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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Table of Content: Volume 10 Number 2, 14 January, 2016

ARTICLES

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Full Length Research Paper

Carbapenem resistance mechanisms among blood isolates of *Klebsiella pneumoniae* and *Escherichia coli*

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The purpose of this study was to characterize the different mechanisms of carbapenem resistance among blood isolates of *Klebsiella pneumoniae* and *Escherichia coli*. Meropenem resistant isolates were included. Antimicrobial susceptibility testing, phenotypic and genotypic detection of carbapenemase production were performed. Genetic relatedness of bla_{NDM-1} producers was determined by pulsed-filed gel electrophoresis (PFGE) typing. The relatedness of bla_{NDM-1} carrying plasmids was studied by plasmid restriction fragment length polymorphisms (pRFLP) and polymerase chain reaction (PCR) based replicon typing (PBRT). For the carbapenem gene negative isolates, other possible mechanism of carbapenem resistance such as role of outer membrane porin loss with ESBL or AmpC production, and efflux pumps were analyzed. Among the 162 isolates studied, 1 (0.6%) was found to be bla_{KPC-3} producer and 42.5% were bla_{NDM-1} producers. All the isolates were multidrug resistant; two isolates carried both bla_{NDM-1} and bla_{VIM-2}. PFGE determined bla_{NDM-1} producers were non-related. The plasmids harbouring bla_{NDM-1}belonged to two major incompatibility plasmid types, IncL/M and IncA/C. IncL/M is a novel plasmid group reported firstly from this region. A clonal outbreak of bla_{IMP-1} *K. pneumoniae* was identified during this study. This is the first report on the emergence of *K. pneumoniae* producing bla_{IMP-1} from South India and bla_{KPC-3} from India. The study suggest including ertapenem in the routine susceptibility screening to find the true rate of KPC producers in Indian hospitals. Colistin and tigecycline are two drugs that have activity but both have developed resistance. Selection of an appropriate initial antibiotic regimen for empiric therapy, rotation of different antibiotic classes and judicious use of antibiotics are essential.

Key words: Blood isolates, carbapenamase, multiplex polymerase chain reaction (PCR), pulse field gel electrophoresis, tigecycline.

INTRODUCTION

Antibiotic resistance in gram-negative bacteria is of increasing concern because of the lack of new antibiotics to treat these infections. Of particular importance from the healthcare epidemiology standpoint is the resistance

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towards carbapenems (Zhanel et al., 2007). Of all the β-lactam antimicrobial drugs, carbapenems (imipenem,meropenem, doripenem and ertapenem) have the most consistent activity against enterobacteriaceae. Activity is retained against isolates that produce AmpC and extended spectrum beta-lactamases (ESBLs) which makes them a drug of choice to treat these resistant infections (Jacoby et al., 1997).

Carbapenems were first introduced in 1980s and are now frequently used as the last choice in treating serious infections caused by multidrug resistant (MDR) strains of gram-negative bacilli (Rodloff et al., 2006). Within a decade, resistance to carbapenems started emerging and has been reported in non-fermenter gram- negative bacilli worldwide over the years with varying frequencies (Spencer et al., 2002). Over the past two decades, while carbapenem resistance has become a serious problem among the non-lactose fermenting bacteria, it has remained uncommon in enterobacteriaceae. Recently however, identification of carbapenem- resistant enterobacteriaceae is increasing (Spencer et al., 2002; Zhanel et al., 2007).

Carbapenem-resistant gram-negative bacteria pose a serious problem due to the genes encoding most of these carbapenemases reside on plasmids or transposons which carry additional genes encoding resistance to other classes of antimicrobial agents (Rasmussen et al., 1996). These transferable structures can readily be acquired by gram-negative pathogens, facilitating the dissemination of these potent resistance mechanisms and, in many cases, conferring on the isolate a MDR profile (Walsh et al., 2005).

The incidence of MDR ESBL producing pathogens is an increasingly difficult problem in hospitals. Carbapenems have been the last line of drug treatment for serious infections caused by these pathogens due to the stability of these agents against the majority of β-lactamases and their high rate of permeation through bacterial outer membranes. This is of great concern as presently carbapenems are considered the last resort especially in ICUs. With *Klebsiella pneumoniae* and *Escherichia coli* remaining the leading pathogen causing infection in our hospital, close monitoring for the possibility of developing antimicrobial resistance is imperative.

With this background, this study was undertaken to identify the different mechanisms of carbapenem resistance, the antimicrobial profile, and the clonal relatedness of the isolates which could provide important information on the future impact of antibiotic resistance.

MATERIALS AND METHODS

Bacterial isolates

In this study, meropenem resistant blood isolates of *K. pneumoniae* and *E. coli* were obtained from patients attending a major tertiary care teaching hospital, Puducherry. Duplicate isolates from the same patients were excluded.

Antimicrobial susceptibility testing

Susceptibility of the isolates to carbapenems (ertapenem, imipenem and meropenem), tigecycline, colistin and polymyxin-B were determined by the standard Kirby Bauer disk diffusion method. Antimicrobial susceptibility of the isolates to other β-lactam and non β-lactam broad spectrum antimicrobials such as amikacin, trimethoprim/sulfamethoxazole, tetracycline, ciprofloxacin, aztreonam, cefotaxime, ceftriaxone, ceftazidime, cefepime, cefperazone/sulbactam, and piperacillin/tazobactam (Hi Media, India) were also determined by disk diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI)(CLSI, 2010). *E. coli* ATCC 25922 was used as the control strain in each series. Determination of MIC was performed by E-test (AB Biodisk, Solna, Sweden) method for carbapenems, polymyxin-B, tigecycline, colistin and by agar dilution method for other β-lactam and non β-lactam antibiotics. The results were interpreted according to the CLSI (CLSI, 2010). Isolates concomitantly resistant to ≥3 different antimicrobial classes were defined as multi drug resistant (MDR).

Phenotypic detection of carbapenemase production

All isolates were screened for metallo-β-lactamase (MBL) and *Klebsiella pneumoniae* carbapenemase (KPC) production by modified Hodge's test (MHT) (Lee et al., 2001). Further, all imipenem resistant isolates were tested by imipenem-I EDTA (I-EDTA) synergy test for MBL production (Lee et al., 2001). The MBL positive isolates were confirmed by the MBL E-test using E-strips (bioMe´rieux). A classAcarbapenemase producer was defined as any isolate which displayed reduced susceptibility to carbapenems and tested positive in MHT, and negative in I-I EDTA synergy test. A MBL producer was defined as, any isolate which displayed reduced susceptibility to carbapenems and tested positive in MHT test and positive in I-IEDTA synergy test.

Molecular detection of carbapenamase and ESBL genes

Polymerase chain reaction (PCR) was used to screen and sequence the following carbapenamase genes: bla_{KPC}, bla_{NDM-1}, $bla_{OXA-48,}$ $bla_{IMP}$and bla_{VIM} genes as described previously by Mulvey et al. *(*2011). The ESBL genes screened were bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA-1} (Nuesch et al., 1996; Boyd et al., 2004). The plasmid mediated AmpC β-lactamase (PMABL) genes bla_{MOX}, bla_{FOX}, bla_{EBC}, bla_{ACC}, bla_{DHA} and bla_{CIT} were also detected as described earlier (Perez and Hanson, 2002).

Determination of genetic relatedness

Molecular typing of bla_{VIM} and bla_{IMP} positive isolates were done by RAPD-PCR technique using the HLWL74 (5'-ACG TAT CTG C- 3') primer (Tribuddharat et al., 2008). Clonal relatedness of bla_{NDM-1} positive isolates was determined by macrorestriction analysis using *Xba*I according to the PulseNet program developed for *E. coli* (Gautom, 1997). Analysis of images was carried out using BioNumerics software (Applied Maths) using the dice coefficient and UPGMA to generate dendrograms with 1.0% tolerance values. Salmonella ser. Braenderup standard strain H9812 was used as the ladder.

Plasmid analysis

Plasmids harbouring bla_{NDM-1} were extracted using commercial kits (Qiagen Inc., Mississauga, ON, Canada). bla_{NDM-1} containing plasmids were transformed in electrocompetent *E. coli* DH10B^{TM}

Table 1. Distribution of carbapenemase genes detected by multiplex PCR from blood isolates of *K. pneumoniae* and *E. coli.*

Carbapenemase gene(s)	*K. pneumonia* (n=108) (%)	*E. coli* (n=54) (%)	Total isolates (n=162) (%)
bla_{NDM-1}	48 (44.4)	19 (35.1)	67 (41.3)
bla_{VIM-2}	7 (6.4)	2 (3.7)	9 (5.5)
bla_{VIM-24}	2 (1.8)	0 (0)	2 (1.2)
bla_{VIM-1}	3 (2.7)	0 (0)	3 (1.8)
bla_{IMP}	5 (4.6)	1 (1.8)	6 (3.7)
bla_{KPC}	1 (0.9)	0 (0)	1 (0.6)
bla_{VIM} and bla_{NDM-1}	2 (1.8)	0 (0)	2 (1.2)
Total	68 (62.9)	22 (40.7)	90 (55.5)

(Invitrogen, Carslbad, CA) (Boyd et al., 2004) and restricted using *Bgl*II (Roche Diagnostics, QC) by standard procedures (Mulvey et al., 2009). PCR based replicon typing (PBRT) was also conducted as per the standard method (Caratolli et al., 2005). Conjugation was carried out by both broth-mating and filter-mating assays at 30°C using NDM-1-producing isolates as donor and azide-resistant *E. coli* J53 as recipient. Transconjugant selection was performed on MacConkey agar containing meropenem (0.5 mg $^{-1}$) and sodium azide (100 mg $^{-1}$). Transconjugants were tested for bla_{NDM-1} and other beta-lactamase genes by PCR as described earlier. Susceptibilities of transformants were performed by disk diffusion method and MIC was determined by agar dilution and/ or E-test as described earlier.

Outer membrane protein profiling

The role of bacterial outer membrane proteins (OMPs) in the mechanism of carbapenem resistance was determined. Bacterial cells in logarithmic phase were lysed by sonication. The outer membrane proteins were extracted and analyzed by SDS-PAGE (Filip et al., 1973). The molecular weight of the OMPs was compared with the carbapenem susceptible strain of *K. pneumoniae* KpCS1. The involvement of potential efflux pumps in carbapenem resistance was analyzed by an MIC based assay. Agar dilution method for meropenem was performed on Muller-Hinton agar plates with 25 µg/ml of reserpine and without reserpine. An MIC ratio of 1:<=3 between reserpine to non-reserpine group suggests inhibition test positive, indicating existence of active efflux mechanism (Ribera et al., 2002).

RESULTS

A total of 3,361 patient blood cultures were tested which yielded 162 (4.8%) meropenem resistant (MEM-R) isolates (108 *K. pneumoniae* and 54 *E. coli*). Among the 162 MEM-R isolates, 95 (58.6%) were positive for MHT. Eighty seven (53.7%) isolates were positive by I-I EDTA synergy test and 67 (41.3%) isolates were negative by both the tests.

Molecular detection of the carbapenemases

Of the 162 isolates tested, 90 (55.5%) were PCR positive for the carbapenemase genes tested and 72 (44.4%) negative. A total of 62.9% *K.pneumoniae* and 40.7% *E. coli* carried carbapenemase genes (Table 1). Two isolates (1.2%) carried both bla_{VIM} and bla_{NDM-1} genes.

Characterization of bla_{VIM} producing isolates

Fourteen (8.6%) isolates were positive for bla_{VIM}: *K. pneumoniae*, n=12 (11.1%) and *E. coli*, n=2 (3.7%). All the isolates were positive for I-EDTA synergy test and MHT. Among the MHT done with different substrates, 10 isolates were positive with meropenem, 12 with ertapenem and all the 14 were positive with imipenem. Antimicrobial susceptibility testing revealed that all were resistant to carbapenems, third generation cephalosporins (3GCs), cephamycins, penicillin inhibitor combinations, fluoroquinolones, aminoglycosides and tetracycline (Table 2). Of the 14 isolates, 9 (64.2%) were resistant to polymyxin-B, 6 (42.8%) to colistin followed by 1 (7.1%) to tigecycline, which was pan resistant. Clonal relatedness of the bla_{VIM}positive isolates was determined by RAPD-PCR using HLWL74 primer. Among the 14 isolates, 7 *K.pneumoniae* (KpV4 to KpV10) showed clonal cluster type I, 2 *K.pneumoniae* (KpV11, KpV12) showed cluster type II, 2 *E. coli* (EcV1, EcV2) were of cluster type III, and 3 *K.pneumoniae* isolates (KpV1, KpV2, KpV3) were of dissimilar types. Nucleotide sequencing of the VIM was done for representative isolates from each cluster. Cluster I and cluster III showed bla_{VIM-2} type, cluster II showed bla_{VIM-24} type and all the three dissimilar clones were of bla_{VIM-1} type variants. In addition, $bla_{CTX-M-15}$ was carried along with the bla_{VIM-24} producing isolate. $bla_{CTX-M-15}$ and bla_{TEM-1} was carried along with bla_{VIM-2} isolates and bla_{SHV-12} was carried in all the three bla_{VIM-1} *K.pneumoniae*.

Characterization of bla_{IMP} producing isolates

Among the 162 isolates tested, 6 (3.7%) were positive for bla_{IMP-1}: *E. coli* (n=1) and *K. pneumoniae*(n=5) (Table 1). All 6 isolates were positive for MHT and I-I EDTA synergy test. All bla_{IMP} containing isolates were MDR. Additionally,

Table 2. Antimicrobial resistance patterns of *bla*VIM, *bla*IMP and *bla*NDM-1 producing isolates of *K. pneumoniae* and *E. coli.*

Antimicrobial agents tested	MIC (µg/mL)		
	***bla*$_{VIM}$ producers (n=14)**	***bla*$_{IMP}$ producers (n=6)**	***bla*$_{NDM-1}$ producers (n=69)**
Piperacillin/tazobactam	≥128 (n=9); 64 (n=5)	≥128 (n=6)	≥256 (n=10); 128 (n=35); ≥64 (n=24)
Cefotaxime	≥256 (n=14)	≥256 (n=6)	≥256 (n=69)
Ceftriaxone	≥256 (n=14)	≥256 (n=6)	≥256 (n=69)
Ceftazidime	≥256 (n=14)	≥256 (n=6)	≥256 (n=69)
Cefoxitin	Not done	≥128 (n=6)	32 (n=37); 64 (n=17); ≥128(n=15)
Cefepime	≥256 (n=14)	≥256 (n=6)	16 (n=9); 32 (n=12); ≥64 (n=48)
Aztreonam	≥128 (n=12)	64 (n=6)	Not done
Meropenem	≥64 (n=14)	≥128 (n=6)	≤32 (n=12); 64 (n=18); ≥128 (n=39)
Ertapenem	32 (n=5); 64 (n=9)	≥128 (n=6)	≤16 (n=8); 32 (n=14); 64 (n=17); >128 (n=30)
Imipenem	32 (n=2); 64 (n=3); ≥128 (n=9)	≥128 (n=6)	≤16 (n=19); 32 (n=7); 64 (n=21) ≥128 (n=22)
Ciprofloxacin	32 (n=3); 64 (n=9); 128 (n=2)	128 (n=6)	32 (n=48); ≥64 (n=21)
Colistin	64 (n=4); ≥128 (n=2)	32 (n=6)	32 (n=12); 64 (n=4); ≥128 (n=10)
Polymyxin-B	16 (n=6); ≥32 (n=3)	≥32 (n=6)	32 (n=9); 64 (n=12); ≥128 (n=3)
Tetracycline	64 (n=9); 128(n=2); ≥128 (n=3)	Not done	Not done
Amikacin	64 (n=3); ≥128 (n=11)	≥128 (n=6)	128 (n=11); ≥256 (n=58)
Gentamicin	≥128 (n=8); ≥256 (n=6)	≥128 (n=6)	128 (n=21); ≥256 (n=48)
Tigecycline	8 (n=1)	≤1 (n=6)	8 (n=6); 16 (n=1)

all were susceptible to tigecycline, polymyxin-B, and 83.3% were susceptible to colistin (Table 2). Genetic relatedness was determined by RAPD-PCR using HLWL74 primer. Among the 5 *K. pneumoniae,* all belonged to a single clone, cluster I. Additionally, all *K. pneumoniae* carried *bla*$_{SHV-12}$ and *bla*$_{TEM-1}$and *E. coli* carried *bla*$_{TEM-1}$ and *bla*$_{OXA-1}$ genes.

Characterization of *bla*$_{NDM-1}$ producing isolates

Among the 162 isolates tested, 67 (41.3%) were *bla*$_{NDM-1}$producers: *K. pneumoniae* (n=48, 44.4% and *E. coli* (n=19, 35.1%) (Table1). Two *K. pneumoniae* isolates were positive for both *bla*$_{NDM-1}$and *bla*$_{VIM}$ genes. All isolates were positive by MHT and I-I EDTA synergy test. All of these isolates were resistant to all antimicrobial groups (Table 2). Seven (7.2%) isolates were resistant to tigecycline, 26 (37.6%) were resistant to colistin and 24 (34.7%) to polymyxin-B. PFGE was performed on a convenience sample of 20 *bla*$_{NDM-1}$ *K. pneumoniae* and 15 *bla*$_{NDM-1}$ *E. coli.* Among *K. pneumonia*, 2 clusters with >80% similarity were observed cluster 1 (n=3) and cluster 2 (n=9), (Figure 1). Among *E. coli*, 10/12 isolates clustered into one of three groups sharing >80% (Figure 2). Two isolates were non-typeable.

Plasmid analysis was conducted on twenty *bla*$_{NDM-1}$ *K. pneumoniae*. Seventeen of the *bla*$_{NDM-1}$ bearing plasmids were successfully transferred to *E. coli* DH10B by electroporation. *bla*$_{NDM-1}$ genes were located on plasmids that ranged in size from 89 to 131 Kb (Figure 3). Replicon typing revealed that the *bla*$_{NDM-1}$ bearing plasmids were distributed among two incompatibility groups: 12 (70.5%) IncA/C and 5 (29.4%) IncL/M. The RFLP patterns of plasmid transformant DNA consisted of 2 clusters corresponding to incompatibility groups (Figure 3). Cluster I consisted of 8 IncL/M plasmids that were genetically similar (>85% homology), varying in size from 89 to 122 Kb. The cluster II consisted of IncL/M plasmids consisted of highly similar RFLP patterns of ~131 Kb in size. Susceptibility testing among the transformants revealed that in addition to carbapenems resistance determinants of other antimicrobial groups was also co-transferred in all the isolates.

Characteristics of both *bla*$_{NDM-1}$ and *bla*$_{VIM}$positive isolates

The two isolates positive for both carbapenemase genes showed by sequencing that they were of *bla*$_{NDM-1}$ and *bla*$_{VIM-2}$ types. PFGE revealed a single clonal type. The isolates were pan resistant including resistance to tigecycline and colistin. Replicon typing revealed they both were on IncA/C plasmids.

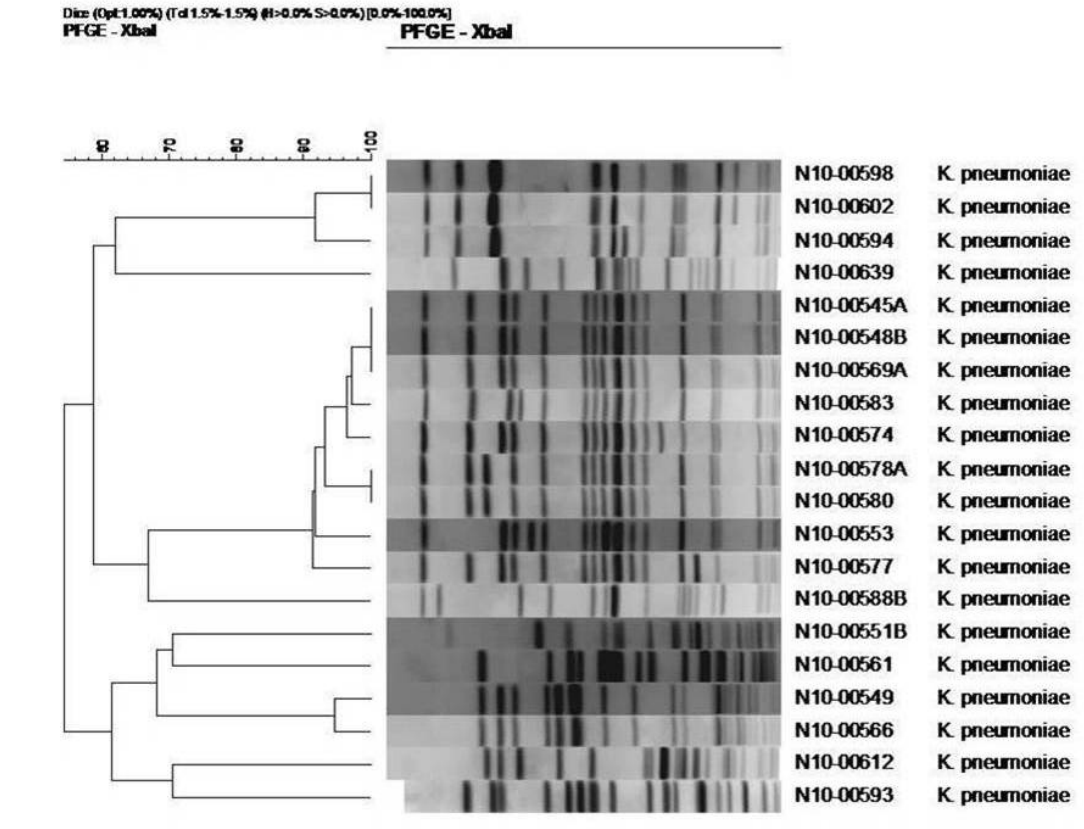

Figure 1. PFGE dendrogram depicting the genetic relatedness of *bla*$_{NDM-1}$ producing blood isolates of *K. pneumoniae.*

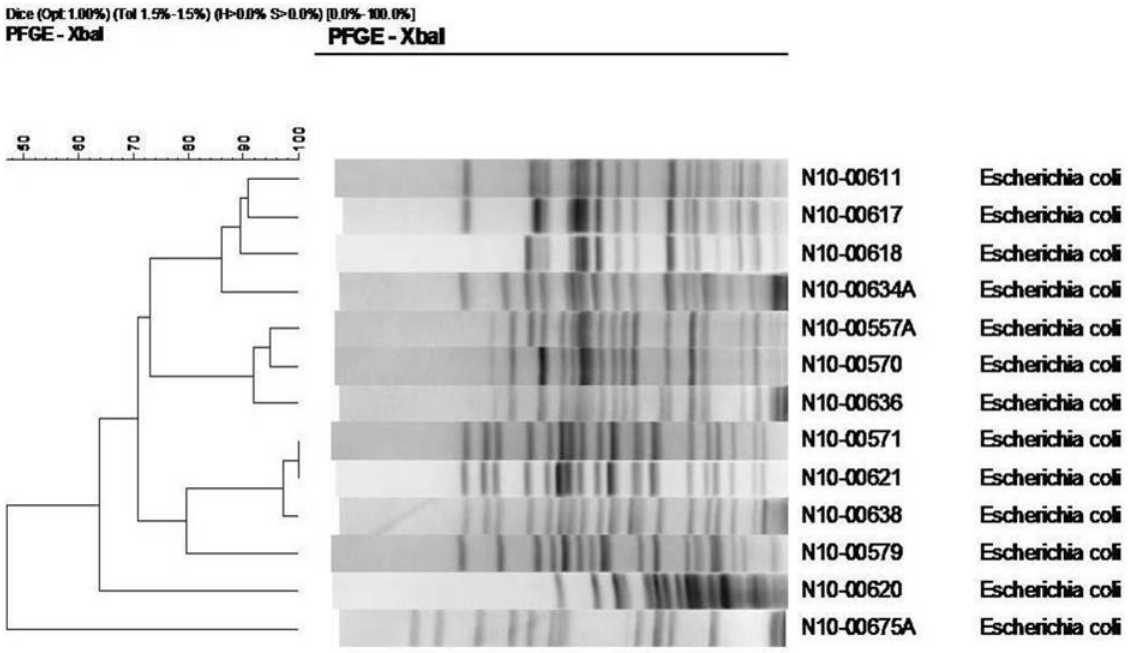

Figure 2. PFGE dendrogram depicting the genetic relatedness of *bla*$_{NDM-1}$ producing blood isolates of *E. coli.*

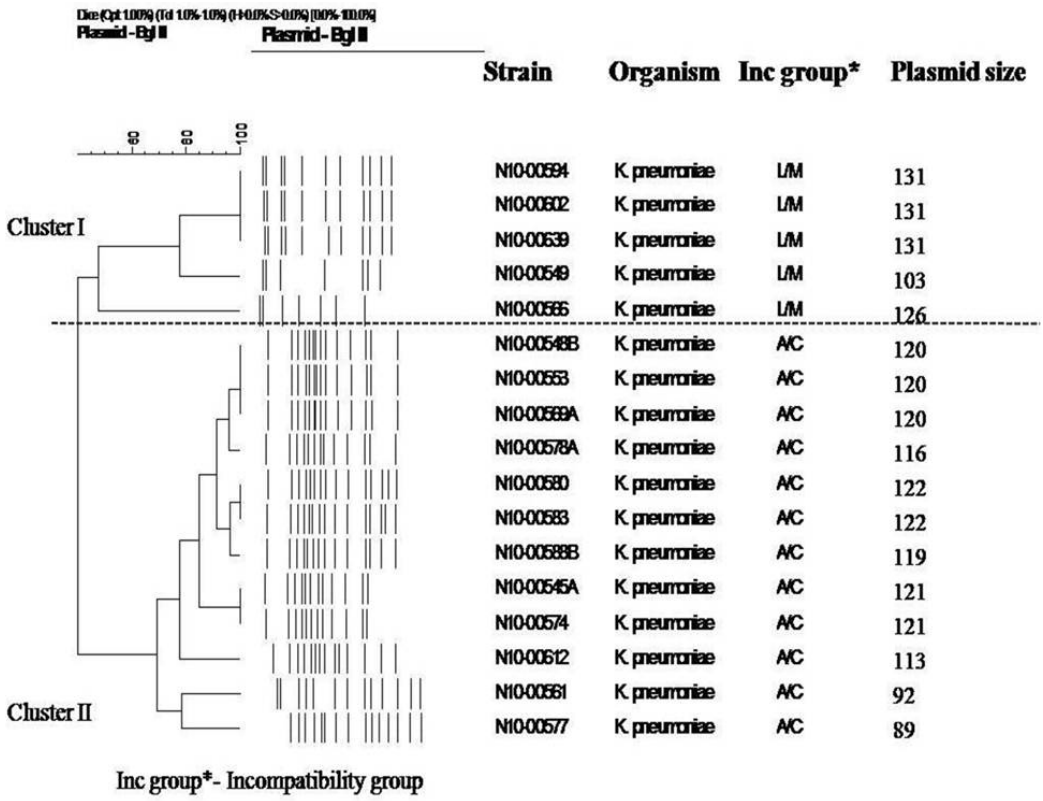

Figure 3. RFLP dendrogram depicting genetic relationships of the *bla*$_{NDM-1}$ bearing plasmids from blood isolates of *K. pneumoniae.*

Non-carbapenemase resistance mechanisms

A total of 44.4% (n=72) of MEM-R *K. pneumoniae* and *E. coli* blood isolates were negative for all carbapenemase genes tested using PCR. A convenience sample of 41 isolates was selected for an efflux assay. Fifteen (36.5%) isolates showed a 1 to 2 dilution decrease in the MICs of meropenem with reserpine, suggesting that an efflux may be responsible, at least in part, for the carbapenem resistance in these isolates. Additional beta-lactamase PCR was conducted on 57 isolates that were negative by both efflux testing and carbapenmase gene PCR. Fourty three (75.4%) possessed ESBL genes and 9 (15.7%) carried PMABL genes. The OMP profile was studied for randomly selected 22 carbapenem resistant, carbapenemase gene negative, efflux negative, ESBL (n=19) and or AmpC positive (n=3) *K. pneumoniae* isolates. OMP profile showed loss of 36kDa porins in 13 ESBL positive and 1 AmpC positive isolate. Thus, 14 (63.6%) showed OMP porin loss.

DISCUSSION

Carbapenems are antibacterial agents with broad spectrum of activity against gram positive, gram negative and anaerobic bacteria. In the last two decades, carbapenems (example, imipenem and meropenem) have become the drugs of choice against ESBL and AmpC producing MDR isolates causing infections in a host of medical centers, but are being compromised by the emergence of carbapenem- hydrolysing β-lactamase (carbapenemase) of molecular classes B and D (Livermore et al., 2006; Lee et al., 2006). This newer antimicrobial resistant species such as carbapenem-resistant enterobacteriaceae (CRE), including *E. coli* and *K. pneumoniae* are emerging which is an increasing therapeutic problem worldwide. Therefore, investigating the mechanisms underlying the resistance has an impact in treatment measures.

MBL production was screened by using the MHT and I - I EDTA synergy test which gave 100% specificity and sensitivity with that of PCR results. MHT detects carbapenemase production and EDTA synergy test confirms MBL production and thus, in the study experience it is suggested to test by both the phenotypic tests which will not miss out any carbapenemase producing isolate. However, specific gene target PCR has to be performed for confirmation.

KPC enzymes are class A carbapenemases that mediate resistance to extended-spectrum cephalosporins in addition to carbapenems and are usually plasmid encoded.

The KPC β-lactamase occurs most commonly in *K. pneumoniae*, but has also been reported in other species of Enterobacteriaceae and in *P. aeruginosa* (Anderson et al., 2007). In this study, a KPC-3 producing *K. pneumonia* was identified from a blood isolate.

In a recent study from this centre, of the 103 meropenem resistant *K. pneumoniae*from blood, urine and exudates samples tested 6 were MHT positive, I-I EDTA synergy test negative and were found to KPC producers phenotypically (Parveen et al., 2010). Among the 9 blood isolates tested, one was KPC positive phenotypically and it was included in this study for further molecular characterization. By PCR and sequencing analysis, the isolate was confirmed to be KPC-3 producer. The phenotypically positive isolates from other sources were not subjected to molecular studies, however by Randomly Amplified Polymorphic DNA (RAPD) typing all the 6 KPC producers showed dissimilar patterns (Parveen et al., 2010). No discernible epidemiological linkage could be seen between these isolates. This finding reveals the diversity of these strains and its non-clonal dissemination in this hospital.

This isolate was found to be resistant to all the antimicrobials except tigecycline and colistin. However, before this information could be used, the patient had died of septic shock, presumably caused by KPC producing *K. pneumoniae*. The source of the isolate could not be found out. Although the study could not determine whether this case was sporadic in nature or attribute to the existence of an outbreak, this enzyme has not been previously identified in this geographic region.

The present study also reveals the presence of VIM MBL producing isolates of *K. pneumonia* and *E. coli* in the study hospital. Fourteen (8.6%) of carbapenem-resistant *K. pneumoniae* and *E. coli* harboured bla_{VIM}. Phenotypic tests indicated that imipenem was inactivated by all the 14 imipenem- resistant isolates by MHT, they were also positive for EDTA synergy test indicating presence of MBL. Sequence identification of bla_{VIM} revealed 9 (64.2%) $bla_{VIM-2,}$ 3 (21.4%) bla_{VIM-1} and 2 (14.2%) bla_{VIM-24} variants**.** To this study knowledge, this is the first report of VIM variants in *K. pneumoniae* and *E. coli* from India.

RAPD analysis of the 12 bla_{VIM}*K. pneumoniae* revealed seven cases of infection by *K. pneumoniae* producing bla_{VIM-2} was found to have been caused by a single clone (data not shown). These findings suggest clonal *K. pneumonia* harbouring bla_{VIM-2}-are contributing to the dissemination of bla_{VIM} in this hospital. The present study reports, 3.7% (n=6) of carbapenem resistant isolates as harbouring bla_{IMP}. All were multidrug resistant and susceptible to tigecycline.

In this study, among the 12 VIM positive *K. pneumoniae*, seven cases of infection by *K. pneumoniae* producing bla_{VIM-2} was found to have been caused by a single clone. During the study period, the other outbreak involved two cases of infection caused by a single clone of bla_{VIM-2} producing *E. coli*. These findings suggest an increasing prevalence rate of bla_{VIM-2} positive isolates in this hospital. Further, three cases of bla_{VIM-1} *K. pneumoniae* of dissimilar clones was also found. This could be in part due to the nosocomial outbreak due to clonal and non-clonal isolates occurring in this hospital over this study period, however, it was not recognized due to the lack of awareness in detecting the MBLs routinely. These results show that bla_{VIM} positive isolates are still confined in different wards and may spread at a low rate and cause sporadic outbreaks in this hospital.

Analysis of other β-lactamases combinations among bla_{VIM} producers showed, bla_{VIM-2}+ $bla_{CTX-M-15}$ + bla_{TEM-1}; bla_{VIM-1}+ bla_{SHV-12} and bla_{VIM-24}+ $bla_{CTX-M-15}$ combinations. The coexistence of two enzymes, an MBL and a non-MBL extended-spectrum β-lactamase, in the same strain has been noteworthy. Kassis et al. (2006) reported bla_{VIM-1} + bla_{SHV-5}*in K. pneumonia* from France and bla_{VIM-4} + bla_{SHV-12} was reported by Luzzaro et al. (2004). From a Tunisian university hospital, a maximum of four enzymes, bla_{VIM-4}, $bla_{CTX-M-15}$, bla_{CMY-4}, and bla_{TEM-1} in a MDR *K. pneumoniae* (Ktari et al., 2006). Since *K. pneumoniae* and *E. coli*are notorious as a hosts' for resistance plasmids and are the major causes of nosocomial infections, they may acquire the resistance genes from non-fermenters. The presence of more than one ESBL or MBL enzymes in the same plasmid may facilitate its potent dissemination to other species or genera.

In this study, five isolates of *K. pneumoniae* possessed the bla_{IMP-1}gene. RAPD-PCR typing showed that all *K. pneumonia* were from a single clone. The source of the infections was unknown. These isolates were collected over a period of three years and from different wards, thus providing evidence that bla_{IMP-1} producing *K. pneumonia* though less in percentage exists close to a state of endemicity in this hospital. A more worrying thing is that bla_{IMP-1} has spread in this region, but has remained undetected to date. To date there are no published data on the identification of IMP harboring *K. pneumoniae* and *E. coli* isolates in India.

The most common carbapenamase identified in this study was bla_{NDM-1} (42.5%) which was approximately 45% of *K. pneumoniae* and 35% of *E. coli* carried bla_{NDM-1}. The first report of bla_{NDM-1} producers from India was by Deshpande et al. (2010) from Mumbai, who found 22 out of 24 isolates to be bla_{NDM-1} producers. The study findings were not surprising given the numerous reports of bla_{NDM-1} from South India.

Antimicrobial susceptibility has shown resistance to all classes with tigecycline and colistin as the only treatment options. However, 10 and 37.6% were resistant to tigecycline and colistin respectively, which narrows the therapeutic choices. This is also evident from a recent study from India that, tigecycline still has effective antimicrobial effects against carbapenemase and ESBL producing *K. pneumoniae and E. coli* from neonatal septicaemia, but with increased MIC levels (Roy et al.,

2013). Molecular typing by PFGE shows dissimilar patterns in both *K. pneumoniae* and *E. coli.* This diverse clonality indicates that parallel and horizontal evolution of resistance which due to carbapenem antibiotic pressure.

Analysis of *bla*$_{NDM-1}$ plasmids indicated large plasmids of 90 to 1130 kbs which also carried co-resistance to aminoglycosides and fluoroquinolones. The plasmids harbouring the *bla*$_{NDM-1}$ gene were found to belong to two major incompatibility group IncA/C (73.9%) and IncL/M (26%). This is similar to the report of Kumarasamy et al. (2010) where, *bla*$_{NDM-1}$ isolates from Chennai carried IncA/C type plasmids thus showing that this is the major plasmid type circulating in South India.

Of interest, 2 isolates with indistinguishable PFGE patterns were identified which carried both *bla*$_{NDM-1}$and *bla*$_{VIM-2.}$ They were extensively drug resistant (XDR) including tigecycline. To this study knowledge, this is the first report on the presence of both *bla*$_{NDM-1}$ and *bla*$_{VIM}$ carbapenemase genes from India. Three strains of *K. pneumoniae*co-producing *bla*$_{VIM-1}$ and *bla*$_{KPC-2}$ from clinical specimens in Greece were reported (Giakoupi et al., 2009). These findings indicate the continued spread of resistance genes among these pathogens.

In the present study, a significant proportion (44.4%) of carbapenem resistance was found to be due to non-carbapenemase producing *K. pneumoniae* and *E. coli*. A total of 63.6% of these isolates were found to be associated with ESBL production along with porin loss OmpK36.

Earlier studies reported the role of both OmpK35 and OmpK36 in increasing the MICs of carbapenems in *K. pneumoniae,* but in this study, lack of OmpK36 seemed to be the only cause of carbapenemresistance (Nikaido, 1989; Nikaido, 1998). It has been previously reported that clinical isolates of *K. pneumoniae* lacking expression of ESBLs express the two porins OmpK35 and OmpK36, whereas most isolates producing these β-lactamases express only the porin OmpK36, while there is either very low expression of the OmpK35 porin or it is not expressed (Doménech-Sánchez et al., 1999). Thus, the study report confirms the specific role of OmpK36 in *K. pneumoniae* clinical isolates with reduced carbapenem susceptibility which produce OmpK35 but with no OmpK36 expression.

False detection of carbapenemase production was observed by the MHT possibly as a result of ESBL production coupled with porin loss (Carvalhaes et al., 2010). However, the study has not experienced this which may be due to the use of ertapenem which is the most sensitive indicator. Hence, keeping this fact in mind when performing MHT for carbapenem resistant strains, especially in high ESBL evidence settings like this study, the study suggest to use ertapenem as the substrate for MHT which gave 100% specific and sensitive results to that of PCR. In analyzing the role of efflux in carbapenem resistance, 36.5% isolates showed that a putative efflux mechanism may be involved in the resistance.

In the study hospital, meropenem use was initiated in 2006. Amikacin and meropenem were the frequently used antibiotics to treat infections by multidrug resistant bacteria in ICUs and soon within a short period resistance to carbapenems developed, thus rendering the treatment options narrow. Similarly, studies have shown that a shift in empirical therapy to the carbapenems, due to the presence of ESBL producers, is associated with emerging resistance and in the ESBL-producing organisms themselves (Kliebe et al., 1985).

Nearly 18% colistin-resistant strains have emerged in this sample collection. The reason for colistin resistance among the isolates in this tertiary care centre, wherein colistin methane sulfnate (CMS) is not in extensive use is inexplicable. Interestingly, none of the patients studied had received colistin prior to the isolation of the resistant strains.

Conclusions

This study records the emergence and rise of *bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{VIM}$, *bla*$_{IMP}$ genes circulating in Puducherry, South India and also the non-carbapenemase resistance mechanisms. These resistance mechanisms create a major public health problem, compounded by a shortage of newer antibiotic options. Physicians must select antibiotics with the specific needs of an individual patient in mind but also in a manner that does not breed further drug resistance. Selection of an appropriate initial antibiotic regimen for empiric therapy, rotation of different antibiotic classes and judicious use of antibiotics are needed.

Conflict of Interests

The authors have not declared any conflict of interests.

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Polymerase chain reaction (PCR) technique compared to conventional bacteriological and serological techniques in diagnosis of human brucellosis in Egypt

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Direct diagnosis of human brucellosis is performed by cultivation with the disadvantage of being time consuming and the increase risk of laboratory acquired infection to laboratory personnel handling and performing culture procedures. Serological techniques as serum agglutination test (SAT) can also be used for diagnosis with the disadvantage of false negative and positive results that may occur. This study aims to evaluate polymerase chain reaction (PCR) technique sensitivity and specificity for diagnosis of brucellosis in comparison with the conventional bacteriological and serological techniques. Blood samples were withdrawn from 40 patients suspected to have brucellosis. Blood culture, SAT, and PCR technique were performed. It was found that PCR had high sensitivity (100%) when culture is the gold standard more than if SAT is considered the gold standard (88%). Specificity of PCR is more (40%) when SAT is considered the gold standard than if culture is the gold standard (37.5%). Molecular diagnosis of human brucellosis by PCR is faster and more sensitive than culture and serology.

Key words: Polymerase chain reaction, human brucellosis, blood culture, standard tube agglutination test.

INTRODUCTION

Brucellosis is one of the world's major zoonotic diseases and responsible for enormous economic losses, as well as considerable human disease in endemic areas (Queipo-Ortuno et al., 2008). Brucellosis was first reported in Egypt in 1939 and is now considered endemic in most parts of the country. Despite its economic and public health importance in recent years, the official Egyptian brucellosis control program does not appear to have been fully implemented (Hegazy et al., 2009).

Brucellosis particularly caused by *Brucella melitensis* (biovar 3) and *Brucella abortus* (Refai, 2002) is endemic in Egypt, presumably affecting large numbers of animals as well as humans. It appears to be of particular risk in rural communities, especially in Upper Egypt (Molina-

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Flores, 2010). In many countries of the region, brucellosis continues to be reported in almost all domestic animals, particularly sheep, goats, and cattle (FAO, 2010).

Concerning its diagnosis, Brucellosis is missed for two reasons. Firstly, like tuberculosis and syphilis, the disease is a great “imitator” and can present with non-specific features in any organ system. Secondly, brucellosis is rare and a neglected disease, so it is often not considered in the differential diagnosis, even in patients with clear risk factors, and mistaken for tuberculosis, malaria, or typhoid fever (Franco et al., 2007). Classically, direct diagnosis is performed by cultivation in artificial media, with identification of the isolates by its morphology and growth characteristics of the colonies, biochemical tests, and phage typing. Disadvantages of these procedures are the high costs, time necessary for growth, and identification of the isolates, apart from high risk for personnel (Queipo-Ortuno et al., 2008). In the absence of culture facilities, various serological tests are available, such as the Rose Bengal test and serum agglutination test (SAT) (Franco et al., 2007). Inaccurate serological results causing incorrect diagnoses are a continuous problem when testing for infectious disease agents as brucellosis. Exposure to cross-reacting microorganisms may cause elevated antibody levels for various periods of time; some prolonged, which can result in a false positive serological reaction which remains a major diagnostic problem in some areas where such microorganisms are endemic. The SAT detects IgG less efficiently, especially IgG1, resulting in low assay specificity. Therefore, the SAT is generally not used as a single test, but rather in combination with other tests. It requires incubation from 8 to 24 h (Nielsen and Yu, 2010).

An alternative to the classic bacteriological methods is the Polymerase Chain Reaction (PCR) that is proving to be faster and more sensitive than the traditional methods. It must be taken into consideration that false-negative reactions can occur through a number of mechanisms, such as specimens that contain EDTA, RNase or DNase, heme or heparin. False-positive reactions resulting from specimen contamination or amplicon carryover also require attention (Yu and Nielsen, 2010).

The present study aimed to evaluate the sensitivity and specificity of polymerase chain reaction (PCR) technique as a rapid method for diagnosis of brucellosis in comparison with conventional bacteriological and serological techniques.

PATIENTS AND METHODS

The study was conducted on 40 patients suspected to have brucellosis who were admitted or from out-patient clinic (OPC) of Abbasia Fever Hospital in the period from July 2012 to May 2013. The study was approved by the ethical committee in Faculty of Medicine, Ain Shams University, Cairo, Egypt. All patients gave informed consent. Patients had symptoms and signs suggestive of acute brucellosis, history of animal contact or ingestion of dairy products from animals or unpasteurized milk. They were investigated routinely for complete blood count (CBC) by Beckman Coulter Counter, erythrocyte sedimentation rate (ESR) by Westergen method, renal function tests (RFT), and liver function tests (LFT). Additional radiological, ultrasonographic or biochemical investigations were done when indicated depending on the clinical picture of the patient.

Sample collection

Ten milliliters of blood were withdrawn by venipuncture. 5 ml of the sample was added to biphasic blood culture bottles, 2 ml was centrifuged to be used in serum agglutination test method, and 3 ml were collected in citrated tubes for performing PCR technique.

I-Blood culture

An inoculum of 5 ml of patient’s blood was added to two-phase system bottle containing both solid (Biolife-Egypt: Tryptic Soy Agar, lot: BA 2301) and liquid medium (Biolife-Egypt: Tryptic Soy Broth, lot: BA 4501). The bottle was incubated at 37°C in 10% carbon dioxide (CO_2) atmosphere. Cultures were not discarded as negative until six weeks had been elapsed. Apparent colonies were sub-cultured on blood agar and chocolate agar for further examination and typing. Colonial morphology, staining, slide agglutination with anti-*Brucella* serum (smooth or rough), urease, catalase, and oxidase tests were the basis for a culture to be identified as belonging to the genus *Brucella*.

II-Serum agglutination test (SAT)

SAT was done for all patients to detect the presence or absence of antibodies using *B. melitensis* Reagent (Atlas Febrile antigens) (Atlas Medical, UK, Lot No.: 12083032). The test was done according to the manufacturer`s instructions. Titer of reactive serum was recorded at the last dilution that gave agglutination.

III-PCR

DNA extraction was done using kits prepared by QIAGN-Italy. The collected DNA was stored at -70°C until the time of assay.

DNA amplification

Primers B4 (5'-TCGGTTGCCAATATCAA-3') and B5 (5'-CGCGCTTGCCTTTCAGGTCTG-3') (prepared by: Sigma chemicals-Egypt) were used to amplify a target sequence of 223 bp present on a gene encoding a 31 kDa *B. abortus* antigen. This sequence has been shown to be common to all *Brucella* biovars (El Kholy et al., 2009).

Fifty microliters of reaction mixture was used. It contained 10 mM tris HCl (pH 8.4), 50 mM KCl, 1 mM magnesium chloride, 200 µM each deoxyribonucleoside triphosphate (dATP, dGTP, dCTP, dTTP), oligonucleotides, B4 and B5 (100 nM each), 1.25 µ of Taq polymerase (Boehringer), 2 to 4 µg of total DNA extracted from blood samples and 100 ng from the positive controls. The reaction was performed in a DNA thermocycler (Thermo-scientific Electrocorporation: model PXE 0.2).

After an initial denaturation at 93°C for 5 min, the PCR profile was set as follows: template denaturation at 90°C for 60 s, primer annealing at 60°C for 30 s, and primer extension at 72°C for 60 s, for a total of 35 cycles, with a final extension at 72°C for 10 min (El Kholy et al., 2009).

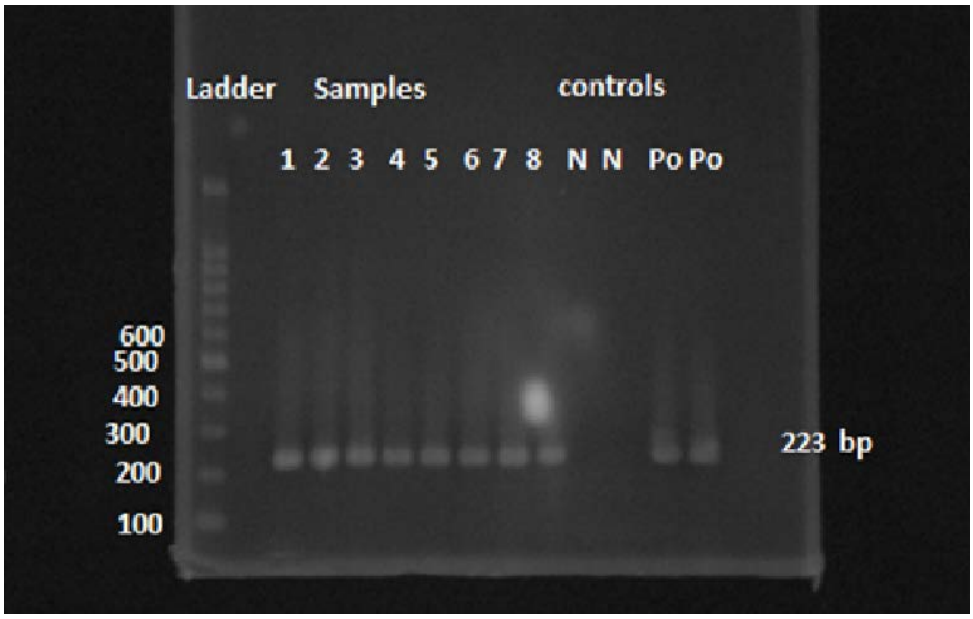

Figure 1. Polymerase chain reaction (PCR) for *Brucella* spp. Agarose gel electrophoresis analysis and ethidium bromide staining for samples 1–9 using B4/B5 primer. From left to right: lane 1 negative PCR sample, lanes 2, 3, 4, 5, 6, 7, 8, 9 positive PCR samples.*Ladder (100 bp ladder): QIAGN cat.no.239045.

The positive control was DNA extracted from *Brucella* species colonies isolated from one patient who suffered from brucellosis and whose laboratory results were positive by serology and blood culture. For the negative control, all reagents were added except for *Brucella* DNA which was replaced by DNA free water.

Gel electrophoresis

The sample was considered positive when DNA with a molecular weight expected for the amplified product was observed after electrophoresis in 2% agarose with fluorescence in the presence of ethidium bromide (2 µg/ml). PCR amplification products were detected through visualization of bands under ultraviolet light.

Statistical methodology

Analysis of data was done by IBM computer using statistical program for social science version 12 (SPSS). Description of quantitative (mean, standard deviation (SD) and range) and qualitative (number and percentage) variables was done. Chi square and unpaired t- tests were used. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were measured.

RESULTS

Forty patients including 23 males and 17 females, aged between 20 and 55 years (mean± SD= 35 ±9.363) were enrolled in the study. All patients gave history of animal contact or ingestion of unpasteurized dairy products.

All patients were presented with fever. Weight loss and sweating followed by headache and arthralgia were the most presenting symptoms other than fever (47.5, 42.5, 35, and 32.5%, respectively). Adenitis was present in 16 (40%) patients. Twenty two patients (55%) showed elevated ESR. Elevated liver function test was present in 20 (50%) patients. Elevated renal function tests and thrombocytopenia were present in 12 (30%) and 10 (25%) patients, respectively.

Regarding statistical analysis, the results showed statistical insignificant difference between PCR positivity and presenting clinical symptom and sign or laboratory tests (weight loss, sweating, headache, arthralgia, adenitis, elevated ESR, elevated liver function tests, elevated renal function tests and thrombocytopenia).

Regarding the occupation of patients, positive PCR results for *Brucella* were in farmers (11 out of 12), housewives (7 out of 9), butchers (6 out of 6), veterinarians (5 out of 10), and workers (2 out of 3).

Among the studied 40 cases, 10 males (10/23, 43.48%) and 6 females (6/17, 35.3%) gave positive culture results. Sixteen males (16/23, 69.6%) and 9 females (9/17, 53%) gave positive SAT results. Seventeen males (17/23, 74%) and 14 females (14/17, 82.4%) gave positive PCR results (Figures 1 and 2).

By comparing culture and SAT results, it was found that 15 samples were negative by SAT (37.5%) and culture (37.5%). 25 samples were positive by SAT (62.5%), 16 of them were positive by culture (40%) and 9 of them were negative by culture (22.5%). These results showed highly statistically significant difference (P value<0.001) (Table 1).

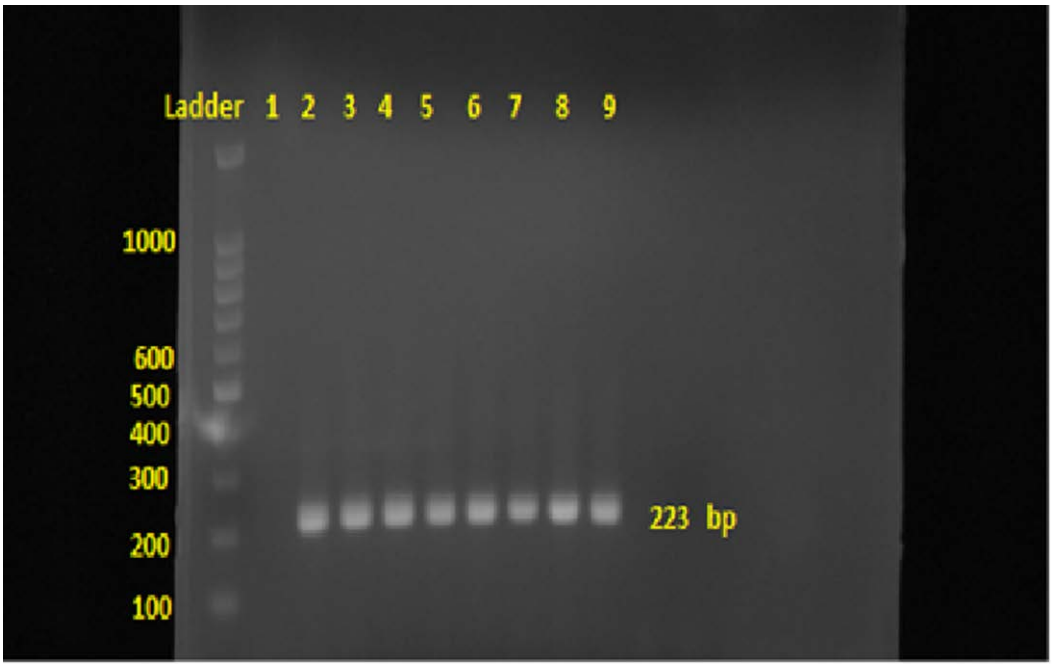

Figure 2. Polymerase chain reaction (PCR) for *Brucella* spp. Agarose gel electrophoresis analysis and ethidium bromide staining for samples 1–8 using B4/B5 primer. From left to right: lanes 1, 2, 3, 4, 5, 6, 7, 8 positive PCR samples; lanes 9, 10 negative control; Lanes 11, 12, positive control. *Ladder (100 bp ladder): QIAGN cat. no. 239045.

Table 1. Comparison between culture results and serum agglutination test results.

Culture		SAT		
		Positive (SAT ≥ 1/320)	**Negative**	**Total**
Positive	N	16	0	16
	%	40.00	0.00	40.00
Negative	N	9	15	24
	%	22.50	37.50	60.00
Total	N	25	15	40
	%	62.50	37.50	100.00
Chi-square	χ^2	13.444		
	P-value	0.0002*		

By comparing PCR with culture results, it was found that 9 samples were negative by PCR (22.5%) and culture (22.5%). 31 samples were positive by PCR (77.5%), 15 of them were negative by culture (37.5%), and 16 of them were positive by culture (40%). These results showed statistically significant difference (P value<0.05) (Table 2).

By comparing SAT with PCR results, it was found that 25 samples were positive by SAT (62.5%), 3 (33.33) of them were negative by PCR and 22 (70.97%) of them were positive by PCR. 15 samples were negative by SAT (37.5%), 6 (66.67%) of them were negative by PCR and 9 (26.03%) of them were positive by PCR. There was no statistically significant difference (P value>0.05) (Table 3).

PCR had high sensitivity (100%) when culture is the gold standard more than if SAT is considered the gold standard (88%). Specificity of PCR is more (40%) when SAT is considered the gold standard than if culture is the gold standard (37.5%) (Tables 5 and 6).

Table 2. Comparison between culture results and PCR results.

Culture		PCR		
		Positive	**Negative**	**Total**
Positive	N	16	0	16
	%	40.00	0.00	40.00
Negative	N	15	9	24
	%	37.50	22.50	60.00
Total	N	31	9	40
	%	77.50	22.50	100.00
Chi-square	X^2		10.898	
	P-value		0.001*	

Table 3. Comparison between serum agglutination test results and PCR results:

SAT		PCR		
		Positive	**Negative**	**Total**
Positive	N	22	3	25
(SAT ≥ 1/320)	%	55.00	7.50	62.50
Negative	N	9	6	15
	%	22.50	15.00	37.50
Total	N	31	9	40
	%	77.50	22.50	100.00
Chi-square	χ^2		2.762	
	P-value		0.0965	

DISCUSSION

The clinical picture of brucellosis alone cannot always lead to diagnosis since the symptoms are non-specific and often atypical; therefore, diagnosis needs to be supported by laboratory tests. Although many serological tests and new automated blood culture techniques have been developed to diagnose brucellosis. There are still significant problems in the diagnosis of the disease. PCR was considered the golden test for diagnosis of brucellosis as was mentioned in a study done for evaluation of different PCR assays. The authors stated that PCR is a very useful tool not only for the diagnosis of acute brucellosis, but also as a predictive marker for the course of the disease and the post treatment follow-up, which is valuable for the early detection of relapses (Mitka et al., 2007).

In the present study, *Brucella* spp. were isolated in biphasic blood culture from 16 patients who had not received antibiotic treatment at the time of sample collection and the range of time for growth was 3 to 8 days. The diagnosed cases using blood culture were only 40% of the studied cases in the present study, while published culture results vary in other studies; 70, 75, and 44.4% (Moreno et al., 1992; Eissa et al., 1990; Shehabi et al., 1990).

In a study done, Mitka et al. (2007) found that the sensitivity of blood cultures is from 53 to 90%, who added that blood cultures were time-consuming, also handling the organism cause a high risk of contagion for laboratory personnel. Although blood cultures are the "gold standard" in the diagnosis of brucellosis; culture may sometimes be negative due to factors inherent in the growth of the microbe. Isolation of the bacteria is hazardous and brucellosis is one of the most common laboratory-acquired infections. This also agreed with Serra and Vinas (2004) who analyzed the importance of blood culture as a diagnostic method and described the limitations of this method in endemic areas of brucellosis (Serra and Vinas, 2004).

SAT test was used as a routine test in the present study to detect anti-*Brucella* antibodies in diagnosis of *Brucella* infection in sera of the patients and this correlates with Mantur et al. (2004) who reported that SAT remains the most popular and yet used worldwide diagnostic tool for the diagnosis of brucellosis, because it is easy to perform, does not need expensive equipments, and training. However, SAT titers above 1/160 are considered diagnostic in conjugation with a compatible clinical presentation. However, in areas of endemic disease using a titer of 1/320 as a cutoff may make the test more specific (Table 4). This agrees with another study whose authors carried out a community-based survey in 2 villages in the Nile Delta. The sero-prevalence of brucellosis among humans was 1.7% and the diagnostic sero-positivity level by SAT was 1/320 (El-Sherbini et al., 2007).

A study from Yazd, Iran, at which 19 out of 300 blood donors had a SAT titer of 1/80 or higher (5.7% of men and 4.3% of women); it was concluded that if a 1/80 titer in the Wright agglutination tube test is considered as diagnostic for brucellosis, the frequency of the disease in blood donors (which are generally a healthy population) is not negligible (Ghilian et al., 2011).

Elfaki et al. (2005) diagnosed much positive brucellosis by agglutination tests, while there were 40 and 70% positive by culture and PCR methods. They believed that producing antibody against *Brucella* was not related to disease condition and for following disease; they had to use blood culture and PCR (Elfaki et al., 2005).

In a study carried out on 263 patients with brucellosis in Kuwait, it was found that 89 cases had positive result in all of the blood culture, SAT, and PCR; 110 cases had positive Wright agglutination tube test and negative blood culture, from which 104 cases showed positive PCR.

Table 4. Comparison between *brucella* antibody titers by SAT and PCR results.

SAT titer	PCR					
	Negative		Positive		Total	
	N	%	N	%	N	%
(1/80) or no agg.	3	33.33	4	12.90	7	17.50
(1/160)	3	33.33	5	16.13	8	20.00
(1/320)	2	22.22	8	25.81	10	25.00
(1/640)	1	11.11	6	19.35	7	17.50
(1/1280)	0	0.00	8	25.81	8	20.00
Total	9	100.00	31	100.00	40	100.00
χ^2			5.325			
P-value			0.255			

Table 5. Sensitivity, specificity, PPV, NPV, and accuracy of PCR test if standard agglutination test was considered the gold standard test.

Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
PCR	88%	40%	70.97%	66.67%	70%

Table 6. Sensitivity, specificity, PPV, NPV and accuracy of PCR test if culture test was considered the gold standard test.

Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
PCR	100%	37.5%	51.6%	100%	62.5%

They concluded that SAT has some false positive results, or it may be that PCR failed to diagnose brucellosis in 6 cases (Al-Nakkas et al., 2005). However, without assuming a method as gold standard, one cannot determine the sensitivity or specificity of other tests.

In the present study, there were 15 samples negative by culture and positive by PCR method and this agrees with studies about detection of *B. abortus* in samples from bovine abortions done by Cortez et al. (2001) who detected 4 positive samples using PCR in 54 samples classified as negative by classic isolation (Cortez et al., 2001). In addition, there was another study whose authors obtained 2 positive samples in 52 negatives for microbiologic cultivation and they stated that it is reasonable suppose the PCR results are not false positives, although isolation showed a negative result (Fekete et al., 1990). The results of the present study found that PCR was more sensitive than culture and this confirmed the results of the study done by Scarcelli et al. (2004) who analyzed samples obtained from 67 aborted bovine fetuses by means of bacteriological methods and PCR and also found that the samples that were positive by PCR (34/67) are more than that of culture (26/67), so they concluded that PCR was more sensitive than culture (Scarcelli et al., 2004). Divergences between PCR and culture results were also observed in a previous study (Marques et al., 2001).

Morata et al. (2001) evaluated 34 human brucellosis cases, in whom 97% had positive PCR test in non-blood specimens, but only 29.4% showed positive culture. They found that 11.4% of the patients had negative serologic test or low antibody titer. So, the authors recommended PCR as a helpful detecting method for brucellosis based on its high sensitivity accompanied by high speed and low contamination risk (Morata et al., 2001).

It was mentioned in a review article on the recent trends in pathogenicity and laboratory diagnosis of brucellosis that timely diagnosis is needed for early management and effective prevention of its consequences in human beings and animals. It was also stated that definite diagnosis of brucellosis is traditionally based on culture of blood, bone marrow, tissues or body fluids, although it does not have high sensitivity (around 40 to 70%) and require long incubation up to 6 weeks (Christopher et al., 2010).

Mitka et al. (2007) stated that PCR is a very useful tool not only for the diagnosis of acute brucellosis, but also as a predictive marker for the course of the disease and the post treatment follow-up, which is valuable for the early detection of relapses.

In the present study, there was statistically insignificant difference as regards results of PCR in relation to SAT (P value>0.05) and this agrees with O'Leary et al. (2006) who suggested that there was no advantage in using PCR methods over standard serological and bacteriological methods in the detection of *B. melitensis* in bovine samples collected from whole blood or lymph nodes. However, molecular techniques have been shown to be more suitable, accurate, highly sensitive, rapid, and simple to require small sample volumes to reduce the risks of handling tissues and more specific for the diagnosis of brucellosis from whole blood samples or from sera (Bounaadja et al., 2009).

The PCR positivity in the present study increased significantly with increasing sero-positivity. This can to some extent explain the false positive serology results at titer 1/160 due to the endemicity of brucellosis in Egypt. This agrees with the recommendation mentioned in a sero-prevalence study done in Gharbia governorate, Egypt; to increase the sero-positivity level of SAT to 1/320 (El-Sherbini et al., 2007), in contrast to Hafez, who used a positive agglutination titer > 1/160 as seropositive for brucellosis (Hafez, 2003).

In the present study when considering culture as the gold standard test, the sensitivity of PCR was 100% and the accuracy was 62.5%. While when considering the SAT as the gold standard test, the sensitivity of PCR technique was 88% and accuracy was 70%.

In the present study, there was highly significant difference as regards, the use of PCR technique compared to using blood culture which showed 100% sensitivity (P value = 0.001) and this finding agrees with Mantur and Mangalgi (2004) who reported that PCR technique showed a sensitivity of 100%. This high sensitivity in culture-positive cases suggests that PCR may replace blood culture as the gold standard for acute brucellosis.

The results of the present study proves that culture method was very specific, but time consuming and less sensitive to detect all positive case. SAT was specific and highly sensitive, but there was false positive result which was not diagnosed by culture or PCR techniques.

In conclusion, PCR-based test using B4-B5 primer is faster and more sensitive than culture and serology for diagnosis of *Brucella* from peripheral blood in suspected cases.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Seroprevalence of hepatitis C virus in HIV seropositive children in Lagos, Nigeria

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Human immunodeficiency virus (HIV) may influence hepatitis C virus (HCV) disease through immunosuppression. The aim of this study was to determine the sero-prevalence of HCV in HIV sero-positive children in Lagos, Nigeria. 132 blood samples of children aged 1-15 years were collected at the HIV clinics of Lagos University Teaching Hospital (LUTH) and Lagos State University Teaching Hospital (LASUTH). Confirmatory analysis of HIV sero-status was done using enzyme-linked immunosorbent assay (ELISA) kit. All the 132 children recruited were HIV sero-positive. Serological assay for HCV was done on the 132 HIV sero-positive serum samples using ELISA technique. Assay procedures were carried out at the Virology Unit, College of Medicine, University of Lagos. Out of the 132 HIV sero-positive samples, 6 were positive for HCV with a prevalence of 4.54% with sex related prevalence of four (6.58%) males and two (2.82%) females, respectively. Zero prevalence was recorded between age groups 1-3 years while a sero-prevalence of 20% was found among age groups 12-15 years. The result of this study implies that HIV positive children are likely to be co-infected with HCV, thus re-affirming the role of this virus in concomitant HIV associated infection in children which may further complicate their immunocompromised state.

Key words: Human immunodeficiency virus (HIV), hepatitis C virus (HCV), enzyme-linked immunosorbent assay (ELISA), Lagos.

INTRODUCTION

Hepatitis is an inflammatory condition of the liver, while viral hepatitis is a conventional term used to denote hepatitis caused by the hepatotrophic viruses (hepatitis A-G). Hepatitis C virus (HCV), one of the agent of the disease in question, is a member of the *flaviviridae* family which has a single stranded RNA

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genome with a size of about 9.4 kb.

According to a review of age-specific sero-prevalence studies carried out between 1990 and 2005, about 184 million people worldwide have anti-HCV antibodies. Also, the prevalence of HCV in the West African Sub-region was 2.8 million with Nigeria contributing to the number of data points in the region (Mohd Hanafiah et al., 2013). Although, HCV infection has been reported in the general Nigerian population, limited studies have reported HCV and HIV co-infection in children.

HCV infection is spontaneously cleared within 6 months. HCV clearance has been suggested to occur in individuals who have overt symptoms of hepatitis, who have non-African descent, and lack HIV infection (Thomas et al., 2000). In about 60 to 85% of persons, spontaneous resolution does not occur, chronic HCV infection is a heterogeneous condition, with individual manifestations and rates of progression (Hadziyannis and Vassilopoulos, 2001).

Patients infected with HIV may be co-infected with HCV because of the similar transmission routes of both etiologic agents. It is estimated that one-third of people living with HIV are co-infected with HCV worldwide (Bruno et al., 2002). The prevalence of HIV/HCV co-infection is high, with 13 times greater risk in HIV patients (Balogun et al., 2010). Evidence that HIV influences HCV disease progression through immunosuppression has been observed. The advent of effective prophylactic drugs highly active anti-retroviral therapy (HAART) has reduced HIV/AIDS related mortality but end-staged liver disease has become a leading cause of death in HIV infected individuals who do not clear HCV infection (Bica et al., 2001).

Nigeria is known to be highly endemic for viral hepatitis. HIV/AIDS among the general population has assumed a prevalence of 4.1% in 2010 (Bashorun et al., 2014). Although, HIV may be a chronic manageable disease for many individuals, but end stage liver disease is an increasing serious concern for people co-infected with HIV and hepatitis. The knowledge of this growing concern initiates the study to determine the burden of HCV in HIV sero-positive children in Lagos, Nigeria because of the associated risk of transmissible HCV infection which may accompany the transmission of HIV.

MATERIALS AND METHODS

This study was a retrospective case control study carried out in two tertiary health facilities in Lagos, Nigeria which are Lagos State University Teaching Hospital (LASUTH) and Lagos University Teaching Hospital (LUTH). Ethical approval was obtained from both institutional ethical review boards. The study was carried out in the HIV/AIDS clinics of the LASUTH and LUTH from December 2007 to July 2008. The HIV/AIDS clinic is one of the President's Emergency Plan Funds for AIDS Relief (PEPFAR) sites, where anti-retroviral regimen are provided free. The attendees at the clinic receive general and specialist care as required. One hundred and thirty two consecutive patients between the age group of one to fifteen years that visited these hospitals for HIV testing and consented to the study were enrolled for monitoring and treatment when positive by rapid immunochromatography kit. Informed consent was obtained from parents/guardians and from children older than 10 years with the assistance of the patients' physicians to avoid stigmatization of enrolled participants.

A sample of 132 was recruited, having calculated the sample size to be 118 using the formula:

$$n = \frac{Z^2pq}{d^2}$$

where n = sample size, z = standard deviation (1.96), p = prevalence, q = 1-p and d = degrees of freedom (0.05), and assuming a prevalence of 8.4% based on the most recent anti-hepatitis C virus antibodies prevalence done in Lagos (Ayolabi et al., 2006).

Structured questionnaire was used to obtain patient's bio-data (age, gender and ethnic group), Occupation, educational status of parents or guardian and questions on possible risk factors for HCV transmission were also obtained. About 3 ml of blood was drawn from the subjects for HIV and HCV assay by a pediatrician. The blood was quickly transferred to a plain sterile bottle and centrifuged within 2 h of collection to obtain serum for serological assays.

HIV sero-status of consecutive participants who were screened and found reactive with HIV antibodies at LASUTH and LUTH were confirmed by a third generation enzyme-linked immunosorbent assay (DIA. PRO. Diagnostic Bioprobes Srl., Italy) for antibodies to both HIV1 and 2 at the Central Research Laboratory, College of Medicine of the University of Lagos. All the 132 children confirmed HIV positive were screened for antibodies to hepatitis C virus by a third generation enzyme-linked immunosorbent assay (DIA. PRO. Diagnostic Bioprobes Srl., Italy) for antibodies to both HIV1 and 2 at the Central Research Laboratory, College of Medicine of the University of Lagos. All assay protocols, cut-offs and interpretation were done according to the manufacturer's instructions.

All data were entered into a Microsoft Excel spreadsheet. Analysis was done using statistical package for social sciences (SPSS) version 13. Prevalence was recorded in a table, simple percentages and bar chart. Mean, median and Chi square analysis was also calculated. A one sided $P<0.05$ was considered statistically significant for Chi-square (used to determine the differences between the groups).

RESULTS

A total of 132 children aged 1-13 years were enrolled in this study. The serum samples of the participants who reacted positive to the rapid test kits used in the hospitals were confirmed sero-positive for HIV. Six out of one hundred and thirty two children (4.54%) had detectable antibodies to HCV. Sex related prevalence showed that 4 out of 61 males and 2 out of females 71 had anti-HCV antibodies as shown in Table 1.

HCV antibodies were detected in children greater than 3 years old. There was an increased detection of antibodies to HCV among age groups 8-15 years in whom 5 participants had detectable anti-HCV antibodies. None of the patients within age groups 1-3 years had antibodies to HCV as shown in Figure 1. The median age group of those who were anti-HCV antibody positive was 8-11 years.

Table 1. Sex related sero-prevalence of HCV antibodies in HIV infected children (1-15years).

Sex	Number screened	Positive antibodies	Percentage infected (%)
Male	61	4	6.58
Female	71	2	2.82

$X^2 = 0.372$ P >0.05.

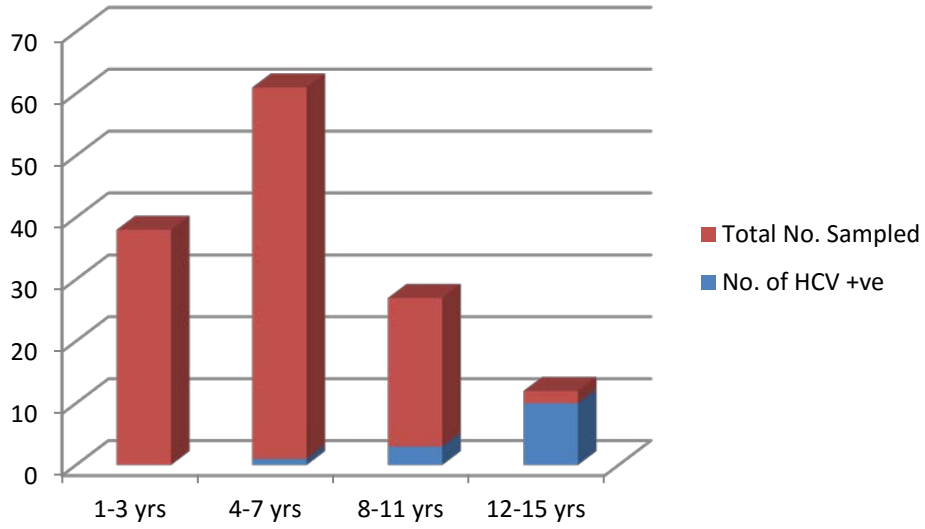

Figure 1. Age related sero-prevalence of HCV antibodies in HIV infected children (1-15 years).

Only blood transfusion was significantly associated with HCV infection in 3 patients (2 males and 1 female P<0.05); other risk factors for acquisition of the infections such as scarification, unsafe injections and surgery were found not to be related to HCV sero-status in this study.

Table 1 shows the females enrolled in this study were more than the males but antibodies were detected among the male participants than the females.

Comparison of anti-HCV antibodies in different age groups shows that only those from age 4-15 years had detectable antibodies while no antibody reaction was detected among age groups 1-3 years.

DISCUSSION

The HCV prevalence recorded in this study was in variance with a similar work conducted in Enugu, Nigeria which recorded a prevalence of 6.8% among adult population (Inyama et al., 2005) and higher than the prevalence of 1.6% recorded among children of age one to fifteen years in Karachi, Pakistan (Jafri et al., 2006). This work was similar to the work done by Sadoh et al. (2011) which reported that 5% of the children below 17 years tested positive for antibodies to hepatitis C virus in Benin City. HCV prevalence was estimated to be 2.1% in the general Nigerian population (Koroney and Sika, 2013). The improvements in health care practices (blood screening for HIV, HCV and other infections, use of disposable syringes) have reduced HCV sero-prevalence.

An increased detection of antibodies to HCV was documented among children in the age groups 8-15 years. This is in consonance with a similar study on HIV infected Tanzanian children (Telatela et al., 2007) which showed high frequency of positivity of HCV in children in the age group of 10–15 years as compared to children within the age groups below them, representing an increase in HCV positivity with increasing age.

Increased prevalence with age can be attributed to greater cumulative exposure to risk factors for HCV infection as a person gets older. The lower prevalence noted in children below 3 year age group found in this study is similar to the findings documented by Agbede et al. (2006) who recorded zero prevalence among pre-school age children in Ilorin. These may suggest a relatively low level of vertical transmission.

More males than females were positive for HCV in this study. The increased activity noticed in male children as they grow may be the reason for degree of transmission

experienced by this gender. Eze et al. (2014) in Enugu also observed this but adduced it to the preferential care of the male child which may have exposed them to more risks of acquiring HCV as they are given both orthodox and non-orthodox treatment when sick.

The significant risk factors for HCV positivity found among the studied cohort were blood transfusion. In a similar study at Jos (Inyama et al., 2005), blood transfusion was found as a significant risk factor. Other risk factors like scarification, circumcision and heterosexual activities have been documented as risk factors for HCV infection in different cohorts. These support the fact that the prevalence and risk factors for each region or age group depends on the predominant socio-cultural activity and life style found among them.

In conclusion, the increasing access to HAART means improved quality of life and increased life expectancy for HIV-infected children. Co-infection with HCV increases the chances of chronic liver disease which in turn reduces life expectancy most importantly when infection is acquired from childhood. More emphasis should be placed on screening of blood for transfusion for HCV, in addition to other blood transmissible diseases. Research should be conducted on larger cohort of patients of pediatric age, followed up over a period of time, to evaluate other possible risk factors and to study further the outcome of HCV/HIV co-infection.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

HCV, Hepatitis C virus; **HIV,** human immunodeficiency virus; **LUTH,** Lagos University Teaching Hospital; **LASUTH,** Lagos State University Teaching Hospital; **ELISA,** enzyme linked immunosorbent assay; **HCC,** hepatocellular carcinoma; **HAART,** highly active anti-retroviral therapy.

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Full Length Research Paper

Species identification of *Candida* isolates in various clinical specimens and their antifungal susceptibility patterns in Côte d'Ivoire

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The aims of the study were to isolate and identify the *Candida* species from clinical cases of candidiasis and to determine their anti-fungal susceptibily. Clinical samples such as vaginal swab, nails scrapings and squama were submitted to the Mycology Unit of Institut Pasteur of Côte d'Ivoire, from 2011 to September, 2015. Samples were screened for the growth of *Candida* species, which were then identified by chromogenic medium CandiSelect®4 and Auxacolor 2® (Biorad). Antifungal susceptibility was performed by ATB Fungus 3® of Biomérieux with the drugs Amphotericin-B, Flucytosine, Fluconazole, Itraconazole and Voriconazole. A total of 924 yeasts were isolated and *Candida albicans* was the common species isolated (40.3%), followed by *Candida glabrata* (29.2%) and *Candida tropicalis* (17.2%). Most of the isolates were collected from vaginal swabs 781 (84.5%) followed by squama 49 (5.67%). The results of the antifungal susceptibility test against vaginal *Candida* species indicated that 96.8 and 96.3% of the vaginal yeast isolated were susceptible to amphotericin B and voriconazole, respectively. The susceptibility rate of the yeasts was 67.7% to flucytosine, 91.8% to fluconazole, and 86.6% to itraconazole. A high proportion of resistance to Itarconazole was observed with all of the isolates tested. The study indicated that the vaginal *Candida* species were less susceptible to the flucytosine than to any azole drugs. In contrast, the susceptibility to flucytosine against *Candida* species isolated from other clinical samples was relatively good. The present study revealed that *C. albicans* was the most commonly found yeast from various clinical specimens; and reported that the fives antifungal drugs tested are still active against the *Candida* species.

Key words: *Candida* species, fluconazole, amphotericin B, itraconazole, flucytosine, voriconazole.

INTRODUCTION

The *Candida* species could infect a wide spectrum of human hosts, ranging from benign colonization of the

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skin and mucosal surfaces to invasion of the bloodstream with dissemination into the internal organs. *Candida* spp. infections are recognized as a major challenge in public health, commonly associated with high morbidity and mortality since its diagnosis and treatment present difficulties and high healthcare costs (Arnold et al., 2010).

The genus *Candida* includes several species involved in human pathology. *Candida albicans* is by far the most common species causing infections in humans. Increase in the prevalence of yeast infections caused by non-albican *Candida* such as *Candida glabrata, Candida krusei, Candida tropicalis and Candida parapsilosis* was reported in many parts of the world (Enwuru et al., 2008). The emergence of drug resistant yeasts, both genic and plasmid borne, reinforces the need to study further these pathogens and survey the susceptibility to drugs commonly used for therapy (Malar et al., 2012).

The azole drugs has been commonly used to treat many forms of candidal infections for a long time. However, their prolonged use selected drug resistance among *C. albicans* and other species. But resistance to the azole is more seen in non *C. albicans* spp. compared to *C. albicans* (Deorukhkar and Saini, 2013). Although several new antifungal drugs were licensed in recent years, antifungal drug resistance is becoming a major concern during treatment of such patients. The clinical consequences of the anti-fungal resistance can be seen either as treatment failure inpatients or as change in the prevalence of the Candida species causing infection. Antifungal resistance is particularly problematic since initial diagnosis of systemic fungal infection can be delayed and few antifungal drugs are available.

The treatments used against *Candida* infections vary substantially and are based on the anatomic location of the infection, the patients' underlying disease and immune status, the patients' risk factors for infection, the specific species of *Candida* responsible for infection, and, the susceptibility of the *Candida* species to specific antifungal drugs (Khan et al., 2015). So, it is of great importance to know the species of *Candida* responsible for the infection as well as its susceptibility patterns. In Cote d'Ivoire, few studies reported varying prevalence of vulvovaginal candidiasis (Konate et al., 2014; Djohan et al., 2012).

C. albicans is by far the most common species causing infections in humans (Djohan et al., 2012; Konate et al., 2014). Meanwhile, non-*albicans Candida* spp. were found to be emergent (Bonouman-Ira et al., 2011). There are few studies reporting yeast types identification and antifungal susceptibility using Fungus 3® with the drugs Amphotericin-B, Flucytosine, Fluconazole, Itraconazole and Voriconazole in Côte d'Ivoire. In addition, there is no National Surveillance Programme to monitor antifungal resistance among yeasts and other pathogenic fungi in Côte d'Ivoire.

The aim of this study was to determine the prevalence of yeasts types and their antifungal susceptibility patterns of yeast isolates obtained from patients attending the Mycology Laboratory of Institut Pasteur of Côte d'Ivoire.

MATERIALS AND METHODS

Study design

This study was a descriptive cross-sectional study conducted from January, 2011 to September, 2015 at the Mycology Laboratory of Institut Pasteur of Côte d'Ivoire. Patients from various hospitals of Abidjan were referred to the Mycology laboratory of Institut Pasteur of Côte d'Ivoire as a reference laboratory for mycological examination of various clinical samples. The informed consent was obtained from the patients. Samples of those who objected were not included in the study. Age, sex and clinical indication of mycology examination were noticed on the medical examination form delivered by the physician.

Samples such as nail scrapings, squama (from feet, back of hands, abdomen, legs, neck, arms), pus of ear and vaginal swab were collected under aseptic conditions in a sterile container and transported immediately to laboratory. Various clinical specimens were collected and processed as per the standard microbiological procedures. Vaginal swab samples were collected from female patients who complained about gynaecological problems such as vaginal discharge or vulvovaginitis and were referred to the laboratory for diagnostic purposes. Two vaginal swabs were taken by medical assistants one after the other by inserting a sterile vaginal speculum into the vagina, and then a sterile cotton wool swab was inserted into the posterior vaginal fornix and rotated gently before withdrawing. The swab was inserted back into the tube from which it was taken. The tube containing the swab was labelled with the patients study number, initials and date and then transported into the Stuart transport medium, to the laboratory.

Cultures of samples

The samples underwent a direct examination by wet mount preparation and Gram stain, inoculated on Sabouraud-Chloramphénicol (SC) and Sabouraud-Actidione-Chloramphénicol (SAC) media. The inoculated media were incubated at 37°C for 24 to 48 h; if no growth was observed, the incubation was extended up to 72 h. The isolates were considered significant if correlated with Gram staining, growth of two consecutive cultures, and the clinical presentation. The suspected *Candida* colonies were processed further for species identification.

Yeast identification

The reference procedures for identifying yeast species comprised germ tube production, micro-fermentation, microscopic morphology, chromogenic medium CandiSelect 4® (CS4; Bio-Rad) and Auxacolor 2® (Biorad) Tests.

Susceptibility testing

Anti-fungal susceptibility testing (Zhang et al., 2014) was done for 490 isolates of *Candida* by using ATB Fungus 3® of Biomérieux. This method enables to determine the susceptibility of the *Candida* isolates to the antifungal agents in a semi-solid medium following the conditions recommended by the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical and

Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 1997).

ATB Fungus 3® was performed following manufacturer's instructions. Briefly, ATB Fungus 3®of Biomérieux strip consists of 16 pairs of cupules including two growth control wells and five antifungal drugs at different concentrations: 5-Flucytosine (4, 16 µg/ml), Amphotericin B (0.5 to 16 µg/ml), Fluconazole (1 to 128 µg/ml), Itraconazole(0.125 to 4 µg/ml) and Voriconazole (0.06 to 8 µg/ml). The inoculated strips were used in duplicate (c and C) and were read visually after incubation at 37°C for 24 h. For each antifungal agent, the reading of the strips was started with the lowest concentration. The growth score was recorded for each of the wells and compared with the control wells as follows: No reduction in growth (4), slight reduction in growth (3), distinct reduction in growth (2), very weak growth (1) and no growth (0).

For Amphotericin B, the minimum inhibitory concentration (MIC) of the *Candida* species corresponded to its lowest concentration, thus enabling complete growth inhibition. For Fluconazole, Itraconazole and Voriconazole, as the possibility of a trailing growth existed, the MIC corresponded to the lowest concentration of the anti-fungal agent, with which a score of 2, 1 or 0 was obtained. For Flucytosine, a growth was looked for and was quantified in both the wells and tested for two concentrations. The results obtained gave an MIC that helps to classify the strain insensitive, intermediate or resistant. The anti-fungal breakpoints used followed the CLSI guidelines (National Committee for Clinical Laboratory Standards, 1997).

Ethical considerations

The study was conducted within the ethical standards and approved by the Comite National d'Ethique de la Recherche of Côte d'Ivoire. Informed consent was obtained by participant prior their inclusion in the study.

Statistical analysis

Drug susceptibility testing was analyzed using WHONET 5.6. Data were analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL). The prevalence of *Candida* species was reported in proportions. The relative frequencies of *Candida* susceptibility, determined by serology, were explored to look for differences between drugs (Chi-square test ($\chi 2$)). Fisher's exact test (at risk 5%) was used for comparison of proportions. Data analysis was conducted using SPSS version 11.5 (SPSS Inc, Chicago, Illinois).

RESULTS

Table 1 show the clinical specimen wise distribution of *Candida* species. Most of the isolates were obtained from vaginal swabs 781 (84.5%), followed by squama 49 (5.67%). Overall *C. albicans* counting was 40.3% (372/924) of the infections, followed by *C. glabrata* (29.2%) and *C. tropicalis* (12.7%). Among the 781 vaginal yeast isolates, *C. albicans* was the most common species and identified in 326 (41.7%) isolates, followed by *C. glabrata* in 253 (32.4%), *Candida tropicalis* in 96 (12.3%), and in 106 (13.6%), representing several species of *Candida*. The distribution of different species of vaginal *Candida* among the different age groups is shown in Table 2. The results obtained also showed that the 15 to 30 years group had the highest frequency of *Candida* species isolated (53.1%) followed by the 30 to 45 years group (39.4%). The age groups above 45 years (5%) and below 15 years (2.4%) had the lowest frequency of *Candida*. Statistical analysis of the data using one sample t- test indicates that there was significant difference in the prevalence among age groups (p-value = < 0.0001). The clinical patterns of vulvovaginitis *Candida* were dominated by leucorrhoea (35.7%) and pruritus (24.3%). The other functional signs were dyspareunia (17.3%), pelvic pain (12.9%) and dysuria (9.3%).

In the present study, antifungal susceptibility testing was performed for 490 *Candida* isolates. The results for the antifungal susceptibility test indicated that 96.8 and 96.3% of the vaginal yeast isolates were susceptible to amphotericin B and voriconazole with 3.2 and 2.9 resistant strains detected, respectively. Various resistant levels were detected against other antifungal drugs. Susceptibility levels to other drugs indicated that 67.7% of the yeasts were susceptible to flucytosine, 91.8% to fluconazole, and 86.6% to itraconazole. High proportion of resistance to Itarconazole were found with all of the *Candida* isolates tested. Intermediate category to flucytosine was shown by 58.8 and 30% of *C. glabrata* and *tropicalis* isolates, respectively. The details of the antifungal susceptibility test results are as shown in Table 3. The results of the antifungal susceptibility of the *Candida* yeast isolates from pus, squama and nails scraping against flucytosine, amphotericin B, fluconazole, itraconazole and voriconazole as determined by the ATB FUNGUS 3 test method are shown in Table 4.

In our study, itraconazole resistance was noted in 13 (11.8%) isolates and fluconazole resistance in 12 (10.9%) of them. Antifungal resistance was more common in *C. albicans* and *C. tropicalis* isolates.

DISCUSSION

Candidiasis is defined as infections caused by *Candida* species. It is considered as commensals in healthy individual and its capacity to produce superficial or systemic infections depends on the host immune system and various risk factors (Kauffman et al., 2011). In the present study, most of the yeast isolates were obtained from vaginal swabs. Clinical manifestations of vulvovaginitis are mainly featured by vaginal discharge and pruritus (Benchellal et al., 2011). Yeast infections of the vagina are common problems that cause significant morbidity and affect the well-being of women. Vaginal yeast prevalence among the patients is common as the organism easily colonizes mucous membranes (Moyes and Naglik, 2011) such as the vagina. In this current study, initial microscopy with wet film was used to assess

Table 1. Yeast species isolated from clinical sample tested.

Yeast isolate	Number (%) of yeast species isolated from sample type					Total	p-values
	VS	Squama	Nail scrapings	Pus	Others		
Candida albicans	326 (41.7)	20 (40.0)	13 (31.7)	3 (12.5)	10 (35.7)	372 (40.2)	<0.0001
Candida glabrata	253 (32.4)	6 (12.0)	5 (12.2)	0 (0)	6 (21.4)	270 (29.2)	<0.0001
Candida tropicalis	93 (12.0)	5 (10.0)	7 (17.1)	7 (29.2)	5 (17.8)	117 (12.7)	<0.0001
Candida parapsilosis	6 (0.8)	8 (16.0)	9 (22.0)	6 (25.0)	0 (0)	29 (3.1)	0.03
Candida krusei	14 (1.8)	2 (4.0)	1 (2.4)	1 (4.2)	1 (3.6)	19 (2.0)	<0.0001
Candida sp	75 (9.6)	3 (6.0)	4 (9.6)	7 (29.2)	3 (10.7)	92 (9.9)	<0.0001
Others species	14 (1.8)	6 (12.0)	2 (4.8)	0 (0)	3 (10.7)	25 (2.7)	<0.0001
Total	781 (100)	50 (100)	41 (100)	24 (100)	28 (100)	924 (100)	
p-values	<0.0001	<0.0001	0.002	0.002	0.003		

VS: vaginal swab, %: percentage isolate in a sample type, N= Number of species isolated.

Table 2. Distribution of different vaginal *Candida* species within different age groups.

Age	Yeast species							Total (%)	p-values
	C. albicans	*C. glabrata*	*C. tropicalis*	*C. parapsilosi*	*C. krusei*	*Candida sp*	Others		
<15	8	5	1	1	0	3	1	19 (2.4)	0.002
15-30	182	144	50	3	5	26	5	415 (53.1)	<0.0001
30-45	120	95	38	1	8	39	7	308 (39.4)	<0.0001
≥45	16	9	4	1	1	7	1	39 (5.0)	<0.0001
Total	326	253	93	6	14	75	6	781 (100)	-
p-values	<0.0001	<0.0001	<0.0001	0.44	0.001	<0.0001	0.01	-	-

whether there were just a few yeast cells, which may be considered a mere colonization. Also when large numbers of yeasts (more than 5 yeast cells per film) were seen with pseudohyphae then the diagnosis of yeast infection is established. The wet film ruled out *Trichomonas vaginalis* and Gram stain ruled out *Neisseria gonorhoeae* and *Gardnerella vaginalis* infections. In this work, *C. albicans* was the most frequently isolated species from vaginal swab followed by *C. glabrata.* However, it seems that non *albicans* species (*C. glabrata* and *C. tropicalis*) of *Candida* appear to be increasing (Salehei et al., 2012*;* Deorukhkar and Saini, 2013) as potential causes of vulvovaginal candidiasis . This increasing detection of non *albicans* species is probably related to the widespread and inappropriate use of antimycotic drugs used in the country.

In a study conducted by Konate et al. (2014), in 172 patients who suffered from vulvovaginal candidiasis, *C. albicans* was most frequently isolated (82.5%). It is worth noting that *C. albicans* is the most prevalent compared with other species, the reason probably being that it is a normal flora that takes advantage of risk factors such as pregnancy, antibiotic therapy, uncontrolled diabetes mellitus, immunosupression due to HIV and others (Kiguli et al., 2015 ; Gandhi et al., 2015).

C. albicans has a high pathogenic potential due to its high capacity to adhere to the vaginal mucosa as a result of the presence of *Candida* ligand vaginal cell inhibitors, thus allowing the manifestation of its virulence factors, germination and transformation from a saprophyte condition as blastospores, into a disease condition in a filamentous form (Anane et al., 2010). Highest frequency of *Candida* species isolated in 15 to 30 years age group could be a vulnerable group of candidal vulvovaginitis probably due to use of contraceptives and increased sexual activity. This results are supported by Abruquah (2012).

The *in vitro* susceptibility testing of antifungal agents is becoming increasingly important because of the introduction of new antifungal agents and the recovery of clinical isolates that exhibit inherent or developed resistance to Amphotericin B, Fluocytosine, and the azole group of drugs or Nystatin during chemotherapy. The sensitivity patterns of *Candida* isolates vary according to studies implemented in different countries (Adjapong et al., 2014; Feglo and Narkwa, 2012).

In this work, *in vitro* susceptibility of vaginal *Candida* species showed that the incidence of Fluconazole

Table 3. Antifungal susceptibility test results of yeast isolates from vaginal swab.

Yeast species isolated	No of isolates	5-FC			AMB			FCZ			VCZ			ITR		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
C. albicans	223	179 (80.3)	30 (14.5)	14 (6.3)	215 (96.4)	0 (00)	8 (3.6)	206 (92.4)	0 (00)	17 (7.6)	214 (96.0)	0 (00)	9 (4.0)	196 (87.9)	0 (00)	27 (12.1)
C. glabrata	97	38 (39.2)	57 (58.8)	2 (2.1)	95 (97.9)	0 (00)	2 (2.1)	88 (90.7)	1 (1.0)	8 (8.2)	94 (97.0)	2 (2.1)	1 (1.0)	82 (84.5)	2 (2.1)	13 (13.4)
C. tropicalis	60	40 (66.7)	18 (30.0)	2 (3.3)	58 (96.7)	0 (00)	2 (3.3)	55 (91.7)	1 (1.7)	4 (6.6)	58 (96.7)	1 (1.7)	1 (1.7)	51 (85.0)	1 (1.7)	8 (13.3)
Total (380)		257	105	18	368	0	12	349	2	29	366	3	11	329	3	48
%Susceptibility		67.7	27.7	4.7	96.8	0.0	3.2	91.8	0.05	7.7	96.3	0.8	2.9	86.6	0.8	12.6
p-values		<0.0001	<0.0001	0.22	0.77	-	0.77	0.88	0.2	0.93	0.9	0.11	0.27	0.66	0.11	0.93

S=number sensitive; I=number intermediate susceptible; R= number resistant. 5-FC=flucytocine; AMB=amphotericin B; FCZ=fluconazole; ITR=itraconazole; VCZ=voriconazol %: percentage.

Table 4. Antifungal susceptibility test results of yeast isolates from other clinical sample (Squama, Pus, Nails scrapings).

Yeast species isolated	No of isolates	5-FC			AMB			FCZ			VCZ			ITR		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
C. albicans	46	35 (76.1)	5 (10.9)	6 (13.0)	45 (97.8)	0 (00)	1 (2.2)	41 (89.1)	0 (00)	5 (10.9)	40 (87.0)	5 (10.9)	1 (2.2)	35 (76.1)	5 (10.9)	6 (13.0)
C. glabrata	17	13 (76.5)	4 (23.5)	0 (00)	17 (100)	0 (00)	0 (00)	11 (64.7)	0 (00)	6 (35.3)	15 (88.2)	0 (00)	2 (11.8)	14 (82.3)	0 (00)	3 (17.7)
C. tropicalis	24	24 (87.5)	2 (8.3)	1 (4.2)	23 (95.8)	0 (00)	1 (4.2)	23 (95.8)	0 (00)	1 (4.2)	19 (79.2)	1 (4.2)	4 (16.7)	20 (95.7)	0 (00)	4 (16.7)
C.parapsilosis	23	22 (95.7)	0 (00)	1 (4.3)	20 (87.0)	0 (00)	3 (13.0)	23 (100)	0 (00)	0 (00)	22 (95.7)	1 (4.3)	0 (00)	22 (95.7)	1 (4.3)	0 (00)
Total (110)		91	11	8	105	0	5	98	0	12	96	7	7	91	6	13
%Susceptibility		82.7	10	7.3	95.5	0.0	4.5	89.1	0.0	10.9	87.2	6.4	6.4	82.7	5.5	11.8
p-values		0.01	0.10	0.23	0.15	-	0.15	0.002	-	0.002	0.40	0.38	0.04	0.24	0.16	0.23

S=number sensitive; I=number intermediate susceptible; R= number resistant. 5-FC=flucytocine; AMB=amphotericin B; FCZ=fluconazole; ITR=itraconazole; VCZ=voriconazole %: percentage.

and Itraconazole resistance were higher than that of Flucytosine, Amphotericin B and Voriconazole. In addition, the decreased susceptibility to Fluconazole was most pronounced in *C. albicans* and *C. glabrata,* which was consistent with other studies (Diekema et al., 2012; Feglo and Narkwa, 2012). Fluconazole is one of the first-line antifungal drugs that are used in the treatment of infections due to *Candida* species other than *Candidakrusei* and some *Candida glabrata* isolates in Côte d'Ivoire.

In the country, Djohan et al. (2012) reported a *C.albicans* susceptibility of 100% to Amphotericin B; 98% to 5-Fluorocytosine; 86.7% to Voriconazole and 80% to fluconazole. Only 46.7% of *C. albicans* strains were sensitive to Itraconazole. Bonouman-Ira et al. (2011) found 65.6% of *C. glabrata* and 68.4% of *C. tropicalis* strains susceptible to Fluconazole. These differences in the country could be explained by antifungal susceptibility test, immunity of participants and different levels of exposure to antifungal drugs. Likewise, the Fluconazole susceptibility rate reported in the present study is high compared to those reported by Mondal et al. (2013) and Khan et al. (2015) who indicated 82 and 87.5% of respective susceptibility rate for Fluconazole.

Fluconazole (FCZ) is the first option for prophylaxis and treatment, due to its good

tolerance, few side effects and low costs (Li et al., 2014). However, the widespread and prolonged use of antifungal agents induces tolerance development as well as collateral resistance to other drugs (Prasad and Rawal, 2014). Continuous exposure to azoles appears to have a major impact in selecting fluconazole-resistant Candida species. The finding of high proportion of *C. glabrata* and *C. tropicalis* isolates in the intermediate category of the sensitivity testing could be considered alarming: it is an indication for a potential selection for flucytosine resistant strains. In Côte d'Ivoire, antifungal drugs are sold over the counter, a practice which encourages self-medication and therefore may contribute to the development and spread of antifungal resistance. Also, the absence of rapid, simple, and inexpensive diagnostic tests continues to result in over diagnosis of vulvovaginal candidiasis with the consequence to extensive and inappropriate use of the drug. Physicians usually give empirical therapy and vaginal cultures are not routinely obtained, and susceptibility testing is rarely performed. Unfortunately, we did not assess usage of antifungal therapy in our patients.

Study showed that the *Candida* species from vaginal were less susceptible to the flucytosine than the azole drugs, likely because of the difference of mechanism between the azole and flucytosine drugs. The mode of action of azoles is less altered by *Candida* species compared to the polyenes drugs. Meanwhile flucytosine was found to be more susceptible to Candida species isolated from pus, squama and Nails scraping. The antifungal susceptibility levels reported are low in many countries (Pfaller et al., 2011), but the differences in susceptibility levels in these countries support the idea that the susceptibility of yeasts to antifungal drugs needs to be monitored.

Our study has limitations. First, risk factors for vulvovaginitis such as pregnancy, antibiotic therapy, uncontrolled diabetes mellitus, immunosupression due to HIV and others were not assessed. Another limitation includes patients being restricted to those referred at the Mycology Unit of Institut Pasteur of Côte d'Ivoire. Therefore we cannot infer our data to the general population. Despite the limitations, the study provided baseline information on the identification of *Candida* species and their antifungal susceptibility in a reference laboratory in Côte d'Ivoire. The study buttresses the fact that most antifungals continue to be active against *Candida* strains from Côte d'Ivoire.

Conclusion

The present study showed that *C. albicans* was the most commonly isolated yeast from various clinical specimens. The results of antifungal susceptibility of drugs tested in this study support the use of these drugs when treating vulvovaginal candidiasis and others candida infections in Côte d'Ivoire. Meanwhile, a continuous monitoring is needed.

Conflict of Interests

The authors have not declared any conflict of interests.

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