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1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)  
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Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. *Afr. J. Biotechnol.* 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant *Staphylococcus aureus* in community-acquired skin infections. *Emerg. Infect. Dis.* 11: 928-930.

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.

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications.* McGraw-Hill Inc., New York, pp. 591-603.

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## Review

## ***Enterococcus faecalis*, a nightmare to endodontist: A systematic review**

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The main goal in endodontics is the prevention and treatment of diseases of the dental pulp and periapical tissues and it can be best achieved if preventive measures and treatment procedures are based on a thorough and detailed understanding of the etiology and pathogenesis of endodontic diseases. There are some cases in which the treatment has followed the highest technical standards and yet failure results. Scientific evidence indicates microbial factors which play an important role. In most cases, failure of endodontic treatment is a result of microorganisms persisting in the apical portion of the root canal system, even in well-treated teeth. *Enterococcus faecalis* is recognized as a pathogen in post-treatment endodontic infections and probably the species that can best adapt to and tolerate the ecologically demanding conditions in the filled root canal. Enterococci are also implicated in infections of the root canal system, however, they make up a small proportion of the initial flora which is dominated by Gram negative species. In contrast, it has been reported that enterococci are frequently isolated from obturated root canals of teeth that exhibit chronic periapical pathology. Eradication of *E. faecalis* from the root canal remains a challenge, while chlorhexidine and combinations of disinfectants show some promise. A better understanding of the role of the virulence factors of *E. faecalis* in endodontic infections may help in the development of new strategies to prevent or eliminate the infection by this species, thereby improving treatment results in endodontics.

**Key words:** *Enterococcus faecalis*, endodontics, periapical tissues, canal, infection.

### INTRODUCTION

The main goal in endodontics is the prevention and treatment of diseases of the dental pulp and periapical tissues. This objective can be best achieved if preventive measures and treatment procedures are based on a thorough and detailed understanding of the etiology and pathogenesis of endodontic diseases (Markus et al., 2003). Root canal treatment usually fails when treatment fall short

of acceptable standards (Seltzer et al., 1963). Undoubtedly, the major factors associated with endodontic failure are the persistence of microbial infection in the root canal system and the periradicular area (Nair et al., 1990). There are some cases in which the treatment has followed the highest technical standards and yet failure results. Scientific evidence indicates that some factors may be associated

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with the unsatisfactory outcome of well-treated cases. They include microbial factors, comprising extraradicular and intraradicular infections (Sjogren et al., 1996; Sundqvist et al., 1998; Lopes et al., 1999). In most cases, failure of endodontic treatment is a result of microorganisms persisting in the apical portion of the root canal system, even in well-treated teeth. To survive in the root-filled canal, microorganisms must withstand intracanal disinfecting measures and adapt to an environment in which there are few available nutrients. Therefore, the few microbial species that have such ability may be involved in the failure of root canal treatment. The ability to survive in such conditions is important for most bacteria because periods of starvation are commonly experienced. Several regulatory systems play an essential role in the ability of bacteria to withstand nutrient depletion. These systems are under the control of determined genes, whose transcription is activated under conditions of starvation. The microbiota associated with failed cases differs from that reported in untreated teeth (primary root canal infection). Whereas the latter is typically a mixed infection, in which Gram negative anaerobic rods are dominant, the former is usually composed of one or a few bacterial species, generally Gram positive bacteria, with no apparent predominance of facultatives or anaerobes.

Moller (1966), after examining failed cases, reported that *Enterococcus faecalis* was found in 29% of the cases. Sundqvist et al. (1998) observed a mean of 1.3 bacterial species per canal and 42% of the recovered strains were anaerobic bacteria. *E. faecalis* was detected in 38% of the infected root canals.

For many years, *Enterococcus* species were believed to be harmless to humans and considered unimportant medically. Because they produce bacteriocins, *Enterococcus* species have been used widely over the last decade in the food industry as probiotics or as starter cultures (Foulquie et al., 2006). Until the mid-1980s, enterococci were not allocated to a separate genus, even though their unique characteristics were recognized among streptococci. The basic observations on staining, cell shape and arrangement as well as lack of catalase placed enterococci in the genus *Streptococcus*. Enterococci are normal human commensals adapted to the nutrient-rich, oxygen-depleted, ecologically complex environments of the oral cavity, gastrointestinal tract, and vagina (Jett et al., 1994).

Enterococci frequently cause a wide variety of infections in humans and commonly infect the urinary tract (Felmingham et al., 1992), bloodstream, abdomen (Graninger and Ragette, 1992), endocardium (Eliopoulos, 1992), biliary tract (Khardori et al., 1991), burn wound, and *in situ* foreign devices. The source of the enterococci found in the root canal system is thus still unclear, but evidence shows an exogenous origin. Recently, enterococci have become one of the most common nosocomial pathogens, with patients having a high mortality rate of up

to 61% (De Fatima et al., 2005). *E. faecalis* is responsible for 80 - 90% of human enterococcal infections.

In the past few years, *E. faecalis* has been the focus of increased interest both in medicine and dentistry. A recognized pathogen in post-treatment endodontic infections, *E. faecalis* is frequently isolated both in mixed flora and in monocultures. *E. faecalis* is probably the species that can best adapt to and tolerate the ecologically demanding conditions in the filled root canal. Enterococci are also implicated in infections of the root canal system, however, they make up a small proportion of the initial flora which is dominated by Gram-negative species. In contrast, it has been reported that enterococci are frequently isolated from obturated root canals of teeth that exhibit chronic periapical pathology (Molander et al., 1998)

This dramatic increase in resistance of *Enterococcus* species worldwide highlights the need for a greater understanding of this genus, including its ecology, epidemiology and virulence.

## ENTEROCOCCI CHARACTERISTICS

In 1930's Lancefield serologically classified Enterococci as group D Streptococci. In 1937, Sherman proposed a classification scheme, in which he recommended that the term 'Enterococcus' should be used specifically for streptococci that grow at both 10 and 45°C, at pH 9.6 and in 6.5% NaCl, survive at 60°C for 30 min and have ability to split esculin. In 1980s, based on genetic differences, enterococci were removed from the genus *Streptococcus* and placed in their own genus, *Enterococcus*. The genus *Enterococcus* consists of Gram positive, catalase negative, non-spore-forming, facultative anaerobic bacteria that can occur both as single cocci and in chains. Enterococci belong to a group of organisms known as lactic acid bacteria (LAB) that produce bacteriocins (Health Protection Agency, 2005). Endospores are not formed and some species can be motile by scanty flagella. They form creamy whitish colonies. Most enterococci are facultative anaerobes, but some species are strict aerobes. Enterococci do not normally reduce nitrate and do not digest pectin or cellulose. They are ubiquitous and potentially pathogenic species that are able to acquire an increased resistance or phenotypic tolerance to many disinfectants or physical agents. *E. faecalis* possess a group D carbohydrate cell wall antigen (Lancefield antigen), which is an intracellular glycerol teichoic acid associated with the cytoplasmic membrane. The cell wall contains a large amount of peptidoglycan and teichoic acid. The peptidoglycan (cross-linked peptide sugar), which is found in most of the bacterial cell walls, helps to maintain the microbe's shape and has a polysaccharide backbone of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acids (MurNAc). The chemical and

structural analyses of the capsular polysaccharides have shown glycerol teichoic acid-like molecules with a carbohydrate backbone structure and sialic acid. These polysaccharides are cross-linked with peptide bridges and contribute to the three-dimensional structure of peptidoglycan. Because of the location of the peptidoglycan on the outside of the cytoplasmic membrane and its specificity, the transglycosylation step has been indicated as a potential target for antibacterial medicaments.

They catabolize a variety of energy sources including carbohydrates, glycerol, lactate, malate, citrate, arginine, agmatine, and many alpha keto acids. *Enterococcus* species live in vast quantities [10<sup>5</sup> -10<sup>8</sup> colony-forming units (cfu) per gram of feces] in the human intestinal lumen and under most circumstances cause no harm to their hosts. Enterococci can survive very harsh environments including extreme alkaline pH and salt concentrations. They resist bile salts, detergents, heavy metals, ethanol, azide and desiccation. They can grow in the range of 10 to 45°C and survive a temperature of 60°C for 30 min (Flahaut et al., 1996).

## VIRULENCE FACTORS

### Aggregation substance (AS)

Aggregation substance (AS) is a pheromone-responsive, plasmid- encoded bacterial adhesin that mediates efficient contact between donor and recipient bacterium, facilitating plasmid exchange AS is expressed by the donor cell, the bacterial conjugation process requires that 'binding substance' (BS, the chromosomally encoded cognate ligand for AS) be expressed on the surface of the recipient cell. AS was also found to mediate binding to extracellular matrix (ECM) proteins, including collagen type I. Binding to collagen type I by bacteria may be of particular importance with respect to endodontic infections, since this is the main organic component of the dentin (Linde A et al., 1993) .AS has been reported to promote direct, opsonin-independent binding of *E. faecalis* to human neutrophils via a complement receptor-mediated mechanism (Vanek et al., 1999). As a consequence of this special type of binding, *E. faecalis*-bearing AS was shown to be resistant to killing by human neutrophils, despite marked phagocytosis and neutrophil activation (Rakita et al 1999). phagosomal oxidant production by the neutrophils may be a possible contribution to tissue damage in case of infection with cells of *E. faecalis* expressing AS. Cell extracts of AS- and BS-positive *E. faecalis* were found to induce T-cell proliferation, with subsequent release of tumor necrosis factor beta and gamma interferon, and to activate macrophages to release tumor necrosis factor alpha. It also stimulates the production of the cytotoxic agent nitric oxide (NO) by a variety of cells, including macrophages and neutrophils,

and may cause undesirable cell and tissue damage.

In a recent study of characterization of virulence factors and clonal diversity of *E. faecalis* isolates from treated dental root canals by phenotyping and Western blotting test, 45% had genes for AS (Archimbaud et al., 2002).

### Surface adhesins (SA)

Enterococcal gene Esp., encoding the high molecular weight surface protein Esp., has been detected in abundance among bacterimia and endocarditis isolates. Esp. is associated with promotion of primary attachment and biofilm formation of *E. faecalis* on abiotic surfaces (Toledo-Arana et al., 2001). In a recent study 90% of virulence genes were efaA and ace genes detected by PCR from treated root canals of teeth. The disruption of the ace gene impaired the conditional binding of *E. faecalis* to the extracellularmatrix proteins (Nallapreddy et al., 2000).

### Sex pheromones

Production of the sex pheromones by strains of *E. faecalis* and its bacterial clumping inducing effect was first described by (Dunny et al., 1978). It was subsequently shown that antibiotic resistance and other virulence traits, like cytolysin production can be passed in strains of *E. faecalis* by sex pheromone system (Clewell and Weaver, 1989). Some of *E. faecalis* sex pheromones were found to be chemotactic for human neutrophils (Sannomiya et al., 1990).

### Gelatinase

*E. faecalis* possesses gelatinase (Hubble et al., 2003) which help it bind to dentin and gelatinase levels were elevated in oral rinses, crevicular fluid and whole saliva samples (Makela et al., 1994) and in gingival biopsy specimens (Soell et al., 2002), from periodontitis patients compared with healthy subjects. High gelatinase production has also been seen in epidemiologic studies with human clinical isolates (Kanemitsu et al., 2001).

### Cytolysin

*E. faecalis* possess cytolysin or hemolysin as a virulence factor. Conflicting studies suggest the role of cytolysin as a possible virulence factor. Initial studies reported that approximately 60% of *E. faecalis* isolates derived from fecal specimens from healthy individuals. However recent studies show that the role of cytolysin as a virulence factor is small or negligible (Coque et al., 1995; Elsner et al., 2000).

**Table 1.** Studies investigating the prevalence of *E. faecalis* in root-filled teeth with an apical periodontitis.

Reference	Number of root filled teeth in study	Number of root filled teeth with bacterial growth	Prevalence of <i>E. faecalis</i> (%)	Method of detection
Engström (1964)	54	21	24	Culture
Möller (1966)	264	120	28	Culture
Molander et al. (1998)	100	68	47	Culture
Sundqvist et al. (1998)	54	24	38	Culture
Peciuliene et al. (2000)	35	20	70	Culture
Peciuliene et al. (2001)	40	33	64	Culture
Hancock et al. (2001)	54	33	33	Culture
Pinheiro et al. (2003)	60	51	53	Culture
Pinheiro et al. (2003)	30	24	46	Culture
Siqueira and Rocas (2004)	22	22	77	PCR
Gomes et al. (2004)	19	19	32	Culture
Rocas et al. (2004)	30	30	67	PCR
Asharf et al. (2005)	37	8	22	PCR
Chiara et al. (2007)	23	9	39.1	PCR
Xiaofei et al. (2010)	32	13	40.6	API20 Strep kits and 16S rRNA sequencing
Isabela et al. (2012)	29	11	38	Quantitative real time PCR

## ***E. FAECALIS* AND APICAL PERIODONTITIS**

*E. faecalis* is associated with different forms of periradicular disease including primary endodontic infections and persistent infections. In the category of primary endodontic infections, *E. faecalis* is associated with asymptomatic chronic periradicular lesions significantly more often than with acute periradicular periodontitis or acute periradicular abscesses. *E. faecalis* is found in 4 to 40% of primary endodontic infections. The frequency of *E. faecalis* found in persistent periradicular lesions has been shown to be much higher. In fact, failed root canal treatment cases are nine times more likely to contain *E. faecalis* than primary endodontic infections (Rocas et al., 2004). Studies investigating its occurrence in root-filled teeth with periradicular lesions have demonstrated a prevalence ranging from 24 to 77% (Table 1)

## **RESISTANCE OFFERED BY *E. FAECALIS***

Enterococci can withstand harsh environmental conditions. As originally defined by Sherman (1937), enterococci can grow at 10 and 45°C, at pH 9.6, in 6.5% NaCl broth, and survive at 60°C for 30 min. *E. faecalis* can adapt to adverse conditions: Following pre-exposure to sublethal stress conditions, *E. faecalis* becomes less sensitive to normally lethal levels of sodium dodecyl sulfate, bile salts, hyperosmolarity, heat, ethanol, hydrogen peroxide, acidity, and alkalinity. Furthermore, 'cross-protection' is pronounced against diverse challenges. Starving *E. faecalis*

cells maintain their viability for extended periods and become resistant to UV irradiation, heat, sodium hypochlorite, hydrogen peroxide, ethanol, and acid (Giard et al., 1996; Hartke et al., 1998). Moreover, *E. faecalis* can enter the viable but non-cultivable (VBNC) state, a survival mechanism adopted by a group of bacteria when exposed to environmental stress, and resuscitate upon returning to favorable conditions (Lleò et al., 2001). The ability of *E. faecalis* to tolerate or adapt to harsh environmental conditions may act as an advantage over other species. It may explain its survival in root canal infections, where nutrients are scarce and there are limited means of escape from root canal medicaments.

*E. faecalis* can adhere to root canal walls, accumulate, and form communities organized in biofilm, which helps it resist destruction by enabling the bacteria to become 1,000 times more resistant to phagocytosis, antibodies, and antimicrobials than non- bio film-producing organisms (Stuart et al., 2006). Upon contamination of the root canal with the bacterium, it can colonize the dentinal walls, adhering to the mineral part, probably through lipoteichoic acids (LTA), and to the collagen through Aggregation substance (AS) and other surface adhesins. These surface adhesins 'Ace', which is expressed by the bacterium under disease conditions and particularly under stress (Rich et al., 1999). Bacteria face a variety of stressful conditions in the root canal, such as nutrient deficiency, toxins of other bacteria, and endodontic medicaments. These conditions may modulate the adhesion expression of the bacterium. In addition, leakage of serum into the root canal can induce the expression of aggregation

substance (AS) and other carbohydrate moieties, thereby increasing the adhesiveness of the bacterium. Adhesion to dentin and penetration along dentinal tubules by *E. faecalis* may serve as a means of protection from endodontic medicaments.

Another mechanism by which *E. faecalis* survives may be through Lipoteichoic acids (LTA), which has been associated with resistance of the bacterium against a variety of lethal conditions. Since *E. faecalis* suppresses the growth of other bacteria with its cytolysin, AS-48 (Aggregation substance), and other bacteriocins, among the target cells of cytolysin are the erythrocytes, PMNs and macrophages, and a broad range of Gram-positive, but not Gram-negative organisms (Jackson RW (1971). It has been hypothesized that if the bacteriocin effect of cytolysin of *E. faecalis* favors colonization of the Gram-negatives, there could be a shift to a bacterial flora usually associated with periodontal disease (Jett and Gilmore, 1990)

The root canal is hardly a nutrient-rich medium, but *E. faecalis* may derive the energy it needs from the hyaluronan present in the dentin through degradation by hyaluronidase. *E. faecalis* may also feed on serum components present in the fluid in the dentinal tubules. Moreover, an inadequate apical seal of root fillings may allow serum to flow into the root canal. Therefore, it seems that, even in a well-debrided and coronally well-sealed root canal, remaining or arriving cells of *E. faecalis* may still grow and utilize local sources of energy and nutrients. Production of extracellular superoxide and release of the lytic enzymes gelatinase and hyaluronidase and the toxin cytolysin by *E. faecalis* can cause direct damage in the dentinal as well as in the periapical tissues. In contrast, *E. faecalis* can also induce host-mediated tissue damage in the periradicular tissues. Since cells of *E. faecalis* in the dentinal tubules cannot be reached and eliminated by the cells of the host defense system, they may elicit a permanent provocative effect on these cells. PMN leukocytes, lymphocytes, monocytes, and macrophages are stimulated by a group of virulence factors of *E. faecalis*, which will contribute to the periradicular damage.

### **E. FAECALIS AND BIOFILM FORMATION**

Many microorganisms are able to form surface-attached microbial communities, known as biofilms. An immature biofilm of *E. faecalis* 12 h on cellulose filters showed variation in the number of viable cells eluted from the biofilm, whereas a pseudo-steady state was developed and maintained from 12 to 96 h. During this time period, the number of cells attached to the biofilm and those shed to the perfusates was constant (Foley and Gilbert, 2001). In contrast Lima et al. (2001) found that 3-day-old biofilms lost more cells than the number of adhering cells. Anaerobic conditions or the presence of 5% CO<sub>2</sub> did not have an effect on the adhesion of enterococci to the

microtiter polystyrene plates, with the exception of *Enterococcus hirae*. However, the presence of carbohydrates in the medium would strongly increase the biofilm formation of *E. faecalis*. *E. faecalis* had a greater ability to adhere to the microtiter polystyrene plates and form a biofilm than *E. faecium* (Baldassarri et al., 2001). With maturation, biofilms on cellulose filters showed a decreased susceptibility to antibiotics and a reduced growth rate than planktonic cultures. Further, biofilms were resistant to vancomycin in a concentration of 4x minimum inhibitory concentration (MIC).

Lima et al. (2001) tested the effect of different chlorhexidine- or antibiotic-containing medicaments on 1 or 3 day biofilms on cellulose nitrate membrane filters of *E. faecalis*. In the presence of clindamycin or clindamycin combined with metronidazole, the number of cells was reduced in the 1 day biofilm. Furthermore, chlorhexidine-containing medicaments were able to reduce strongly the number of bacterial cells of *E. faecalis* in the 1 and 3 day biofilm. Spratt et al. (2001) showed that 2.25% NaOCl was the most effective medicament on a 2 day old *E. faecalis* biofilm, whereas 10% povidone iodine (Betadine) required 60 min to eliminate 100% of the cells, and in the presence of 0.2% chlorhexidine gluconate most of the cells survived after 60 min.

Quorum sensing occurs when a bacterial population produces a signal via an auto inducing peptide (AIP), regulated by a two-component system. AIP then accumulates in the environment by increased expression of the communication signal, or by increased numbers of cells producing the signal. Once the AIP reaches a threshold concentration, it interacts with a cell-surface receptor or reenters the cell and causes a cascade of transcriptional regulation (Alksne and Projan, 2000).

### **ADHESION OF E. FAECALIS TO DENTINAL TUBULES**

Although the mechanism of bacterial invasion is not completely understood, bacterial adhesion to dentinal tubule walls (TWs) is a logical early step in the process. Adherence is considered to be the first step for bacterial colonization of host tissue, including tubule invasion, and is mediated by bacterial specific cell-surface components (adhesins) (Patti et al., 1994). Collagen is widely considered to be the primary substrate for specific binding of *E. faecalis* to dentine, and the collagen binding protein of *E. faecalis* (Ace) and a serine protease (Spr) have been proposed to play significant roles in binding to the root canal wall (Nallapareddy et al., 2000; Hubble et al., 2003). Ace also promotes *E. faecalis* binding to collagen type I and in vitro ace gene expression at 37C was enhanced in the presence of collagen (Nallapareddy and Murray, 2006). The ability of *E. faecalis* to grow as chains has been suggested as another explanation for the moderate to high extent of tubule invasion. After initial attachment to

the poorly non mineralized pre-dentine at the tubule orifices, deeper penetration may not require specific binding as invasion may result from intratubular cell growth (Love and Jenkinson, 2002).

### **STEPS THAT CAN BE CONSIDERED FOR THE ELIMINATION AND PREVENTION OF *E. FAECALIS***

Treatment regimens should aim at prevention and elimination of *E. faecalis* during treatment in between appointments and after completion of treatment. We can prevent its re-entry by following certain norms. That includes, ensuring that the patient rinses with 0.2% chlorhexidine prior to the treatment, disinfecting the tooth and rubber dam with chlorhexidine or sodium hypochlorite and disinfecting gutta-percha points with sodium hypochlorite before insertion in the canal. A study by Sukawat et al. (2002) suggested a combination of calcium hydroxide mixed with camphorated paramonochlorophenol completely eliminated *E. faecalis* within dentinal tubules. Mickel et al. (2003) showed the addition of stannous fluoride to calcium hydroxide is also more effective than calcium hydroxide by itself. Gomes et al. (2003) concluded that Two percent chlorhexidine gel combined with calcium hydroxide achieves a pH of 12.8 and can completely eliminate *E. faecalis* within dentinal tubules. Shur et al. (2003) chlorhexidine-impregnated and iodoform-containing gutta-percha points have shown little inhibitory action against *E. faecalis*. In Nagayoshi et al. (2004), ozonated water has been shown to have the same antimicrobial efficacy as 2.5% sodium Hypochlorite. In Mickel et al. (2003), the antimicrobial activity against *E. faecalis* of various sealers has also been studied. Roth 811 (Roth International Ltd., Chicago, IL), a zinc-oxide eugenol based sealer, has been shown to exhibit the greatest antimicrobial activity against *E. faecalis* as compared to other sealers. Saleh et al. (2004) concluded that AH Plus and Grossman's sealer are effective in killing *E. faecalis* within infected dentinal tubules.

So, the following treatment protocol can be followed to eliminate *E. faecalis* from root canal: Adequate apical preparation; use of canal irrigants such as (6% sodium hypochlorite, 17% EDTA and 2% chlorhexidine); use of intracanal medicaments such as (2% chlorhexidine gel or 2% chlorhexidine gel + calcium hydroxide); Considering AH plus or Grossman's sealer and Proper coronal seal are given.

### **CALCIUM HYDROXIDE, AN INACTIVE INTRACANAL MEDICAMENT AGAINST *E. FAECALIS***

Calcium hydroxide, a commonly used intracanal medicament, has been shown to be ineffective in killing *E. faecalis* on its own, especially when a high pH is not maintained (Lin et al., 2003; Tronstad et al., 1981). The

following reasons have been proposed to explain why *E. faecalis* is able to survive intracanal treatment with calcium hydroxide: (a) *E. faecalis* passively maintains pH homeostasis. This occurs as a result of ions penetrating the cell membrane as well as the cytoplasm's buffering capacity. (b) *E. faecalis* has a proton pump that provides an additional means of maintaining pH homeostasis. This is accomplished by "pumping" protons into the cell to lower the internal pH. (c) At a pH of 11.5 or greater, *E. faecalis* is unable to survive (McHugh et al., 2004). However, as a result of the buffering capacity of dentin, it is very unlikely that a pH of 11.5 can be maintained in the dentinal tubules with current calcium hydroxide utilization techniques. Studies using the dentin powder model have shown that the presence of dentin has an inhibitory effect on various concentrations of root canal medicaments including calcium hydroxide, sodium hypochlorite, chlorhexidine and iodine potassium iodide. Diverse components of dentin including dentin matrix, type-I collagen, hydroxyapatite and serum are responsible for altering the antibacterial effects of these medicaments (Portenier et al., 2001).

### **NEWER STRATEGIES OF TREATMENT AGAINST *E. FAECALIS***

#### **MTAD. Bio Pure MTAD**

MTAD. Bio Pure MTAD (Dentsply, Tulsa, OK) is a mixture of a tetracycline isomer, an acetic acid, and Tween 80 detergent (MTAD) was designed to be used as a final root canal rinse before obturation (Torabinejad et al., 2003). MTAD mixture is effective against *E. faecalis*, and it is also less cytotoxic than a range of endodontic medicaments, including eugenol, hydrogen peroxide (3%), EDTA, and calcium hydroxide paste. Newberry et al. (2007) determined the antimicrobial effect of MTAD as a final irrigant on eight strains of *E. faecalis*. After irrigating with 1.3% NaOCl, the root canal and the external surfaces were exposed to MTAD for 5 min. Roots or dentin shavings were cultured to determine the growth of *E. faecalis*. The results showed that this treatment regimen was effective in completely eliminating growth in seven or eight strains of *E. faecalis* (Zhang et al., 2003).

#### **Tetraclean**

Tetraclean (Ogna Laboratori Farmaceutici, Muggio (Mi), Italy), like MTAD, is a mixture of an antibiotic, an acid and a detergent. However, the concentration of the antibiotic, doxycycline (50 mg/mL), and the type of detergent (polypropylene glycol) differ from those of MTAD (Giardino et al., 2006). In another study, they compared the antimicrobial efficacy of 5.25% NaOCl, MTAD and Tetraclean against an *E. faecalis* biofilm generated on cellulose nitrate membrane filters. Only the NaOCl could

disaggregate and remove the biofilm at every time interval tested although treatment with Tetraclean caused a high degree of biofilm disaggregation at each time interval when compared with MTAD (Giardino et al., 2007).

### QMIX

QMIX is a novel endodontic irrigant for smear layer removal with added antimicrobial agents. It contains EDTA, CHX and a detergent. QMIX is a clear solution, ready to use with no chair-side mixing. Mixing EDTA and CHX is known to produce a white precipitate. In QMIX, this is avoided because of its chemical design. Another recent concern in endodontic irrigation is a potentially carcinogenic precipitate between sodium hypochlorite and CHX. A recent study by Ma et al. (2011) showed QMIX to be as effective as 6% sodium hypochlorite against *E. faecalis* in dentinal tubules.

### Electrochemically activated solutions

Electrochemically activated (ECA) solutions are produced from tap water and low-concentrated salt solutions. The ECA technology represents a new scientific paradigm developed by Russian scientists at the All-Russian Institute for Medical Engineering (Moscow, Russia, CIS). Principle of ECA is transferring liquids into a metastable state via an electrochemical unipolar (anode or cathode) action through the use of an element/reactor ("Flow-through Electrolytic Module" or FEM). ECA is showing promising results due to ease of removal of debris and smear layer, nontoxic and efficient in apical one third of canal. It has a potential to be an efficient root canal irrigant (Solovyeva and Dummer, 2000).

### Ozone

Ozone is a very powerful bactericide that can kill microorganisms effectively. It is an unstable gas, capable of oxidizing any biological entity. It was reported that ozone at low concentration, 0.1 ppm, is sufficient to inactivate bacterial cells including their spores (Broadwater et al., 1973).

### Photon-activated disinfection

The use of photodynamic therapy (PDT) for the inactivation of microorganisms was first shown by Oscar Raab who reported the lethal effect of acridine hydrochloride on *Paramecia caudatum* (Raab, 1900). PDT is based on the concept that nontoxic photosensitizers can be preferentially localized in certain tissues and subsequently activated by light of the appropriate wavelength to generate singlet

oxygen and free radicals that are cytotoxic to cells of the target tissue (Dougherty et al., 1998). Methylene blue (MB) is a well-established photosensitizer that has been used in PDT for targeting various gram-positive and gram-negative oral bacteria and was previously used to study the effect of PDT on endodontic disinfection (Harris et al., 2005). Several studies have shown incomplete destruction of oral biofilms using MB-mediated PDT due to reduced penetration of the photosensitizer. Soukos et al. (2003) used the combined effect of MB and red light (665 nm) exhibited up to 97% reduction of bacterial viability. Pagonis et al. (2010) studied the in vitro effects of poly (lacticoglycolic acid) (PLGA) nanoparticles loaded with the photosensitizer methylene blue (MB) and light against *E. faecalis* (ATCC, 29212). The study showed that utilization of PLGA nanoparticles encapsulated with photoactive drugs may be a promising adjunct in antimicrobial endodontic treatment.

### HERBAL IRRIGANTS AGAINST *E. FAECALIS*

A wide variety of synthetic antimicrobial agents have been used over the years as endodontic irrigants against *E. faecalis*. Because of the increased antibiotic resistance to these antimicrobial agents, toxic and harmful side effects of few common antibacterial agents, there is a need for alternative agents which are affordable, nontoxic and effective. It has been found that natural plant extracts could be used as effective endodontic irrigants (Sharad et al. (n.d.). The major advantages of herbal irrigants are safety, easy availability, increased shelf life, cost effectiveness and lack of microbial resistance so far. The *in vitro* studies conducted so far have shown that herbs can have a promising role as root canal irrigants.

### Propolis

This is prepared from resins collected by bees from trees of poplars and conifers or from flowers of genera *clusia*. It also contains viscidone. It is used as intracanal medicaments, root canal irrigant. In a study conducted by Al-Qathami and Al-Madi (2003), the antimicrobial efficacy of propolis, sodium hypochlorite and saline as endodontic irrigants was compared and it was found that propolis showed antimicrobial activity equal to that of sodium hypochlorite. Another study by Sudha and Deepak (2012) confirmed the antibacterial action of propolis against *E. faecalis*.

### *Morinda citrifolia* (noni)

*M. citrifolia* also known as Indian mulberry has a wide range of uses due to its biocompatibility, and antibacterial, anti-inflammatory, anti-viral, anti-oxidant and

analgesic effects. It is one of the first herbal alternatives given for an intra-canal irrigant. In a study conducted by Prabhakar et al (2013), *M. citrifolia* was compared with Chlorhexidine as anti-microbial endodontic irrigants. From this study, *M. citrifolia* was found to have significant antibacterial activity which is attributed to its contents of alizarin, scopoletin, aucubin and asperuloside. *M. citrifolia* juice and  $\text{Ca(OH)}_2$  has been compared on *E. faecalis* infected root canal dentin at two different depths and three intervals. It was concluded that it was effective against *E. faecalis* in dentin on extracted teeth.

### ***Acacia nilotica* (Babool)**

*A. nilotica* also known as the gum Arabic tree, possesses good anti-microbial anti-oxidant, anti-fungal, anti-viral and antibiotic activity. It has been shown by Rosina et al. (2009) that this tree possesses anti-bacterial activity against *Streptococcus mutans* and *E. faecalis*. In another study by Dhanya and Preena (2010), extracts of liquorice, clove, cinnamon, babool were investigated for their anti-microbial activity. It was shown that babool at a concentration of 50% had the highest activity against *E. faecalis*.

### ***Curcuma longa* (turmeric)**

Curcumin, a member of a ginger family possesses anti-inflammatory, anti-oxidant, anti-microbial and anti-cancer activity. In an *in vitro* study conducted by Prasanna et al. (2011), it was shown that curcumin has significant antibacterial activity against *E. faecalis* and can be used as an alternative to sodium hypochlorite for root canal irrigation. Thus, this herb can be used especially in endodontics for root canal failure cases.

### ***Azadirachta indica* (neem)**

Neem's anti-viral, anti-fungal, anti-bacterial and anti-carcinogenic activity makes it a potential agent for root canal irrigation. Neem leaf extract is also used to treat dental plaque and gingivitis. Being a bio-compatible anti-oxidant, use of neem is advantageous as it is not likely to cause the severe harms to patients that might occur through sodium hypochlorite accidents. Aarati et al. (2011) observed that ethanolic extract of neem had significant anti-microbial activity against *E. faecalis*.

### ***Aloe vera* (*Aloe barbadensis* miller)**

*Aloe vera* possesses good anti-bacterial and anti-fungal activity. In a study conducted by Sureshchandra and

Kumar (n.d.), anti-microbial effect of water, alcohol, chloroform extracts of aloe vera gel were investigated and it was found that chloroform extract of aloe vera had significant anti-microbial effect against *E. faecalis*. It also has been found to be effective against the resistant microorganisms commonly found in the pulp.

### **Triphala and green tea polyphenols**

Triphala's fruit is rich in citric acid which helps in removing the smear layer. Its chelating property makes it an effective alternative to sodium hypochlorite for root canal irrigation. Green tea polyphenols have significant anti-oxidant, anti-cariogenic, anti-inflammatory, thermogenic, probiotic and anti-microbial properties. Triphala and Green tea polyphenols are preferred over the traditional root canals irrigants due to their curative properties such as anti-oxidant, anti-inflammatory and radical scavenging activities. In an *in vitro* study conducted by Prabhakar et al. (2010), Triphala and green tea polyphenols were found to have significant anti-microbial activity against *E. faecalis* biofilm formed in tooth substrate. In another study by Madhu et al. (2011), antimicrobial efficiency of triphala, green tea polyphenols and 3% sodium hypochlorite were compared against *E. faecalis* and it was observed that triphala and green tea polyphenols showed significantly better antibacterial activity against 2 week biofilm.

### **German chamomile and tea tree oil**

German chamomile has anti-inflammatory, analgesic and anti-microbial properties. Tea tree oil also has many properties such as being an antiseptic, anti-fungal agent, anti-bacterial and a mild solvent. The active component in tea tree oil is terpinen-4-ol which is responsible for the above properties. In a SEM study conducted to overcome the undesirable effects of sodium hypochlorite, it was observed that chamomile when used as an irrigant was more effective in removing smear layer when compared to sodium hypochlorite used alone but less effective than sodium hypochlorite combined with EDTA (Milind and Nitin, 2006). In another study by Uday et al. (2013), antibacterial efficacy of tea tree oil was compared with 3% sodium hypochlorite and 2% chlorhexidine against *E. faecalis*. It was found that maximum anti-microbial activity was shown by chlorhexidine followed by tea tree oil and then sodium hypochlorite.

### **Aroeira-da-praia and Quixabeira**

In an *in vitro* study conducted by Edja (2012), anti-microbial activity and root canal cleaning potential of Aroeira-da-praia and Quixabeira against *E. faecalis* was

evaluated. It was concluded that Aroeira-da-praia showed anti-microbial activity at all concentrations tested whereas Quixabeira was effective only at 100 and 50% concentrations.

### ***Spilanthes calva* DC**

*S. calva* DC is an important herb for oral health care. It is nontoxic to human beings and has anti-bacterial and anti-fungal activities. Moulshree (2013) compared the anti-bacterial efficacy of methanolic extract of *S. calva* DC roots with 2% chlorhexidine, 3% sodium hypochlorite and doxycycline at different concentrations against *E. faecalis*. From the study, it was concluded that *S. calva* DC root extract had comparable anti-bacterial activity to sodium hypochlorite. Thus, it may have potential as a root canal irrigant.

### **CONCLUSION**

Studies indicate that the prevalence of *E. faecalis* is low in primary endodontic infections and high in persistent infections. *E. faecalis* is also more commonly associated with asymptomatic cases than with symptomatic ones. Eradication of *E. faecalis* from the root canal remains a challenge, while chlorhexidine and combinations of disinfectants show some promise. A better understanding of the role of the virulence factors of *E. faecalis* in endodontic infections may help in the development of new strategies to prevent or eliminate the infection by this species thereby improving treatment results in endodontics.

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## Full Length Research Paper

# Prevalence and antibiotic resistance pattern of blood culture isolates from human immuno-deficiency virus (HIV) patients on highly active anti-retroviral therapy (HAART) in Nigeria

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Bloodstream infections remain public health problem and have resulted to morbidity and mortality in HIV/AIDS patients. This study was therefore aimed at determining the prevalence of bacteremia in HIV infected patients on highly active anti-retroviral therapy (HAART) attending Federal Medical Centre, Ido-Ekiti, Ekiti State. A total of 105 blood samples were cultured using brain heart infusion and subcultured on bismuth sulphite agar, MacConkey agar and chocolate agar. Isolates were identified using cultural and biochemical characterization. Antibiotic susceptibility test was done using disc diffusion method. The prevalence of bloodstream infection in HIV infected patients on HAART was found to be 22.9% and they were having the CD4<sup>+</sup> T-cell counts of <300 cells/ $\mu$ l. There were five males and 19 females that had bloodstream bacterial infection. A total number of 24 isolates were recovered with *Escherichia coli* being the most prevalent bacterial isolates 12(50.0%), followed by *Staphylococcus aureus*, 8 (33.3%) and *Streptococcus pneumoniae*, 4 (16.7%). The antibiotic susceptibility test revealed a high level of resistance with highest resistance seen in Cotrimoxazole (87.5%), followed by ciprofloxacin (83.3%), ampicillin (79.2%), levofloxacin (62.5%) while ceftazidime, ceftriaxone and gentamycin (54.2% each) showed the least resistance. The overall percentage of antibiotics resistance of the 7 selected antibiotics tested against isolated bacterial had 71.4, 67.9 and 66.1% resistance for *S. pneumoniae*, *Escherichia coli* and *S. aureus*, respectively. However, the prevalence of bloodstream infection among HIV patients on HAART in Ekiti was relatively high and resistance to cephalosporins drugs was lower as compared to fluoroquinolones. Therefore, cephalosporin group of antibiotics is recommended for the treatment of bacteria bloodstream infection in HIV/AIDS patients.

**Key words:** Bacteremia, human immunodeficiency virus (HIV), antibiotics resistance, Nigeria.

## INTRODUCTION

Bacteria bloodstream infections constitute a significant public health problem (Adeleye et al., 2010) and it occurs more frequently in HIV infected patients than in HIV-negative patients and is associated with higher rates of morbidity and mortality (Ojo-Bola and Oluyeye, 2014).

Mortality rates in HIV patients as compared to those without HIV differ between reports. It has been reported that bacterial infections are the leading cause of deaths in patients with the acquired immune deficiency syndrome (AIDS) (Gordon, 2008).

Bloodstream infections have long been one of the hallmarks of the acquired immune deficiency syndrome since the beginning of the epidemic (Whimbey et al., 1986; Bekele et al., 2003) and it can be defined as the presence of viable bacteria in the blood with a documented positive blood culture and the presence of chemical symptoms of systemic infection (Garner et al., 1998). However, it was responsible for 8% of all AIDS deaths (Stein et al., 1992). Bekele et al. (2003) reported that of 1,225 hospitalized patients at the university of health science centre, 88 (7%) had blood stream infection and 73 of the infections were community acquired. Consequently, the presence of bloodstream infections is associated with an increased mortality rate, length of hospital stay and intensive care unit admission rate. Also, a high percentage of bloodstream infections, ranging from 10 to 63% were observed in hospitalized HIV infected individuals presenting with fever in a number of studies conducted in sub-Saharan Africa (Archibald and Den Dulk, 1998; McDonalds and Archibald, 1999; Peters et al., 2004). Another study showed that bloodstream infections were associated with recent HIV diagnosis. Lower CD<sub>4</sub><sup>+</sup> T-cell counts were also strongly associated with the patients having blood stream infections (Verma and McCarthy, 2010).

Symptoms of abdominal illness such as nausea, vomiting and loss of appetite were also associated with blood stream infections caused by bacterial pathogens (Verma and McCarthy, 2010). HIV-infected patients may be predisposed to blood stream infections due to several conditions such as defective cell-mediated immunity, altered B-cell function and qualitative and quantitative deficits of neutrophils leading to an increase in the susceptibility of the patient to bacterial infections (Mertins and Ortona, 1990; Zurlo and Lane, 1997).

However, Kiertiburanakul et al. (2012) reported that opportunistic infections are still the leading causes of blood stream infection (BSI) among HIV infected patients in the HAART era in Thailand. Out of 140 patients tested 91 (65%) who had CD<sub>4</sub><sup>+</sup> T cell median of 32 cells/ $\mu$ l had blood stream infection. Common bacterial associated with bloodstream infection in HIV infected individuals include: *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *klebsiella pneumoniae*, *Pseudomonas aeruginosa* (Verma and McCarthy, 2010). The frequent presence of bacteria in blood stream of HIV/AIDS patients compare with the HIV negative patients was said to be due to the debilitated immune system which can leave individual vulnerable to bacterial infections (Ojo-Bola and Oluyeye, 2014).

The clinical manifestation of HIV secondary infections in developing countries, including Nigeria, shows a high prevalence of infections of blood, skin, gut, respiratory

tract, tuberculosis and malnutrition. The virus has a specific capacity to infect the CD<sub>4</sub><sup>+</sup> T-cells, resulting in progressive decline in their CD<sub>4</sub> + T-cells number. This has serious health consequences, since CD<sub>4</sub><sup>+</sup> T-cell counts may almost be absent (Adeleye et al., 2010).

However, antibiotics is fundamental to illness control in these patients; unfortunately the multiple antibiotic resistance among bacterial isolates from various study groups are frightening (Adeleye et al., 2010; Ojo-Bola and Oluyeye, 2014). This is because such organisms can become endemic within the environment and pose serious public health threats to the treatment and management of HIV/AIDS patients (Ojo-Bola and Oluyeye, 2014).

Although, HIV patients are at higher risk of BSI, nevertheless, the introduction of HAART was said to have reduced the rate of bacteria associated blood stream infection; yet HIV/AIDS patients who are on HAART still has BSI, it then becomes imperative to know in this study area, the prevalence and antibiotics resistance pattern of bacterial isolated from HIV/AIDS on HAART. This will provide a more recent update on the treatment regimes for effective management of BSI.

## METHODOLOGY

### Study area

The study was conducted among HIV infected individuals attending Federal Medical Centre (FMC), Ido Ekiti, Ekiti State, Nigeria. FMC is located in Ido Ekiti, the principal town in Ido/Osi Local Government of Ekiti State with an estimated population of 107,000. The hospital is serving five contiguous states (Ekiti, Osun, Ondo, Kogi and Kwara State).

### Study subject

A total of 105 blood samples were collected from the HIV/AIDS patients on HAART presenting with fever (axillary temperature  $\geq$  37.5°C) and seek medical attention in the above-named hospital between March, 2013 to December, 2013.

### Ethical consideration, questionnaire and informed consent

The study was approved by the Medical and Research Ethics Committee of the Federal Medical Centre, Ido Ekiti, Ekiti State. The consent of the patients was sought prior to collection of blood samples. The samples were collected by medical laboratory scientist and dispensed unto brain heart infusion broth under aseptic condition.

### Isolation and identification of bacterial isolates from culture of HIV infected patients

Five milliliters of blood samples were collected from the HIV

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**Table 1.** Demographic data of the HIV infected patients on highly active antiretroviral therapy (HAART) attending Federal Medical Centre, Ido-Ekiti.

Age group (years)	Male	Female	Total (%)
15- 25	6	10	16 (15.2)
26-35	10	19	29 (27.6)
36-45	5	19	24 (22.9)
46-55	6	14	20 (19.0)
56-65	6	5	11 (10.5)
66-75	5	0	5 (4.8)
Total	38	67	105 (100.0)

infected individuals by venous puncture from the antecubital foci on the arm after disinfecting the area with 70% of alcohol. The blood samples were inoculated into pre warmed brain heart infusion broth and incubated at 37°C for seven days. Subcultures were made on Bismuth Sulphite agar, MacConkey agar and Chocolate agar respectively from cultures showing signs of bacterial growth. The suspected colonies from Bismuth Sulfite Agar plates, MacConkey agar plates and Chocolates agar paltes were isolated using their morphology and biochemical characteristics using standard methods as described by Cheesbrough (2000). The following biochemical tests were used for the identification; catalase, coagulase, indole, motility, substrate utilization test while optochin test for *Streptococcus pneumoniae*. All the reagents were purchased from reputable company (LAB M, Lancashire, UK).

#### Antibiotic susceptibility test

The antibiotics susceptibility test was performed using Kirby-bauer disc diffusion method as described by CLSI (2011). Standardized inoculum was inoculated onto sterile Mueller Hinton agar plates using standard techniques for all the isolates except *S. pneumoniae* that was done on chocolate agar. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculums from the swab. The dried surface of the Mueller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface to ensure even distribution of inoculums. The concentration of the antibiotics in the test discs were as follows: levofloxacin (5 µg/ml), ciprofloxacin (5 µg/ml), ceftazidime (30 µg/ml), ceftriaxone (30 µg/ml), ampicillin (25 µg/ml), cotrimoxazole (1.25+23.75 µg/ml) and gentamycin (10 µg/ml). The discs were dispensed onto the surface of the surface of the inoculated agar plate with a sterile forcep. Each disc were pressed down to ensure complete contact with the agar surface and distributed evenly so that they were no closer than 24 mm from centre to centre. The plates were inverted and placed in an incubator set to 37°C for 24 h. The resulting zones of inhibition were measured to the nearest whole millimeter using a meter rule and were interpreted using interpretative standards of CLSI M100-S12. Performance standards for antimicrobial susceptibility testing and the organisms were reported as susceptible, intermediate or resistant to the antibiotic being tested. The antibiotics discs were purchased from Oxoid (Basingstoke, Hampshire, UK).

#### Statistical analysis

Statistical procedure for social science (SPSS) version 16.0 (SPSS Inc. Chigago IL) using frequency tables was used to analyse the data generated from this study.

## RESULTS

The study population comprised of one hundred and five HIV infected patients on HAART of which sixty seven were females and thirty eight (38) males. They were aged 15-75 years with a mean age of 45.4 years. Distribution according to age range showed that the majority of the HIV infected patients were in the age group of 26-35 years followed by those whose age ranged between 36-45 years and the least number was between the age group of 66-75 years (Table 1). Twenty four (24) of the HIV infected patients had a CD4<sup>+</sup> T-cell counts of less than or equal to 299 cells/µl and had positive bacterial growth while the remaining eighty-one (81) HIV infected patients had no bacterial growth with the CD4<sup>+</sup> T-cell of ≥ 500 cells/µl (Table 2).

The bacterial profile of isolates from blood culture of HIV infected patients on HAART revealed that *E. coli* had the highest percentage of 50% followed by the *S. aureus* with 33.3% while *S. pneumoniae* had the lowest percentage of 16.67% (Table 3).

*S. pneumoniae* exhibited the highest percentage resistance of 71.4% to the seven antibiotics tested followed by *E. coli* with a percentage resistance of 67.9%, *S. aureus* had the least percentage resistance of 66.1%. Cotrimoxazole had the highest percentage of 87.5 followed by ciprofloxacin with 83.3% and ampicillin with 79.2% and the least was ceftazidime. Both ceftriaxone and gentamycin had the same percentage resistance of 54%.

The antibiotic resistance pattern of bacterial isolates from blood culture showed that *E. coli* had ten different antibiotic resistance patterns (Table 4). *S. pneumoniae* had the least antibiotic resistance pattern of 4 (Table 5).

## DISCUSSION

Blood stream infection is more common among HIV-infected individuals and remains World Health Organization (WHO) stage 4 defining condition (Gordon, 2008). Bloodstream infections constitute a significant public health problem and represent an important cause of morbidity and mortality in HIV/AIDS patients (Adeleye et al., 2008). However, from this study, the prevalence of bacteria in the blood samples of HIV infected patient on HAART attending Federal Medical Center Ido-Ekiti was 22.9%. The prevalence observed in this study is higher as compared to the study conducted in University Teaching Hospital during October 2005-2006 Lagos State, Nigeria which was 12.9% (Adeleye et al., 2010). Also, study conducted in Malawi showed 30% prevalent rate of HIV infected patients with blood stream infections (Bonadio et al., 1998).

The prevalence of bacteria in relation to sex of the HIV/AIDS patients revealed that there were more females than males that had bacteria in their blood and they are in ratio 13:11 in the study subject and this is a reflection of

**Table 2.** The CD4<sup>+</sup> T-cells count in relation to sex of the HIV infected patients on HAART Attending Federal Medical Centre, Ido-Ekiti.

CD4 <sup>+</sup> T-cells count (cell/ ml)	Sex distribution		Total (%)	Bacteria growth
	Male	Female		
100-199	6	7	13 (12.3)	Positive
200-299	5	6	11(10.5)	Positive
300-399	0	0	0 (0.0)	-
400-499	0	0	0 (0.0)	-
500-599	15	21	36 (34.3)	Negative
600-699	7	18	25 (23.8)	Negative
700-799	5	13	18 (17.1)	Negative
800-899	0	2	2 (1.9)	Negative
Total	38	67	105 (100.0)	

**Table 3.** Bacteria profile of isolates from blood culture of HIV patients on highly active anti-retroviral therapy (HAART).

Organism	No. of isolates (%)
<i>Staphylococcus aureus</i>	8 (33.3)
<i>Escherichia coli</i>	12 (50.0)
<i>Streptococcus pneumoniae</i>	4 (16.7)
Total	24 (22.9)

**Table 4.** Antibiotic resistance in bacterial isolates from blood culture of HIV infected patients on highly active anti-retroviral therapy (HAART).

Organism	lev	cip	cefta	ceftr	amp	cot	gen	Total average
<i>Escherichia coli</i> (n=12) (%)	8(66.6)	10(83.3)	5(41.7)	5(41.7)	10(83.3)	11(91.6)	8(66.6)	8.1 (67.9)
<i>Staphylococcus aureus</i> (n= 8) (%)	5(62.5)	6(75.0)	5(62.5)	5(62.5)	5(62.5)	7(87.5)	4(50.0)	5.3 (66.1)
<i>Streptococcus pneumoniae</i> (n=4) (%)	2(50.0)	4(100.0)	3(75.0)	3(75.0)	4(100.0)	3(75.0)	1(25.0)	2.9 (71.4)
Total Average (n =24) (%)	15(62.5)	20(83.3)	13(54.2)	13(54.2)	19(79.2)	21(87.5)	13(54.2)	(54.2)

Cip = ciprofloxacin, lev = levofloxacin, cefta = ceftazidime, cot = cotrimoxazole, ceftr = ceftriaxone, gen = gentamicin, amp = ampicillin.

disproportionate impact of HIV on women and girls than on men. Obi et al. (2007) established that there were more HIV positive females than males in the studies in South Africa, indicating a gender bias. The vulnerability of women to HIV in sub-Saharan African stem from their greater physiological susceptibility and the severe social, legal and economic disadvantage that confront them. In sub-Saharan African region, as worldwide, female population is a key factor in the epidemiology of HIV and AIDS because 50% of all adults with HIV infection are predominantly women infected via heterosexual transmission. Furthermore, females are the most severely affected (Mitchell et al., 2003; Hickey and Shanson, 1993).

There are twenty four (UNAIDS /WHO, 2009) bacterial isolates of three different generals from one hundred and five samples examined with total prevalence rate of

22.9%. There were 12 *E. coli* with prevalent rate of 50% followed by *S. aureus*, 8 (33.3%) and *S. pneumoniae*, 4 (16.67%). This agreed with the study conducted by Kiertburanakul et al. (2012) that reported *E. coli* and *S. pneumoniae* as the most common Gram negative and positive organisms isolated from the bloodstream of hospitalized HIV-infected patients in Thailand. Other studies have suggested an increased risk of bloodstream infections due to aerobic Gram negative bacilli such as *E. coli* in HIV/AIDS patients particularly in those having invasive devices (Zurlo and Lane, 1997; WHO, 2005; Tumbarello and Tacconelli, 1998). Also, the most recent study conducted by Ojo-Bola and Oluyeye (2014) reported that *E. coli* had the highest prevalence of bacterial associated with pneumonia in HIV/AIDS patients and this can be disseminated to bacterial bloodstream infection.

**Table 5.** Resistance pattern of bacteria isolates from blood culture of HIV patients on highly active anti-retroviral therapy.

Bacterial species	Resistance pattern	Number of organism showing pattern
<i>Escherichia coli</i>	lev, cip, amp, cot, gen,	1
	Amp, Cot, Gen	1
	lev, cip, cefta, ceftri, amp, cot, gen	5
	cip, amp, cot, gen	1
	cip, ceftri, amp, cot	1
	lev, cip, cefttr, amp, cot	1
		10
<i>Staphylococcus aureus</i>	lev, cip cefta, cefttr, amp, cot, gen	2
	cefta, cefttr, cot	1
	lev, cip, cefttr, cot	1
	cip, cefta, ceftri, amp, cot, gen	1
	lev, cip, amp, cot, gen,	1
	lev, cip, cefta, cot, gen	1
		7
<i>Streptococcus pneumonia</i>	cip, cefta, cefttr, amp, cot	1
	lev, cip, cefta, cefttr, amp, cot, gen	1
	cip, cefta, cefttr, Aamp, cot, gen	1
	lev, cip, amp	1
		4

cip = ciprofloxacin, lev = levofloxacin, cefta = ceftazidime, cot= cotrimoxazole, cefttr = ceftriaxone, gen= gentamicin, amp = ampicillin

However, HIV has a specific capacity to infect the CD4+ T-cells lymphocyte, resulting in progressive decline in their CD4+ T-cells number. This has serious health consequences, since CD4+ T-cells constitute about 10% of the total T-cell pool. In HIV/AIDS patients, the number of CD4+ T-cells may be almost absent. Nevertheless, the era of HAART has greatly reduced the secondary infection in these groups of patients (Adeleye et al., 2008). In this study, the twenty four HIV infected patients with bacterial growth had a CD4<sup>+</sup> T-cell counts of less than or equal to 299 cells/μl and the remaining 81 (eighty one) patients without bacterial growth had a CD4<sup>+</sup> T-cell counts ≥ 500 cells/μl. This study agreed with a report that there is a steady decline in the CD4<sup>+</sup> T-cell counts of patients and by the time opportunistic infections set in, there may be no more CD4<sup>+</sup> T-cells present in the immune system (UNAIDS/WHO, 2009).

The antibiogram of the isolates were tested with seven antibiotics commonly prescribed in the hospital. *E. coli* showed 66.6% resistance to gentamycin and levofloxacin, 83.3% resistance to ciprofloxacin, 41.7% resistance to ceftazidime and ceftriaxone, 83.3% resistance to ampicillin, 91.6% resistance to cotrimoxazole, *S. aureus* showed 62.5% resistance to levofloxacin, ceftazidime, ceftriaxone and ampicillin, 75% resistance to ciprofloxacin, 87.5% to cotrimoxazole and 50% resis-

tance to gentamycin. *S. pneumoniae* showed 100% resistance to ciprofloxacin and ampicillin, 75% resistance to ceftazidime, ceftriaxone and cotrimoxazole, 50% resistance to levofloxacin and 25% resistance to gentamycin. It has been observed in this study that the resistance of bacteria to some used antibiotics (ciprofloxacin, ceftazidime, cotrimoxazole, levofloxacin and gentamycin) was relatively high. Of a total of seven antibiotics employed in the antibiotic susceptibility test, *E. coli* exhibited a higher percentage of 91.6% resistance to cotrimoxazole, while *S. aureus* also exhibited 81.5% percentage resistance to ciprofloxacin and ampicillin.

The multiple antibiotics resistance is a resistance to more than 3 groups of different antibiotics. *E. coli* showed 10 different pattern of multiple resistance while *S. aureus* and *S. pneumoniae* has 7 and 4 patterns, respectively.

In conclusion, the prevalence of isolation of bacteria from blood culture of HIV infected patients on HAART in this study was found to be 22.9% and was among HIV patients whose CD4+ T-cell falls with ≥ 299 cells/μl; the antibiotic resistance of the bacteria was relatively high to the antibiotics used. This may be as a result of unnecessary use or overuse of antibiotics to treat bacterial infections. However, based on this study, cephalosporins drugs had a lower resistance as compared to fluoroquinolones and it should be used for the treatment

of bacteria associated bloodstream infections.

### Conflict of interest

There is no conflict of interest.

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Full Length Research Paper

## Differentiation of *Stemphylium solani* isolates using random amplified polymorphic DNA markers

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*Stemphylium solani* isolates infect two varieties of eggplant, *Solanum aethiopicum* and *Solanum melongena* grown in Senegal (West Africa) and are morphologically quite similar. Using the random amplified polymorphic DNA (RAPD) procedure with arbitrary 10-mer primers, we were able to differentiate these *S. solani* isolates into two groups directly related to their plant host origin. A RAPD product of approximately 480 bases pairs obtained with OPF-20 primer were polymorphic between the two groups. Four new primers (F20F1, F20F2, F20R1 and F20R2) based on nucleotide sequence analysis of this 480 bases pairs RAPD fragment were developed. Such primers used pairwise amplified a single fragment from the DNA of *S. solani* isolates whatever their host origin. However, DNA extracted from *Fusarium oxysporum* (f. sp. *vasinfectum*, f. sp. *elaeidis*), *Verticillium dahliae* and *Phyllosticta* sp. isolates did not amplify using these primers. Our results indicate that these primers sets were good tools for specific identification of these two eggplants *S. solani* isolates by polymerase chain reaction (PCR).

**Key words:** *Stemphylium solani* isolates, *Solanum aethiopicum*, *Solanum melongena*, random amplified polymorphic DNA (RAPD) markers, identification.

### INTRODUCTION

The genus *Stemphylium* Wallr. was established in 1833 (Wallroth, 1833) with *Stemphylium botryosum* Wallr. as the type species. There were 33 published taxa of *Stemphylium* in 2002 (Câmara et al., 2002). Since then,

many new species have been described (Simmons, 2004; Pei et al., 2009, 2011; Wang et al., 2009). Species of *Stemphylium* genus are dematiaceous hyphomycetes, many of them are economically important pathogens on a

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**Table 1.** Geographic and host origin of *Stemphylium* isolates used in this study.

Isolates code	Localities	Hosts origin
S0	Kourouar	<i>Solanum aethiopicum</i>
PJ1	Pikine	<i>Solanum aethiopicum</i>
PJ2	Pikine	<i>Solanum aethiopicum</i>
S2	Thiaroye	<i>Solanum aethiopicum</i>
S2'	Thiaroye	<i>Solanum aethiopicum</i>
S3	Thiaroye	<i>Solanum aethiopicum</i>
C2	Kolda	<i>Solanum aethiopicum</i>
C3	Kolda	<i>Solanum aethiopicum</i>
C23	Ziguinchor	<i>Solanum aethiopicum</i>
C31	Ziguinchor	<i>Solanum aethiopicum</i>
C4	Kolda	<i>Solanum melongena</i>
C5	Kolda	<i>Solanum melongena</i>
C8	Kolda	<i>Solanum melongena</i>
C13	Kolda	<i>Solanum melongena</i>
C15	Kolda	<i>Solanum melongena</i>
C16	Kolda	<i>Solanum melongena</i>
C21	Ziguinchor	<i>Solanum melongena</i>
C25	Ziguinchor	<i>Solanum melongena</i>
C27	Ziguinchor	<i>Solanum melongena</i>
C28	Ziguinchor	<i>Solanum melongena</i>

wide range of plants (Mehta, 2001; Câmara et al., 2002; Tomioka and Sato, 2011). Until the emergence of molecular phylogenetic analysis, the taxonomy of *Stemphylium* species was based primarily on conidial morphology, including variation in conidial shape, size, length/width ratio, color, septation and ornamentation (Simmons, 1985, 2001). However, many of these characters overlap among species, making species determination difficult. The emergence of polymerase chain reaction (PCR) techniques has enhanced traditional approaches to fungal taxonomic investigations. DNA sequence data are now being commonly used to verify morphological concept and other taxonomic hypothesis (Hsiang and Goodwin, 2001; Hunter et al., 2006; Pei et al. 2011). The ITS-rDNA sequence is being widely used to identify phylogenetic relationships among fungal taxa, especially at the species level (Callac and Guinberteau, 2005; Sotome et al., 2009). In a previous study, ITS-rDNA sequence analysis confirmed the identification of 45 fungal isolates as *Stemphylium solani* (Ndir et al., 2008), but was not able to reveal polymorphism among them. Random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990) has been used successfully to detect genetic variability in many groups of plants fungi (Assigbetse et al., 1994; Alfonso et al., 2000; Macdonald et al., 2000; Collopy et al., 2001). In the present paper, we used RAPD procedure on a sample of twenty isolates to find polymorphism between 10 *S. solani* isolates from

*Solanum aethiopicum* and 10 others from *Solanum melongena* and we used the sequence information of some RAPD marker to develop a PCR-specific assay for these *S. solani* isolates identification.

## MATERIALS AND METHODS

### Fungal materials

Out of 45, a set of twenty *S. solani* isolates collected from naturally infected hosts: *S. aethiopicum* and *S. melongena* in different vegetables growing areas throughout the Senegal were used as listed in Table 1. Using morphological characters and molecular analysis of the ITS regions including 5.8S rDNA (RFLP and sequencing), we identified these 45 isolates as closely related to members of *S. solani* species by comparing our ITS sequence data with reference isolates in GenBank (data not shown). Isolate May S3 was therefore chosen from our collection and deposited in GenBank database under accession number AF 426739. Four *Fusarium* and three *Verticillium* strains were obtained from IRD-Montpellier, one strain from INRA-Montfavet was identified as *Phyllosticta* sp. All cultures were single-spored and stored on potato dextrose agar slants (PDA, Difco) at 4°C until further processing.

### Fungal culture and DNA extraction

Isolates were grown in 200 ml of potato-dextrose-broth (PDB, Difco) for 7 days at 25°C. The mycelium was harvested by filtration, frozen 48 h at -80°C and lyophilized for 24 h. Total DNA was extracted following the method of Lee et al. (1988) with modifications and was performed by phenol-chloroform-isoamyl alcohol (25:24:1) procedure. The DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA) to a final concentration of 5 ng  $\mu\text{l}^{-1}$ .

### DNA amplification

#### RAPD primers

The primers used are listed in Table 2 and were obtained from kit F, Operon Technologies (Alameda, CA, USA).

#### Amplification conditions

RAPD reactions were performed in a total volume of 25  $\mu\text{l}$  containing 2.5 mM  $\text{MgCl}_2$  (1.5  $\mu\text{l}$ ), 10 mM dNTPs (0.25  $\mu\text{l}$ ), 20 p.mol. of primer (4  $\mu\text{l}$ ), 25 ng of genomic DNA, 1 U of *Taq* DNA polymerase (Promega Charbonnières, France) and 2.5  $\mu\text{l}$  of 10X *Taq* polymerase buffer. Negative controls without DNA were included in all reactions. The amplifications were conducted as follows: 4 min at 94°C, 30 cycles (1 min at 94°C, 1 min at 36°C and 1 min 30 s at 72°C), 5 min at 72°C. PCR reactions using sequence-specific primers were performed in the same conditions using 5 Units of *Taq* DNA polymerase, with an initial denaturation step at 94°C for 4 min followed by 24 cycles (30 s at 94°C, 1 min at 56 to 60°C and 1 min at 72°C), 5 min at 72°C. Amplification products were separated by electrophoresis on 1.4 % agarose gels stained with ethidium bromide and visualized under UV light. A 1 kb molecular weight ladder (Eurogentec, France) was used as size standard.

#### DNA templates

RAPD amplification reactions were conducted with each primer on

**Table 2.** Codes and sequences of the 6 primers tested for RAPD analysis of *S. solani* isolates, with total number of amplified DNA fragments and number of polymorphic DNA fragments obtained with each primer.

Primer code	Sequences 5' to 3'	Amplified fragment	Polymorphic fragment
OPF-02	GAGGATCCCT	7	2
OPF-04	GGTGATCAGG	5	2
OPF-10	GGAAGCTTGG	10	1
OPF-13	GGCTGCAGAA	8	2
OPF-14	TGCTGCAGGT	5	1
OPF-20	GGTCTAGAGG	10	1

the DNA of 10 *S. solani* isolates from *S. aethiopicum* and 10 others from *S. melongena*. PCR assays using specific primers were conducted with the DNA from *Stemphylium*, *Fusarium*, *Verticillium* and *Phyllosticta* isolates. All amplification reactions were repeated in separate experiments. RAPD data were analyzed considering the presence or the absence of bands.

#### Specific primers selection

An OPF-20 RAPD fragment of about 480 bp obtained from the amplification was extracted and purified from a low melting point 1% agarose gel by using QIA quick Gel Extraction Kit Protocol (QIAGEN) and ligated into the *EcoRI* site of pGEM-T Easy Vector (Promega). After transformation of *Escherichia coli* (TM 109-High Efficiency Competent Cells), white colonies were selected. Plasmid DNA was prepared and after restriction digest, clones containing the expected size insert were sequenced (Genome Express, France). From the sequence obtained, two sets of primers were defined and used to amplify DNA from different fungal species.

## RESULTS

### RAPD analysis

No banding pattern was observed in negative controls without DNA (data not shown). RAPD patterns were established for the 20 isolates of *S. solani* with the six primers listed in Table 2. These primers were chosen from the 16 tested because of the clear amplification pattern they produced. The profiles were reproducible from one experiment to another. The size of amplified DNA fragments generated with the 6 primers ranged from 500 to 2500 bases pairs.

Table 2 showed the total number of amplified products and the number of polymorphic fragments produced with each primer. Amplification with primer OPF-14 resulted in a single marker band (OPF14-1500 bp), which was specific for *S. solani* isolates from *S. melongena*. Of the 10 amplified products generated with primer OPF-20, one of approximately 480 bp was amplified strongly with *S. solani* isolates only from *S. melongena*. Figure 1 shows amplification products generated with primers OPF-14 (A) and OPF-20 (B).

All six primers generated two distinct amplicotypes within *S. solani* isolates. These primers revealed polymorphism

useful to classify the isolates. Using RAPD approach, we were able to identify two groups according to their plant host origin. The RAPD-480 bp polymorphic between the two groups was chosen for its specificity, its strong intensity and its size, which would facilitate its cloning.

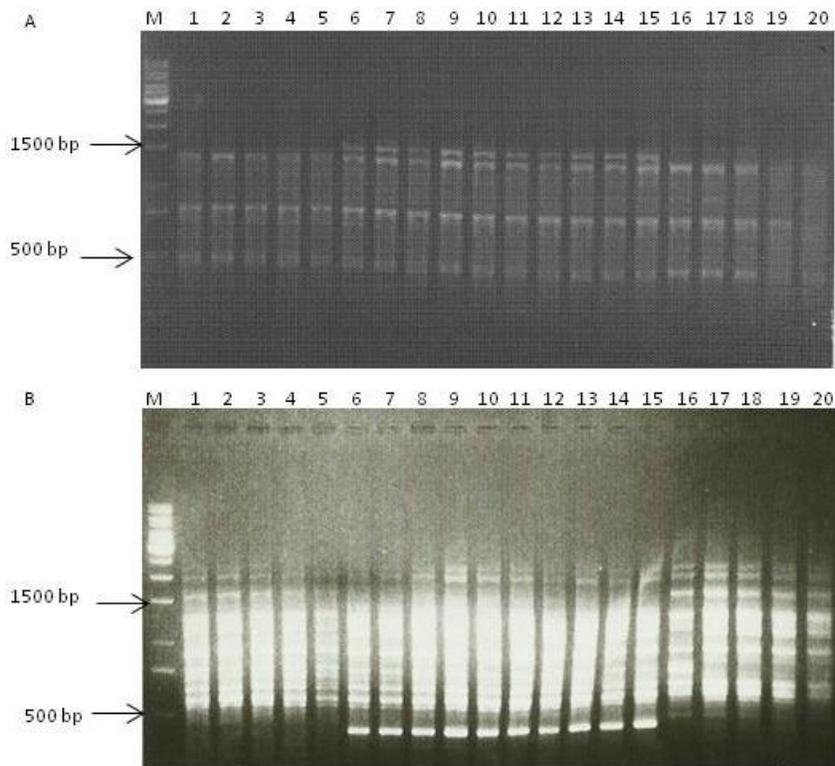
### Characterization of the 480 bp RAPD fragment

The RAPD fragment amplified with OPF-20 primer from only *S. melongena* isolates revealed a 502 bp sequence length. Using the BLAST search program (National Center for Biotechnology Information), there were no obvious similarities with any of the sequences available in the GenBank database.

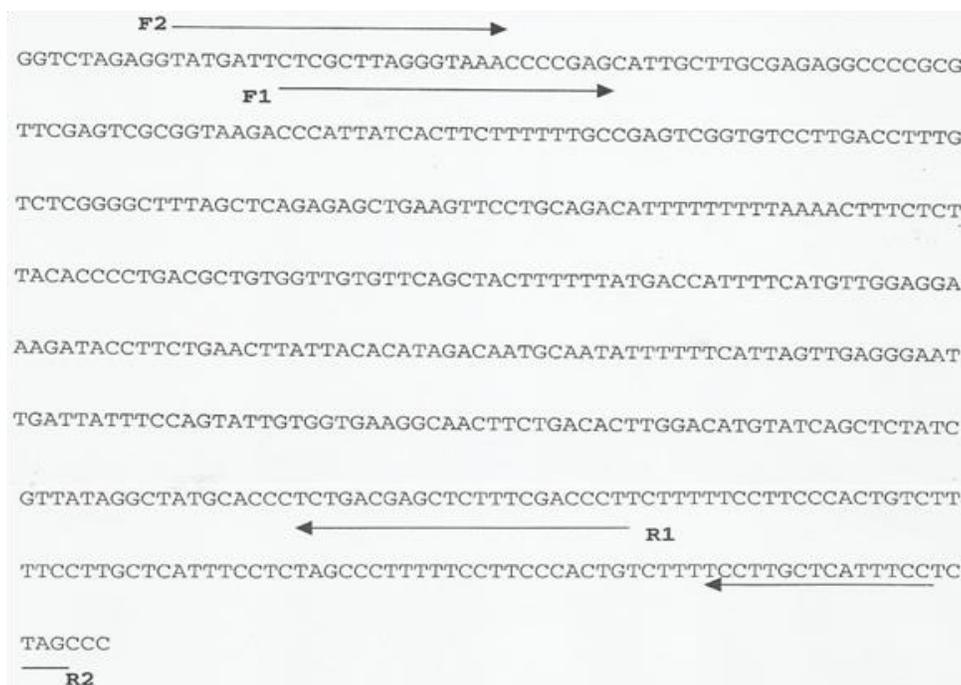
Using this sequence information (Figure 2), two sets of specific primers were synthesized (F20F1-F20R1 and F20F2-F20R2) (Table 3). These primers were designed without any percent of the primer OPF-20 sequence (ten first nucleotides).

### PCR identification of *Stemphylium solani* isolates

Amplification of entire genome of all *S. solani* isolates tested using these new primers resulted in a single PCR product for all whatever their plant host origin. The length of the single amplicon produced was of 400, 406, 485 and 492 bp, with the new primers in the following association: F20F1-F20R1, F20F2-F20R1, F20F1-F20R2 and F20F2-F20R2 (Table 4). The Figure 3 showed a DNA fragment of 400 bp yielded with one of these sets of primers for all *S. solani* DNA samples (F20F1-F20R1). To confirm the PCR identification of the isolates collected in our vegetables growing areas, one *S. solani* isolate from the two previous groups were associated with DNA samples from three other fungal species in a second amplification test using the same set of primers. As shown in Figure 4, a DNA fragment of expected size (400 bp) was amplified for *S. solani* isolates. However, DNA from *Verticillium dahliae*, *Fusarium oxysporum* and *Phyllosticta* sp. isolates did not amplify with F20F1-F20R1 primer pair.



**Figure 1.** Random amplified polymorphic DNA (RAPD) patterns obtained for 20 isolates of *S. solani* with: A) primer OPF-14 and B) primer OPF-20. Lanes 1-5, 16-20 are isolates from *Solanum aethiopicum*, and lanes 6-15 are isolates from *Solanum melongena*. Lane M shows the molecular weight marker (1kb DNA ladder).



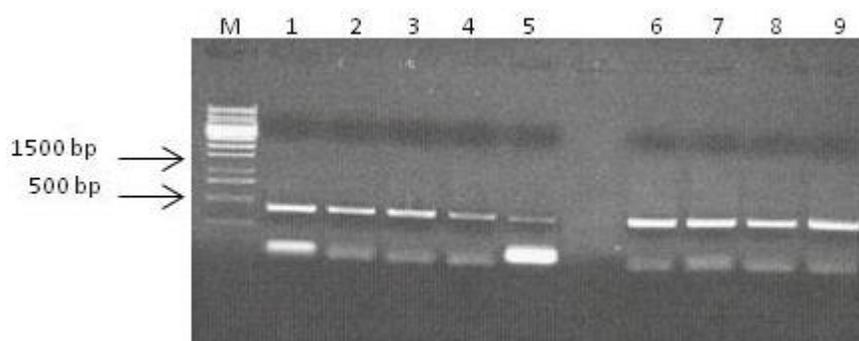
**Figure 2.** Nucleotide sequence of OPF20-RAPD fragment. The new primers for specific amplification used in this study are indicated by arrows.

**Table 3.** Nucleotide sequences of the four primers developed from the 502 bp RAPD fragment and their annealing Temperature (°C).

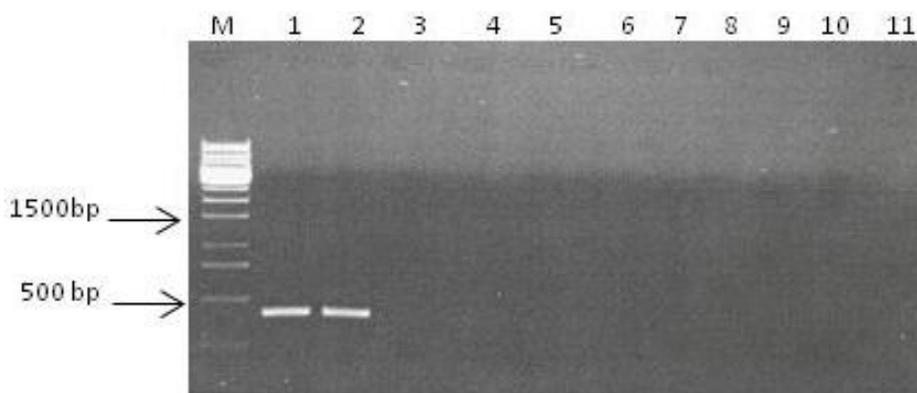
Primer	Sequence	Annealing temperature (°C)
F20F1	5' CTCGCTTAGGGTAAACCCCGAGC 3'	64
F20R1	5' GAAGGGTCGAAAGAGCTCGTCAG 3'	62
F20F2	5' TATGATTCTCGCTTAGGGTAAACC 3'	57
F20R2	5' GCTAGAGGAAATGAGCAAGG 3'	56

**Table 4.** Length of PCR fragments amplified with the new primers.

Primer	Fragment length (bp)	
	F20F1	F20F2
F20R1	400	406
F20R2	485	492



**Figure 3.** Specific PCR amplification of *S. solani* isolates with primers set F20F1-F20R1. Lane M: 1-kb marker; lanes 1, 2, 3, 4, 5 show amplification produced from *Solanum aethiopicum* isolates (S3, C2, C3, C31, C32), lanes 6, 7, 8, 9 isolates from *S. melongena* (C4, C5, C8, C13).



**Figure 4.** Specific PCR assay on selected fungal species using primers set F20F1-F20R1. Lane M: 1 kb marker; lane 1 isolate S3 (*S. solani* from *S. aethiopicum*), lane 2 isolate C4 (*S. solani* from *S. melongena*), lane 3-5 (V6, V14, V7) (*Verticillium dahliae*), lanes 6-7 (455R23, US1) (*Fusarium* o.f.sp. *vasinfectum*) lanes 8-9 (6F, PB32) (*Fusarium* o.f.sp. *elaeidis*) lane 10: T5 (*Phyllosticta* sp.), lane 11: negative control.

## DISCUSSION

RAPD analyses used in this study appear to be extremely powerful and can separate individuals having intra specific variability. It gives more comprehensive information regarding the genetic variability among the fungal populations as it is based on the entire genome of an organism (Zimand et al., 1994; Achenbach et al., 1997; Mehta, 2001). In a previous study, RAPD data were also used to separate cotton and tomato *S. solani* isolates in Brazil (Mehta, 2001). Similar RAPD analyses have been successfully applied to characterize genomic variability in numerous fungal pathogens (Adebrhan and Furtek, 1994; Alfonso et al., 2000; Pollastro et al., 2000). In many phytopathogenic fungi, RAPD analyses have proved useful for detecting genomic polymorphisms directly related to host specialization (Hamelin et al., 1993; Assigbetse, 1994). Data presented here may form the basis for further studies using larger samples to assess host specialization among *S. solani* isolates.

As a result of this study, RAPD markers generated with *S. solani* isolates from *S. melongena* but never observed with *S. aethiopicum* isolates were identified. Such markers were exploited in an attempt to set up a new diagnostic technique based on PCR. Using the RAPD OPF-20 fragment sequence information, we synthesized two sets of specific oligonucleotide primers that could identify *S. solani* isolates from two senegalese eggplants among other fungal species. The PCR primers we designed with these data were successfully used to identify these *S. solani* isolates, however a step of fungal culture still remains necessary (Tooley et al., 1997; Le Cam et al., 2001; Pollastro et al., 2000; Zimand et al., 1994). In order to use these specific primers in the detection of this organism, further development is needed to detect the fungus from infected host plant as recommended by Le Cam et al., (2001). DNA-based techniques recently developed, enabled many uses in mycology and provided tools for both discrimination of closely related fungi and also identification of fungal species. The RAPD analyses have proved useful for distinguishing the isolates of *S. solani* used in this study. Additionally, the specific primers and PCR assay we have described may be used as a quick and reliable protocol for Senegalese *S. solani* isolates identification. Further studies need to be conducted to test these primers on other *Stemphylium* species and related fungi in order to assess the extent and limits of their specificity.

## Conflict of interests

The authors did not declare any conflict of interest.

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## Full Length Research Paper

# Optimization of flavor ester synthesis catalysed by *Aspergillus niger* lipase

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The performance of *Aspergillus niger* lipase in catalyzing of butyl butyrate synthesis was studied as a function of reaction parameters temperature (°C), substrate molar ratio and added water (% (v/v)). A face-centered design (FCD) and response surface methodology (RSM) were applied in order to optimize the esterification yield (Y). The optimal conditions for butyl butyrate synthesis were found at 40°C, substrate molar ratio of 1:2.41 butyric acid:n-butanol and added water content of 1.05% (v/v). Under these conditions, over 94.5% of esterification was obtained in 20 h. It was verified a favorable effect of alcohol excess in relation to acid. High temperatures (above 40°C) were detrimental to the synthesis of butyl butyrate. Addition of water also provided a strong increase in esterification yield (%). This high esterification yield represents an improvement to previously reported results and confirms the potential application of *Aspergillus niger* lipases (ANL) in processes for producing flavor ester.

**Key words:** Lipase, *Aspergillus niger*, esterification, butyl butyrate, flavor ester, optimization.

## INTRODUCTION

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are hydrolytic enzymes that reversibly catalyze the cleavage of ester bonds of triglycerides (Hasan et al., 2006; Guillén et al., 2012). These enzymes present numerous industrial applications, such as the synthesis of food ingredients, enantiopure drugs, refined products, additives to detergents and others. Nevertheless, few enzymes have demonstrated adequate stability and activity in organic media to be considered industrially applicable (Hasan et al., 2006; Dandavate and Madamwar, 2007; Martins et al., 2013).

Among microorganisms that produce lipase, *Aspergillus niger* is one of the most notable producers because its extracellular lipase is highly stable and recognized as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA) (Mhetras et al., 2009; Contesini et al., 2010). Lipase from *A. niger* also presents high positional specificity and lower cost when compared with other sources (Mhetras et al., 2009; Contesini et al., 2010). Although this enzyme has already been applied to reactions such as some esters synthesis (Mhetras et al., 2010; Romero et al., 2012), hydrolysis of

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triacylglycerol, alcoholysis (Chowdary and Prapulla, 2002) and interesterification, few works have been performed to exploit the potential from these lipases for synthesis of short chain aliphatic esters such as butyl butyrate (Langrand et al., 1990; Welsh and Williams, 1990; Chowdary and Prapulla, 2002; Contesini et al., 2010; Mhetras et al., 2010), an important flavor ester with great application in food, beverage and cosmetic industries with sweet fruity flavor similar to that of pineapple (Martins et al., 2013).

Butyl butyrate can be obtained by direct extraction from fruits or by chemical synthesis. The production of flavors by direct extraction from fruits is dependent on environmental factors which make the extraction process expensive and chemical synthesis often results in the formation of undesirable byproducts, reducing process efficiency and increasing downstream costs (Guillén et al., 2012; Martins et al., 2013). Therefore, the use of lipases in flavor ester synthesis results in lower cost as compared to the direct extraction process from fruits and higher selectivity than chemical synthesis, as well as acquiring of compounds considered "natural" (Martins et al., 2011; Guillén et al., 2012).

Thus, for utilization of *A. niger* lipase (ANL) in specific applications, knowledge on the reaction parameters for determining the high conversion yield is essential.

The aim of this study was optimize the synthesis of butyl butyrate catalyzed by *A. niger* lipase, using a face-centered design (FCD) coupled with the response surface methodology (RSM). Additionally, the butyl butyrate synthesis under the best conditions obtained was evaluated in order to determine the optimal reaction time for the industrial application of ANL.

## MATERIALS AND METHODS

### Materials and reagents

Lipase from *A. niger* was kindly provided by Prozyn BioSolution (São Paulo, Brazil). Specific hydrolytic activity of *A. niger* lipase (95.84 U/g<sub>protein</sub>) was estimated by hydrolysis of p-nitrophenyl palmitate (p-NPP) at pH 8.0 and 45°C (Winkler and Stuckmann, 1979). One unit (U) of hydrolytic activity is defined as 1 μmol of p-nitrophenol released per minute. Total protein content was determined according to the Bradford (1976) method. Butyl butyrate (CAS number 109-21-7), butyric acid (CAS number 107-92-6), n-butanol (CAS number 71-36-3) and hexane (CAS number 110-54-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

### Assay of synthesis activity

The synthesis reactions of butyl butyrate in organic media were carried out in screw-capped vials using n-butanol and butyric acid as substrates. The standard mixture was composed of 5 mL of hexane as the reaction medium. Temperature, substrate molar ratio (butyric acid: n-butanol) and added water (% by volume of reaction medium) were selected according to each defined experimental condition (Table 1). In all experiments, the butyric acid concentration was maintained constant at 0.120 M and the n-butanol con-

centration was varied as 0.182, 0.289 and 0.706 M.

The enzyme (approximately 0.08 g d.wt and 4.6 U) was initially weighted in the reaction vial and then added to the reaction mixture. This sample was incubated at the selected temperature for 24 h with continuous shaking. Sample blank were made at the same each substrate solution composition but without the enzyme.

Butyric acid consumption and the butyl butyrate formed were analyzed by high performance liquid chromatography (HPLC) using a liquid chromatograph (Shimadzu, Japan) with a diode array detector (Shimadzu, Japan), at a wavelength set at 210 nm. A reverse phase column (C18 apHera™, 250 × 4.6 mm, Supelco Analytical) was employed for chromatographic separation. The mobile phase was composed of acetonitrile/water (75:25, v/v) at a flow rate of 0.8 mL/min. A volume of 20 μL was injected into the chromatograph and the esters were eluted from the column using an isocratic method (Chen, 1996). The retention times of butyric acid and butyl butyrate under these conditions are 4.0 and 6.0 min, respectively. Each sample was previously filtered through a 0.45 μm membrane filter (Millipore, Bedford, USA).

The esterification yield ( $Y$ , %) in butyl butyrate synthesis was based on the amount of butyric acid consumed (Equation 1) (Martins et al., 2011):

$$Y\% = \frac{(C_0 - C_f)}{C_0} \times 100 \quad (1)$$

Where,  $C_0$  is the initial molar concentration of butyric acid (M) and  $C_f$  is the final molar concentration of butyric acid (M).

### Experimental design and statistical analysis

A face-centered design (FCD) with three independent variables, specifically temperature ( $X_1$ ), substrate molar ratio (butyric acid: n-butanol) ( $X_2$ ) and added water ( $X_3$ ) coupled with RSM was carried out in order to obtain the optimal conditions for synthesis reactions of butyl butyrate. RSM includes factorial designs and regression analysis and it is applied to evaluate the significance of several affecting factors even in the presence of complex interactions. For this design setup, the original and coded variables as well as each level are shown in Table 1.

The experimental data obtained from the FCD (Table 1) was analyzed by the response surface regression procedure (RSREG, SAS Institute Inc., v. 9.0, Cary, NC, USA) using the second order polynomial equation (Equation 2):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + e \quad (2)$$

Where,  $Y$  is the response variable,  $b_0$ ,  $b_i$ ,  $b_{ii}$  and  $b_{ij}$  are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively and  $X_i$  and  $X_j$  are the independent variables.

Statistical significance of the model was evaluated by analysis of variance (ANOVA) and the suitability of the model was assessed using Fisher's statistical test ( $F$ -test) by testing for significant differences between sources of variation in experimental results, that is, the significance of the regression, the lack of fit, and the coefficient of multiple determination ( $R^2$ ). For each parameter estimator ( $\beta_i$ ), a Student's  $t$ -test was performed. Parameters with less than 95% significance ( $p > 0.05$ ) were pooled into the error term. Results associated with the RSM were used to visualize the effect of the factors on the esterification reaction and to optimize this

**Table 1.** Experimental design and results of the face-centered design (FCD).

Assay	Temperature $X_1$	Substrate molar ratio $X_2$	Added water $X_3$	Esterification yield (%)
1	-1 (40.0) <sup>a</sup>	-1 (1: 5.88)	-1 (0)	12.27
2	-1 (40.0)	-1 (1: 5.88)	+1 (1.4)	86.82
3	-1 (40.0)	+1 (1:1.52)	-1 (0)	23.97
4	-1 (40.0)	+1 (1:1.52)	+1 (1.4)	99.98
5	+1 (50.0)	-1 (1: 5.88)	-1 (0)	15.55
6	+1 (50.0)	-1 (1: 5.88)	+1 (1.4)	19.75
7	+1 (50.0)	+1 (1:1.52)	-1 (0)	45.52
8	+1 (50.0)	+1 (1:1.52)	+1 (1.4)	79.99
9	-1 (40.0)	0 (1:2.41)	0 (0.7)	98.40
10	+1 (50.0)	0 (1:2.41)	0 (0.7)	97.32
11	0 (45.0)	-1 (1: 5.88)	0 (0.7)	70.13
12	0 (45.0)	+1 (1:1.52)	0 (0.7)	99.38
13	0 (45.0)	0 (1:2.41)	-1 (0)	44.66
14	0 (45.0)	0 (1:2.41)	+1 (1.4)	98.22
15	0 (45.0)	0 (1:2.41)	0 (0.7)	92.72
16	0 (45.0)	0 (1:2.41)	0 (0.7)	98.09
17	0 (45.0)	0 (1:2.41)	0 (0.7)	98.70
18	0 (45.0)	0 (1:2.41)	0 (0.7)	98.23

<sup>a</sup>Numbers in parenthesis represents the uncoded experimental values.

response, that is, to determine conditions that maximize yield of the esterification reaction. All statistical analyses were carried out using the SAS v.9 software (SAS Institute Inc., v. 9.0, Cary, NC, USA). All contour plots were generated by the SigmaPlot software version 11.0 for Windows (Systat Software, Inc., Point Richmond, California, USA).

## RESULTS

### Experimental design, model fitting and ANOVA

Experimental results obtained from the FCD are shown in Table 1. Statistical analysis of the esterification yield using the full quadratic model was tested by ANOVA using a Fisher statistical test. The results of the ANOVA can be visualized in a Pareto chart (Figure 1), in which the standardized estimated effect of each factor is plotted in decreasing order and compared with the minimum magnitude of a statistically significant factor with 95% confidence, represented by the vertical dashed line.

According to Figure 1, the variable that showed the highest estimated linear effect on esterification yield was the added water followed by its quadratic effect. The next linear significant effect was the substrate molar ratio and lastly the negative effect of temperature which points out the importance of working at low temperature reaction.

After removing the factors that were not statistically significant at a 95% confidence level, new reduced models were obtained for the response variable by regression analysis using only the significant factors

listed before. The ANOVA performed for the reduced models indicated that the model is statistically valid, with significant  $p$ -values ( $p < 0.0001$ ). These reduced models can be described by Equation 3, in terms of coded values.

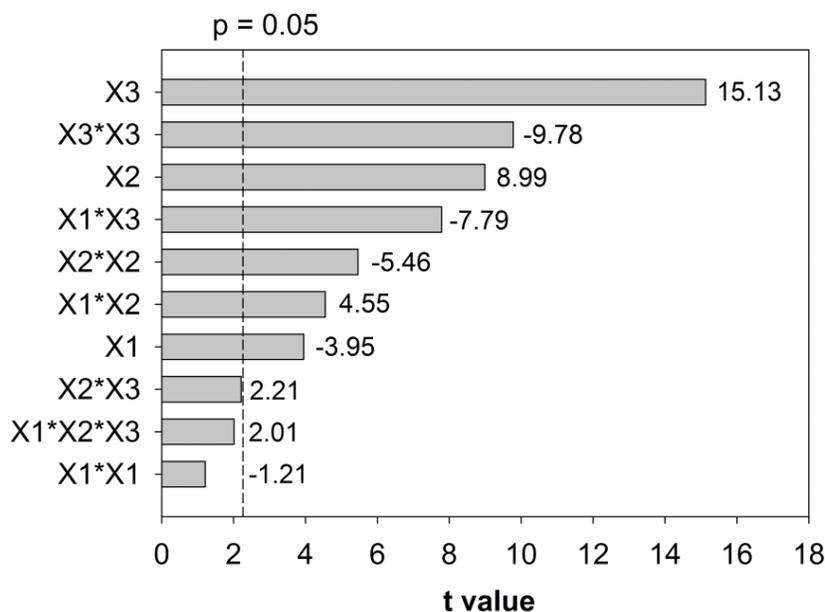
$$Y = 98.656 - 6.33X_1 + 14.43X_2 + 24.28X_3 - 18.15X_2^2 - 31.46X_3^2 + 8.17X_1X_2 - 13.98X_1X_3 \quad (3)$$

Where,  $Y$  is the percentage of esterification yield and  $X_1$ ,  $X_2$  and  $X_3$  are the coded values of temperature, substrate molar ratio and added water, respectively.

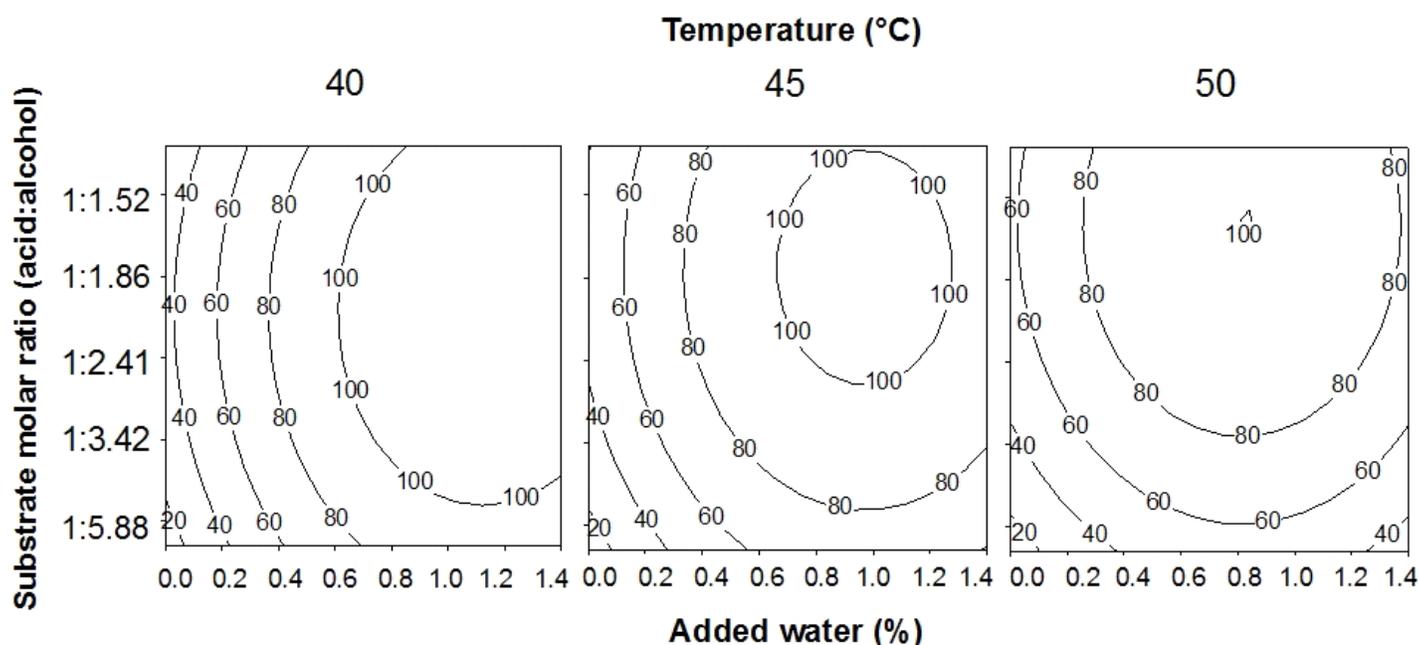
The coefficient of determination ( $R^2$ ) was found to be 0.98 and there are also a non-significant lack of fit ( $p > 0.06$ ). This result showed the suitability of the model for an appropriate representation of the real relationship among the reaction variables. The whole effects of the studied factors on esterification yield can be better visualized by examining the contour plots presented in Figure 2.

Observing the contour plots (Figure 2), it can be verified that at low temperatures and medium levels of substrate molar ratio and added water, the esterification yield was over 90%. The optimal experimental conditions for butyl butyrate synthesis corresponded to the reaction mixture incubated at 40°C, molar ratio of 1:2.41 butyric acid: n-butanol and 1.05% (v/v) of added water. Under these conditions, the experimental esterification yield was 98.8%.

Additionally, experiments were performed at the



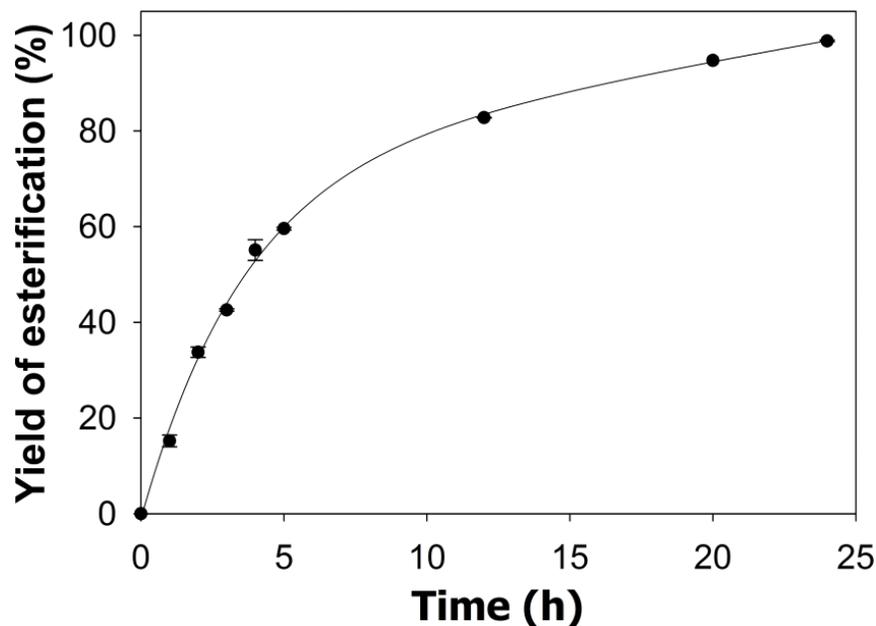
**Figure 1.** Pareto chart for the standardized effects of the variables: temperature ( $X_1$ ), substrate molar ratio ( $X_2$ ) and added water ( $X_3$ ) on the esterification yield.



**Figure 2.** Effects of reaction parameters: temperature ( $^{\circ}\text{C}$ ), substrate molar ratio (acid:alcohol) and added water (%) on esterification yield (%). The numbers inside the contour plots indicate esterification yields (%) at given reaction conditions.

optimal conditions defined by the experimental design in order to evaluate the reaction progress of butyl butyrate synthesis catalyzed by ANL. This is considered an important indicator of the ester synthesis since reaction time is related to efficiency and productivity. In this work, 60% of esterification yield was achieved after only 5 h of

synthesis reaction (Figure 3). Others works reported approximately 18, 24 and 25 h for maximal yields (74, 56 and 69.6%) of butyl butyrate synthesis using lipase of *Aspergillus* sp. (Langrand et al., 1990), *A. niger* (Chowdary and Prapulla, 2002) and *A.niger* ADF 75,000 (Welsh and Williams, 1990), respectively. This result is



**Figure 3.** Esterification yield (%) as a function of time for butyl butyrate synthesis catalyzed by *Aspergillus niger* lipase at 40°C, substrate molar ratio of 1:2.41 butyric acid:n-butanol and added water content of 1.05 % (v/v).

interesting for industrial applications, since most of previous literature reports longer times of synthesis reaction and smaller esterification yield.

## DISCUSSION

### Effect of water on esterification yield

As can be seen in Figure 2, the esterification yield increased with the increasing quantity of water added, whereas in the reaction system with no initial added water, a poor esterification yield (less than 46%) was observed.

Based on these results, it can be confirmed that the presence of water strongly contributed to ester synthesis. In fact, these results agreed with the studies of other researchers (Welsh and Williams, 1990; Monot et al., 1991) who suggested that an optimum amount of water greater than that found in the anhydrous solvent might be required to ensure enzyme activity, especially for reaction mixtures containing hydrophilic substrates. In the reaction media in which hydrophilic substrates are employed (butyric acid) the added water should be increased to prevent undesirable interactions between the hydrophilic substrates and enzyme hydration water, which are fundamental to maintain its active three-dimensional conformational form and mobility (Monot et al., 1991; Chen, 1996; Martins et al., 2011). However, because water is one of the products of the esterification reaction employing a primary aliphatic monoalcohol and an

aliphatic monoacid, it has been reported that an excess of water can decrease the esterification yield by shifting the equilibrium towards hydrolysis rather than synthesis (Goldberg et al., 1990; Guillén et al., 2012; Lorenzoni et al., 2012).

In the present study, it was found that the optimal content of added water was 1.05% (v/v) which was suitable for maintaining a proper enzyme hydration level during the full reaction time. Other researchers found that the optimum amount of water added to the reaction mixtures was 0.25% (w/w) for butyl acetate synthesis catalyzed by Novozym® 435 (Martins et al., 2011) and 5% (v/v) in butyl butyrate synthesis (Monot et al., 1991) catalyzed by *Mucor miehei* lipase.

### Effect of substrate molar ratio on esterification yield

Several researchers have suggested that high initial alcohol concentrations can shift the esterification reaction from hydrolysis to ester biosynthesis because the equilibrium of the reaction is directed toward product formation as the nucleophile (alcohol) concentration is elevated (Gubicza et al., 2000; Hari Krishna et al., 2001; Romero et al., 2005; Han et al., 2009; Mhetras et al., 2010; Martins et al., 2011; Romero et al., 2011). Thus, it was chosen to work with high initial alcohol concentrations, with a substrate molar ratio around 1:5 to 1:1.5 (acid : alcohol) and maintaining the butyric acid molar concentration fixed at 120 mM. The substrate molar ratios utilized are within the ranges commonly

studied (Yadav and Lathi, 2003; Melo et al., 2005; Mhetras et al., 2010; Guillén et al., 2012).

As shown in Figure 2, the extent of esterification was negatively affected by an alcohol excess, especially when the initial n-butanol concentration increased from approximately 1:3.42 to 1:5.88 (butyric acid:n-butanol).

This excess of alcohol content was detrimental to ester synthesis and could be attributed to the inhibitory effect of n-butanol on the reaction, since alcohol acts as a dead-end inhibitor of the enzyme in the synthesis of other esters (Yadav and Lathi, 2003). This same inhibitory effect was observed by others (Hari Krishna et al., 2001; Romero et al., 2005; Martins et al., 2013) when working with a substrate molar ratio of approximately 1:4 (acid:alcohol).

Therefore, it could be hypothesized that alcohol excesses in the reaction medium led to the formation of an inactive complex between the second substrate n-butanol and the binary enzyme-acyl complex, or affected acyl transfer due to alcohol binding, as other studies have reported (Chen, 1996; Hari Krishna et al., 2001; Romero et al., 2007). The best molar ratio of butyric acid to alcohol was found to be 1:2.41.

### Temperature effect on esterification yield

As reported in literature, the temperature parameter may favor the reaction kinetics or negatively affect the stability of the enzyme (Yadav and Lathi, 2003; Guillén et al., 2012). As is visualized in Figure 2, the esterification yield decreased with increases in temperature. This negative effect was also observed in biosynthesis of butyl butyrate (Santos and Castro, 2006) and ethyl butyrate (Guillén et al., 2012), and may be due to thermal inactivation of the enzyme which negatively affects its stability (Mahapatra et al., 2009).

It is interesting to note that the interaction among the variables temperature and substrate molar ratio presented a positive effect on the esterification yield. Thus, the reduction of butanol content in relation to fixed content of butyric acid (120 mM) and the raise of temperature promoted increases in the esterification yield.

This behavior can be explained based on the fact that high temperatures tend to make the substrate more diffusible and hence easily acceptable to the enzyme (Kontkanen et al., 2004). Therefore, at low levels of substrate molar ratio (1:2.41 to 1:5.88), the excess of butanol diffuses more easily into the catalytic site of the enzyme making their microenvironment toxic.

In the present study, the optimum temperature for butyl butyrate synthesis was found to be 40°C (Figure 2). Some authors reported the best results for butyl butyrate synthesis using lipase from *A. niger* (Welsh and Williams, 1990) and *Thermomyces lanuginosus* (Martins et al., 2013) at 50°C, and when using lipase from *Rhizomucor miehei* at 40°C (Lorenzoni et al., 2012).

### Conclusions

This work demonstrated that ANL is a suitable choice to catalyze the synthesis of butyl butyrate due to its high esterification yields (94.7% after 20 h of reaction). It was also concluded that the added water played an important role in maintaining the enzyme stability and mobility. This high esterification yield represents an improvement to previously reported results and confirms the potential application of ANL in processes for producing flavor ester.

### Conflict of interests

The authors declare that there is conflict of interest.

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## Full Length Research Paper

# Comparative study of ready-to-eat foods from road-side and eateries in Benin City, Nigeria

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The microbiological qualities of ready to eat foods sold in road-side and eateries were studied using the standard microbiological methods. The mean viable bacterial counts in food samples obtained from eateries ranged from  $2.4 \times 10^3 \pm 0.23$  to  $4.8 \times 10^4 \pm 0.23$  cfu/g, while the mean viable bacterial counts in food samples obtained from road side canteen ranged from  $9.0 \times 10^4 \pm 0.43$  to  $2.20 \times 10^5 \pm 0.40$  cfu/g. The fungal counts in the food samples obtained from eateries ranged from  $3.0 \times 10^3 \pm 0.15$  to  $3.5 \times 10^4 \pm 0.18$  cfu/g while the fungal counts in food samples obtained from road side ranged from  $2.5 \times 10^4 \pm 0.33$  to  $5.3 \times 10^4 \pm 0.22$  cfu/g. The microorganisms isolated were identified based on their cultural characteristics, Gram staining and biochemical tests. A total of eight bacterial isolates were obtained from the salad samples which included *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Micrococcus* sp., *Escherichia coli*, *Bacillus licheniformis* and *Klebsiella* sp. The fungi isolated were: *Saccharomyces cerevisiae*, *Mucor mucedo*, *Aspergillus flavus*, *Fusarium* sp., *Aspergillus niger*, *Penicillium* sp. and *Rhizopus* sp. Therefore, good personal hygiene, proper sanitation practice and the use of clean utensils during the preparation of ready to eat foods are recommended to avoid food poisoning and spoilage associated with the isolated microorganisms.

**Key words:** Ready-to-eat foods, hygiene, microbial analysis, food poisoning, canteen.

## INTRODUCTION

Ready-to-eat foods can be described as foods ready for immediate consumption at the point of sale. They could be raw or cooked, hot or chilled and can be consumed without further heat treatment (Clarence et al., 2009; Tsang, 2002; Oranusi and Braide, 2012; Mahakarnchanakul et al., 2010). There are certain factors that make street foods popular. These include familiarity, taste, low cost and convenience (Mahakarnchanakul et al., 2010). Our

society shows a social pattern characterized by increased mobility, large number of itinerant workers and less family or home centered activities. This situation, however, has resulted in more ready-to-eat foods taken outside the home. Thus, food vendor services are on the increase and the responsibility of good manufacturing practices of food including good sanitary measures and proper handling have been transferred from individuals/families

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to the food vendors who rarely enforce such practices (Musa and Akande, 2002; Clarence et al., 2009).

Food consumption is an important pathway for bacteria to infect humans, hence the presence of antimicrobial resistant bacteria in foods warrants particular attention. Antimicrobial resistant bacteria have been recovered from both healthy humans (Okeke et al., 2000) and a wide variety of foods, which include vegetables (Osterblad et al., 1999), confectionary (Pinegar and Cooke, 1985) meat and meat products and poultry (Schoeder et al., 2004). Hence food contaminated by faecal material from healthy humans may be an important source of antibiotic resistant organisms that later cause human infections (Teuber, 1999; Schoeder et al., 2004).

Contamination of food may occur during and after processing of such food. Contamination of ready-to-eat food is of primary concern because such organisms may be pathogenic thereby leading to outbreak of food-borne illness. Food-borne illnesses may occur when food sources that contain pathogenic microorganisms are consumed raw or improperly cooked (Sangoyomi et al., 2012). The health implications cannot be over-emphasized. *E. coli* for instance can induce gastroenteritis (Olowe et al., 2008); *Staphylococcus aureus* isolates have been implicated in a number of clinical cases (Adegoke and Komolafe, 2009; Serrano et al., 2004; Adegoke et al., 2010). Ready-to-eat foods have been reported to be easily available, affordable, provide diverse/variable food source, employment and with a potential for improving food security and nutritional status and general social security (Draper, 1996). It is, however, a variable source of food-borne pathogen (Abdussalam and Kaferstein, 1993; Arambulo et al., 1994; Mensah, 2002; Patricia and Azanza, 2005). Moreover, non-pathogenic organisms that may contaminate man's food chain from time to time may serve as reservoir of genes for antimicrobial resistance in organisms. These genes are encoded by integrons that occur on plasmids or that are integrated into the bacterial chromosome. Antimicrobial resistant strains of animal or human commensals that do not produce disease may transmit their resistance genes to pathogenic organisms whenever they occur in humans.

In Nigeria, most of these products are stored under unhygienic conditions. They are often displayed in open trays or container in the market or are hawked along the street and major runways. Contamination of food can occur at any point in the production chain (from the raw materials, processing, packing, transportation, storage or marketing) to consumption. Because of improper processing, handling and storage of these foods could be subject to contamination by microorganism (Musa and Akande, 2002).

In Nigeria, there is little or no knowledge of transmission of foodborne diseases among food handlers working in ready-to-eat food centers. Most proprietors of ready-to-eat food centers are not duly licensed and their staff properly selected. Therefore, this work was designed to

examine the microbiological quality of ready-to-eat foods from road-side and eateries in Benin City, to reduce the risk of food poisoning.

## MATERIALS AND METHODS

### Sample collection

A total of 18 ready-to-eat food samples were purchased from road-side local food centers and high class eateries within Benin metropolis. All samples were collected in sterile containers and were taken under aseptic condition to the laboratory for microbiology analysis.

### Sample preparation

Ten grams of each sample were weighed and mashed in a sterile laboratory mortar and pestle and aseptically introduced into 90 ml of sterile distilled water, properly shaken before a 10 fold serial dilution was prepared.

### Preparation of culture media

All media were prepared accordingly to manufacturer's instruction. The media used in this study were nutrient agar (used for heterotrophic bacterial count) and potato dextrose agar (used for fungal count).

### Isolation and enumeration of microorganisms

One milliliter from appropriate dilutions was plated out by pour plate method on nutrient agar and potato dextrose agar. The nutrient agar plates were incubated at 37°C for 24 h while the potato dextrose agar plates were incubated at room temperature 28°C for 72 h. After incubation, discrete colonies of culture on nutrient agar and potato dextrose agar plates were counted and expressed in cfu/ml.

### Characterization and identification of bacterial isolates

Bacterial isolates were identified on the basis of cultural morphological and biochemical tests according to Jolt et al. (1994) and Cheesbrough (2006). The fungal colonies were identified as described by Harrigan (1998).

### Total coliform count

From the homogenate, 1 ml of serially diluted (1:10, 1:100 and 1:1000) sample was transferred into 9 ml sterilized lauryl sulphate tryptose (LST) broth in triplicate. For each dilution, the tubes were incubated at 35°C for 48±2 h to evaluate tryptose broth used as a pre enrichment medium. After primary incubation, one (0.3 mm) loopful of positive tube (gas formation by the action of chloroform bacterial in fermenting lactose medium tube) was transferred to Brilliant Green Lactose Bile (BGLB) broth, further incubated at 35°C for 48 ± 2 h for total coliforms count. Inverted Durham fermentation tubes were added into test tubes before the addition of BLGB broth to allow easy identification of gas production. Then, the number of tubes with positive gas production was counted. Most probable number (MPN) of coliform bacteria per gram sample was calculated from MPN table based on number of tubes of BGLB broth producing

**Table 1.** Total viable bacterial counts (cfu/g) in the ready-to-eat food samples.

Samples	Eateries	Road side canteen
	Mean±SD	Mean ±SD
Egusi soup	8.0 x10 <sup>3</sup> ±0.10	7.2 x10 <sup>5</sup> ±2.92
Salad	4.9 x10 <sup>4</sup> ±1.37	1.22 x10 <sup>5</sup> ±0.40
Pounded yam	0.0	7.4 x10 <sup>4</sup> ±0.63
Vegetable soup	1.29 x10 <sup>4</sup> ±0.16	2.20 x10 <sup>5</sup> ±0.40
Moimoi	6.1 x10 <sup>3</sup> ±0.39	1.58 x10 <sup>3</sup> ±0.70
Beans	8.2 x10 <sup>3</sup> ±0.13	2.13 x10 <sup>5</sup> ±0.63
Porridge yam	4.8 x10 <sup>4</sup> ±0.23	1.43 x10 <sup>5</sup> ±0.23
Jollof rice	2.4 x10 <sup>3</sup> ±0.23	1.03 x10 <sup>5</sup> ±0.43
Amala	3.1 x10 <sup>3</sup> ±0.40	9.0 x10 <sup>4</sup> ±0.43

**Table 2.** Total fungal counts (cfu/g) in the ready-to-eat food samples.

Samples	Eateries	Road side canteen
	Mean±SD	Mean±SD
Egusi soup	8.0 x10 <sup>3</sup> ±0.01	5.3 x10 <sup>4</sup> ±0.22
Salad	3.0 x10 <sup>3</sup> ±0.15	4.3 x10 <sup>4</sup> ±0.29
Pounded yam	9.0 x10 <sup>3</sup> ±0.10	2.5 x10 <sup>4</sup> ±0.33
Vegetable soup	3.5 x10 <sup>4</sup> ±0.18	9.5 x10 <sup>4</sup> ±0.20
Moimoi	6.6 x10 <sup>3</sup> ±0.05	3.2 x10 <sup>4</sup> ±0.27
Beans	1.4 x10 <sup>4</sup> ±0.10	4.7 x10 <sup>4</sup> ±0.21
Porridge yam	4.0 x10 <sup>3</sup> ±0.08	3.5 x10 <sup>4</sup> ±0.19
Jollof rice	3.0 x10 <sup>3</sup> ±0.15	2.1 x10 <sup>4</sup> ±0.30
Amala	1.5 x10 <sup>3</sup> ±0.04	7.0 x10 <sup>4</sup> ±0.15

**Table 3.** Microbial isolates from food samples.

Bacterial isolates	Fungal isolates
<i>Staphylococcus aureus</i>	<i>Mucor mucedo</i>
<i>Bacillus subtilis</i>	<i>Aspergillus flavus</i>
<i>Enterobacter aerogenes</i>	<i>Saccharomyces</i>
<i>Pseudomonas aeruginosa</i>	<i>Penicillium</i> sp.
<i>Micrococcus</i> sp.	<i>Aspergillus niger</i>
<i>Escherichia coli</i>	<i>Fusarium</i> sp.
<i>Bacillus licheniformis</i>	<i>Rhizopus</i> sp.
<i>Klebsiella</i> sp.	

gas at the end of incubation period.

## RESULTS AND DISCUSSION

The mean viable bacterial counts in food samples obtained from eateries ranged from 2.4 x10<sup>3</sup>±0.23 to 4.8 x10<sup>4</sup>±0.23 cfu/g while the mean viable bacterial counts in

food samples obtained from road side canteen ranged from 9.0 x10<sup>4</sup>±0.43 to 2.20 x10<sup>5</sup>±0.40 cfu/g (Table 1). The highest bacterial counts was recorded in vegetable soup obtained from road side canteen while the lowest bacterial counts was recorded in *jollof* rice obtained from eateries. The fungal counts in the food samples obtained from eateries ranged from 3.0 x10<sup>3</sup>±0.15 to 3.5 x10<sup>4</sup>±0.18 cfu/g while the fungal counts in food samples obtained from road side ranged from 2.5 x10<sup>4</sup>±0.33 to 5.3 x10<sup>4</sup>±0.22 cfu/g (Table 2). The salad samples obtained from eateries had the lowest fungal counts while *egusi* soup obtained from road side canteen had the highest fungal counts. The microbial counts in the food samples obtained from road side were comparably higher than the microbial loads of food samples obtained from eateries. The microorganisms isolated from the ready to eat food samples analyzed included eight bacterial isolates and seven fungal isolates. The bacterial isolates were *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Micrococcus* sp., *Escherichia coli*, *Bacillus licheniformis* and *Klebsiella* sp. The fungal isolates include *Saccharomyces cerevisiae*, *Mucor mucedo*, *Aspergillus flavus*, *Fusarium* sp., *Aspergillus niger* and *Rhizopus* sp., this is seen in Table 3. The occurrence of the microorganisms isolated from the ready to eat food samples is presented in Tables 4 and 5.

Despite the high level of contamination of the ready-to-eat foods in this study, most of the sampled foods contained total aerobic plate counts of 10<sup>3</sup> to 10<sup>5</sup> cfu/g. These foods are therefore considered fit for human consumption (ICMSF, 1974; FAO/WHO, 2005). The ICMSF (1974) identified foods with counts >10<sup>5</sup> cfu/g as unacceptable for consumption. The high microbial load recorded for food samples obtained from road side canteen could be associated with the poor hygienic practices and handling of the product by the sellers. Lack of storage facilities could have heightened the chances of contamination (Baro et al., 2007). The ready-to-eat foods are maintained throughout the day under room temperature, this encourages proliferation of contaminants. The microorganisms isolated from the ready to eat food samples analyzed included eight bacterial isolates and seven fungal isolates. Our results revealed that the bacterial isolates were *S. aureus*, *B. subtilis*, *E. aerogenes*, *P. aeruginosa*, *Micrococcus* sp., *E. coli*, *B. licheniformis*, *Klebsiella* sp. This finding is similar to previous reports by Desmarchelier et al. (1994), Nichols et al. (1999) and Mensah et al. (2002). The fungal isolates include *S. cerevisiae*, *M. mucedo*, *A. flavus*, *Fusarium* sp., *A. niger* and *Rhizopus* sp. This result was in agreement to that reported by Wogu et al. (2011), who isolated similar fungi from ready-to-eat foods, although few data are available on the occurrence of fungi in ready-to-eat food. Some of the microorganisms isolated from the ready to eat have been reported to be associated with food spoilage and food poisoning (Jay, 1996). Organisms implicated in

**Table 4.** Distribution of the bacterial isolates in the food samples.

Bacterial isolates	Egusi soup	Salad	Pounded yam	Vegetable soup	Moimoi	Beans	Porridge yam	Rice	Amala
<i>S. aureus</i>	-	+	+	+	+	+	-	+	+
<i>B. subtilis</i>	+	+	+	-	+	+	-	+	+
<i>E. aerogenes</i>	+	-	-	+	-	-	+	-	-
<i>P. aeruginosa</i>	+	+	-	+	-	-	+	-	-
<i>Micrococcus</i> sp	-	-	-	-	+	+	+	-	+
<i>E. coli</i>	-	+	-	-	-	-	-	-	-
<i>B. licheniformis</i>	-	-	+	-	+	+	-	+	-
<i>Klebsiella</i> sp	+	+	+	+	-	-	+	-	-

+ = Present; - = Absent.

**Table 5.** Distribution of the fungal isolates in the food samples.

Fungal isolates	Egusi soup	Salad	Pounded yam	Vegetable soup	Moimoi	Beans	Porridge yam	Rice	Amala
<i>Mucor mucedo</i>	+	+	-	+	-	+	+	-	+
<i>Aspergillus flavus</i>	+	-	+	+	+	+	-	-	+
<i>Saccharomyces cerevisiae</i>	-	+	-	-	-	-	-	-	+
<i>Penicillium</i> sp	+	+	+	-	+	-	+	+	-
<i>Aspergillus niger</i>	+	+	+	+	+	+	-	+	-
<i>Fusarium</i> sp	+	+	+	+	-	-	+	-	-
<i>Rhizopus</i> sp	-	-	-	-	-	-	-	+	-

+ = Present; - = Absent.

food-borne diseases are numerous and diverse bacteria, fungi, viruses and parasites (Jay, 1996; Chukwu et al., 2010; De Rover, 1998). *E. coli*, *S. aureus*, *Bacillus* species and *Pseudomonas* sp. were isolated from the ready to eat foods indicating poor sanitary control and practices. These organisms are known food borne pathogens and opportunistic pathogens that have been implicated in food-borne disease outbreaks (Oranusi and Braide, 2012; Mudgil et al., 2004; Oranusi et al., 2006, 2007; Yadav et al., 2011; Tambeker et al., 2008).

Fungi are common environmental contaminants due to their ability to produce spores; this could explain their presence in ready to eat food. They have been implicated in ready to eat foods. Species of *Aspergillus*, *Penicillium* and *Fusarium* are known to produce deleterious mycotoxins under favourable conditions (Oranusi and Braide, 2012), their presence in ready to eat food must therefore be treated with caution. *Aspergillus* species which were the most predominant in this study are among the most abundant and widely distributed organisms on earth. Members of this genus are saprophytic moulds. Most live in the environment without causing disease. Virtually, all the common *Aspergillus* species have been recovered at some time from agricultural products. *A. niger* and *A. flavus* were isolated from the ready to eat food samples analyzed.

The occurrence of *S. cerevisiae* may be due to its role in fermentation of agricultural products. *S. cerevisiae* is

well known for converting carbohydrates into alcohol and other aroma compounds such as esters, organic acids and carbonyl compounds. Their presences in food sometimes represent assimilation of glucose, galactose and lactic acid by them. Examination for the presence of pathogens in ready-to-eat food contributes to food safety (Roberts and Greenwood, 2003; Elviss et al., 2009; Willis et al., 2009). Although low numbers of food pathogens probably represent a low risk, their presence can suggest fault(s) in the production and/or subsequent handling which, if not controlled, could lead to an unacceptable increase in risk (Health Protection Agency, 2009). The presence of *Bacillus* sp. in these foods indicate the possibility that spices such as pepper have been added after the main cooking process (Health Protection Agency, 2009). Evidence show that food can be a reservoir of extra-intestinal infections caused by epidemic strains of *E. coli* causing uncomplicated urinary tract infections (Manges et al., 2008) and other severe infections (Johnson et al., 2002; Manges et al., 2006).

## Conclusion

The results of this present study indicated that the ready-to-eat food samples sold in road side canteen and eateries had microbial contaminants. Hence, it is recommended that a more close supervision of ready-to-eat

food should be carried out by relevant authorities.

### Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

## Characterization of *Pleurotus* sp. of mushroom based on phenotypic, biochemical and yield parameter

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The present investigations were carried out to study the cultural characteristics, morphological characteristics and protein percent of 10 different species of genus *Pleurotus* namely *Pleurotus sajor caju*, *Pleurotus flabellatus*, *Pleurotus platypus*, *Pleurotus fossulatus*, *Pleurotus florida*, *Pleurotus citrinopileatus*, *Pleurotus sapidus*, *Pleurotus djamor*, *Pleurotus ostreatus* and *Hypsizygus ulmarius*. Results obtained show that the fruiting bodies of the 10 species were of seven different colors. Diameter of the fruiting bodies ranges between 5.4 and 8.8 cm whereas length ranges between 4.9 and 13.9cm. *H. ulmarius* was found to be the best yielder (97.50% B.E.) with highest percentage (33.6) of protein content.

**Key words:** Oyster mushroom, morphological character, cultural character, protein, biological efficiency, yield.

### INTRODUCTION

For vegetarian's mushrooms are considered as healthy food because their mineral content is higher than that of meat or fish. Besides their nutritional value, they have medicinal properties also. Mushrooms are an important source of income and nutrition in both developed and developing countries (Chan, 1981). There are more than 5000 varieties of mushrooms present which could be employed as a source of food and medicine (Chang and Miles, 1991; Boa, 2004). Almost all edible mushrooms are members of Basidiomycotina and Ascomycotina (Sharma, 1989). Mushrooms have low fat content, high fiber and all essential amino acids (Sadler, 2003). On

exposure to UV light, mushrooms produce large amounts of vitamin D. it has been found that mushrooms have some beneficial effects on cancer, HIV-1, AIDS and other diseases (Beelman et al., 2003). The three primary factors which are essential of mushroom production are temperature, compost component and humidity (Dung, 2003). Cultivation process of oyster mushrooms include 3 main steps- Isolation of mushrooms from fruiting bodies, preparation of primary and secondary spawn and cultivation of oyster mushrooms from these spawns to harvest fruiting bodies (Dung et al., 2012). The genus *Pleurotus* (Fr.) comprises various edible mushroom

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species and has important medical and biotechnological properties and environmental applications (Nelson et al., 2010). *Pleurotus* mushroom generally called oyster mushroom has gained prominence as a type of edible mushroom due to their culinary properties and wider adaptability.

There are 38 species of the genus *Pleurotus* recorded throughout the world (Singer, 1986). In recent year, 25 species were commercially cultivated in different parts of the world, such as follows: *Pleurotus ostreatus*, *Pleurotus flabellatus*, *Pleurotus florida*, *Pleurotus sajor caju*, *Pleurotus sapidus*, *Pleurotus eryngii*, *Pleurotus fossulatus*, *Pleurotus opuntiae*, *Pleurotus australis*, *Pleurotus purpurcoolivaceus*, *Pleurotus populinus*, *Pleurotus levis*, etc. The present study was conducted to assess the cultural and morphological characters along with yield and protein content in ten *Pleurotus* species.

## MATERIALS AND METHODS

### Cultural characteristics

The cultures of 10 species of oyster mushroom in which *P. djamor* and *H. ulmarius* were first time included for such typical study were obtained from Mushroom Research Laboratory of Chandra Shekhar Azad University of Agriculture and Technology, Kanpur for the present study. Cultural characteristics such as growth pattern, colors, diameter and length of fruiting bodies were observed on potato dextrose agar medium and wheat straw substrate, respectively.

### Spawn production

Grain spawn of all ten species was prepared using the standard methodology suggested by Garcha (1994). Healthy, uncrumpled wheat grains were washed and boiled (grain: water 1:25 w/v) to tender without rupturing of the seed coat. Extra water was drained off and the grains were allowed to dry on sieve. Commercial grade gypsum and calcium carbonate were mixed at 3% of grain to maintain pH level. The grains were filled in clean glass bottles and the bottles were plugged with non-absorbent cotton and sterilized at 22 lbs steam pressure for 90 min. Sterilized bottles were taken out of the autoclave, while still hot and were shaken to avoid clumping of the grains. The next day, the bottles were inoculated with bits of agar medium colonized with mycelium of pure culture (7-10 days old). Inoculated bottles were incubated at 25°C. After 7 and 10 days of inoculation, bottles were shaken vigorously so that mycelial threads were broken and become well mixed with the grains. Entire grains were covered with fine mycelial growth after 18 days of inoculation.

### Substrate preparation

The substrate wheat straw was filled in gummy bags and these bags were soaked in a tank with water chemically treated with Bavistin 7 g + formalin 115 ml per 100 L water for 12 h (tank was covered with polythene sheet to prevent the evaporation of formalin). Thereafter, substrate were taken out of tank and spread on cemented floor treated with 2% formalin solution for 2-4 h to drain out excess water. The correct water content of the substrate was determined by squeezing the substrate in the palm; about 67% moisture was maintained (Savalgi and Savalgi, 1994).

### Spawning and spawn run

Spawning was done under aseptic conditions. The grain spawn of different species of *Pleurotus* were mixed thoroughly @ 2% in the substrate containing 65-70% moisture filled up in polythene bags @ 4 kg each. After spawning, bags were kept in the crop room temperature (24 to 28°C) and relative humidity (80 to 85%) was maintained for spawn run. Humidity was maintained by spraying water twice a day.

After the completion of spawn run in the straw, it became a compact mass, which is also sticking to the polythene bags. The polythene bags were then cut by sharp and sterilized blade and opened for sporophore formation. At the time of sporophore formation, the windows were kept open for 1-2 h to provide fresh air inside the crop room and release of CO<sub>2</sub> and maintaining the relative humidity at 80-90%. Total harvesting period given was 40 days. The required care was taken to avoid the occurrence of pests by spraying Dimacron @ 0.2%.

### Biological efficiency

Biological efficiency of the substrate was calculated by using following formula:

$$\text{Biological efficiency} = \frac{\text{Fresh weight of fruit body}}{\text{Dry weight of substrate}} \times 100$$

The protein content was estimated through Kjeldahl method (A.O.A.C., 1970). The estimation was done thrice, to compare the protein content of different species of oyster mushroom.

### Protein estimation

Total protein content of different species of selected mushrooms was estimated by Kjeld plus nitrogen analyzer. Total protein nitrogen was estimated by this method, multiplied by a factor for estimating the total protein content.

## RESULTS AND DISCUSSION

### Cultural characteristics

Observations on the morphological characteristics *viz.* growth pattern, colour, diameter and length of fruiting bodies are summarized in Table 1. The pattern of mycelial growth on potato dextrose agar in Petri plate in the case of *P. sajor caju* and *P. platypus* were compact whereas in *P. citrinopileatus* was highly fluffy. Similarly pattern of mycelium in *P. fossulatu*, *P. flabellatu* and *P. sapidus* were slightly fluffy, sparse growth in *P. djamor* and cottony growth in *H. ulmarius* were observed. Colour of fruiting bodies of all 10 species was of seven types (off white to bluish grey). As far as diameter and length of fruiting bodies is concern, the ranges are from 5.4 to 8.8 cm and 4.9 to 13.9 cm, respectively as given in Table 1.

### Yield performance

From the data presented in the Table 2, it is clear that *H. ulmarius* gave significantly higher yield (975.0 g/kg of

**Table 1.** Cultural and morphological characteristics of *Pleurotus* species.

Name of species	Growth pattern on PDA	Characteristics of fruiting bodies on Wheat straw substrate		
		Colour	Diameter (cm)	Length (cm)
<i>P. sajor caju</i>	Compact	Grey	5.4	11.0
<i>P. citrinopileatus</i>	Highly fluffy	Milky white	8.5	12.8
<i>P. fossulatus</i>	Slightly fluffy	Off white	7.6	11.4
<i>P. florida</i>	Sparse	Milky white	6.7	12.5
<i>P. flabellatus</i>	Slightly fluffy	Milky white	8.2	13.9
<i>P. ostreatus</i>	Fluffy	Cream white	8.8	13.2
<i>P. sapidus</i>	Slightly fluffy	Off white	5.9	11.6
<i>P. djamor</i>	Sparse	Pink	7.5	4.9
<i>P. platypus</i>	Compact	Cream	6.1	9.5
<i>H. ulmarius</i>	Cottony	Bluish grey	8.1	13.0

**Table 2.** Yield performance and protein analyses of different *Pleurotus* species used under the study.

Name of Species	No. of fruiting bodies	Yield in g/kg substrate	Biological efficiency (%)	Protein (%)
<i>P. sajor caju</i>	46	638.5	63.85	32.3
<i>P. citrinopileatus</i>	88	685.2	68.52	31.5
<i>P. fossulatus</i>	57.2	270.0	27.00	28.8
<i>P. florida</i>	96.2	531.7	53.17	33.5
<i>P. flabellatus</i>	17.7	538.7	53.87	30.3
<i>P. ostreatus</i>	86.5	755.5	75.55	27.6
<i>P. sapidus</i>	68.0	501.2	50.12	32.3
<i>P. djamor</i>	48.2	750.5	75.05	30.6
<i>P. platypus</i>	77	390.0	39.00	28.4
<i>H. ulmarius</i>	48	975.0	97.50	33.6

substrate) followed by *P. ostreatus* (755.5 g/kg of substrate), *P. djamor* (750.5 g/kg of substrate) *P. citrinopileatus* (685.2 g/kg of substrate). *P. sajor caju* (638.5 g/kg of substrate), *P. flabellatus* (558.7 mg/kg substrate) *P. florida* (531.7 g/kg substrate), *P. sapidus* (501.2 g/kg of substrate), *P. platypus* (390.0 g/kg of substrate) and *P. fossulatus* (270 g/kg of substrate).

These results are in partial agreement and disagreement with the findings of the earlier worker (Dundar et al., 2008) who determined that fresh mushroom yield from WS, CS, MS and SS substrate media were 17.9, 14.3, 22.7 and 31.5 g, respectively. *P. djamor* and *H. ulmarius* were included in such type a study for the very first time. Singh et al. (2010) also found *H. ulmarius* as the best yielder with the highest percentage of protein (33.6).

### Protein analysis

The protein content of oyster mushrooms was estimated by Kjeldahl method on dry weight basis, the data is pre-

sented in the Table 2. *Hypsizygus ulmarius* gave significantly higher protein (33.6%) followed by *P. florida* (33.5%), *P. sajor caju* and *P. sapidus*, which both have equal amount of protein (32.3%), *P. citrinopileatus* (32.5%) *P. djamor* (30.6%), *P. flabellatus* (30.3%) *P. fossulatus* (28.8%), *P. platypus* (28.4%) and *P. ostreatus* (27.6%) had different protein contents. In a study conducted by Khan et al. (2008), it was found that protein content was highest in *P. sajor-caju* (24.5 g/100 g of dry weight). Dundar et al. (2008) conducted a study with three species of mushrooms and found that *P. sajor-caju* is best among all the other species.

Studies carried out on crude mushroom protein by Lintzel (1943) suggested 34 - 89% of the protein digest easily. Further, Fitzpatrick et al. (1946) and Gilbert and Robinson (1957) indicated a digestibility of 60 - 70%. Various scientists such as Bose and Bose (1940), Bano et al. (1964) and Chang (1990) observed the protein content of various species of mushrooms.

It is concluded from the present study that the cultivation of *H. ulmarius* could be popularized at large scale due to greater biological efficiency (97.5) and higher

protein content (33.6).

### Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

## Isolation of *Escherichia coli* from cattle and lechwe antelopes at the livestock/wildlife interface area of the Kafue flats in Zambia

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This study was conducted at the livestock/wildlife interface areas of national parks and traditional cattle grazing areas. The main aim of the study was to establish the occurrence of *Escherichia coli* serogroups isolated from Kafue lechwe antelopes (*Kobus leche kafuensis*) and pastoral cattle. A total of 593 faecal samples from lechwe (232) and pastoral cattle (361) were conveniently picked from the grazing pastures in the interface areas and cultured for *E. coli*. From each faecal sample, two or three presumptive *E. coli* isolates were picked to constitute 1,283 isolates with 574 from lechwe and 708 from cattle. Some of these isolates were found to be similar to Shiga toxin-producing *E. coli* O157 on CHROMagar and sorbitol MacConkey agar. Only 18 *E. coli* isolates from Kafue lechwe antelopes were grouped into eight serogroups while 32 from pastoral cattle were grouped into 16 serogroups. The most prevalent type-able serogroups from lechwe antelopes and pastoral cattle were O125 (5.8%) and O29 (4.2%), respectively. On further analysis by fermentation of various sugars, some isolates showed a similar pattern suggesting that the strains were indistinguishable.

**Key words:** *Escherichia coli*, diarrheagenic, Shiga toxin, *Kobus leche kafuensis*, pastoral cattle.

### INTRODUCTION

The genus *Escherichia coli* comprise several serotypes among which are the most important bacterial public

health hazards. The serotypes are known to cause diverse intestinal and extra intestinal diseases by means

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of different mechanism that affect a wide range of cellular processes (Kaper et al., 2004). The serotypes that are associated with disease of humans are classified under six recognized categories; Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC) and Enteroaggregative *E. coli* (EAEC) (Garcia and Bouguenec, 1998; Nataro and Kaper, 1998; Presterl et al., 2003). Each category has distinct virulence factors, serogroups, epidemiological features and produces characteristic clinical symptoms (Levine, 1987). Diarrheagenic outbreaks are mainly because of consumption of contaminated food of animal origin and water. Meat from food animals (wild and domestic) has been identified as an occasional vehicle of enteric infections (Keene et al., 1997). Various studies have shown meat from deer and cattle as being responsible for some reported cases (Rabatsky-Her et al., 1987; Hornitzky et al., 2005; Fairbrother and Nadeau, 2006).

In Zambia, meat from wildlife is a major source of animal protein, with the Kafue lechwe (*Kobus lechwe kafuensis*) antelope being one of the most sought after game animal for consumptive utilization (Stafford, 1991; Siamudaala et al., 2003). It is for this reason that it becomes imperative to know the microbiological safety of meat and meat products derived from Kafue lechwe antelopes given the less than optimal handling and hygienic standards during evisceration and general processing. Other investigators suggest that poorly processed wildlife meat could serve as a source of human infections (Rabatsky-Her et al., 1987; Keene et al., 1997). Therefore, this study was aimed at elucidating evidence that Kafue lechwe antelopes which are in close contact with pastoral cattle in the livestock/wildlife interface areas may be asymptomatic carriers of diarrheagenic *E. coli* (DEC) serogroups. Human infections can occur from the ingestion of contaminated food of animal origin (Angulo et al., 2000). For instance, ETEC serotype 078 is known to cause severe diarrhea outbreaks (Ryder et al., 1976; Germani et al., 1985) while EPEC serogroup O125 is also known to cause a profuse watery diarrheal disease in developing countries (Regua et al., 1990). EPEC-like organisms have also been isolated from animals such as rabbits, pigs and dogs (Zhu et al., 1994; Nataro et al., 1998) and therefore knowledge on the occurrence of pathogenic *E. coli* in animal species meant for food is important.

## MATERIALS AND METHODS

### Study area

The study was conducted in the livestock/wildlife interface areas of Lochinvar (410 km<sup>2</sup>) and Blue-Lagoon (420 km<sup>2</sup>) National Parks on the Kafue Flats in Zambia that provide a unique interaction between livestock and wildlife as over 300,000 herds of cattle are moved from the upland areas to the wetlands during the drier months of the year. The Blue Lagoon and Lochinvar National Parks provide a

rich lucustrine habitat for the Kafue lechwe antelopes. GMAs are interface areas where interaction between wildlife and humans is facilitated through transhuman livestock herding activities.

### Sample collection

Samples were collected from Kafue lechwe antelope and cattle that shared grazing pasture on the Kafue flats. Faecal samples from lechwe antelopes were collected in two categories. The first category, involved 2 g faecal contents from the rectum of the 77 hunter harvested animals from Lochinvar ( $n = 52$ ) and Blue-lagoon ( $n = 25$ ). The second category involved 155 faecal samples of about 5 g each from freshly voided grazing lechwe antelopes in Lochinvar ( $n = 110$ ) and Blue-lagoon ( $n = 45$ ). The collected samples were placed in a 150 x 100 mm polythene self adhesive bag and then stored at 4°C till laboratory analysis. Hunter harvested lechwe antelopes faecal samples were from animals that were cropped on special permission from Zambia Wildlife Authority (ZAWA). The sample size was pre-determined as authorized by the ZAWA research quota system for scientific research. In the case of pastoral cattle, a total of 361 freshly voided faecal samples were picked from the grazing pastoral cattle in Lochinvar ( $n = 261$ ) and Blue-lagoon ( $n = 100$ ).

### Isolation and identification of *Escherichia coli*

All faecal samples were processed according to the method described by Seker and Yardimci (2008) with slight modification. One gram of faeces was transferred into tubes containing 9 ml peptone water and homogenized by vortexing for 5 min. The vortexed mixture was incubated at 37°C for four hours and then a loopful of the mixture was sub-cultured on Desoxycholate Hydrogen Sulfide Lactose agar (DHL) (Nissui Pharmaceutical Co., Tokyo, Japan). About four suspicious *E. coli* colonies from each sample were transferred to Eosine Methylene Blue agar (EMB) (Eiken Chemical Co., Tokyo, Japan) and then incubated at 37°C for 24 h as described by Balows et al. (1991). Suspected isolates on EMB were confirmed as *E. coli* according to previous workers (Carter and John, 1990; Barrow and Feltham, 1993; Quinn et al., 2002). Screening for the presence of Shiga toxin (*Stx*) producing *E. coli* serogroups was done using Sorbitol MacConkey agar (SMAC) (March and Ratnam, 1986; Boyce et al., 1995) and CHROMagar O157 (Bettelheim, 1998; Hirvonen et al., 2012). The *E. coli* isolates were serogrouped with commercially available *E. coli* antisera test kit (Denka Seiken Co. Tokyo, Japan) for specific O antigens. Following serogrouping, 12 isolates (5 belonging to O27, 2 isolates from O78 and 5 isolates from O125 serogroup) were selected randomly for fermentative reaction tests to a range of 18 sugars as described by O'Sullivan et al. (2006).

### Statistical analysis

The database was established in Excel Spread sheets and for statistical analysis it was transferred to STATA SE/11 for windows statistical package (Stata Corp. College Station, Texas, USA). A p-value of <0.005 was considered indicative of a statistically significant difference.

## RESULTS

A total of 1,283 *E. coli* presumptive isolates from 593 faecal samples comprising lechwe ( $n = 232$ ) and pastoral cattle ( $n = 361$ ) were picked for analysis. In lechwe, 575

**Table 1.** Distribution of *E. coli* isolates from Kafue lechwe and cattle by selective media and serogrouping

Serogroup	Lechwe isolates (n = 104)		Cattle isolates (n = 144)	
	Non-sorbitol fermenting isolates on SMAC	Mauve coloured isolates on CHROMagar O157	Non-sorbitol fermenting isolates on SMAC	Mauve coloured isolates on CHROMagar O157
O8	0	0	1	2
O8/O27	0	0	0	1
O8/O27/O125	0	0	1	0
O8/O27/O55/O125	0	0	2	0
O8/O27/O55/O125/O169	0	0	3	0
O15	2	0	0	0
O27	2	4	3	1
O27/O115	0	0	1	0
O27/O55/O125	0	0	1	0
O29	0	0	2	4
O55	0	0	1	0
O55/O125/O169	0	0	2	0
O78	0	1	0	1
O8/O125	1	0	0	0
O29/O143	0	1	0	0
O55/O125	0	2	0	0
O125	6	0	4	0
O125/O169	0	0	2	0
O159	0	0	0	1
None-typeable	69	27	45	84
Total (%)	80 (76.9)	35 (33.6)	68 (47.2)	94 (65.2)

*E. coli* isolates were recovered and identified with 104 (18.1%) being non-sorbitol-fermenting (NSF) isolates on SMAC and appeared mauve coloured on CHROMagar O157 (Table 1). Eighty (76.9%) isolates out of 104 presumptive STEC O157 isolates from Kafue lechwe antelopes were NSF, while 35 (33.7%) were mauve coloured. Of these isolates, 11 exhibited both characteristics. All suspected STEC isolates exhibited a typical green metallic sheen on EMB agar. Out of 104 *E. coli* isolates, 79 (76%) were from Lochinvar NP, while 25 (24%) were from Blue-lagoon NP (Table 2). All the lechwe isolates from Blue-lagoon examined were NSF while only 69.6% (55/79) of *E. coli* isolates from Lochinvar were NSF. Thirty-four of 35 (97.1%) mauve coloured isolates were from Lochinvar while only one mauve coloured isolate was from Blue-lagoon. Of the 35 mauve coloured isolates, 18 (51.4%) were from the faecal samples picked from the pastures while 17 (48.6%) were coming from the rectal samples. The proportion of mauve coloured isolates from Lochinvar (43%) was higher than those from Blue-lagoon. Based on the serogrouping results, the isolates were classified into 8 O serogroups (Table 1). Serogrouping of 104 isolates revealed that 18 (17.3%) isolates were typeable while the rest were non-typeable. All isolates belonging to serogroups O15 and O125 were NSF isolates on SMAC, whereas the isolates belonging to serogroup O78 and the

majority of the strains belonging to serogroup O27 (P=0.004) were mauve coloured on CHROMagar O157. There was a significant association (P = 0.004) between serogroups and the choice of the selective media used. All isolates belonging to serogroups O15 and O125 were NSF isolates on SMAC, whereas the isolates belonging to serotype O78 and the majority of the strains belonging to serotype O27 (P=0.004) were mauve coloured on CHROMagar O157. The most frequently isolated DEC serogroups were EPEC O125 (5.8%) followed by ETEC serogroup O27 (4.8%) (Table 3).

In pastoral cattle, *E. coli* isolates were cultured and selected from 361 cattle faecal samples. Altogether 708 isolates were isolated from these samples, out of which, 144 (20.3%) were either NSF on SMAC and appeared mauve coloured on CHROMagar O157 (Table 1). Seventy-one (19.7%) faecal samples out of 361 samples were found to harbour the suspected STEC O157 isolates. The area distribution of the 71 faecal samples were 52 (73.2%) from Lochinvar and 19 (26.8%) from Blue-lagoon. Of the 144 suspected STEC O157 isolates, 110 (76.4%) were from Lochinvar, while 34 (23.6%) were from Blue-lagoon NP (Table 2). Of 144 isolates, 68 (47.2%) were NSF while 94 (65.3%) were mauve coloured on CHROMagar O157 with eighteen isolates exhibiting both characteristics. Seventy-five out of 94 (79.8%) mauve coloured isolates were from Lochinvar,

**Table 2.** Distribution of *E. coli* serogroups from Kafue lechwe and pastoral cattle by area of sampling.

Serogroup	Pastoral cattle (n = 144)		Kafue Lechwe (n = 104)	
	Lochnivar	Blue Lagoon	Lochnivar	Blue Lagoon
O15	0	0	1	1
O125	4	0	3	3
O125/O169	1	1	0	0
O159	0	1	0	0
O27	1	3	5	0
O27/O115	0	1	0	0
O27/O55/O125	1	0	0	0
O8/O125	0	0	0	1
O8/O27	0	1	0	0
O29	0	6	0	0
O55	0	1	0	0
O55/O125/O169	0	2	0	0
O55/O125	0	0	2	0
O78	1	0	1	0
O8	1	1	0	0
O8/O27/O125	1	0	0	0
O8/O27/O55/O125	2	0	0	0
O8/O27/O55/O125/O169	3	0	0	0
O29/O143	0	0	1	0
Non-typeable	95	17	66	20
Total	110	34	79	25

**Table 3.** Occurrence estimates of *Escherichia coli* serovars from Kafue lechwe and pastoral cattle.

Serogroup	Category	Frequency of isolation	
		Pastoral cattle (n = 144)	Kafue lechwe (n = 104)
O15	ETEC	0	2 (1.9)
O125	EPEC	4 (2.8)	6 (5.8)
O125/O169	EPEC/ETEC	2 (1.4)	0
O159	ETEC	1 (0.7)	0
O27	ETEC	4 (2.8)	5 (4.8)
O27/O115	ETEC	1 (0.7)	0
O27/O55/O125	ETEC/EPEC	1 (0.7)	0
O8/O125	ETEC/EPEC	0	1 (0.96)
O8/O27	ETEC	1 (0.7)	0
O29	EIEC	6 (4.2)	0
O55	EPEC	1 (0.7)	0
O55/O125/O169	EPEC/ETEC	2 (1.4)	0
O55/O125	EPEC	0	2 (1.9)
O78	ETEC	1 (0.7)	1 (0.96)
O8	ETEC	2 (1.4)	0
O29/O143	EIEC	0	1 (0.96)
O8/O27/O125	ETEC/EPEC	1 (0.7)	0
O8/O27/O55/O125	ETEC/EPEC	2 (1.4)	0
O8/O27/O55/O125/O169	ETEC/EPEC	3 (2.1)	0
Non-typeable	-	112 (77.8)	86 (82.7)
Total		144	104

**Table 4.** Comparison of fermentative reaction results of selected *E. coli* biovars from Kafue lechwe and pastoral cattle ( $n = 12$ ).

Test	<i>E. coli</i> serogroup tested											
	O27					O78				O125		
	Lechwe			Cattle		Lechwe		Cattle		Lechwe		Cattle
	1	2	3	4	5	6	7	8	9	10	11	12
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-
Dextran	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	+	+	-	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	+	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
Meliobiose	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+
Resorcinol	-	-	-	-	-	-	-	-	-	-	-	-
Ribitol	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	+	+	+	+	+	+	+	-	-	+	-	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	-	+	+	+	+	+	-	-	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	+	+	+	+	+	+
Barbitol	-	-	-	-	-	-	-	-	-	-	-	-

+ = Positive reaction, - = negative reaction.

while 19 (20.2%) were from Blue-lagoon. Thirty-two (22.2%) of these isolates were grouped into 16 O serogroups (Table 1). Most of the defined serogroups were from Lochinvar (Table 2). The most frequent serogroup was the non-typeable *E. coli* which accounted for 66% (95/144) and 11.8% (17/144) of the isolates from Lochinvar and Blue-lagoon, respectively (Table 2).

The metabolic fingerprint of *E. coli* serogroups from cattle and lechwe was determined by biochemical profile. Twelve *Escherichia coli* isolates belonging to serogroups O27 (5), O78 (2) and O125 (5) respectively were randomly selected from lechwe antelopes and pastoral cattle. These isolates were subjected to fermentation of various carbohydrates (Table 4). The results of sugar utilization from various source suggested that isolates belonging to serogroup O27 were of three distinctive fermentative groups with one group comprising isolates numbers 1 and 4, while the second group were isolate number 3 and 5 from lechwe antelopes and pastoral cattle, respectively. The third group is of isolate number 2. The O78 serogroup had 2 distinct fermentative groups while in the O125 serogroup, 3 distinct groups were recorded (Table 4).

## DISCUSSION

This study has shown that Kafue lechwe antelopes and domestic cattle on the Kafue Flats are potential carriers

of food-borne pathogens. *E. coli* is generally used as an indicator organism because of the ease of isolation (Anonymous, 2003). In this study, we used the culture method followed by serogrouping and biochemical typing to confirm the presence of *E. coli*. According to our observations, the use of SMAC agar was specific in the proportion of recovery of *E. coli* serogroup O125 than CHROMagar O157. The use of SMAC agar and CHROMagar O157 to screen for presumptive STEC O157 strains was cost effective in the sense that they helped to reduce the material costs and the number of *E. coli* isolates needed to be confirmed by serogrouping. Though it is most likely that some unusual sorbitol positive *E. coli* O157 strains could have been missed which could not be differentiated from normal intestinal flora as observed by Ammon et al. (1999).

Kafue lechwe antelopes are carriers of at least seven diarrheagenic *E. coli* serogroups which include enterotoxigenic *E. coli* (O15, O27 and O78), enteropathogenic *E. coli* (O55 and O125) and enteroinvasive *E. coli* (O29/O143). It is worth noting that the ETEC serogroup O27 and O78 were most predominant in Lochinvar, while EPEC serogroup O125 and ETEC serogroup O15 were evenly distributed between Lochinvar and Blue-lagoon, suggesting that the Kafue lechwe antelopes may be the probable reservoir host. The present study attempted for the first time to classify *E. coli* from faecal material of Kafue lechwe and

pastoral cattle into O serogroups. The most commonly association was between serogroups O27, O78 and O125 from Kafue lechwe and cattle, respectively or vice versa. The isolation of pathogenic *E. coli* serogroups in the present study is in agreement with reports that wild animals may be involved in the epidemiology of zoonoses and may serve as reservoirs (Kruse et al., 2004). We reported O15, O27, O78 and O125 serogroups from Kafue lechwe, which could be potential sources of human gastroenteritis. Elsewhere, studies focusing on the Shiga toxin – producing *E. coli* isolates from wildlife meat as a threat to public health have been conducted (Miko et al., 2009).

There were more *E. coli* serogroups from faeces of pastoral cattle than Kafue lechwe antelopes, as pastoral cattle tend to graze varied epidemiologically in different areas because of their transhumant nature and are thus exposed to pasture with different microbiological profiles. Our finding may be in agreement with the existing knowledge which has always implicated cattle as a major reservoir of DEC (Trevena et al., 1999; Bach et al., 2002; Cookson et al., 2006). The presence of typeable DEC serogroups from pastoral cattle was higher in Blue-lagoon than Lochinvar. This could be attributed to the fact that Blue-lagoon is more transhumance in nature than Lochinvar, because pastoralist villages are much closer to the livestock/wildlife interface area. Furthermore, *E. coli* serogroups O29 and O125 could be ecologically adapted to respective area animal sanctuaries. Collectively, these results strongly intimate that the eventual predominance of ETEC O27 and EPEC O125 on the pasture may have important public health implications in the study area. Interestingly, we also noted that ETEC serogroup O15 and EIEC serogroup O29 are only confined to faecal samples from Kafue lechwe antelope and pastoral cattle, respectively; despite the latter being the most frequently isolated DEC serogroup from the pasture. We are tempted to assume that serogroup O29 was perhaps host specific though we would like to suggest that more research be conducted to substantiate these findings. A heightened interaction between wild animals and cattle is likely to lead to bacteria interspecies transfer (Gilbreath et al., 2009). In this study, serogrouping and biochemical profiling of the bacterial isolates provided information on the relatedness between *E. coli* serogroups from Kafue lechwe and pastoral cattle. This study has shown that ETEC (O27 and O78) and EPEC (O125) are common serogroups harboured by Kafue lechwe antelopes and pastoral cattle. This is in agreement with the report by Rice et al. (1995) that wild animals and cattle sharing common areas would likely experience interspecies transfer. Therefore, current findings in our study may confirm that bacterial strains from Kafue lechwe antelopes are somewhat related to some strains from pastoral cattle posing a risk to the pastoral communities living in these areas.

## Conflict of interests

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Molecular characterization of group A rotaviruses circulating in Senegal over a 7-year period (2005-2011)

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Group A rotaviruses are characterized by large genomic diversity that is typically studied by genotyping of the VP7 glycoprotein, which defines G-type, and the protease-sensitive VP4, which defines P-type. The aim of this study was to examine the genotypes of group A rotavirus strains circulating in Senegal over a 7-year period between 2005 and 2011. ELISA positive stool specimens were analyzed by PAGE and were genotyped by multiplex hemi-nested RT-PCR. Data were analyzed with Epi Info 7 software. Rotavirus was found in 222 samples of stools of which 32 (14.41 %) were positive on PAGE. Five electropherotype patterns were identified, three long (L1, L2 or L3) and two short (S1 or S2). Twenty-three (23) samples had a long electropherotype and nine had a short electropherotype. One hundred strains (45.04%) were analyzed by VP7 genotyping, which identified five different genotypes: G1, G2, G3, G8 and G9. Ninety-three (93) samples (41.89%) were analyzed by VP4 genotyping which showed four different genotypes: P[4], P[6], P[8] and P[11] and mixed genotypes (1.8%). Seventy seven (34.68%) samples were genotyped for both VP7 and VP4. The G1P[8] strain was the most predominant strain followed by the G9P[8] strain. Rotavirus strains circulating in Senegal are genetically diverse. Genotypes G1P[8] and G9P[8] are the predominant strains. We also found mixed infections that could favor the emergence of new viral strains. Thus, regular monitoring of genotypes circulating in the country is required.

**Key words:** Rotavirus, diarrhea, genotypes, children, Senegal.

## INTRODUCTION

Human group A rotaviruses (RVA) are ubiquitous and are the leading cause of diarrhea in children under five years old. In Senegal, rotavirus is the principal cause of pediatric diarrhea during the dry season (Sambe-Ba et al., 2013). The virus appears as a wheel-shaped particle on electron microscopy. These non-enveloped viruses have an icosahedral capsid composed of a triple layer of

proteins (VP2, inner layer; VP6, middle layer; VP7+VP4, outer layer) and a genome made of 11 segments of double-stranded RNA (dsRNA) (Estes and Greenberg, 2013). The viral RNA migration pattern obtained by polyacrylamide gel electrophoresis (electropherotype) is used as a marker in epidemiology, with differences in migration of one or more RNA segments distinguishing

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rotavirus strains (Estes and Greenberg, 2013). However, the method of choice for RVA classification today is genotyping by reverse transcription-polymerase chain reaction [RT-PCR] for classification of RVAs into groups/species (Matthijnssens et al., 2012). The three main antigenic proteins of the virus can be used to classify rotavirus into seven groups labeled A to G (VP6), and into G (VP7) and P (VP4) genotypes (Estes and Greenberg, 2013). At least 27 G-types and 37 P-types have been characterized so far (Trojnar et al., 2013), and various combinations of G- and P-types exist (Estes and Greenberg, 2013; Rahman et al., 2005). The diversity of strains circulating in different regions is important for vaccine development and clinical assessment (Gentsch et al., 2005; Santos and Hoshino, 2005).

Although the G9 G-type is considered one of the major human genotype of RVA worldwide (Ianiro et al., 2013), G8, G9 and G12 genotypes, which are not included in the vaccines, have appeared more regularly over the past few years (Matthijnssens et al., 2010). The emergence of these new genotypes reveals the importance of monitoring rotavirus infections before and after the introduction of vaccines. The major aim of this study was to study the genotypes of RVA strains circulating in Senegal over a 7-year period, between 2005 and 2011.

## MATERIALS AND METHODS

### Samples collection

This was a prospective study carried out between January 1st, 2005 and December 31st, 2011. Stool samples were collected from children with diarrhea, who were treated as inpatients or outpatients at five hospitals: three in Dakar (Albert Royer Children's Hospital, Pediatric Institute of Pikine and Abass Ndao Hospital) and one in Saint-Louis (263 km from Dakar) and one in Diourbel (146 km from Dakar). Most children were under 5 years old.

Samples were collected in an airtight container and taken immediately to the laboratory. Containers were placed in an icebox in case transportation was delayed.

### Detection of RVA

A commercial ELISA kit (IDEIA Rotavirus, DAKO Ltd, Glostrup, Denmark) was used to detect RVA. This kit contains a polyclonal antibody detecting proteins specific to RVA, notably the internal capsid protein VP6. It is a sandwich-type ELISA test that can be read either with the naked eye or through a spectrophotometer. Positive samples were frozen at -80°C for PAGE analysis and genotyping at one of three reference laboratories: the Pasteur Institute virology laboratory in Dakar and two WHO collaborative centers, the National Health Laboratory Service (NHLS) in South Africa and the Medical Research Council (MRC) in The Gambia. Statistical analysis was carried out with Epi Info. 7 software.

### Extraction of viral RNA with TRI-Reagent1

Viral RNA was extracted with TRI-Reagent1 (Sigma) according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi (1987).

### Separation of viral genome by Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was performed on two polyacrylamide gels of different concentrations: - a 10 % gel, which enables most of the viral genome to be separated into various segments; - a 3 % gel, which facilitates the separation of each segment. Extracted RNA was mixed with bromophenol blue and glycerol before loading onto the migration gel.

The migration was performed in Tris-glycine buffer for 22 h at 100 V. Bands were detected by silver staining and the gel was subsequently placed between two cellophane sheets and put into a gel dryer for 90 min.

### Molecular typing of rotavirus strains

#### *Denaturation of double-strand RNA (dsRNA) segments and annealing of external primers on single-strand RNA*

Five (5) µl of dsRNA extract was added to 1 mmol of each primer (sense and antisense) with 3 µl of DEPC-treated water in an Eppendorf tube. The mixture was put into a thermocycler at 97°C for 5 min then immediately cooled on ice.

#### *Reverse transcription*

A total of 40 µl of a reaction mixture containing 24 µl of DEPC-treated water, 2 mmol of dNTPs, 5 µl of reverse transcriptase buffer, 2 mmol of Dithiothreitol (DTT), 4 mmol of MgCl<sub>2</sub> and 0.05 U of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega1) was added to the denatured RNA. Tubes were then put into the thermocycler at 42.8°C for 45 min.

#### *First PCR*

PCR was carried out with all of the RT product. A total of 25.7 µl of sterile distilled water, 20 µl of Taq polymerase buffer, 10.02 mmol of dNTPs, 0.25 mmol of MgCl<sub>2</sub>, and 1.5 U of Taq polymerase (Go-Taq, Promega1) were added to each tube, and PCR was performed with 30 cycles of 1 min denaturation at 95.8°C, 1 minute hybridization at 42°C and 1 minute elongation at 72°C. These cycles were preceded by a denaturation step of 5 min at 95.8°C, and then followed by an elongation step for 7 min at 72°C.

#### *Hemi-nested multiplex PCR*

The PCR product was subjected to a second amplification by hemi-nested multiplex PCR with internal primers (Table 1) (Iturriza-Gomara et al., 2004). The quantity of DNA used at this step depended on the band intensity resulting from the first amplification, and ranged from 0.5 µl at high band intensity up to 6 µl at low band intensity. Afterwards, 0.5 mmol of MgCl<sub>2</sub>, 0.2 mmol of dNTPs, 0.2 mmol of each primer, 10 µl of PCR buffer, 1.5 U of Taq polymerase and enough sterile water to obtain a final volume of 50 µl were added to the first PCR product. The mixture was prepared on ice, and then placed into the thermocycler for a second round of amplification similar to the protocol described above.

#### *Detection of PCR products*

The resulting PCR products were subjected to electrophoresis in a 2% agarose gel containing 0.0015% of ethidium bromide. A molecular weight marker (100 bp ladder; Promega1) was also

**Table 1.** G and P consensus and type-specific recommended primers.

Primer	Sequences (5'–3')	nt Position	Amplicon (bp)
<b>G-typing (a)</b>			
<b>1st round</b>			
VP7-F	ATGTATGGTATTGAATATAACCAC	(nt 51–71)	881
VP7-R	AACTTGCCACCATTTTTTCC	(nt 914–932)	
<b>2nd round</b>			
G1	CAAGTACTCAAATCAATGATGG	(nt 314–335)	618
G2	CAATGATATTAACACATTTTCTGTG	(nt 411–435)	521
G3	ACGAACTCAACACGAGAGG	(nt 250–269)	682
G4	CGTTTCTGGTGAGGAGTTG	(nt 480–499)	452
G8	GTCACACCATTGTAAATTCG	(nt 178–198)	754
G9	CTTGATGTGACTAY <sup>a</sup> AAATAC	(nt 757–776)	179
G10	ATGTCAGACTACAR <sup>b</sup> ATACTGG	(nt 666–687)	266
VP7-R	As above		
<b>P-typing (b)</b>			
<b>1st round</b>			
Con-3	TGGCTTCGCCATTTTATAGACA	(nt 11–32)	876
Con-2	ATTTCCGACCATTATAACC	(nt 868–887)	
<b>2nd round</b>			
P[4]	CTATTGTTAGAGGTTAGAGTC	(nt 474–494)	483
P[6]	TGTTGATTAGTTGGATTCAA	(nt 259–278)	267
P[8]	TCTACTGGR <sup>b</sup> TTR <sup>b</sup> ACN <sup>c</sup> TGC	(nt 339–356)	345
P[9]	TGAGACATGCAATTGGAC	(nt 385–402)	391
P[10]	ATCATAGTTAGTAGTCGG	(nt 575–594)	583
P[11]	GTAACATCCAGAATGTG	(nt 305–323)	312
Con-3	As above		

<sup>a</sup>Y= C or T; <sup>b</sup>R= A or G; <sup>c</sup>N= A, G, C or T.

included. The migration buffer was Tris–Borate–EDTA (composed of 0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3). DNA bands were visualized by ultraviolet (UV) light.

## RESULTS

### Detection rate and distribution of rotavirus according to year

Rotaviruses were found in 222 stool samples. The distribution of the positive samples according to year is showed in Table 2.

### Electrophoretic migration profiles of RVA found between 2005 and 2011

Among the 222 positive samples, 32 (14.41 %) were positive on PAGE. Five different electropherotypes were found during this six year period: 23 samples had a 'long' electropherotype (L1, L2 or L3) and 9 had a 'short' electropherotype (S1 or S2) (Figure 1).

### Composition of VP7 genotypes

Among the 222 positive stools, 100 (45.04 %) were typed for VP7, which revealed five different genotypes: G1, G2, G3, G8 and G9. In 2005, G1 and G2 genotypes were predominant whereas most genotypes found in 2011 were G1 or G9 (Figure 2).

### Composition of VP4 genotypes

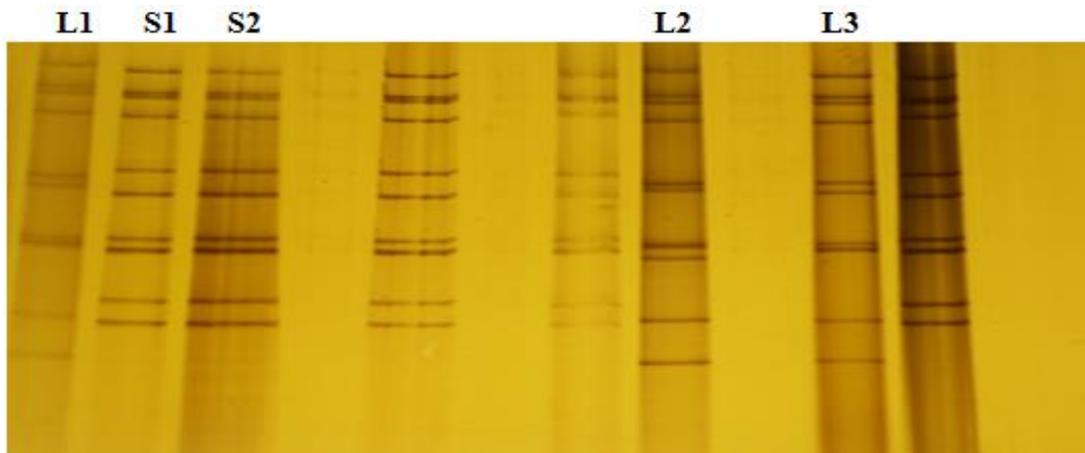
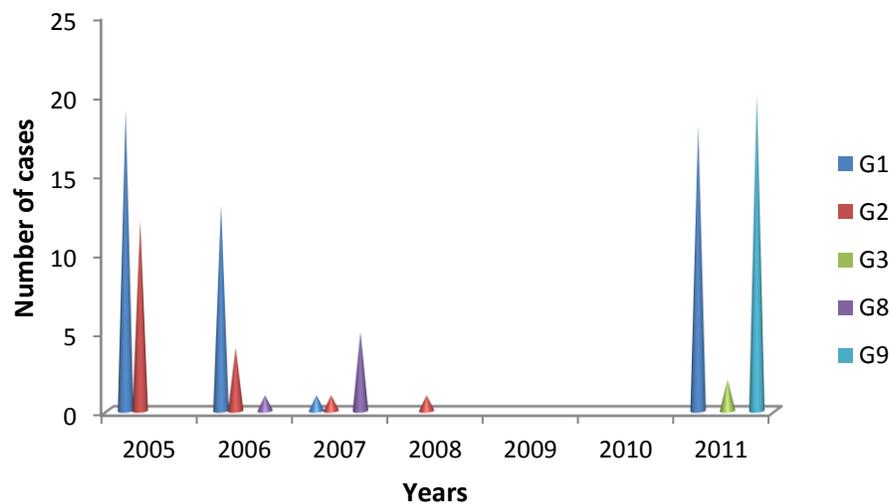
Ninety-three (41.89 %) strains were typed for VP4, which showed four different genotypes: P[4], P[6], P[8] and P[11], and four mixed genotypes P[8]+P[6] (1.8%). Genotypes P[8] and P[6] were the predominant ones (Figure 3).

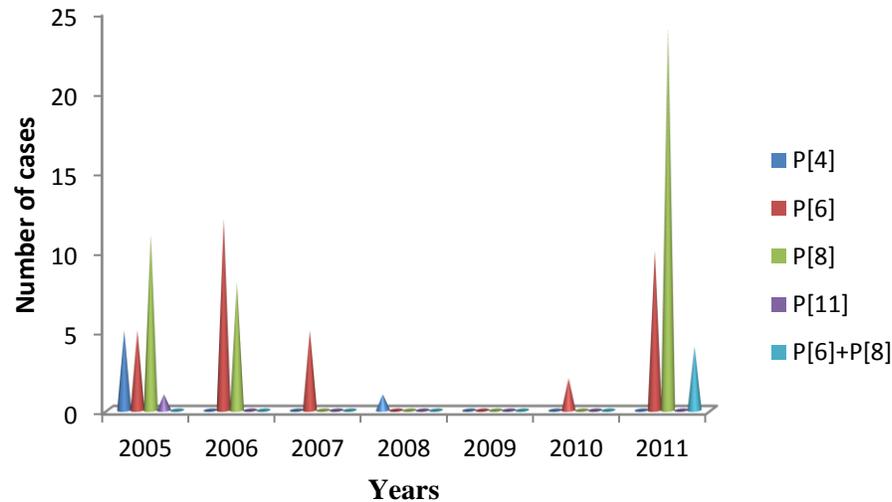
### VP7/VP4 associations

Seventy-seven (34.68%) samples were typed for both VP7 and VP4, which identified several associations between genotypes (Table 2). The G1P[8] strain appears

**Table 2.** Evolution of VP4/VP7 genotypes according to years.

Genotype	2005	2006	2007	2008	2009	2010	2011	Total
G1P6	2	6				1	2	11
G1P8	10	7					9	26
G1P11	1							1
G2P4	5			1				6
G2P6	3	5						8
G2P8	1							1
G8P6		1	3					4
G9P6		1				1	4	6
G9P8							14	14
GP typed	22	20	3	1	0	2	29	77
Out of	39	29	22	82	0	5	45	222

**Figure 1.** Electrophoretic profile with Polyacrylamide gel electrophoresis (PAGE). The polyacrylamide gel was visualized after silver staining. The RNA segments show the classic group A rotavirus distribution of bands. Three long electropherotypes (L1, L2 or L3) and two short (S1 or S2) are represented.**Figure 2.** Composition of VP7 genotypes during the study period.



**Figure 3.** Composition of VP4 genotypes during the study period.

to be the predominant strain of RVA in Senegal (26 strains), followed by the G9P[8] strain (14 strains). This analysis also identified an unusual association between G2 and P[8].

## DISCUSSION

### Polyacrylamide gel electrophoresis

This is the first study of its kind to report the strains of RVA circulating in Senegal. We found that rotaviruses circulating during a 7-year study period were characterized by five different electrophoretic migration profiles; three long electropherotypes and two short electropherotypes. Long electropherotypes were more common than short electropherotypes in Senegal during the study period ( $n=23$  versus  $n=9$ , respectively), consistent with a report from Tunisia (Chouikha et al., 2011). The co-circulation of many electrophoretic migration profiles has been described by other studies (Dzikwi et al., 2008), and is a characteristic of the epidemiology of rotavirus infections.

### Composition of VP7 genotypes during the study period

Our study shows that the composition of circulating rotavirus G genotypes has changed throughout the years in Senegal. In 2005, the G1 genotype was predominant. The G3 genotype, which was absent in Senegal between 2005 and 2010, appeared in our study in 2011. The G2 genotype was present between 2005 and 2008. This genotype is the predominant G genotype in Tunisia, accounting for 52% of the strains found in this country

(Chouikha et al., 2011). It was also highly prevalent in Africa from 2007 through 2011, accounting for 16, 8% (Seheri et al., 2014).

The G8 genotype emerged in 2006 but disappeared quickly afterwards in 2007. We did not find the G4 genotype in our study, despite the fact that the G2, G3 and G4 genotypes are the most frequent genotypes worldwide after the G1 genotype and are circulating in variable proportions across many regions (Iturriza-Gomara et al., 2000).

The G9 genotype appeared in 2011 in our study. This genotype have been detected sporadically and in localized outbreaks in various African countries, including South Africa, Botswana, Malawi, Kenya, Cameroon, Nigeria, Ghana, Guinea-Bissau, Libya, and Mauritius (Page et al., 2010). Moreover, the G9 genotypes are "humanized" since the early 2000s, before they were a typical porcine RVA VP7 genotype. Today they are still one major VP7 genotype of RVA infecting pigs (Donato et al., 2012; Theuns et al., 2015).

### Composition of VP4 genotypes during the study period

In 2005, the P[4], P[6] and P[8] genotypes were co-circulating in Senegal. These three genotypes are the most frequent VP4 genotypes worldwide (Iturriza-Gomara et al., 2011).

The P[8] genotype was the predominant form in 2005. In the following year, the P[6] genotype was the most abundant type, but was superseded by the P[8] genotype in 2011. The P[8] genotype is the most common genotype worldwide: it accounts for 97.4% of P genotypes in Japan (Phan et al., 2007), 83.7% in Tanzania (Moyo et al., 2007) and 31% in India (Jain et al., 2001).

The P[11] genotype, which is rarely found in other countries, was isolated in our study in 2005. This genotype is currently emerging in several regions of the world (Esona et al., 2010). The prevalence of this genotype has been reported as 3% (Jain et al., 2001) and 8.5% (Banerjee et al., 2006) in studies conducted in India. It has also been described in Indonesia with a prevalence of 0.5% (Putnam et al., 2007).

Four samples had a mixed profile (P[8]+P[6]), which have been previously reported in several studies (Mwenda et al., 2010; Serravalle et al., 2007). Mixed profiles are particularly frequent during epidemics, because they are derived from genome assortment that occurs during mixed infections in the same child (Martella et al., 2010; Matthijssens and Van Ranst, 2012).

### Association between VP7/VP4 genotypes

The G1P[8] strain was predominant in Senegal between 2005 and 2011, consistent with the findings of other authors (Banyai et al., 2009). This association is one of the most frequent worldwide (Da Silva et al., 2015). However, the abundance of this strain declined in the following years and was replaced by an unusual association between G9 and P[8] in 2011. We also noted the emergence of another unusual combination between G8 and P[6] in 2006. However, the most frequently found associations involve stable combinations of genotypes with strong affinities for each other. For example, the P[4] genotype is often linked to the G2 genotype, whereas the P[8] genotype is most frequently associated with the G1, G3 and G4 genotypes (Abdel-Haq et al., 2003).

We found rotavirus strains with uncommon VP7/VP4 associations such as G2P[8]. Such associations have been previously reported in other studies, showing the variability of rotaviruses and re-assortment within rotavirus strains (Esona et al., 2010). Indeed, our data provide evidence of likely *in vivo* reassortment, for G/P constellations G1P[6], G1P[11], G2P[6], G2P[8], G9P[6], that is for 27/77 (35.1%) of fully G/P typed isolates. This is a high number which is not untypical in RVA strains collections from Africa (Iturriza-Gomara et al., 2001; Iturriza-Gomara et al., 2011). Furthermore, there may be RVA isolates carrying a G or P protein which is not covered by the primers used and therefore not genotyped. This is a frequent finding in African RVA strain (Seheri et al., 2014).

Few data about the P[11] genotype are available in literature (Matthijssens et al., 2010). In our study, this genotype was associated with the G1 genotype, which is an unusual association. Indeed, a study carried out in India showed that P[11] genotypes are always associated with G10 genotypes (Jain et al., 2001). However, a Tunisian study detected the same G1P[11] association (Chouikha et al., 2011). We found the G9 genotype in association with the P[8] and P[6] genotypes. This genotype is also associated with P[8] and P[4] in Cameroon

(Esona et al., 2010). Indeed, the G9 genotype has been reported to be in association with these VP4 genotypes, as well as the P[4] and P[19] genotypes. However, the G9 genotype is most frequently associated with P[8] (Zhou et al., 2001).

Overall, 1.8% of infections were mixed infections. This percentage is lower than that reported in other countries, including Brazil (29%) (Serravalle et al., 2007) and India (21%) (Jain et al., 2001). The high frequency of mixed infections in these countries facilitates the emergence of new strains (Fischer et al., 2005).

### Conclusion

Our findings reveal a high diversity of rotavirus strains in Senegal during a 7-year period. Among these strains, the genotypes G1P[8] and G9P[8] were predominant. We also recorded mixed infections, which could favor the emergence of new strains, demonstrating the need for regular monitoring of genotypes circulating in the country. The coverage of both, sample collection and RVA genotyping has been very uneven over the years and this study is the start of more comprehensive work to be undertaken in Senegal.

### Conflict of interests

The authors did not declare any conflict of interest.

### ACKNOWLEDGMENTS

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## Full Length Research Paper

# Genotyping and antifungal susceptibility of *Candida albicans* strains from patients with vulvovaginal and cutaneous candidiasis in Palestine

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The aims of this study was the genotyping of *Candida albicans* strains isolated from patients with invasive cutaneous and vulvovaginal infections and to investigate the fluconazole, flurocytosine (5-FC), and amphotericin B susceptibility of the strains genotypes. For this purpose, 151 *C. albicans* isolates [from 68 candidiasis (VVC) and 83 cutaneous (CC) infections] were genotyped by using specific PCR primers designed to span the transposable group I of the 25S rDNA gene. Susceptibility of genotypes A, B, and C to the antifungal agents amphotericin B, flurocytosine and fluconazole was determined by disk-diffusion, and broth microdilution methods. Eighty-three of the 151 isolates were genotype C (83.55 %), 49 were genotype A (32.4%), and 19 were genotype B (12.6%). Genotypes D and E which represent *C. dubliniensis* were not found. Antifungal susceptibility testing showed that isolates of *C. albicans* genotype A were more resistant to fluconazole and flurocytosine than B and C genotypes (A>B>C), and also more resistant to amphotericin than C and B genotypes (A>C>B). These results indicate that there may be a relationship between *C. albicans* genotypes and resistance to antifungals. The presence or absence of the transposable group I intron in the 25S rDNA gene may be important in determining the resistance of *C. albicans* to antifungals. To our knowledge, this is the first study that reports *C. albicans* genotypes in Palestine and its differential resistance to fluconazol, flurocytosine, and amphotericin B.

**Key words:** *Candida albicans*, genotyping, antifungal susceptibility, Palestine.

## INTRODUCTION

*Candida albicans* is the major etiologic species of candidiasis; it is responsible for 50-70% of all disseminated *Candida* infections (Dalle et al., 2000). In recent years, *C. albicans*, as a common opportunistic yeast, has

caused an increasing number of human cutaneous (CC) as well as vaginal candidiasis (VVC) (Ri, 1988; Xiao-dong et al., 2008).

Since the pathogenicity and antifungal susceptibility of

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*C. albicans* often vary among strains, identification of the disease-causing strains is crucial for diagnosis, clinical treatment and epidemiological investigation. Furthermore, a few studies have indicated the presence of correlation of genetic diversity of *C. albicans* strains recovered from VVC and CC with antifungal susceptibility (Antonopoulou et al., 2009; Krawczyk et al., 2009; Costa et al., 2010).

A study conducted by Fan and colleagues (Fan et al., 2008) to determine the genotypic variations of *C. albicans* in patients with various conditions of VVC revealed that genotypes of *C. albicans* strains correlate with the severity of VVC. The study also shows that the strains with the dominant genotypes are more virulent than others in causing VVC, and that strain differences may play a significant role in the etiology of VVC. Zhu et al. (2011) and Emmanuel et al. (2012) also confirmed that susceptibility varies among the various genotypes of *C. albicans*. Hence in the management of candidiasis it is important to take into consideration the genotype of the Candida strain causing VVC or CC as genotypes show correlation to sensitivity.

To the best of our knowledge, this is the first study aimed at determining the genotypic variation among *C. albicans* causing VVC and CC in Palestine and testing whether genotypes correlate with patients' demographic factors.

Studying the genetic relatedness of clinical strains of Candida species that cause different CC locations and different VVC conditions may have significance in clinical management. Genotyping of strains may also help to distinguish candidiasis relapse due to inadequate treatment or from de novo infection by a new strain, or distinguish resistant or susceptible strains to antifungals.

Molecular typing of *C. albicans* is important for epidemiological studies and for the development of appropriate infection control strategies (McCullough et al., 1999). Using ribosomal sequences for genetic typing, McCullough et al. (1999) used a pair of PCR primers designed to span the region that includes the site of the transposable group I intron of the 25S rRNA gene (rDNA), and can classify *C. albicans* strains into three genotypes on the basis of the amplified PCR product length: genotype A (approx. 450 bp product), genotype B (approx. 840 bp product), and genotype C (approx. 450 and 840 bp products). The two other observed genotypes with this method, genotypes D (approx. 1080 bp product) and E (approx. 1400 bp product), are found to belong to the same taxon as *C. dubliniensis* (Tamura et al., 2001). This method has shown to be easy to perform, give clear and reliable results, be able to differentiate appropriate number of strains, and to be adapted to large number of isolates (Karahan et al., 2004).

In this study, the 25S ribosomal DNA (rDNA) based PCR method (Hattori et al., 2006), which is easy and quick to be performed with low cost (Karahan et al., 2004) was used to characterize the genotypic distribution

of *C. albicans* affecting women with VVC and infants with CC in Northern West Bank (Palestine). We also examined whether there was an association between these genotypes and susceptibility to antifungals.

## MATERIALS AND METHODS

### Patients and clinical specimens

The study population comprised 120 infant patients (62 males, 58 females; aged 1 day- 18 months) with cutaneous candidiasis (lesion locations: groin, armpit, perianal, hip, neck and back), and 126 pregnant women (aged 17-44 years) with vulvo-vaginal candidiasis (underlying conditions: intense vulvar pruritus, erythema, burning and dyspareunia associated with a creamy discharge). Scales and macerated skin of infected lesions, and vaginal secretions were collected from CC, and VVC patients, respectively, as clinical specimens by sterile swabs.

All clinical specimens were submitted to direct microscopy with 20% KOH and cultured on yeast extract-peptone-dextrose medium (YPD) agar plates containing 50 µg/ml chloramphenicol, and incubated at 37°C for 48 h. Each colony was identified as *C. albicans* by colony morphology, Chromogenic agar (CHROMagar Candida; Oxoid Co., UK) and germ tube test. A total of 151 strains of *C. albicans* (83 from CC specimens, and 68 from VVC specimens), were recovered and subsequently used for genotyping.

### Genomic DNA isolation from Candida

Genomic DNA extraction from Candida isolates was carried as described by Harju et al. (2004). Briefly, a single colony of *C. albicans* was added to 2 ml of YPD broth medium and grown in a shaking incubator for approximately 48 h at 30°C. The overnight cultures were pelleted in a microcentrifuge tube and the pellets were resuspended in 200 µl of lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] (Hoffman and Winston, 1987). The tubes were then placed in a -80°C freezer until completely frozen, then immersed in a 95°C hotplate for 1 min to thaw quickly. The process was repeated once, and the tubes were vortexed vigorously for 30 s. Chloroform (200 µl) was added and the tubes were vortexed for 2 min and then centrifuged 3 min at room temperature at 20,000 x g. The aqueous layer was transferred to a tube containing 400 µl of ice-cold 100% ethanol. The samples were allowed to precipitate 5 min at room temperature and centrifuged. DNA pellets were washed with 0.5 ml of 70% ethanol, dried and resuspended in 20 µl TE [10 mM Tris, 1 mM EDTA (pH 8.0)] (Harju et al., 2004).

### Genotyping of *C. albicans* isolates using PCR primers for the 25S rDNA (P-I)

For genotype determination of *C. albicans* on the basis of 25S rDNA, primers CA-INT-L (5'ATAAGGGAAGTCGGCAAATAGATCCGTA3') / CA-INT-R (5'CCTTGGCTGTGGTTTCGCTAGATAGTAGAT3') (referred to as P-I) were used (Hattori et al., 2006). *C. albicans* was grouped into five genotypes (A, B, C, D, and E) on the basis of the sizes of PCR products. Genotype D corresponds to *C. dubliniensis* (Hattori et al., 2006; McCullough et al., 1999).

### PCR conditions and agarose gel electrophoresis

Genomic DNAs were amplified in a reaction mixture (25 µl) containing

genomic DNA (10-100 ng), forward primer and reverse primer (Hylabs, Israel), each (10 µmol/L), 10x buffer, MgSO<sub>4</sub>, dNTPs (10 mM), Taq DNA polymerase 0.2 µl (2.5 U/ml; Hylab, Ltd., Israel), and sterile distilled water. The PCR cycle parameters were as follows: preheating at 96°C for 120 s; followed by 35 cycles of 96°C for 30 s, 60°C or 65°C for 30 s, and 72°C for 60 s, and final extension at 72°C for 5 min. The annealing temperature of the primers was 65°C. All reaction mixtures were amplified using a thermal cycler (TC-Plus, Techne, UK).

The PCR products were electrophoresed in a 1% agarose gel for identification and genotyping of *C. albicans* on the basis of 25S rDNA.

### Antifungal susceptibility testing

A total of 11 *C. albicans* isolates, selected on the basis of their genotype, were tested, including 3 strains of each of genotype A (N43, M22, N96), B (N72, M97, M19), and C (N66, M35, M29). Type cultures *C. albicans* CBS9120, and *C. albicans* CBS6589 (Centraalbureau voor Schimmelcultures CBS, The Netherlands) were used as quality controlled strains in each run of the experiment.

The susceptibilities in vitro to fluconazole, flucytosine (5-FC), and amphotericin B were carried out using disc diffusion and broth microdilution methods, described here.

### Inoculum preparation

The inoculum was prepared using 24 h plate cultures of *C. albicans*. The colonies were suspended in 0.85% saline and the turbidity was compared with the 0.5 McFarland standard, to produce a yeast suspension of 1x10<sup>6</sup> to 5x10<sup>6</sup> cells/mL. The cell suspension was diluted 1:100 in the media to obtain a final concentration of 1 × 10<sup>4</sup> to 5 × 10<sup>4</sup> colony-forming units per milliliter (CFU/mL).

### Disk diffusion method

The disk diffusion method was used as outlined in NCCLS document (M44-A) (NCCLS, 2004). Stock solutions (640 µg/ml) of antifungal agents were prepared in water (fluconazole, Pfizer; and flucytosine, Alfa Aesar, A Johnson Matthey Company, GB) or dimethylsulphoxide (DMSO) for amphotericin B (Sigma Chemicals, St. Louis, MO). Inoculums (10<sup>6</sup> yeast cells/mL) were spread on Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/mL methylene blue dye (GMB) medium plates. The final concentrations of the antifungal agents ranged from 64 to 0.125 µg/ml. Filter paper discs (6 mm in diameter) were individually impregnated with 50 µL of each drug, and controls, placed onto the surface of inoculated Petri dishes, and incubated at 35°C (± 2 °C) within 15 min after the disks are applied. After 24 h incubation, the diameters (mm) of inhibition zones were measured. All the experiments were done in triplicates. DMSO or water served as a negative control.

### Micro-dilution test

The broth microdilution method was performed according to the document M27-A3 of the Clinical and Laboratory Standards Institute (CLSI, 2008).

The antifungal agents used were amphotericin B, flucytosine and fluconazole, and the susceptibility cut offs were in accordance to the parameters established by Yang et al. (2008) with MIC values

≤1 µg/mL considered susceptible and ≥2 µg/mL resistant to amphotericin, and by the supplement document M27-A3–M27-S3 (CLSI, supplement 2008) to fluconazole and flucytosine.

### Statistical analysis

All statistical analyses were conducted using SPSS. 16.0 statistical software. The chi-square test was performed to determine the differences between the *C. albicans* genotypes and associations with other variables such as site of infection, gender, age, residence and predisposing factors. *P* < 0.05 was considered as significant.

## RESULTS

### Genotype analysis of *C. albicans* strains

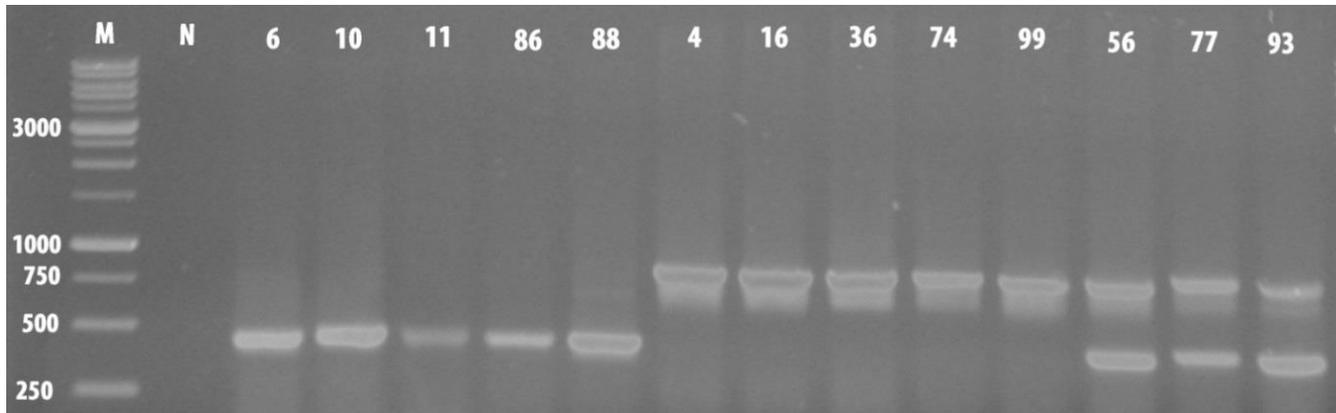
The genomic DNAs of the *C. albicans* strains obtained from different body locations of patients with CC and from patients with VVC were amplified by PCR using P-I to determine the genotypes based on variations in the 25S rDNA. The PCR profiles amplified with P-I defined DNA products of 450 bp for genotype A, 840 bp for genotype B, and 450, and 840 bp for genotype C (Figure 1). Of the one hundred and fifty one *C. albicans* strains, 83 (55%), 49 (32.4%), and 19 (12.6%) were recognized as genotypes C, A, and B, respectively (Table 1).

### Distribution of *C. albicans* genotypes

Genotypic distribution of the strains isolated from different CC locations or different conditions of VVC is presented in Tables 2 and 3. The frequency and distribution of genotypes among subgroups (location or condition) of each sample group were variable. Analysis of genotype distribution of *C. albicans* in every subgroup showed no obvious association between the strains of a certain genotype colonizing a specific condition of VVC or cutaneous location, gender, age, predisposing factors, or residence (Tables 2 and 3). However, genotypes A, B, and C showed their highest frequency in infants with younger age (up to four months), in infants with recent administration of antibiotics, younger women, and presence of VVC at pregnancy. Also, genotype A seemed to have higher association with infant's mouth, genotype B with the perianal region, and intense vulval pruritis and burning sensation conditions, and C with groin and hips sites (Tables 2 and 3).

### Relationship between genotypes and antifungal susceptibility

According to the analysis with a method of Multiway ANOVA, among the three antibiotics drugs of fluconazole, flucytosine, and amphotericin B, the susceptibility of the tested isolates to flucytosine and



**Figure 1.** Ethidium bromide-stained UV transilluminated PCR products of the transposable group I intron of the 25S rDNA gene of *C. albicans* isolates. Molecular size marker is the lane marked M and corresponding sizes as base pairs are given on the left. Lanes 6, 10, 11, 86, and 88 are genotype A (approximately 450 bp PCR product), lanes 4, 16, 36, 74, and 99 are genotype B (approximately 840 bp PCR product), and lanes 56, 77, and 93 are genotype C (approximately 450 and 840 bp PCR products). N refers to negative control.

**Table 1.** The frequency and distribution of genotypes among cutaneous (CC) and vulvovaginal (VVC) candidiasis groups.

Primer	Genotype	Number of isolates			Sig.
		CC (%)	VVC (%)	All lesions (%)	
P-I	A	29 (34.9)	20 (29.4)	49 (32.5)	0.686
	B	11 (13.3)	8 (11.8)	19 (12.6)	
	C	43 (51.8)	40 (58.8)	83 (55)	
Total		83 (100)	68(100)	151	

**Table 2.** *C. albicans* 25S rDNA genotypes distribution among CC infant patients in relation to different cutaneous locations and demographic characteristics of study population

Variable	Genotype			Total	P value*
	A Num (%)	B Num (%)	C Num (%)		
<b>Site of infection</b>					
Neck	4 (33.3)	3 (25.0)	5 (41.7)	12 (100)	0.329
Groin	1(9.01)	1 (9.01)	9 (81.8)	11 (100)	
Mouth	8 (47.1)	2 (11.8)	7 (41.2)	17 (100)	
Hips	4 (28.6)	1 (7.1)	9 (64.3)	14 (100)	
Perianal	4 (33.3)	3 (25.0)	5 (41.7)	12 (100)	
Armpit	2 (28.6)	0 (0.0)	5 (71.4)	07 (100)	
Back	6 (60.0)	1 (10.0)	3 (30.0)	10 (100)	
<b>Gender</b>					
Males	16 (37.2)	5 (11.6)	22 (51.2)	43 (100)	0.854
Females	13 (32.5)	6 (15.0)	21 (52.5)	40 (100)	
<b>Age (months)</b>					
(0-4)	15 (29.4)	8 (15.7)	28 (54.9)	51 (100)	0.751
(5-9)	10 (43.5)	2 (8.7)	11 (47.8)	23 (100)	
(10-14)	4 (50.0)	1 (12.5)	3 (37.5)	08 (100)	
(15-19)	0 (0.0)	0 (0.0)	1 (100)	01 (100)	

Table 2. Contd.

<b>Predisposing factors</b>					
Low weight birth	3 (33.3)	2 (22.2)	4 (24.4)	9 (100)	0.806
Recent administration of antibiotics	11 (32.4)	4 (11.8)	19 (55.9)	34 (100)	
Intravenous catheters	0 (0.0)	1 (50.0)	1 (50.0)	2 (100)	
Diaper	3 (30.0)	2 (20.0)	5 (50.0)	10 (100)	
Tight clothes	3 (60.0)	1 (20.0)	1 (20.0)	5 (100)	
Iatrogenic immune suppression	1 (33.3)	0 (0.0)	2 (66.7)	3 (100)	
No predisposing factors	8 (42.1)	1 (5.3)	10 (52.6)	19 (100)	
<b>Residence</b>					
City	29 (35.0)	11 (13.30)	43 (51.8)	83 (100)	
Village	0	0	0	0	
Camp	0	0	0	0	
<b>Total</b>				<b>83</b>	

\*Statically significant at ( $\alpha = 0.05$ ).

**Table 3.** Frequency of *C. albicans* P-I genotypes by VVCs conditions, patients age, residence and predisposing factors.

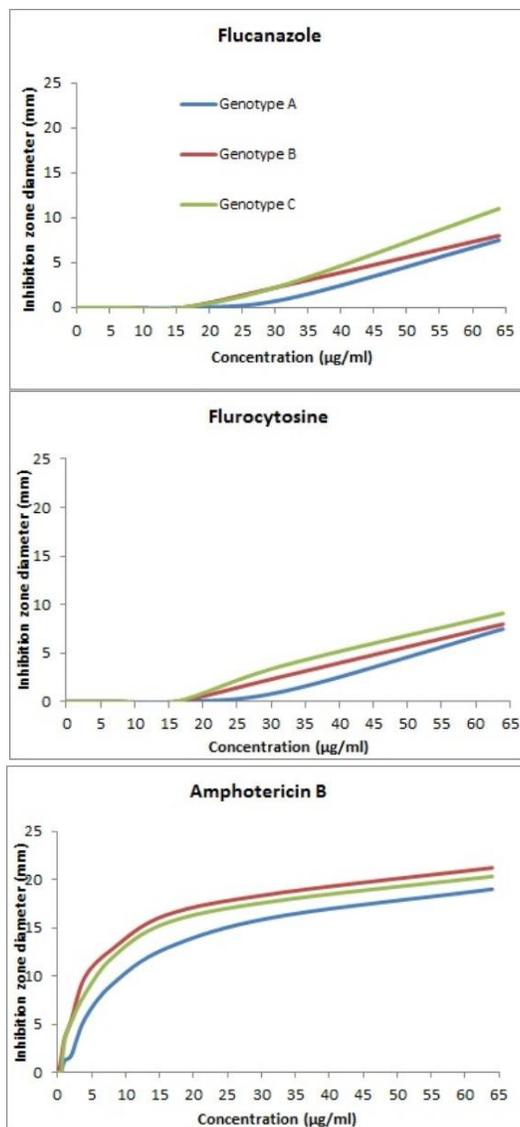
Variable	Genotype			Total	p Value*
	A Num (%)	B Num (%)	C Num (%)		
<b>Age</b>					
17-23	8 (40.0)	2 (10.0)	10 (50.0)	20 (100)	0.854
24-30	11 (27.5)	5 (12.5)	24 (60.0)	40 (100)	
31-37	1 (14.3)	1 (14.3)	5 (71.4)	7 (100)	
38-45	0 (0.0)	0 (0.0)	1 (100)	1 (100)	
<b>Residence</b>					
Jenin	5 (25.0)	4 (20.0)	11 (55.0)	20 (100)	0.206
Tubas	6 (30.0)	3 (15.0)	11 (55.0)	20 (100)	
Tamon	4 (21.1)	1 (05.3)	14 (73.7)	19 (100)	
Aqaba	5 (71.4)	0 (0.0)	2 (28.6)	7 (100)	
Tayaser	0 (0.0)	0 (0.0)	2 (100)	2 (100)	
<b>Predisposing factors</b>					
Tight clothes	2 (22.2)	1 (11.1)	6 (66.6)	9 (100)	0.973
Prolonged administration of antibiotics	3 (23.1)	1 (7.7)	9 (69.2)	13 (100)	
Presence of vaginal candidiasis at pregnancy	12 (32.4)	5 (13.5)	20 (54.1)	37 (100)	
No predisposing factor	3 (33.3)	1 (11.1)	5 (55.6)	9 (100)	
<b>Conditions (Symptoms)</b>					
Intense vulval pruritis	6 (37.5)	3 (18.8)	7 (43.8)	16 (100)	0.110
Burning	2 (25.0)	3 (37.5)	3 (37.5)	8 (100)	
Erythema	3 (18.8)	1 (6.3)	12 (75.0)	16 (100)	
Dyspareunia	9 (32.1)	1 (3.6)	18 (64.3)	28 (100)	

fluconazole differed considerably ( $P > 0.05$ ); there were differences among the susceptibilities of the three genotypes of *C. albicans* to flurocytosine and fluconazole (Figure 2).

The *C. albicans* genotype A was considerably less susceptible to flurocytosine and fluconazole than genotype B and C (both  $P < 0.05$ ). However, to the

susceptibility of genotype B and genotype C to flurocytosine and fluconazole, there was no statistical difference ( $P > 0.05$ ). On the other hand, the susceptibility of genotypes A, B, and C to amphotericin B was similarly high.

The distribution of the MICs of the isolated *C. albicans* to the three antifungal agents was presented in Table 4.



**Figure 2.** Susceptibility of *Candida albicans* genotypes to antifungal drugs.

**Table 4.** Minimum inhibitory concentration (MIC) of antifungal drugs against *Candida albicans* genotypes.

Antifungal agent	Genotype (Number of strains)	MIC (µg/ml)
Flucanazole	Genotype A (4)	1
	Genotype B (4)	0.44
	Genotype C (3)	0.5
Flurocytosine	Genotype A (4)	3.5
	Genotype B (4)	1
	Genotype C (3)	1
Amphotericin B	Genotype A (4)	0.5
	Genotype B (4)	0.5
	Genotype C (3)	0.5

## DISCUSSION

Molecular typing of *C. albicans* provides fundamental techniques for studying the epidemiology of this frequently isolated causative agent of candidiasis in humans, and for developing rational therapeutic strategies to combat the disease (Zhu et al., 2011; Redding et al., 1999).

In this study, the prevalence of *C. albicans* in the northern West Bank area of Palestine from 2012 to 2013 was investigated. Totally, 151 isolates of *C. albicans* in the northern West Bank area, Palestine was obtained from clinical specimen with a standard method.

PCR targeting 25S rDNA, which has frequently been used for genotype analysis of *C. albicans*, allows, on the basis of the length of the amplified PCR product, *C. albicans* isolates to be grouped into three main genotypes A, B, and C (Tamura et al., 2001; Hattori et al., 2006; McCullough et al., 1999; Iwata et al., 2006; Millar et al., 2002). In our study, the most prevalent *C. albicans* genotype among all isolates on the basis of amplification of 25S rDNA was genotype C (55%), followed by genotype A (32.4%), and B (12.6%). The ratios of genotypes A, B and C, of *C. albicans* varied among the reports, where the ratio of genotype B or C to genotype A *C. albicans* varied in each group of clinical specimens (Gurbuz and Kaleli, 2010; McCullough et al., 2004). These findings may be affected by the kinds of clinical specimens colonized by *C. albicans* (Iwata et al., 2006). However our results differed from those of previous researches carried out on VVC strains (Xiao-dong et al., 2008; Emmanuel et al., 2012; Zhu et al., 2011; Karahan and Akar, 2005), where it was shown that genotype A was represented with the highest frequency followed by genotype B and C. These variations of genotypes of *C. albicans* among these studies may be attributed to differences in type of strain, patient's age, and geographic locations (Clemons et al., 1997).

In this study, we could not detect a clear association between candidiasis strains and their invasive body sites, or predisposing factors, gender, age or conditions of infection of patients. This finding is in agreement with that of Xiao-dong et al. (2008) who also found no clear association between genotypes and the site of cutaneous infections, probably due to the fact that the influences of different skin surfaces in the cutaneous locations were minor and could not influence the genotype of strains. Our results are also in agreement with those of Xia-dong et al. (2008) who found no noticeable differences in genotypes of isolates from various VVC conditions, or significant association between genotypes of *C. albicans* and predisposing factor. The influences of residence and predisposing factors were minor and could not influence the genotype of strains.

Furthermore, based on the present findings, it was proposed that the influences of clearly different environments of cutaneous and vaginal mucous membranes,

patients with different age and sex, different skin surfaces in CC, and the different conditions of the VVC were minor and could not significantly influence the genotype of strains.

In conclusion, no clear correlation was found between the genotypes of *C. albicans* and patient's gender, age, underlying conditions of VVC, location of the CC lesions, residence, or predisposing factors. In the same patient there was usually one genotype of *C. albicans* colonizing various CC locations.

Many genotyping methods mainly focused on the study of the cloning origin of *C. albicans* and the patterns of transmission. Little is focused on the relationship between the genotyping and virulence (Jain et al., 2001). Early reports demonstrated that there was correlation between the group I intron in the 25S rDNA of *C. albicans* and the susceptibility to flurocytosine (Mercure et al., 1997; Zhu et al., 2011).

Our results of the antifungal susceptibility testing showed that there were differences among the antifungal susceptibilities of genotype A, B and C to flurocytosine. Genotypes B and C of *C. albicans* were more susceptible to flurocytosine and fluconazole than genotype A, but no differences were found in the antifungal susceptibilities of genotype A, B and C to amphotericin. It has also been shown by other researchers that genotype A is significantly resistant to fluconazol and flurocytosine, than genotypes B and C (Emmanuel et al., 2012; Tamura et al., 2001; McCullough et al., 1999).

The classification of genotypes employing this PCR relies on the presence of group I intron of varying size in the 25S rDNA, and group I intron has self-splicing capability which is necessary for the formation of mature 25S rRNA (Mercure et al., 1993). The self-splicing capability can be inhibited by base analogs (Mercure et al., 1997), so the strains harboring group I intron will be more susceptible to base analogs, such as flurocytosine (Zhu et al., 2011).

The antifungal mechanisms of amphotericin B are different from flurocytosine, therefore there was no association between the presence of group I intron in the 25 S rDNA of *C. albicans* and strains susceptibility to amphotericin B, and reliable markers of drug sensitivity or resistance are needed. Thus, identifying *C. albicans* at the genotype level help in prescribing the suitable antifungal drugs by specialists, and help in the control of *C. albicans*.

## Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

# Detection of *Mycobacterium tuberculosis* by rapid molecular methods augments acid fast bacilli (AFB) smear microscopy in a non-culture tuberculosis laboratory

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Laboratory diagnosis of tuberculosis (TB) has evolved into simplified molecular procedures that are adaptable in routine settings. We compared the diagnostic performance of smear microscopy by Ziehl-Neelsen (ZN) for detection of acid fast bacilli (AFB) with two different rapid *Mycobacterium tuberculosis* (MTB) specific molecular methods; Xpert MTB/RIF (Xpert) and Geno Type MTBDR*plus* (MTBDR), using a total of 194 consecutive sputum specimens from cases of pulmonary tuberculosis (PTB) in Jos, Nigeria. AFB was detected in 20% of cases by SM; while MTB was detected in 21 and 24% cases by Xpert and MTBDR, respectively. Fifty two (27%) of 194 specimens tested, were positive for AFB, MTB or both. One of 52 (2.0%) AFB positive result was MTB negative while 11/52 (21%) MTB positive cases were AFB negative. There was concordance in 65% of positive results detected by the three methods. MTB drug resistance by the molecular methods occurred in 26 MTB positive cases, of which 8/26 (31%) were MDR-TB. The simultaneous detection of MTB and the associated drug resistance, using Xpert and MTBDR improved TB diagnosis in Jos, Nigeria. We suggest the use of parallel testing of sputum specimens by SM and Xpert, with the retesting of rifampicin (RIF) resistant presumptive MDR-TB cases by MTBDR, as algorithm for TB diagnosis in high TB burden countries with limited TB culture laboratories.

**Key words:** *Mycobacterium tuberculosis* acid fast bacilli, Ziehl-Neelsen, GeneXpert MTB/RIF, Geno Type MTBDR*plus*.

## INTRODUCTION

Tuberculosis (TB) is one of the leading infectious diseases prevalent in low income countries where impoverished persons are at high risk of infection. The

World Health Organization (WHO) estimates that about 3 million people living with TB are unreached annually while 450,000 infected persons mainly in Eastern Europe

and Asia develop multidrug resistance tuberculosis (MDR-TB) globally (WHO, 2013). Strategic interventions by global health organizations aimed at reducing TB mortality and improving methods of laboratory diagnosis resulted in the endorsement of two rapid molecular methods; GenoType MTBDR*plus* (MTBDR; Hain Lifescience GmbH, Nehren, Germany) and GeneXpert MTB/RIF (Xpert; Cepheid; Sunnyvale, CA), that simultaneously detect the presence of *Mycobacterium tuberculosis* (MTB) and the associated drug resistance from clinical specimens and culture isolates (WHO, 2008, 2011). Several studies have validated the diagnostic performances and suitability of the methods (Hillemann et al., 2005; Scott et al., 2011; Crudu et al., 2012; Caws et al., 2006; Barnard et al., 2012; Sekadde et al., 2013; Ocheretina et al., 2014). Consequently, Xpert has been recommended for use in HIV high-incidence, low-income settings (Steingart et al., 2013; Balcha et al., 2014).

Nigeria ranks 11<sup>th</sup> among the 22 high TB burden countries that account for 80% of global TB (WHO global TB report, 2013). With high burdens of TB, HIV and MDR-TB, the nation is scaling up implementation of Xpert at different tiers of diagnostic laboratories to serve the nation's population of about 170 million and TB incidence of 108/1000000 people (WHO, 2012). In Jos, North central region of Nigeria, implementation of Xpert commenced in February 2013 while MTBDR was in July 2010. Xpert serves only for routine diagnosis of TB in HIV positive patients, presumptive drug resistant cases and other risk groups while MTBDR is reserved for further testing of rifampicin (RIF) resistant cases detected by Xpert, presumptive MDR and clinical specimens that do not comply with Xpert technology.

Only few laboratories in Nigeria have capacity for TB culture. Thus, the national TB Directly Observed Treatment Short Course (DOTS) algorithm stipulates the commencement of TB treatment with positive AFB SM results despite the known limitations.

This study compared the detection of acid fast bacilli (AFB) using smear microscopy (SM) by Ziehl-Neelsen with two different MTB specific molecular methods: Xpert MTB/RIF and MTBDR in a non TB culture laboratory.

## METHODOLOGY

A total of 194 consecutive sputum specimens were tested at the AIDS Prevention Initiative in Nigeria (APIN) laboratory Jos, Nigeria between February 2013 and April 2014. Specimens were registered for the study if patients were: referred from the DOTS-TB clinics in Jos, Nigeria, provisionally diagnosed for pulmonary TB and eligible for testing by Xpert. Of the total number of specimens tested, 151 were new or presumptive TB, 30 presumptive drug resistant TB and 13 treatment failed cases. Each of the specimens was tested using SM, Xpert and MTBDR methods.

## Smear microscopy

Non homogenized sputum (direct) was used for smear microscopy as recommended by the National DOTS-TB (NTBLCP, 2009). Smears were prepared directly from un-concentrated sputum specimens and left to dry overnight in a class 11 biosafety cabinet, fixed with 5% phenol alcohol, stained by ZN method and examined for AFB.

## MTBDR

DNA extraction with GenoLyse was performed by suspending 1 ml of 4% NaOH treated sputum specimen in 1.5 ml screw capped micro centrifuge tube and centrifuged for 15 min at 10,000 rcf. Pellet was re-suspended in 100 µl of lysate buffer, vortexed intermittently and incubated in a heat block at 95°C for 5 min and allowed to cool for 5 min after brief spinning. 100 µl of neutralization buffer was added to the lysate, vortexed and centrifuged for 5 min at full speed in a tabletop centrifuge. A positive control specimen (in-house verified positive specimen), was treated along with test specimens. Amplification mixture (43 µl) was dispensed into amplification tubes and 7 µl each of supernatant (DNA) of test and positive control specimens was added. Nuclease free water (7 µl) was used in place of DNA for negative control. PCR and detection were performed according to manufacturer's instruction (Hain Lifescience, 2014).

## Xpert

Following the manufacturer's instruction, (Cepheid, 2010) reagent buffer containing NaOH and isopropanol was added in a 2:1 ratio to sputum to a final volume of at least 2 mL. After 15 min incubation at room temperature and intermittent hand mixing for liquefaction and inactivation, 2 ml of treated specimen was added to the cartridge containing DNA amplification mixture and fluorescent detection probes and then loaded onto the Xpert module. The semi nested real time amplification and detection in integrated reaction proceeded to completion in 2 h with results displayed as MTB negative or MTB positive (with semi-quantification) and RIF sensitive or resistant.

## Statistical analysis

Statistical analysis of results was performed using the Statistical Package for Social Sciences (SPSS) version 17 for descriptives and Chi square distribution. P value < 0.05 was considered significant.

## RESULTS

### Detection of AFB and MTB

Fifty two of 194 (27%) specimens were positive by: SM; 40/194 (21%), Xpert; 41/194; (21%) and MTBDR; 46/194 (24%). A total of 34 (65%) positive results were concordantly detected by the three methods against 18 (35%) discordance of which; 13/52 (25%) were AFB negative-

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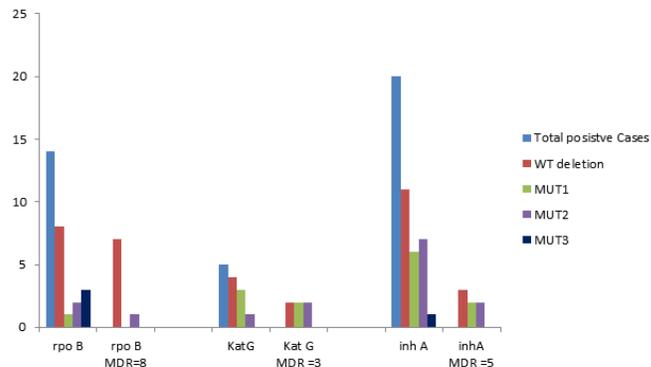
**Table 1.** Acid fast bacilli and *M. tuberculosis* detected using smear microscopy, Xpert and MTBDRplus. N=52 (%).

Assay	Number positive	%
<b>Concordant results (AFB, MTB)</b>		
smear microscopy		
Xpert MTB/RIF	34	65
GenoType MTBDRplus		
Total positive cases	34	65
<b>Discordant results (AFB, MTB)</b>		
Smear microscopy		
Xpert MTB/RIF	3	6
Smear microscopy		
GenoType MTBDRplus	1	2
Smear microscopy	1	2
Total positive cases	5	10
<b>Discordant results (MTB)</b>		
Xpert MTB/RIF		
GenoType MTBDRplus	2	4
Xpert MTB/RIF	2	4
GenoType MTBDRplus	9	17
Total positive cases	13	25

MTB positive, 1/52 (2%), AFB positive- MTB negative and 5/52 (10) AFB positive-MTB positive but detected by either Xpert or MTBDR (Table 1). TBDR detected significantly higher positive results in each case than SM and Xpert (p=0.006) (Chi square analysis).

**Drug susceptibility**

Twenty six of the 51 (51%) MTB positive cases were resistant to drugs. MDR was detected in 8/26 (31%) cases and mono resistance detected in 6/26 (23%); RIF and 12/26 isoniazid (INH); (46%) (Table 2).



**Figure 1.** Resistance profiles of Rifampicin and isoniazid by GenoType MTBDRplus (N=26).

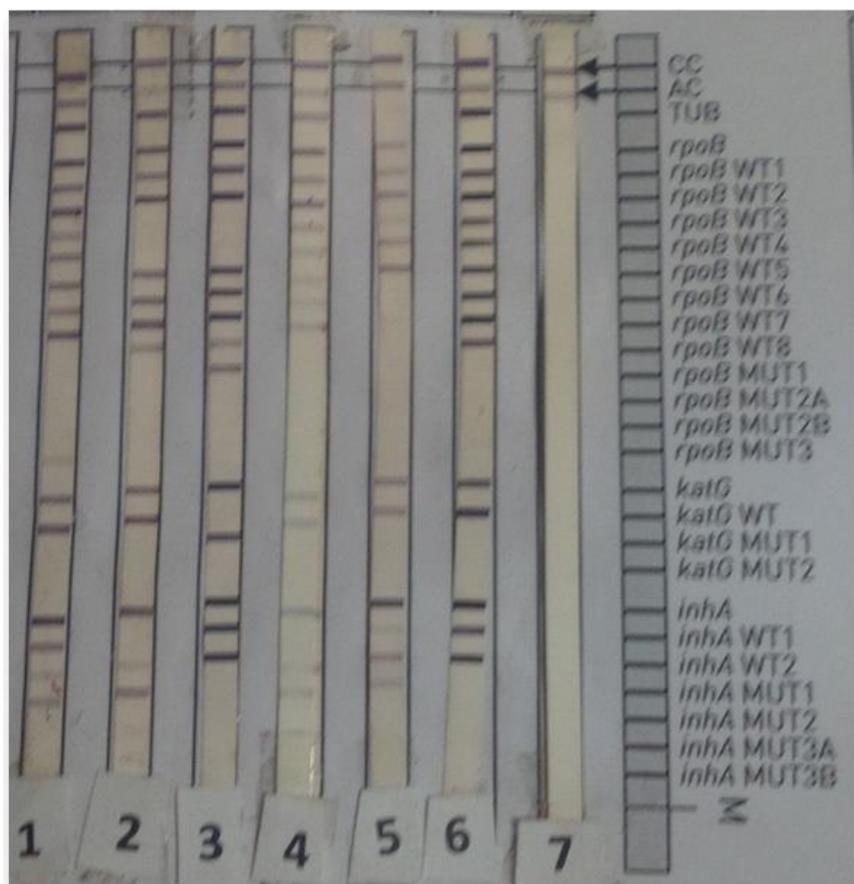
**Table 2.** Rifampicin and isoniazid resistant *M. tuberculosis* detected using Xpert MTB/RIF and GenoType MTBDRplus N=26(%).

Resistant <i>M. tuberculosis</i>	XPert	MTBDR*
Rifampicin (mono- resistance)	3	6
Isoniazid (mono-resistance)	N/A	12
<i>InhA</i> =11, <i>KatG</i> =1		
MDR	3	8
Total	6	26

\*Three additional cases of rifampicin mono resistant and 5 MDR were detected by MTBDR. N/A= Not applicable.

**Mutation types detected by MTBDR**

Mutation (MUT) type 3 on *rpoB* gene region conferring RIF resistance occurred in 3/14 (21%) followed by MUT2B 2/14 (14%) cases (Figure 1). Other RIF resistance types on the *rpoB* wild type regions occurred with no noticeable corresponding mutations in 8/14 (57%) cases (Figure 2, Lanes 2, 3, 4 & 5). INH mono resistance was more frequent on the *inhA* gene region; 11/20 (55%) than on *katG* gene; 1/20 (5%). Three of 5 (60%) *katG* gene associated INH resistance were MDR (Table 2, Figure 1). MTBDR test strips of some results showing RIF and INH resistance profiles are shown in Figure 2.



**Figure 2.** Test strips showing some results of Geno Type MTBDR $plus$ . M = Results evaluation template, CC = Conjugate control, AC = Amplification control, TUB = *Mycobacterium tuberculosis* complex, WT = Wild type probe, MTU = Mutation; Lane 1. *rpoB* WT 8 deletion, *rpoB* MUTs 1 and 3 insertion = MDR. Lane 2. *rpoB* WTs 3 and 4 deletion, *inhA* WT 1 deletion, *inhA* MUT 1 insertion = MDR. Lane 3. *rpoB* WTs 3 and 4 deletion, *rpoB* MUT 1 insertion, *katG* WT deletion, *katG* MUT 1 insertion = MDR. Lane 4. *rpoB* WT 8 deletion, *inhA* WT 1 missing, *inhA* MUT 1 insertion = MDR. Lane 5 *rpoB* WTs 6, 7 and 8, deletion, *inhA* MUT 1 = MDR. Lane 6. Positive control; RIF sensitive, INH sensitive. Lane 7. Negative control; NIL DNA.

## DISCUSSION

Direct SM is generally used for TB diagnosis in most low income high TB burden countries due to the limited availability of the gold standard TB culture methods. This study compared SM for detection of AFB with Xpert and MTBDR for MTB. Our results indicated that 25% of the positive results were AFB negative while 2% AFB were MTB negative. Due to limitations of this study, further identification tests were not performed to ascertain the specific identities of “positive” and “negative” results detected. The results however suggested low prevalence of AFB positive non *M. tuberculosis* (NTM) infection in Jos, Nigeria. A previous study on the genetic diversity of *Mycobacterium* species in Jos Nigeria reported *M. tuberculosis* as the dominant genotype (Ani et al., 2010).

There was agreement in the detection of RIF resistance

by Xpert and MTBDR (6/14; 43%) though MTBDR detected more MTB and RIF resistant cases. The finding was significant in the study setting considering the fact that decisions on treatment were based on the drug resistant profiles of either or both assays.

MTBDR also detected genetic profiles on respective gene regions of RIF and INH. Mutations (MUT 3 and MUT 2B) on *rpoB* gene region detected in this study have been reported in other geographical regions (Hirano et al., 1999; Mokrousov et al., 2004; Aparna et al., 2010). RIF resistance on the *rpoB* gene region with no observable corresponding mutation (Figure 2, Lanes 2, 3, 4 and 5) has been associated with mutations that may have occurred outside the region of analysis. (Ramasamy and Musser 1998; Huong et al., 2005; Hillmann et al., 2007) (Figure 2, Lanes 2 to 5).

The results also show that INH mono resistance

occurred more frequently on the *inhA* gene 10/20 (50%) regions than *katG* region; 1/20 (5%). Complete *katG* deletion is associated with a high level of INH resistance (MIC >5 µg/mL) (Tomasz et al., 2013). A total of 3/5 *katG* related INH resistance in this study were MDR.

The use of the Xpert and MTBDR enhanced TB diagnosis in Jos, Nigeria by providing prompt TB diagnosis, especially in AFB negative cases, HIV patients and presumptive MDR-TB. The simultaneous detection of MTB and drug resistance was relevant for early and improved patients' management in the absence of MTB culture and drug susceptibility test (DST).

In conclusion, the molecular methods used in this study are simple to perform and adaptable in routine settings. We recommend that the assays be considered for routine use in the absence of TB culture laboratories. However due to the high cost of the equipment for molecular assays and issues of sustainability, the assays may not be readily affordable in most of the low income countries. These limitations underscore the need for increased funding to support the implementation and sustainability of the methods.

### Conflict of interests

The authors did not declare any conflict of interest.

### ACKNOWLEDGEMENTS

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