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Characterization, *In-vitro* Detection of Extracellular Hydrolytic Enzymes and Antifungal Susceptibility of Faecal Candida Isolates from Diarrhoeal Patients

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ABSTRACT

Mycological analysis of 60 diarrhoeal stool samples from patients attending different hospitals / clinics in Uyo was carried out using standard procedure. Characterization of extracellular hydrolytic enzymes and antifungal susceptibility of candida isolates obtained were determined using appropriate culture media and disc diffusion technique. Of the 60 stool samples collected, 70.0 % samples were only watery, 18.3 % samples were watery and mucoid, 6.7 % samples were watery and bloody, while 5.0 % samples were watery, mucoid and bloody. Of the 42 stool samples (watery only), 21.4 % showed candida overgrowth (counts of $>10^4$ CFU/ml) and the five candida species isolated were *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei*. The results showed that 41.7 % and 33.3 % stool samples from male patients aged $\leq$ 20 yrs and $\geq$ 51yrs had candida isolates, respectively. Among the female patients with diarrhoea, the highest occurrence of candida isolates was obtained from age group < 20 yrs and the lowest occurrence was obtained among age group 31-40 yrs. There was no significant relationship (P >0.05) between the age group and occurrence of candida isolates in the diarrhoeal stool of patients. $\geq$ 60.9 %, 47.8 % and 65.2 % isolates were sensitive to fluconazole, nystatin and voriconazole, respectively. The results showed that 39.1 % isolates produced haemolysin and 26.1 % produced protease only, 17.4 % isolates produced both haemolysin and lipase, 13.4 % were both haemolysin and protease producers, 8.7 % isolates produced both lipase and protease, while 11.1 % *isolates* produced all the three extracellular hydrolytic enzymes. This study has revealed the emergence of candida associated diarrhoea in the study area and voriconazole as the first drug of choice for the treatment.

Key words: Candida, Overgrowth, Antifungal, Enzymes, Susceptibility, Diarrhoea.

INTRODUCTION

Diarrhoea accounts for an estimated 3.6 % of the global burden of disease (Murray and Lopez, 1997). Although mortality attributable to diarrhoea has declined substantially over the past 25 years globally, morbidity from diarrhoea in sub-Saharan Africa has not reduced (Okeke *et al*., 2003). The increase in diarrhoeal diseases can be attributed to poor hygiene and sanitation, limited access to safe drinking water, insufficient promotion of breastfeeding, as well as inadequate education of health care providers and recipients (Curtis *et al*., 2000; Okeke *et al*., 2003; Thapar and Sanderson, 2004). Various pathogens such as enteric bacteria, parasites, fungi and viruses have been incriminated in diarrhoea and their involvement in causing diarrhoea vary considerably between regions depending on local meteorological, geographical and socio-economic conditions (Babaniyi, 1991; Akinjogunla *et al*., 2009; Imade and Eghafona, 2015). *Candida* spp form parts of the normal flora in the alimentary tract and mucocutaneous membranes, and their occurrences in the faecal samples are considered non-pathogenic (Warren *et al*., 1991). Occasionally, *Candida* spp become pathogenic after proliferation in the gastrointestinal tract owing to alterations in gut flora and other host predisposing factors (Sardi *et al.,* 2013; Imade and Eghafona, 2015). *Candida*, *Trichosporon* and *Geotrichum* are fungi that have been reported to cause diarrhoea and the role of *Candida* spp in pathogenesis of antibiotic-associated diarrhoea in elderly patients have also been well documented (Talwar *et al*., 1990; Danna *et al*., 1991). *C albicans* is the predominant yeast in human faeces, being identified in high concentrations in the diarrhoeal stools of healthy adults and malnourished children (Enweani *et al.*, 1994). Dimorphism, adhesion, production of extracellular hydrolytic enzymes and antigenic modulations are important virulence factors that contribute to the pathogenicity of *Candida* spp. Extracellular hydrolytic enzymes such as lipase, secreted aspartyl proteinases, phospholipases and haemolysin play an essential role in candidal overgrowth and facilitate adherence, tissue penetration and invasion of the host tissue (Schaller *et al*., 2005; Tsang *et al*., 2007). The aim of this study was to determine the extracellular hydrolytic enzymes and antifungal susceptibility profile of faecal candida isolates from diarrhoeal patients in Uyo, Nigeria.

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MATERIALS AND METHODS

Collection of Diarrhoeal Stool Samples

Sixty (60) stool samples from diarrhoeal patients were aseptically collected from patients (aged ≤ 20 yrs to ≥ 51 yrs) using sterile wide mouth containers between April and July, 2017. Verbal informed consents were obtained from the patients who had not taken antifungal drugs seven days prior to the time of samples collection. The stool samples were properly labelled, kept on ice and transported to the microbiology laboratory for mycological analysis.

Mycological Analysis of Diarrhoeal Stool Samples

One (1) ml of each stool sample was aseptically diluted in 9 ml of sterile physiological saline and serial dilution was made up to 10^{-4}. One (1) ml of each aliquot was aseptically inoculated onto each plate of Sabouraud Dextrose Agar (SDA) containing chloramphenicol and aerobically incubated at 35ºC for 48 hrs. After incubation, the colonies on each plate were enumerated and counts of $>10^4$ CFU/ml were considered as over-growth and thus, significant for candida diarrheal infection. The candida isolates in the primary plates were subcultured onto plates of SDA, aerobically incubated at 35ºC for 48 hrs, maintained on SDA slant at 4ºC, characterized and identified based on their cultural and morphological characteristics. The candida isolates were further subcultured onto plates of CHROM agar Candida (Difco BBL., USA), aerobically incubated for 48 hrs at 35°C, and pigmentation was observed and used for species differentiation. Gram staining, germ tube, chlamydospores production, sugar fermentation and assimilation tests were also carried out.

Antifungal Susceptibility Testing of candida Isolates

In vitro susceptibility of the candida isolates to fluconazole (FLU, 25 µg), nystatin (NYS, 100 units) and voriconazole (VOR, 1 µg) was determined by disc diffusion method (CLSI, 2012). Suspension (10 µl) of each candida isolates, prepared directly from an overnight agar plate using physiological saline, visually adjusted to turbidity of 0.5 McFarland Standard, was inoculated and spread over the dried surface of each plate containing Glucose - Methylene Blue - Mueller Hilton Agar (GMBMHA, composition: 0.5 g/ml methylene blue, 2 % glucose and Mueller Hilton Agar) using sterile pipettes. The antifungal discs were aseptically placed onto the surface of each GMBMHA plate and incubated for 48 hrs at 35 °C. Inhibitory zones after incubation were observed and measured in millimetre. The interpretation of the measurement as sensitive (S), dose dependent susceptible (DDS) and resistant (R) was made as follows: NYS and VOR (S: ≥ 16, DDS: 10-15, R ≤ 9) and FLU (S: ≥19, DDS: 15-18, R ≤ 14).

Detection of Haemolysin Producing Candida Isolates

Suspension (10 µl) of each candida isolate, adjusted to turbidity of 0.5 McFarland Standard, was spot inoculated onto plate of human blood SDA (3 % glucose, 5% human blood and SDA) and aerobically incubated for 48 hrs at 35 ºC. Translucent zone around the isolate was considered positive for haemolytic activity (Manns *et al*., 1994; Akinjogunla *et al.*, 2016)

Detection of Proteinase Producing Candida Isolates

Suspension (10 µl) of each candida isolate, adjusted to turbidity of 0.5 McFarland Standard, was spot inoculated onto plates of gelatin agar (1 % gelatin, SDA) and aerobically incubated for 48 hrs at 35 ºC. Transparent zones around the isolates indicated production of proteinase (Nachimuthu *et al*., 2011; Akinjogunla *et al.*, 2014)

Detection of Lipase Producing Candida Isolates

Suspension (10 µl) of each candida isolate, adjusted to turbidity of 0.5 McFarland Standard, was spot inoculated onto plate of Tributyrin-SDA (1 % tributyrin and SDA) and aerobically incubated at 35 ºC for 48 hrs. Clear zone around the isolate indicated the production of lipase (Nachimuthu *et al*., 2011).

Statistical Analysis

All statistical analyzes were performed using Statistical Package for Social Science (SPSS, Version 20)**.** Chi-square test was used and a P-value < 0.05 was considered as significant.

RESULTS

The macroscopic examination and candida overgrowth of stool samples of diarrhoeal patients are shown in Table 1. Of the 60 stool samples collected, 42 (70.0%) samples were only watery, 11 (18.3%) samples were watery and mucoid, 4 (6.7 %) samples were watery and bloody, while 3 (5.0 %) samples were watery, mucoid and bloody. Among the 42 stool samples (watery only) from the diarrhoeal patients, 9 (21.4 %) showed candida overgrowth (counts of $>10^4$ CFU/ml), 13 (31.0 %) samples were without overgrowth of candida (counts of $< 10^4$ CFU/ml), while 20 (47.6 %) samples had no candida growth on the SDA used. Only 1 (9.1 %) SDA plate inoculated with watery and mucoid stool samples showed candida overgrowth, while mucoid and bloody stool samples (n=4) and watery, mucoid and bloody stool samples (n=3) had no candida growth (Table 1). A total of twenty three (23) candida isolates were obtained from the sixty (60) stool samples of patients with diarrhoea (Table 3). The occurrence of *C. albicans* and non - albicans candida (NAC) in stool samples of diarrhoeal patients in descending order was as follows: *C. albicans* (39.1%, n=9) > *C. tropicalis* (21.7 %, n=5) > *C. glabrata* (17.4 %, n=4) > *C.*

parapsilosis (13.0, n=3) > *C. krusei* (8.7 %, n=2) (Table 2).

Table 3 shows the age and gender-specific occurrence of candida isolates in stool samples of diarrhoeal patients. The results showed that 5 (41.7%) stool samples from male diarrhoeal patients (aged < 20 yrs) had candida isolates, while 2 (33.3 %) , 2 (40.0 %) and 1 (33.3 %) samples from male diarrhoeal patients with age groups of 21-30 yrs, 31-40 yrs and > 51yrs had candida isolates, respectively. Among the female patients with diarrhoea, the highest occurrence of candida isolates was obtained from age group < 20 yrs with 7 (46.7 %) and the lowest occurrence was obtained among age group 31-40 yrs with 1 (25.0 %) (Table 3). There was no statistically significant difference ($P > 0.05$) between the occurrence of candida isolates in the stool samples of diarrhoeal patients with respect to age and gender (Table 3).

Of the 23 candida isolates tested, 14 (60.9 %), 11 (47.8 %) and 15 (65.2 %) were sensitive to fluconazole, nystatin and voriconazole, respectively. The percentage resistance of the isolates to the antifungal assayed ranged from 21.7 % to 30.4 %. *C. glabrata* were highly sensitive to fluconazole having 75.0 %. *C. albicans* and *C. tropicalis* were highly sensitive to voriconazole with percentage susceptibility ranging from 80.0 % to 88.8 %, while 50.0 % of *C. krusei* were resistant to nystatin and voriconazole. ≥ 3 (13.0 %) candida isolates were dose dependent susceptible to fluconazole, nystatin and voriconazole (Table 4). The virulence factors of *Candida albicans* and NAC isolated from the stool samples of diarrhoeal patients are shown in Table 5. Of the twenty (23) candida isolates, 9 (39.1 %) produced haemolysin, 5 (21.7 %) produced lipase and 6 (26.1 %) produced protease only. The results also showed 4 (17.4 %) isolates as haemolysin and lipase producers, 3 (13.4 %) were haemolysin and protease producers, while 2 (8.7 %) isolates produced both lipase and protease. Only 1(11.1 %) *C. albicans* produced all the three extracellular hydrolytic enzymes (Table 5).

Table 1: Macroscopic Examination and *Candida* Overgrowth of Stool Samples of Diarrhoeal Patients

Nature of Stool Samples	No of Samples Collected	Samples With *Candida* Overgrowth No (%)	Samples Without *Candida* Overgrowth No (%)	Samples Negative for *Candida* No (%)
Watery	42	9 (21.4)	13 (31.0)	20 (47.6)
Watery + Mucoid	11	1 (9.1)	0 (0.0)	10 (90.9)
Watery + Bloody	4	0 (0.0)	0 (0.0)	4 (100)
Watery + Mucoid + Bloody	3	0 (0.0)	0 (0.0)	3 (100)
Total	60	10 (16.7)	13 (21.7)	37 (61.7)

Table 2: The Occurrence of Candida Isolates in Stool Samples of Diarrhoeal Patients

Candida Isolates	No of Occurrence	Percentage of Occurrence
C. albicans	9	39.1
C. tropicalis	5	21.7
C. krusei	2	8.7
C. glabrata	4	17.4
C. parapsilosis	3	13.0
Total	23	100

Table 3: Age and Gender-Specific Occurrence of Candida Isolates in Stool Samples of Diarrhoeal Patients

Ages (Yrs)	Male			Female			X^2	P-value
	No of Samples	No (%) Positive	No (%) Negative	No of Samples	No (%) Positive	No (%) Negative		
< 20	12	5 (41.7)	7 (58.3)	15	7 (46.7)	8 (53.3)		
21-30	6	2 (33.3)	4 (66.7)	7	3 (42.9)	4 (57.1)	1.50	0.826
31-40	5	2 (40.0)	3 (60.0)	4	1 (25.0)	3 (75.0)		
41-50	2	0 (0.0)	2 (100)	3	1 (33.3)	2 (66.7)		
>51	3	1 (33.3)	2 (66.7)	3	1 (33.3)	2 (66.7)		
Total	28	10 (35.7)	18 (64.3)	32	13 (40.6)	19 (59.4)		

Table 4: Antifungal Susceptibility of Candida Isolates from Stool Samples of Diarrhoeal Patients

Yeast Isolates	No of Isolates	Fluconazole			Nystatin			Voriconazole		
		S No. (%)	DDS No. (%)	R No. (%)	S No. (%)	DDS No. (%)	R No. (%)	S No. (%)	DDS No. (%)	R No. (%)
C. albicans	9	6(66.7)	1(11.1)	2(22.2)	4(44.4)	2(22.2)	3(33.3)	8(88.9)	1(11.1)	0(0.0)
C. tropicalis	5	2(40.0)	2(40.0)	1(20.0)	2(40.0)	1(20.0)	2(40.0)	4(80.0)	0(0.0)	1(20.0)
C. krusei	2	1(50.0)	1(50.0)	0(0.0)	1(50.0)	0(0.0)	1(50.0)	0(0.0)	1(50.0)	1(50.0)
C. glabrata	4	3(75.0)	0(0.0)	1(25.0)	2(50.0)	1(25.0)	1(25.0)	2(50.0)	0(0.0)	2(50.0)
C. parapsilosis	3	2(66.7)	0(0.0)	1(33.3)	2(66.7)	1(33.3)	0(0.0)	1(33.3)	1(33.3)	1(33.3)
Total	23	14(60.9)	4(17.4)	5(21.7)	11(47.8)	5(21.7)	7(30.4)	15(65.2)	3(13.0)	5(21.7)

Keys: S: Sensitive; DDS: Dose Dependent Susceptible; R: Resistant

Table 5: Extracellular Hydrolytic Enzymes of Candida Isolates from Stool Samples of Diarrhoeal Patients

Candida Isolates	No of Isolates	HAE No (%)	LIP No (%)	PRO No (%)	HAE + LIP No (%)	HAE + PRO No (%)	LIP + PRO No (%)	HAE + LIP + PRO No (%)
C. albicans	9	4(44.4)	1(11.1)	2(22.2)	2(22.3)	0(0.0)	1(11.1)	1(11.1)
C. glabrata	5	1(20.0)	2(40.0)	2(40.0)	1(20.0)	1(20.0)	0(0.0)	0(0.0)
C. krusei	2	1(50.0)	0(0.0)	1(50.0)	1(50.0)	0(0.0)	0(0.0)	0(0.0)
C. tropicalis	4	2(50.0)	2(50.0)	0(0.0)	0(0.0)	1(25.0)	1(25.0)	0(0.0)
C. parapsilosis	3	1(33.3)	0(0.0)	1(33.3)	0(0.0)	1(33.3)	0(0.0)	0(0.0)
Total	23	9(39.1)	5(21.7)	6(26.1)	4(17.4)	3(13.0)	2(8.7)	1(4.3)

Keys: HAE: Haemolysin; LIP: Lipase; PRO: Protease

DISCUSSION

Diarrhoea is one of the main causes of morbidity and mortality among individuals in socio-economically developing and developed countries. In this study, 16.7 % diarrhoeal stool showed candida overgrowth indicating candida diarrhoeal infection and this value was lower than 30.0 % reported by Imade and Eghafona (2015) in Benin City. The occurrence of candida overgrowth in diarrhoeal stool samples corroborates the reports of Nkuo-Akenji *et al.* (2002) who found an association between *Candida* spp and diarrhoea. The occurrence of *C. albicans* and *C. krusei* in the stool samples of diarrhoeal patients substantiates the previous results of Chaudhury *et al*. (1996) and Beena *et al*. (2016). The NAC are also gaining clinical importance and their emergence is probably related to selection of less susceptible species by the pressure of antifungal agents (Vaideeswar *et al*., 1999). The predominant *Candida* isolate obtained was *C. albicans* and this agrees with the results of Enweani *et al*. (1994) who reported *C. albicans* as the most common candida isolate in diarrhoeal stool.

The findings in this study showing *C. albicans* as the most common fungal yeast in the stool samples was dissimilar with that of Beena *et al*. (2016) who reported C. *krusei* as the prevalent yeast isolate. The variability in the occurrence of different *Candida* spp in different individuals from different geographical areas might be attributed to age factor and immunity (Vazquez and Sobel, 2002). The candida isolates from the diarrhoeal stool in this study were highly sensitive to voriconazole and fluconazole, and this validates the previous results of Beena *et al*. (2016) who reported exceedingly high sensitivity of candida isolates to voriconazole and fluconazole. The emergence of fluconazole as the principal treatment option for practically all forms of susceptible candida infections in both immune competent and immune compromised hosts have been reported (Pfaller *et al*., 1999). The fluconazole sensitive *C. albicans* were more than fluconazole sensitive *C. tropicalis* and this result was similar to that of Beena *et al*. (2016) who observed 57.2 % *C. albicans* and 13.6 % *C. tropicalis* sensitivity to fluconazole. In our study, 66.7 % *C. albicans* and 40. 0 % *C. tropicalis* were sensitive to fluconazole and these values were higher than the values reported by Patel *et al.* (2012) in which 25.5 % *C. albicans* and 18.7 % *C. tropicalis* were sensitive to fluconazole. Findings from this study showed that 50 % *C. krusei* were resistant to fluconazole. Although, this value was lower than 100% obtained by Hamza *et al.* (2008) who reported in Tanzania that all isolate of *C. krusei* tested were resistance to fluconazole. It however, confirmed the well-established reports that *C. krusei* is intrinsically resistant to fluconazole. The occurrence of nystatin resistant candida isolates in our study agrees with Kashid *et al.* (2011) and Sajjan *et al*. (2014) who also obtained nystatin resistant candida isolates. Nystatin act by binding polyene to sterols in the yeast plasma membrane resulting in a change in their permeability, thus, the fungal cells lose potassium, sugar and phosphate ions, which leads to the impairment of glycolysis and cellular respiration.

The pathogenicity of *Candida* spp is attributable to extracellular hydrolytic enzymes that act synergistically under favourable conditions (Silva *et al*., 2011). The occurrence of haemolysin, lipase and protease producing *Candida* spp in this study corroborates the previous reports of Ying and Chunyang (2012) and Rossoni *et al.* (2013). The haemolysin aids the isolates to lyse host erythrocytes and strips iron from haemoglobin molecules (Manns *et al*., 1994). Proteinase enzyme facilitates adherence and phenotypic switching of *Candida* spp by hydrolyzing the peptide bonds in proteins (Naglik *et al*., 2003).

Conclusion: This study showed that voriconazole should be considered as the first drug of choice for treatment of candida associated diarrhoea and further supports the association between *Candida* spp and diarrhoea notwithstanding the undetermined mechanisms by which faecal candida overgrowth causes diarrhoea.

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