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

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Full Length Research Paper

Antifungal activity of nanofungicide Trifloxystrobin 25% + Tebuconazole 50% against *Macrophomina phaseolina*

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This study was aimed to develop the nanoform of a commercial fungicide Trifloxystrobin 25% + Tebuconazole 50% (75 WG) with broad spectrum of action for improving its antifungal activity against *Macrophomina phaseolina*. The fungicide commercially available as Trifloxystrobin 25% + Tebuconazole 50% (75 WG) was converted into its nanoform using ball milling method and assessed for its efficacy against the soil borne fungal pathogen *M. phaseolina* at various concentrations, namely 5, 10, 15, and 25 ppm using poisoned food technique. Nanoform of the fungicide was characterized using Scanning Electron Microscopy (SEM) and Particle Size Analyzer (PSA). The average particle size of nano Trifloxystrobin 25% + Tebuconazole 50% (75 WG) was about 108 nm. Fungicidal potential of nanoform was better in comparison to the conventional ones. Nanoform of the fungicide was effective at 10 ppm and it exerted hyphal abnormality, hyphal lysis and abnormality of sclerotial formation on *M. phaseolina* when tested under *in vitro* than control. This study suggests the possibility to enhance the antifungal activity of fungicide Trifloxystrobin 25% + Tebuconazole 50% (75 WG) towards the control of *M. phaseolina.*

Key words: Antifungal, nanoformulations, chilli, Trifloxystrobin 25% + Tebuconazole 50% (75 WG), *Macrophomina phaseolina*.

INTRODUCTION

Soil-borne fungal pathogens cause diseases in economically important crops resulting in huge monetary losses to the farmers. Among them, *Macrophomina phaseolina*a has very wide host range in the tropics and subtropics that causes charcoal rot, seedling blight, dry root rot, wilt, leaf blight and ashy stem blight in more than 500 cultivated and wild plant species including economically important crops as soybean, common bean, sorghum, maize, cotton, peanut, and cowpea (Hall, 1991; Diourte et al., 1995; Javaid and Saddique, 2011). It forms microsclerotia in senescing shoot tissues and survive in the soil for a long period (Mayek-Pérez et al., 2002).

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Changes in agricultural technology have been a major factor shaping modern agriculture. The development of nanodevices and nanomaterials could open up novel applications in plant biotechnology and agriculture (Scrinis and Lyons, 2007). Nanotechnology permits broad advances in agricultural research, such as reproductive science and technology, conversion of agricultural and food wastes to energy and other useful by-products through enzymatic nano-bioprocessing, disease prevention and treatment in plants using various nanofungicides (Patolsky et al., 2006). Improved properties of the nanoparticles compared to the application of bulk materials have greater opportunity to reduce the load of unwanted chemicals especially plant protectants. The fungicidal efficacy of nano-sulphur and commercial sulphur at 1000 ppm against *Erysiphe cichoracearum* of okra has been studied. The sulphur fungicides significantly reduced the germination of conidia of *E. cichoracearum* than the control. Nano-sulphur was more effective than the commercial formulations and could be applied at lower amount for controlling powdery mildew disease for its better efficacy (Gogoi et al., 2013). In the present scenario, *M. phaseolina* is managed by seed and soil application of benzimidazole group fungicides. Bulk use of fungicides, not only causes environmental pollution, but also results in the development of resistance in pathogens.

To avoid the risk of phytotoxicity, threat to non-targeted organisms and the environment, an idea was conceived to have better control of *M. phaseolina* using lower or safe doses of fungicides. Recently, nanoformulations (particle size <100 nm) of fungicides have been able to draw much attention due to their higher efficacy even at very low doses, because nanoparticles could be more chemically reactive and bioactive than larger particles (Gogoi et al., 2009).

Hence, the present study was aimed to increase the efficacy of commercially available Trifloxystrobin 25% + Tebuconazole 50% (75 WG) by converting into nanoform and nanoform of fungicide had higher antifungal activity against *M. phaseolina* even at low concentration.

MATERIALS AND METHODS

The experiment was carried out at the Department of Nano Science and Technology, Tamil Nadu Agricultural University, Coimbatore, during 2015 under *in vitro* condition.

Chemicals

Commercially available Trifloxystrobin 25% + Tebuconazole 50% (75 WG) fungicide of Bayer Crop Science Ltd. was purchased from the pesticide shop from Coimbatore, Tamil Nadu, India.

Synthesis and characterization of nanofungicide

The nanofungicide was size reduction using FRITSCH-High Energy Ball Milling Method (Shyla, 2014). Characterization of the synthesized nanoparticles was performed by the techniques described subsequently.

Particle size analyzer (PSA)

Particle size and the distribution pattern of synthesized sample suspensions were determined using Horiba Scientific Nanopartica SZ-100 (Nanoparticle analyzer), Japan. MALVERN, Zetasizer Ver.6.01. particle size analyzer was used accurately, 0.5 mg sample was dispersed in 20 ml distilled water, sonicated for 15 min and the suspension was analyzed under dynamic light scattering method using 90° or 173° at 25°C (Anandraj, 2013).

Zeta potential

Zeta potential measurement for synthesized inorganic NPs was determined using a zeta analyzer (Horiba, SZ-100). In the zeta analyzer, zeta potential measurement varied from -200 to 200 mV and the data acquisition time is usually less than 1 min for zeta potential measurement and the laser light is divided into two beams as input light and reference light. Scattered light by sample particles and reference light modulated by the modulator interfere in the prism are detected and the detected signals are changed into digital signal to be calculated (Sridhar, 2012).

Scanning electron microscopy (SEM)

SEM (FEI QUANTA 250) was used to characterize the size and morphology of the nanoparticles. Sample of test nanoparticles (0.5 to 1.0 mg) was dusted on one side of the double sided adhesive carbon conducting tape, and then mounted on the 8 mm diameter aluminum stub. Sample surface were observed at different magnification and the images were recorded (Shyla, 2014).

In vitro assay of antifungal activity of nanofungicides

The antifungal activity of nanofungicides with active ingredient Trifloxystrobin 25% + Tebuconazole 50% (75 WG) were evaluated against *M. phaseolina* by using poisoned medium technique (Packia Lekshmi et al., 2012) using potato dextrose agar (PDA) medium amended with different concentrations (5, 10, 15, and 25 ppm). Non-amended medium served as a control. A nine millimeter disc of the actively growing *M. phaseolina* from a 7 days old culture was placed at the centre in each of the nanoparticles amended medium as well as in the untreated check. The mycelial growth of the pathogen was measured after five days of inoculation by incubating the Petri plates at 28±2°C. The percent inhibition of the mycelial growth over control was calculated to express the antifungal activity.

Ultra microscopic changes on *M. phaseolina* induced by nanofungicides

Structural abnormality, hyphal lysis and inhibition of sclerotial formation induced by Trifloxystrobin 25% + Tebuconazole 50% (75 WG) nanofungicides were examined under SEM (Model FEI Quanta 250) at various resolutions (2500 to 6000X). The stub of SEM fixed with double-side adhesive carbon tape was gently placed over the mycelia mat of Petri dishes having hampered growth and removed immediately upon visual confirming for the presence of hyphae, then was fixed in the appropriate location of the SEM and observed for hyphal characters under low vacuum condition.

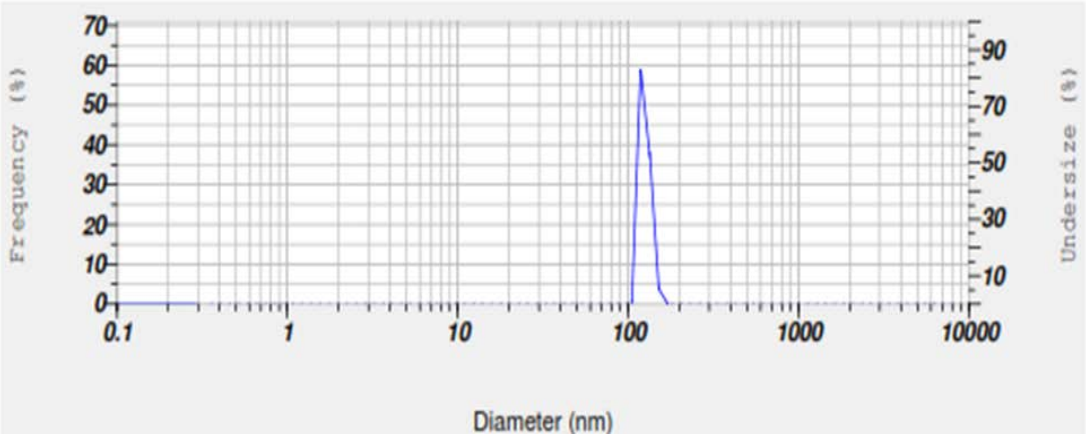

Figure 1. Particle size of ball milled nano fungicide.

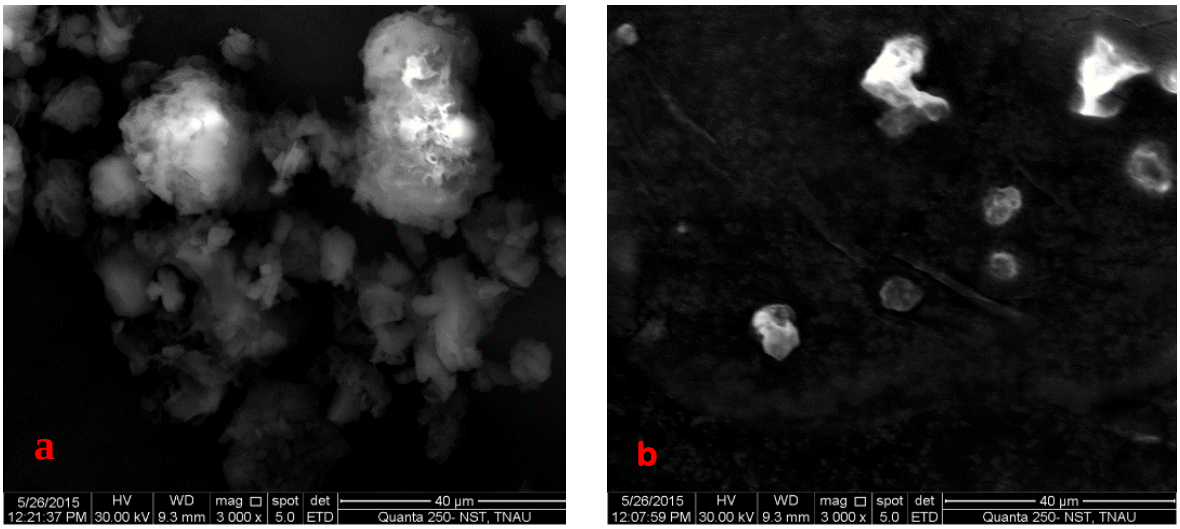

Figure 2. SEM image: (a) Commercially available fungicide at 3000x; (b) After ball milled nano formed fungicide at 3000x.

RESULTS AND DISCUSSION

Characterization of nanofungicide Trifloxystrobin 25% + Tebuconazole 50% (75 WG)

The surface morphology of the nanofungicides synthesized and examined under particle size analyzer (PSA) revealed that the particle size range from 108 to 130 nm diameter (Figure 1). But the size of traditionally available Trifloxystrobin 25% + Tebuconazole 50% (75 WG) was of 1.3 to 3.0 µm diameter.

To confirm the results of PSA, the same nanoformulated Trifloxystrobin 25% + Tebuconazole 50% (75 WG) fungicide was characterized under SEM. Irregular shaped nanoparticles was observed and it ranged from 108 to 130 nm (Figure 2).

Antifungal activity of nanofungicide

The poisoned medium technique was employed to determine inhibitory efficacy of different concentrations of nanoform of Trifloxystrobin 25% + Tebuconazole 50% (75 WG) as compared to commercial fungicide against *M. phaseolina* (Table 1). Among the different concentrations, the maximum inhibition of the mycelial growth was obtained with 15 ppm concentration which accounted for 99.6% reduction of mycelial growth over control in *M. phaseolina*. This was followed by complete inhibition of mycelial growth at 25 ppm which accounted for 100% reduction of mycelial growth over control (Figure 3).

Exploitation of nanofungicides in future may pave the way for effective management of seed borne and soil borne pathogens like *M. phaseolina*; thereby may reduce

Table 1. Effect of nano form of Trifloxystrobin 25% + Tebuconazole 50% (75 WG) fungicide on the mycelial growth of *M. phaseolina in vitro*.

Nanofungicide (concentration, ppm)	Mycelial growth inhibition over control (%)*	
	Fungicide (without ball milling)	Nano fungicide (ball milled)
5	26.7[f]	73.7[d]
10	40.0[e]	84.1[c]
15	60.4[d]	99.6[b]
25	76.3[c]	100.0[a]
50	87.8[b]	100.0[a]
100	100.0[a]	100.0[b]
Uninoculated control	0.0[g]	0.3[e]

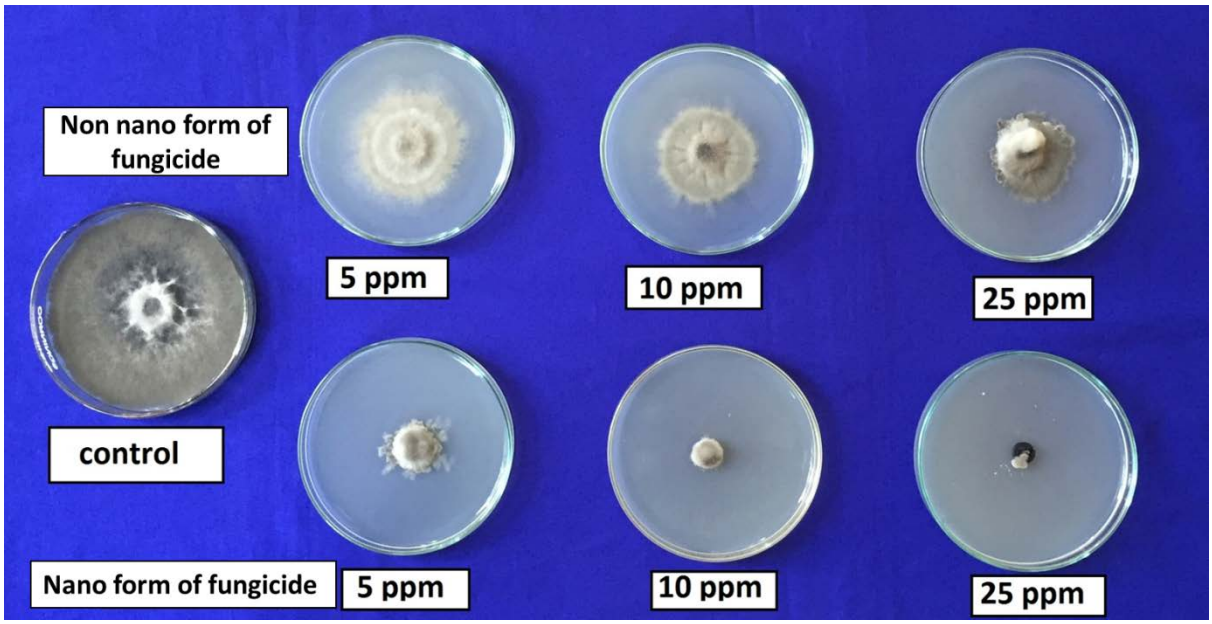

Figure 3. Inhibition zones caused by nanofungicide and conventional micro sized fungicide against *M. phaseolina*

the bulk pesticide use in the environment. Exploitation of nanoparticles as antifungal agents is relatively as being reported by recent workers (Kumar, 2011; Sridhar, 2012). Nanoparticles interactions with fungal pathogen are dependent on the size and shape of the nanoparticles (Pal et al., 2007). Metal nanoparticles are an obvious choice due to their effective antimicrobial effects (Duncan, 2011). The results of the present study clearly revealed that the maximum level of inhibition zone was observed with the increasing concentration of nanoparticles. Which was comparatively superior in its antifungal activity against non nanoform of Trifloxystrobin 25% + Tebuconazole 50% (75 WG).

Nanoparticles are highly antimicrobial and antioxidant to several species of bacteria, fungi and viruses. Antimicrobial property of nanoparticles may be due to penetration of the cell wall and modulation of the cellular level signaling by dephosphorylating putative key peptide substrates, which are critical for cell viability and cell division (Shrivastava et al., 2007). Nanoparticles are believed to inactivate microbial enzymes, facilitating production of reactive oxygen species that leads to microbial cell death (Allahverdiyev et al., 2011).

Morphological modification of mycelia

The mycelial fragment from nanoparticles treated and untreated plates were examined under SEM at various resolutions. Hyphal filament was smooth walled and equal in thickness throughout the length with blunt tips and found to bear sclerotial bodies (Figures 4a and 5a). However, in nanoparticles treated plates, hyphae were found broken and sclerotial formation either lacking or

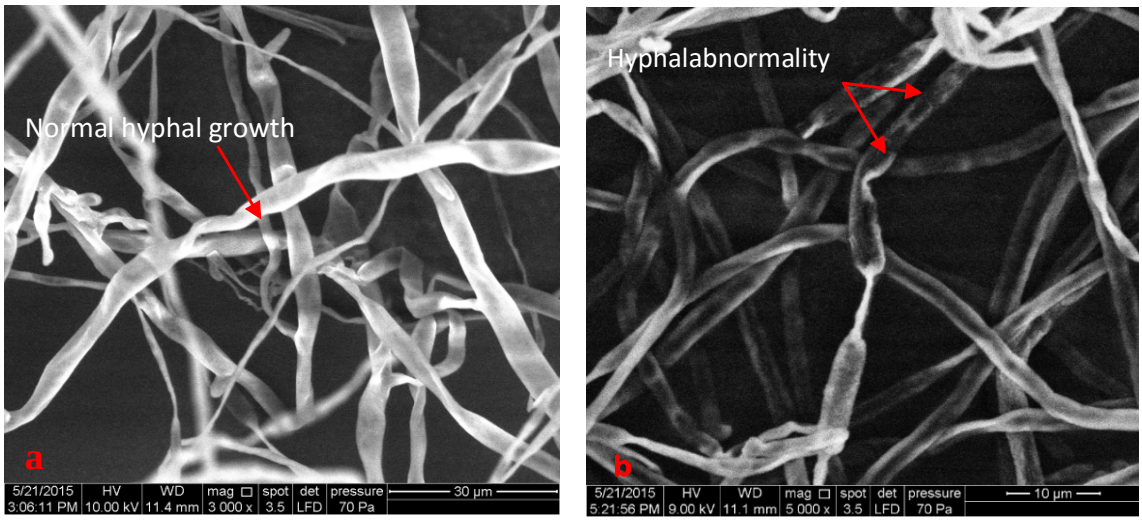

Figure 4. SEM image of *M. phaseolina* surface ultrastructure in (a) control normal hyphal formation at 3000x and (b) hyphal abnormality at 5000x.

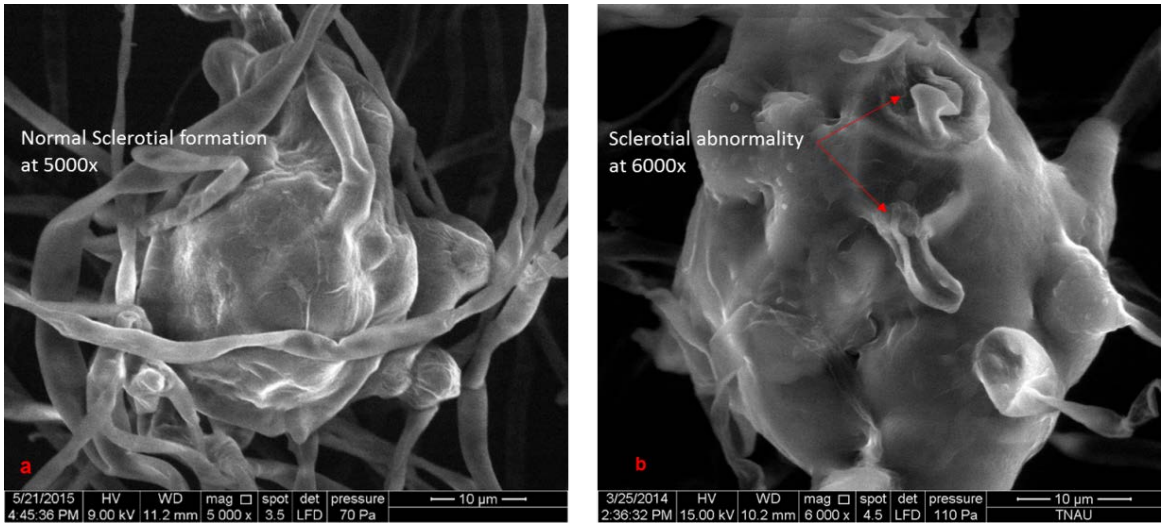

Figure 5. SEM image of *M. phaseolina* surface ultrastructure in NPs treate: (a) control normal sclerotial formation at 5000x; (b) sclerotial abnormality at 6000x magnification.

abnormal, if formed. In addition, the cell surface of hyphae was observed to be crinkled in *M. phaseolina* (Figures 4b and 5b).

The metallic nanoparticles are most promising as they have remarkable antibacterial properties due to their large surface area to volume ratio (Gong et al., 2007; Rai et al., 2009).These results lead us to consider that nanofungicide may be the most effective for controlling *M. phaseolina*. Size reductions involve the increase of contact surface, which is an important condition for the effects of nanofungicide. Another reason for considering nanofungicide as superior is its broad antimicrobial activity (Spacciapoli, 2001).

Conclusion

The present investigation is an offshoot of the main research on exploring the possibilities of nanofungicide for increasing the seed qualities especially in vegetables where the maintenance of seed viability is difficult. In the present work, it was demonstrated that, nanoformulation of Trifloxystrobin 25% + Tebuconazole 50% (75 WG) has significant fungicidal property against the fungi *M.*

phaseolina. Thus, it can be effectively explored against the soil borne pathogen *M. phaseolina* to protect various crop seeds, instead of using the commercially available synthetic fungicides, which shows higher toxicity to humans. Moreover, this report opens up for further research; field experiments are to be carried out in order to recommend the nanofungicide against the disease and the area of mode of action of nanocomposites against phytopathogenic fungi.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Foot-and-mouth disease virus isolates: Candidate strains for trivalent vaccine development in Nigeria

Hussaini Ularamu G.[1]*, John Ibu O.[2], Jerry Abenga N.[2], David Lazarus D.[1], Yiltawe Wungak S.[1], Anthony A. Chukwuedo[1], David Shamaki[1] and Mohammed Adah I.[2]

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A quality foot and mouth disease (FMD) vaccine is a prerequisite for effective control in addition to other zoosanitary measures and effective biosecurity practices in disease endemic sub-Saharan countries like Nigeria. To ensure an effective control programme by vaccination, countries that practice mass vaccination campaigns need to conduct vaccine matching studies to establish a relationship between prevalent field isolates with available vaccine for effective control. To this effect, a research was conducted in order to select foot-and-mouth disease viruses (FMDV) that will give a quality vaccine containing relevant serotypes and matching strains as a pre-requisite for effective vaccine. The study was conducted using two dimensional virus neutralization assays to determine the antigenic relationship 'r' value between the candidate vaccine strains and the field isolates. A total of forty-two specimens (epithelial tissue) were send to the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) for virus detection and antigen serotyping and some of the field isolates were selected for vaccine matching based on geographic location and topotypes/subtype. The isolates selected were two each of serotype O, A and SAT 2 from bovine species. The selected field isolates revealed high antigenic similarity with the vaccine strains tested showing 'r' value greater than 0.3 which suggests a close relationship between field isolates and vaccine strain tested. A potent vaccine containing the vaccine strain is likely to confer protection in vaccinated candidates. A vaccine match with 'r' value less than 0.3 suggests poor protection against challenge with the isolates. The result of this study has indicated that the selected field isolates could be used as vaccine strains for a candidate trivalent FMD vaccine production in Nigeria.

Key words: foot and mouth disease (FMD) virus, 'r' value, vaccine strain, Nigeria.

INTRODUCTION

Foot-and-mouth disease (FMD) is an economically challenging disease affecting all ruminants and cloven hoofed domestic and wild animal species (OIE, 2008). The disease is characterized by vesicles and ulcer in the

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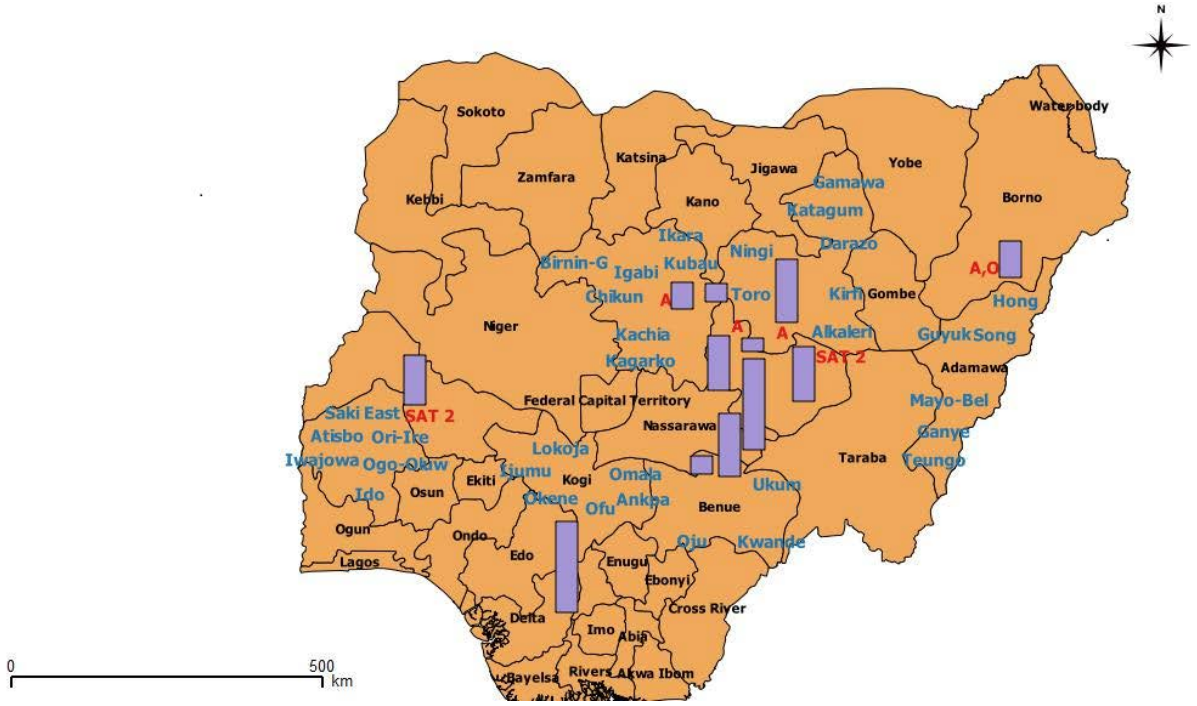

Figure 1. A Map showing the origin of the vaccine and field Isolates used in the study.

mouth, muzzle, feet and udder of lactating cows. FMD incurs huge economic losses to the livestock industry because of high morbidity in adult animals resulting in loss of production, loss of draught power, retarded growth, abortion in pregnant animals and mortality in young calves and lambs (Ayelet et al., 2009).

FMD is an endemic trans-boundary animal disease (TAD) in Nigeria with outbreaks occurring seasonally in both pastoral and sedentary husbandry. It is one of the major animal diseases that impact negatively on trades in livestock and livestock products in most developing countries (Jamal et al., 2014). To date, four of the seven serotypes had been found in circulation in Nigeria (Fasina et al., 2013). These include serotypes O, A, SAT 1 and SAT 2 and each serotype has many subtype variants. This antigenic variation is a cause of major setback in the control of FMD, as infection or vaccination with one serotype of FMDV does not protect against other serotypes and may fail to confer protection against other subtypes within the same serotype (Ayelet et al., 2009). The disease is caused by a small positive sense ssRNA virus (approx. 8.3 kb), which belongs to the *Aphthovirus* genus of the family *picornaviridae* (Ayelet et al., 2009).

A good quality vaccine containing relevant serotypes and matching strains is vital for effective control programme. However, the protective efficacy of FMD vaccine can be evaluated through vaccine matching using indirect serological methods (Rweyemamu 1984a; Rweyemamu 1984b) and it can also be calculated using the relatedness between the field isolate and available vaccine strains using *in vivo* challenge tests (Brehm et al., 2008; Goris et al., 2008). Selection of viruses for vaccine matching is very important and should be based on epidemiological information, including stages of an epidemic, geographical locations or range of host species (Alonso et al., 1993; Paton et al., 2005) by incorporating a minimum of two isolates from outbreak (OIE, 2008).

Since vaccination is important in the control of FMD in West Africa, Nigeria in particular, and the best vaccination program should involve those that target the topotypes, strains and serotypes circulating within the West and Central Africa sub-region (Knowles and Samuel, 2003). It is necessary to compare field viruses against vaccine strains as it has been shown that some variants within a serotype were unable to break through immunity and therefore more antigenic characterization is important (Knowles and Samuel, 2003). More so, the presence of multiple serotypes of FMD and the inability to cross protect among serotypes and subtypes has necessitated the need to develop a vaccine having strains that can confer sufficient immunity (Jamal et al., 2014). In this regard this study was designed to determine FMD vaccine strains that are most appropriate for serotypes/topotypes currently circulating in Nigeria.

MATERIALS AND METHODS

FMD virus candidate strains for trivalent vaccine

This study was conducted on virus isolates from different region of the Country of Nigeria (Figure 1) between 2011 and 2014. The samples were sent to the World reference laboratory for foot-and mouth disease (WRLFMD), United Kingdom for virus detection and

antigen serotyping. A total number of 42 specimens (epithelial tissue) were sent for virus detection and antigen serotyping, of which ten serotype O, nine serotype A, and 13 SAT 2 were detected respectively (Table 1). The vaccine candidates were selected according to specific geographic locations, endemic regions, topotypes/subtype, source species and the period of occurrence (OIE, 2008). Some vital determinants that may affect the efficacy of a vaccine and determine whether it may protect or not are: the ability of the vaccine strain to elicit antibodies that will cross-react and protect against the field or outbreak virus in question (defined as the vaccine or antigenic match), and the potency of the vaccine to elicit a strong and long-lasting immune response (Doel, 2005). Two isolates were selected from each of the field circulating serotype representing serotypes O, A and SAT 2. The isolates used were A NIG. 03/13, A NIG. 07/13, SAT 2 NIG 03/12, SAT 2 NIG 17/11, O NIG 03/14 and O NIG 04/14 (Table 2). All samples were isolated in primary cell culture and serotyped by ELISA (Table 2).

Vaccine preparation

The preparation of inactivated monovalent FMD vaccine from selected vaccine strains was based on the OIE recommended protocol (OIE, 2008). The virus isolate after primary isolation using ZZR continuous cell line was adapted to BHK-21 cell line and passage to 7th passage on BHK-21 monolayer. The isolates were inactivated using 0.04% of 10% buffered Formalin and the effect of the formalin was neutralized by 2% each of sodium thiosulphate (20%) and sodium bisulphite (20%) (Iyer et al., 2000). After the inactivation process equal volume of Montanide ISA 206 was added and mixed thoroughly. The prepared vaccine was kept at +4 ° C until use.

Antiserum preparation

A total of ten adult rabbits weighing averagely 2.5 kg were used after been screened for FMD Non-structural proteins antibodies by 3ABC-ELISA (PrioCHECK®FMDV NS prionics Lelystad B.V. Netherlands). The rabbits were inoculated intramuscularly using 1.0 ml of the inactivated antigen and four booster doses were given to achieve a high antibody titre. The rabbit were bled at day 56th and serum harvested and stored at -20°C until used.

FMD virus titration

The FMDV vaccine candidates and field isolates were titrated using a tenfold serial dilution beginning with 10^{-1} by taking 0.5 ml of the isolate to 4.5 ml of the diluent minimum essential medium (MEM). Using a sterile pipette tips, 0.5 ml from the first dilution was taken and transferred to the next and continued serially to the tenth dilution using different sterile pipette tips at each transfer. Fifty microliter of each virus dilution (10^{-1} to 10^{-8}) was distributed in the wells of respective rows on microtiter plates containing established cell layers of baby hamster kidney (BHK-21). Then 100 µl/well MEM was added and incubated at 37° C for 24 h and the titer was determine (Ayelet et al., 2013)

Vaccine matching by two-dimensional virus neutralization

The vaccine matching was performed at the WRLFMD by using the two-dimensional virus neutralization test according to protocol of WRLFMD United Kingdom. Briefly, both field isolates and vaccine strains were passaged on monolayer of BHK-21 cell culture until adapted to give 100% CPE within 24 h. The infected BHK-21 monolayer cells were subjected to three times freeze-thaw cycles to release the viral particles from the cells. Fifty microliter of serum raised against the reference vaccine strain was added on row A wells (1-10) and serially diluted starting with ½ in microtiter plates (OIE, 2008) and a constant amount (50 µl) of pre-titrated field isolates of 100 $TCID_{50}$ dose was added in each well using two columns for each antigen. Columns 1 and 2 of each microtiter plate were used for homologous virus of the candidate vaccine strain. After 1 h incubation at 37°C, 50 µl of virus/serum mixture was transferred into their respective microplate wells containing established monolayer BHK-21 cells, sealed with a semi permeable sealer and incubated at 37°C in a 5% CO_2 incubator for 48 h. After 48 h incubation, plates were observed for cytopathic effect using an inverted microscope. Finally, titers of the reference antiserum against the heterologous or field isolates and titer of reference antiserum against homologous virus was calculated for each test viruses and candidate vaccine strains (OIE, 2008).

Statistical analysis

The data obtained in two dimensional sero-neutralization assays were used in order to calculate the antigenic similarity between candidates of vaccinal strains and field isolates. Antibody titers of the vaccine serum against the field isolate for each virus dose used were calculated using the Spearman-Karber method (OIE, 2008). The titer of the vaccine serum against 100 $TCID_{50}$ of each virus was then estimated by regression analysis. The relationship between the field isolate and the vaccine strain is then expressed as:

$$r = \frac{\text{Reciprocal arithmetic titer of reference serum against field virus}}{\text{Reciprocal arithmetic titer of reference serum against vaccine virus}}$$

It is generally accepted that in case of neutralization, r values greater than 0.3 indicates that the field isolate is sufficiently similar to the vaccine strain (OIE, 2008).

RESULTS

The interpretation of the results for the vaccine matching suggests that there is a close relationship between field isolates studied and vaccine strains tested. And a potent vaccine containing the vaccine strain is likely to confer protection against challenge with a homologous or related virus. The isolates with ‘r’ value less than 0.3 suggest that the field isolate is so different from the vaccine strain and that the vaccine is unlikely to protect.

The results as shown in Table 3 indicates that there is a close antigenic relationship between the vaccinal A ERI98 and A TUR06 and the field isolates of A NIG 03/13 and A NIG 07/13. This is because the calculated ‘r’ value was greatly higher than the minimum required value (>0.3).

For serotype O (Table 4) the results indicated that O NIG 03/14 was antigenically related to O TUR 5/09.

The SAT 2 serotype of SAT 2 NIG 03/12 and SAT 2 17/11 were antigenically related to that of SAT 2 ERI and SAT 2 ZIM but the SAT 2 NIG17/11 showed slight decrease in ‘r’ value compare to SAT 2 NIG 03/12 as shown on Table 5.

DISCUSSION

Foot-and-mouth disease is an endemic disease in West

Table 1. FMD detection and serotyping results.

NIG. Reference	WRL Reference	Description of Sample	PCR Result	Serotyping Result by Cell Culture/ELISA
MKD/FMD2011/04E	NIG1/2011	BOVINE, epithelium, collected 11/06/20111	FMDV (GD)	O
KG/M5	NIG2/2011	BOVINE, epithelium, collected 26/06/2011	FMDV (GD)	A
KG/OKEBUKU/5	NIG3/2011	BOVINE, epithelium, collected 26/06/2011	FMDV (GD)	O
BN/MKD/18	NIG4/2011	BOVINE, probang, collected 07/07/2011	(NGD)	NVD
PL/DN/001/E	NIG5/2011	BOVINE, epithelium, collected 20/07/2011	FMDV (GD)	SAT 2
PL/DN/006/E	NIG6/2011	BOVINE, epithelium, collected 20/07/2011	FMDV (GD)	SAT 2
KD/KCH/07	NIG7/2011	BOVINE, epithelium, collected 22/07/2011	FMDV (GD)	SAT 2, O
PL/JS/005	NIG9/2011	BOVINE, epithelium, collected 02/08/2011	FMDV (GD)	SAT 2
PL/JS/001	NIG10/2011	BOVINE, epithelium, collected 02/08/2011	FMDV (GD)	SAT 2, O
NS/DM/008	NIG11/2011	BOVINE, probang, collected 02/08/2011	NGD	NVD
PL/TNK/01	NIG12/2011	BOVINE, epithelium, collected 06/08/2011	FMDV (GD)	SAT 2
PL/TNK/05	NIG13/2011	BOVINE, epithelium, collected 06/08/2011	FMDV (GD)	SAT 2
14/UD/EP/2/1/11	NIG14/2011	BOVINE, epithelium/homogenate, collected 10/08/2011	FMDV (GD)	NVD
TR/IB/29/P	NIG15/2011	BOVINE, probang, collected 09/09/2011	FMDV (GD)	NVD
PL/BK/08185	NIG16/2011	BOVINE, epithelium, collected 03/11/2011	FMDV (GD)	SAT 2
PL/BK/08196	NIG17/2011	BOVINE, epithelium, collected 03/11/2011	FMDV (GD)	SAT 2
OY/IGB/4	NIG1/2012	BOVINE, epithelium, collected 22/06/2012	FMDV (GD)	SAT 2

Table 1. Cont'd.

OY/IGB/02C	NIG2/2012	BOVINE, epithelium, collected 22/06/2012	FMDV (GD)	SAT 2
OY/IGB/03b	NIG3/2012	BOVINE, epithelium, collected 22/06/2012	FMDV (GD)	SAT 2
AD/MDG/2012	NIG4/2012	BOVINE, epithelium, collected 03/08/2012	FMDV (GD)	O
AD/SH/6	NIG5/2012	BOVINE, epithelium, collected 03/08/2012	FMDV (GD)	O
PL/SH/2012	NIG6/2012	BOVINE, epithelium, collected 08/08/2012	FMDV (GD)	NVD
PL/KA/12M	NIG7/2012	BOVINE, epithelium, collected 09/09/2012	FMDV (GD)	NVD
PL/BLD/02B	NIG8/2012	BOVINE, epithelium, collected	FMDV (GD)	A
PL/BLD/01A	NIG9/2012	BOVINE, epithelium, collected 06/11/2012	FMDV (GD)	A
PL/BLD/01B	NIG10/2012	BOVINE, epithelium, collected 06/11/2012	FMDV (GD)	A
NS/WAM/03	NIG 11/2012	BOVINE, epithelium, Collected 07/11/2012	FMDV GD	NVD
KD/KAU/01	NIG 12/2012	BOVINE, Epithelium, Collected 13/1/2012	FMDV GD	A
ABJ, TISS/03	NIG 1/2012	BOVINE, epithelium,/homogenate, Collected 03/01/2013	FMDV GD	NVD
KD/KAU/1	NIG 2/2013	BOVINE, epithelium, Collected 26/06/2013	FMDV GD	NVD
PL/BLD/02/13	NIG 3/2013	BOVINE, Lab isolated sample/cell culture, Collected 17/09/2013	FMDV GD	A
PL/BLD/04/13	NIG 4/2013	BOVINE, Lab isolated sample/cell culture, Collected 17/09/2013	FMDV GD	NVD
PL/BLD/03/13	NIG 5/2013	BOVINE, Lab isolated sample/cell culture, Collected 17/09/2013	NGD	NVD
BAU/T/B2/13	NIG 6/2013	BOVINE, Epithelium, Collected 21/11/2013	FMDV GD	A
BAU/T/C3/13	NIG 7/2013	BOVINE, Epithelium, Collected 21/11/2013	FMDV GD	A

Table 1. Cont'd.

BAU/T/A1/13	NIG 8/2013	BOVINE, Epithelium, Collected 21/11/2013	FMDV GD	A
PL/JS/KA/1	NIG 1/2014	BOVINE, Epithelium, Collected 03/01/2014	FMDV GD	O
PL/JS/KA/2	NIG 2/2014	BOVINE, Epithelium, Collected 03/01/2014	FMDV GD	O
PL/KA/03	NIG 3/2014	BOVINE, Epithelium, Collected 03/01/2014	FMDV GD	O
PL/KA/4/14	NIG 4/2014	BOVINE, Epithelium, Collected 14/01/2014	FMDV GD	O
PL/KA/7B	NIG 5/2014	BOVINE, Epithelium, Collected 18/01/2014	NGD	NVD

NVD, No virus Detected; FMDV GD, FMDV Genome Detected; NGD, No Genome Detected.

Table 2. FMDV candidate vaccine strains selected for vaccine matching.

Name of candidate vaccine	Site of isolation	Year of isolation	Serotype	topotype
O NIG 03/2014(PL/KA/03)	Plateau	2014	O	EA-3
O NIG 04/2014(PL/KA/4/14)	Plateau	2014	O	EA-3
A NIG 03/2013(PL/BLD/02/13)	Plateau	2013	A	Africa
A NIG 07/13(BAU/T/C3/13)	Bauchi	2013	A	Africa
SAT 2 NIG 03/12(OY/IGB/03b)	Oyo	2012	SAT2	VII
SAT 2 NIG 17/11(PL/BK08196)	Plateau	2011	SAT2	VII

Africa and Nigeria in particular which has remained a major economic challenge for livestock production. FMD has hampered the export of livestock and livestock products to more international market due to strict zoosanitary regulations. The genetic diversity of FMD and its endemic nature in Nigeria and the current circulating serotypes/subtypes in the Country have necessitated research into the production of trivalent vaccine. Therefore, in order to have good quality, FMD vaccine that will have a protective capacity, the candidates vaccine need to be evaluated through vaccine matching using indirect serological methods (Rweyemamu, 1984b; Paton et al., 2005). The viruses selected for vaccine matching in this research was based on epidemiological information, including the stages of an epidemic and the geographical locations as reported by (Alonso et al., 1993; Paton et al., 2005).

From the findings, the A NIG 03/13 ('r'=0.41) and A NIG 07/13('r'=0.46) field isolates showed a good serological matching with A ERI 98 but a decrease in 'r' value A NIG 03/13 (0.35) and A NIG 07/13(0.28) to A TUR 06. The serological match of the field isolates to A ERI 98 could be attributed to the topotype similarities which suggest that there is close relationship between field isolate and the vaccine strain. But the low 'r' value recorded with the vaccine strain of A Iran 2005 and A22 IRQ suggest that the field isolate is so different from the vaccine strain that the vaccine is unlikely to protect (Ayelet et al., 2009).

The O NIG 03/14 and 04/14 also showed a good serological match with O TUR 5/09 which also suggest that there is a close relationship between the field isolates and the vaccine strain. But the result with O Manisa vaccine strain was far lower and these suggest that the Nigeria serotype O 2014 field isolate is so different from the vaccine strain and the vaccine is unlikely to protect.

The 'r' value obtained for SAT 2 serotype indicate that the SAT 2 Eri and SAT 2 ZIM showed a close relationship between field isolate and vaccine strain. However, the result of SAT 2 Nig17/11 indicated 'r' value less than 0.3 which suggest that the field isolate of SAT 2 NIG17/2011 is so different from the SAT 2 ZIM strain and that the vaccine is unlikely to protect (Rweyemamu, 1984a). The likely explanation of this difference in 'r' value (0.25) of

Table 3. 'r' values obtained between serotype A field isolates and vaccine strains.

Field Isolates	2dm VNT			
	Vaccines			
	A ERI98	**A IRAN 2005**	**A22 IRQ**	**A TUR06**
A NIG 03/13 (mean)	0.41	0.07	0.26	0.35
A NIG 07/13 (mean)	0.46	0.06	0.17	0.28

Table 4. 'r' values obtained between serotype O field isolates and vaccine strains.

Field Isolates	2dm VNT		
	Vaccines		
	0.3039	**O Manisa**	**O TUR. 5/09**
O NIG 03/14 (mean)	0.65	0.10	0.40
A NIG 07/13 (mean)	0.53	0.06	-

Table 5. 'r' values obtained between serotype SAT 2 field isolates and vaccine strains.

Field Isolates	2dm VNT	
	Vaccines	
	SAT 2 ERI	**SAT 2 ZIM**
SAT 2 NIG 03/12 (mean)	0.36	0.47
SAT 2 NIG 17/11 (mean)	0.37	0.25

the Nigerian isolate (SAT 2 NIG 17/11) with that of SAT 2 ZIM strain could be as the differences in strain, because the two Nigerian isolates are from different geographical locations and could have different strain.

In conclusion all the tested candidate vaccine strains that had 'r' value greater than 0.3 indicate strong antigenic match. This also suggests that there is a close relationship between the field isolate and vaccine strain. But the candidate vaccine strains that had 'r' value less than 0.3 suggest that the field isolate is so different from the vaccine strain that the vaccine is unlikely to protect. Therefore the candidate vaccine strain selected could be included in the trivalent vaccine formulation so as to effectively control FMD outbreaks in Nigeria. Finally vaccine matching should be routinely carried out in order to have a potent vaccine strain that is likely to confer protection in FMD endemic Country like Nigeria.

Conflict of interests

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

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Full Length Research Paper

Sensitivity and lower protease activity of *Candida albicans* species isolated from Egyptian cancer patients after exposure to cytotoxic and/or radiotherapy treatment

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Oropharyngeal candidiasis is a common disease among cancer patients receiving chemo or radiotherapy which precede systemic candidemia, a life threatening infection. This study investigated the diversity and prevalence of different *Candida* species among Egyptian cancer patients, evaluated the sensitivity of *Candida albicans* to the frequently administered antifungal therapies and the effect of different radio and chemotherapeutic agents on its virulence. A total of 119 *Candida* spp. isolates were identified out of 399 clinical samples, of which 72 isolates were *C. albicans*, 15 were *Candida tropicalis,* 22 were *Candida krusei*, and 10 were *Candida glabrata*. 98.6% of the *C. albicans* isolates were sensitive to fluconazole; on the other hand, only 8.3% out of the tested isolates were sensitive to amphotericin B. No significant differences were observed in the ability of biofilm formation among *C. albicans* isolates exposed to chemo, radio or both therapies when compared with standard *C. albicans ATCC 60193.* Surprisingly, the protease activities in isolates obtained from cancer patients were significantly lower than that of the reference strain after exposure to chemo, radio or both therapies. Thus, it is concluded that radio and chemotherapies may not be in some cases a predisposing factor for the virulence of *C. albicans* strains.

Key words: Candidiasis, antifungal agents, virulence, radio and chemotherapy.

INTRODUCTION

Cancer is one of the most serious health problems faced by many individuals in the course of their lives and it is usually associated with high incidence of microbial infections (Bodey, 1986). Diseases involving *Candida*

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species are common in cancer patients (Ramirez-Garcia et al., 2014). The penetration of this fungal species into the bloodstream and its dissemination, causing candidemia, is a life threatening infection, being responsible for 30 to 50% of mortality rates among cancer patients (Nucci and Marr, 2005).

Candida albicans is the main resident flora of the digestive mucosa and the genital area, identified in approximately 10 to 20% of healthy adults, followed by *Candida glabrata* and *Candida tropicalis* (Zadik et al., 2010). *C. albicans* is responsible for about 80% of oropharyngeal infections (Bensadoun et al., 2011). Oropharyngeal candidiasis (OPC) is common among patients undergoing intensive chemo or radiotherapy and is known to precede systemic infections (Al-Attas and Amro, 2010). It is a major cause of morbidity in cancer patients (Bodey, 1986). The development of OPC results from the imbalance between fungal virulence factors and host defenses. Several known virulence factors contribute to the pathogenicity of *C. albicans*, which include adherence to host tissues, phenotypic switching, dimorphism conversion, and enzymes that are integral to its pathogenesis (Calderone et al., 2000; Liu, 2001). Proteinases and phospholipase B are the main enzyme categories secreted by different *Candida* spp. (De Bernardis et al., 2001).

The common applied therapy, in case of candidiasis, is fluconazole, where its effectiveness is highly proven and superior to other treatments, such as nystatin and clotrimazole with a broad therapeutic range and little toxicity (Sheehan et al., 1999). However, increasing fluconazole resistance in cancer patients is reported (Tortorano et al., 2004). The mechanism of resistance mainly depends on either mutation or over expression of the *erg11* gene leading to reduced drug affinity for the target enzyme or increase in ergosterol synthesis, respectively (Lupetti et al., 2002; Maebashi et al., 2003). Caspofungin, is the first member of a new class of antifungal agents targeting the fungal cell wall. It showed high effectiveness in the treatment of candidiasis (Letscher-Bru and Herbrecht, 2003), but unfortunately, its high cost limits its use in Egypt.

Chemotherapy may lead to damage to the mucosal barrier that may result in epithelial atrophy and mucosal ulceration, which may be associated with increased adherence and invasion of *Candida* (Bensadoun et al., 2011). On the other hand, antineoplastic agents may negatively affect morphogenesis, fungal growth, and virulence of *Candida* spp. as reported in *in vitro* studies (Chen et al., 2011). Hence, the present study aimed at the identification of different *Candida* spp. causing OPC infections and evaluating the sensitivity of *C. albicans*, the most common infective species in cancer patients, to the frequently administered antifungal therapies and the effect of different chemotherapeutic agents on the virulence of *C. albicans*. These data are urged to be continuously updated in order to tailor treatment and update prevention guidelines.

MATERIALS AND METHODS

Sample collection

A total of 399 samples were collected from different cancer patients of both sexes and from adults as well as infants. Patients were submitted in Kasr El-Einy, Center of Radiation, Oncology and Nuclear Medicine, Faculty of Medicine, Cairo University (NEMROCK). All cases were diagnosed by physicians at the hospital. Samples were collected from the patients' oral cavity using sterile sealed swabs. The work was carried in accordance with the code of ethics of the world association (declaration of Helsinki) for experiments involving humans. A written consent was signed by the studied subjects or their parents after full explanation of the study. The ethical approval was obtained from the medical ethics committee at the Faculty of Pharmacy, Cairo University. The clinical data collected included the personal data, age, residence, clinical diagnosis and predisposing factors, such as diabetes mellitus, pregnancy, use of antibiotics, previous surgical operations, the use of immunosuppressant drugs, exposure to radiation and the type of this radiation.

Identification and maintenance of isolates

All samples were streaked on Sabouraud Dextrose Agar, Oxoid, CM0041 (SDA, pH 6.5) and incubated for 48 h at 37°C. A total of 119 isolates of *Candida* spp. were identified out of the 399 samples by Gram stain microscopic examination. Further identification was done by isolating the 119 samples on surface of *Candida* Ident Agar media, modified (Biochemika, Fluka, Sigma Aldrich, 94382), and incubated for 48 h at 37°C. Each isolate represents a unique strain from a single patient. All isolates were scraped from the media, suspended in Brain Heart broth; equal volumes of 30% sterile glycerol were added and mixed evenly using a vortex. The mixtures were then distributed into sterile Eppendorf tubes and stored at -80°C for long time preservation (Prasad et al., 2010). Identified *C. albicans* samples were subjected to susceptibility, biofilm formation, and protease activity assays in comparison to a standard strain of *C. albicans,* ATCC 60193.

Susceptibility testing

Antibiotic susceptibility was tested by disc diffusion method described in the Clinical and Laboratory Standards Institute (CLSI). Fluconazole (10 and 25 µg) and amphotericin B (100 units) disks were obtained from Hi-media (India) and Oxoid (Sparks, Md., UK). The standard *C. albicans* ATCC 60193 and each isolated *C. albicans* strain were sub-cultured on Sabouraud Dextrose Agar (SDA) plates for 48 h at 37°C. Three to four colonies were transferred aseptically into 5 ml of Sabouraud Dextrose broth and incubated at 25 to 30°C for 8 h. The turbidity of the suspension was adjusted to 0.125 at λ = 550 nm which is equivalent to 0.5 Macfarland standard (approximately 1.5×10^8 CFU/ml). Plates filled with SDA to a depth of 4.0 mm were used. The SDA surface was inoculated by using a swab dipped in the prepared cell suspension of each isolate in addition to the reference strain, then the antibiotic discs were placed on the surface of the SDA plates. The plates were inverted and incubated at 25 to 30°C for 40 to 48 h. Subsequently, the plates were examined and the zone diameters were measured in mm. The test was repeated twice for each isolated strain and the average diameter was calculated.

Table 1. Distribution of *Candida* spp. according to age and sex of patients.

Candida spp.	Patients				Total	Percentage of *Candida* spp.
	Male		Female			
	Adult	Infant	Adult	Infant		
C. albicans	38	3	31	0	72	60.5
C. tropicalis	9	0	6	0	15	12.6
C. krusei	13	0	9	0	22	18.5
C. glabrata	5	0	5	0	10	8.4
Total	65	3	51	0	119	-
Percentage of patients	54.6	2.5	42.9	0	100	-

Infant: 0-12 years; Adult: Above 12 years.

Biofilm formation test

C. albicans samples were inoculated in yeast nitrogen base media (YNB) supplemented with 100 mM glucose and cultured overnight. The yeasts were then harvested, washed twice with phosphate buffer saline (PBS, pH 7.2, Ca^{+2} and Mg free), suspended to 10^7 cells/ml by adjusting the optical density of the suspension to 0.38 at 520 nm, and used immediately. A volume of 100 µl of standardized cell suspension (10^7 cells/ml) of each sample was transferred into sterilized micro titer plate and incubated in a shaker at 75 rpm, for 1.5 h at 37°C to allow the yeast to adhere to the surfaces of the wells. The plate was washed; fresh media was added and incubated again at 37°C in a shaker at 75 rpm. The biofilms were allowed to develop up to 66 h and then the yeasts were quantified by the crystal violet assay as described earlier (Djordjevic et al., 2002).

Quantitative protease assay

Protease activity of *C. albicans* was assessed by a commercial kit (Pierce Quantitative Protease Assay Kit, Thermoscientific, 23263, Hyclone, USA) according to the method described in Rao et al. (1997) and modified by Tian et al. (2004). The inoculums were adjusted to 0.8 at 550 nm to get 10^8 cells/ml as described by the manufacturer's protocol. The protease activity was measured using a plate reader at 450 nm.

Statistical analysis

Data of protease activity and biofilm formation experiments were expressed as mean ± standard error of means (SEM). Significant difference was calculated by one way analysis of variance (ANOVA) for both biofilm formation test and quantitative protease assay using Graphpad prism 5 for windows. Differences in results with $p<0.05$ were considered to be significant. The demographic distribution data were analyzed using Chi square test. The data were expressed as absolute count number. Significance at p value<0.05 was considered.

RESULTS

Demographic distribution of samples

Out of 399 screened patients, only 119 (29.8%) were confirmed to have OPC. The presented samples were distributed according to sex to 57% males and 43% females, of which 29.4% were identified as smokers and 61.3% were non-smokers. Out of 119 samples, *C. albicans* represented 60.5% (72 isolates) of the total identified *Candida* isolates. The percentage of other isolates were 12.6% *C. tropicalis* (15 solates), 18.5% *C. krusei* (22 isolates), and 8.4% *C. glabrata* (10 isolates) (Table 1).

Of the *C. albicans* identified patients, 32 received chemotherapy before sampling and 14 patients were on radiotherapy, while 24 patients received both chemo- and radio-regimens. Two patients did not receive either chemo or radiotherapies and were referred to as unknown treatment in the present study.

Figure 1 shows the incidence and prevalence of each *Candida* spp. among haematological and solid cancer types. It was observed that *C. albicans* is more prevalent in solid cancer types (64%) followed by C. *krusei*, then C. *glabrata* and finally *C. tropicalis.* Moreover, in the haematological cancer, *C. albicans* was also the most prevalent (53%), followed by *C. tropicalis, C. krusei* and *C. glabrata.* The prevalence of *C. albicans* and *C. krusei* was much higher in solid cancer types than in haematological cancer, while the opposite is true for *C. tropicalis* and *C. glabrata.*

Figure 2 shows the incidence and prevalence of each *Candida* spp. among haematological and solid cancer types in relation to different cancer treatments. It was observed that in chemotherapy, the heamatological cancer type was more prevalent than the solid cancer type in all *Candida* spp. While in radiotherapy, only solid cancer type was observed in all *Candida* spp. except C. *glabrata* which was found neither in solid nor in heamatological cancer types.

Susceptibility testing

For fluconazole (10 and 25 µg), 98.6% of the isolated *C. albicans* were sensitive, 1.4% were of intermediate susceptibility and none of the samples showed resistance. Trailing phenomena was observed and

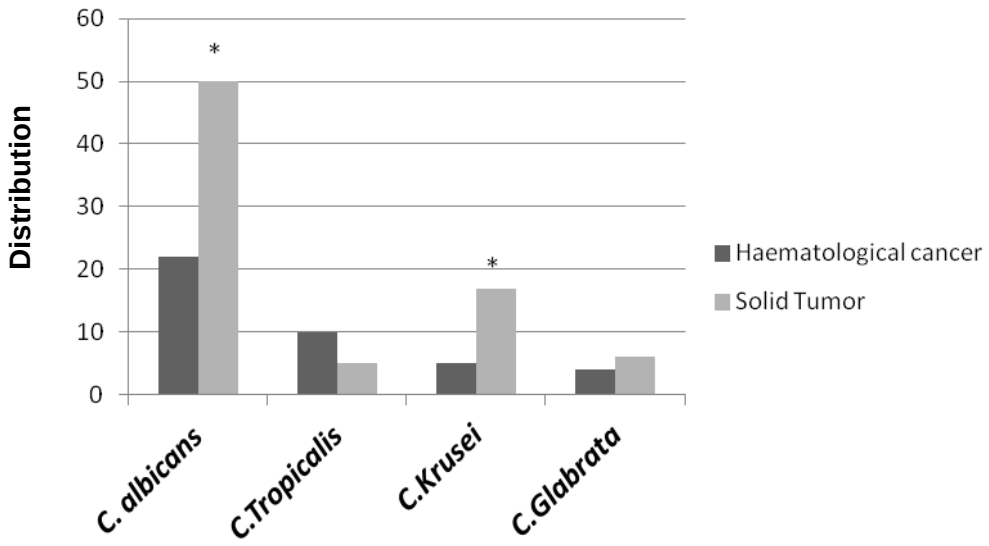

Figure 1. Distribution of *Candida* spp. among different cancer types. Significance is calculated using Chi square test P value<0.05.

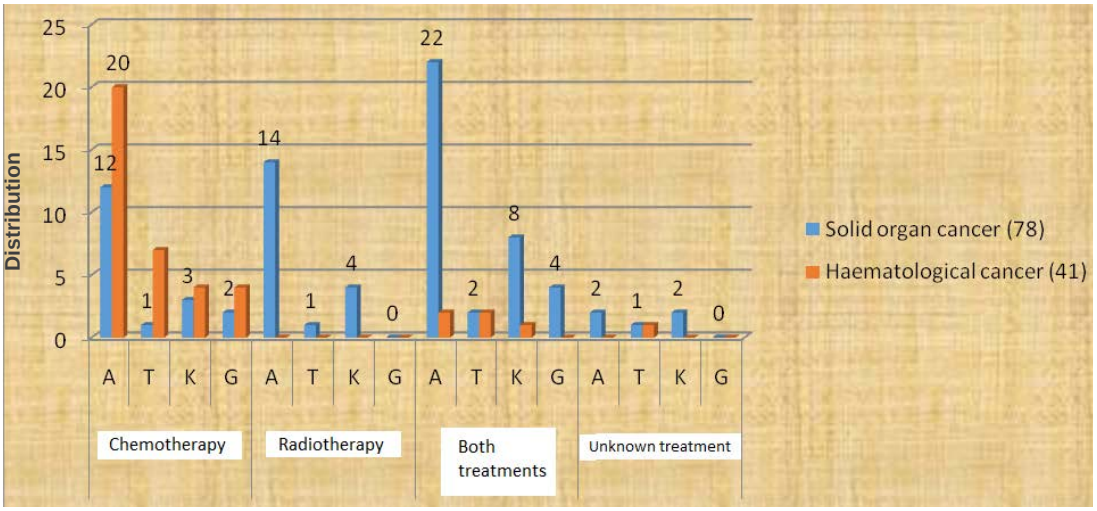

Figure 2. Distribution of *Candida* spp. among different types of cancer treatment and cancer types. Significance is calculated using Chi square test P value<0.05.

considered as sensitive results. For amphotericin B, 8.3% of the tested *C. albicans* isolates were sensitive, while 87.5% were of intermediate susceptibility and 4.2% were resistant. The standard ATCC 60193 was sensitive to fluconazole and of intermediate susceptibility to amphotercin B. The interpretation of the results was based on the criteria specified by the CLSI. All results are shown in Table 2.

Biofilm formation

The biofilm formation of *C. albicans* isolates from each of the 4 groups of cancer patients was compared to that of the standard ATCC 60193. No significant differences were observed among chemotherapy (0.449±0.002), radiotherapy (0.450±0.003), both therapies (0.446±0.001), or unknown treatment (0.459±0.01) groups as compared to the standard ATCC 60193. Results are demonstrated as show in Figure 3.

Protease production

The protease concentrations in *C. albicans* isolates obtained from cancer patients were significantly lower

Table 2. Susceptibility testing of isolated *C. albicans* strains by disc diffusion method.

Antifungal agent	Disc content	Breakpoint (mm)			Resistant		Intermediate		Sensitive		ATTC
		R	I	S	Frequency	%	Frequency	%	Frequency	%	
Fluconazole (FU^{10})	10 µg	≤18	19-21	≥22	0	0	1	1.4	71 (T)	98.6	T
Fluconazole (FCA)	25 µg	≤25	26-29	≥30	0	0	1	1.4	71 (T)	98.6	T
Amphotericin B (AP^{100})	100 Units	≤10	11-17	≥18	3	4.2	63	87.5	6	8.3	I

R: Resistant; S: Sensitive; I: Intermediate; T: Trailing.

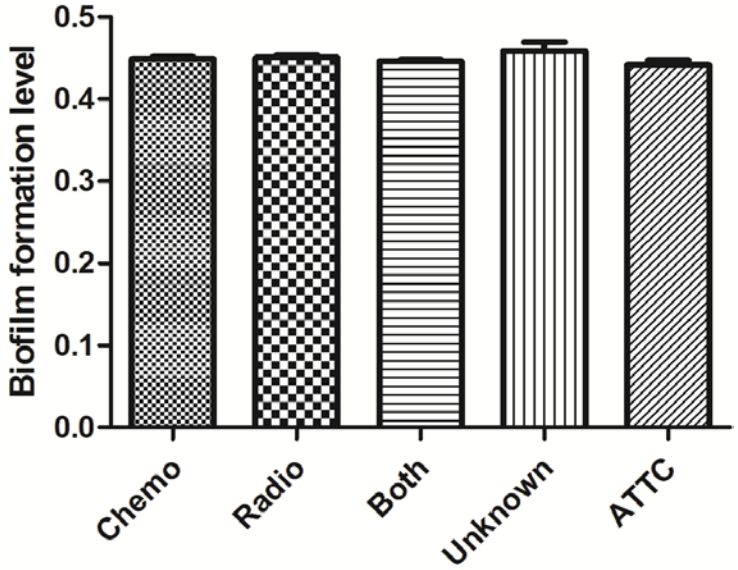

Figure 3. Biofilm formation of *C. albicans* among different therapy groups. Data represent mean ± standard error of mean (SEM). Statistical analysis was carried out by one-way analysis of variance (ANOVA).

than that of *C. albicans* ATCC 60193 reference strain after exposure to chemotherapy, radiotherapy, both treatments, or unknown treatment (Table 3).

DISCUSSION

Patients who undergo chemo and/or radiotherapy are at increased risk of developing fungal infection. In the case of changes in the mucous membrane, fungi can move into the blood and develop into disseminated fungal infection, often leading to death. Hence, it is important to determine

Table 3. Protease enzyme levels of *C. albicans* in cancer patients received chemotherapy, radiotherapy or both, compared to *C. albicans* ATCC 60193 reference strain.

Group	Protease level (ng/ml)	Number of samples
Chemotherapy	185.9±10.2*	20
Radiotherapy	204.2±17.1*	11
Both therapies	178.7±8.4*	18
ATCC 60193	249.3±0.8	1

Data represent mean ± standard error of mean (SEM). Statistical analysis was carried out by one-way analysis of variance (ANOVA). *p<0.05 vs. ATCC 60193 standard sample.

the presence of fungi in this group of patients before the beginning of chemo- or radiotherapy to enable early treatment. The current study discussed the prevalence and the virulence of *C. albicans* among this group of patients. A total of 60.5% of candidiasis subjected in this study was identified as *C. albicans*, which is a large proportion despite the widespread use of fluconazole. On the other hand, other *Candida* spp. were found in minor proportions. This complies with a previous report, where *C. albicans* constituted 56.3% of patients receiving radiotherapy for head and neck neoplasms, while each of *C. glabrata* and *C. tropicalis* was present in 12.5% of the total patients (Tudela et al., 2002). Furthermore, most of the OPC samples from cancer patients in different regions were also identified as *C. albicans* with a range of 33 to 76% of the total isolates (Laverdiere et al., 2002; Al-Abeid et al., 2004; Belazi et al., 2004).

This study also showed higher prevalence of *C. albicans* in both solid and haematological cancer types, followed by *C. krusei*, then *C. glabrata* and finally, *C. tropicalis* in solid cancer types. While in the haematological cancer types, *C. albicans* was found to be followed by *C. tropicalis*, *C. krusei* and *C. glabrata*, respectively. The prevalence of *C. albicans* and C. *krusei* was much higher in solid cancer types than in haematological cancer, while the opposite is true for *C. tropicalis* and *C. glabrata.* This was also the case in a study made by Slavin et al. (2010) where the prevalence of *C. ablicans* in solid organ malignancies was higher than in haematological malignancies, 51 and 33%, respectively.

The present study showed that chemotherapy discloses the most incidence of *C. albicans* infection (44%), followed by the combined chemo-radiotherapy, a result that coincides with the study made by Al-Abeid et al. (2004) where the chemotherapy patients were colonized more frequently by *C. albicans* than radiotherapy patients.

The adherence level of the studied isolates was found not to be significantly different from that of ATCC control strain. This result does not agree with that obtained from the study made by Al-Abeid et al. (2004), where there was a statistically significant difference between the number of adhered *C. albicans* and the ATCC control strain.

Our findings also revealed that the protease production was lowered in isolates from different cancer patients than that of ATCC control strain. The same result was suggested by Al-Abeid et al. (2004). This may be explained by the fact that, some cytotoxic agents can lower the protease production in *C. albicans* as various anticancer agents, such as glucocorticoids, cytotoxic agents and calcineurin inhibitors have direct inhibitory effects on and/or have altered the biology of fungal cells and in some cases can also be used as a combined therapy with antifungal agents (Chen et al., 2011). Although the virulence of the *C. albicans* is lowered, the infection and colonization persists in cancer patients, this may be due to the immune-suppression effect of anticancer treatments.

Disk diffusion method was used to determine the susceptibility of *C. albicans* to fluconazole and amphotericin B as it represents a reliable and reproducible method (Rodrí´Guez-Tudela et al., 1996). *C. albicans* isolates in the current investigation were susceptible to fluconazole and of intermediate sensitivity to amphotericin B suggesting the prime role the fluconazole has in fungicidal activity. These results are in accordance with other studies reporting susceptibility of *C. albicans* in cancer patients (Al-Abeid et al., 2004; Slavin et al., 2010). Additionally, it was noted that strains exhibiting trailing growth, as seen in the present experiment, responds to low doses of fluconazole (Revankar et al., 1998).

In conclusion, the effective management of *C. albicans* infections prior to any anti-cancer treatment is highly recommended in order to avoid the complications that might arise from the fungal infection upon starting the anticancer treatment. Although the current study indicates that cancer therapy, either chemotherapy or radiotherapy, has affected the virulence of *C. albicans*, it is highly recommended to keep caution when dealing with powerful antifungal agents in the prophylaxis or the management of OPC especially in immune-compromised patients to avoid the possible emergence of resistant strains.

Conflict of Interests

The authors have not declared any conflict of interests.

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