pulmonary tuberculosis.

KEY WORDS: PULMONARY TUBERCULOSIS, HIV, DOTS, AFB.

INTRODUCTION

Despite the attention and financial resources it had attracted to itself over the years, pulmonary tuberculosis still remains a major global public health problem. Approximately 2 billion people (one third of the world's population) are already infected with the TB bacillus (1). About 8.8 million new TB cases were estimated in 2005; 7.4 million in Asia and sub Sahara Africa. A total of

1.6 million people die of TB including 195,000 patients infected with HIV (1).

The World Health Organization (WHO) declared tuberculosis a world emergency in 1993 (2). In 2006, Nigeria was ranked as having the fourth largest tuberculosis burden in the world out of 22 high burden countries (3). (India and China ranked first and second respectively (4)). Subsequently, the Federal Ministry of Health declared tuberculosis a national emergency.

In 2005 report, the TB incidence rate was stable or in decline in all six WHO regions, however, the total number of new TB cases was still rising slowly because the case-load continued to grow in Africa, Eastern Mediterranean and South-East Asia regions (5). The recommended strategy was for countries to strive to detect at least 70% of new smear-positive cases and ensure a total cure rate of at least 35% of these detected cases. To achieve this, WHO in 1994 recommended that the national TB programmes adopt the strategy of 'Directly Observed Therapy Short course' (DOTS) (1). The two most important, out of the 5 main components of DOTS being 'improved cases detection by sputum-smear microscopy of symptomatic patients and standardized short-course chemotherapy to all sputum smears positive cases with direct observation of drug intake in at least the first 2 months of treatment' (1). By 2004, 182 countries had adopted and were implementing DOTS in their National programme, and in 2005 it increased to 187 countries (5).

More than 90 million TB patients were reported to WHO between 1980-2005; 26.5 million patients were notified by DOTS programmes between 1995-2005, and 10.8million new smear-positive cases were registered for treatment by DOTS programme between 1994 and 2004 (5).

Globally, WHO reported 2.3 million and 2.1million new smear-positive case notifications and 60% and 65% new smear-positive case detections under DOTS in 2005 and 2006 respectively (5). Under this report WHO reviewed the number of new smear-positive

cases successfully treated under DOTS at 1.7 million and 1.8 million in 2005 and 2006 respectively.

The proportion of HIV-positive people screened for TB rose from 8.8% in 2005 to 61% in 2006 and the number of TB cases found to be HIV positive rose from 0.083% in 2005 to 0.5% in 2006 (5).

Presently, above 89% of the world's population lived in areas where DOTS is being implemented by public health services. Global data clearly indicates that most of the resource poor countries are facing some limitations in the implementation of their DOTS programmes (6).

A number of factors contribute to the tuberculosis epidemic in various parts of the world. Severely deficient general healthcare infrastructure is the driving force. Among other factors, poverty (7), ignorance (2) and HIV co-infection (8, 9, 10) stand out as the disturbing pre-disposing factors for the spread of tuberculosis. Previous studies have revealed that HIV infected patients have increased risk for reactivation of latent tuberculosis, of rapid progression to disease of a newly acquired infection, and of death from tuberculosis infection (4, 12, 13, 14). The increase in the number of patients with HIV/TB co-infection has resulted in significant rise in the transmission of multidrug resistant *Mycobacterium tuberculosis* strains (15).

The six principal components highlighted by the 'stop TB strategy' (5), a new global vision include: addressing TB/ HIV co-infection, controlling and combating MDR-TB,

contributing to the strengthening of healthcare system, engaging all healthcare providers, empowering patients and, communities and enabling and promoting research. All efforts are geared towards improving overall global TB control and reduce morbidity and mortality particularly in rural communities such as the one under review.

Several studies in Nigeria in the past two decades have tried to present the picture of the tuberculosis situation in some cities of the country such as Portharcourt (16), Lagos (17, 18, 19), Benin City (20), and Ibadan (21). These were studies based in urban cities. Reports of the TB situation in rural communities of the country with low socio-economic status and poor healthcare facilities, where majority of the patients reside, is scarce.

Therefore, this study was designed to assess the extent of realization of the 2005 global targets of 70% cases detection and 85% cure rate proposed by the WHO assembly in 2000 (22) in a rural community. From this point, we x-ray the need to brace-up to partner with WHO on the policies and strategies to address the major constraints towards achieving global TB control and the primary vision of the stop TB strategy “ the global plan to stop TB, 2006-2015” launched in Jan. 2006 (5).

MATERIALS AND METHODS

THE STUDY AREA

The assessment was conducted at Osina Community Hospital, a secondary healthcare facility located in Osina, Ideato North Local

Government Area of Imo State. The hospital was the only Directly Observed Therapy Short course (DOTS) centre for tuberculosis in Ideato North and South LGAs and a few others nearby LGAs in Imo State as at the time of this study. The presence of German expatriate specialist medical officers from the inception of the hospital up to the late 90's gave the hospital a wide scope of patient in-take from all over Imo State and the neighboring states of Abia, Anambra and Enugu.

The DOTS centre commenced in 1996 and follows the guidelines of the National Tuberculosis/Leprosy Control Programme in conjunction with German Leprosy Relief Association (GLRA).

THE PATIENTS

All patients attending the out-patients department of the Osina Community Hospital with symptoms of pulmonary disorders either as primary patients or referred from other hospitals and clinics and whose 3 sputum (one spot and two other consecutive) samples were submitted to the lab for testing between Jan 2001 to Dec. 2006 were assessed for this report.

A total of 1219 patients were tested for pulmonary tuberculosis from January 2001 to December, 2006.

METHOD OF TESTING

Three sputum samples were collected from the patients according to the National Tuberculosis/Leprosy Control Programme guidelines as follows: 1st spot sample on arrival at the

laboratory, 2nd early morning sample the next day and 3rd sample produced on submission of the 2nd sample. For follow-up laboratory testing of smear-positive patients on treatment, 2 consecutive early morning sputum samples were collected. Smears of the samples were made according to standard procedures (23) and stained by Ziehl-Neelsen technique. The films were thoroughly examined under oil-immersion for the acid-fast bacilli. Patients were classified as smear-positive if at least 2 samples out of the 3 examined were positive for AFB. Culture facilities for *Mycobacterium sp* were not available in the laboratory within this period of review. Patients who have only 1 smear-positive result were asked to repeat the test after a short while. Smear-positive patients were counseled by the medical officer to enroll in the NBTL/GLRA Directly Observed Therapy Short course (DOTS) which includes 2 months intensive phase and 6 months continuation phase. Treatment followed the NBTL/ World Health Organization guidelines.

Patients were re-tested in the laboratory for AFB at the end of the 2nd month, 5th month and 7th month respectively. Treatment outcome for each of the patients was recorded after the final AFB result and completion of the treatment.

HUMAN IMMUNODEFICIENCY VIRUS (HIV) TESTING

HIV testing was performed for some of the patients based on the selection criteria of the medical officer. A total of 235 sputum-smear positive patients were co-tested for HIV

antibodies. Testing was performed using Immunocomb 11 HIV 1 & 2 Bispot (Organics, Israel) and confirmed by HIV-1 Western Blot kit (Epitope Inc, Beaverton, Oregon) and Immunocomb 11 HIV 1 & 2 Combfirm (Organics, Israel).

No anti-retroviral drug (ARV) was provided in the treatment centre for HIV-infected Tb patients at the time of the study. With this period under review data for this retrospective study were gathered from the laboratory and clinical case notes of the patients respectively.

RESULTS

From January 2001 to December 2006, a total of 1219 patients were tested for pulmonary tuberculosis by the Ziehl-Neelsen staining technique for acid fast bacilli. Out of this number, 350 (28.7%) were positive. This includes 198 (56%) males and 152 (43%) females, male to female ratio 1.3:1. The year 2004 has the highest number of pulmonary tuberculosis patients, 76(21.7%): 39 males and 37 females, followed by the year 2003 with 62 smear positive patients (17.7%): 31 males and 31 females. The year 2005 had the least smear positive patients (43, 12.3%) (Table 1). Mean age of occurrence of pulmonary tuberculosis was 35.4 ± 14.7 years. Peak age frequency was in the age range 20 to 29 (108, 30.9%). This was followed by the age range 30 to 39 (91, 26%) as seen in table 2.

A total of 235 sputum smear positive patients were tested for the HIV antibodies.

TABLE 1: SPUTUM SMEAR RESULTS AND TREATMENT OUTCOMES

| YEAR | TOTAL NO EXAMINED | POSITIVE NO M F T | NO TREATED | TREATMENT COMPLETED | CURED | FAILED | DEFAULTED | DIED |
|-------|----------------------|------------------------|----------------|------------------------|-----------------|-------------|---------------|--------------|
| 2001 | 414(34.0%) | 35 24 59 | 42 | 31 | 31 | 0 | 8 | 3 |
| 2002 | 179(14.7%) | 29 24 53 | 43 | 37 | 37 | 0 | 2 | 4 |
| 2003 | 162(13.3%) | 31 31 62 | 48 | 35 | 33 | 2 | 7 | 6 |
| 2004 | 219(18.0%) | 39 37 76 | 59 | 41 | 40 | 1 | 9 | 9 |
| 2005 | 135(11.1%) | 25 18 43 | 28 | 13 | 12 | 1 | 10 | 5 |
| 2006 | 110(9.0%) | 38 18 57 | 50 | 44 | 42 | 2 | 3 | 3 |
| TOTAL | 1219 | 198 152 350 (28.7%) | 270 (77.1%) | 201 (57.4%) | 195 (55.7 %) | 6 (1.7%) | 39 (11.1%) | 30 (8.6%) |

M= male

F= female

T= Total

Of this, 63 (26.8%) were positive for HIV. There were more female HIV positive patients (34, 54%) than males (29, 46%). Again, the year 2004 had the highest HIV positive tuberculosis patients (20, 35.1%). There were more male HIV positive tuberculosis patients in the years 2001 and 2006 and more females in 2002, 2003 and 2005 (figure 1). Of the 350 sputum smear positive patients, 270 (77.1%) enrolled for the DOTS treatment while 80 (22.9%) did not show up despite extensive counseling by the medical officer. Out of the 270 patients that started the treatment, 201 (74.4%) completed the treatment.

Of those that started the treatment, 39 (14.4%) defaulted and were lost in the study and 30 (1.1%) of those that started the treatment died before completion of the treatment. Of the 201 that completed the treatment, 195 (97%) were declared cured after follow-up sputum smear negative results at the end of treatment while 6 (3%) were declared failed following persistent sputum smear positive results at the end of the treatment period. Out of the 195 that were declared cured, 5 (2.6%) had under one year repeat episodes (i.e. presented back about a year later with recurrent sputum smear positive tuberculosis).

TABLE 2: AGE DISTRIBUTION OF PULMONARY TUBERCULOSIS PATIENTS.

| Year | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | Total |
|-------|------|------|------|------|------|------|-------|
| AGE | | | | | | | |
| 10-19 | 7 | 3 | 6 | 6 | 5 | 4 | 31 |
| 20-29 | 19 | 23 | 23 | 13 | 11 | 19 | 108 |
| 30-39 | 13 | 13 | 17 | 23 | 12 | 13 | 91 |
| 50-59 | 5 | 3 | 3 | 6 | 5 | 6 | 28 |
| 60-69 | 4 | 4 | 1 | 11 | 1 | 1 | 22 |

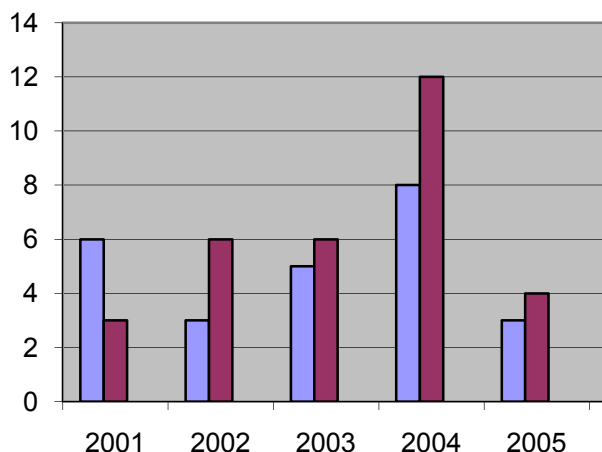


FIGURE 1: MALE- FEMALE DISTRIBUTION OF HIV/TB CO-INFECTION

DISCUSSION

PTB detection rate of 28.7% was recorded in this study. This was detected by sputum smear. The WHO national case detection rate of pulmonary tuberculosis by sputum smear in Nigeria was 27% in 2004. But it has been reported that pulmonary tuberculosis patients with less than 10^4 bacilli/ml of sputum cannot be detected by smear AFB microscopy (24). Furthermore, this rate was detected among patients with symptoms of pulmonary disorders. A good number of latent and asymptomatic tuberculosis patients were left undetected. The male-to-female ratio for pulmonary tuberculosis was 1.3:1. This correlates with the findings of Nwokoma in Portharcourt (16). Several other studies in the country have reported similar higher male pulmonary tuberculosis cases (17, 19) contrary to the findings at Ibadan where it was reported to be higher females than males (21). In the 6 year period, the year 2004 had the highest number of tuberculosis patients (76, 21.7%). This was followed by the year 2003. A cursory look at the WHO data for Africa shows the same trend

(3). The mean age of patients infected by tuberculosis in this study was 35.4 ± 14.7 . This falls between the age ranges discovered in Lagos (19) and Portharcourt (16). The peak age frequency was in the age range 20-29 followed by 30-39 years. This was the same peak age discovered in Ibadan (7) and closely related to the findings in Lagos (17) and Portharcourt (16) and corroborates reports that majority of people affected by tuberculosis in the developing countries were below the age of 50 while those of the developed countries were above the age of 50 (17, 25).

Of the 350 smear-positive patients, 235 were tested for HIV infection. 63 (26.8%) of these were positive. This agrees with the WHO estimate that about 27% of tuberculosis patients in Nigeria were also co-infected with HIV (3), but below the reported finding in Malawi that nearly two-thirds of tuberculosis patients were HIV positive (9). However, the number of patients tested for HIV in this study depended on mere clinical presentations and not a routine measure. This implies that those who were

tested manifested AIDS symptoms. This method of selection is obviously a source of bias since high risk patients could have escaped the clinical acumen of the medical officer. The synergistic effect of HIV/TB co-infection has been on the increase and accounts for more deaths associated with tuberculosis. Unfortunately, in this TB treatment centre, as in many others within some region of Africa, no antiretroviral drug is provided for the HIV infected TB patients. This will definitely compromise the treatment of these patients and could even increase the potential for the transmission of multi-drug resistant (MDR) (10) and extensive drug resistant (XDR) (27) tuberculosis.

As with the age distribution of tuberculosis patients in this study, most of the HIV infected TB patients were in the age range of 20-29 and 30-39. These are the sexually active age groups which are known to be at high risk of HIV infection. Earlier studies have revealed that TB patients less than 50 years of age are more frequently HIV infected (26). There were more female HIV infected TB patients than males, the same trend that was reported in Lagos (17) and Benin City (20) and which the researcher in Benin City attributed to higher female sexual activities. The bio-anatomical nature of female genito- urinary system is also a contributing factor.

Of the 350 sputum smear positive patients, 270 (77.1%) enrolled for the DOTS treatment while 80 (22.9%) absented after counseling. This is worrisome because these people went back into

the community without receiving appropriate treatment and continued to shed the bacilli in the environment to the detriment of the general populace. Some of these patients erroneously believe that tuberculosis is 'witchcraft' or 'poison' sent to them by their enemies, relatives or neighbors. They therefore go to seek 'solution' from traditional medicine or 'spiritual houses'. The proprietors of these non-scientific medicine centres give the patients false assurance of spontaneous cure after their rituals and prayers. One of such patients traced was even assured that the blood he was coughing out was a way of getting rid of the 'poison' he swallowed from his 'wicked uncle'. However, this patient was abandoned by this 'miracle worker' when his condition worsened but after two months at the DOTS centre, he made a dramatic recovery. Many of this kind of case abound in these rural communities and some could not muster the courage to come back to the hospital after their initial refusal of treatment, unless compelled by some enlightened relative or friends for the fortunate ones. Some come when it was too late owing to ignorance.

Furthermore, out of the 270 patients that started the DOTS treatment, 201 (74.4%) completed the approved treatment regiment, 39 (14.4%) defaulted (abandoned the treatment) and were lost in the study. This could be as a result of the tendency among many patients especially the less informed in the rural communities to abandon treatment once their health seemed to have improved. Some of these patients could

also have died without proper document. The defaulter-retrieval function of the local government Tb supervisor is not sincerely done due to dereliction of duty by the officer concerned or as a result of paucity of funds, or because the patients or their relatives do not give correct and detailed contact addresses, or the patient may have relocated.

Of those that started the treatment, 30 (11.1%) died before the completion of the treatment. This could be as a result of the delay by patients in seeking medical attention at the onset of the symptoms. The non inclusion of antiretroviral drugs to the treatment regimen of the HIV infected Tb patients could also have caused the death of some of the patients as some of the dead were HIV positive.

Out of the 201 patients that completed the treatment, 195 (97%) were declared cured. This is better than the reported success rates in Nigeria of 79% between 2001 and 2002, 59% in 2003 (3) and the WHO target of 85% in Nigeria at these periods.

Six patients (3%) out of those that completed the treatment were declared failed. These could be patients infected with multidrug resistant (MDR) and extensive drug resistant (XDR) strains of *Mycobacterium tuberculosis* (10, 27), although there is presently no scientific report on MDR/XDR in Nigeria to the knowledge of these authors.

Furthermore, 5 patients (2.6%) of those that were declared cured had repeated episodes. This is quite alarming and could be endogenous reactivation of the previous tuberculosis or

exogenous re-infection of the patient (28). Previous studies have noted that patients cured of Tb infection by one strain of *Mycobacterium tuberculosis* could be re-infected by another strain as the immunity by the primary strain does not protect the patient against a later infection by another strain (3). Rate of re-infection has been reported to be higher than that of new infections in South Africa (29).

RECOMMENDATION AND CONCLUSION

From the results obtained in this study, it could be seen that the global targeted case detection and cure rates are achievable even in the rural areas with a little more efforts. An improvement in the case detection rate could be achieved by provision of culture facilities at the DOTS centre or at a nearby referral centre. Defaulter retrieval should be intensified through provision of adequate funds to provide incentives to local retrieval officer and to mobilize the patients to visit the clinic days this will save the community from being infected by fleeing infected patients who hide in the village and continue to shed the infective bacilli to the environment. Antiretroviral drugs must be provided for HIV infected Tb patients to reduce the death rate attributed to tuberculosis. Apart from the radio and television jingles on Tb, other means of enlightenment of the people in the rural areas should be adopted such as house to house visitation by the local authorities and rallies at the village squares to let the people know what tuberculosis is and what it is not in order to reduce morbidity, mortality and stigma

otherwise the global emergency is yet to commence.

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THE USE OF RAP-PCR IN STUDYING *MYCOBACTERIUM TUBERCULOSIS* INTRACELLULAR GENE DURING MACROPHAGE INFECTION.

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Running title: *RAP-PCR in studying Mycobacterium tuberculosis intracellular gene expression*

ABSTRACT

Mycobacterium tuberculosis is the second leading cause of death from infectious agent. This study sought to detect *M. tuberculosis* genes, which were specifically expressed, or upregulated during intracellular infection of J774 murine macrophages; as such genes may be potential targets for novel drug action. J774 murine macrophage cell line was infected with *M. tuberculosis* (H37Rv strain) at 10:1 multiplicity of infection (MOI). RNA was differentially extracted from *M. tuberculosis* infecting J774 macrophage cell line. The control in this case was RNA from extracellular broth grown bacteria. Approximately 50 ng of RNA from intracellular derived bacteria and extracellular derived bacteria (control) were subjected to random arbitrarily primed PCR (RAP-PCR) using 50 primer combinations. Eleven differential RAP-PCR products were observed. All RAP-PCR products were cloned into pCR®2.1 and sequenced in order to determine the identity of the products. Four of the eleven products were derived from macrophage genes and another 4 products were derived from the *M. tuberculosis* rRNA genes (three 23S and one 16S rRNA). The 3 remaining RAP-PCR products were found to be mycobacterial genes other than ribosomal genes. The three products were genes encoding enzyme involving in a shikimate pathway, a putative carboxyphosphoenolpyruvate phosphonmutase and a serine protease with homology to HtrA. Of the 3 mycobacterial genes other than ribosomal genes detected, none were specifically expressed during intracellular infection but competitive RT-PCR showed that *aroF* gene was upregulated two-fold in intracellular derived bacilli.

Keywords: - RAP-PCR/*Mycobacterium tuberculosis*/ macrophage infection/RAP-PCR

INTRODUCTION

Mycobacterium tuberculosis is the second leading cause of death from any infectious agents in developed and developing countries after deaths due to human immunodeficiency virus (HIV), accounting for one-third of the mortality due to infectious. Over the past few years, there has been an increased in the incidence of tuberculosis partly due to the HIV

infection pandemic [1]. About 9.2 million people was estimated to be new cases of tuberculosis (TB) worldwide in 2006 – an increase of 0.6 % from 2005 with 1.7 million died from the disease [2]. The emergence of multi-drug resistant strains of *M. tuberculosis* [3] also complicated the treatment of this infection. In addition, there is no adequate vaccine for prevention of the disease. The BCG vaccine - a

vaccine of choice in most of the countries has variable degree of vaccine efficacy ranging from 0 to 90% [4-6]. Therefore, there is need for new intervention in terms of new chemotherapeutic agents and design of effective vaccines. Design of new chemotherapeutic agents and vaccines require better understanding of the basic biology of the organism including the metabolism. How *M. tuberculosis* survives the early interaction with the alveolar macrophages still remains a mystery. Understanding the survival mechanisms of *M. tuberculosis* could only come from the use of molecular biological techniques. Many of the molecular biological techniques require the use of a well-developed genetic system for mycobacteria. Progresses have been made in this area over the last few years with the development of transposon mutagenesis [7], homologous recombination [8], and *in vivo* complementation test [9, 10]. The difficulty in applying some of these techniques called for the use of other techniques that do not rely on a well-developed genetic system such as RNA based approach. The expression of a gene is indicated by the transcription of a particular segment of DNA into RNA representing the template carrying the necessary information for protein synthesis. RNA synthesis is in a continuous state of flux, which could be a reflection of environmental changes or demand for a particular gene to carry out a specific function. Plum and Clark-Curtiss (1994) had used RNA-cDNA subtractive hybridisation to identify a macrophage induced gene that was expressed by *M. avium* during tissue culture infection [11]. Another approach using the RNA based approach is arbitrary-primed polymerase chain reaction (AP-PCR) or differential display reverse transcription PCR (DDRT-PCR). AP-PCR or DDRT-PCR developed by Liang and Pardee (1992) is based on the use of random primers to identify differences in gene expression between two

target cell populations [12]. Wong and McClelland (1994) used this approach to study differential gene expression when *S. typhimurium* was exposed to oxidative stress (H_2O_2) [13]. Kwaik and Pederson (1996) had used a similar technique to identify a gene specifically expressed (*eml* – early stage macrophage-induced locus) by *Legionella pneumophila* during macrophage residency [14]. RNA extraction techniques have improved in mycobacteria over the last few years [15, 16], which makes it feasible for anybody who wants to study gene expression via transcription. This study was aimed at using RAP-PCR to detect genes that are differentially expressed by *M. tuberculosis* during the intracellular infection of macrophages.

MATERIALS AND METHODS

Maintenance and culture of mycobacteria.

Mycobacteria species used in this study was *M. tuberculosis* H37Rv (ATCC 9360) obtained from the National Culture Type Collection, Colindale, UK. Mycobacteria were grown to mid log phase in Middlebrook 7H9 broth (Difco Laboratories Ltd., Surrey, UK) supplemented with 10% albumin-dextrose catalase enrichment (ADC; Difco) and 0.02% Tween 80 (Sigma, Dorset, UK) at 37°C (in the presence of 5% CO_2 for *M. tuberculosis*) before harvesting at 0.5-1.0 OD_{600} . *M. tuberculosis* H37Rv cultures were also grown for 3 weeks at 37°C on thick 7H10 agar plates supplemented with 10% oleic acid-albumin-dextrose catalase enrichment (OADC; Difco) and 0.2% glycerol and then stored at 4°C.

Maintenance and culture of macrophage cell line.

The murine macrophage cell line J774.2 (obtained from European Collection of Cell Cultures, CAMR, Porton Down, Salisbury, UK) was used in all infection experiments. J774.2 macrophages were cultured in

Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated foetal bovine serum (Life Technologies, Paisley, UK) with no antibiotic supplements. Macrophage monolayers were maintained at 37°C in humidified air containing 5% CO₂ before and after infection. For storage purposes, cell pellets were suspended in 9% dimethyl sulphoxide (DMSO) in foetal bovine serum (FBS) at a concentration of approximately 4×10^6 cells/ml and aliquoted into 1 ml cryo-vials (Nunc, UK). The vials were placed in a polystyrene box and then placed at -20°C overnight before transferring to liquid nitrogen.

Macrophage infections and preparation of RNA.

J774.2 macrophages were synchronously infected overnight with non-opsonised static cultured of 10:1 *M. tuberculosis* bacilli in mid log phase [17]. After 4 hr the medium was discarded and washed in three changes of Hanks' balanced salt solution (HBSS) to remove the extracellular bacilli before the infected cell line layer was resuspended in DMEM. A control comprised *M. tuberculosis* bacilli growing in Middlebrook 7H9/ADC/Tween 80 broth without shaking. RNA was prepared from macrophages infected with *M. tuberculosis* using a method based on a differential lysis after overnight incubation. Briefly, the culture medium was discarded and the infected macrophage monolayer was re-suspended in 25 ml guanidine thiocyanate (GTC) solution per flask to lyse the macrophages. In order to reduce the viscosity of the solution, a long thin nosed plastic Pasteur pipette was used to squirt the lysate solution in and out with force to shear the macrophage nucleic acids. The lysates were transferred to 30 ml sterile universal tubes and centrifuged at 2,500×g for 20 min to concentrate the intracellular bacilli. The pellets of intracellular bacilli were combined using 1 ml of wash solution (1 ml 0.5 % Tween 80) and transferred to a

1.5 ml Eppendorf tube and centrifuged in a microcentrifuge at 12,000×g for 30 sec. The supernatant (wash solution) was saved and placed in a fresh Eppendorf tube and stored at -80°C. The pellet of bacteria was re-suspended in 200 µl of sterile DEPC treated water. The RNA was extracted from the harvested intracellular bacilli and the extracellular bacilli control using Mangan et al's method [16]. All RNA samples were DNase I (Pharmacia, UK) treated to destroy any contaminating DNA by incubating the RNA samples in the presence of the enzyme for 30 min.

RAP-PCR.

Two different concentrations of the RNA (approximately 50 ng and 25 ng) extracted from the intracellular bacilli and the controls (extracellular bacilli control and the saved Tween 80 wash from the intracellular bacilli) were reverse transcribed in a 20 µl reaction volume containing 0.5 µM of arbitrary primer (15-20-mer) with 0.5 mM each of dATP, dGTP, dCTP and dTTP with 100 U Superscript™ II RNase H- reverse transcriptase (Invitrogen) in the presence of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂ and 10 mM dithiothreitol (DTT) following the manufacturer's instructions. Briefly, the RNA sample and the arbitrary primer were incubated together for 15 min at 70°C and chilled on ice before the addition of dNTPs. This was incubated for 2 min at 42°C. Thereafter, reverse transcriptase was added. The reaction mixes were first incubated at 25°C for 15 min to allow for non-specific hybridization and finally incubated for 50 min at 42°C to allow for cDNA synthesis. Following cDNA synthesis, the reverse transcriptase was denatured for 15 min at 70°C. One tenth of the cDNA representing 2 µl was amplified in a 20 µl PCR reaction volume containing 0.5 µM of arbitrary primer (15 – 20 mer), 10 mM Tris-HCl (pH

8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, and 62.5 μM of dNTPs, and 1 U of *Taq* polymerase. The PCR parameters used were denaturation (94°C, 30 sec), annealing (35°C, 1 min), and extension (72°C, 2 min) for 45 cycles in a Perkin-Elmer Gene Amp 9600 thermal cycler. With RAP PCR strategy, 50 primer combinations were used. Following RAP-PCR, 7 μl portions of the RAP-PCR products were added to 2 μl of sample loading buffer and the samples were electrophoresed along with a 100 bp DNA ladder marker through a Clean gel using the Multiphor system. Following electrophoresis, the gel was silver stained. Differential RAP-PCR products were excised from the gel. The excised DNA bands were eluted and re-amplified as before. The reproducibility of the RAP product was re-assessed using the same cDNA, new cDNA preparation from the same RNA samples and also cDNA from independent infection experiment prior to cloning and sequencing.

Cloning and Sequencing.

RAP products were re-amplified using the same cycling parameters which generated the original product except that the final extension step at 72°C was increased to 10 min to provide TA overhangs. The re-amplified RAP product was cloned using TA cloning kit (Invitrogen, The Netherlands.) into a plasmid cloning vector (pCR2.1) following manufacturer's instructions. Plasmid DNA was extracted using the method of Birnboim and Doly [18]. PCR was used to screen the plasmid for the cloned RAP product and the plasmid containing the desired RAP product was used in sequencing. Sequencing was undertaken using the ABI PRISM™ dye terminator cycle sequencing kit (Applied Biosystems) following manufacturer's conditions. The sequencing products were sent to Alta Bioscience,

University of Birmingham, Birmingham, UK for analysis.

Computer analysis of sequence data.

Nucleotide sequences were analysed using the University of Wincosin's Genetics Computer Group (GCG) software package [19] on the UNIX system of the University of Birmingham Bioinformatics Unit. The BLAST programs [20] were carried out at the National Institute for Biotechnology Information, National Institute of Health, The Institute of Genetic Research, USA and Sanger Centre, Cambridge, UK.

Quantitative Competitive RT-PCR.

RNA (0.5 μg) was reverse transcribed in a 20 μl containing 0.5 μM of downstream primer AROFB (5'-GGATCACATGCCGTCTCATA-3') for *aroF* gene, 50 mM Tris-HCl pH 8.3; 75 mM KCl; 3 mM MgCl₂ and 10 mM DTT with 10 units of Superscript RNase H- reverse transcriptase. The reaction was incubated at 42°C for 50 min. The reaction was stopped by incubating at 70°C for 15 min. Quantitative RT-PCR was performed using the method described by Celi *et al* [21] in preparing the competitive template. In this assay, the concentration of the cDNA was adjusted based on 16s cDNA relative concentration and fixed in a competitive PCR containing series of two fold dilution of the competitive template for *aroF*, 15% glycerol, 0.125 mM of dNTP, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 1 μM AROFA (5'-GTCACCTTCGATCGCATTGTC-3') and 1 μM AROFB (5'-GGATCACATGCCGTCTCATA-3'). The cycling parameters used are as following: 94°C - 5 min for 1 cycle; 94°C - 30 s; 50°C - 30 s; 72°C - 30 s for 35 cycles and 72°C - 10 min. for 1 cycle. The expected product sizes for the competitive and cDNA amplicons were 120 bp and 180 bp, respectively.

RESULTS

The RNA extracted from the bacterial pellets represented majority of the intracellular *M. tuberculosis* following selective removal of macrophage nucleic acids as indicated by the presence of 16S and 23S bands on agarose gel (data not shown). Using RAP-PCR, eleven products were identified of which three RAP products were identified from *M. tuberculosis* (H37Rv) during intracellular infection that appeared not to be present in the extracellular bacilli (mycobacteria grown in Middlebrook's medium) using 50 primers combination. Four out of the eleven were macrophage genes with another 4 RAP products being mycobacterial ribosomal RNA genes (three 23s rRNA and one 16s rRNA genes). The three remaining RAP products were found to be mycobacterial genes apart from the ribosomal genes earlier mentioned. The three RAP Products were designated RAP 61, RAP 136 and RAP 148. The first RAP product - RAP61-1 (figure 1) was found to be about 400 bp. Two hundred and seventy bases were sequenced and subjected to

computer analysis. The DNA search on NCBI using the BLAST programme showed that the DNA has homology to a gene on *M. leprae* cosmid B1559 ($p=9.2e^{-63}$) and also to SEC101 gene of *Streptomyces lividans* ($p=2.1e^{-36}$). At amino acid level using the BLASTX programme, the gene was found to show strong homology to other *aroF* from plants and other bacteria. Apart from this particular gene, it also showed homology to phenazine F of *Pseudomonas aureofaciens* ($p=7.0e^{-06}$). The gene annotation in Sanger centre database showed the gene coded for a product with 462 amino acid residues with strong homology to *aroF* gene of plants. The *aroF* gene has 48% identity over 444 amino acid residues with the *aroF* of *Helicobacter pylori*, 90% identity to *aroF* of *M. leprae* over 462 amino acid residues, 50% identity to *Arabidopsis thaliana* over 436 amino acid residues and 43% identity to phenazine F of *Pseudomonas aureofaciens* over 429 amino acid residues. The sequence alignment of the putative AroF in figure 2 shows how related the AroF of *M. tuberculosis* to AroF of plants and bacteria.



Figure 1. An autoradiograph of the ^{32}P labeled RAP-PCR products derived from cDNA of intra- Cellular *M. tuberculosis* after 15 hr of infection of J774.2 macrophage like cells (lane 1), *in-vitro* grown *M. tuberculosis* (lane 2) and from J774.2 macrophage (lane 3). The arrowhead shows the RAP-61 whose RAP-PCR product was sequenced and found to be *aroF* gene.

The second differential RAP product - RAP 136-9 (data not shown) was found to be about 300 bp in size. Sequencing result showed that this gene has homology with a gene on MTCY39 ($p=0.0$) using the FASTA program on GCG at DNA level. The function of the gene is not known but it has homology with carboxyphosphoenolpyruvate

phosphonmutase gene of *Streptomyces hygroscopicus* ($p=0.17$) and unknown protein of *Saccharopolyspora erythraea* ($p=1.9e^{-56}$) at amino acid level using BLASTP programme at NCBI. This unknown protein of *Saccharopolyspora erythraea* has been implicated in the final hydroxylation step in erythromycin biosynthesis [22]. Carboxyphosphoenolpyruvate

phosphonomutase has been characterised *hygroscopicus* SF1293 [23]. The general clue that we could derived from the result of the database search is that this gene might be involved in antibiotic synthesis as an enzyme catalyzing the formation of an unusual C-P bond that is involved in the biosynthesis of the antibiotic bialaphos (BA) in *Streptomyces*.

The third differential RAP-145 (data not shown) obtained using this approach with new primers set gave about 200 bp in size. The sequencing result showed that this product was found to have 100% homology to a cosmid MTCY16A12 on searching the *M. tuberculosis* DNA database at Sanger centre. The whole sequence (about 6,400 bp) of the cosmid was pulled out for further analysis at BCM using the program called BCM gene finder. Using this program the RAP 145 was within one of the two potential genes on the cosmid. The putative translated sequence of the gene which was 464 amino acids was subjected to further computer analysis to find the likely identity of the gene on the gene database at NCBI. This gene was found to show homology to most of the high temperature response (*htrA*) genes from other bacteria including *M. tuberculosis* and *M. leprae htrA* gene.

The degree of homology to the *htrA* gene of the *M. tuberculosis* was not 100% suggesting that this gene is not *htrA* but has similar properties to the *htrA* gene. At the upstream of the *htrA*-like gene was the two component regulatory gene with homology to other two component regulatory genes in the GENBANK database. The *htrA*-like gene on Sanger's database is referred to as serine protease gene. Further computer analysis revealed that this protein is a membrane bound protein as indicated by the presence of the myristoyl domain.

To confirm that genes detected by RAP-PCR were differentially expressed a PCR based strategy had to be developed because of the small amount of RNA obtained from intracellular bacilli (typically, 20 ng from a flask of infected macrophages) negating the possibility of using Northern analysis or RNase protection assays to confirm upregulation of gene expression. It was essential that for any comparative gene expression study that the input RNA or cDNA from the test and control samples were equal.

| | | | | | |
|-----------------|-------------|------------|-----------------------------------|-----------------------|-----|
| | 101 | | 150 | | |
| M.leprae | MRTVLESVPP | VTVPSEIIRL | QEQLALVANG KAFLLQGGDC AETFDVNTPEP | | |
| M.tuberculosis | MRTVLESVPP | VTVPSEIVRL | QEQLAQVAKG EAFLLQGGDC AETFMNDTEP | | |
| A.thaliana | VLKKTIEAFPP | IVFAGEARNL | EEERLADAAGV KAFLLQGGDC AESFKGFNAT | | |
| S.tuberosum | VLKKTLEMNPP | LVFAGEARSL | EEKLGAAALG KAFLLQGGDC AESFKGFNAN | | |
| H.pylori | VKKELHSYPP | LVFAGEARNL | QERLAQVIDN KAFLLQGGDC AESFSQFSAN | | |
| P.auereofaciens | AQAYLRDSAS | LIRVEDILVL | RATLARVAAG EAMVIQSGDC AEDMDESTPD | | |
| Consensus | ---L---PP | L---E---L | QE-LA---A-G -AFLQGGDC AE-F-E--- | | |
| | 151 | | 200 | | |
| M.leprae | HIRSNVRTLL | QMAVVLTGGA | SLPVVKVARI AGQYAKPRS. .ADVDALGL | | |
| M.tuberculosis | HIRGNVRALL | QMAVVLTGGA | SMPVVKVARI AGQYAKPRS. .ADIDALGL | | |
| A.thaliana | NIRDTFRVLL | QMSIVLTFGG | QVPVIKVGRM AGQFAKPRSD AFEEKDGVKL | | |
| S.tuberosum | NIRDTFRILL | QMSVVLTFGG | QVPVIKVGRM AGQFAKPRSD PLEEDINGVKL | | |
| H.pylori | RIRDMFKVMM | QMAIVLTFAG | SIPIVKVGRI AGQFAKPRSN ATEMLDNEEV | | |
| P.auereofaciens | HVARKAAVLD | ILAGTFRLVT | QQPVVRVGRI AGQFAKPRSN NNERIGDVLE | | |
| Consensus | -IR---R-LL | QM-VVL-F-- | --PVVKV-R- AGQFAKPRS- ----I----L | | |
| | 201 | | 250 | | |
| M.leprae | KSYRGDMING | FAPDAAAREH | DPSRLVRAYA NSSAAMNLVR ALTSSGLASL | | |
| M.tuberculosis | RSYRGDMING | FAPDAAAREH | DPSRLVRAYA NSSAAMNLVR ALTSSGLASL | | |
| A.thaliana | PSYKGDNING | DTFDEKSRI | DPNRMIRAYT QSAATLNLLR AFATGGYAAI | | |
| S.tuberosum | PSYKGDNING | DTFDEKSRI | DPHRLIRAYM QSAATLNLLR AFATGGYAAI | | |
| H.pylori | LSYRGDIING | .ISKKERE | NPERMLKAYH QSVATLNLLR AFATGGYAAI | | |
| P.auereofaciens | PVYRGDMVNG | REAVCGHRQH | DAQRLVRGY. | | |
| Consensus | -SYRGD-ING | -----R-- | DP-RLVRAY- ---A-LNL-R A---G-A-L | | |
| | 251 | | 300 | | |
| M.leprae | HLVHDWNRFF | VRTSPAGARY | EALAGEIGRG LAFMSACGVA DRN...LQTA | | |
| M.tuberculosis | HLVHDWNRFF | VRTSPAGARY | EALATEIDRG LRFMSACGVA DRN...LQTA | | |
| A.thaliana | QRVTQWNLDF | VEQSEQADRY | QELANRVDEA LGFMSACGLG TDH.FLMTTT | | |
| S.tuberosum | QRVTQWNLDF | VENCEQGDY | QELANRVDEA LGFMAAAGLT VDR.FIMSTT | | |
| H.pylori | EQVHRFNLD | VKNNDFGQKY | QQIADRITQA LGFMRACGVE IERTPIILREV | | |
| P.auereofaciens | | | .SAARDIMQH LGWKASAS. ...QEQLSGS | | |
| Consensus | --V---N-DF | V-----RY | Q-LA--I--- L-FM-A-G-- ----L--- | | |
| | 301 | | 350 | | |
| M.leprae | EIYASHEALV | LDYERAMLRL | AGAPEGPDGG LQLYDLSAHT VWIGERTROL | | |
| M.tuberculosis | EIYASHEALV | LDYERAMLRL | S...DGDDGE PQLFDLSAHT VWIGERTROI | | |
| A.thaliana | DFYTSHECLL | LPYEQSLTRL | DSTSG.... .LYYDCSAHM VWCERTROL | | |
| S.tuberosum | DFWTSHECLL | LPYEQALTR | DSTSG.... .LYYDCSAHM VVWCERTROL | | |
| H.pylori | EFYTSHEALL | LHYEPLVRK | DSLTN.... .QFYDCSAHM LWIGERTROP | | |
| P.auereofaciens | PAWNHEMLV | LDYELPQLRQ | D.....EQ GRVFLGSTHW PWIGERTROL | | |
| Consensus | E-Y-SHE-L- | L-YE--L-R- | ----- --YD-SAH- -WIGERTROL | | |
| | 351 | | 400 | | |
| M.leprae | DGAHVAFAEV | IANPIGVKMG | ATMTEPELAVE YVERLDPHNK FGRLTLVSRL | | |
| M.tuberculosis | DGAHIAFAVQ | IANPVGKVLG | PNMTPELAVE YVERLDPHNK FGRLTLVSRM | | |
| A.thaliana | DGAHVEFLRG | IANPLGIKVS | NKMDPFELVK LVEILNPNK PGRITVIVRM | | |
| S.tuberosum | DGAHVEFLRG | VANPLGIKVS | QKMDPNELIK LIDILNPANK PGRITVIVRM | | |
| H.pylori | KGAHVEFLRG | VGNPIGVKIG | PNASVSEVLE LCDVLNPNRI KGRNLNIVRM | | |
| P.auereofaciens | TGAHVTLLE | VLPNFAVKVG | PDITQDQLLS LCERLDAKRE FGRLTLIARM | | |
| Consensus | -GAHV-F--- | --NP-GVKV- | -----V- --E-L-P-N- FGRLT-I-RM | | |
| | 401 | | 450 | | |
| M.leprae | GNNKVRDVL | PIVEKVATG | HQVIWQCDPM HGNTHESSSTG YKTRHFDRV | | |
| M.tuberculosis | GNNKVRDVL | PIVEKVATG | HQVIWQCDPM HGNTHESSSTG FKTRHFDRV | | |
| A.thaliana | GAENMRVKLP | HLIRAVRRSG | QIVTWVCDPM HGNTIKSTCG LKTRAFDSIL | | |
| S.tuberosum | GAENMRVKLS | HLIRAVRGAG | QIVTWVCDPM HGNTIKAPCG LKTRAFDSIL | | |
| H.pylori | GSKMIKERLP | KLLQGVLEEK | RHLWSIDPM HGNTVKTSLG VKTRAFDSVL | | |
| P.auereofaciens | GAQKVAERLP | PLVEAVRQAG | HKIIWLSIDPM HGNTIVAPCG NKTRMVQAIT | | |
| Consensus | G---R--LP | -LV--V---G | --V-W--DPM HGNT-----G -KTR-FD-I- | | |
| | 451 | | 500 | | |
| M.leprae | DEVQGFVEVH | RALGTYPGGI | HVEITGEDVT ECLGGAQDIS DTDLAGRYET | | |
| M.tuberculosis | DEVQGFVEVH | RALGTHPGGI | HVEITGENVT ECLGGAQDIS ETDLAGRYET | | |
| A.thaliana | AEVRAFLDVH | EQEGSHAGGI | HLEMTGQNV | ECIGGSRTVT YDDLSSRYHT | |
| S.tuberosum | AEVRAFLDVH | EQEGSHPGGI | HLEMTGQNV | ECIGGSRTVT YDDLSSRYHT | |
| H.pylori | DEVKSFFEI | RAEGSLASGV | HLEMTGENVT | ECIGGSQAIT EGLSCHYIT | |
| P.auereofaciens | EEIAAFKHAV | TSAGGVAAGL | HLETPDDVS | ECASDAAGLH Q..VASRYKS | |
| Consensus | -EV--F-EVH | ---G---GI | H-E-TGE-VT | ECIGG---I- ---L--RY-T | |
| | 501 | | | | |
| M.leprae | ACDPRLN | TQQ | SLELAFLVAE | MLRD----- | --- |
| M.tuberculosis | ACDPRLN | TQQ | SLELAFLVAE | MLRD----- | --- |
| A.thaliana | HCDPRLN | ASQ | SLELAFLVAE | RLRRRTGSQ | RVS |
| S.tuberosum | HCDPRLN | ASQ | SLELSFIVAE | RLRRRMSTQ | RL~ |
| H.pylori | QCDPRLN | ATQ | ALELAFLIAD | MLKKQHA~ | --- |
| P.auereofaciens | ICDPRLN | FWQ | AITAVMAWKN | QPSSTLASF~ | --- |
| Consensus | -CDPRLN- | -Q | -LEL-FLVAE | -LR----- | --- |

Figure 2. Alignment of the deduced amino acid sequence of the *M. tuberculosis* DAHP synthase with homologs from *Pseudomonas auereofaciens*, *M. leprae* (AL022602), *M. tuberculosis* (AL021957), *Arabidopsis .thaliana* (M74819), *Solanum .tuberosum* (P37822), *H.pylori* (024947) and *P. auereofaciens* (L488339). The positions and identities of

amino acids common to all the six proteins are in bold letters. The sequences for all DAHP synthase were obtained from GenBank at NCBI. For protein alignments, we used programs within the Genetics Computer Group sequencing Analysis Software package (GCG, Madison, USA).

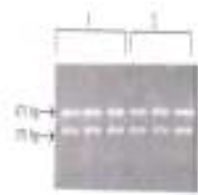


Figure 3 Competitive PCR for 16S cDNA to confirm equal proportion of cDNA used in the competitive PCR for *aroF* gene homolog competed in figure 4.



Figure 4. Competitive PCR for cDNA of *aroF* gene in intracellular bacilli and broth cultures of *M. tuberculosis*. Various amounts of *aroF* competitive DNA (2-fold dilutions of the competitive template) competed against a fixed concentration of *aroF* cDNA from intracellular bacilli and broth cultured *M. tuberculosis*. Lanes 1 - 4: Intracellular bacilli (in duplicate); lanes 5 - 8: broth cultured bacilli (in duplicate); lanes 9 & 10: PCR negative and positive controls, respectively and lane 11: 100 bp DNA ladder marker.

Determining the concentration of RNA in the test sample was particularly problematic owing to the contribution of the macrophage RNA despite the removal of host nucleic acids during selective lysis of the macrophages. Competitive RT-PCR was employed in order to rule out some background inherited using RNA dot blot hybridisation and the insensitivity of the technique to detect small variation in the samples. Using this approach, putative *pepp* gene was found not to be upregulated using limiting dilution PCR and *htrA*-like gene was found also not to be upregulated after 15 hr of intracellular infection by quantitative competitive PCR (data not shown). There was at least 2 fold (2.3 ± 0.8) in the level of expression of *aroF* gene in *M. tuberculosis* as shown in figure 3 during intracellular infection of macrophages compared to the broth grown organism,

DISCUSSION

Our strategy in using synchronised method of infecting the macrophages was to force all the mycobacteria into the same stage in the infectious cycle with the harvesting of the RNA representing 15 hr of infection. The infection process was to favour the uptake of the bacilli by the macrophages in order to be able to detect the genes that are expressed in order to survive inside the macrophage on a long term basis i.e. the genes that are very important for the parasitic existence of the bacilli inside the macrophages which might include the metabolic genes. Differential lysis of the macrophages and *M. tuberculosis* (H37Rv) was adopted with the hope of removing majority of the macrophage nucleic acids.

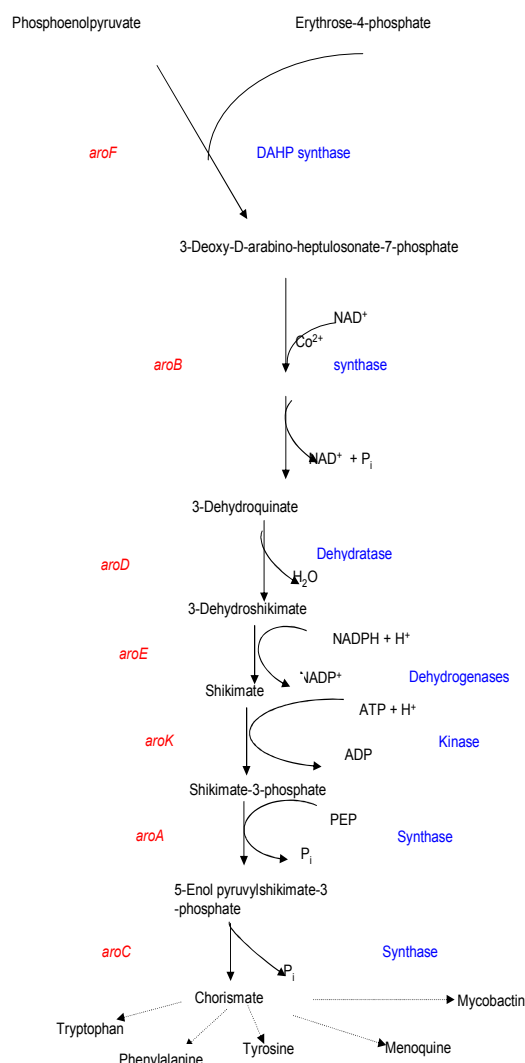


Figure 5: The pre-chorismate pathway of bacteria.

This method developed by Philip Butcher's group [16] exploited the differences in the cell architecture of the host and mycobacterium. The tender nature of the macrophage cell membrane makes it more susceptible to lysis than mycobacteria. This process of lysis has also taken into consideration of preserving the

mycobacterial RNA with guanidine thiocyanate solution. Macrophage induced gene homologue has been found to be upregulated in *M. tuberculosis* based on the RNA extraction method [24]. With this strategy, the quantity of the RNA from mycobacteria within the intracellular environment could be roughly assessed for RAP experiment without a wide departure from the actual concentration. All together, three RAP products of interest were identified after eliminating the host genes and ribosomal genes for mycobacteria using 50 primer combinations. The low detection of differentially expressed gene might be as a result of using conventional PCR in amplifying cDNA which does not favour the amplification of DNA fragment with high G:C content. We are currently addressing this issue.

The *htrA-like* (serine protease) gene identified in this study was found not to be differentially expressed suggestive that this gene play little role in the intracellular survival of *M. tuberculosis*. This does not come as a surprise because of the presence of more than one related genes which could complement each other in functions. HtrA mutants of bacteria namely *Brucella abortus*, *Yersinia enterocolitica* showed decrease resistance to killing within macrophages [25, 26], but it is most likely that *htrA* mutant of *M. tuberculosis* will not behave in a similar way as with the other bacteria. Further characterisation of the *htrA-like* gene along with

the neighboring gene (putative two component regulatory gene) will throw some light on the pattern of expression and whether two-component regulatory gene does have some roles to play in controlling the expression of *htrA-like* gene in the early stage of infection. In addition to the genes identified in this study, we found upregulation of the *aroF* gene during intracellular infection of macrophages. The product of this gene is 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase (DAHP) which is the first enzyme in the shikimate pathway with the final product of this pathway being chorismate - a precursor of many aromatic compounds (figure 5). The upregulation of *aroF* coding for this protein shows the importance of this particular pathway to the intracellular survival of *M. tuberculosis*. We could speculate that the increased in metabolic demand for the final product of this pathway in the synthesis of 2, 3-dihydroxybenzoic acid (a component of certain siderophores, which participate in the entry of iron into the cell) and p-hydrobenzoic acid (a precursor of the quinones) could lead to upregulation of *aroF* gene. For effective bacterial pathogenesis, the organism must sense its environment appropriately and respond with coordinate alterations in the expression of virulence genes. A number of environmental factors, including pH, osmolarity, temperature, and amino acid concentration, co-ordinately regulate the expression of virulence genes [27]. Since free iron is extremely limited in the mammalian host, a shift from a high to a low-

iron environment is an important environmental signal to bacteria for coordinate regulation of gene expression. And to acquire this bound iron, bacteria have evolved a system for acquiring this element, in case of mycobacteria - a mycobactin and the precursors for the synthesis of this compound is the chorismate. We further speculate that in order to carry out this function i.e. iron acquisition from transferrin, it makes sense that the pathway leading to the synthesis of this compound is upregulated. Another condition that could lead to the upregulation of this gene is the differences in the oxygen tension between intracellular environment of the macrophage and broth culture environment. Although standing culture was used as the control but there would still be a subtle difference in the oxygen tension. In order to maximise the survival chance of *M. tuberculosis*, the menaquinone synthesis has to be increased of which the precursor is chorismate and this could directly or indirectly control the expression of *aroF* gene. *Arabidopsis thaliana aroF* gene has been shown to be upregulated in response to pathogenic attack to *Pseudomonas syringae* [28] which is as a result of the induction of phenylalanine ammonia lyase - the first enzyme in phenylpropanoid pathway and other enzymes specific to lignin synthesis and secondary metabolism in order to provide aromatic precursors for the synthesis of defensive secondary metabolites to contain bacterial proliferation. Analogy of this phenomenon could happen in *M. tuberculosis* in order to produce precursors for the synthesis of

defensive secondary metabolites which could be antibiotic in nature. The *aroF* gene was also found to be upregulated in *Escherichia coli* grown in glucose, acetate, and glycerol media [29]. Chorismate/shikimate pathway is very important for bacteria survival, this probably explain why most preventable or curative approaches have been geared towards this pathway. The shikimate pathway in *M. tuberculosis* has long attracted attention as the potential target of finding a new vaccine or chemotherapeutic agent [30, 31]. In micro-organisms and plants, chorismic acid is a central precursor for the biosynthesis of an array of biochemically important and structurally diverse aromatic compounds. These include folic acid, vitamin K, ubiquinone and the three aromatic acids - phenylalanine, tyrosine and tryptophan [32]. The enzymes of the aromatic biosynthetic pathway are attractive targets for inhibitors since this pathway is absent in mammals. 6-Fluoroshikimic acid has been shown to be converted *in vivo* by the pre-chorismate pathway enzymes to ultimately produce inhibition of p-aminobenzoic acid (PABA) synthesis, and thus folic acid production [33, 34]. Mutants defective in the pre-chorismate pathway have vaccine potential. Aromatic-dependent mutants of the pathogenic bacteria *Salmonella spp.* which shares similarity to *M. tuberculosis* in being intracellular pathogen [35, 36], *Bordetella pertusis* [37], *Yersinia enterocolitica* [38], *Bacillus anthracis* [39] and *Aeromonas salmonicida* [40] have been shown to be avirulent and stimulate protective immunity. Para amino-

salicylic acid is one of the drugs used in the treatment of tuberculosis; the drug mode of action is on this pathway by mimicking the compound p-aminobenzoic acid and thereby leads into wrongful incorporation of this agent in the folate synthesis thereby thwarting nucleotide synthesis. This drug is also known to have some inhibitory effect at some stage in iron metabolism [41]. This study confirms the importance of pre-chorismate pathway to the survival of *M. tuberculosis* during intracellular infection of macrophages. Before meaningful intervention can take place, there is need for detail knowledge of the enzymes of this pathway. Searching the mycobacterial DNA database, it appears that there is only one copy of *aroF* gene, as it is in *Streptomyces coelicolor* [42] which in contrast to *Staphylococcus aureus* which has more than one copy. This indicates that the disruption of this gene could be lethal to the organism since there is no gene to complement the function of this gene. Also, means the control of the *aroF* gene may be complicated in this organism since the product of the compounds it catalyses is a precursor of many metabolites including secondary metabolites. The other genes of pre-chorismic pathway are in clusters with the exception of *aroE* gene that codes for shikimate-5-dehydrogenase on the cosmid MTCY159 on the Sanger's sequencing database. In this study, we show that *aroF* gene expression is upregulated in *M. tuberculosis* during macrophage infection. In addition we provide plausible reasons for the importance of this pathway to the survival of *M. tuberculosis*

and demonstrate the value of the RAP-PCR in detecting some of the genes that might play important or additional roles during intracellular infection. Work is under progress in characterising the condition inside the phagocytes which could be multifactorial, in promoting the expression of this gene.

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