

International Journal of Medicine and Medical Sciences

Volume 5 Number 3 March 2013

ISSN 2006-9723



*Academic
Journals*

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Case Report

Isolated cleft of the anteroir leaflet of the mitral valve: Case report in a nine month old boy at Delta State University Teaching Hospital, Oghara, Niger Delta, Nigeria

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Accepted 31 January, 2013

Isolated cleft of the anterior leaflet of the mitral valve is the occurrence of mitral cleft without ostium primum or ostio secundum defect and it is rare. We present a nine month old boy who presented in our clinic with a two month history of fast breathing, cough, and loss of weight. He was found not to be cyanosed but pale, tachypnoeic, dyspnoeic with basal crepitations. Cardiovascular system examination showed that he had a precordial bulge with tachycardia, cardiomegaly and a holosystolic murmur maximum at the apex. There was tender hepatomegaly. He was diagnosed as having congestive cardiac failure with broncho pneumonia secondary to an acyanotic congenital heart disease. Plain chest X-ray confirmed the cardiomegaly and bronchopneumonia while an electrocardiography showed bilateral atrial enlargement with right ventricular hypertrophy. The cardiac failure was treated but he represented with recurrent heart failure upto three times within six months. An echocardiogram done on the third admission showed an isolated cleft of the anterior leaflet of the mitral valve and he has been referred for surgery. Isolated cleft of the mitral valve commonly presents with mitral incompetence and eventual heart failure as in our patient. Early surgical intervention is advised as the width of the cleft tend to increase as the child grows leading to worsening of the mitral insufficiency.

Key words: Cleft, anterior leaflet, mitral valve.

INTRODUCTION

Isolated cleft of the anterior leaflet of the mitral valve is the occurrence of mitral cleft without ostium primum or ostium secundum defect (Mohanty et al., 1999). It is a rare occurrence and a rare cause of mitral insufficiency (Kaan et al., 2003). Clinically significant congenital mitral valve lesions are rare and estimated to affect 0.4% of those with congenital heart disease or 5/100,000 of the general population (Hoffman and Laplan, 2002). Usually, mitral cleft with or without ostium primun defect is associated with other congenital heart defects including

ventricular septal defect, tetralogy of Fallot, tricuspid atresia, and double-inlet left ventricle (Van Praagh et al., 2003). Its occurrence with an anomalous origin of the left coronary artery leads to death in over 90% of patients in the first year of life without treatment (Rathinam et al., 2005). Congenital cleft of the mitral valve is a rare cause of mitral incompetence, resulting from various degrees of failure of fusion of the embryonic atriventricular (AV) endocardial cushions (McDonald et al., 1994). Sigfussion et al. (1995) suggested that a cleft in an otherwise normal

Table 1. Haematology.

Item	Results (normal ranges in brackets)
Haemoglobin (g/dl)	9.3 (14-18)
PCV (%)	28.9 (40-54)
MCV (fl)	78.5 (80-95)
MCH (pg)	25.2 (27-32)
MCHC (pg/dl)	32.1 (30-35)
Total white cells	34,900 (4000-11000)
Granulocytes (%)	85.4
Lymphocytes (%)	9.4
Monocytes (%)	5.2
Platelets count	529 (150-400)
Blood group	B+ve

PCV: Packed cell volume; MCV: mean cell volume; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration.

mitral valve should be classified separately from atrioventricular canal defects (AVCD) with a common junction. In isolated cleft of the anterior mitral leaflet, the annulus is in the normal position and incompetence is caused by flail segments of the anterior leaflet (Nadas, 1972). The most common clinical presentation is congestive cardiac failure secondary to mitral incompetence (Tamura et al., 2000). We present an infant boy who presented with congestive heart failure whom we diagnosed to have acyanotic congenital heart disease, ventricular septal defect, but which came out to be cleft of the anterior leaflet of the mitral valve. The presentation and literature review of isolated cleft of the anterior leaflet of the mitral valve is presented here, and to the best of our knowledge this is the first documentation in Nigeria.

CASE PRESENTATION

This baby was first seen in our clinic at the age of nine months on 12/12/2011 with 3-month history of cough, fast breathing, diarrhoea. He was found to be dyspnoeic tachypnoeic with diaphoresis and pale. There was no cyanosis, but was underweight with a weight of 7 kg (expected is 9 kg). He had tachycardia with a heart rate of 150 beats/min. He had a precordial bulge with apex beat at the 5th left intercostal space mid-clavicular line. The first and second heart sounds were heard and normal, but he had a grade 3/6 holosystolic murmur maximum at the apex and radiating to the back. The respiratory rate was 84 breaths/min with flaring of the alar nasi, intercostal and subcostal recessions. He also had crepitations at the lower zone of the right lung anteriorly. There was tender hepatomegaly on abdominal palpation. A diagnosis of acyanotic congenital heart disease with congestive cardiac failure with bronch pneumonia was made and the following investigations ordered for: full blood count, plain chest X-ray (CXR), electrocardiography (ECG) and echocardiography.

RESULTS

The results show anaemia with leucocytosis, and absolute granulocytosis with thrombocytosis. The CXR

showed cardiomegaly with patchy opacities in both lung fields. The ECG showed a sinus rhythm with normal axis considering the age, bilateral atrial enlargement with right ventricular hypertrophy with repolarization abnormality (Table 1).

He was treated with frusemide then later with spirinolactone and hydrochlorothiazide, ceftriazone which was later changed to cefixime suspension and was discharged after two months admission. However, he was readmitted just one week after being discharged with congestive cardiac failure and the weight had dropped to 6.5 kg. He stayed for a week and was discharged due to parental pressure. He was readmitted for the third time for congestive heart failure in April 2012, which was four months from the initial admission. The echocardiogram showed he had cleft of the anterior leaflet of the mitral valve. He was treated and referred to India for surgery after two weeks of admission. While waiting and arranging for the logistics, he represented for the fourth time congestive cardiac failure two months later and was last seen on 27th June 2012, that is a week after discharge for follow up.

DISCUSSION

Cleft, derived from the verb *to cleave*, is defined as a space or opening made by splitting (Anderson et al., 1985). A cleft mitral valve has a split anterior with each part of the leaflet typically attaching to a different papillary muscle (Anderson et al., 1985). This patient presented early in life, in infancy. Just as in this patient, it commonly presents with congestive heart failure secondary to mitral incompetence. Early presentation in this patient may be as a result of associated other cardiac anomalies which were not detected with the 2-D echocardiography. It has been documented that there are at times discordance between the echocardiographic and surgical/postmortem findings in mitral cleft (Smalhorn et al., 1982). Congenital

cleft malformation in an otherwise normal mitral valve usually presents with concomitant cardiac defects, mainly an atrial septal defect, and Down syndrome is the commonest noncardiac anomaly (Fraisie et al., 2002). It is known that its association with anomalous origin of the left coronary artery presents early in infancy with congestive cardiac heart failure and death occurs in infancy in over 90% of cases if surgery is not done (McDonald et al., 1994). Cleft in the anterior mitral leaflet is best visualized from a subcostal or a parasternal axis view (Sugeng et al., 2008). From that parasternal long axis view, the presence of the cleft could be suspected based on an abnormal orientation of the anterior mitral leaflet towards the outflow septum (Sugeng et al., 2008). When available, colour Doppler mapping clearly demonstrates the location and extent of mitral regurgitation (Di Segni et al., 1992). Also, colour and septal Doppler identified left ventricular outflow obstruction caused by the mitral cleft attachments (Di Segni et al., 1992). The width of the cleft in some cases with normally related great arteries appear to increase with age and because the mitral regurgitation in isolated cleft of the mitral valve is usually progressive, early surgical intervention is recommended even when the mitral regurgitation is mild (Van Praagh et al., 2003; Zias et al., 1998). Our patient presented more frequently recurrence of congestive cardiac failure. Direct suture of the cleft is the preferred procedure, but glutaraldehyde-treated autologous pericardium can be used if there is lack of valvular tissue (Stelin et al., 2010). Mitral valve replacement is performed in adult patients whose valves cannot be repaired initially (Perier and Clausnizer, 1995). The most important complication of cleft repair is the need for reoperation (Van Praagh et al., 2003; Ohno et al., 1999). Complete correction of the mitral valve insufficiency is the most important factor affecting long-term complication (Perier and Clausnizer, 1995; Ohno et al., 1999). A rare long term complication which occurs when the regurgitation is not completely corrected which can occur if the surgeon fears mitral stenosis, is continued mitral regurgitation with accompanying marked left ventricular hypertrophy and dysfunction. This will then require cardiac transplantation (Van Praagh et al., 2003; Aharon et al., 1994).

Conclusion

This child would have benefited from early surgical intervention, but because of lack of facilities and poor financial power, the child has continued to suffer recurrent congestive cardiac failure.

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Case Report

Malignant peritoneal mesothelioma: Two cases and literature review

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Accepted 4 February, 2013

Malignant peritoneal mesothelioma (MPM) is a rare aggressive tumor arising from the mesothelial lining of the peritoneum. Only 20 to 30% of all mesotheliomas arise from the peritoneum itself. In the present paper, 2 cases of MPM were discussed, in light of the literature review. One of the cases is a 73 years old male admitted with complaints of abdominal swelling and ascites. Peritoneal biopsy was reported as epitheloid malignant mesothelioma. Further radiologic examination revealed that it was primarily of peritoneal origin. He was evaluated as inoperable. After the completion of systemic chemo, he is still on follow-up with stable disease on 10th month of diagnosis. The second case is a 51 years old female with a familial history of mesothelioma with the complaints of abdominal pain and weight loss. Because of the diffuse peritoneal carcinomatosis, she was diagnosed as inoperable peritoneal mesothelioma and after the start of chemotherapy she got clinically worse and died. MPM is a highly lethal neoplasm and given the rarity, it is difficult to obtain precise information regarding incidence, history and optimal management. Treatment approaches have traditionally been largely unsuccessful in this disease, but newer techniques are promising.

Key words: Peritoneal, mesothelioma, treatment.

INTRODUCTION

Malignant peritoneal mesothelioma (MPM) is a rare aggressive tumor arising from the mesothelial lining of the peritoneum. The natural history of peritoneal mesothelioma is that of rapid progression with fatal outcome without treatment. Only 20 to 30% of all mesotheliomas arise from the peritoneum itself (Bijelic et al., 2012). The clinical and radiologic presentation is nonspecific. In the present paper, 2 cases of MPM Were discussed in light of the literature review.

CASE REPORTS

Case 1

A 73 years old male, with a history of asbestos exposure,

presented with abdominal swelling. Abdominal tomography revealed massive ascites diffuse peritoneal thickenings and a subcutaneous nodular lesion on left thoracal area. Upper and lower gastrointestinal endoscopic examinations were normal. Peritoneal biopsy pathology was reported as epitheloid malignant mesothelioma staining positively with calretinin, mesothelin and CK-7. Further radiologic examination revealed that it was primarily of peritoneal origin. Since whole omental and peritoneal surfaces were invaded, the case was reported as inoperable and systemic chemotherapy consisting of cisplatin and pemetrexed combination was started. After 3 courses, partial response was detected and six courses of chemotherapy were completed. At the 10th month of diagnosis, he is still on follow-up with stable disease.

Case 2

A 51 years old female, having a family history of mesothelioma in her father, admitted with the complaints of abdominal pain and weight loss. Since radiologic imaging techniques did not reveal significant findings, laparoscopic examination was performed. Diffuse peritoneal carcinomatosis was detected, the case was inoperable and biopsies were taken during the procedure. The pathologic examination revealed a biphasic type of malignant mesothelioma staining positively for calretinin and pan-CK. After additional procedures, the case was evaluated as primary peritoneal mesothelioma. Systemic chemotherapy consisting of cisplatin and pemetrexed was started. After the third chemotherapy, on the fourth month of diagnosis, the patients clinic became worse and she died.

DISCUSSION

MPM is a highly lethal neoplasm and given the rarity, it is difficult to obtain precise information regarding incidence, history and optimal management. Despite the similarities between pleural and peritoneal mesotheliomas, clinical and prognostic features and precise nature of MPM may be different (Taşkın et al., 2012). There is a strong relationship between asbestos exposure and the development of mesothelioma at any location. The lifetime risk of developing mesothelioma among asbestos workers is thought to be as high as 10%, and the latency period between exposure and the development of mesothelioma is approximately 30 years. Although, asbestos exposure is the predominantly defined risk factor, there are also case reports of MPM arising in irradiated fields; Exposure to other mineral fibers (e.g. erionite, a silicate fiber of the zeolite family) is reported to be a risk factor for peritoneal as well as pleural mesothelioma (Baris et al., 1987). There are no signs or symptoms that are specific for MPM. Although most cases are symptomatic, a few are diagnosed incidentally, after inquiry into an unrelated process, such as infertility, or recognized during a routine physical examination. Diffuse MPM is highly aggressive, in contrast, patients with a localized MPM usually have a good prognosis following complete surgical excision.

Treatment approaches have traditionally been largely unsuccessful in this disease and consisted of systemic chemotherapy with surgery for palliation of symptoms. The median survival ranged from 9 to 14 months with these strategies (Antman et al., 1988; Sridhar et al., 1992). Alternative treatment strategies with cytoreductive surgery with heated intraoperative intraperitoneal chemotherapy in selected cases, has been used in a number of centers showing median survivals of 36 to 92 months (Brigand et al., 2006).

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Full Length Research Paper

Faecal carriage of extended-spectrum beta-lactamase (ESBL)-producing commensal *Klebsiella pneumoniae* and *Escherichia coli* from hospital out-patients in Southern Nigeria

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Accepted 8 February, 2013

Normal intestinal microflora is the major source from which common hospital- and community-acquired infections originate. This study aimed to determine faecal carriage of extended-spectrum beta-lactam (ESBL) resistant genes from commensals of out-patients in Nigeria. Non-duplicate *Klebsiella pneumoniae* and *Escherichia coli* from different hospitals were investigated for susceptibility to a panel of antibiotics, carriage of plasmid mediated β -lactam resistance, and analysis of plasmids present, including replicon typing. The minimum inhibitory concentrations (MICs) for β -lactam antibiotics showed MIC₉₀ of ≥ 256 $\mu\text{g/ml}$ for all antibiotics. CTX-M carriage was 36.8% for the 114 strains; 30 of these were CTX-M-15 and 12 carried CTX-M-2. TEM-1 genes were present in 102 isolates (89.5%), SHV-1 genes in 24 (21.1%), OXA-1 in 36 (31.6%) and 10 (8.8%) in AmpC genes detected. There was no significant difference in the proportion of ESBL genes detected in *E. coli* and *K. pneumoniae* (*t* test; $p = 0.64$; $p > 0.05$) and between hospitals ($\chi^2 = 0.35$; $p = 0.84$; $p > 0.05$). *IncF* was the common plasmid encoding β -lactamases. High faecal carriage of ESBL genes in commensals, importantly classical CTX-M-15 in out-patients is a reflection of the prevalence from clinical specimens in diseased conditions in Nigeria.

Key words: Faeces, bacteria, plasmid, extended-spectrum beta-lactam (ESBL) genes, Nigeria.

INTRODUCTION

Production of extended-spectrum β -lactamases (ESBLs) is the most common mechanism of resistance to third-generation cephalosporins among *Enterobacteriaceae* including *Klebsiella pneumoniae* and *Escherichia coli* (Paterson and Bonomo, 2005; Pitout and Laupland, 2008). ESBL determinants have been detected not only in clinical isolates but also in commensal bacteria from humans and animals and in isolates from products of the food chain and sewage, revealing distribution and suggesting the presence of environmental reservoirs for these resistance determinants (Brinas et al., 2004).

Reducing the spread of plasmid-mediated resistance

genes in hospitals requires the identification of the genes involved in order to control the movement of this resistance mechanism. Over the last 6 years, CTX-M (especially CTX-M-15) β -lactamase-producing *E. coli* have increased rapidly in number both within and outside the hospital environment and make up the vast majority of isolates (Woodford et al., 2004; White, 2008). CTX-M β -lactamases have been shown to be currently the most common cause of multidrug resistance in *E. coli* from other areas of the world; in particular, the Far East and South-East Asia, where rates can be as high as 50 to 70% (Hawkey, 2008), are a growing problem in some

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Table 1. Primers used to amplify genes for ESBLs.

Forward primer	Sequence (5' to 3')	Reverse primer	Sequence (5' to 3')	Annealing temp. (°C)	Product size (bp)
CTXM	CGATGTGCAGTACCAGTAA	CTXM	TTAGTGACCAGAATAAGCGG	60	585
OXA	ATATCTCTACTGTTGCATCTCC	OXA	AAACCCCTTCAAACCATCC	56	620
SHV1	AGGATTGACTGCCTTTTTG	SHV1	ATTTGCTGATTTTCGCTCG	56	393
TEMH	CCCCGAAGAACGTTTTTC	TEMC	ATCAGCAATAAACCCAGC	51	517
CTXM1	GACGATGTCACCTGGCTGAGC	CTXM1	AGCCGCCGACGCTAATACA	60	499
CTXM2	TGATACCACCACGCCGCTC	CTXM2	TATTGCATCAGAAACCGTGGG	60	341
CTXM8	CGCTTTGCCATGTGCAGCACC	CTXM8	GCTCAGTACGATCGAGCC	60	307
CTXM9	GCTGGAGAAAAGCAGCGGAG	CTXM9	GTAAGCTGACGCAACGTCTG	60	293
CTXM25	CGCTTTGCCATGTGCAGCACC	CTXM25	GCTCAGTACGATCGAGCC	60	307

parts of Europe (Hawser et al., 2011), Latin America (Pallecchi et al., 2007) and Senegal in Africa (Ruppe et al., 2009). To the best of our knowledge, no definitive studies have been done to determine faecal carriage of ESBLs-producing bacterial isolates in Nigeria in spite of report of high level of β -lactamases resistance genes in this environment including *TEM*, *SHV*, *OXA*, *CTX-M* and *AmpC* types (Ogbolu et al., 2011). This study was conducted to determine the carriage of ESBLs resistant genes in faecal isolates of commensal *E. coli* and *Klebsiella* species in out-patients from South Western hospitals in Nigeria.

MATERIALS AND METHODS

Bacterial isolates

In total, 114 strains of *K. pneumoniae* (60) and *E. coli* (54) were obtained from 122 non-duplicate faecal specimens of out-patients submitted to Medical Microbiology and Parasitology laboratory, for routine medical examination between 2010 and 2011. It was assumed they would not have taken any antibiotics nor had any contact with hospitals or health care facilities prior to submission of their samples since they were not sick or have any obvious illness. Single isolate from specimens without positive results of bacterial intestinal pathogens were retained and without intestinal disorder or other diseases based on the information on the requisition form. Isolates were from three teaching hospitals in South-Western Nigeria, namely University College Hospital, Ibadan (33), Obafemi Awolowo University Teaching Hospital, Ile-Ife (51) and Ladoko Akintola University of Technology Teaching Hospital, Osogbo (30). All isolates were confirmed using API 20E strips (bioMérieux, Marcy l'Etoile, France).

Antibiotic susceptibility testing

Antimicrobial disc susceptibility tests were carried out on the isolates on freshly prepared Mueller-Hinton agar (Oxoid, England) and were standardized by the method of Clinical and Laboratory Standard Institute (CLSI, 2007). The antibiotics and the disc contents are shown in Table 1. All susceptibility testing runs included the control organisms *E. coli* NCTC 10418. Plates with antibiotic discs were incubated for 24 h at 37°C and sensitivity pattern was compared with that of the control strain.

Minimum inhibitory concentrations (MICs) of a panel of β -lactam antibiotics were determined and interpreted using the agar dilution method according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC). All susceptibility testing runs included the control organism *E. coli* NCTC 10418.

Detection of ESBL

ESBL production was confirmed by double disc synergy test (Jarlier et al., 1988). ESBL-positive *K. pneumoniae* ATCC 700603 and ESBL negative *E. coli* ATCC 25922 control strains were used in these experiments.

Detection of AmpC

AmpC β -lactamase production was detected using cefepime and cefpodoxime discs alone or in combination with clavulanic acid as previously described (Derbyshire et al., 2009). AmpC β -lactamases were detected using a difference of ≥ 14 mm between cefepime/cloxacillin and cefpodoxime/cloxacillin discs. Difference of > 5 mm between cefepime/clavulanate and cefepime or cefpodoxime/clavulanate and cefpodoxime discs was used to detect ESBL in the presence of AmpC which would have been missed with double disc synergy test mentioned earlier.

Amplification of β -lactam genes

Polymerase chain reaction (PCR) was used to detect genes encoding resistance to β -lactams; *blaOXA*, *blaSHV*, *blaTEM*, *blaCTX-M* (Table 1) (Maynard et al., 2003) and *blaAmpC* (Table 2) (Tan et al., 2009) as previously described. All the amplimers resulting from these PCR reactions were sequenced to confirm the identity and specific variant of each gene identified and sequences were aligned to known reference sequences using ClustalW.

Conjugational transfer of antibiotic resistance

Mating was done for β -lactamases on selected *E. coli* or *K. pneumoniae* using *E. coli* DH5 α with a chromosomal mutation conferring rifampicin resistance as recipient cells. All mating procedures were done on filters for 18 h at 37°C, 200 μ l of mixed cultures were plated onto selective plate containing rifampicin (100 μ g/ml) and ampicillin (50 μ g/ml). Transconjugants were confirmed by susceptibility and amplification of ESBL and *AmpC* genes.

Table 2. Primers used for amplification of *ampC* genes.

Primer	DNA sequence (5' to 3')	Target(s)	Amplicon size (bp)
MOXMF MOXMR	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	520
CITMF CITMR	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	462
DHAMF DHAMR	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	DHA-1, DHA-2	405
ACCMF ACCMR	AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC	ACC	346
EBCMF EBCMR	TCG GTA AAG CCG ATG TTG CGG CTT CCA CTG CGG CTG CCA GTT	MIR-IT, ACT-1	302
FOXMF FOXMR	AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG	FOX-1 to FOX5b	190

Table 3. Antimicrobial disc susceptibility pattern of 114 bacterial strains.

Antibiotic (µg/ml)	Sensitive strains (%)	Resistant strains (%)	Intermediate strains (%)
Ceftazidime (30)	78 (68.4)	21 (18.4)	15 (13.2)
Amoxyclav (30)	45 (39.5)	30 (26.3)	39 (34.2)
Cefotaxime (10)	6 (5.3)	66 (57.9)	42 (36.8)
Cefpodoxime (10)	27 (23.7)	81 (71.1)	6 (5.3)
Gentamicin (10)	45 (39.5)	66 (57.9)	3 (2.6)
Ciprofloxacin (5)	54 (47.4)	42 (36.8)	18 (15.8)
Pefloxacin (5)	60 (52.6)	42 (36.8)	12 (10.5)
Amoxicillin (25)	22 (19.3)	83 (72.8)	9 (7.9)
Tetracycline (30)	6 (5.3)	106 (93.0)	2 (1.8)

Digestion of plasmid DNA with restriction enzymes

Plasmids from transconjugants were digested with Eco RV (Promega MADISON, WI U.S.A.) to determine their physical characteristics. Reactions were carried out in samples that contained 8 µl of plasmid DNA, 2 µl of 10X buffers, 2 µl of EcoRV, and made up to 20 µl with sterile nuclease free water. They were incubated at 37°C for 3 h; 20 µl of each digestion mixture was subjected to electrophoresis on 0.7% agarose gel. They were visualised under ultra violet (UV) light on a 'Gene Genius' image analyser (SYNGENE, Cambridge, U.K.). The restriction digestion patterns were compared to each other.

Estimation of plasmid size

Plasmid size was estimated as previously described (Wang et al., 2003).

Identification of plasmids by PCR-based replicon typing

Incompatibility/replicon PCR-based typing was used in order to

trace plasmids conferring drug resistance representing the major plasmid incompatibility groups circulating among the *K. pneumoniae* and *E. coli* as was previously described (Carattoli et al., 2005). Total DNA was generated for PCR using the Wizard genomic DNA purification System (Promega, Madison, WI).

Statistics analysis

Data were analysed using the statistical package within Microsoft Excel. Student *t*-test was done to determine the significant difference between distribution of ESBL genes in *E. coli* and *K. pneumoniae*, while Chi square was used to determine the association between distribution of ESBL genes and hospitals. In both cases, *p* value less than 0.05 was considered to be significant.

RESULTS

Table 3 shows the disc susceptibility of 114 bacterial isolates. Ceftazidime and pefloxacin had the highest susceptibility of 68.4 and 52.6%, respectively, while least

Table 4. MIC of bacterial strains Panel I: MIC of 114 bacterial strains.

Organism (no. of strains)	Panel I: MIC by strains				
	Antimicrobial agent	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Range (µg/ml)	
<i>E. coli</i> (54)	Ceftazidime	0.5	256	0.5 – 512	
	Cefpodoxime	1	256	0.5 - 512	
	Cefotaxime	1	512	0.5 – 512	
<i>K. pneumoniae</i> (60)	Ceftazidime	0.5	256	0.5 – 512	
	Cefpodoxime	1	512	0.5 - 512	
	Cefotaxime	1	512	0.5 – 512	
Hospitals (no. of strains)					
	<i>E. coli</i> (10)	Ceftazidime	0.5	2	0.5 - 512
		Cefpodoxime	0.5	4	0.5 - 512
Cefotaxime		0.5	4	0.5 - 512	
<i>K. pneumoniae</i> (12)	Ceftazidime	0.25	1	0.5 - 512	
	Cefpodoxime	0.5	1	0.5 - 512	
	Cefotaxime	0.5	2	0.5 - 512	

MIC: Minimum inhibitory concentration.

Table 5. Faecal carriage of ESBL genes.

Species	N	Phenotypic detection			PCR positive for ESBL genes				
		n (%)			n (%)				
		ESBL	AmpC	TEM-1	SHV-1	OXA-1	CTX-M-15	CTX-M-2	AmpC
<i>E. coli</i>	54	12 (22.2)	3 (5.6)	44 (81.5)	9 (16.7)	12 (22.2)	17 (31.5)	5 (9.3)	3 (5.6)
<i>K. pneumoniae</i>	60	6 (10)	8 (13.3)	58 (96.7)	15 (25)	24 (40)	13 (21.7)	7 (11.7)	7 (11.7)
Total	114	18 (15.8)	11 (9.6)	102 (89.5)	24 (21.1)	36 (31.6)	30 (26.3)	12 (10.5)	10 (8.8)

susceptibility of 5.3% was found in cefotaxime and tetracycline. Appreciable level of intermediate was also recorded especially with cefotaxime, 36.8% and augmentin, 34.2%. The MICs for β -lactam antibiotics are shown in Table 4. The results showed a high degree of resistance, with MIC₉₀ values (MIC for 90% of the organisms) of ≥ 256 µg/ml for all antibiotics. It is noteworthy, the low value of MIC₅₀ in spite of the high MIC₉₀.

Of the 114 bacteria strains, 18 (15.8%) produced ESBL by double disc diffusion test, of which 12 of 54 (22.2%) *E. coli* and 6 of 60 (10%) *K. pneumoniae* are ESBL producers. Similarly, phenotypic detection of AmpC revealed that AmpC enzymes were found in 11 strains (9.6%), 3 (5.6%) in *E. coli* and 8 (13.3%) in *K. pneumoniae*.

All 114 strains were amplified by PCR; CTX-M carriage (Figure 1) was found in 42 strains (36.8%). Sequencing identified these as 30 CTX-M-15 and 12 CTX-M-2. OXA-1 genes (Figure 2) were present in 36 isolates (31.6%), TEM-1 genes (Figure 3) in 102 (89.5%) and SHV-1 (Figure 4) in 24 (21.1%). Multiplex PCR for detection of AmpC genes (Figure 5) confirmed 10 (8.8%) isolates

carry non-chromosomal AmpC enzymes (Table 5), these genes were only found in isolates with CTX-M genes. Of the 10 AmpC-positive genes, 5 amplified using ACC group primers, 3 by DHA group primers and 2 by FOX primers. Sequencing identified these genes as ACC, DHA-1 and FOX-1, respectively. The ESBL genes cut across the three hospitals in varying proportions (Table 6). There was no significant difference in the proportion of positive ESBL genes detected in *E. coli* and *K. pneumoniae* (*t* test; $P = 0.64$; $P > 0.05$). Furthermore, no association was found between hospital and distribution of ESBL genes in patients' faeces ($\chi^2 = 0.35$; $p = 0.84$; $p > 0.05$).

Transfer of the ESBL resistance phenotype was successful for all the selected strains. Restriction digestion products to further type plasmids showed several bands having similar patterns (Figures 6 and 7). The transconjugant plasmids sizes were estimated to be 108 kb. Replicon type of the common plasmids for all β -lactamases was found to be *IncF*. This confirmed presence of a common plasmid in these strains.

Table 6. Distribution of ESBL genes in hospitals.

Hospital	N	ESBL genes [n (%)]					
		<i>TEM-1</i>	<i>SHV-1</i>	<i>OXA-1</i>	<i>CTX-M-15</i>	<i>CTX-M-2</i>	<i>AmpC</i>
UCH	33	30 (90.9)	8 (24.2)	12 (36.4)	10 (30.3)	2 (6.1)	3 (9.0)
OAUTH	51	46 (90.2)	11 (21.6)	16 (31.4)	14 (27.5)	6 (11.8)	3 (5.9)
LTH	30	26 (86.7)	5 (16.7)	8 (26.7)	6 (20.0)	4 (13.3)	4 (13.3)

N: Frequency of strains in hospital; UCH: University College Hospital, Ibadan; OAUTH: Obafemi Awolowo University Teaching hospital; LTH: Ladoke Akintola University Teaching Hospital. Chi square= 0.35; $p = 0.84$, $p > 0.05$.

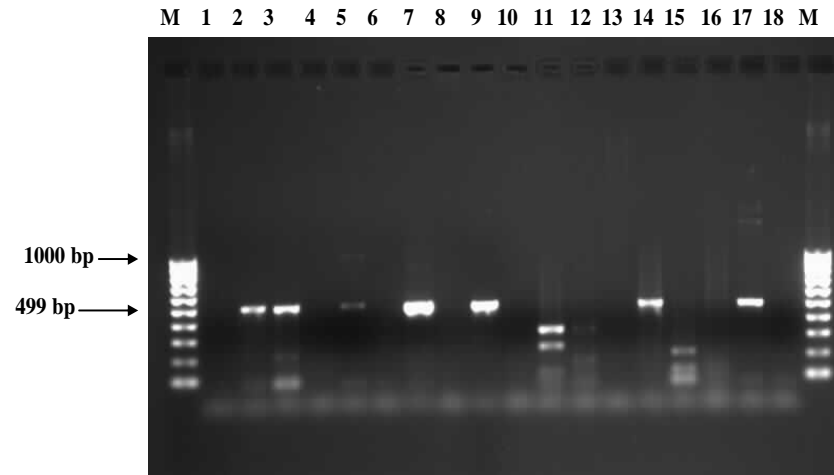


Figure 1. PCR amplification of CTX-M group 1. Lane m: Hyperladder IV; 9 Bands from 100-1000bp, lane 25: positive control, lane 26: negative control, lanes 1 to 24: tests. Product size is 499 bp.

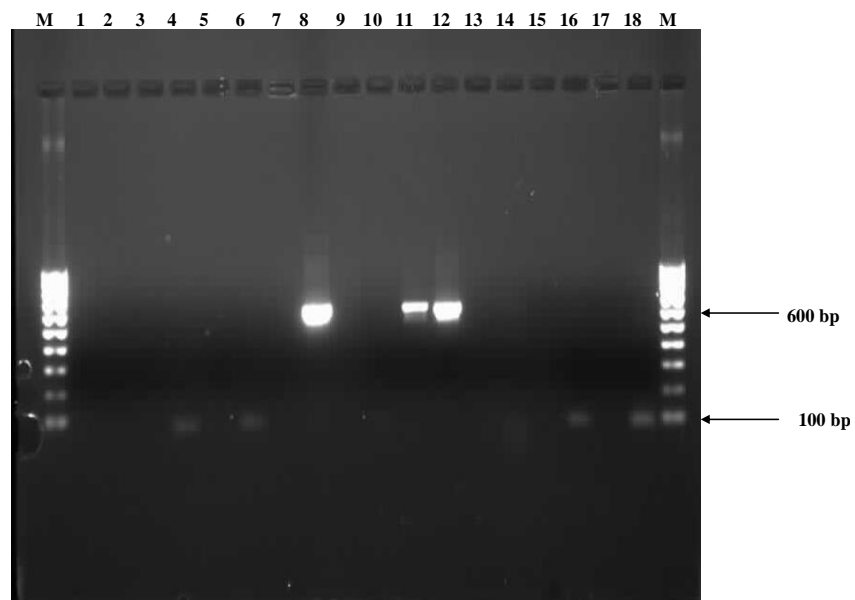


Figure 2. PCR amplification of OXA gene type for ESBL. Lane M: Hyperladder IV; 9 Bands from 100-1000bp, lane 18: Negative control, lanes 1 to 17: tests. Product size is 620 bp.

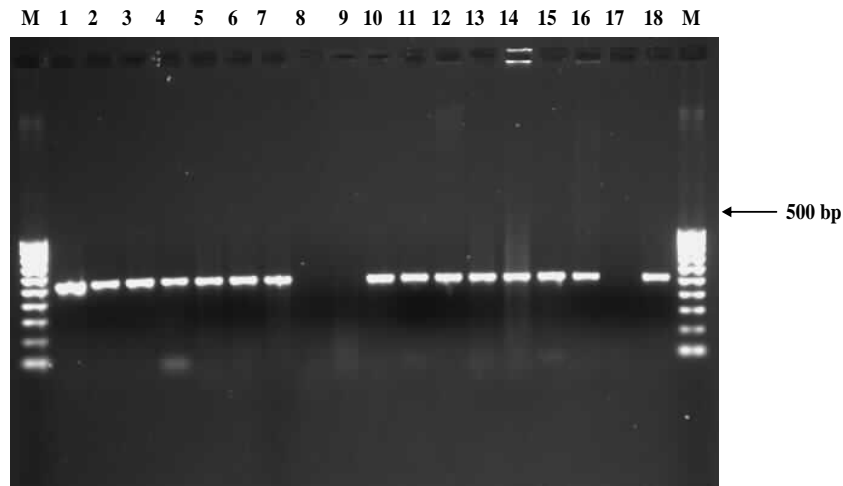


Figure 3. PCR amplification of TEM gene type for ESBL. Lane M: Hyperladder IV; 9 Bands from 100-1000 bp, lanes 1 to 18: tests. Product size is 517 bp.

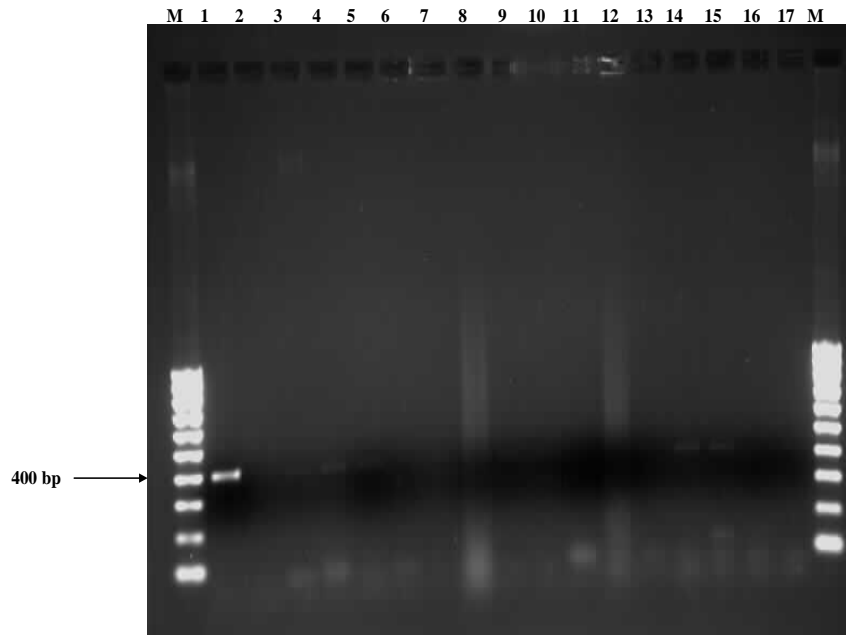


Figure 4. PCR amplification of SHV gene type for ESBL. Lane M: Hyperladder IV; 9 Bands from 100-1000 bp, lanes 1 to 17: tests. Product size is 393 bp.

DISCUSSION

The faecal strains demonstrated high level of resistance to all the drugs tested, including the cephalosporins and fluoroquinolones. These faecal strains can serve as reservoir to pathogens in the spread of resistance. *E. coli* and *K. pneumoniae* are known to be the leading cause of primary and opportunistic infections in human. Hence, they can be incriminated in virtually any type of infectious disease. ESBL phenotype is lower, 18 (15.8%) than previous reports from Nigeria, where a previous study that found 8 (20%) of 40 *Enterobacter* isolates to produce

an ESBL by the double disc diffusion test (DDDT) (Aibinu et al., 2003) and 28 (28.9%) of 134 Gram negative bacteria carried an ESBL (Ogbolu et al., 2011). This lower rate may not be unconnected with the fact that the strains are commensals in view of their origin, though they may cause various types of infection in other sites. Similarly, 8.8% occurrence of AmpC producers was obtained, this was predominantly found in *K. pneumoniae*. High level faecal carriage ESBL genes was found in our environment, previous studies from other countries showed Senegal, 10% (Ruppe et al., 2009) and Europe, 21.6% travellers with resistant *E. coli* post-travel

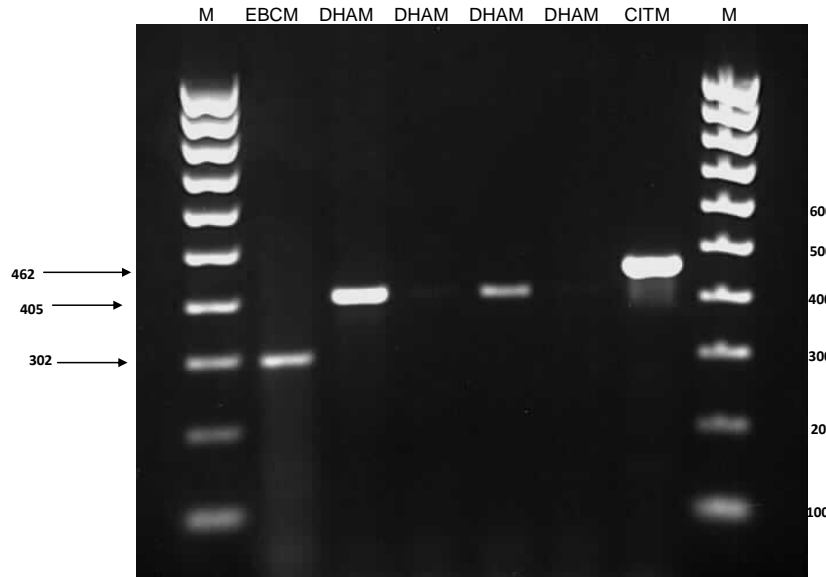


Figure 5. Positive ampC multiplex PCR products. Lanes are labeled with the ampC primers used. Lane M: Hyperladder IV. The amplified product size is indicated on the left. EBCM and CITM are positive controls, while DHAMs are tests

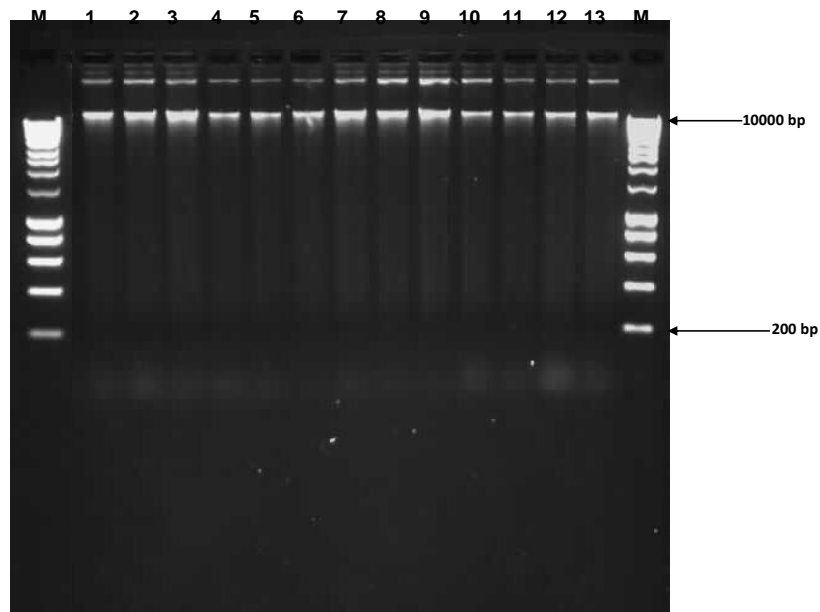


Figure 6. Transformants plasmid DNA. Lane M Hyperladder I; 14 bands from 200-10,000 bp. Lane 1 to 13 are transformants.

(Kennedy and Collignon, 2010). Extremely high rates from 50 to 70% have been found in the Far East and South-East Asia (Hawkey, 2008); particularly India, 100% (Muzahed et al., 2009). There has been an increase in the isolation of CTX-M-producing bacteria in the last 20 years globally and these genes are spread worldwide. Classical CTXM-15 has been described in Nigeria from

clinical specimens (Ogbolu et al., 2011) and its presence confirms the rapid spread and global phenomenon (Ruppe et al., 2009; Oteo et al., 2010). It is however surprising that CTX-M-3 that was first reported in Nigeria (Ogbolu et al., 2011) in the same environment was not found, instead CTX-M-2 was detected, a new variant to Nigeria strains. DHA-1 was the only AmpC in this study

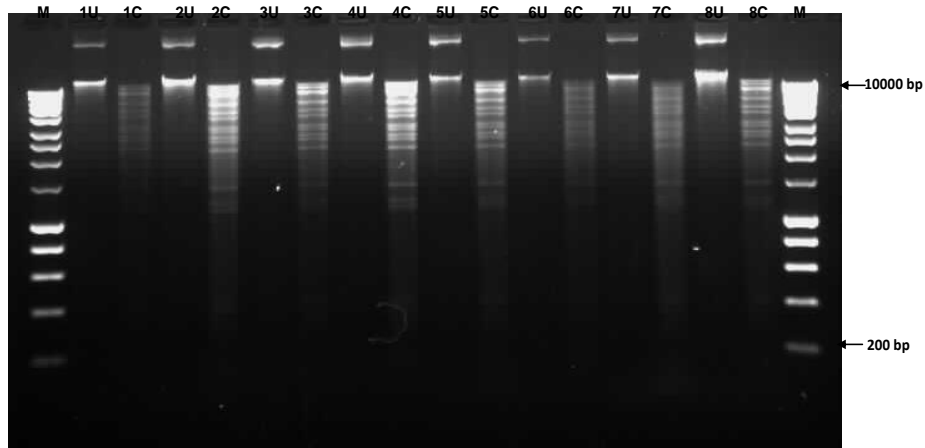


Figure 7. Restriction enzyme *EcoRV* digest of transformants plasmid DNA. Each transformant has two lanes, the first uncut and second cut DNA. Lane M: Hyperladder I; 14 bands from 200-10,000 bp. U-Uncut; C-Cut.

that was previously identified in our environment (Ogbolu et al., 2011) and was the most common. In accordance with our previous study, *AmpC* genes were found in isolates co-producing other ESBL genes such as *CTX-M-15*, *CTX-M-2*, *TEM-1*, *SHV-1* and *OXA-1* genes. This is different from the study of Muzahed et al. (2009) where *TEM* and *SHV* were not detected in *K. pneumoniae* faecal strains of patients with acute gastroenteritis in India.

There was no significant difference in the proportion of positive ESBL genes detected in *E. coli* and *K. pneumoniae* and between hospitals. This may be due to the fact that these hospitals are within the South-West region of Nigeria about 100 km to each other and obviously there are contacts among these inhabitants within this region, including inter-hospital patients transfer. Also, orally administered antimicrobials including fluoroquinolones are available over-the-counter and frequently used for the purpose of self-medication in Nigeria. There is a common plasmid conferring different antimicrobial resistance in *bla* genes in the region evident by restriction digestion and *IncF* obtained from PCR-based replicon typing.

The discordance found between ESBL phenotype and PCR method could be that bacteria also reduce the fitness cost of resistance by silencing the genes when not required. There is little documented evidence for gene silencing in bacteria in general or for silencing of resistance genes in particular (Yarmolinski, 2000). In clinical settings, carriage of antibiotic resistance genes is generally assumed on the basis of phenotype, and in most genotypic investigations, only resistant isolates are screened for the presence of particular genes conferring antibiotic resistance. Accordingly, if silent genes were present, most surveys of resistant bacteria would fail to detect them. Expression of intact antibiotic resistance gene systems can be switched off in bacteria, that is,

resistance genes can be silenced (Enne et al., 2006), and this process is reversible.

The existence of faecal carriage of *CTX-M* genes has clinical implications, as intestinal tract colonization is prerequisite for infection by ESBLs-producing organisms. What is less clear is the route by which community infections arise. On one hand, many patients with 'community' infections with *CTX-M*- β -lactamase-producing *E. coli* have a history of recent hospitalization (Soge et al., 2006), where they may have been colonized. Not all colonized individuals have a history of hospitalization and it may be that low-level gut colonization occurs in the community, via the food chain. This study showed that β -lactam determinants were located in transferable plasmids. The transferable nature of these resistance genes is particularly worrisome, and treatment options for infections caused by these organisms are very limited, and this may account in part for the association between fluoroquinolones resistance and expanded-spectrum cephalosporins. The selection of such highly resistant isolates in countries such as Nigeria may act as a reservoir of resistant strains that can be transferred to other countries in the era of global travel.

Conclusion

There is high faecal carriage of ESBL genes in commensal isolates, importantly classical *CTX-M-15* in out-patients in Nigeria. Screening populations for faecal or rectal carriage would be the obvious way to resolve these issues, but has not yet been undertaken on any wide scale. This calls for enhanced infection control and a better understanding of resistance mechanisms, molecular epidemiology and the means by which spread occurs.

ACKNOWLEDGEMENTS

Molecular biology works were all carried out in the Molecular Biology Laboratory, Department of Biomedical Sciences, Ladoke Akintola University of Technology, Mercyland Campus, Osogbo. We acknowledge Mr. Oyenike of the Molecular Biology Laboratory for his technical assistance. We would also like to thank the Medical Laboratory Scientists of the various hospitals.

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Full Length Research Paper

Status of urinary schistosomiasis among primary school children in Abeokuta, Nigeria

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Accepted 11 January, 2013

A study was carried out to determine the current status of urinary schistosomiasis on 552 pupils from seven primary schools in Abeokuta North and Abeokuta South Local Government Areas of Ogun State, Nigeria using haematuria and parasitological tests. Males that complied were 75% to 25% females. Children within the age-group of 10 to 14 years were more (65%) when compared with other age groups. Out of the 552 samples examined, 35 (6%) tested positive for blood in urine while 20 (3.6%) tested positive for *Schistosoma haematobium* ova. The percentage of the females that tested positive were higher than male; however, the difference in prevalence was not significant ($p>0.05$). Though, the prevalence of the infection was relatively low, there is still need for coordinated public enlightenment of the pupils on the danger of contacting the infection from contaminated rivers and streams in their surrounding areas. Government also needs to provide more social amenities to reduce or eradicate the infection in the study area.

Key words: Schistosomiasis, school children, Abeokuta, Nigeria.

INTRODUCTION

Schistosomiasis is a human disease condition which is caused by infection from one of several species of parasitic trematodes of the genus: *Schistosoma* (WHO, 1993). Schistosomiasis affects about 200 million people and poses a threat to 600 million people in more than 76 countries of the world, including Nigeria (Ekpo and Mafiana, 2004). Schistosomiasis is next to malaria as a source of human morbidity and mortality in Africa, South America, the Caribbean, the Middle East and Asia with varying prevalence's (WHO, 1993).

Water contact activities and traditional agricultural practices are reported as the factors in the distribution of the disease and its snail vectors (Ukoli, 1990). Humans become infected when they come in contact with the water containing the cercaria that penetrates through the

skin. The larva then attaches itself to the nearest blood vessel where it undergoes larval migration.

For a large population, the efficacy of the reagent strip might be low. However, in screening of communities, reagent strip-detected haematuria might be a reliable predictor for a *Schistosoma haematobium* infection than previously reported, although stratification by sex and age tends to increase their validity (WHO, 1993). Therefore, the objective of this study was to determine the prevalence and intensity of urinary schistosomiasis among primary schools children in some selected schools in Abeokuta North and Abeokuta South Local Government Areas of Ogun State. This will provide information for preventive chemotherapeutic treatment towards elimination of the disease.

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Table 1. Demographic data of pupils.

Description	Frequency	%
Age (years)		
10 - 14	361	65
15 - 19	180	33
>19	11	2
Total	552	100
Sex		
Male	415	75
Female	137	25
Total	552	100

Table 2. Prevalence of haematuria among the study participants.

Blood	Frequency	%
Negative	517	94
Positive	35	6
Total	552	100.0

MATERIALS AND METHODS

Study area

This study was carried out in Abeokuta, Ogun State, Nigeria. The city is located on approximately 7°11'N and 3°21'E in the rain forest belt, with an annual rainfall of 963.3 mm. 552 pupils were selected from 5 primary schools in Abeokuta North Local Government Area and 2 primary schools in Abeokuta South Local Government Area based on a previous survey for urinary schistosomiasis by Ekpo et al. (2008) that classified primary schools in Abeokuta areas into high risk, low risk and negative. Eighty pupils from each school in primary 4 to 6 classes of aged 10 to 20 years were enrolled for the study.

The schools in Abeokuta North Local Government Area are NUD Primary School, Ago Ika, Abeokuta North Local Government Primary School; Iberekodo, Abeokuta North Local Government Primary School; Olomore, Abeokuta North Local Government Primary School, Oke Ago-Owu, and Muslim Primary School, Ago-Oko. The schools in Abeokuta South Local Government Area are St Paul's Primary School, Igboire and Methodist Primary School, Ogbe. These schools are situated in areas of Abeokuta that are close to Ogun river that people utilized for domestic, agricultural and recreational activities.

Ethical consideration

Consent was obtained from the local government authorities before the commencement of the study. Letters were obtained from the Ogun State Primary Education Board (SPEB) which granted the request for permission to conduct the study in the schools. The letters were tendered at the Abeokuta North and Abeokuta South Local Government Education Authorities. These letters were approved and the approved letters were distributed to the schools accordingly before the study began.

Specimen collection

Dark (black), sterile, plastic universal containers (labelled) were given to the pupils to collect terminal urine samples. This was done between the hours of 11.00 am to 1.00 pm. The collected urine was then taken immediately to the laboratory for analysis. A total of 552 pupils from the 7 schools returned their samples for the study.

Urine examination

The terminal urine samples collected were immediately tested for blood using reagent test strip, combi-9-screen made by Analyticon Biotechnologies AG, Germany. The reagent strip was carefully dipped into the sample bottle and the readings were recorded within 60 s. Ten millilitres of the urine sample (which was duly labelled) was measured into the centrifuge tube and spun for 5 min at 500 rpm. Afterwards, about 9 ml (the supernatant) was removed from the spanned sample using a syringe. The other 1 ml (the deposit) left was prepared for microscopy. Small quantity of the deposit was placed at intervals onto a clean, grease-free slide; a cover slip was placed gently on it in a slanting form to avoid air-bubbles. It was then observed under the microscope and viewed with different magnifications. The haematobium eggs were identified, analyzed, counted and recorded as eggs/10 ml of urine (Sam-Wobo et al., 2011).

RESULTS

A total of 552 pupils were enrolled which consist of 415 (75%) males and 137 (25%) females. The ages of the pupils range from 10 to 20 years with the mean age of 15 years. The 10 to 14 years group had the highest number of pupils (65%) (Table 1).

Prevalence of microhaematuria and *S. haematobium* ova

Out of the 552 samples examined, 35 (6%) tested positive for blood in urine while 94% were negative (Table 2). The results of the prevalence of *S. haematobium* ova in the urine samples showed that 20 (3.6%) were positive with higher prevalence in females than males. Though, the difference in prevalence was not significant ($p > 0.05$) (Table 3). The age-wise consideration showed that there was no significant difference in the infection across the age, even though, the pupils in age group 10 to 14 were more infected than the other groups (Table 3). Only 2 pupils (15%) of the 20 infected pupils passed out more than 20 eggs per 10 ml of urine at a time. Majority of the infected pupils (55%) had the least occurrence or intensity of the egg, excreting 1 to 10 eggs per 10 ml of urine (Table 4).

DISCUSSION

The results of the present study showed reduction in the prevalence of *S. haematobium* in Abeokuta from 11.9%

Table 3. Prevalence of *S. haematobium* by age and sex.

Age	Male			Female			Total		
	Number examined	Number infected	%	Number examined	Number infected	%	Number examined	Number infected	%
10-14	292	9	3.1	69	5	7.2	361	14	3.9
15- 19	115	4	3.5	65	2	3.1	180	6	3.3
>19	8	0	0	3	0	0	11	0	0
Total	415	13	3.1	137	7	5.1	552	20	3.6

Table 4. Egg Counts by sex among the infected persons.

Egg count/10 ml of urine	Male	Female	Total
	No. infected (%)	No. infected (%)	No. infected (%)
1-10	7 (53.8)	4 (57.1)	11 (55.0)
11-20	4 (30.8)	2 (28.6)	6 (30.0)
>20	2 (15.4)	1 (14.3)	3 (15.0)
	13 (100.0)	7 (100.0)	20 (100.0)

(Mafiana and Beyioku, 1998) to 3.6%. The reduction in the prevalence of the infection may be due to the availability of alternative sources of water for domestic uses and improved waste disposal system. The degree of prevalence of schistosomiasis has been known to depend largely on frequency of man contact with contaminated water and the environmental sanitation activities (Hassan et al., 2012).

Though, females infected were higher than males, statistical analysis showed no significant difference in the occurrence of the infection in both sexes. This result is at variance to previous results (Mafiana and Beyioku, 1998; Akinwale et al., 2009) and the pattern of the results may be due to variation in the number of participating sexes as more males participated than the females. The higher infection among pupils of the 10 to 14 years group could be attributed to degrees of exposure. This result is in agreement with previous studies that the prevalence of urinary schistosomiasis usually increases with age, reaching a peak at age 15 (Fajewonyomi and Afolabi 1994, Hassan et al., 2012). This is because children within the age of 10 to 15 usually engage in many outdoor activities including swimming and fishing due to the youthful exuberance.

Despite the prevalence, the average count was relatively low as only 3 (15%) of the total infected persons had more than 20 eggs per 10 ml of urine. The study observed that the number of pupils that tested positive for microhaematuria using the reagent test strip were higher than those who were actually infected with disease. This result shows that the positive haematuria may not necessarily signifies the presence of urinary schistosomiasis. The pupils may also be infected with other urinary-tract diseases. This therefore calls for caution when using

haematuria results to interpret the level of endemicity of a community to urinary schistosomiasis.

In conclusion, this study showed that the prevalence of urinary schistosomiasis has reduced in Abeokuta metropolis. However, there is still need for more concerted efforts in public enlightenment of the pupils on the route on the danger of contacting contaminate rivers and streams in their surrounding areas. Government also needs to provide more social amenities to reduce or eradicate the infection in the study area.

ACKNOWLEDGEMENTS

The authors thank the Heads and pupils of all the schools that participated in this study.

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Full Length Research Paper

Percutaneous bone marrow grafting in delayed union and non-union

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Accepted 5 November, 2012

Percutaneous bone marrow injection is a simpler method of performing autologous bone grafting with less morbidity than standard technique. Autologous bone grafting has been a standard operative method used for the treatment of delayed union and non-union for decades but has been associated with numerous complications. In recent times, percutaneous bone marrow grafting has emerged as a successful alternative to traditional method of open bone grafting. The medical records and radiographs of 50 patients of delayed and non-union were analysed between 2007 to 2010 in our institution. Among those, 38 cases were of delayed union and 12 of non-union involving different bones of the body, with tibia as the most common site (54%). 39 cases were males and 11 females. Mode of injury was road traffic accident in 70% of the cases. Duration of injury was 20 to 24 weeks in 22% of cases followed by 24 to 28 weeks in 18% of cases. Among 50 cases, local anaesthetic was used in 39 cases. Only one injection was given in 30 cases (60%) and two injections in 20 cases (40%). 73.3% cases of delayed union required only one injection and out of 12 cases of non-union, 10 cases (83.3%) required two injections. The cases were evaluated radiologically after a variable period of time for callus formation. Mean time taken for union in successful cases was 14.6 weeks in delayed union and 18.4 weeks in non-union. Out of 50 cases, union was achieved in 46 cases. Union rate was more in case of delayed union (97.37%). In case of non-union, percutaneous bone marrow injection was successful in only 75% of the cases. We concluded that percutaneous autologous bone marrow grafting has been most useful for preventive treatment of non-union by early injection in delayed union, with less complications and morbidity. The general idea is that the minimally invasive technique of percutaneous bone marrow grafting is worth exploring before embarking on a more extensive open surgery.

Key words: Percutaneous bone marrow grafting, delayed union, non-union, injection.

INTRODUCTION

Autologous bone grafting has been a standard operative method used for the treatment of delayed union and non-union for decades but has been associated with numerous complications. Autologous bone potentially contributes three vital components for healing which are

osteoconduction, osteoinduction and osteogenic cells but operative harvesting is associated with numerous complications both at donor and recipient sites. In addition, the need to open the recipient site has added to the risk of devascularisation of the fracture where healing

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is already impaired. The non-operative methods include the use of low intensity ultrasound, electrical stimulation and electromagnetic stimulation but all these procedures are tedious, require sophisticated equipments, expertise and anaesthesia for surgery and are time consuming, hence a continuous search has been made to find out such an alternative method of treating delayed and non-union which is safe, easy and economical.

In recent times, percutaneous bone marrow injection has emerged as a successful alternative to traditional methods of treatment. Bone marrow injection has shown to have stimulated callus formation where autologous bone grafting has failed. Though all of these methods have shown varying degrees of excellent to good results but most of the techniques are tedious and require sophisticated equipments, expertise, anaesthesia and are time consuming, with added risk of infection (Garg et al., 1993). Osteogenic precursor cells which are capable of producing bone have been demonstrated among the stromal and endosteal cells of bone marrow, which are the key elements in the process of bone formation and fracture healing (Friedenstein, 1973). The determined and inducible marrow cells supplement periosteal and primitive mesenchymal cells to form cellular component of bone healing (Gray and Elves, 1979). It is a minimally invasive procedure with negligible complications.

Encouraged by the simplicity and minimal complication rate of bone marrow grafting for delayed and non-union in experimental studies and clinical trials, the prospective study has been taken in our institution to evaluate the efficacy of this procedure.

MATERIALS AND METHODS

50 cases of post-traumatic delayed and non-union (out of 50 cases 38 were delayed and 12 were non-union), irrespective of their age and sex, were selected from orthopaedic out patient department from 2007 to 2010 after which they were examined clinically and radiologically to establish the diagnosis on the basis of following criteria.

Inclusion criteria

Clinical criteria include: 1. Age of fracture more than 12 weeks; 2. Abnormal mobility at the fracture site; 3. Tenderness at the fracture site; 4. Pain on applying bending stresses.

Radiological criteria include: 1. Gap at the fracture site; 2. Insufficient amount of callus; 3. Sclerosis of fracture ends; 4. Obliteration of bone marrow cavity at the fracture sites.

Exclusion criteria

Patients with infection and local malignancy were excluded from the study.

Operative technique

The procedure was performed as an outdoor/indoor procedure under local/short general anaesthesia. Under all aseptic

precautions, patient was put in supine position and donor and recipient sites were prepared separately but simultaneously to prevent cross contamination of needles. Bone marrow was aspirated from the donor iliac crest with a bone marrow aspiration needle connected to a 20 ml syringe. About 10 to 15 ml of marrow was aspirated from one site and to obtain more, multiple aspirations were done. The aspirated marrow was injected percutaneously immediately at the recipient fracture site with the help of a 16 gauge spinal needle under image intensifier. On random basis, aspirate slides were made to confirm bone marrow cells under microscope. After bone marrow injection, the recipient site was immobilised either by plaster of Paris cast or with the help of braces. Donor site was dressed and sealed. Serial X-rays were taken at interval of 4 to 6 weeks (including special views to see callus formation) and if needed second injection was given.

Assessment of results

All cases were followed after an interval of 4 to 6 weeks for 6 months following the bone marrow injection and then after a variable period of time depending on callus formation. The clinical as well as radiological assessment of union was done.

Criteria for union

Clinical criteria for union include: 1. No abnormal mobility at the fracture site; 2. No pain at the fracture sites on applying bending stresses; 3. No tenderness.

Radiological criteria for union include: 1. No gap at the fracture site; 2. Sufficient amount of callus.

RESULTS

Percutaneous bone marrow grafting was done in 50 patients of delayed union and non-union over a period of two years and was followed for an average period of 18 months. Out of 50 patients, 33 (66%) were found in the age group of 21 to 40 years. One case was above 60 years and 2 patients were below 21 years of age. 39 (78%) cases were males and 11 (22%) were females. 35 (70%) of our cases had road traffic accident as mode of injury, 11 (22%) cases as fall and 4 (8%) cases as assault. Duration of injury is shown in Table 1. Mean duration of injury was 28.1 weeks.

The study included 38 (76%) cases of delayed union (Case 1) (Figures 1, 2, and 3) and 12 (24%) cases of non-union. Prior to bone marrow injection various modes of internal fixation (Case 2) (Figures 4, 5, and 6) were done in 17 (85%) cases and external fixation in 3 (15%) cases and rest of 30 cases were treated conservatively before bone marrow injection. Out of 50 cases, local anaesthesia was used in 39 cases and in 11 cases, procedure was done in short general anaesthesia. Only one injection was needed in 30 (60%) cases and in 20 (40%) cases, injection was repeated. In none of the cases, more than two injections were given. Out of 12 cases of non-union, 10 of them required second injection. Results were finalised after careful clinical and radiological examination and final inference was made as given in Table 2.



Figure 1. Delayed union humerus.

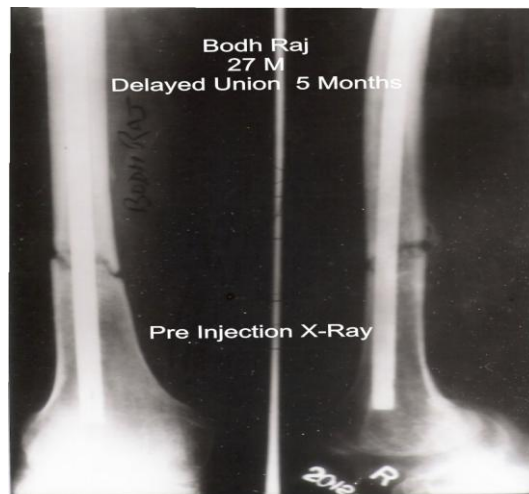


Figure 4. Delayed Union distal femur, 5 months.



Figure 2. 6 Weeks after bone marrow injection.

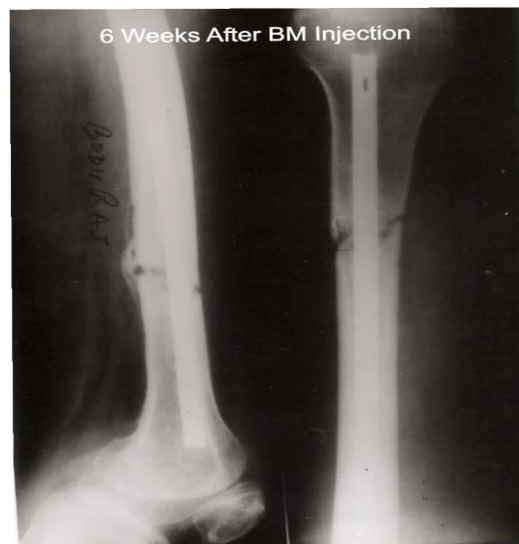


Figure 5. Distal femur delayed union, 6 weeks after bone marrow injection.



Figure 3. 12 weeks after bone marrow injection.

Out of the 4 cases in which union was not achieved, three of them were non-union and one delayed union. The mean time for appearance of callus in successful cases was 4.9 weeks and most of the cases united within 12 to 22 weeks after bone marrow injection. Mean time taken for union in successful cases is given in Table 3. In our series of 50 cases of delayed union and non-union grafted with autologous bone marrow by percutaneous complication was seen. There was no complication at recipient site. No sign of infection at donor or recipient was encountered in any patient included in this study. It was observed that at times, the injection of bone marrow injection except for little pain during aspiration of bone marrow (that too under local anaesthesia), no at fracture site became difficult whenever there was slight delay in



Figure 6. Distal femur delayed union, 10 weeks after bone marrow injection.

injecting the aspirated marrow at the fracture site. This technical difficulty was avoided by inserting the 16-gauge spinal needle at the fracture site under image intensifier prior to aspiration of bone marrow from iliac crests that the procedure could be completed as rapidly as possible.

DISCUSSION

Delayed union and non-union are rare but well known complications of long bone fractures which present a major challenge to an orthopaedic surgeon despite continued advances in their treatment. For long, autologous cancellous bone grafting has been the standard of treatment. But this bone grafting has not been without complications such as painful scar, hematoma, infection, fracture or subluxation and gait disturbances. Various methods of treating delayed and non-unions have been described such as Onlay bone grafting with or without internal fixation (Campbell, 1939; Phemister, 1947), cancellous insert grafts (Nicoll, 1956), subcortical iliac bone grafts (Forbes, 1961), dual only bone grafts (Boyd, 1941), stimulation by direct current through implanted electrodes (Peterson and Lewis, 1980) and by an electro magnetic field about the site of fracture (Sharrard et al., 1982). In bone grafting, the bone grafts mainly act as a scaffold with most of the cellular elements dying out and being replaced by creeping substitution (Charls, 1992). Bone marrow injection has shown to have stimulated callus formation where autologous bone grafting has failed. Though all of these methods have shown varying degrees of excellent to good results, but most of the techniques are tedious and require sophisticated equipments, expertise, anaesthesia and are time consuming with added risk of infection (Garg et al., 1993).

McGaw and Habin (1934) were among the first to demonstrate the osteogenic activity of bone marrow. They

Table 1. Duration of injury.

Duration of injury (weeks)	No. of cases	Percentage
12-16	5	10
16-20	8	16
20-24	11	22
24-28	9	18
28-32	3	6
32-36	2	4
36-40	5	10
40-44	3	6
44-48	1	2
48-52	1	2
>52	2	4
Total	50	100

Mean duration of injury was 28.1 weeks.

Table 2. Final results after careful clinical and radiological examination.

Outcome	No. of cases	Percentage
Union achieved (success)	46	92
Union not achieved (failure)	4	8
Total	50	100

Table 3. Mean time taken for union in successful cases.

Parameter	No. of successful cases	Time in weeks
Delayed union	37	14.6
Non-union	9	18.4
Total	46	15.3

Table 4. The types of cases in our study were both delayed and non-union in comparison to other studies.

Parameter	Delayed union	Non-union
Our study	38	12
Healey et al. (1990)	8	0
Connolly et al. (1991)	0	15
Verma and Kulshershtha (1997)	0	35
Goel et al. (2005)	0	20

They grafted bone defect in dog fibulae with bone marrow alone and compared this with contralateral ungrafted defect. Only the bone marrow grafted defect filled the gap with bone. The osteogenicity of bone marrow has been traced to the stromal and endosteal cells of the marrow. Two types of osteoprogenitor cells (OPC) have been demonstrated. One that is induced to produce bone (IOPC) and the other that is determined to produce bone

Table 5. Comparison of repeated bone marrow injections with other studies.

Parameter	Injections			
	1	2	3	4
Our study	30	20	0	0
Garg et al. (1993)	0	15	0	0
Verma and Kulshershta (1997)	0	0	0	35
Goel et al. (2005)	2	9	4	0

In our study, we repeated bone marrow injection for the second time after interval of 6 weeks in 20 cases which included 10 cases of non-union out of 12 and 10 cases of delayed union out of 38 in comparison to other studies as given in Table 6.

Table 6. Comparison of results with other studies.

Study	Success rate% (union achieved)
Healey et al. (1990)	62.5 (5 out of 8 patients)
Connolly et al. (1991)	90 (18 out of 20 patients)
Goel et al. (2005)	75 (15 out of 20 patients)
Our series	92 (46 out of 50 patients)

The results achieved are comparable to other studies.

(DOPC) (Sharrard et al., 1982). The former (IOPC) exists in all connective tissue and the latter (DOPC) is found only in the marrow. Because bone marrow is the only tissue that contains an abundance of both determined and inducible osteoprogenitor, it is a logical graft choice (Budenz and Bernard, 1980).

The concept of bone grafting percutaneously was introduced by Herzog (1951). He used a large bore needle and small cancellous chips to graft a non-union. Since bone marrow has a liquid texture, combining the percutaneous grafting technique introduced by Herzog (1951) and the bone marrow graft introduced by McGaw and Habin (1934) seemed a logical step. An experimental study on percutaneous bone marrow grafting of fractures and bony defects was conducted by Paley et al. (1986) on thirty adolescent white rabbits. Bone marrow and saline control was injected percutaneously in to fracture site of bony defects. Results showed that bone marrow grafting sites had earlier and more abundant callus. Similarly, the bony defect which were grafted with bone marrow united by a bony bridge whereas the saline control did not. These effects were optimal when used early in the fracture healing process.

The types of cases in our study were both delayed and non-union in comparison to other studies as given in Table 4. We included cases in which either internal or external fixation was done prior to bone marrow injection in comparison to study by Verma and Kulshershta (1997) and Goel et al. (2005). The mean duration of fracture in our study was 7 months in comparison to studies by Goel et al. (2005). In our study we repeated

bone marrow injection for the second time after interval of 6 weeks in 20 cases which included 10 cases of non-union out of 12 and 10 cases of delayed union out of 38 in comparison to other studies as given in Table 5. Union was achieved in 92% cases (46 out of 50 cases) after a mean time of 15.3 weeks. The results achieved are comparable to other studies as given in Table 6. This high success rate proves beyond doubt the high osteogenicity of bone marrow and it has established that bone marrow grafting by percutaneous injection is equally effective as open bone grafting and that too, with numerous advantages. So percutaneous bone marrow grafting is a simple, minimally invasive technique, which can be done as an outdoor procedure. It is safe, easy, practical and time saving. Moreover, it is economical and shortens the hospital stay of the patient. It is an easy treatment for a difficult problem.

Conclusion

Our study has established that bone marrow has high osteogenic potential and can be grafted percutaneously successfully. This procedure of bone marrow grafting by percutaneous injection has tremendous clinical potential with no complications. This minimally invasive procedure is a biological method of bone grafting as it does not disturb the vascularity at the fracture site. It is an easy, safe, simple, economical and short procedure that can be performed as an outdoor procedure under local anaesthesia. So, it is a very useful procedure for those patients who are not fit for general anaesthesia. It is both patient friendly as well as surgeon friendly procedure. In short, it is an easy solution for a complex problem.

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Full Length Research Paper

Correlation between elevated homocysteine levels and insulin resistance in infertile women with or without polycystic ovary syndrome in North Indian population

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Accepted 31 January, 2013

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting about 5 to 10% women of reproductive age group worldwide. This study was designed to examine the relationship between elevated homocysteine levels and insulin resistance (IR) in infertile women with or without PCOS. This cross sectional case control study was conducted in Department of Obstetrics and Gynaecology, Pathology and Medicine, CSM Medical University (Erstwhile KGMU), Lucknow, UP India. After informed consent, a total 90 infertile women were enrolled which included 50 diagnosed cases of PCOS according to Rotterdam consensus meeting 2003 and 40 healthy non PCOS as controls. Mean plasma homocysteine levels were significantly higher in PCOS women (11.8796 ± 5.5) as compared to non PCOS women (7.8095 ± 2.2). Obesity was found in 64% PCOS women and 37.5% of non PCOS women which was significant. Serum testosterone levels were elevated in 42% of PCOS women, but none of the women of non PCOS group had elevated testosterone levels. Amongst the PCOS patients (32/50), 64% were IR and (18/50) 36% were non insulin resistance (NIR) PCOS group which was stratified by HOMA-IR. Thirty-two women had mean HOMA-IR (5.95 ± 1.27) designated PCOS IR and 18 had mean HOMA-IR (1.86 ± 0.028) designated PCOS NIR. Elevated body mass index (BMI) was not significantly associated with IR in PCOS patients. BMI in PCOS IR was 27.43 ± 3.096 and BMI in PCOS NIR was 25.728 ± 2.609 ($P < 0.07$). Homocysteine levels were significantly elevated in PCOS patients (mean 11.87 ± 5.5) (All PCOS), IR PCOS (13.82 ± 5.69 pmol/l) versus controls (7.80 ± 2.29 pmol/L) ($P < 0.001$). In conclusion, obesity is not an independent risk factor to increase plasma homocysteine levels in PCOS women.

Key words: Homocysteine, polycystic ovary syndrome, insulin resistance, infertility.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting about 5 to 10% women of reproductive age group worldwide. It is also estimated to be the major cause of anovulatory infertility accounting for about 73% of cases. Variance in prevalence among population is

thought to be dependent on ethnic origin, race and other environmental factors on the phenotype Ehrmann (2005). The exact aetiology of PCOS remains unknown, but hyperandrogenism was thought to be a main underlying factor. Now, the syndrome is thought to have wider

metabolic and cardiovascular implications. Plenotypic manifestations of the syndrome vary from patient to patient and a Rotterdam diagnostic criteria for PCOS is based on the clinical identification of at least two of the three defined criteria which include: (1) oligo/anovulation, (2) chemical and/or biochemical evidence of hyperandrogenemia and (3) ultrasonographic findings of polycystic ovaries (Pehlivanov and Orbetzova, 2007; The Rotterdam ESHRE/ASRIVI sponsored PCOS consensus workshop group revised, 2003).

Elevated levels of plasma homocysteine have been implicated as a significant risk factor for cardiovascular disease, preeclampsia and recurrent pregnancy loss (Del Bianco et al. 2004).

Homocysteine is an intermediate substance formed during the breakdown of the amino acid methionine and may undergo remethylation to methionine or trans-sulfuration to cystathionine and cysteine. Recent research has pointed to many non-enzymatic factors which may influence homocysteine levels including age, gender, or sex-steroid environment (IVlcCarty 2000a).

Insulin levels have also been implicated as a modulating factor of homocysteine in which insulin inhibits hepatic cystathionine P-synthase activity (Schneede et al. 2000). Hence, elevated levels of homocysteine have been positively associated with insulin levels in a number of clinical situations. PCOS a common endocrinopathy in women of reproductive age group is a multifaceted metabolic disease (IVlcCarty 2000a; Schneede et al. 2000). Plasma levels of insulin seem to influence homocysteine metabolism, possibly through effects on glomerular filtration or by influencing the activity of key enzymes in homocysteine metabolism (Giltay et al. 1998; Gallistl et al. 2000). Thus, it seems logical to hypothesize that elevated homocysteine levels could be another feature of PCOS both being associated with IR and reproductive failure (Craig et al. 2002). Insulin resistance (IR) has been found in up to 70% of women with PCOS and is a risk factor for development of type II diabetes mellitus in these women (Laivuoriet al.1999). Several studies have investigated the association of homocysteine levels in PCOS patients. Because of complex limitations of these studies, such as lack of uniformity in the definition of PCOS and information on levels of other cofactors, the results vary. Thus, this study was designed to examine the relationship between elevated homocysteine levels and IR in infertile women with or without PCOS.

MATERIALS AND METHODS

This cross sectional case control study was conducted in Department of Obstetrics and Gynaecology, in collaboration with Department of Pathology and Medicine, CSM Medical University, Lucknow, UP, India. After informed consent, a total of 90 infertile women were enrolled which included 50 diagnosed cases of PCOS according to Rotterdam European Society of Human Reproduction and Embryology Consensus Meeting 2003 guideline and 40 healthy non PCOS infertile women were taken as controls. Ethical clearance

was obtained from Institutional Ethics Committee.

Exclusion criteria for all subjects including current or previous use of oral contraceptive pills (within 6 months), drugs like metformin, phenytoin, folic acid, vitamins, antiandrogens, antidiabetics, statin, glucocorticoids, cigarette smoking, chronic alcohol consumption, coffee consumption more than 2 cups/day known case of hypertension, diabetes mellitus and cardiovascular disease. All subjects were asked to keep their normal diet and not to perform any sport like activity; same exclusion criteria as case group were used for control group. The entire women were subjected to thorough physical examination and laboratory tests.

All blood samples were obtained in the morning on the 2nd day of menstruation after an overnight fasting. During the same visit, all subjects underwent anthropometric measurement. The serum concentration of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, prolactin and thyroid-stimulating hormone (TSH) were measured by Chemiluminescent enzyme immunoassay (Immulate 2000 diagnostic product corporation LA, CA). Serum glucose was measured using glucokinase technique. Plasma insulin levels were measured by Chemiluminescent enzyme immunoassay (Immulate 1000 analyzer with inter-assay and intra-assay coefficient of variation did not exceed 6.4%). Blood samples for homocysteine measurement were collected, and were immediately placed on ice and were centrifuged at 4°C. Plasma was separated within 30 min and was stored at -70°C. Plasma homocysteine concentration was measured by fluorescence polarization immunoassay by using ABBOTT diagnostic kit (USA). Normal reference range of our laboratory were 5 to 11 pmol/L. Hyperhomocysteinemia was defined as plasma homocysteine level >11 pmol/L. IR was determined by a number of different methods including, fasting insulin, fasting glucose to insulin ratio and HOMA- IR. HOMA-IR >2.5 was considered as IR. The estimation of IR by HOMA score was calculated with the formula, fasting serum insulin (pU/ml X fasting plasma glucose (mmol/L)/ 22.5). BMI was calculated as the weight in kilograms divided by the square of the height in metres (kg/m).

The data were subjected to statistical analysis by Statistical Package for Social Sciences (SPSS-16) version. The difference between two groups was assessed by independent 't' test and probability was calculated on the basis of associated degree of freedom. 95% confidence level has been chosen and P value <0.05 was considered as significant. Pearson's correlation coefficients were used to calculate correlation between paired data sets. Significance of correlation and the relative contribution of each variable were calculated.

RESULTS

All the subjects included in this study were matched for age, religion, dietary habits and socioeconomic status. Hirsutism is one of the common clinical features of PCOS. In our study 56% of PCOS women had hirsutism as compared to 12.5% controls had hirsutism ($P<0.001$). Mean plasma homocysteine levels were significantly higher in PCOS women (11.88 ± 5.5) as compared to non PCOS women (7.81 ± 2.2) ($P<0.001$). Obesity was found in 64% PCOS women as compared to 37.5% of non PCOS women which was significant ($P<0.001$). Serum testosterone levels were elevated in 42% of PCOS women, but none of the women in the control group had elevated testosterone levels ($P<0.001$) Table 1.

IR definitions

IR was defined as an abnormal result in fasting insulin,

Table 1. Clinico-demographic profile of the subjects.

Profile	Case (%)	Control (%)	P value
Normal	50	40	-
Mean age (years)	26.10±4.08	27.01±4.28	0.299
Infertility Primary	84.4	77.5	0.08
Secondary	15.6	22.5	
Religion wise Hindu	74	77.5	0.7
Muslim	26	22.5	
Dietary habits vegetarian	66	60	0.557
Non vegetarian	34	40	
Socio-economic status wise distribution	80	77.5	0.773
Middle and higher lower	20	22.5	
Distribution of subjects according to menstrual abnormality			
Oligomenorrhea	38	-	-
Prolong	42	-	
Normal	20	100	
Distribution of subjects according to hirsutism			
Present	56	12.5	0.001
Absent	44	87.5	
Occupation			
Housewife	82.2	70.0	-
Working	17.8	30.0	
Mean plasma homocysteine level	11.88±5.55	7.80±2.29	<0.001
BMI			
<25	36	62.5	<0.001
≥25	64	37.5	
S.LH/FSH ratio			
<2.5	48	97.5	<0.001
≥2.5	52	2.5	
S. Testosterone (ngm/dL)			
<80	58	100	<0.001

glucose: insulin ratio or HOMA-IR value, determined by calculating threshold values >95th percentile of the normal control group. These were fasting insulin (>20mIU/L), glucose insulin ratio (GI<4.5) and HOMA-IR value (>2.5). Thus, IR was found in 56% of PCOS women by fasting insulin and in 60% of PCOS patients by glucose insulin ratio and in 64% of PCOS patients by

HOMA-IR all statistically significant as compared to normal controls (P<0.001).

Thus, HOMA-IR is one of the most sensitive indicators to determine IR. Amongst the PCOS patients (32/50), 64% were insulin resistant and (18/50) 36% were non insulin resistant (NIR) PCOS group which was stratified by HOMA-IR. 32 women had mean HOMA-IR (5.95±1.27)

Table 2. Clinical and biochemical data of all patient, PCOS stratified by insulin resistance.

Parameter	No.	Age	BMI	LH/FSH	Insulin	G/I ratio	HOMA	Plasma Hcy
Reference range	-	-	≤25	<2.5	<20 mIU/L	>4.5	<2.5	5-11<11 μmol/L
Case (all PCOS)	50	26.10±4.08	26.82±3.665	2.294±0.963	21.07±9.69	5.34±3.02	4.48±2.23	11.87±5.55
PCOS, NIR	18	26.56±4.48	25.7289±2.60906	1.67±0.65	9.25±1.35	9.09±1.59	1.86±.28	8.24±.77
PCOS, IR	32	25.84±4.39	27.438±3.996	2.64±0.89	27.62±4.71	3.23±.57	5.95±1.27	13.82±5.69
P value IR								
PCOS versus NIR PCOS	-	NS	0.07 ^{NS}	0.000	0.000	0.000	0.000	<0.001
Control	40	27.02±4.28	24.65±2.62	0.98±.60	7.53±3.55	11.68±3.02	1.83±2.25	7.80±2.29
P value PCOS IR versus control	-	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P value PCOS NIR versus control	-	NS	0.237 ^{NS}	<0.002	0.110 ^{NS}	<0.001	0.947 ^{NS}	0.700 ^{NS}

designated PCOS IR and 18 had mean HOMA-IR (1.86±0.028) designated PCOS NIR (Table 2).

After statistical analysis, it was found out that there was no association of IR with age and BMI in PCOS women. However, elevated HOMA index was found to be significantly associated with higher LH/FSH ratio, insulin levels, glucose:insulin ratio and elevated plasma homocysteine (Table 2). In other words, IR correlated positively with homocysteine in PCOS women. Cystathionine-β-synthase, the key enzyme of the transsulfuration pathway in homocysteine metabolism, is down regulated in an insulin resistant state.

Body weight and other variables

PCOS patients were found to be more obese than controls. When patients were stratified by BMI, 64% (32/50) PCOS patients had a BMI ≥25 kg/m², whereas only 37.5% (16/40) of controls group had a BMI ≥25 kg/m². Elevated BMI was not significantly associated with IR in PCOS patients. BMI in PCOS IR was 27.43±3.096 and BMI in PCOS

NIR was 25.728±2.609 (P<0.07) (Table 2). Elevated LH/FSH ratio was significantly associated with PCOS state whether obese or non obese, IR or NIR (Tables 2 and 3). Homocysteine as related to other variables Homocysteine levels were significantly elevated in all PCOS patients (mean 11.87±5.5 μmol/L), in IR PCOS (13.82 ± 5.69 μmol/L) versus controls (7.80±2.29 μmol/L), (P<0.001) (Table 2). Elevated homocysteine was noted in both non obese and obese PCOS as opposed to non obese and obese controls (9.4±3.10; 13.2±6.17 versus 7.5±1.53; 8.29±3.1; P<0.001) (Table 3). The 95th percentile for homocysteine in our control group was 11.0 μmol/L, when a level of 11 μmol/L was used as a threshold, 36% of PCOS patient had homocysteine value more than normal cutoff value, (P<0.001) (Table 3). Out of 18 NIR PCOS patients, only 5 (22.7%) had elevated homocysteine level (>11 μmol/L), whereas 32(40.6%) IR PCOS had an elevated homocysteine levels demonstrating the differential effect of IR on homocysteine (Table 4). When we had compared non obese control with non obese PCOS patients,

these two groups were found to have insignificant different BMI, but significant differences in IR and homocysteine levels. Non obese PCOS patients had higher homocysteine levels than non obese controls (9.4±3.10; 7.50±1.53) (Table 3). Thus, body weight was not found to be predictor of homocysteine, rather IR regardless of body weight correlated with homocysteine levels.

Multiple comparison analysis was done among plasma homocysteine, IR (PCOS), NIR (PCOS) and controls. Comparison analysis strongly suggests that IR have strong correlation for high plasma homocysteine in patients of PCOS (Table 5). Correlation of different clinical and laboratory parameters was examined. Clinical and biochemical criteria were examined. A strong positive correlation was found between HOMA IR and insulin levels (r~0.987) and moderate positive correlation was observed between HOMA IR and BMI, homocysteine levels and serum LH and FSH ratio. Positive correlation was also observed with glucose levels and serum testosterone levels (Table 6). No association was found between age and PCOS, age and IR or age and homocysteine

Table 3. Comparison of clinical and biochemical properties of infertile women as stratified as body mass index (BMI).

Parameter [no.]	Reference range						
	Mean age \pm SD	BMI < 24.9	LH/FSH < 2.5	Insulin < 20 (mIU/L)	G/I > 4.5	HOMA < 2.5	HCY \leq 11
PCOS (Non-obese) [18]	26.0 \pm 4.07	23.0 \pm 1.58	2.2 \pm 0.94	17.8 \pm 8.1	5.8 \pm 2.69	3.94 \pm 1.81	9.4 \pm 3.10
PCOS (Obese) [32]	26.2 \pm 4.15	38.9 \pm 2.56	2.3 \pm 0.99	22.6 \pm 10.3	5.2 \pm 3.19	4.69 \pm 2.43	13.2 \pm 6.17
Control (Non-obese) [24]	27.1 \pm 4.88	23.0 \pm 1.14	0.95 \pm 0.56	6.8 \pm 1.34	12.3 \pm 2.42	1.34 \pm 0.28	7.50 \pm 1.53
Control (Obese) [16]	26.9 \pm 3.3	27.2 \pm 2.07	1.03 \pm 0.67	8.7 \pm 5.27	10.8 \pm 3.64	1.95 \pm 1.36	8.27 \pm 3.11
Statistical Significance "p"	0.773	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PCOS obese versus PCOS non-obese	0.898	<0.001	0.696	0.097	0.494	0.260	0.018
PCOS obese versus control obese	0.500	0.028	<0.001	<0.001	<0.001	<0.001	0.004
PCOS obese versus control non-obese	0.427	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PCOS non-obese versus control non-obese	0.433	0.987	<0.001	<0.001	<0.001	<0.001	0.011

Table 4. Clinical and Biochemical Data for all patients using normal homocysteine as illustrated values are Mean \pm SD.

Group	No. (%)	BMI	LH/FSH	Insulin (mIU/L)	G/I	HOMA	HCy
HCy<11 (Control)	36 (90)	24.17 \pm 2.06	0.98 \pm 0.63	6.79 \pm 1.29	12.04 \pm 2.50	1.43 \pm 0.65	7.23 \pm 1.52
HCy<11 (PCOS)	32 (64)	25.52 \pm 3.40	1.93 \pm 0.91	18.56 \pm 8.64	6.09 \pm 3.04	3.75 \pm 1.84	8.29 \pm 1.71
HCy \geq 11 (Control)	4 (10)	29.53 \pm 2.20	0.97 \pm 0.12	14.29 \pm 8.80	8.42 \pm 5.48	2.97 \pm 1.81	13.03 \pm 1.01
HCy \geq 11 (PCOS)	18 (36)	29.09 \pm 2.88	2.94 \pm 0.69	24.87 \pm 10.56	4.14 \pm 2.59	5.62 \pm 2.43	18.26 \pm 4.01
"P"	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Clinical and biochemical data for patients with different homocysteine levels in different groups revealed a statistically significant intergroup difference for all the parameters being studied (P<0.001).

Table 5. Multiple comparison analysis: Plasma homocysteine comparison among IR (PCOS), NIR (PCOS) and control **1 (NIR, PCOS), 2 (IR, PCOS), 3 (Control).

Dependent variable	Homagr	Homagr	Mean difference (I-J)	Standard error	Sig.	95% Confidence interval	
						Lower bound	Upper bound
NIR versus IR	1	2	-5.68601*	1.15737	0.000	-7.986	-3.3856
		3	0.43106	1.11492	0.700	-1.785	2.6471
IR versus Control	2	1	5.68601*	1.15737	0.000	3.3856	7.9864
		3	6.11706*	0.93166	0.000	4.2653	7.9688
NIR versus control	3	1	-0.43106	1.11492	0.700	-2.6471	1.7850
		2	-6.11706*	0.93166	0.000	-7.9688	-4.2653

The statistics value shown in the table strongly suggest insulin resistance have strong correlation for high plasma homocysteine in patient of PCOS.

Table 6. Correlation of different clinical and laboratory parameters with HOMA index.

Parameter	Value
Insulin	r~0.987
BMI	r~0.50
Homocysteine	r~0.51
S.FSH/LH ratio	r~0.57

level.

DISCUSSION

PCOS associated infertility has been attributed to various factors including oligo-anovulation, dysfunctional gonadotropin secretion, hyperandrogenemia and dysfunction of any or several ovarian growth factors and their binding proteins. The clinic-demographic profile of both PCOS and non PCOS patients were similar except that the prevalence of obesity was more amongst PCOS patients (62.5%) as compared to controls (37.5%).

Recent research has focused on systemic and local effect of IR and homocysteine and its secondary effects on reproductive system. Recently in literature, it has been reported that PCOS is not only the most common reproductive disorder, but also a pleurimetabolic syndrome (Scarpitta and Sinagra 2000). Previous studies have found an association between IR and elevated homocysteine in specific patients groups in women of reproductive age. It is of interest to note that in our study, elevated homocysteine was best correlated to HOMA index out of three indices used for IR. Other authors have examined the relationship between plasma homocysteine levels and IR in patients with PCOS.

Elevated homocysteine was related to IR in women with preeclampsia, but not in normal controls (Laivuori et al. 1999). In this study, IR were higher in PCOS women as compared to non PCOS, but no significant difference was found in IR between obese and non obese PCOS women, this might be due to small sample size. While others reported that mean fasting insulin level was significantly higher in women with PCOS than controls. IR indices were significantly different between PCOS and controls, as well as obese PCOS patients as opposed to non obese PCOS, although both obese and non obese PCOS patients were more IR than normal healthy control (Ilhan et al. 2005).

Few studies reported a positive correlation between BMI and IR in premenopausal women; however, in this study IR was not related with BMI in PCOS women and it was independently associated with PCOS (Tanrikulu-Kilic et al. 2006). In our study, IR indices were not significantly different in obese PCOS as compared to non obese PCOS, but there were more insulin resistant as compared to healthy controls. This means IR in patients with PCOS is associated with elevated plasma homocysteine

regardless of body weight.

In this study, the mean serum fasting insulin, HOMA IR and homocysteine were significantly higher in PCOS group and results were highly similar to study done by other author (Mesut et al. 2009).

Moderate hyperhomocysteinemia has been found to be a risk factor for recurrent early pregnancy loss. Despite several pathophysiological hypotheses including impaired cell proliferation, increased oxidative stress, apoptosis, reduced extra-embryonic vascular development and hypomethylation, it is not clear whether hyperhomocysteinemia is causally related to recurrent early pregnancy loss or whether it is only a marker of increase risk of recurrent early pregnancy loss (Latacha and Rosenquist 2005). Elevated homocysteine may impair implantation by interfering with endometrial blood flow, vascular integrity and has been documented to increase the probability of early pregnancy loss (Del Bianco et al. 2004).

Both impaired implantation and increased rates of miscarriage are more frequent in PCOS women even after controlling ovulatory abnormalities, increased LH, and hyperandrogenism, which might be due in part to elevated homocysteine in these patients. Various studies have examined the association between plasma homocysteine levels and IR in his specific population of patients with PCOS. They reported that mean serum homocysteine concentrations are increased in women with PCOS as compared to controls (Ilhan et al. 2005).

Similarly, mean homocysteine levels were higher in PCOS women as compared to non PCOS here. When patients were stratified by body mass index, the homocysteine levels were significantly higher in obese and non obese PCOS as compared to controls (PCOS obese=13.2±6.17, PCOS non obese=9.4±3.10, obese controls=8.27±3.11, non obese controls=7.50±1.53) and similar conclusion were drawn by other study (Ilhan et al. 2005). Another study reported elevated homocysteine levels in PCOS patients and no significant difference was found regarding IR in obese and non obese PCOS. However, in PCOS women serum insulin levels and HOMA IR values are significantly higher as compared to controls, similar to those in this study (Ahmed et al. 2007). Yarali et al. (2001) examined the cardiac diastolic dysfunction of PCOS patients as detected by echocardiography and have shown significantly higher plasma homocysteine concentration in both lean and obese PCOS patients than control group and this was related to IR (Yaraliet al. 2001). Schachter et al. (2003) also reported that IR and hyperinsulinemia in patients with PCOS was associated with elevated plasma homocysteine levels regardless of body weight (Schachter et al. 2003). Another study has shown that PCOS patients had elevated plasma homocysteine levels independent from their BMI (Loverro et al. 2002). In this study, significant association between plasma homocysteine levels and HOMA index was present; however, this is not in accordance with the findings of studies performed on large

healthy population Abbasi et al. 1999; Godsland et al.2001; Rosolova et al. 2002). The results of this study are in corroboration with the study conducted by (Giltay et al, 1998) which was carried out on smaller population, in which they found significant association between high insulin levels and elevated homocysteine levels in healthy non obese subjects. One study reported elevated homocysteine levels in patients with PCOS and this correlated significantly with fasting insulin (Wijeyaratne et al. 2004). While other did not find any association between PCOS and plasma homocysteine level. In this study, patients were selected on the basis of ultrasound morphology only, not incorporating other components of the syndrome (Sills et al. 2001). An Italian study including 70 PCOS patients with low folate intake and vary high prevalence of the mutated 677T allele, did not show elevated homocysteine concentration in patients with PCOS, as is usually observed in that population (Orio et al. 2003).

Conclusion

In the present study, we examined the association between IR and elevated homocysteine in women with PCOS and found that obesity is not an independent risk factor to increase plasma homocysteine levels in PCOS women. IR was common in PCOS patients and was practically more common in obese PCOS sub groups. In non obese PCOS, the mean plasma homocysteine levels were significantly higher than in obese controls. Thus, we conclude that IR was a significant risk factor for hyperhomocysteinemia. IR could be a part of metabolic syndrome in polycystic ovarian syndrome. Other variables that influence the homocysteine concentration (Vitamin B12 and folate levels) were not examined in this study. However, in this study, sample size is small in metabolic terms, PCOS may possibly be considered a variant of IR.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Vinita Das for supporting in patient selection. This work is financially supported by the Talents India Pharma Pvt. Ltd.

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C677T polymorphism of methylenetetrahydrofolate reductase in women with polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* 88(2):673-9.

Full Length Research Paper

Safety and effectiveness of BIAsp 30 treatment: Data from the Moroccan cohort of the A₁chieve study

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Accepted 11 December, 2012

This sub-analysis aimed to determine the safety and effectiveness of biphasic insulin aspart30 (BIAsp30) therapy in people with type 2 diabetes (T2D) from Morocco as a part of the global, 24-week, non-interventional A₁chieve study. A total of 770 Moroccan T2D patients, both insulin-naïve patients and prior insulin users, who started BIAsp 30 therapy at their physicians' discretion, were included. Baseline glycaemic control was poor in the Moroccan cohort with a mean (\pm SD) glycated haemoglobin A_{1c}(HbA_{1c}) of $9.8 \pm 1.8\%$. After 24 weeks, mean HbA_{1c} significantly reduced by $-2.2 \pm 1.7\%$ in the Moroccan cohort ($-2.5 \pm 1.8\%$ in insulin-naïve patients and $-1.9 \pm 1.5\%$ in prior insulin users, all $p < 0.001$). In the entire cohort, there was no significant difference from baseline to Week 24 in the percentage of patients with at least one event of overall or minor hypoglycaemia, while there was a significant reduction in the percentage of patients reporting at least one event of major or nocturnal hypoglycaemia ($p < 0.0001$). Mean body weight increased (1.4 ± 4.3 kg), while the lipid profile and systolic blood pressure improved over 24 weeks. Overall, BIAsp 30 therapy was well-tolerated and improved glycaemic control in Moroccan T2D patients.

Key words: Type 2 diabetes, BIAsp 30, Morocco, non-interventional study, glycaemic control.

INTRODUCTION

The International Diabetes Federation forecasts a global increase in the number of diabetes cases from 366 million affected people in 2011 to 552 million in 2030, with type 2 diabetes (T2D) comprising between 90 and 95% of all cases (Whiting et al., 2011; Das and Chakrabarti, 2005). The Middle East and North Africa

(MENA) region is heavily impacted by the disease burden with a predicted 83% increase in diabetes prevalence over the next 20 years (Whiting et al., 2011).

In Morocco, as in other developing countries, increasing urbanization has led to lifestyle and dietary changes that adversely affect metabolism, resulting in the

rapid emergence of diabetes during the last decade (Rguibi and Belahsen, 2006; Benjelloun, 2002). A total of 1.4 million Moroccan adults have been diagnosed with diabetes and Morocco ranks seventh among the 10 countries with the highest diabetes prevalence in the MENA region (IDF Diabetes Atlas, 2011). A survey by the Ministry of Health in 2000 showed that diabetes equally affected men and women nationwide with a higher prevalence in urban areas compared to rural areas (Benjelloun, 2002).

Regular assessment of glycaemic control status plays an important role in the management of T2D. Maintaining a glycated haemoglobin A_{1c}(HbA_{1c}) level of <53 mmol/L (<7.0%) is recommended for optimal glycaemic control and to minimize the risk of experiencing diabetes-related complications later in life (Saydah et al., 2004; Nathan et al., 2009). Appropriate intensification of treatment regimens, including the timely initiation of insulin, is highly recommended to maintain adequate glycaemic control (Inzucchi et al., 2012).

However, despite the known effectiveness of insulin treatment in T2D management, patients and physicians tend to delay initiating or intensifying insulin therapy due to apprehensions of weight gain, hypoglycaemia, high number of injections and lifestyle restrictions (Peyrot et al., 2005). Premixed insulin formulations were designed to improve the convenience and practicality of dosing for patients as they combine a long-acting basal component with a fast-acting mealtime component, thus requiring fewer daily injections than basal-bolus regimens (Raja Khan et al., 2007). The premixed insulin analogue, biphasic insulin aspart 30 (BIAsp 30), was developed to provide a more predictable pharmacological profile than biphasic human insulin. BIAsp 30 has demonstrated efficacy in controlling HbA_{1c} and PPPG levels with a low incidence of major hypoglycaemia and is commonly prescribed (Raja Khan et al., 2007; Valensi, 2009a).

The A₁chieve study aimed to determine the safety and effectiveness of insulin analogues prescribed in local clinical care settings in diverse populations in 28 countries (Home et al., 2011). Data from such large observational studies can provide important evidence to support prescribing decisions and guidelines for T2D management. Currently, there is limited efficacy and safety information regarding the routine clinical use of insulin analogues such as BIAsp 30 in treating T2D in Morocco; hence, this sub-analysis was performed to evaluate outcomes data from Moroccan patients on BIAsp 30 therapy. This sub-analysis was also expected to provide information on the current scenario of T2D management in this region.

MATERIALS AND METHODS

Study design

The 24-week, international, prospective, multi-centre, non-interventional A₁chieve study aimed to assess the clinical safety

and effectiveness of BIAsp 30 (NovoMix 30[®], Novo Nordisk A/S, Denmark), insulin detemir (Levemir[®], Novo Nordisk A/S, Denmark) or insulin aspart (NovoRapid[®], Novo Nordisk A/S, Denmark) in routine clinical use outside the Western economies (Home et al., 2010). In this sub-analysis, we examined the clinical safety and effectiveness of BIAsp 30 in 770 Moroccan T2D patients, recruited between 15 October, 2009 and 15 July, 2010, at 76 centres in Morocco.

BIAsp 30 was commercially available and funded according to local practice in clinical care. The choice of BIAsp 30 was decided jointly by the patient and physician. The physician determined the dose and frequency of administration of BIAsp 30, in accordance with the licensed approval from the local regulatory authority. Concurrent oral glucose-lowering drugs (OGLDs) were permitted, at the discretion of the physician, throughout the study.

All measurements were made by the treating physician during routine clinical care; there were no pre-defined study-related procedures. Data were collected from the physicians' records and from the patients' recall and self-monitoring diaries/blood glucose meters at baseline and Week 24. All information was transferred to standard case report forms (CRFs).

Patient population

Any Moroccan T2D patient prescribed BIAsp 30 at the discretion of the physician was eligible for this sub-analysis. Patients who were treated with insulin analogues (alone or in combination) for more than 4 weeks prior to the study were excluded. Pregnant women and women who were breast-feeding or had the intention of becoming pregnant were also excluded. Signed informed consent was obtained from all patients and ethics committee approval was obtained for Morocco. Patients could withdraw at any time. Following withdrawal, the data collected were used for analysis until the point when consent was withdrawn. All investigators were trained on the study protocol, CRF completion, informed consent and safety reporting procedures.

Assessments and outcome measures

The primary objective was to evaluate the clinical safety of BIAsp 30 based on the number of serious adverse drug reactions (SADRs), including major hypoglycaemic events, considered related to BIAsp 30 from baseline to Week 24. The secondary safety assessments included changes in the occurrence and frequency of hypoglycaemic events and the number of adverse drug reactions (ADRs) and adverse events from baseline to Week 24.

Efficacy assessments included the change from baseline to final visit in HbA_{1c}, pre-breakfast fasting plasma glucose (FPG), post-breakfast postprandial plasma glucose (PPPG), body weight, systolic blood pressure (SBP) and lipid profile.

Local laboratories were used for all laboratory measurements with local standardisation and quality control procedures.

Health-related quality-of-life (QoL) was assessed using the five-dimensional EQ-5D questionnaire at baseline and after 24 weeks of therapy with BIAsp 30. Current health-related QoL was measured on a standard vertical 20 cm visual analogue scale (VAS) with scores ranging from 0 (worst imaginable health) to 100 (best imaginable health).

Statistical methods

Statistical analyses were performed for the entire Moroccan cohort, insulin-naïve patients and prior insulin users. No statistical analyses were performed to compare differences between insulin-naïve patients and prior insulin users.

Table 1. Patient numbers and characteristics for the entire cohort and by pre-study therapy.

Parameter	Entire cohort	Insulin-naïve	Prior insulin users
n (%)	770 (100)	435 (56.5)	335 (43.5)
Sex, M/F (%)	47.4/52.6	49.7/50.3	44.5/55.5
Mean (SD)			
Age (years)	57.2 (12.4)	57.3 (11.9)	57.0 (13.0)
Body weight (kg)	72.9 (12.4)	72.0 (13.1)	74.1 (11.4)
BMI (kg/m ²)	26.7 (4.3)	26.0 (4.0)	27.5 (4.5)
Diabetes duration (years)	9.6 (7.0)	8.2 (6.4)	11.3 (7.4)
HbA _{1c} (mmol/mol)	84 (20)	88 (22)	79 (17)
HbA _{1c} (%)	9.8 (1.8)	10.2 (2.0)	9.4 (1.6)
Prior OGLDs; n (%)			
Metformin	339 (66.5)	248 (64.9)	91 (71.1)
Sulfonylureas	360 (70.6)	308 (80.6)	52 (40.6)
Thiazolidinediones	13 (2.5)	13 (3.4)	-
One/two/>two	229 (44.9)/249 (48.8)/32 (6.3)	137 (35.9)/215 (56.3)/30 (7.9)	92 (71.9)/34 (26.6)/2 (1.6)

BMI: body mass index; F: female; M: male. Data are n (%), % or mean (SD).

Continuous and discrete variables were summarized using descriptive statistics and frequency tables (number and percentage), respectively. All statistical analyses were conducted using two-sided tests with a pre-specified 5% significance level, unless otherwise stated.

The change from baseline in HbA_{1c}, FPG, PPPG, SBP, body weight, blood lipids and QoL was analyzed using a paired t-test with baseline and Week 24 values. The change from baseline to Week 24 in the percentage of patients reporting at least one hypoglycaemic event was analyzed using Fisher's exact test. Data were analyzed by Novo Nordisk using SAS (Version 9.1.3).

RESULTS

Study patients

A total of 770 Moroccan T2D patients started BIAsp 30 treatment. Patient characteristics and prior OGLD use are presented in Table 1. Prior to study enrolment, 49.6% of patients were on OGLDs alone, 16.6% on OGLDs + insulin therapy, 26.9% on insulin only and 6.9% did not report any prior T2D medication.

Fewer males (47.4%) than females (52.6%) were enrolled. The average age was 57.2 years and the mean body mass index (BMI) was 26.7 kg/m² with a mean diabetes duration of 9.6 years.

The most common reasons reported by the physicians for starting BIAsp 30 therapy were to improve glycaemic control (94%), patient dissatisfaction with current therapy (48%), and to reduce plasma glucose variability (43%).

A total of 126 (16.4%) patients withdrew from the study; the most common reason being failure to maintain contact with their physician, 95 patients (12.3%), and the

remaining 31 patients (4.0%) withdrew for a variety of other reasons. No withdrawals due to ADRs occurred.

Entire cohort and by prior insulin use

Blood glucose lowering and insulin dose

The total daily insulin dose at 24 weeks was titrated up to 47.1 ± 14.8 U/day in the entire cohort and up to 45.2 ± 14.9 U/day for insulin-naïve patients. In prior insulin users, the pre-study insulin dose was 41.0 ± 17.3 U/day², the total starting insulin dose was 43.6 ± 14.4 U/day titrated up to 49.5 ± 14.5 U/day over 24 weeks.

Blood glucose parameters improved significantly after 24 weeks (Table 2: HbA_{1c} -24 mmol/mol [-2.2%], FPG -5.5 mmol/L, PPPG -6.8 mmol/L, all p<0.001).

Overall, the percentage of patients achieving an HbA_{1c} level of <53 mmol/mol (<7.0%) increased from 2.8% at baseline to 19.7% at Week 24.

The OGLDs most commonly continued at baseline by insulin-naïve patients after initiating BIAsp 30 treatment were metformin (83.9%), alpha-glucosidase inhibitors (15.1%) and sulphonylurea (6.5%). The OGLDs most commonly continued by prior insulin users after transferring to BIAsp 30 treatment were metformin (86.0%), alpha-glucosidase inhibitors (9.3%) and glinides (8.1%).

Hypoglycaemia

The incidence of hypoglycaemia in the entire cohort and

Table 2. Safety and effectiveness outcomes for the entire cohort and by pre-study therapy at baseline and after 24 weeks of BIAsp 30 therapy

Parameter		Entire cohort	Insulin-naïve	Prior insulin users
Insulin dose (U/day)	n	770	435	335
	Pre-study	41.0 (17.3)	-	41.0 (17.3)
	Baseline	39.5 (12.8)	36.4 (10.3)	43.6 (14.4)
	Week 24	47.1 (14.8)	45.2 (14.9)	49.5 (14.5)
HbA _{1c} (mmol/mol/%)	n	443	241	202
	Baseline	84 (20)/9.8 (1.8)	88 (22)/10.2 (2.0)	79 (17)/9.4 (1.6)
	Week 24	60 (10)/7.6 (0.9)	60 (10)/7.6 (0.9)	58 (10)/7.5 (0.9)
	Change, p	-24 (19)/-2.2 (1.7), <0.001	-27 (20)/-2.5 (1.8), <0.001	-21 (16)/-1.9 (1.5), <0.001
FPG (mmol/L)	n	532	308	224
	Baseline	12.8 (4.4)	13.8 (4.4)	11.5 (4.0)
	Week 24	7.4 (2.1)	7.5 (2.2)	7.3 (2.0)
	Change, p	-5.5 (4.5), <0.001	-6.4 (4.6), <0.001	-4.2 (4.1), <0.001
PPPG (mmol/L)	n	349	184	165
	Baseline	16.5 (4.7)	17.4 (4.5)	15.5 (4.8)
	Week 24	9.7 (2.8)	9.8 (2.9)	9.6 (2.8)
	Change, p	-6.8 (5.2), <0.001	-7.6 (5.3), <0.001	-5.9 (5.0), <0.001
Weight (kg)	n	576	323	253
	Baseline	73.2 (12.3)	72.2 (12.7)	74.5 (11.6)
	Week 24	74.6 (11.3)	74.0 (11.3)	75.2 (11.2)
	Change, p	1.4 (4.3), <0.001	1.9 (4.6), <0.001	0.7 (3.7), 0.002
SBP (mmHg)	n	461	249	212
	Baseline	135.4 (17.0)	136.0 (18.1)	134.6 (15.7)
	Week 24	131.9 (13.3)	131.6 (13.2)	132.2 (13.4)
	Change, p	-3.5 (16.7), <0.001	-4.4 (16.6), <0.001	-2.4 (16.8), 0.037
Total cholesterol (mmol/L)	n	142	74	68
	Baseline	5.2 (1.2)	5.3 (1.2)	5.2 (1.1)
	Week 24	4.7 (0.7)	4.7 (0.7)	4.7 (0.8)
	Change, p	-0.6 (1.1), <0.001	-0.6 (0.9), -	-0.5 (1.2), -
Triglycerides (mmol/L)	n	142	73	69
	Baseline	1.8 (1.0)	2.0 (1.1)	1.7 (0.8)
	Week 24	1.6 (0.5)	1.6 (0.5)	1.6 (0.5)
	Change, p	-0.2 (0.9), 0.002	-0.3 (1.0), -	-0.1 (0.7), -
HDL cholesterol (mmol/L)	n	118	61	57
	Baseline	1.2 (0.3)	1.1 (0.3)	1.2 (0.3)
	Week 24	1.2 (0.4)	1.2 (0.4)	1.2 (0.3)
	Change, p	0.0 (0.4), 0.211	0.1 (0.4), -	-0.0 (0.3), -
LDL cholesterol (mmol/L)	n	123	62	61
	Baseline	3.3 (1.1)	3.3 (1.3)	3.3 (0.9)
	Week 24	2.9 (0.6)	2.8 (0.6)	2.9 (0.6)
	Change, p	-0.4 (0.9), <0.001	-0.5 (1.0), -	-0.4 (0.8), -

Table 2. Contd.

Hypoglycaemia (event per patient-year/percent with at least one event)				
Overall	Baseline	10.08/24.7	6.49/17.9	14.75/33.4
	Week 24	5.77/23.4	5.17/24.6	6.55/22.0
	p ^a	0.6177	0.0232	0.0017
Minor	Baseline	7.60/23.9	4.66/17.9	11.41/31.6
	Week 24	5.75/23.4	5.14/24.6	6.55/22.0
	p ^a	0.8508	0.0232	0.0084
Nocturnal	Baseline	4.17/18.1	2.93/14.0	5.78/23.3
	Week 24	1.43/ 9.2	1.33/9.4	1.57/8.9
	p ^a	<0.0001	0.0483	<0.0001
Major	Baseline	2.48/11.7	1.82/9.9	3.34/14.0
	Week 24	0.02/0.2	0.04/0.3	0
	p ^a	<0.0001	<0.0001	<0.0001

FPG: fasting plasma glucose; HbA_{1c}: glycated haemoglobin A_{1c}; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; PPPG: postprandial plasma glucose; SBP: systolic blood pressure. ^ap-value is for difference in percent of patients with at least one event. ^bData are mean (SD) unless otherwise specified.

by pre-study therapy is presented in Table 2. The incidence of major hypoglycaemia decreased in the entire cohort (with a statistically significant difference in the percentage of patients reporting at least one event, $p < 0.0001$). In the insulin-naïve patients, the reported rate of overall hypoglycaemia decreased marginally from 6.49 to 5.17 events/patient-year, with a statistically significant increase in the percentage of patients reporting at least one event ($p < 0.05$). In the prior insulin users, the reported rate decreased from 14.75 to 6.55 events/patient-year after 24 weeks, associated with a statistically significant reduction ($p < 0.005$) in the percentage of patients reporting at least one event.

For insulin-naïve patients, the rate of minor hypoglycaemic events increased from 4.66 to 5.14 events/patient-year (percentage of patients reporting at least one event, $p < 0.05$) while the rate of nocturnal hypoglycaemia decreased from 2.93 to 1.33 events/patient-year (percentage of patients reporting at least one event, $p < 0.05$). In the prior insulin users, the incidence of minor and nocturnal hypoglycaemic events decreased from 11.41 to 6.55 events/patient-year (percentage of patients reporting at least one event, $p < 0.01$) and 5.78 to 1.57 events/patient-year (percentage of patients reporting at least one event, $p < 0.0001$), respectively, during the study period.

Body weight, blood lipids and blood pressure control

Mean body weight change over 24 weeks was statistically significant (Table 2, $p < 0.001$ for the entire cohort and insulin-naïve patients; $p < 0.005$ for prior insulin

users). The mean body weight increase for the entire cohort was 1.4 kg (1.9 kg for insulin-naïve patients and 0.7 kg for prior insulin users).

Total cholesterol levels reduced in the entire cohort from a mean of 5.2 mmol/L at baseline to 4.7 mmol/L at Week 24 (Table 2, -0.6 mmol/L, $p < 0.001$). Low-density lipoprotein cholesterol levels reduced from a mean of 3.3 to 2.9 mmol/L after 24 weeks (-0.4 mmol/L, $p < 0.001$) and a statistically significant reduction was also seen in triglycerides levels (-0.2 mmol/L, $p < 0.005$) in the entire cohort. No change in high-density lipoprotein cholesterol levels was noted during the study.

Mean SBP reduced in the entire cohort, from 135.4 mmHg at baseline to 131.9 mmHg after 24 weeks of treatment (Table 2, -3.5 mmHg; $p < 0.001$).

SADRs and serious adverse events

Overall, the incidence of SADRs was low; 4 SADRs (all hypoglycaemic events) were reported by 4 patients (0.01 events/patient-year).

The event rate of serious adverse events (SAEs) was also low (0.02 events/patient-year); 8 patients had 11 SAEs and 7 events had fatal outcomes. The majority of SAEs were reported under metabolism and nutrition disorders (4 events in 4 patients), of which all events were hypoglycaemia-related.

Quality of life assessments

As measured by the VAS from the EQ-5D questionnaire (on a scale of 0–100), QoL increased significantly by 16

points from 60.1 points at baseline to 76.1 points at Week 24 for the entire cohort ($p < 0.001$).

DISCUSSION

This sub-analysis demonstrated the safety and effectiveness of BIAsp 30 treatment in T2D in routine clinical practice in Morocco.

International data from routine clinical practice reveal that glycaemic control in T2D patients is generally poor (Davidson et al., 2008). In the IMPROVE observational study, almost 50% of patients had an $HbA_{1c} > 9.0\%$ at baseline (Valensi et al., 2009b). This was reflected in the global A₁chieve study where the mean baseline HbA_{1c} value was 9.5% (Home et al., 2011) and in the Moroccan data in this paper where the mean baseline HbA_{1c} value was 9.8%.

For the Moroccan cohort, BIAsp 30 therapy significantly improved overall glycaemic control over 24 weeks, as measured by HbA_{1c} , pre-breakfast FPG and post-breakfast PPPG values. These results are similar to the global A₁chieve study results (Home et al., 2011) and the results of two other large observational studies, IMPROVE and PRESENT (Valensi et al., 2009b; Khutsoane et al., 2008). Overall, 19.7% of Moroccan patients achieved HbA_{1c} levels < 53 mmol/mol ($< 7.0\%$) with BIAsp 30 treatment after 24 weeks. It is possible that earlier insulin initiation and more active intensification of treatment may be helpful in allowing a greater proportion of T2D patients to attain long-term glycaemic goals.

The marked improvement in glycaemic control was accompanied by a low incidence of SADR and hypoglycaemia. The risk of hypoglycaemia typically increases when insulin is used to attain better glycaemic control and defined glycaemic targets (Gerstein et al., 2008). As expected following the initiation of BIAsp 30 therapy, overall and minor hypoglycaemia increased slightly in the insulin-naïve patients. For prior insulin users, overall and minor hypoglycaemia reduced significantly with BIAsp 30 therapy as also observed in the IMPROVE and PRESENT studies (Valensi et al., 2009b; Khutsoane et al., 2008). In the entire Moroccan cohort, the percentage of patients reporting nocturnal hypoglycaemia decreased significantly and the reduction in major hypoglycaemia was also marked.

Statistically significant reductions in blood lipids and SBP were also noted in the entire cohort. These results are similar to the findings from the global A₁chieve study (Home et al., 2011) and other studies on BIAsp 30 (Gero et al., 2009; Schmoelzer et al., 2005). It is possible that physicians and patients may have taken advantage of the start of insulin analogue therapy to augment self-management behaviours (Home et al., 2011); however, as diet, lifestyle and concomitant disease and medication were not controlled, detailed information on the likely changes initiated is not available.

Initiating BIAsp 30 was shown to positively impact QoL

as significant improvements in QoL were reported after 24 weeks for Moroccan patients, as also observed in the global A₁chieve study (Home et al., 2011).

As this observational study was not randomized and lacked a standardized design, limiting factors such as the absence of a control group, retrospective data collection methods and the potential for recall bias in observations such as the occurrence of hypoglycaemia may not be excluded. However, all measurements were performed in accordance with local regulations and by methods that are NGSP-certified. The 24-week study duration was considered suitable to evaluate patient responses to the regulatory-approved BIAsp 30 therapy. In contrast to a more stringent randomized clinical trial setting, this study provided a valuable opportunity to collect real-life data on the safety and effectiveness of BIAsp 30 from a heterogeneous patient group in Morocco.

Conclusions

In conclusion, starting BIAsp 30 therapy was well-tolerated in this Moroccan cohort and resulted in significant improvements in glycaemic control. The poor baseline glycaemic control evinced in this cohort also points to the urgent need to reconsider current healthcare practices for T2D management in Morocco.

ACKNOWLEDGEMENTS

The authors would like to thank all participants who provided data, and all investigators involved in the A₁chieve study. The authors would also like to thank Anjali Philip of Cognizant for writing assistance, funded by Novo Nordisk.

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Full Length Research Paper

Efficacy of praziquantel for the treatment of schistosomiasis in Ethiopia

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Accepted 4 February, 2013

Schistosomiasis is a tropical disease caused by blood-dwelling fluke of the genus *Schistosoma*. The main schistosomes infecting human beings are: *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. *Schistosoma intercalatum* and *Schistosoma mekongi* are only of local importance. Two clinical forms of human schistosomiasis occur in Ethiopia: *S. mansoni* which is transmitted by *Biomphalaria pfeifferi* and *Biomphalaria sudanica*; and *S. haematobium* which is transmitted by *Bulinus abyssinica* and *Bulinus africanus*. The national policy on schistosomiasis control has adopted praziquantel as the main drug of use to reduce morbidity. It is widely preferred owing to its safety, present low cost, accepted single dose with improved patient compliance, and efficacy against all five schistosome species. While global use of praziquantel is scaling up, there is also a growing concern regarding low cure rate and drug resistance. Data regarding the efficacy of praziquantel are still missing at this time when there is increased concern that schistosomes might develop resistance to the drug. Results from infected patients, not cured by multiple doses of praziquantel, have been reported from different geographic locations, suggesting that resistance to the drug may be present. In Ethiopia, field report shows that praziquantel is efficacious. The purpose of this review is to summarize results from field and laboratory studies on efficacy of praziquantel mainly in Africa and to relate the findings to the Ethiopian situation.

Key words: Praziquantel, schistosomiasis, drug efficacy, drug resistance, toxicity, dose and drug administration

INTRODUCTION

Schistosomiasis is a tropical disease caused by blood-dwelling fluke worms of the genus *Schistosoma*. Soil-transmitted helminth (STH) and schistosome infections are recognized as a major public health problem in developing countries. For schistosomiasis alone, more than 200 million people are affected worldwide, of whom more than 30 million suffer from associated severe morbidity causing 155,000 deaths annually. World Health Organization (WHO) estimates that 600 million people are at risk of infection and 120 million display symptoms. The disease is a major growing health problem in Ethiopia (Jemaneh, 2000).

The main schistosomes infecting human beings are:

Schistosoma mansoni, which is transmitted by *Biomphalaria* species snails and causes intestinal schistosomiasis in Africa, Arabian Peninsula, and South America; *Schistosoma haematobium*, transmitted by *Bulinus* species and causes urinary schistosomiasis in Africa and Arabian Peninsula; and *Schistosoma japonicum*, transmitted by *Oncomelania* species and causes intestinal and hepatosplenic schistosomiasis in China, Philippines, and Indonesia. *Schistosoma intercalatum* and *Schistosoma mekongi* are only of local importance.

Two clinical forms of human schistosomiasis occur in Ethiopia: *S. mansoni* which is transmitted by *Biomphalaria*

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pfeifferi and *Biomphalaria sudanica*; and *S. haematobium* which is transmitted by *Bulinus abyssinica* and *Bulinus africanus*.

The devastating effects resulting from schistosomiasis include impaired cognitive potential among primary school-age children, hepatosplenomegaly, anemia, bladder cancer and stunted growth.

Three compounds are currently in use, that is, metrifonate, oxamniquine, and praziquantel (PZQ) for treatment of schistosomiasis and all are included in the World Health Organization list of essential drugs. PZQ is currently the drug of choice for the treatment of schistosomiasis and is rapidly becoming the only commercially available antischistosomal drug. It is widely preferred owing to its safety, present low cost, accepted single dose with improved patient compliance, and efficacy against all five schistosome species (Webbe and James, 1977). Since the mid 1980s, along with a significant cost reduction, PZQ has become the drug of choice for morbidity control due to schistosomiasis (WHO, 2002). The superiority of PZQ over other antischistosomal compounds that have been available for some time, including metrifonate and oxamniquine, has been proven (Ferrari et al., 2003). While global use of PZQ is scaling up (Fenwick et al., 2003), there is also a growing concern regarding low cure rate and drug resistance. PZQ resistance has emerged, or will soon emerge, in human parasites.

Requirements for large amounts of PZQ are anticipated as new efforts are underway for mass treatment in several African countries (Fenwick and Webster, 2006). Countries, which have a high prevalence of schistosomiasis, have devised an action plan for the control of schistosomiasis that includes regular treatment of infected people using a school-based approach. However, data regarding the efficacy of PZQ are still missing at this time when there is increased concern that schistosomes might develop resistance to the drug (WHO, 2005).

Results from infected patients, not cured by multiple doses of PZQ, have been reported from different geographic locations, suggesting that resistance to the drug may be present. This has been coupled with several *in vivo* (e.g. studies on mice infected with 'resistant isolates') and *in vitro* tests (e.g. direct application and measurement of the effect of the drug on schistosomes maintained in culture) demonstrating a significant reduction in the drug's efficacy.

Currently, population based target/mass chemotherapy with PZQ is the cornerstone in schistosomiasis control strategies. In Ethiopia, PZQ is administered for treatment of cestodes and trematode at different regimen. After epidemiological survey, PZQ therapy for school children and community is common. However, the efficacy of PZQ against Ethiopian strain at different foci is not well investigated and documented. Therefore, the purpose of this review paper is to summarize results from field and

laboratory studies on efficacy of PZQ mainly in Africa and to relate the findings to the Ethiopian situation.

PZQ

PZQ, discovered in the 1970s, was subsequently introduced for the treatment of schistosomiasis. Its chemical formula is C₁₉H₂₄N₂O₂ and has a molecular mass of 312.411. PZQ, a broad spectrum schistosomicide, is a pyrazinoisoquinoline derivative. It is a pyrazinoisoquinoline with an asymmetric center, and standard preparations are composed of equal proportions of the active, levo (-) and the inactive, dextro (+) optical isomer. It is a white to nearly white crystalline powder of bitter taste, melting at 136 to 140°C with decomposition. It is stable under normal conditions and it is practically insoluble in water, sparingly soluble in ethanol and soluble in organic solvents like chloroform and dimethylsulfoxide.

PZQ has activity against all species of schistosomes and shows minimal side effects. As a consequence, it has become the drug of choice against schistosomiasis. Indeed, with the added benefit of dramatic reductions in price, PZQ has in essence become the sole antischistosomal agent that is available commercially (Fenwick et al., 2003; Hagan et al., 2004). PZQ is also active against other trematode and cestode infections, though generally not against nematodes (Andrews, 1985). Schistosomes show stage-dependent differences in PZQ sensitivity (Pica-Mattocchia and Cioli, 2004).

Both *in vivo* and *in vitro* test shows that immature stages of schistosomes are not sensitive to PZQ. Experimental studies have shown that immature (2 to 4 weeks old) worms are refractory to a number of schistosomicidal drugs, including PZQ (Xiao et al., 1985).

MODE OF ACTION/MOLECULAR TARGETS OF PZQ

Despite the widespread use of PZQ and nearly three decades of research, the exact mechanism of PZQ action is still unresolved (Day et al., 1992; Redman et al., 1996; Harder, 2002; Cioli and Pica-Mattocchia, 2002). The detailed molecular mechanism of action of PZQ has not yet been elucidated (Day et al., 1992; Cioli, 2000), but a few phenomena connected with its effects are well known.

The most obvious and immediate modification that can be observed in schistosomes exposed to the drug either *in vitro* or *in vivo* is a spastic paralysis of the worm musculature. This contraction is accompanied and probably caused by a rapid Ca²⁺ influx inside the schistosome (Cioli and Pica-Mattocchia, 2002). Another early effect of PZQ consists in morphological alterations that can be observed in the worm tegument, initially represented by vacuolization at the base of the tegumental syncytium and blebbing at the surface (Cioli

and Pica-Mattoccia, 2002; Mehlhorn et al., 1981). These morphological alterations are accompanied by an increased exposure of schistosome antigens at the parasite surface (Harnett and Kusel, 1986). Some of the drug exposed antigens have been identified and appear to be connected with the host immune response that is required for the complete activity of PZQ (Doenhoff et al., 1987; Brindley et al., 1989).

An interesting report drew attention to schistosome calcium channels as the possible molecular target of PZQ (Kohn et al., 2001). The β -subunits of these channels appear to have a different structure from other known β -subunits and, when expressed together with heterologous α -subunits, can confer to the latter a previously nonexistent sensitivity to PZQ.

There are recent advances in identifying the molecular target of PZQ. PZQ acts selectively against members of the phylum Platyhelminthes. Accordingly, the molecular target (or targets) for PZQ might be encoded by a novel gene found exclusively in the flatworms. Schistosome genomes and transcriptomes contain several sequences that show no clear cut homology with genes found in other phyla (Hu et al., 2004; LoVerde et al., 2004; McManus et al., 2004; Verjovski-Almeida et al., 2004). On the other hand, the target for PZQ might be a member of a gene family found in other phyla as well as in the Platyhelminthes, but with platyhelminth-specific structural signatures required for interaction with the drug. Even minor differences in critical domains of a protein, including single amino acid alterations, can have major consequences for the functional and pharmacological properties of typical receptors and channels (Greenberg, 2005; Satin et al., 1992).

Though, elucidating the mode of action of PZQ has proved a daunting task, the effects of the drug on adult schistosomes do provide clues to potential targets for the drug. PZQ produces a well-documented effect on intracellular Ca^{2+} levels in adult schistosomes (Andrews, 1985; Day et al., 1992; Redman et al., 1996). Within seconds of exposure to the drug, adult schistosomes exhibit a rapid, sustained contraction of the worm's musculature (Greenberg, 2005) and vacuolization and disruption of the parasite tegument (Cioli and Pica-Mattoccia, 2002; Mehlhorn et al., 1981), an effect associated with the subsequent exposure of parasite antigens on the surface of the worm (Harnett and Kusel, 1986). Both of these responses are thought to be linked to a PZQ-dependent disruption of Ca^{2+} homeostasis (Day et al., 1992; Redman et al., 1996). PZQ elicits a rapid uptake of Ca^{2+} in adult schistosomes. The effects of PZQ on both contraction of the worm's musculature and disruption of the parasite tegument are Ca^{2+} -dependent processes. Removal of Ca^{2+} from the medium blocks both responses (Cioli and Pica-Mattoccia, 2002; Mussie et al., 1982; Xiao et al., 1984). However, neither of these inhibitory effects appears immediately. For example, inhibition of the PZQ-dependent contraction of the

musculature requires at least 10 min to occur, a delay thought to correspond to the time required for depletion of sequestered intracellular Ca^{2+} stores. These results indicate that though extracellular Ca^{2+} is not required for the initiation of PZQ-dependent action, it is required for maintenance of the response.

Based on comparisons between PZQ response in intact and detegumented parasites, it appears that both the tegument and the sarcolemma contain PZQ-sensitive sites (Blair et al., 1992). Thus, intact worms that are bathed in a medium with high magnesium (Mg^{2+}): Ca^{2+} ratio exhibits a PZQ-dependent biphasic muscle contraction instead of the tonic contraction that occurs in standard media. Detegumented worms continue to respond to PZQ, but they show only a single, pronounced phasic contraction in high Mg^{2+} , indicating that a tegumental site is necessary for the full response. Furthermore, unlike intact worms, which show a transient response to PZQ in Ca^{2+} -free medium, application of PZQ to detegumented worms in Ca^{2+} -free medium produces no muscular contraction. Interestingly, PZQ (1 to 2 μM) has been reported to interact with both sarcolemmal and intracellular sites to produce a sustained Ca^{2+} -dependent contraction in the penile retractor muscle from the mollusc *Lymnaea stagnalis* (Greenberg, 2005).

The effects of PZQ on Ca^{2+} homeostasis could point to a direct action of the drug on membrane permeability to Ca^{2+} . However, early experiments indicated that PZQ is not acting as a Ca^{2+} ionophore (Cioli and Pica-Mattoccia, 2002). On the other hand, it has been reported that PZQ alters the structure of membrane bilayer phospholipids or membrane fluidity (Greenberg, 2005), which could result in changes in membrane permeability to Ca^{2+} or to indirect effects on membrane receptors and channels.

Recently, voltage-gated Ca^{2+} channels have been identified as candidate targets of PZQ action (Kohn et al., 2003). As important entry sites for extracellular Ca^{2+} , voltage-gated Ca^{2+} channels play a critical role in regulating levels of intracellular Ca^{2+} . However, until recently, the role of voltage-gated Ca^{2+} channels in PZQ action had not been tested directly, as Ca^{2+} currents had never been recorded from schistosome cells. Nevertheless, pharmacological studies (Blair et al., 1992) on PZQ-induced contraction in both intact and detegumented worms led them to suggest that Ca^{2+} channels might be involved in the action of the drug. Interestingly, high concentrations (50 μM) of PZQ prolong the Ca^{2+} -dependent plateau phase of the cardiac action potential in rats, which is carried by voltage-gated Ca^{2+} channels (Greenberg, 2005). On the other hand, methoxyverapamil (D-600), an inhibitor of one class of mammalian Ca^{2+} channels (L-type), does not block the PZQ-dependent Ca^{2+} influx in schistosomes, though it does block the tonic contraction of these cells resulting from increased K^{+} concentrations (Greenberg, 2005). However, recent results from expression of cloned Ca^{2+} channel proteins indicates a significant role for voltage-gated Ca^{2+} channels

in PZQ action. Voltage-gated Ca^{2+} channels are membrane protein complexes that form Ca^{2+} -selective pores gated by depolarization. Like other voltage-gated channels, Ca^{2+} channels contribute to impulse propagation, but they are also essential regulators of intracellular Ca^{2+} levels. By providing a pathway for rapid Ca^{2+} influxes, Ca^{2+} channels couple depolarization of the cell to a wide array of Ca^{2+} -dependent responses including muscle contraction and neurosecretion in muscles, nerves, and other excitable cells (Greenberg, 2005).

PHARMACOKINETICS AND CLEARANCE

PZQ is well (approximately 80%) absorbed from the gastrointestinal tract. PZQ and its metabolites are mainly excreted in the urine, and within 24 h after a single oral dose, 70 to 80% are found in urine, but less than 0.1% is found as the unchanged drug. PZQ is metabolized through the cytochrome P450 pathway 3A4. Orally administered PZQ is rapidly absorbed, measurable amounts appearing in the blood as early as 15 min after dosing (Valencia et al., 1994). Maximum plasma concentration after a standard dose of 40 mg/kg shows wide inter-individual variations in the range of 200 to 2,000 ng/ml (Mandour et al., 1990). PZQ undergoes a pronounced liver first pass metabolism, with rapid disappearance from the circulation and a plasma half-life generally ranging between 1 and 3 h. Elimination occurs essentially through the urine and the feces and it is more than 80% complete after 24 h (Cioli and Pica-Mattocchia, 2002).

The main metabolites of PZQ are represented by mono-, di- and tri-hydroxylated compounds that are produced in the liver by microsomal cytochrome P450, particularly by those isoforms (2B1 and 3A) that are experimentally inducible by phenobarbitone (Masimirembwa and Hasler, 1994; Giorgi et al., 2001). The most abundant metabolite is the 4-hydroxycyclohexylcarbonyl analog (that is, the compound with a single hydroxyl group in the 4'-position of the cyclohexane ring), which represents about two thirds of total urinary metabolites. The bioavailability of PZQ is increased by the simultaneous administration of substances that inhibit cytochrome P450 activities. For instance, cimetidine causes a 100% increase (Metwally et al., 1995; Cioli and Pica-Mattocchia, 2002) and has been used in association with PZQ especially for the treatment of neurocysticercosis, where high drug concentrations are required. Similar increases can be effected by 17 alpha-ethynylestradiol and diphenylhydramine, whereas the opposite effect is observed after the simultaneous administration of antiepileptics or corticosteroids, especially carbamazepine, phenytoin or dexamethasone (Na-Bangchang et al., 1995; Cioli and Pica-Mattocchia, 2002).

The hepatic dysfunction accompanying the late stages of schistosomal disease was found to be associated with slower PZQ metabolism and disposition (El Guiniady et

al., 1994).

Agents that induce or inhibit Cyp450 3A4 (that is, phenytoin, rifampin, azole antifungal) will have an effect in the metabolism of PZQ. In tropical areas, PZQ may be administered together with the antimalarial chloroquine, an association that was found to decrease the bioavailability of PZQ and to reduce its maximum serum concentration to a significant extent in rats and in humans (Cioli and Pica-Mattocchia, 2002).

DOSE AND ADMINISTRATION

According to special dosing schedules for each different indication, one single dose or a one-day treatment with divided doses may be sufficient. The recommended dose is 40 to 60 mg/kg body weight, the lower amount being generally used for *S. mansoni* and *S. haematobium*, while the higher dose (generally split into two administrations a few hours apart) is especially recommended for Asian schistosomes (*S. japonicum* and *Schistosoma mekongi*) (WHO, 2002). But 40 mg/kg is the most commonly administered dose. It has been repeatedly reported that the bioavailability of PZQ increases with the concomitant administration of food (Mandour et al., 1990; Homeida et al., 1994), a procedure that should be considered whenever possible. The increased bioavailability of PZQ upon simultaneous food administration may be mediated by modifications in microsomal enzyme activities.

PZQ has not been formally tested in pregnant or lactating women. Although administration to pregnant women has been avoided in general practice (Kusel and Hagan, 1999), concerns have been expressed that withholding treatment may actually involve more detrimental effects than substantial risks. An ad hoc committee recently convened by WHO (2002) has indeed recommended that PZQ treatment be offered to pregnant and lactating women as well. No significance differences has been found in the occurrence of adverse birth outcomes (abortion, stillbirth, birth defect) between women inadvertently exposed to PZQ and women not exposed to the drug (Adam et al., 2004). In areas where schistosomiasis are endemic, risk-benefit analysis have revealed that the health advantages of treating women of reproductive age and pregnant women far outweigh the risks to their health and to the health of their babies (Savioli et al., 2003). PZQ therapy is eligible for school children (6 to 15 age) but ineligible to children under 4 years of age (WHO, 2006).

EFFICACY OF PZQ

Since the early animal studies, it was apparent that PZQ is equally effective against *S. mansoni*, *S. haematobium*, *S. japonicum*, *Schistosoma intercalatum* and *Schistosoma mattheei* (Webbe and James, 1977).

Neurological syndromes caused by *S. mansoni* and *S. haematobium* also respond well, possibly in association with corticosteroids (Cioli and Pica-Mattoccia, 2002). Acute toxemic forms (Katayama fever) are also treated with PZQ (Monson, 1987; Farid et al., 1987).

The major weakness of PZQ is its lack of efficacy against juvenile schistosomes. This has been clearly shown in *in vitro* tests (Xiao et al., 1985) and it has been confirmed by clinical data (Gryseels et al., 2001). The sensitivity of schistosomes to PZQ has a peculiar biphasic profile, with the earliest stages (from cercariae to the first few days after infection) being susceptible, followed by progressive insensitivity down to very low levels around 3 to 4 weeks after infection (Gryseels et al., 2001). This age-dependence of activity is probably the source of most treatment failures experienced with PZQ in clinical practice. In endemic areas with active transmission of schistosomiasis, any patient at the time of treatment has a given probability of having been infected in the previous 3 to 5 weeks. Such a patient would thus harbor immature schistosomes that are not killed by PZQ and that will mature and deposit eggs in the subsequent weeks, thus resulting in an apparent drug failure. To overcome this problem, a protocol has been proposed that contemplates two PZQ doses spaced 3 weeks apart and a follow-up examination 2 weeks after the second dose (Renganathan and Cioli, 1998). Another possibility would be to administer PZQ together with artemether, a drug that has been found to be active against immature schistosomes, with an age-activity profile that is exactly complementary to that of PZQ (De Clercq et al., 2000; Utzinger et al., 2001).

Researches have been done to assess the efficacy of PZQ in different epidemiological settings. Cure rates are recorded using the recommended dosages: 75 to 85% for *S. haematobium*; 63 to 85% for *S. mansoni*; 80 to 90% for *S. japonicum*; 89% for *S. intercalatum* and 60 to 80% for double infections with *S. mansoni* and *S. haematobium* (Cioli and Pica-Mattoccia, 2002). These values help to evaluate the efficacy of PZQ in different schistosomiasis endemic areas. Cure rate of PZQ against *S. haematobium* in Northern Senegal was 30%, 5 weeks after treatment and it remained low until the end of the study, although the cure rates at 12 weeks (55%) and 24 weeks (44%) were higher than those at 5 weeks (De Clercq et al., 2002). Since early stages of schistosomes are not susceptible to PZQ, the maturation rate and fecundity of the parasite should be considered at time of assessing PZQ efficacy. Fallon et al. (1997) have examined the fecundities and drug susceptibilities of *S. mansoni* isolates from Senegal, Puerto Rico, and Kenya in mice. The Senegal parasite, obtained from the field in 1993, was shown to have a longer prepatent period (eggs first recovered in the faeces on day 46 after infection) than those of two isolates, that had been maintained for a long period in the laboratory (faecal eggs recovered on days 38 and 36 after infection, respectively). A Kenyan

isolate, also collected from the field in 1994, was shown to mature more slowly than the laboratory-maintained Kenyan isolate. Tissue egg counts confirmed that early in infection, the fecundity of the recently collected isolates from Senegal and Kenya was significantly lower than that of the long-term laboratory-maintained Kenyan isolate. King et al. (2000) examined the long-term efficacy of PZQ (Biltricide, Bayer, Leverkusen, Germany) against *S. haematobium* during a school-based treatment program in the Msambweni area of Coast province, Kenya. Results indicated substantial year-to-year variation in drug efficacy, from a cure rate of 96% in 1990 to a cure rate of 65% in 1986. Kihara et al. (2007) studied the efficacy of PZQ (Prazitel Cosmos) against *S. mansoni* in school children in Mwea (Kenya), it was 92.6% and indicate a good reduction in parasite burden. Tchuem-Tchuente et al. (2004) in Cameroon studied to determine the efficacy of PZQ (Shin Poong, Seoul, South Korea) against *S. haematobium*. Their results indicated that a single treatment with PZQ possesses a high efficacy since the sixth and ninth weekss post-treatment cure rate was 83 to 88.6% and the egg reduction rate was 98%. PZQ is efficacious against *S. haematobium* in Zimbabwe with overall cure rate of 88.5% and the egg reduction rate was 98.2% (Midzi et al., 2008). Raso et al. (2004) assessed the efficacy of PZQ against *S. mansoni* in a rural community of Western C^ote d'Ivoire. They reported overall cure rate, assessed 6 weeks post-treatment, of 60.9%, which indicated that the drug is not efficacious in such area under a given epidemiological settings.

In Ethiopia studies on PZQ efficacy and drug resistance is not much pronounced. Degu et al. (2002) studied PZQ (brand not mentioned) efficacy against *S. mansoni* in North West Ethiopia (Gorgora village) and they found that the average egg reduction rate was 97% and cure rate was 94%, six weeks after single PZQ treatment. They conclude that there is no evidence for PZQ resistance in this area. Here, the single dose treatment is not recommended to assess efficacy instead of two PZQ doses spaced 3 weeks apart and a follow-up examination 2 weeks after the second dose is the recommended protocol (Renganathan and Cioli, 1998). Berhe et al. (1999) studied the efficacy of PZQ (Laboratoria Wolfs N.V., Antwerp, Belgium) against *S. mansoni* in North-east Ethiopia in Borkena valley (Bati, Harbu and Kemise). The cure rate of PZQ among 541 children who had stool examination 5 weeks after treatment was 83.2%. Since maturation rate of the strain is not studied in detail; assessment after 5 week therapy with single dose is not a reliable data. The other study on efficacy of PZQ for the treatment of *S. mansoni* was tested on four groups of Ethiopian sugar estate workers. The cure rates were 96, 93 and 74% at one, three and six months post-treatment for patients receiving a single dose (40 mg/kg body weight) of PZQ (Taddese et al., 1988). Although the studies showed PZQ to be efficacious, single dose administration is not currently recommended for assessment

of PZQ efficacy. Birhanu et al. (2008) evaluate the current efficacy of PZQ (Biltricide, Bayer AG, Germany) against *S. haematobium* in Dulshatalo village (Kurmuk) in Western Ethiopia and reported the cure rate and parasitological egg reduction rate to be 86.8 and 84.67%, respectively. They conclude that, PZQ (40 mg/kg body weight) is still effective for the control of *S. haematobium* in Ethiopia.

TOXICITY STUDIES AND SIDE EFFECTS

In general, the toxicity of PZQ in animals was found to be very low, both in acute and long-term experiments (Cioli and Pica-Mattocchia, 2002). No genotoxic risks could be demonstrated from various mutagenicity studies in bacterial, yeast, *Drosophila* and mammalian systems (Cioli and Pica-Mattocchia, 2002). Occasional and somewhat conflicting reports have claimed clastogenic, co-clastogenic or anticlastogenic effects of PZQ. No signs of mutagenicity were detected in patients treated with the high doses employed for neurocysticercosis (Cioli and Pica-Mattocchia, 2002). A wary review of all possibly suspicious data (Montero and Ostrosky, 1997) argues for more genotoxic and/or carcinogenic studies, based mainly on the consideration that there might be some human genetic polymorphism leading to the accumulation of potentially mutagenic metabolites.

Relatively new and at times serious side effects continue to be reported. The majority of side-effects develop due to the release of the contents of the parasites as they are killed and the consequent host immune reaction. The heavier the parasite burden, the heavier and more frequent the side effects normally. Stomach discomfort, dizziness, diarrhea, nausea, headache, vomiting, itchy skin, lethargic and sleepy swollen face are reported side effects by Midzi et al. (2008). Berhe et al. (1999) had reported that abdominal cramps, dizziness, nausea, weakness and headache are PZQ treatment associated side effects.

RESISTANCE TO PZQ

Since PZQ serves as the only antischistosomal treatment in widespread use, there might be the possibility of emerging drug resistance. The first alarming reports of possible PZQ resistance came from an intensive focus in Northern Senegal, where the drug had produced very low cure rates (18 to 39%) (Cioli and Pica-Mattocchia, 2002; Stelma et al., 1995). The most common interpretation of these findings is that they were mainly due to the peculiar epidemiological situation of the focus, that is, high numbers of worms present in each patient, high probability of immature parasites and rapid re-infection (Cioli, 2000; Gryseels et al., 2001).

Additional evidence for resistance to PZQ was collected

in Egypt, where a number of schistosome isolates were established in the laboratory from the eggs excreted by patients who had been unsuccessfully treated (three times) with PZQ (Ismail et al., 1996). Some of the isolates obtained from easily cured patients showed a decreased sensitivity to PZQ *in vivo* (Bennett et al., 1997) and *in vitro* (Ismail et al., 1999). Differences in ED50 (the dose of PZQ required to kill 50% of adult worm) between sensitive and resistant schistosomes are relatively small (2 to 6 folds), and no practical clinical problems have been detected so far in the area.

The common principle that researchers agreed up on, is the development of drug resistance in the course of long time treatment. Ismail et al. (1996) reported that the extensive use of PZQ in the Nile Delta region of Egypt has not resulted in a dramatic change in the efficacy of PZQ. Liang et al. (2001) look for possible evidence of the development of resistance in *S. japonicum* to PZQ in China. The results indicate that there was no evidence for reduced susceptibility of *S. japonicum* to PZQ despite its extensive use in the main endemic areas of China for more than 10 years. But schistosomes that have been repeatedly subjected to drug pressure in the laboratory have been found to be less sensitive to PZQ than the original not subjected strain (Fallon and Doenhoff, 1994; Liang et al., 2001). *S. mansoni* subjected to drug pressure may develop resistance to schistosomicidal drugs over the course of relatively few passages (Fallon and Doenhoff, 1994).

PZQ resistance survey carried out in 3 villages of Egypt and the results revealed that PZQ was effective and reduced egg count significantly; however, at the end of the study, some cases remained infected. Several factors that can be responsible among them are the presence of resistance strains (Ismail et al., 1996). The aggressive use of PZQ to combat schistosomiasis in Egypt raises concern about the possible emergence of resistance.

One of the hallmark effects of PZQ on schistosomes *in vitro* is a disruption of the worm's outer surface, the tegument. PZQ-induced tegumental damage is observed in 3 distinct isolates, 2 derived from resistant infections and 1 from an infection cured by a single dose. The isolates from the resistant infections were less susceptible to PZQ-induced tegumental damage *in vitro*, suggesting that the worms are in some way less responsive to the drug (William et al., 2001)

The potential for resistance to PZQ was demonstrated by Fallon and Doenhoff (1994). They showed in the laboratory that applying drug pressure to successive mouse passages of a hybrid isolate raised from a pool of cercariae of four geographically separate *S. mansoni* isolates produced worms that were less sensitive to PZQ. In human infections, there have been no confirmed cases of resistance to PZQ, and yet reports of failure to cure all adult patients and Kenyan children treated twice a month apart (Coles et al., 1987) were reported in the 1980s. Testing the response of *S. mansoni* isolates from uncured

children after their placement in mice revealed normal susceptibility to PZQ (Bruce et al., 1987).

The first strand of evidence for possible resistance to PZQ was from Northern Senegal, an area of high transmission with 41% of the subjects excreting >1000 eggs/g faeces. In this area, treatment conducted during 1994 to 1995 with the standard PZQ dose (40 mg/kg) recommended for population based chemotherapy gave cure rates of 18 to 39% only (Gryseels et al., 1994; Stelma et al., 1995), which were alarmingly low when compared with the normally expected cure rates of 60 to 90% in the 1980s (Cioli and Pica-Mattocchia, 2002). Increasing the dose of PZQ from 40 to 60 mg/kg did not significantly improve the cure rates (Guisse et al., 1997). In the same area, a cure rate of 58.1% after the first treatment with no change after a second treatment was also recorded (Tchuem-Tchuente et al., 2001). When the parasite line collected from the same focus was tested in the laboratory, it showed significantly less response to PZQ (Fallon et al., 1995, 1994; Liang et al., 2001). In addition, when the routine dose of oxamniquine (20 mg/kg) was tested in the same area, a 79% cure rate was recorded, when compared with 36% in a group that was simultaneously treated with PZQ (Stelma et al., 1997).

The second source of evidence for possible resistance to PZQ has come from The Nile Delta region of Egypt, which examined the response of *S. mansoni*-infected villagers to PZQ, demonstrated a treatment failure of 1.6% after three PZQ treatments. Specifically, uncured patients after an initial dose of 40 mg/kg were given two additional treatments; one at 40 mg/kg followed by a second at 60 mg/kg (Ismail et al., 1996). Each dose was separated by 4 to 6 weeks to eliminate immature parasites maturing to egg-producing adult worms between treatment and follow-up. Eggs were detected from patients still excreting *Schistosoma* ova following the three doses of PZQ and were used to generate infection specific isolates in mice. A total of 80% of the resultant murine infections/isolates were significantly more difficult to cure when compared with mice infected with isolates from humans that responded to the drug. This diminished responsiveness was demonstrated as a significant increase in the dose that normally reduces worm burden by 50% (ED₅₀) (Ismail et al., 1999). The increase in ED₅₀ value did not exceed 3 to 5 folds of the ED₅₀ value recorded in control *S. mansoni* isolates that were collected, either before the introduction of PZQ or from patients successfully responding to a single dose of the drug. This contrasts sharply with values that have been reported for mice resistant to hycanthonone or oxamniquine. Here, a 1000-fold difference was recorded between resistant and sensitive parasites (Pica-Mattocchia et al., 1993). The sensitivity/insensitivity of these Egyptian isolates to PZQ after their placement into animals, away from human host confounding factors, was tested using well established *in vitro* effects of PZQ. These effects correlate with PZQ's *in vivo* action in

infected mice and include diminished *S. mansoni* worm motility (Ismail et al., 1999), tegument disruption (William et al., 2001) and calcium influx (William and William, 2004). King et al. (2000) examined the long-term efficacy of *S. haematobium* to PZQ, which may be more disastrous in terms of morbidity incidence, during a school-based treatment program in Kenya and reported year to year variation in response to PZQ.

Conclusion

PZQ consumption is expected to grow rapidly. Fenwick et al. (2003) expect a consumption >40 million tablets a year by the end of 2005. In view of the expected future increase in PZQ usage, researchers must remain vigilant.

In Senegal, it has been argued that subjects in the high transmission area of North of Senegal were probably facing heavy re-infections and they may have had many immature parasites, which are known to be insensitive to PZQ, at time of treatment. With regards to Egypt, and despite the reassuring data obtained from the field in 2005, *S. mansoni* isolates retrieved from Egyptian villages during the late 1990s showed resistance, or at least a decreased susceptibility to PZQ.

Further work is needed to elucidate the mode of action of PZQ, because no firm knowledge concerning PZQ's mode of action is available. Diminished responsiveness to PZQ was based upon examination of all documented effects for PZQ, including worm spastic paralysis (Ismail et al., 1999), tegumental disruption (William et al., 2001) and changes of Ca²⁺ influx (William and Botros, 2004), in addition to estimation of the drug ED₅₀ value (Ismail et al., 1999). These diminished responses were evident in isolates collected from a small percentage of villagers that could not be cured after three doses of PZQ. *In vivo* and *in vitro* tests on *S. mansoni* isolates showed that resistance to PZQ had occurred not only in some Egyptian isolates (Ismail et al., 1996, 1999; William et al., 2001, William and Botros 2004), but also in some Senegal isolates and a laboratory maintained isolate subjected to therapeutic pressure (Fallon et al., 1994, 1995, 1997; Liang et al., 2001). Although examination of the response of *S. mansoni*-infected Egyptian villagersto PZQ after a decade of drug pressure revealed a normal range of cure rates, one should take into account the drastic reduction in the intensity of the infections that occurred between the first and second observation. It is very difficult to put the existing data from the field and the laboratory findings into a meaningful context, because the efficacy of PZQ has not been monitored on a systematic basis with no base line data from the field.

Experimental studies have shown that immature (2 to 4-week-old) worms are refractory to a number of schistosomicidal drugs, including PZQ (Xiao et al., 1985; Sabah et al., 1986). Therefore, the state of maturation of a schistosome infection at the time when it is subjected to

drug treatment has implications for the evaluation of drug efficacy.

Several explanations considered to explain low cure rates of PZQ in the treatment of schistosomes: (i) diagnostic factor/method to assess cure; (ii) extremely high intensity of infections in this focus, so that, even if treatment was 99% effective, a sufficient number of schistosome pairs would survive and continue laying eggs; (iii) intense transmission and high reinfection rates, so that many individuals would harbour immature schistosomes, which are not susceptible for PZQ, at the time of treatment (Shaw, 1990); (iv) repeated infection in the interval between treatment and parasitological assessment; (v) immaturity of the human's anti-schistosome immune response in this recently established focus, it has been proposed that PZQ acts synergistically with the immune response of the host (Sabah et al., 1985); (vi) possible resistance of the strain to PZQ.

In Ethiopia, efficacy of PZQ was studied in limited foci in the field and all field reports show that PZQ is efficacious. But, no laboratory data that show the efficacy of PZQ against Ethiopian strains exist. Fecundity, maturation rate of the parasite, re-infection rate, etc., of Ethiopian schistosome parasite strain exposure to PZQ should be studied in the laboratory by using animal model systems to obtain a more precise information on the state of PZQ sensitivity in Ethiopia.

Abbreviations: PZQ, Praziquantel; STH, soil-transmitted helminthes.

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Full Length Research Paper

Liaison of tuberculosis and human immunodeficiency virus (HIV) co-infection in the progression to AIDS: Prognostic value of cluster differentiation 4 (CD4+) cell as marker of disease progression

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Accepted 24 January, 2013

This biphasic study investigated the prevalence and co-infection of the human immunodeficiency virus (HIV) and tuberculosis (TB) along major transport routes of Enugu State; their consortium in active HIV disease and the role of cluster differentiation 4 (CD4) cells and other haematologic parameters as markers of HIV progression. Prevalence and co-infection with HIV and TB were studied in the preliminary phase (Phase 1: 1999 to 2001), among 12,000 individuals who were screened for HIV using determine rapid test kits and TB by the Ziehl-Neelsen staining technique. Effect of TB on HIV progression using CD4 cell (estimated by cytometry using Partec Cyflow SL-3 counter) as surrogate marker was studied in Phase 2 (2005 to 2008) among 50 of those subjects presenting with AIDS-related clinical symptoms. Other investigated parameters included white blood cells (WBC) and haemoglobin/packed cell volume (PCV). The rate of co-infection with HIV and TB was 49.6%. The effect of TB on the progression of HIV to acquired immune deficiency syndrome (AIDS) was highly significant at $\alpha = 0.05$. The study confirmed the role of TB in furthering the progression of HIV to AIDS as well as the importance of the surrogate markers as indicators of immune system deterioration and subsequent decline.

Key words: Prevalence, co-infection, cluster differentiation 4 (CD4) cells, tuberculosis (TB), human immunodeficiency virus (HIV), surrogate markers.

INTRODUCTION

It is not easy to decide which of the deadly duo (human immunodeficiency virus (HIV) and tuberculosis (TB)) precedes the other in the precipitation of acquired immune deficiency syndrome (AIDS) condition. TB has already been recognized as the most common cause of death from a single pathogen worldwide and most of these deaths occur in the developing countries of Africa, Asia and South America (WHO, 1977; UNAIDS, 1977; Sunderman et al., 1986). The relationship between TB and HIV is thought to be synergistic, thus, the combined

effect of both is worse than their separate effects added together (Sunderman et al., 1986; Murray et al., 1990). HIV multiplies the problems of TB for individuals and entire communities; TB complicates the management and course of HIV infection (Nunn et al., 1990; Bonecici et al., 1998). Hence, the HIV-TB link has a dramatic impact, particularly in developing countries where 95% of people with TB and HIV live.

One of every four HIV-1-infected persons in the world is diagnosed with active TB, making TB the most frequent

life-threatening co-infection in HIV-1-infected patients (Harris, 1995). TB is associated with increased HIV-1 viral load, a fall in cluster differentiation 4 (CD4) lymphocyte (LYM) counts, and increased mortality. In a prospective epidemiologic study in Uganda on the impact of pulmonary TB on survival of HIV-infected individuals matched for CD4 cell count, the course of HIV-1 infection was found to be accelerated after TB diagnosis (Whalen et al., 2000). Pulmonary TB occurs before the onset of severe immunodeficiency at a relatively higher CD4+ counts (336 to 441 ml⁻¹) than in other opportunistic infections such as *Pneumocystis carinii* or *Toxoplasma gondii* (Mildvan and Muthur, 1987).

With the staggering worldwide growth of HIV pandemic, the Center for Disease Control and Prevention (CDC) has defined set of guidelines and recommendations for HIV-infected adolescents and adults on the basis of clinical conditions associated with the HIV infection and CD4+ T-lymphocyte counts (CDC, 1997). These CDC guidelines are based on studies done in developed countries, but are under trial in developing countries (Kam and Wong, 1998). Low CD4 T-cell count is considered to be a marker of the progression of HIV, and is associated with a variety of conditions, including many opportunistic infections, burns, trauma, etc. The low CD4 counts caused by some of these conditions often fall below 200 mm⁻³, which is the level needed to diagnose AIDS in someone who was previously positive for antibodies to HIV (CDC, 1999). The daily increasing incidence of HIV and subsequent diagnostic and management failure especially in the remote and resource poor communities of Nigeria, resulting from lack of trained personnel and infrastructure necessitates this study which investigated the prevalence HIV and TB co-infection in high risk communities of Nigeria, in the geographical area along the major North-South trucking/stop over transport routes of Enugu State, and to evaluate the association of the deadly duo in AIDS progression. This study attempts to assess the prognostic value of CD4 cells and other haematologic surrogate markers in disease progression with the view to establishing a globally accepted, cost effective and efficient tool for effective diagnosis and management of people living with HIV/AIDS in the rural communities of Nigeria.

MATERIALS AND METHODS

Study design

This biphasic study investigated the prevalence and co-infection of HIV and TB along major transport routes of Enugu State; their consortium in active HIV disease (Phase 1, 1999 to 2001), and the role of CD4 cells and other haematologic parameters as markers of HIV progression (Phase 2, 2005 to 2008).

Selection of study areas

This work was a survey of sub-groups in specific locations in part of South-Eastern Nigeria, namely, Enugu North and Enugu Urban,

located between Longitude 56° North and Latitude 17° South. Earlier studies by the Federal Ministry of Health had recorded certain locations in this zone as HIV/AIDS high prevalence area on the basis of the following characteristics: (i) geographical location as truck routes/stop-over of commuters from the Northern to the Southern parts of Nigeria; (ii) proliferation of commercial activities (occasioned by the Federal Ministry of Health) including commercial sex working in the specified locations; (iii) high incidence of AIDS cases, as well as the report which indicated that HIV cases are more predominant in transport routes and urban centers (Federal Ministry of Health, 1986; Uwakwe, 1994; Essex, 1992).

Sample size

The sample population was self-selected because of the peculiar characteristics that are relevant or tend to predispose to HIV/AIDS such as sexual promiscuity in forms of concubinage, woman to woman marriage, child-marriage, etc. HIV studies in these areas are therefore very sensitive issues and require a high level of confidentiality and informed consent. Study participants therefore consisted of only those who gave their consent, opting for inclusion following explanation of the study and agreed to have HIV testing. However, a sample size of 4,000 individuals was taken (for consistency and reliability) for each of the 3 years comprising HIV-positive and negative persons. Thus, a total sample size of 12,000 subjects was used in Phase 1 of the study.

Ethical consideration

The consent of the various hospitals and clinics used in the study was sought for and obtained prior to the investigation. Informed consent was obtained from all study participants prior to enrollment. Subject confidentiality was ensured by strict adherence to the University of Nigeria Ethical Guidance for Human Subjects including ensuring privacy of participants by using codes in place of person's names to avoid observation, intrusion or attention of others; storing samples or data in locked cabinets and not disclosing available information.

Laboratory analysis

HIV Screening

A 10-ml blood sample was collected from each participant by venepuncture into a Vacutainer K_3 ethylenediaminetetraacetic acid (EDTA) bottle using automated BD Vacutainer eclipse needle (Vacutainer CPT; Beckton Dickinson, Basel, Switzerland) after pre-test counseling and informed consent. Plasma was separated within 2 h and either processed immediately or frozen at -80°C until use. Screening for HIV-1/2 antibodies was carried out according to manufacturers' instructions using a rapid *in vitro* test kit; the Determine (Abbot Laboratories Japan).

Screening for *Mycobacterium TB*

Collection of sputa and other nasal secretion: Early morning sputa and nasal secretions from participants were collected in sterile wide-necked leak proof disposable containers soon on waking before mouth wash was done. Sputum production by some patients was enhanced by inhalation of mist. Three different samples were collected from each patient fortnightly and analyzed within 24 h of collection. These were first checked macroscopically by the investigator, and reported as purulent, muco-purulent, mucoid or muco-salivary, or bloody.

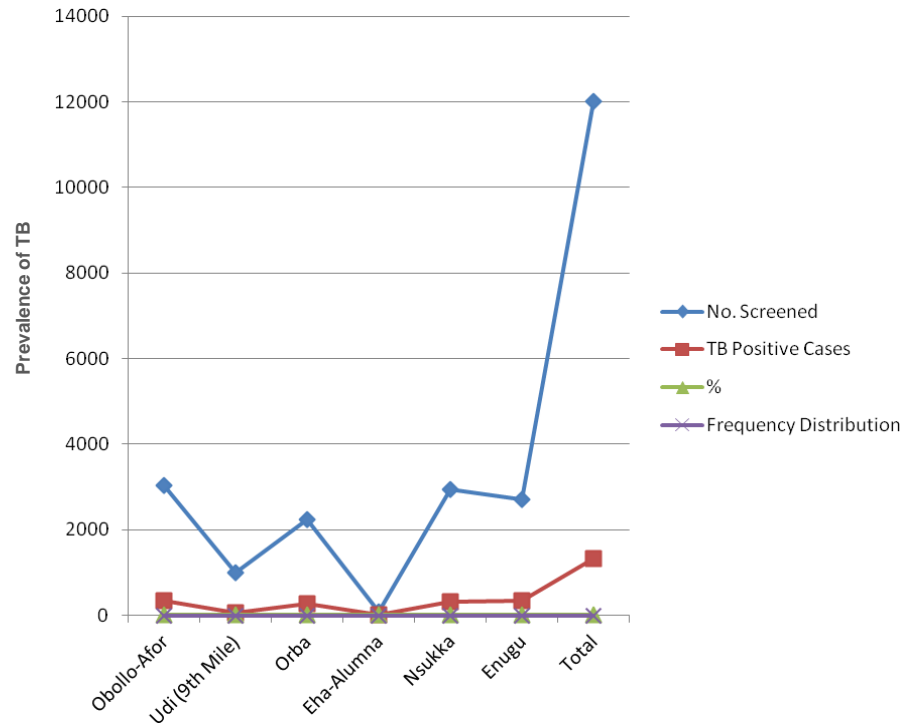


Figure 1. Prevalence of TB cases in the surveyed locations (1999-2001).

Preparation and microscopic examination of sputum smear:

Screening for *Mycobacterium tuberculosis* was done using the Ziehl-Neelsen (ZN) method for acid-fast-bacilli (AFB). One drop (50 μ l) of purulent, muco-purulent or cheese-like specimens was digested with two drops (100 μ l) of 4% potassium hydroxide solution and held at room temperature for 1 h until the viscous samples became fluid (Stokes, 1970). The digest was then smeared on a clean greaseless microscope slide, air-dried and stained by ZN method (Cheesbrough, 1991). The stained preparation was examined microscopically for AFB.

Phase 2, a follow-up exercise was conducted between June, 2005 to July, 2008, using sera and sputa samples from 75 asymptomatic HIV positive people from the cohort. These people were re-tested to determine their HIV and TB status and followed up with regular clinical observation and laboratory screening to determine the effect of TB on HIV progression. Fifty (50) of these subjects came up with AIDS-defining conditions: recurrent fever, weight loss >10% body weight, oral candidiasis, etc. These subjects were subsequently used as a sub-cohort for determining the effect of TB on HIV progression. Patients were also classified into a CDC matrix on the basis of clinical symptoms and CD4 counts. Haematological markers of HIV progression including CD4 were fitted as time-dependent covariates, adjusting for age, sex, transmission category, and risk, using Cox proportional hazards models. Other haematological indicators of disease including white blood cell (WBC), haemoglobin and erythrocyte sedimentation rate (ESR) were used to ascertain the level of HIV disease progression.

HIV screening: Participants were re-screened for HIV1/2 and TB as earlier indicated for HIV and TB screening.

CD4 cell count: CD4 count was done by flow cytometry using an automatic portable single solid-state laser machine (the Partec Cyflow SL-3 counter; wavelength: 30nW @ 532 (green), with flow

cell size, (250 \times 350 μ m)) synthetic quartz flow cuvette for luminal transport with sheet fluid sample and a built-in thermoprinter. The concentration or volume of fluorescent cell was measured at 0.2 ml by the volume detector, while the ploidy analyzer determined the number of cells per milliliter. CD4 cell counts were monitored at 6 monthly intervals. AIDS was defined as clinical stage C of the 1993 classification system (CDC, 1993).

Haematological parameters

An automated Coulter counter T540 machine, standardized against a 4C plus blood control was used for haematological parameter estimation. The machine automatically diluted 29.6 μ l whole blood samples, lysed, counted and printed out the result of absolute numbers of WBCs, RBCs and lymphocytes (all expressed as number of cells per liter). ESR was done using the Western Green technique.

Statistics

Data were analyzed using the Statistical Package for the Social Scientist (SPSS, Version 18): the analysis of variance (ANOVA) where appropriate. The mean and standard deviation (SD) values were calculated for the CD4 ratios.

RESULTS

Prevalence of TB

The overall prevalence of TB among the studied population was 11.1%. This ranged from 5.3% among screened subjects in Eha-Alumona and the 9th Mile Corner to 12%

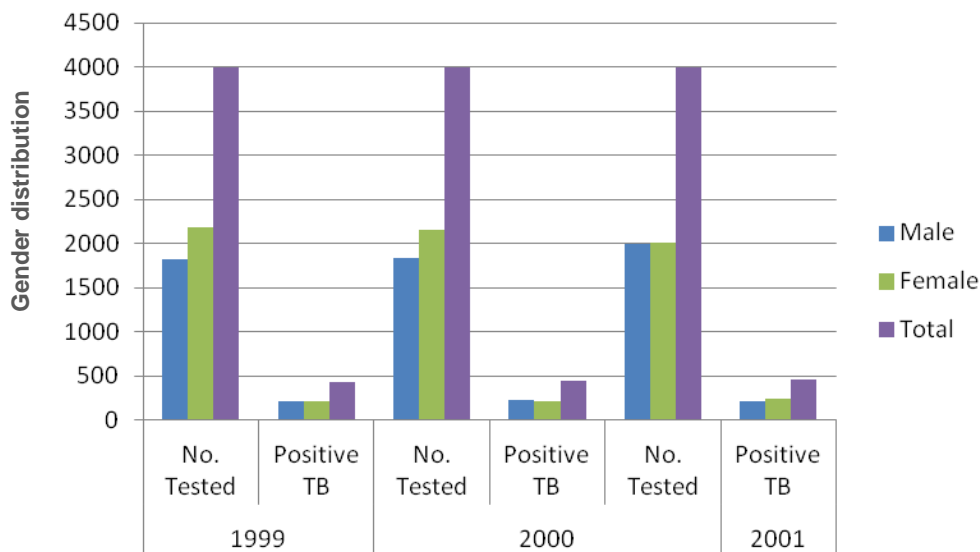


Figure 2. Gender specific distribution of TB infection (1999-2001).

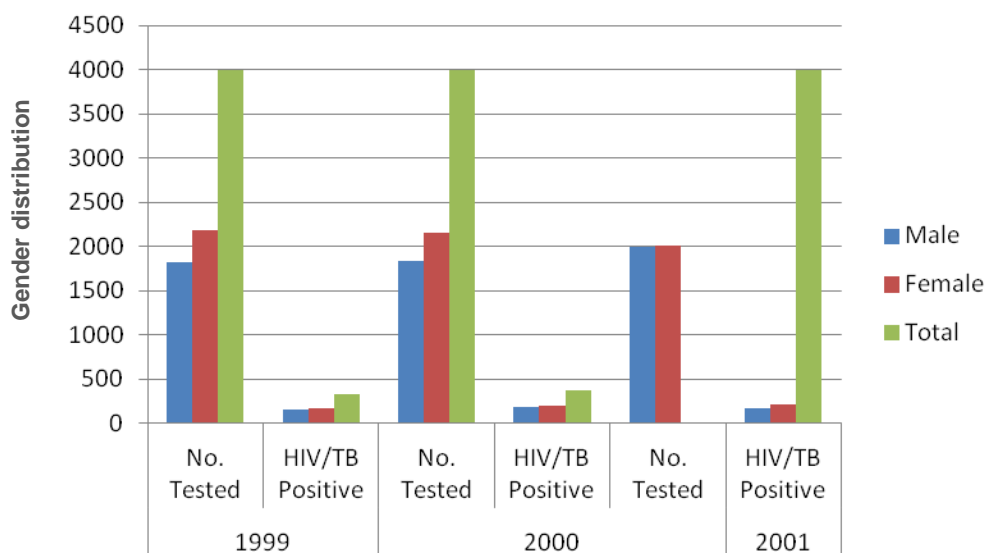


Figure 3. Gender distribution of multiple HIV/TB infections (1999-2001).

in Enugu and Orba, respectively (Figure 1). The pattern of TB infectivity among males and females in the study area for the 1999 to 2001 study period indicated that 667 (12%) of the 5662 males and 668 (11%) of the 6338 females screened were positive for TB (Figure 2). Co-infections with HIV and TB were apparent in the study. Out of the overall 2199 HIV positive subjects, 1,092 (49.6%) were infected by both HIV and TB (Figure 3). Single and pairwise infections are presented in Table 1.

Categorization of the 50 patients followed up after the preliminary screening based on their initial (baseline) and final CD4 count at immunodeficiency is shown Table 2.

There was a significant decrease in the final values of all surrogate markers at immunodeficiency. However, there was no association between the decrease and age or gender ($P > 0.05$). A significant increase was nevertheless observed in the AFB status of patients (from + to ++ or +++) at immunodeficiency ($P < 0.05$), indicative of progression to active TB.

The five (5) surrogate markers used in this study were CD4 count, WBC, haemoglobin (Hgb), packed cell volume (PCV) and lymphocyte counts. These markers were assessed on the sub-cohort 50 HIV patients who were followed up after the preliminary study. Descriptive

Table 1. Prevalence of dual HIV/TB singly and pair-wise in surveyed area, 1999-2001.

Location	1999									
	No. Screened	HIV positive	TB Positive	HIV Singly	%	TB Singly	%	HIV/TB	%	
Enugu	901	161	102	73	8.1	14	1.5	88	9.7	
Nsukka	984	148	106	60	0.6	26	2.6	80	8.1	
(UDI) 9 th Mile	330	61	23	45	13.6	7	2.1	16	4.8	
Obollo-Afor	1010	170	109	91	9.0	30	2.9	79	7.8	
Orba	750	125	89	58	7.0	22	2.9	67	8.9	
Eha-Alumona	25	2	1	1	4.0	1	4.0	1	4.0	
2000										
Enugu	901	182	105	89	9.0	12	1.3	93	10.3	
Nsukka	984	187	108	90	9.1	11	1.1	97	9.8	
(UDI) 9 th Mile	330	64	28	44	13.3	8	2.4	20	6.0	
Obollo-Afor	1010	199	115	109	10.7	25	2.4	90	8.9	
Orba	750	119	92	45	6.0	18	2.4	74	9.8	
Eha-Alumona	25	5	2	1	4.0	1	4.0	1	4.0	
2001										
Enugu	901	190	106	99	10.9	15	1.6	91	10.0	
Nsukka	984	154	104	66	6.7	16	1.6	88	8.9	
(UDI) 9 th Mile	330	65	31	43	13.0	9	2.7	22	6.6	
Obollo-Afor	1010	223	119	115	11.3	11	1.0	108	10.6	
Orba	750	139	94	63	8.4	18	0.2	76	10.1	
Eha-Alumona	25	5	2	3	12.0	-	-	2	8.0	

Table 2. Baseline and final CD₄ count, AFB status and blood profile of follow-up group.

Code	Age	Sex	CD4 Count (µl) at immunodeficiency syndrome				Other blood profile								AFB status	
			Initial/Baseline CD4 count (µl)			<200 cells/µl	Baseline				At immunodeficiency				AFB status initial/Baseline	AFB at immunodeficiency
			<200 cells/µl	200-300 cells/µl	≥300 cells/µl		Total WBC cells/µl	HB (g/dl)	PCV (%)	LYM (%)	Total WBC cells/µl	HB (g/dl)	PCV (%)	LYM (%)		
1	41	M	-	-	305	102	12,800	14.0	42.2	60	3,200	4.9	20.2	28	+	+++
2	46	F	-	-	402	113	13,900	9.8	30.6	47	2,400	6.7	19.6	26	+	+++
3	31	F	-	290	-	101	11,000	13.1	39.9	31	2,601	7.2	18.0	30	-	++
4	25	F	-	252	-	153	15,002	11.1	36.0	36	3,500	5.6	19.5	31	+	++
5	44	M	-	-	335	96	11,900	9.4	36.7	54	2,600	6.0	17.2	29	++	+++

Table 2. Contd.

6	37	F	-	-	301	77	14,400	12.3	35.7	35	2,500	4.9	18.9	30	+	+++
7	31	F	-	-	375	106	14,800	11.7	37.6	27	2,450	5.2	17.0	26	-	+
8	28	F	-	-	384	194	13,700	12.8	36.7	61	3,000	6.3	19.0	32	-	-
9	27	F	-	-	355	131	14,600	11.4	32.8	57	4,000	5.6	20.2	27	++	+++
10	33	M	-	239	-	89	15,700	13.9	40.3	41	2,800	6.5	18.9	30	-	-
11	32	M	-	-	386	102	16,400	13.2	42.5	39	3,700	6.3	19.8	30	+	++
12	42	F	-	-	312	94	17,700	13.5	38.5	35	2,800	5.3	18.1	28	+	+++
13	31	F	-	277	-	189	16,000	11.4	32.4	33	2,520	7.1	17.7	35	-	+
14	30	F	-	296	-	162	15,600	8.1	35.6	55	3,100	5.3	19.0	29	++	+++
15	33	F	-	225	-	82	15,500	11.6	36.0	33	2,500	5.0	19.2	30	+	+++
16	55	M	-	-	395	100	16,800	12.3	36.7	30	2,650	6.0	18.5	26	-	+++
17	40	F	-	-	377	74	14,400	10.6	33.4	26	4,100	6.0	27.0	22	++	+++
18	31	F	-	202	-	68	16,800	10.0	33.5	34	2,700	5.6	20.4	31	+	+++
19	36	F	-	-	333	101	14,500	11.7	36.1	38	2,500	5.3	18.1	27	+	++
20	38	M	191	-	-	77	14,100	14.3	42.5	35	2,700	4.9	19.8	33	++	+++
21	31	F	-	-	300	95	14,600	9.2	34.0	18	4,000	5.7	17.2	20	++	+++
22	39	F	-	-	261	74	14,200	9.8	30.2	61	3,250	5.0	18.7	46	-	++
23	32	M	-	-	333	88	17,000	11.0	32.2	33	2,600	7.3	17.9	32	-	+
24	51	M	-	-	311	103	13,008	11.7	36.5	53	3,450	7.6	17.0	27	+	++
25	34	F	-	290	-	160	14,900	6.6	35.5	35	4,000	5.7	19.1	23	++	+++
26	30	F	-	-	326	118	14,900	11.1	36.1	50	3,500	7.2	20.4	29	-	-
27	38	F	-	290	-	152	15,100	12.2	33.7	63	4,150	7.4	19.6	52	-	+
28	49	F	-	266	-	110	14,000	8.8	37.8	43	3,450	6.3	20.3	40	+	++
29	48	M	-	-	310	150	15,900	12.0	33.8	33	3,500	5.3	19.5	35	+	++
30	27	F	-	-	325	166	11,200	11.9	35.7	44	2,900	5.0	18.9	40	-	+++
31	57	M	-	-	315	81	14,300	10.6	32.6	24	3,820	6.6	17.4	29	-	++
32	58	M	199	-	-	72	16,000	8.0	25.0	06	3,300	7.5	18.2	19	-	-
33	28	F	-	281	-	88	18,200	6.5	36.0	08	2,900	6.0	20.0	25	+++	+++
34	42	F	-	-	307	109	16,600	9.8	37.3	27	2,780	5.8	18.5	22	+	+++
35	39	F	-	282	-	159	14,900	6.1	33.9	43	4,200	7.0	18.9	20	-	+
36	27	F	-	-	309	127	17,200	8.2	35.3	19	2,600	4.9	17.9	22	+	+++
37	34	F	-	-	317	111	16,800	10.5	33.6	39	3,900	5.0	19.8	25	+	++
38	39	F	-	-	302	96	14,700	11.2	36.8	31	4,200	7.6	20.4	30	-	++
39	45	F	-	265	-	108	12,300	9.3	28.1	22	3,610	5.7	18.6	27	+	++
40	35	F	-	285	-	130	16,800	10.1	34.2	47	3,000	5.0	20.0	31	+++	+++
41	55	M	-	275	-	174	11,100	9.3	39.2	32	4,100	7.7	18.8	26	-	-
42	65	F	-	225	-	104	14,500	7.3	33.5	41	2,750	5.3	19.2	20	++	+++

Table 2. Contd.

43	45	M	-	-	388	100	15,000	14.7	38.4	33	2,800	6.3	18.6	32	+	+++
44	25	F	-	-	345	73	12,900	11.5	40.5	41	3,300	6.0	19.5	29	-	++
45	48	M	-	-	306	113	15,100	10.0	30.8	31	2,850	5.6	17.9	30	++	+++
46	34	F			342	120	16,800	10.8	38.7	51	3,000	4.9	18.	27	++	+++
47	48	F	-	260	-	126	14,900	10.0	39.5	48	3,950	5.0	20.	24	+	++
48	35	F	-	215	-	72	10,900	10.9	30.4	64	4,380	6.0	19.	31	-	-
49	34	F	189			68	19,200	5.8	38.1	12	2,810	5.5	19.	28	+	+++
50	64	M		-	385	101	17,000	7.4	36.4	41	2,550	5.0	18.	25	++	+++

Table 3. Descriptives of the surrogate markers on the degree of infection of HIV.

Parameter	CD4		WBC		HB		PCV		LYM	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
HIV	300.7	55.21	14912	1875.7	10.57	2.159	35.59	3.575	38.00	13.78
AIDS	111.2	32.57	3196	594.6	5.93	0.876	19.07	1.488	28.92	6.15
Total	206.0	105.4	9054	6048	8.25	2.849	27.33	8.738	33.46	11.55

statistics of the surrogate markers on the degree of infection of HIV as well as the means and standard deviations of the patients, computed at both HIV and AIDS levels on the 5 surrogate markers are shown in Table 3. The descriptive analysis indicated a higher mean value for the HIV than that for the AIDS level using the markers as variables. The standard deviations of the variables at AIDS level were all lower than those at HIV levels ($P < 0.00$) (Table 4).

The correlation between AFB results (categorized as negative, +, ++ and +++) and HIV progression to AIDS, determined by using the surrogate markers described, showed a steady decline in the mean value of CD4 count from 256.13 to 119.37 across the four categories of AFB from negative (at baseline) to +++ (at

immunodeficiency), respectively (Table 5). However, there were differences in SD across the categories; values with most uniformity had least SD of 54.21, and were indicated by +++. The mean WBC level increased from negative to +, but decreased steadily from + to +++, indicating an inverse relationship. Both the mean haemoglobin level and their SD were observed to increase from negative (-) to positive (+), but decreased steadily from + to +++. Similarly, category +++ showed values of haemoglobin with most uniformity, with SD of 1.03. The mean PCV count showed similarity to mean haemoglobin. However, their SD varied at random with least value of 4.59 occurring at +++. The mean lymphocyte values as well as their standard deviations decreased steadily across the four categories.

A test of significance (ANOVA) to determine the effect (influence) of TB on the progression of HIV to AIDS, using the surrogate markers as test variables is shown in Table 6. CD4, WBC, haemoglobin and PCV tests showed P-value as 0.00 ($P < 0.05$); the lymphocyte count had P-value as 0.012 (Table 6). The effect of TB on the progression of HIV to AIDS is very highly significant at $\alpha = 0.05$.

DISCUSSION

This biphasic study investigated the prevalence of co-infection with HIV and TB in the geographical area along major transport routes of Enugu State. Their consortium in active HIV disease and the

Table 4. ANOVA test on the difference between baseline and syndrome levels.

Parameter		Sum of squares	df	Mean square	F	Sig.
CD4	Between groups	898135.290	1	898135.290	437.145	0.000
	Within groups	201345.460	98	2054.546		
	Total	1099480.750	99	-		
WBC	Between groups	3431487525.210	1	3431487525.210	1772.476	0.000
	Within groups	189726586.180	98	1935985.573		
	Total	3621214111.390	99	-		
HB	Between groups	537.776	1	537.776	198.222	0.000
	Within groups	265.874	98	2.713		
	Total	803.650	99	-		
PCV	Between groups	6824.412	1	6824.412	910.004	0.000
	Within groups	734.934	98	7.499		
	Total	7559.346	99	-		
LYM	Between groups	2061.160	1	2061.160	18.107	0.000
	Within groups	11155.680	98	113.833		
	Total	13216.840	99	-		
AFB	Between groups	37.210	1	37.210	40.726	0.000
	Within groups	89.540	98	0.914		
	Total	126.750	99	-		

Table 5. Descriptives of the surrogate markers on the effect of AFB on progression from HIV to AIDS.

Parameter	CD4		WBC		HB		PCV		LYM	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Negative	256.13	96.28	11511.7	5026.5	10.0	2.46	31.01	7.89	36.75	14.55
+	263.92	85.14	12826.3	5310.9	10.1	2.61	32.53	8.13	35.46	11.42
++	195.64	111.25	8485.2	5848.3	7.6	2.39	26.26	8.59	34.96	10.57
+++	119.37	54.21	4044.1	3913.8	5.7	1.03	20.43	4.57	27.37	6.95
Total	205.95	105.38	9054.3	6048.0	8.3	2.85	27.33	8.74	33.46	11.55

role of CD4 cells and other haematologic parameters as markers of HIV progression was similarly evaluated. The steady increase in TB and HIV prevalence rates observed has serious public health implications. HIV tends to complicate the problems of TB patients and of course endangers the entire community by increasing the rate of multi-drug resistant strains of the bacterium as well as further compromising the immune status of infected persons as earlier reported (Nunn et al., 1990; Bonecici et al., 1998). Co-infection with HIV and TB investigated in this study has a dramatic impact on the quick progression of HIV/TB patients to full-blown AIDS as well as the possible activation of latent TB and subsequent progression to active TB among the multiply infected persons. Immune suppression or degeneration

resulting from the multiple HIV/TB infections will automatically increase the risk for opportunistic infections and AIDS-related malignancies in the population. The reality that HIV/TB co-infection has caused more deaths than the individual infections is confirmed in this study. This view is supported by the reports on the synergistic effect of the co-infections (Murray et al., 1990). The reported occurrence of multiple HIV/TB infection further suggests that an epidemic of TB is going on alongside HIV/AIDS disease among the people within the area being studied, including the transport (truck/stopover) communities of Enugu State. This evidence is supported by the reports on the presence of co-infection in immune suppressed individuals (Lucas, 1992). Co-infection with these pathogens is therefore particularly devastating in

Table 6. ANOVA test on the effect of AFB on progression from HIV to AIDS.

Parameter		Sum of squares	df	Mean square	F	Sig.
CD4	Between Groups	346114.235	3	115371.412	14.702	0.000
	Within Groups	753366.515	96	7847.568		
	Total	1099480.750	99	-		
WB	Between Groups	1172249741.145	3	390749913.715	15.317	0.000
	Within Groups	2448964370.245	96	25510045.523		
	Total	3621214111.390	99	-		
HB	Between Groups	342.869	3	114.290	23.811	0.000
	Within Groups	460.781	96	4.800		
	Total	803.650	99	-		
PCV	Between Groups	2287.833	3	762.611	13.888	0.000
	Within Groups	5271.513	96	54.912		
	Total	7559.346	99	-		
LYM	Between Groups	1413.125	3	471.042	3.831	0.012
	Within Groups	11803.715	96	122.955		
	Total	13216.840	99	-		

the rural communities under study where the burden of disease is high. Though the paradigm of the dual HIV/TB infections and distribution does not seem to show any significant difference, there are however associated socio-economic and socio-cultural implications. On the socio-economic level, the constant rise in HIV/TB infection in the area indicates a mutual interaction between the two infections and also portrays the level of socio-economic status of the area such as poverty. Several people are living below subsistence level which further sustains risk behaviours predisposing to HIV, including sex working (commercial and clandestine) and multiple sex partnering (Dibua, 2010). In addition is overcrowding, a consequence of harsh economy which facilitates transmission of TB and promotes HIV infection thereby further weakening the vulnerability and susceptibility of the entire community.

The surrogate markers used in this study have proven prognostic value in evaluating the influence of TB on HIV progression and the development of AIDS-related conditions. This view is confirmed by previously published reports on the importance of surrogate markers in evaluation of HIV infection (Deyton, 1996). The absolute CD4 T-lymphocyte count is one of the best surrogate markers for assessing the risk of progression to AIDS among HIV-infected individuals. The general tendency for the CD4⁺ count to decline with time among HIV-infected patients despite the variability among individuals was demonstrated in the study; the decrease in CD4 count is thus indicative of the HIV progression to AIDS, authenticating the categorization of the AFB as indicated.

The idea that CD4⁺ T-lymphocyte numbers are predictive of AIDS-defining illnesses was yet strengthened by the observation of significant differences in CD4⁺ levels associated with increased AFB (+++). This observation is confirmed by the reports of World Health Organization, in defining its staging system for HIV disease, in which it proposed the utilization of total lymphocyte count as predictor of HIV disease (WHO International Collaborating Group for the Study of the WHO Staging System, 1993). The results of this study confirm the value of using the surrogate markers in limited resource settings, for the diagnosis and management and/or follow-up of infected subjects.

Among the other cellular measures used in the study that could reflect immune defense processes in the HIV disease progression, no one appears to be consistently irrelevant; the reported low haemoglobin and PCV were predictive of steeper decline of CD4⁺ counts; though did not seem predictive of the onset of AIDS: a typical normal number of RBCs found in a blood specimen can range from four to 6 million cells/ μ l (one thousandth of a milliliter) of blood depending upon gender and the altitude at which the person lives. Hemoglobin varies with altitude (male: 13.8 to 17.2 gm/dl; female: 12.1 to 15.1 gm/dl). The impact of a decline in the haemoglobin/PCV levels as observed in the study is severe anaemia. This is in conformity with the reports on the association of changes in haemoglobin levels in infants with low birth weight and fetal anaemia (Cessie, 2002).

The statistical analysis (descriptive) of the surrogate markers indicated a higher mean value for the HIV than

that for the AIDS level using the markers as variables. However, the SD of the variables at AIDS level was all lower than those at HIV levels. A test of significance using the analysis of variance to determine the difference between baseline and syndrome levels (the mean HIV and AIDS level) using each of the surrogate markers as test variables, indicated a P-value of 0.00, implying a significant difference between the mean values at HIV and AIDS levels: the more the progression from HIV to AIDS, the more the values of the surrogate markers tend towards decline. This confirms the influence of TB in HIV disease progression. The AFB value, +++, was therefore observed to be the most uniform value of all investigated parameters at immunodeficiency state. The analysis of variance ($P < 0.05$), confirmed the view that TB is a significant factor in furthering the progression of HIV to AID; this is very significant at $\alpha = 0.05$. The analysis further indicates the role of the surrogate markers as indicators of immune system deterioration and subsequent decline.

Conclusion

Co-infection of HIV and TB has intrinsically been linked to acceleration of the course of HIV and subsequent progression to AIDS, particularly among the local population in Nigeria. The study confirmed the individual role of TB in furthering the progression of HIV to AIDS, and in addition, emphasized the importance of CD4 counts as prognostic marker, as well as the role of other surrogate markers as indicators of immune system deterioration and subsequent decline. The use of these markers in HIV disease monitoring and diagnosis was proposed especially in the local communities where diagnostic resources are limited and the burden of the disease is high.

ACKNOWLEDGEMENTS

The author is indebted to several individuals whose kind suggestions were very useful in the execution of this research particularly Prof. Oboegbulem of the Faculty of Veterinary Medicine, Prof. G. C. Mbah of the Department of Mathematics, and Mr. Mbanefo of the Department of Statistics, University of Nigeria, Nsukka.

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Full Length Research Paper

Technician capability for blood screening in hospitals of Islamabad

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Accepted 28 February, 2013

The study was conducted with the objective to access the technician capability for blood screening in hospitals of Islamabad. The parameters pertaining to technicians' capability under investigation were; qualification, skills, experience, on job training, knowledge and capability to use microscope, centrifuge and computers. The results of the study revealed that the technician capability for blood screening in hospitals of Islamabad was far below the World Health Organization (WHO) standards and also not compatible to National Health Policy guidelines. In order to adopt modern blood screening practices, the technician capability may be increased by imparting regular, periodical in-service training to the technician, so that safe blood is available for transfusion and as such, "more blood more life" slogan attain a reality in real perspective.

Key words: Technician capability, blood screening, hospitals, Islamabad.

INTRODUCTION

World blood day, 2011 was celebrated in Pakistan as in other parts of the world (Jang, 2011). The slogan of 2011 blood day was "more blood more life". This is absolutely valid in Pakistan perspective, where blood transfusion is an absolute necessity in cases of various hematological disorders, cancer, hepatitis, thalesemia, heart transplant, trauma and other diseases, and also for patients of road accidents, which occur as a routine rather than exception in Pakistan.

Access to safe blood transfusion is a basic human right and also integral part of modern medicine system. According to Red Crescent Society of Pakistan (2011), for 18 persons, only 1 blood bag is available. On daily basis, 8,000 blood bags in the country are needed as against 4,100 bags supplied, as reported by Fatmide Foundation (2011). Moreover, almost 50% of the blood is either not screened at all or not properly screened,

according to WHO standard operating procedures or national health care guidelines, because of poor capability of technicians and other impediments involved in blood screening (Atta-ur-Rehman, 2011; 2012).

The capability of blood technicians is dependent on many variables including knowledge, skills practices and other factors that impart sufficient experience to technicians for blood screening. The technical training prior to induction in job is prerequisite. The theoretical, as well as practical aspects ought to be a focal point of pre job training. As per World Health Organization (2009) and National Health Care Policy, on job periodical training is a must, and this should be imparted twice a year, and no technician should be left untrained. This aspect is quite important but an unattended segment; health is a neglected sector in the whole country. The lack of in-service training of technicians is a major hurdle in the

upgrading of our blood banks to international standards.

The quality management and quality assurance of blood is a responsibility of the quality control officer and the technician involved in blood screening. Blood quality management includes documentation, internal and external quality validation, accreditation schemes, haemeovigilance and proper disposal of wastes, infection material. The technician ought to be conversant with all these aspects for proper screening of blood, which is usually not the case in most blood banks in Pakistan.

In Pakistan, Blood transfusion authorities (BTAS) have been established to regulate safe blood transfusion practices. However, it has been observed that BTAS have not been instrumental in safe blood transfusion, as fragmental structure and lack of on-the-job training of technicians were main impediments in this regard. The quality management and the quality assurance of blood are far from the desired standard as reported by WHO (2009). The transfusion of poor quality blood has resulted in wide spread of hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) and other blood related diseases. The intensity of these diseases may be well realized as according to Pakistan Medical Research Council (2009), the spread of hepatitis B and C in a general population of Pakistan is 11.84 million. It has been reported (Zaheer, 2009) that transfusion of contaminated blood caused up to 16 million new infections of HBV, 5 million of HCV and 16,000 HIV globally.

In recent medical practices, the trend is to use blood components rather than the use of whole blood for transfusion. Shamshi (2001) narrated that blood platelets are used for cancer and dengue fever, plasma is used for hepatitis patient and red blood cells are injected to patient of thalesemia. All blood technicians in Pakistan are adequately and properly trained in fractionating of blood. There is dire need of time that technicians may be adequately trained in proper screening of blood and blood components. The health sector can be strengthened specifically if technicians are well versed in blood screening practices, hence periodical training of technicians in modern blood screening techniques and transfusion technology is essential. From the preceding, it can safely be concluded that in proper blood screening and in safe blood transfusion, the role of a blood technician is vital. Hence, this study was conducted with the objective to access the capability of technicians for blood screening in hospitals of Islamabad.

METHODOLOGY

The current survey was carried out in the capital city of Pakistan, Islamabad. For the study purpose, three government administered

hospitals were selected at random. One or two rooms were designated as blood banks, and these blood banks were associated/located within the premises of hospitals. The blood banks taken for the study were, Federal Government Services Hospital, Polyclinic Hospital, Pakistan Institute of Medical Sciences and Social Security Hospital. The blood banks were coded as blood bank facility 1 (BBF1), blood bank facility 2 (BBF2) and blood bank facility 3 (BBF3), and code number of each blood bank and name of respective hospital were kept confidential. A structured questionnaire was developed to assess the knowledge, skills and practices, and also experience of technicians of the three blood banks. The questionnaire of the study includes the following:

1. Qualification;
2. Experience;
3. Job description;
4. On job training;

Have complete knowledge regarding blood screening procedures/ standard operating procedures available at workstation:

1. Skills pertaining to blood screening/transfusion;
2. Knowledge.
3. Awareness of WHO standards/Standard Operating Procedures (SOP);
4. Awareness of National Health Policy guidelines;
5. Ability/skills to screen blood for HBV, HCV and HIV;
6. Capability to use microscope and centrifuge;
7. Ability of technicians to use computers;
8. Capability of flood management and quality assurance.

The permission for the study was taken from the managers of all blood banks under investigation. The information was also collected regarding blood banks' capacity and the techniques and technology used by the technicians. The technicians and all others concerned were informed about the objectives of the study. The interviewee responded in affirmative or otherwise. The data so obtained was tabulated and transformed in percent and frequencies, and inferences were drawn accordingly. The limitations in technicians' capability were highlighted and suggestions were imparted to improve the capability of technicians for blood screening in hospitals of Islamabad, and also, implication of study elsewhere in Pakistan will be documented.

RESULTS AND DISCUSSION

The results pertaining to technicians' capability for blood screening in hospitals of Islamabad are presented in Table 1 and 2. As regards qualification, all the technicians in blood bank facility 1 (BBF1), 2 (BBF2) and 3 (BBF3) had a diploma in medical technology. The experience of technicians in BBF1 and BBF2 was more than ten years. This indicates that working experience of technicians in these two blood banks was adequate but was hampered by use of old routine, stereoscopic blood screening methods. There was no urge and even no opportunity for adoption of modern medical technology for update of blood banks according to international standards. However, the experience of technicians in BBF3

Table 1. Technicians' capability for blood screening in hospitals of Islamabad.

S/No.	Technician's capability	BBF1	BBF2	BBF3
1	Qualification: Diploma in medical technology	Yes	Yes	Yes
2	Experience	>10 years	>10 years	<10 years
3	Job description	Yes	Yes	Yes
4	Technician vaccination of HBV	Yes	No	Yes
5	Formal training-prior to posting	Yes	Yes	Yes
6	On Job Training	No	No	No
7	Training pertaining to transfusion transmitted infection	4 days	7 days	4 days
8	Knowledge of storage temperature	Yes	Yes	Yes
9	Shelf life	Yes	Yes	Yes
10	Usage of disposable tube	Yes	Yes	Yes
11	Knowledge updated as per WHO standards	No	No	No
12	National update as per national blood policy	No	No	No
13	Ability to screen blood for HIV, HBV and HCV	Yes	Yes	Yes
14	Use of microscope and centrifuge	Yes	Yes	Yes
15	Use of computers	Yes	Yes	Yes

BBF1: Blood bank facility 1, BBF2: blood bank facility 2, BBF3: blood bank facility 3.

Table 2. Technicians' capability for blood screening in hospitals of Islamabad.

S/No.	Technician's capability	Frequency (%)
1	Qualification: Diploma in medical technology	100
2	Experience	66.7
3	Job description	100
4	Technician vaccination of HBV	66.7
5	Formal training-prior to posting	100
6	On Job training	0
7	Training pertaining to transfusion transmitted infection 4 days	66.7
8	Training pertaining to transfusion transmitted infection 7 days	33.3
9	Knowledge of storage temperature	100
10	Shelf life	100
11	Usage of disposable tube	100
12	Knowledge updated as per WHO standards	0
13	National update as per national blood policy	0
14	Ability to screen blood for HIV, HBV and HCV	100
15	Use of microscope and centrifuge	100
16	Use of computers	100

Data pertaining to three hospitals of Islamabad, Pakistan.

technicians in BBF3 was less than 10 years. All the technicians had job description in three blood banks.

Technicians were vaccinated against HBV in BBF1 and BBF3, but were not vaccinated in BBF2. It is worth mentioning that all technicians in the three BBFs had received formal training during the study period; there was

lack of in-service training facilities or capabilities but no on-the-job in-service training facility was provided to any technicians in all the blood banks. This was contrary to WHO standards (WHO, 2009) and the National Health Policy guidelines that envisaged that in-service training of technicians ought to be a regular feature. In order to ensure

Table 3. Difference in the key characteristics of the technicians across BBF1 and BBF2.

Characteristic	BBF1	BBF2	Difference	t-value
Age	46	55	-9**	-1.97
Experience (years)	18	13	5*	1.68
Technician vaccination of HBV (dummy)	1	1	0.00	0.00
Training pertaining to transfusion transmitted infection (days)	4	7	3***	2.73

The results are significant at ***, **, * at 1, 5 and 10% levels, respectively.

that technicians are well versed with modern blood screening practices, it is imperative that on job, in-service training should be imparted at least twice a year and no technicians should be left untrained. Though the technicians did not have opportunity of training in blood screening techniques yet the training pertaining to transfusion transmitted infection was given as a workshop of 3 to 4 days in BBF1 and BBF3, and training was for one week in BBF3.

The knowledge of technicians in respect of blood screening was also a subject of investigation. The technicians in all the blood banks had knowledge of temperature at which blood was to be stored. This is a desired attribute as blood is to be stored at optimum temperature, otherwise blood and blood component may deteriorate and even may prove fatal to the patient to whom such blood and blood component are transfused. The technicians were also aware of the shelf life of blood component. If the shelf life of blood component is not kept in view, the use of blood components beyond shelf life may be of little use on transfusion to the patient. The technician had the knowledge that disposable tube was to be used for blood each time, otherwise infection may prevail, causing spread of blood related infectious diseases.

The technicians in all three blood banks were able to screen blood for HBV, HCV and HIV occurrences. The technicians also knew how to use microscope and also how to centrifuge the blood samples. They also had the capability to use computers. The study evidently indicate that technicians in all the blood banks had basic knowledge of blood screening, however at the same time it was visualized that technicians' knowledge was not updated according to WHO standards or the National Blood Policy guidelines in the three blood banks under study. This revealed that outdated methods of blood screening may not cope with modern developments in blood technology and calls for in-service training of technicians in respect of blood screening, and it should be imparted regularly/sequentially.

Table 3 presents the difference in key characteristics of BBF1 and BBF2. The difference in age was negative and

significant at 5% level of significance, indicating that technician of BBF2 were more aged as compared to BBF1. The experience difference was positive and significant at 10% level of significance. The technician vaccination of HBV was included as a dummy variable and there was no difference in technician vaccination of HBV. The training pertaining to transfusion transmitted infection difference was positive and highly significant at 1 % level of significance.

Table 4 presents the difference in key characteristics of BBF1 and BBF3. The difference in age was positive and highly significant at 1% level of significance. Similarly, the difference in experience was also positive and highly significant at 1% level of significance, indicating that technicians of BBF1 were more experienced as compared to BBF3. There was no difference in technician vaccination of HBV and training pertaining to transfusion of transmitted infection. Table 5 presents the difference in key characteristics of BBF2 and BBF3. The difference in age was positive and highly significant at 1% level of significance. The difference in experience was also positive and significant at 10% level of significance. There was no difference in technician vaccination of HBV. The difference in training pertaining to transfusion of transmitted infection was also positive and significant at 5 % level of significance.

The residents of Islamabad Capital Territory are well educated, and quite aware of principles of healthy living. Above all, health care and blood bank situations in the Federal Capital are far better than in the remote rural areas of the country. If the capability of technicians in the Federal Capital is not up to the mark and far below WHO standards or the National Health Policy guidelines, then the situation in far flung areas of the country may be well imagined. Moreover, in remote areas, mostly private blood banks operate with little concern about technicians' capability and also about blood screening practices. Hence, it is of paramount importance that technicians' capability may be enhanced by regular and periodical training, so that proper blood screening is guaranteed and the 2011 World blood day slogan, "more blood, more life" may be realized in true perspective.

Table 4. Difference in Key characteristics of BBF1 and BBF3.

Characteristic	BBF1	BBF3	Difference	t-value
Age	46	35	11***	9.85
Experience (years)	18	06	12**	2.14
Technician vaccination of HBV (dummy)	1	1	0	0.00
Training pertaining to transfusion transmitted infection (days)	4	4	0	0.00

The results are significant at ***, **, * at 1, 5 and 10% levels, respectively.

Table 5. Difference in key characteristics of BBF2 and BBF3.

Characteristic	BBF2	BBF3	Difference	t-value
Age	55	35	20***	12.44
Experience (years)	13	06	7*	1.76
Technician vaccination of HBV (dummy)	1	1	0	0.00
Training pertaining to transfusion transmitted infection (days)	7	4	3**	2.34

The results are significant at ***, **, * at 1, 5 and 10 % levels, respectively.

CONCLUSION AND RECOMMENDATIONS

The study conducted revealed that technicians capability in hospitals of Islamabad was not up to prescribed level but rather far below the WHO standards or National Health Policy guidelines. The technicians need training regarding the safe blood screening. The blood screening is confronted with awful situation that needs to be rectified. This may be done by regular, periodical training of technicians in order to enhance their capability of blood screening. By so doing, the health conditions in Pakistan will certainly improve and people will relish better and healthy life.

It is suggested that technician's capability may be increased in Islamabad and also in remote rural areas of Pakistan to supply safe screened blood for blood transfusion to the patients. Such studies may be extended to other areas of the country as well.

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UPCOMING CONFERENCES

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