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Review

Detection of aflatoxigenic *Aspergillus* strains by cultural and molecular methods: A critical review

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Aflatoxin contamination of food and feed commodities, caused by *Aspergillus* section *Flavi* group of fungi, is a serious problem worldwide. Exposure through consumption of contaminated food and feed has deleterious effects on human and animal health. Therefore, aflatoxin contaminated products are a barrier to international trade of agricultural commodities. Not all fungi from *Aspergillus* section *Flavi* produce aflatoxins. Hence it is important to differentiate *Aspergillus* spp. into toxigenic and atoxigenic species to better understand their population structure in a specific environment. A range of methods are available today, including cultural, analytical and molecular methods, to identify the toxin producing ability of isolates from section *Flavi*. A comprehensive review of these methods would be of great use for researchers in developing nations where fully equipped aflatoxin detection laboratories are lacking. In this paper we critically reviewed the cultural and molecular methods of detecting aflatoxigenic *Aspergillus* species and their precision.

Key words: Toxigenic *Aspergillus*, atoxigenic *Aspergillus*, cultural methods, PCR based methods.

INTRODUCTION

Aflatoxins are secondary metabolites produced by *Aspergillus* section *Flavi* group of fungi. These aflatoxins are potent carcinogenic, teratogenic, mutagenic, hepatotoxic and immunosuppressive agents that cause significant damage to human and animal health (Moss, 2002; Saleemullah et al., 2006). The *Aspergillus* section *Flavi* comprises two groups of species; first group include *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii* and *A. bombycis*. Of these, the *A. flavus* and *A. parasiticus* are the predominant aflatoxin producers (Cary and Ehrlich, 2006; Ehrlich et al., 2007). Other *Aspergillus* spp. reported to be aflatoxigenic in this section are *A. pseudocaelatus* (B1, B2 and G1, G2 aflatoxins), *A. pseudonomius* (B1 and not G-type aflatoxins) (Varga et al., 2011) and *A. parvisclerotigenus* (Godet and Munaut, 2010). The second group comprises atoxigenic species such as *A. oryzae*, *A. sojae* and *A. tamarii* (Kumeda and Asao, 2001). The atoxigenic species such as *A. sojae*

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and *A. oryzae* are generally used as starters in fermentation of foods (Chang et al., 2007). Particularly, *A. sojae* and *A. oryzae* are perceived as atoxigenic variants of *A. flavus* and *A. parasiticus* (Klich and Pitt, 1988). Other species of *Aspergillus* not included in section *Flavi* are also reported to be aflatoxigenic (Chang et al., 2007). For example, *A. ochraceoroseus* (from section Ochraceroesi); the ascomycete fungi *Emericella astellata* and *E. venezuelensis* (*Aspergillus* section Nidulantes) also produce B1 (Cary et al., 2005; Klich et al., 2000; Frisvad et al., 1999 , 2005).

The predominant aflatoxigenic species of *Aspergillus*; *A. flavus* and *A. parasiticus* produce aflatoxins, that are a group of 20 closely related secondary metabolites (Liu and Wu, 2010; Snigdha et al., 2013). These fungi are ubiquitous, they are common soil inhabitants, air-borne, and are also found in crops and foods at both pre-and post-harvest stages (Waliyar et al., 1994; Jaime-Garcia and Cotty, 2004; Williams et al., 2004). Among different aflatoxins, the naturally occurring and well-known ones are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) (Gimeno, 2004; Saleemullah et al., 2006; Strosnider et al., 2006). Of them, AFB1 is categorized as a group 1 carcinogen (Castegnaro and McGregor, 1998), and is the most prevalent aflatoxin. Predominantly, these toxins affect peanuts, corn, cotton seed, tree nuts, pea, sorghum, rice, pistachio, oilseed rape, sunflower seed, figs, spices, meats, dairy products, and fruit juices (apple, guava) (Abdin et al., 2010). Further, to this, in occupational settings, mycotoxin exposure is one of the major health concerns. Monitoring for mycotoxin exposure is therefore mandatory so as to take adequate precautionary steps. Presently, monitoring on Aflatoxin B1 is rare at occupational settings. In this context, occupational settings with potential residents of aflatoxigenic *A. flavus* strains needs enumeration. It is also at this juncture, the application aspects of the current review would be prudent. Knowledge on differentiation of atoxigenic and toxigenic strains would finally contribute to enhancement of precautionary safety systems in various occupational settings.

There are morphological similarities between aflatoxigenic and non-aflatoxigenic *Aspergillus* species. For example, *A. sojae* is morphologically similar to *A. parasiticus* and it is believed that *A. sojae* is a domesticated strain of *A. parasiticus* (Chang et al., 2007). However, few distinguishing characters such as color, texture and conidial diameter separate these two species (Klich, 2002). Precise detection of aflatoxigenic *Aspergillus* species is important for both research and mitigation. In this paper, we have comprehensively reviewed cultural and molecular methods of detection of aflatoxigenic *Aspergillus* spp. and their differentiation using cultural and molecular methods. Current analytical methods used to validate the aflatoxin production and quantification include thin layer chromatography (TLC) (Stroka and Anklam, 2000), high-performance liquid chromatography (HPLC) (Seitz, 1975; Sobolev and Dorner, 2002; Trucksess et al., 1991), liquid chromatography /mass spectroscopy (LC/MS), enzyme linked immunesorbent assay (ELISA) (Patey et al., 1989) and immunoaffinity with fluorescence (Nasir and Jolley, 2002). Each of these methods has advantages and limitations. For example, cultural methods though inexpensive, but are less sensitive, affecting accuracy (Abbas et al., 2004a). On the other hand, molecular techniques provide rapid diagnosis because of their high sensitivity, specificity, and are currently in use for detection of aflatoxigenic strains of *A. flavus* and *A. parasiticus* (Shapira et al., 1996; Sweeney et al., 2000).

Since, the toxigenic profiles of both *A. flavus* and *A. parasiticus* are mostly different; adopting a single method has not yet been reliable in differentiation. The compiled information may be useful in devising a polyphasic, cost effective and robust approaches using one or more methods that allow accurate differentiation between aflatoxin producing and non-producing strains of *Aspergillus*.

CULTURAL METHODS

Cultural methods for detecting aflatoxins either rely on quantification of purified extracts (Filtenborg and Frisvad, 1980; Shotwell et al., 1966), or on qualitative assessments of fluorescence (Bennett and Goldblatt, 1973; de Vogel et al., 1965; Hara et al., 1974; Lin and Dianese, 1976) or UV absorption (Yabe et al., 1987). They include a) blue fluorescence (FL) (particularly in the presence of an enhancer in the medium such as β-cyclodextrin (Fente et al., 2001; Ordaz et al., 2003); b) yellow pigmentation (YP) on the undersides of colonies (Gupta and Gopal, 2002; Lin and Dianese, 1976; Odhiambo et al., 2014); and c) color change of the yellow pigment to plum-red on exposure of the culture to ammonium hydroxide vapor (AV) (Saito and Machida, 1999; Abbas et al., 2004a; Odhiambo et al., 2014).

Different media are in use for growing aflatoxigenic *Aspergillus* spp. They include *Aspergillus flavus* and *parasiticus* agar (AFPA) (Pitt et al., 1983), Czapek's yeast extract agar (CYA), yeast extract sucrose agar medium (YES), coconut agar medium (CAM), aflatoxin producing ability medium (APA) (Jaimez et al., 2003b). However, the toxin production in these media varies with extraneous factors such as pH, temperature and time. Incubation for a period of five days is necessary for toxin production in YES (Gqaleni et al., 1996; Leontopoulos et al., 2003). Aflatoxin production (AFB1) by *A. flavus* and *A. parasiticus* in cheese and rice for toxigenic isolates can be peak at 7, 10, 14, 21 and 28 days. Further, maximum production of AFB1 will be at 14 days (Park and Bullerman, 1983).

Different reports on the potential of media in supporting toxin production are available. Ritter et al. (2011) showed

that YES media is better compared to CYA, whereas Abranson and Clear (1996) proved that CYA agar was slightly better than YES agar. Reddy et al. (2009) reported that all toxigenic isolates of *A. flavus* produced AFB1 on YES media, whereas, 65%, and 53% of the same isolates produced aflatoxin on Czapek's and PDA agar, respectively. However, none of the toxigenic isolates produced AFB1 in AFPA medium. In another study, Fente et al. (2001) showed that YES media was comparatively superior in supporting aflatoxin production over Czapek's, aflatoxin producing ability (APA) media, and different coconut agars. In contrast, Desai and Ghosh (2003) reported high AFB1 production in Czapek's agar over APA and CAM.

A combination of media can be of great advantage in determining the toxigenic potential of fungi. For example, combined use of CYA and YES media is advantageous since both these media allow production of different mycotoxins (Bragulat et al., 2001; Frisvad and Filtenborg, 1983). A new media containing Czapek's yeast extract agar (CYA) and YES + 0.6% of YCSD (commercial YES medium supplemented with 0.3% Mβ-cyd and 0.6% sodium desoxycholate) is used to detect aflatoxigenic potential of *A. flavus* strains. When grown on this media, previously verified toxigenic strains have developed a fluorescent ring under UV light. Other media that have potential to detect aflatoxigenic *A. flavus* and *A. niger* are PDA + NaCl and APA media that produce fluorescence around the colonies. Further, the diameter of the beige ring and the intensity of its fluorescence under UV increased over time up to four days (Almoammar et al., 2013). Coconut agar medium (CAM) is also generally used for rapid detection of aflatoxin production by *Aspergillus* spp. (Lin and Dianese, 1976). Cultivation on CAM is generally a preliminary tool to detect aflatoxin production (de Vogel et al., 1965; Arseculeratne et al., 1969; Lin and Dianese, 1976; Davis et al., 1987). Liquid formulation of CAM is also used for detecting aflatoxins by a microplate fluorescent reader-based assay (Degola et al., 2011). A visible beige ring can be observed, under UV light, in cultures of aflatoxigenic fungi (Figure 1). Further, a blue fluorescence surrounding aflatoxigenic colonies under UV light is also seen on the reverse of the plates (Almoammar et al., 2013) (Figure 2). Isolates thought to be atoxigenic also produced toxins in CAM under optimal conditions (Hoeltz, 2005). However, due to the frequent false negative results, CAM is an unreliable method for detecting the toxigenic potential of *A. flavus* and *A. parasiticus* (Taniwaki, 1996). Other coconut culture media in use for detecting toxigenic strains include coconut milk agar (CMA), coconut extract agar, coconut cream agar (Fente et al., 2001; Davis et al., 1987; Dyer and McCammon, 1994), fresh coconut extract (FCE) and commercial coconut extract (CCE) as substrates (Lin and Dianese, 1976). Coconut cream agar is relatively more effective than other synthetic coconut media due to desiccated coconut as the ingredient (Dyer

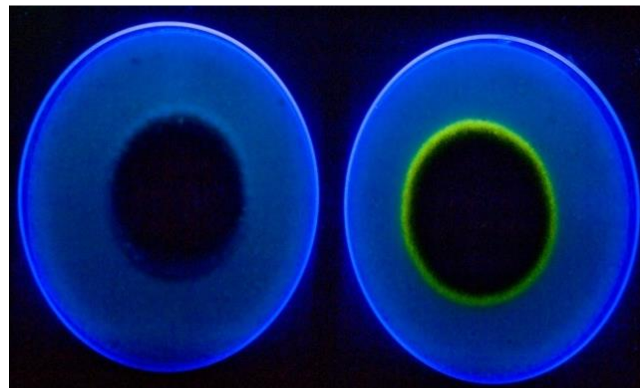

Figure 1. Characteristic beige ring shown by aflatoxigenic *Aspergillus flavus* (right side) on coconut agar medium

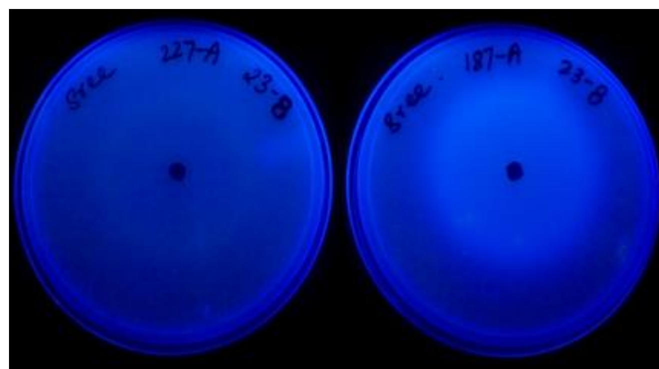

Figure 2. Characteristic blue fluorescence exhibited by aflatoxigenic *Aspergillus flavus* (right side) on reverse side of coconut agar medium.

and McCammon, 1994). Another reliable substrate for rapid detection of aflatoxigenic fungi is palm kernel. Yellow pigmentation of toxigenic isolates in palm kernel media is indicative of the presence of aflatoxins. Compared to desiccated coconut agar, the fluorescence nature, intensity of diffusion of water soluble fluorescent compounds of isolates was unique in palm kernel media (Atanda et al., 2005).

Presence of a beige ring around the colonies on media and its fluorescence under UV light is an indication of aflatoxin-producing ability of strains. Quantitative measure of agar medium fluorescence for estimating the aflatoxins are also available. Simple fluorescence is one such rapid estimation of aflatoxin levels in a solid culture medium (Cotty, 1988). In this procedure, glass test tubes containing 5 ml of agar are inoculated with spores of *Aspergillus* spp. and incubated for 3 days at 30°C. Fluorescence in the agar by UV light is observed. Further, the agar fluorescence is directly correlated with concentration of aflatoxins (Cotty, 1988). Hara et al. (1974) described in detail the fluorescence method of

detecting aflatoxigenic strains of *A. flavus* by utilizing UV induced fluorescence of toxin in a modified Czapek's solution agar containing corn steep liquor, $HgCl_2$, and $(NH_4)H_2PO_4$ instead of $NaNO_3$. Further, the toxin presence is confirmed by thin layer chromatography (TLC) of $CHCl_3$ extracts of the fluorescing agar. In another study, Atanda et al. (2005) reported that aflatoxigenic isolates exhibit a characteristic blue or blue green fluorescence of agar under long wave UV light against a pink background as confirmed by TLC. However, certain non-aflatoxigenic isolates also fluorescence under UV light (Almoammar et al., 2013). For example, strains of *A. flavus* and *A. oryzae* produce several substances other than aflatoxins that give a blue fluorescence under UV light. They include asperopterin A or B (Kaneko and Sanada, 1969), flavacol and deoxy-hydroxy-aspergillic acid (Yokotsuka et al., 1967).

Several other cultural media used for detection of toxigenic strains through observations on fluorescence or visible color of pigments are potato dextrose agar (PDA) and coconut agar (Davis et al., 1987; Gupta and Gopal, 2002; Lemke et al., 1988; Lin and Dianese, 1976; Saito and Machida, 1999), corn steep liquor (Hara et al., 1974; Wicklow et al., 1981) and glucose-yeast extract medium (GY-agar) (Filtenborg and Frisvad, 1980; Yabe et al., 1987; Cotty, 1988), Sabouraud dextrose and yeast extract agar when fortified with cyclodextrin (β-CD) was also a screening substrate for detecting aflatoxigenic strains (Fente et al., 2001). Complex agar medium containing sucrose, various salts, and an aqueous extract of aflatoxin-free groundnut (de Vogel et al., 1965); synthetic liquid medium (Adye and Mateles, 1964) and silica gel medium (Torrey and Marth, 1976) are also used (Fente et al., 2001). Dichloran 18% glycerol agar, DG18; dichloran rose bengal agar, DRBC; malt extract agar, MA; oxytetracycline glucose yeast extract agar, OGY; tryptic soy with lecithin and polysorbate 80, TSA; Dichloran chloramphenicol peptone agar, DCPA are also used in certain investigations on phosphorescence phenomenon to allow the detection of aflatoxigenic strains in culture media (Rojas-Duran et al., 2007).

Enrichment of culture media with cyclodextrins

Fluorescence enhancers such as cyclodextrins (CDs) are generally used to confirm the production of fluorescence by aflatoxigenic *A. flavus* strains. Fluorescence by AFB1 and AFG1 is greatly enhanced in presence of CDs. These CDs are cyclic oligosaccharides consisting of (α-1, 4)-linked α-D-glucopyranose units. The behaviour of AFB1 in presence of CDs was investigated by several researchers (Blais et al., 1988; Cepeda et al*.,* 1988; Francis et al*.,* 1988; and Vazquez et al., 1991). However, the exact mechanism behind fluorescence enhancement by aflatoxins through β-CD complexation is not clearly understood (Vazquez et al., 1992). Recently, a theoretical mechanism on fluorescence enhancement of AFB1 by β-CD was proposed (Fente et al., 2001). The β-CD form is relatively more effective in the formation of aflatoxin inclusion complexes when both α and β-CD are used (Cepeda et al., 1996). However, no significant differences on intensity of color change at different concentrations of β-CD and methylated β-CD derivatives in different media were noticed. Further, the intensity of fluorescence had increased with time (Fente et al., 2001). Conjunctive use of hydroxypropyl-β-cyclodextrin (HBC) (Cavasol®) and bile salts (cholic acid, sodium taurocholate and sodium dehydrocholate) also resulted in better detection of aflatoxigenic strains (Fente et al., 2001; Jaimez et al., 2003a, b; Rojas et al., 2005).

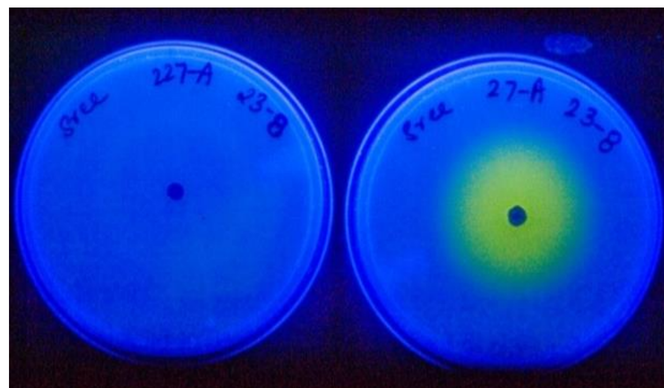

Figure 3. Characteristic golden yellow pigmentation by aflatoxigenic *Aspergillus flavus* (right side) on reverse side of coconut agar medium.

Pigments as indicators

Production of yellow to orange pigments by aflatoxigenic *A. flavus* strains was observed for the first time by Wiseman et al. (1967). Yellow pigment formation in mycelia and media is also a basis for diagnosis of aflatoxigenic isolates (Figure 3) (Abbas et al., 2004a; Shier et al., 2005; Odhiambo et al., 2014). Aflatoxin producing *A. flavus* cultures isolated from insect pests on coconut produced a bright yellow pigmentation (Gupta and Gopal, 2002). The degree of yellow pigmentation is proportional to blue fluorescence in culture media (Lin and Dianese, 1976).

Vapor tests for color change

A new and rapid method for detecting toxigenic strains of *A. flavus* and *A. parasiticus* is through vapor tests. By exposing the aflatoxigenic colonies to ammonium hydroxide vapors using standard procedures (Abbas et al., 2004b), will result in quick color change of the reverse side from brownish/yellowish color to plum-red (Saito and Machida, 1999) (Figure 4). Biochemical basis of vapor

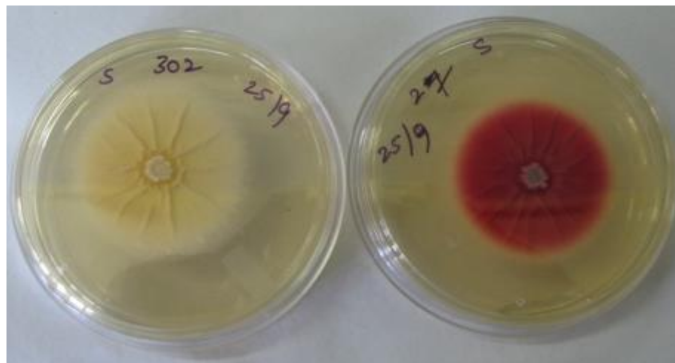

Figure 4. Characteristic plum red color change by aflatoxigenic *Aspergillus flavus* (right side) on reverse side of Yeast extract sucrose (YES) medium when exposed to ammonium hydroxide vapors.

tests was investigated by extracting pigments from lyophilized cultures of toxigenic strains grown on PDA. Further mixing of these pigments with ammonium hydroxide or other bases (sodium hydroxide, potassium hydroxide, sodium carbonate, and sodium bicarbonate) resulted in color change to plum-red (Abbas et al., 2004b). The pigments associated with color change are norsolorinic acid, averantin, averufin, versicolorin C, versicolorin A, versicolorin A hemiacetal, and nidurufin (Aucamp and Holzapfel, 1970; Cole, 1981; Heathcote and Dutton, 1969). All these pigments are anthraquinone intermediates in aflatoxin biosynthetic pathway (Bhatnagar et al., 2003). Of these, averufin was produced by a non-aflatoxigenic mutant of *A. parasiticus* (Donkersloot et al., 1972).

MOLECULAR METHODS

Application of molecular techniques in aflatoxin research is multitude. For example, molecular approaches have been used for differentiating the *Aspergillus* section *Flavi* complex, determining the phylogenetic analysis, characterization of isolates, identification of aflatoxigenic isolates from different food and feeds, diversity studies and in understanding the non-aflatoxigenicity of certain *Aspergillus* spp. (Montiel et al., 2003; Almoammar et al., 2013; Chang et al., 2007; Baird et al., 2006; Hatti et al., 2010; Yin et al., 2009). Applications of such studies can be useful in bringing out effective atoxigenic strains rapidly for their further use as potential biocontrol agents (Yin et al., 2009). Differentiation of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* group through molecular approaches is important because conventional approaches are not entirely reliable (Criseo et al., 2001). Moreover, the molecular mechanisms responsible for loss of aflatoxigenicity of *Aspergillus* are not clearly understood (Schmidt-Heydt et al., 2008). Earlier reports indicated that atoxigenic *A. flavus* isolates were found to be majorly associated with the deletions of a part or the entire aflatoxin gene cluster (Chang et al., 2005). Defects in the aflatoxin gene, *pksA* in *A. flavus* AF36 isolate of cotton seed is also responsible for its atoxigenicity (Ehrlich and Cotty, 2004). Other reasons for atoxigenicity of *Aspergillus* can be attributed to large deletions in the aflatoxin gene cluster (Prieto et al., 1996). Jiang et al. (2009) reported a large fragment deletion in the aflatoxin gene cluster and further replacement of its location by a heterologous insert. PCR assays have revealed that *Aspergillus* isolates with entire aflatoxin gene cluster could not produce aflatoxins (Yin et al., 2009). Hence, analysis of deletion within aflatoxin gene cluster can be an effective method for rapid identification of true non-aflatoxigenic *Aspergillus* strains (Yin et al., 2009).

Based on molecular characterization studies, it is concluded that both toxigenic and atoxigenic *A. flavus* isolates are genetically similar, but some atoxigenic isolates having deletions within the aflatoxin gene cluster can be identified readily by PCR assays (Yin et al., 2009). Complete differentiation of atoxigenic and toxigenic strains is however not feasible with molecular methods such as random amplified polymorphic DNA (RAPD), Microsatellite-primed PCR (MP-PCR) profiles and DNA sequences of internal transcribed spacer (*ITS*) regions, *pksA* and *omtA* genes (Yin et al., 2009). Previous research has also indicated that molecular techniques such as RAPD and AFLP (amplified fragment length polymorphism) could not differentiate toxigenic and atoxigenic strains (Tran-Dinh et al., 1999; Montiel et al., 2003; Barros et al., 2007). For example, the atoxigenic *A. sojae* and the toxigenic *A. parasiticus* could not be differentiated through sequencing of the ribosomal DNA ITS (internal transcribed spacers) (Montiel et al., 2003). However, this technique could separate the *A. flavus/A. parasiticus* subgroup from the *A. oryzae/A. flavus* subgroup. In another study, Baird et al. (2006) reported that DNA amplification fingerprinting (DAF) was not effective in distinguishing aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*. However, use of mini-hairpin primers using the arbitrary signatures from amplification profiles (ASAP) technique successfully distinguished the majority of aflatoxigenic from non-aflatoxigenic isolates (Baird et al., 2006). AFLP fingerprints however can be used for genetic differentiation of *Aspergillus* section *Flavi* complex fungi. In a study by Montiel et al. (2003), analysis of AFLP data consistently and clearly separated *A. sojae*/*A. parasiticus* isolates from the *A. oryzae*/*A. flavus* isolates. Further studies by Montiel et al. (2003) have identified polymorphic sequences that could be developed into markers for aflatoxigenic *A. parasiticus* isolates.

Use of multiplex PCR with three sets of primers specific for three structural genes of the aflatoxin pathway (*nor-1*, *ver-1* and *omt-A*) could differentiate only the aflatoxin producing fungi, *A. flavus* and *A. parasiticus*

from others, but not aflatoxin producing and non-producing strains of the same species (Geisen, 1996). In another study, Rashid et al. (2008) categorized *A. flavus* and *A. parasiticus* isolates of stored wheat into aflatoxigenics and non-aflatoxigenics using multiplex PCR based assay. Their studies on detection of four genes, *AflR* (regulatory gene) and structural genes such as *Nor 1* (norsolorinic acid), *Ver 1* (Versicolorin) and *Omt* (O methylsterigmatocystin) indicated that only one of the tested strains of *A. parasiticus* (AP4) out of 36 strains has shown the presence of these four genes. Further, it was also observed that strains with presence of *aflR* gene also did not produce aflatoxins (Rashid et al., 2008). Criseo et al. (2001) worked with quadruplex PCR using primers for *aflR*, *nor-1*, *ver-1* and *omt-A* genes of the aflatoxin biosynthetic pathway for differentiating aflatoxigenic and non-aflatoxigenic strains. Their results indicated that aflatoxigenic strains gave a quadruplet pattern, indicating the presence of all the genes involved in the aflatoxin biosynthetic pathway. However, for non-aflatoxigenic strains, the results are variable with one, two, three or four banding patterns. Further, a banding pattern in few non-aflatoxigenic strains resulted in non-differentiation between these two strains.

Aflatoxin production by *A. flavus* is more stable in nature than in culture (Bayman and Cotty, 1993). Gene expression between *Aspergillus* spp. can be different despite their close relatedness at the DNA level according to microarray analysis (Wilkinson et al., 2007). Hence, understanding the aflatoxigenicity and non-aflatoxigenicity through molecular means at gene level is an important step for further rapid and precise detection of these species. Developing rapid, precise, cost-effective and less cumbersome methods in this direction will further aid in devising strategies that prevent aflatoxin contamination in agriculturally important crops.

CONCLUSIONS

For precise differentiation of toxigenic and atoxigenic strains of *A. flavus*, a polyphasic approach involving the use of different cultural and molecular methods is apt. False positives with respect to aflatoxin production are also not uncommon in aflatoxigenic strain detection. In this regard, analytical methods such as HPLC, TLC and ELISA that detect toxin production in the substrate can also be confirmative when used in polyphasic approach along with cultural and molecular methods. An elaborative and updated review in this area would be useful for aflatoxin researchers especially in detecting and differentiating atoxigenic and toxigenic *Aspergillus* spp. in crop soils that are posing severe health hazards and trade losses in several major crops.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Abbas HK, Shier WT, Horn BW, Weaver MA (2004a). Cultural methods for aflatoxin detection. Toxin Rev. 23:295-315.

Abbas HK, Zablotowicz RM, Weaver MA, Horn, BW, Xie W, Shier WT (2004b). Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. Can. J. Microbiol. 50(3):193-199.

Abdin MZ, Ahmad MM, Javed S (2010). Advances in molecular detection of *Aspergillus*: an update. Arch. Microbiol. 192(6): 409-425.

Abranson D, Clear RM (1996). A convenient method for assessing mycotoxin production in cultures of *Aspergilli* and *Penicillia*. J. Food Prot. 59:142-144.

Adye J, Mateles RI (1964). Incorporation of labelled compounds into aflatoxins. Biochim. Biophys. Acta 86:418-420.

Almoammar H, Bahkali AH, Abd-Elsalam KA (2013). A polyphasic method for the identification of aflatoxigenic *Aspergillus* species isolated from Camel feeds. Aust. J. Crop Sci. 7(11): 1707-1713.

Arseculeratne SN, De Silva LM, Wijesundera S, Bandunatha CHSR (1969). Coconut as a medium for the experimental production of aflatoxin. Appl. Microbiol. 18(1):88-94.

Atanda I, Rati ER, Ozoja M (2005). Palm Kernel: A potential substrate for rapid detection of aflatoxigenic fungi. Food Sci. Technol. Int. 11(1):67-74.

Aucamp PJ, Holzapfel CW (1970). Polyhydroxy anthraquinones from *Aspergillus versicolor*, *Aspergillus nidulans* and *Bipolaris* sp. Their significance in relation to biogenetic theories on aflatoxin B1. J. S. Afr. Chem. Inst. 23: 40-56.

Baird RE, Trigiano RN, Windham G, Williams P, Kelley R, Abbas HK, Moulton JK, Scruggs ML (2006). Comparison of aflatoxigenic and non aflatoxigenic isolates of *Aspergillus flavus* using DNA amplification fingerprinting techniques. Mycopathologia 161: 93-99.

Barros GG, Chiotta ML, Reynoso MM, Torres AM, Chulze SN (2007). Molecular characterization of *Aspergillus* section Flavi isolates and *Aspergillus flavus* S and L morphotype isolates. Int. J. Food Microbiol. 108:172-177.

Bayman P, Cotty P (1993). Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. Can. J. Bot. 71:23-31.

Bennett JW, Goldblatt LA (1973). The isolation of mutants of *Aspergillus flavus* and *A. parasiticus* with altered aflatoxin producing ability. Sabouraudia 11: 235-241.

Bhatnagar D, Ehrlich KC, Cleveland TE (2003). Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol. 61: 233-93.

Blais J, Prognon P, Mahuzier G, Vigny P (1988). Relative affinity of 5-methoxypsoralen and 8-methoxypsoralen towards β-cyclodextrin: a fluorescence, circular dichroism and chromatographic study. J. Photochem. Photobiol. B 2(4): 455-465.

Bragulat MR, Abarca ML, Cabanes FJ (2001). An easy screening method for fungi producing ochratoxin A in pure culture. Int. J. Food Microbiol. 71:139-144.

Cary JW, Ehrlich KC (2006). Aflatoxigenicity in *Aspergillus*: molecular genetics, phylogenetic relationships and evolutionary implications. Mycopathologia 162(3):167-177.

Cary JW, Klich MA, Beltz SB (2005). Characterization of aflatoxin-producing fungi outside of *Aspergillus* section *Flavi.* Mycologia 97: 425-432.

Castegnaro M, McGregor D (1998). Carcinogenic risk assessment of mycotoxins. Rev. Med. Vet-Toulouse 149: 671-678.

Cepeda A, Franco CM, Fente CA, Vazquez BI, Rodriguez JL, Prognon

P, Mahuzier G (1996). Post column excitation of aflatoxins using cyclodextrins in liquid chromatography for food analysis. J. Chromatogr. A. 721(1): 69-74.

Cepeda A, Prognon P, Mahuzier G, Blais J (1988). Improvement in the fluorometric detection of 5-methoxypsoralen by using β-cyclodextrin column. Anal. Chim. Acta 211: 333-337.

Chang PK, Horn BW, Dorner JW (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in non aflatoxigenic *Aspergillus flavus* isolates. Fungal Genet. Biol. 42: 914-923.

Chang PK, Matsushima K, Takahashi T, Yu Jiujiang, Abe K, Bhatnagar D, Yuan GF, Koyama Y, Cleveland TE (2007). Understanding non aflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. Appl. Microbiol. Biotechnol. 76: 977-984.

Cole RJ (1981). Versicolorin group. In: Handbook of toxic fungal metabolites. New York: Academic Press, pp. 94-127.

Cotty PJ (1988). Simple fluorescence method for rapid estimation of aflatoxin levels in a solid culture medium. Appl. Environ. Microbiol. 54: 274-276.

Criseo G, Bagnara A, Bisignano G (2001). Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. Lett. Appl. Microbiol. 33: 291-295.

Davis ND, Iyer, SK, Diener UL (1987). Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 53: 1593-1595.

De Vogel P, Van Rhee R, Koelensmid W (1965). A rapid screening test for aflatoxin-synthesizing *Aspergilli* of the *flavus-oryzae* group. J. Appl. Bacteriol. 28: 213-220.

Degola F, Berni E, Restivo, FM (2011). Laboratory tests for assessing efficacy of atoxigenic *Aspergillus flavus* strains as biocontrol agents. Int. J. Food Microbiol. 146(3): 235-243.

Desai MR, Ghosh S (2003). Occupational exposure to airborne fungi among rice mill workers with special reference to aflatoxin producing *A. flavus* strains. Ann. Agric. Environ. Med. 10(2):159-162.

Donkersloot JA, Mateles RI, Yang SS (1972). Isolation of averufin from a mutant of *Aspergillus parasiticus* impaired in aflatoxin biosynthesis. Biochem. Biophys. Res. Commun. 47(5): 1051-1055.

Dyer SK, McCammon S (1994). Detection of toxigenic isolates of *Aspergillus flavus* and related species on coconut cream agar. J. Appl. Bacteriol. 76: 75-78.

Ehrlich KC, Cotty PJ (2004). An isolate *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. J. Microbiol. Biotechnol. 65: 473-478.

Ehrlich KC, Kobbeman K, Montalbano BG, Cotty PJ (2007). Aflatoxin-producing *Aspergillus* species from Thailand. Int. J. Food Microbiol. 114: 153-159.

Fente CA, Ordaz JJ, Vazquez BI, Franco CM, Cepeda A (2001). New additive for cultural media for rapid identification of aflatoxin producing *Aspergillus* strains. Appl. Environ. Microbiol. 67: 4858-4862.

Filtenborg O, Frisvad JC (1980). A simple screening method for toxigenic fungi in pure cultures. Lebensm. Wiss-Technol. 13:128-130.

Francis OJ, Kirschenheuter GP, Ware GM, Barman AS, Kuan SS (1988). β-Cyclodextrin post-column fluorescence enhancement of aflatoxins for reverse-phase liquid chromatographic determination in corn. J. AOAC Int. 71: 725-728.

Frisvad JC, Filtenborg O (1983). Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. Appl. Environ. Microbiol. 46(6): 1301-1310.

Frisvad JC, Houbraken J, Samson RA (1999). *Aspergillus* species and aflatoxin production: a reappraisal, In: A.C.J. Tuijtelaars, R.A. Samson, F.M. Rombouts, S. Notermans (Eds.), Food Microbiology and Food Safety into the Next Millennium, Foundation Food Micro '99, Zeist, pp.125-126.

Frisvad JC, Skouboe P, Samson RA (2005). Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-*O*-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. Syst. Appl. Microbiol. 28(5): 442-453.

Geisen R (1996). Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Syst. Appl. Microbiol. 19(3): 388-392.

Gimeno A (2004). Aflatoxina M1 no leite. Riscos para a saúde pública, prevenção e controlo. Alimentação Animal (Revista de la Associação Portuguesa dos Industriais de Alimentos Compostos para Animais (IACA)) 49: 32-44.

Godet M, Munaut F (2010). Molecular strategy for identification in *Aspergillus* section *Flavi*. FEMS Microbiol. Lett. 304: 157-168.

Gqaleni N, Smith JE, Lacey J (1996). Co-production of aflatoxins and cyclopiazonic acid in isolates of *Aspergillus flavus*. Food Addit. Contam. 13(6): 677-685.

Gupta A, Gopal M (2002). Aflatoxin production by *Aspergillus flavus* isolates pathogenic to coconut insect pests. World J. Microbiol. Biotechnol. 18(4): 329-335.

Hara S, Fenell DL, Hesseltine CW (1974). Aflatoxin producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. Appl. Microbiol. 27: 1118-1123.

Hatti AD, Taware SD, Taware AS, Pangrikar PP, Chavan AM, Mukadam DS (2010). Genetic diversity of toxigenic and non-toxigenic *Aspergillus flavus* strains using ISSR markers. Int. J. Curr. Res. 5: 61-66.

Heathcote JG, Dutton MF (1969). New metabolites of *Aspergillus flavus*. Tetrahedron 25(7): 1497-1500.

Hoeltz M (2005). Estudo da Influencia de Manejos Pos-Clolheita na Incidencia de Fungos e Micotoxinas no Arroz (*Oryza sativa* L.). Dissertacao (Mestrado em Microbiologia Agricola e do Ambiente)-Universidade Federal do Rio Grande do Sul. Porto Alegre.

Jaime-Garcia R, Cotty PJ (2004). *Aspergillus flavus* in soils and corn cobs in South Texas: Implications for management of aflatoxins in corn-cotton rotations. Plant Dis. 88: 1366-1371.

Jaimez J, Fente CA, Franco CM, Cepeda A, Vazquez B (2003a). Application of a modified culture medium for the simultaneous counting of molds and yeasts and detection of aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. J. Food Prot. 66: 311-318.

Jaimez J, Fente CA, Vazquez BI, Franco CM, Cepeda A (2003b). Development of a method for direct visual determination of aflatoxin production by colonies of the *Aspergillus flavus* group. Int. J. Food Microbiol. 83:219-225.

Jiang H, Yan L, Ma Z (2009). Molecular characterization of an atoxigenic *Aspergillus flavus* strain AF051. Appl. Microbiol. Biotechnol. 83(3): 501-505.

Kaneko Y, Sanada M (1969). Studies on the fluorescent substances produced by *Aspergillus* fungi. VII. Purification and isolation of asperopterin B and chemical properties of asperopterin B and A. J. Ferment. Technol. 47:8-19.

Klich MA (2002). Identification of common *Aspergillus* species. Centralbureau voor Schimmelcultures, The Netherlands.

Klich MA, Mullaney EJ, Daly CB, Cary JW (2000). Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *Aspergillus ochraceoroseus*. Appl. Microbiol. Biot. 53(3): 605-609.

Klich MA, Pitt JI (1988). A laboratory guide to common *Aspergillus* species and their teleomorphs. CSIRO Division of Food Processing, North Ryde, NSW.

Kumeda Y, Asao T (2001). Heteroduplex panel analysis, a novel method for genetic identification of *Aspergillus* section *Flavi* strains. Appl. Environ. Microbiol. 67(9): 4084-4090.

Lemke PA, Davis ND, Lyer SK, Creech GW, Diener UL (1988). Fluorometric analysis of iodinated aflatoxin in mini cultures of *Aspergillus flavus* and *Aspergillus parasiticus*. J. Ind. Microbiol. 3: 119-125.

Leontopoulos D, Siafaka A, Markaki, P (2003). Black olives as substrate for *Aspergillus parasiticus* growth and aflatoxin B1 production. Food Microbiol. 20(1):119-126.

Lin MT, Dianese JC (1976). A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. Phytopathology 66: 1466–1469.

Liu Y, Wu F (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. Environ. Health Perspect. 118(6): 818-824.

Montiel D, Dickinson MJ, Lee HA, Dyer PS, Jeenes DJ, Robertis IN, James S, Fuller LJ (2003). Genetic differentiation of the *Aspergillus* section *flavi* complex using AFLP fingerprints. Mycol. Res. 107:1427-

1432.
Moss MO (2002). Risk assessment for aflatoxins in food stuffs. Int. Biodeter. Biodegradation 50(3-4): 137-142.
Nasir MS, Jolley ME (2002). Development of a fluorescence polarization assay for the determination of aflatoxins in grains. J. Agric. Food Chem. 50(11): 3116-3121.
Odhiambo BO, Murage H, Wagara IN (2014). Screening for atoxigenic *Aspergillus* species and evaluating their inhibitory potential against growth and sporulation of aflatoxigenic *Aspergillus* species. Egerton J. Sci. Technol. 14: 61-80.
Ordaz JJ, Fente CA, Vazquez BI, Franco CM, Cepeda A (2003). Development of a method for direct visual determination of aflatoxin production by colonies of the *Aspergillus flavus* group. Int. J. Food Microbiol. 83: 219-225.
Park KY, Bullerman LB (1983). Effect of cycling temperatures on aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus* in rice and Cheddar cheese. J. Food Sci. 48: 889-896.
Patey AL, Sharman M, Wood R, Gilbert (1989). Determination of aflatoxin concentrations in peanut butter by enzyme linked immunosorbent assay (ELISA): study of three commercial ELISA kits. J. Assoc. Off. Anal. Chem. 72(6): 965-969.
Pitt JI, Hocking AD, Glenn DR (1983). An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. J. Appl. Bacteriol. 52: 109-114.
Prieto R, Yousibova GL, Woloshuk C (1996). Identification of aflatoxin biosynthesis genes by genetic complementation in an *Aspergillus flavus* mutant lacking the aflatoxin gene cluster. Appl. Environ. Microbiol. 62: 3567-3571.
Rashid M, Khalil S, Ayub N, Ahmad W, Khan AG (2008). Categorization of *Aspergillus flavus* and *Aspergillus parasiticus* isolates of stored wheat grains into aflatoxinogenics and non-aflatoxinogenics. Pak. J. Bot. 40: 2177-2192.
Reddy KRN, Saritha P, Reddy CS, Muralidharan K (2009). Aflatoxin B1 producing potential of *Aspergillus flavus* strains isolated from stored rice grains. Afr. J. Biotechnol. 8(14): 3303-3308.
Ritter AC, Hoeltz M, Noll IB (2011). Toxigenic potential of *Aspergillus flavus* tested in different culture conditions. Ciencia e Tecnologia de Alimentos Campinas 31(3): 623-628.
Rojas TR, Fente CA, Vazquez BI, Franco CM, Cepeda A (2005). Use of twelve fungal culture media with added cyclodextrin for the rapid determination of aflatoxigenic strains common in foods. Food Control 16: 445-450.
Rojas-Duran TR, Fente CA, Vazquez BI, Franco CM, Sanz-Medel A, Cepeda A (2007). Study of a room temperature phosphorescence phenomenon to allow the detection of aflatoxigenic strains in culture media. Int. J. Food Microbiol. 115(2): 149-158.
Saito M, Machida S (1999). A rapid identification method for aflatoxin producing strains of *A. flavus* and *A. parasiticus* by ammonia vapor. Mycoscience 40:205-221.
Saleemullah AI, Khalil IA, Shah H (2006). Aflatoxin contents of stored and artificially inoculated cereals and nuts. Food Chem. 98: 699-703.
Schmidt-Heydt M, Magan N, Geisen R (2008). Stress induction of mycotoxin biosynthesis genes by abiotic factors. FEMS Microbiol. Lett. 284: 142-149.
Seitz LM (1975). Comparison of methods of aflatoxin analysis by high-pressure liquid chromatography. J. Chromatogr. 104:81-91.
Shapira R, Paster N, Eyal O, Menasherov M, Mett A, Salomon R (1996). Detection of atoxigenic molds in grains by PCR. Appl. Environ. Microbiol. 62: 3270-3273.
Shier WT, Lao Y, Steele TW, Abbas HK (2005). Yellow pigments used in rapid identification of aflatoxin-producing *Aspergillus* strains are anthraquinones associated with the aflatoxin biosynthetic pathway. Bioorg. Chem. 33(6): 426-438.
Shotwell OL, Hesseltine CW, Stubblefield RD, Sorenson WG (1966). Production of aflatoxin on rice. Appl. Microbiol. 14(3):425-428.
Snigdha M, Hariprasad P, Venkateswaran G (2013). Mechanism of aflatoxin uptake in roots of intact groundnut (*Arachis hypogaea* L.) seedlings. Environ. Sci. Pollut. Res. Int. 20 (12): 8502-8510.
Sobolev V, Dorner J (2002). Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. J. AOAC Int. 85(3):642-645.
Stroka J, Anklam E (2000). Development of a simplified densitometer for the determination of aflatoxins by thin-layer chromatography. J. Chromatogr. A 904(2): 263-268.
Strosnider H, Azziz-Baumgartner E, Banziger M, Bhat RV, Breiman R, Brune MN, DeCock K, Dilley A, Groopman J, Hell K, Henry SH, Jeffers D, Jolly C, Jolly P, Kibata GN, Lewis L, Liu X, Luber G, McCoy L, Mensah P, Miraglia M, Misore A, Njapau H, Ong CN, Onsongo MTK, Page SW, Park D, Patel M, Phillips T, Pineiro M, Pronczuk J, Rogers HS, Rubin C, Sabino M, Schaafsma A, Shephard G, Stroka J, Wild C, Williams JT, Wilson D (2006). Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. Environ. Health Perspect. 114: 1898-1903.
Sweeney MJ, Pamies P, Dobson AD (2000). The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in *Aspergillus parasiticus* 439. Int. J. Food Microbiol. 56(1): 97-103.
Taniwaki MH (1996). Meios de cultura para contagem de fungos em alimentos. Bol. SBCTA 30 (2): 132-141.
Torrey GS, Marth EH (1976). Silica gel medium to detect molds that produce aflatoxin. Appl. Environ. Microbiol. 32(3): 376-380.
Tran-Dinh N, Pitt JI, Carter DA (1999). Molecular genotype analysis of natural toxigenic and nontoxigenic isolates of *Aspergillus flavus* and *A. parasiticus*. Mycol. Res. 103: 1485-1490.
Trucksess MW, Stack ME, Nesheim S, Page SW, Albert RH, Hansen TJ, Donahue KF (1991). Immunoaffinity column coupled with solution fluorometry or liquid chromatography postcolumn derivatization for determination of aflatoxins in corn, peanuts and peanut butter: collaborative study. J. Assoc. Off. Anal. Chem. 74(1): 81-88.
Varga J, Frisvad JC, Samson RA (2011). Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud. Mycol. 69: 57-80.
Vazquez ML, Cepeda A, Prognon P, Mabuzier G, Blais J (1991). Cyclodextrins as modifiers of the luminescence characteristics of aflatoxins. Anal. Chim. Acta 255: 343-350.
Vazquez ML, Franco CM, Cepeda A, Prognon P, Mabuzier G (1992). Liquid-chromatographic study of the interaction between aflatoxins and beta-cyclodextrin. Anal. Chim. Acta 269: 239-247.
Waliyar F, Ba A, Hassan H, Bonkoungou S, Bosc JP (1994). Sources of resistance to *Aspergillus flavus* and aflatoxin contamination in groundnut genotypes in West Africa. Plant Dis. 78: 704-708.
Wicklow DT, Shotwell OL, Adam GL (1981). Use of aflatoxin producing ability medium to distinguish aflatoxin-producing strains of *Aspergillus flavus*. Appl. Environ. Microbiol. 41: 697-699.
Wilkinson JR, Yu J, Bland JM, Nierman WC, Bhatnagar D, Cleveland TE (2007). Amino acid supplementation reveals differential regulation of aflatoxin biosynthesis in *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* SRRC 143. Appl. Microbiol. Biotechnol. 74:1308-1319.
Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am. J. Clin. Nutr. 80: 1106-1122.
Wiseman HG, Jacobson WC, Marmeyer WC (1967). Note on removal of pigments from chloroform extracts of aflatoxin cultures with copper carbonate. J. Assoc. Off. Anal. Chem. 50: 982–983.
Yabe K, Ando Y, Ito M, Terakado N (1987). Simple method for screening aflatoxin-producing molds by UV photography. Appl. Environ. Microbiol. 53: 230-234.
Yin Y, Lou T, Yan L, Michailides TJ, Ma Z (2009). Molecular characterization of toxigenic and atoxigenic *Aspergillus flavus* isolates, collected from peanut fields in China. J. Appl. Microbiol. 107: 1857-1865.
Yokotsuka T, Sasaki M, Kikuchi I, Asao Y, Nobuhara A (1967). Studies on the compounds produced by moulds. 1. Fluorescent compounds produced by Japanese industrial moulds. Nippon Nogei Kagaku Zasshi 41:32-38.

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Full Length Research Paper

Characterization of the quorum quenching activity of *Streptomyces minutiscleroticus*: A new approach for infection control

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By playing a major role in the virulence of several pathogenic bacteria, N-Acyl homoserine-lactone (AHL) -dependent quorum sensing, is now considered a useful target for antimicrobial therapy. In the present study a total number of 63 *Streptomyces* isolates recovered from different soil samples collected from different localities in Egypt were screened for quorum quenching activity using a fast, reliable, simple screening method and *Chromobacterium violaceum* mutant strain (CV026) as a biosensor. Primary screening against synthetic hexanoyl homoserine lactone (HHL) revealed that 8 isolates showed quorum quenching activity while secondary screening against the extracted naturally produced acyl homoserine lactone (AHL) signals of seven *Pseudomonas aeruginosa* clinical isolates showed a variable profile of activity. Characterization of activity showed that isolate that coded St62, showing highest activity among other *Streptomyces* isolates, could degrade 2 µM HHL in 4 h. The crude enzyme of this isolate was found to have high thermal stability retaining >83% of its activity when pre-incubated at 80°C for 60 min. It also retained >90% of the activity when pre-incubated at pH 8, pH 9 and pH 10 and >80% when pre-incubated at pH 6. All the tested metal ions and EDTA had no effect on activity except for Cu^{++} which caused partial reduction in activity. Enzyme extract was found to have acylase activity equal to 5.2 U/mg total protein. Vmax and Km were found to be equal to 19.92 $nM.min^{-1}$ per mg protein and 23.05 nM, respectively. Maximum catalytic activity was observed at pH 8 over a temperature range of 20-50°C. The enzyme also showed preference in hydrolyzing AHLs with long acyl chains than short acyl chains but yet digested all the tested AHL standards. Finally, identification of the selected isolate using 16S ribosomal RNA gene analysis and phylogenetic analysis showed it to be a *Streptomyces minutiscleroticus* which is, to the best of our knowledge, the first identified quorum quenching bacterial species of its type.

Key words: Quorum sensing, *Streptomyces minutiscleroticus*, Antipathogenic drugs, Acyl homoserine lactones.

INTRODUCTION

The increasing occurrence of multi-resistant pathogenic bacterial strains has gradually rendered the traditional antimicrobial treatment ineffective. Today, a global concern has emerged that we are entering a post-antibiotic era with a reduced capability to combat microbes, and, hence, the development of novel therapeutic approaches for the treatment of bacterial infections constitutes a focal point in modern research (Hentzer and Givskov, 2003).

The term quorum sensing (QS) describes a process in which a signal, known as an autoinducer, accumulates in the environment as the bacterial population grows. At some threshold concentration, the autoinducer affects a

change in the gene expression of the population leading to transcriptional regulation (Fuqua et al., 1994). QS-controlled genes often encode virulence factors and gene products required for bacteria-host interactions (Pirhonen et al., 1993; Parsek and Greenberg, 2000; Pearson et al., 2000). QS was also found to be involved in many other bio-chemical processes in the bacterial cell (Diggle et al., 2007).

The approach of targeting quorum sensing systems is referred to as 'quorum quenching' and compounds with abilities to stop bacterial virulence through targeting QS system are termed antipathogenic drugs as opposed to antibacterial drug (Hentzer and Givskov, 2003). This approach has the advantage over the traditional treatments which are based on compounds that kill or inhibit bacterial growth and which suffer from problems such as: side effects of chemotherapeutic agents, limited number of newly discovered antibiotics and increasingly emerging resistance to existing antibiotics. There are basically three different targets for QS inhibition which include: the signal generator; the signal receptor and the signal molecule (Rasmussen and Givskov, 2006)

Many Gram negative bacteria, like *Pseudomonas aeruginosa*, have Acyl homoserine lactone (AHL)-dependent quorum sensing systems (Fuqua et al., 2001). Accordingly, inactivation of this signal molecule represents a new effective approach for the control of infection of such pathogenic highly resistant bacteria.

Many different bacteria belonging to various genera have been reported to express quorum quenching through enzymes degrading AHLs. Examples include the degradation of acyl homoserine lactone molecule by lactonases which are produced by some *Bacillus* species (Dong et al., 2001; Lee et al., 2002). O ther example include degradation by *Arthrobacter* sp. (Park et al., 2003), *Variovorax paradoxus* (Zhang et al., 2002; Carlier et al., 2003) and acylases produced by some *Streptomyces* species (Park et al., 2005). A search for bacteria degrading the Autoinducers of QS systems is promising for designing agents to effectively suppress bacterial infections (Khmel and Metlitskaya, 2006)

The goal of the present study was to screen soil for *Streptomyces* with quorum quenching activity against synthetic and naturally produced AHL from clinically isolated *P. aeruginosa* isolates together with the characterization of the activity and the identification of the promising isolate.

MATERIALS AND METHODS

Chemicals

All chemicals were of high quality from available grades purchased from El-Nasr Chemicals (Adwic), Egypt. Acyl homoserine lactone standards (bytanoyl (C4), haxanoyl (C6), heptanoyl (C7), and octanoyl (C8) homoserine lactone) were purchased from Sigma-Aldrich, Germany. Reagents for DNA extraction and PCR were a product of Fermentas, USA.

Bacterial strains

Chromobacterium violaceum CV026

CV026 is a mutant strain of *C. violaceum* that produces the characteristic purple pigment violacein only in response to the presence of acyl homoserine lactone with C4-C8 side chain and it acts as (AHL) dependent biosensor (McClean et al., 1997). CV026 was subcultured in Luria Bertani (LB) broth for maintenance. For purification, 20 µg/ml kanamycin were added in the growth medium as it is kanamycin resistant (Ravn et al., 2001). It was stored in slant medium or in lyophilized form for long term preservation.

Pseudomonas aeruginosa isolates

Seven *P. aeruginosa* isolates were used in this study. These isolates were previously recovered from clinical specimens, studied for their AHL productivities and had their AHL signals analyzed using thin layer Chromatography (TLC) (Sakr et al., 2014). They were used to study the spectrum of quorum quenching activity of the recovered *Streptomyces* isolates.

Streptomyces isolates

Sixty three *Streptomyces* isolates were recovered onto starch nitrate agar (Waksman, 1959) from 18 soil samples previously collected from different localities in Egypt and pre-enriched with CaCO3 at the Microbiology and Immunology Department, Faculty of pharmacy, Ain shams University. These isolates were routinely maintained onto starch nitrate agar and some selected isolates were subject for long term preservation in slant medium or as spores glycerol stocks (Kieser et al., 2000; Shepherd et al., 2010).

Growth of test isolates for collection of the cell free supernatants

Streptomyces isolates were grown in two steps, first a preculture that was prepared by suspending a fresh slant of 4 days age of *Streptomyces* isolate in 5 ml distilled H_2O from which 1 ml was used to inoculate 5 ml YMG medium (yeast extract 0.4%w/v, malt extract 1%w/v, glucose 0.4%w/v) contained in a test tube. The tube was then incubated for 4 days at 28°C with shaking at 160 rpm. Second, the main culture was done according to (Sun-Yang et al. (2005), with some modifications. From the preculture, 1 ml (about 10^7 cfu/ml) was transferred into 20 ml YMG medium contained in 100 ml conical flask to provide appropriate aeration. Then, the flask was incubated for 7 days at 28°C with shaking at 160 rpm. The *Streptomyces* cells were then removed by centrifugation at 15000 rpm for 15 min and the resulting supernatant was collected. Because AHLs (test substrate) are easily hydrolyzed under alkaline conditions (Yates et al., 2002), the pH of the culture supernatants of the test isolates was adjusted to 7.0 before being tested for AHL degrading activity.

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Primary screening using the synthetic signal molecule (HHL)

Screening was done according to Jafra and his coworkers ((Jafra and van-der-Wolf, 2004), with some modifications. Briefly, *C. violaceum* CV026 strain was grown overnight in 5 ml LB broth at 28°C with shaking at 160 rpm then the optical density (OD_{640}) of the growth produced was adjusted to equal to 0.1-0.2 (about 10^8cfu/ml, Blosser and Gray, 2000). Then, in each well of the microtitre plate, about 50 µl of 0.5 µM hexanoyl homoserine lactone (HHL) were overlaid by 50 µl of collected supernatant, incubated for 4 h at 28°C, then the bacterial cultures were exposed to UV germicidal lamp (working at 254 nm) for 90 min after which, 50 µl of the adjusted count CV026 culture was added to each well. The plates were incubated at 28°C overnight where CV026 produced purple pigment in wells where HHL was present. While, in the wells where HHL was degraded by bacterial isolates, no color was produced. Positive control was prepared by adding 50 µl plain LB broth instead of the collected supernatant.

Secondary screening of the positive isolates using *P. aeruginosa* extracts

Secondary screening were done for the collected supernatants that proved to degrade HHL in the primary screening and was carried out against the extracts of seven *P. aeruginosa* isolates that were previously screened for their abilities to produce acyl homoserine lactone signal molecules and analyzed using TLC. This was performed according to the protocol described by Jafra and coworkers (Jafra and van-der-Wolf, 2004). Briefly, About 0.3 ml aliquots of the *P. aeruginosa* extract was mixed with 1.5 ml M63 buffer ,pH 7.0, prepared according to Heidemarie and coworkers (Heidemarie et al., 1980). About 50 µl of the resulting solution (1.8 ml) was added to each well of the microtitre plate overlaid with 50µl of the supernatants of and the assay was carried out just as described in the primary screening. Positive control for each *P. aeruginosa* extract was done using plain LB broth instead of collected supernatants. Secondary screening was done in triplicates for confirmation of the results.

Testing the activity of tested isolates at different levels of substrate concentration

In an experiment, two dilutions of the collected cell free supernatant of positive *Streptomyces* isolates were tested against different concentrations of synthetic HHL (substrate); 0.5, 2, 5 and 10 µM and this was carried out as described in primary screening.

Measuring the total protein concentration in the crude enzyme extracts of positive isolates

Growth and preparation of cell free supernatants of test isolates were done as previously described. The protein concentrations of the prepared cell free supernatants, termed as crude enzyme extracts, were measured using bovine serum albumin as a standard (Lowry et al., 1951). A standard curve of absorbance at 660 nm as a function of protein concentration of the standard solutions was plot and used to determine the samples' protein concentrations. Then, protein concentration in the crude enzyme was adjusted to 3 mg/ml.

Measuring the activity of the crude enzyme extracts of positive isolates using well diffusion method

This assay was done according to Ravn and his coworkers (Ravn et al., 2001) where 10 ml LB agar was overlaid by 10 ml semis olid LB agar supplemented with HHL at a concentration 100 nM and seeded with the biosensor strain (CV026) . When the overlaid agar had solidified, wells were punched into the agar with a sterile Cork borer of diameter 10 mm. After that, 150 µl of the crude extract of the positive isolates were pipetted into the wells. The plates were then incubated for 24 hours at 28°C. The growth of CV026 showed the purple color in the whole plate except the zones around the wells.

Physical parameters that affect the enzyme stability of the selected isolate

Thermal stability

In this assay, the enzyme was incubated at 50, 80 and 90°C for 30 and 60 min. Then, 150 µl of each aliquot was pipetted into the wells of semisolid LB agar prepared as previously described in the well diffusion method (Ravn et al., 2001).The residual activity was then measured by comparing the inhibition zone diameters of the test to that of the control incubated at room temperature. The experiment was done in triplicates and the means and standard deviations were calculated.

Stability at different pH

The assay was carried out according to Cao and his coworkers (Cao et al., 2012) with modifications as follows: 1 ml aliquots of the enzyme extract were transferred to wasserman tubes and the pH of each was changed to the required pH using either McIlvaine buffer (prepared by mixing 19.6 ml 0.1M citric acid and 0.4 ml 0.2M Na_2HPO_4) or Glycine-NaOH buffer (0.1 M glycine-NaOH adjusted to pH 12). The tubes were incubated at room temperature for 30 min and then neutralized to pH 7 using the counter buffer. Afterwards, the volumes of all the aliquots were adjusted equally. Then, 150 µl of each aliquot was pipetted into the wells and the activity was measured using the well diffusion method as previously described. The residual activity was then measured by comparing the inhibition zone diameters at different pH to that of pH 7. The experiment was done in triplicates and the means and standard deviations were calculated. The stability of the enzyme was studied over a pH range of 4 to 10.

Effect of some divalent metals and EDTA on activity

Concentrations of 1 and 10 mM of Ca^{++}, Fe^{++}, Mg^{++} and EDTA were added to the enzyme extract and incubated for 30 min. For Cu^{++} and Zn^{++}, a concentration of 10mM was found to have an inhibitory effect on the growth of CV026, so they were used in concentrations 1 and 2 mM. A sample without an additive served as a control. Then, 150 µl of the tested aliquots and the control were pipetted into the wells of semisolid LB agar, as previously described, and the inhibition zone diameters compared to that of the control. The experiment was done in triplicates and the means and standard deviations were calculated.

Catalytic parameters of crude enzyme of the selected isolate

Catalytic activity rate

This was done according to Cao and his coworkers (Cao et al., 2012) with modifications as follows: aliquots of the enzyme extract (0.5 ml) were incubated with 10 µM HHL at 28°C with shaking at 160 rpm and the reaction was terminated using 2% SDS after 4, 8, 12, 16 and 20 h. The control was done using plain medium of pH 7

containing HHL of 10 μM final concentration incubated under the same conditions, for the same time period and also treated with SDS. Afterwards, the residual amount of HHL was measured by well diffusion assay as follows: 10 ml LB agar were overlaid by 10 ml semisolid agar seeded with CV026 and the plates were left to solidify. Wells were punctured into the agar and 60 μl of the tested samples were transferred into them. The plates were then incubated for 24 hours at 28°C for color development. The HHL in the wells form zones of purple color around the well, the diameter of which was proportional to the HHL concentration. Measured zones were then compared to standard curve showing the diameters of the purple zones as a function of the different HHL concentrations (with 2% SDS) pipetted into the wells. One unit of AHL degrading enzyme activity was defined as: the amount of enzyme that hydrolyzed 1nM HHL in 1 min and was determined after 8 h reaction time.

Effect of temperature on catalytic activity

This was done as described for the determination of the catalytic activity rate of the enzyme but with the incubation temperature of the reaction mixture adjusted at 5, 20, 40, 50 or 70°C. A control was prepared and incubated at 28°C. The rate of reaction was determined for both the control and the test after 8 h and compared.

Effect of pH on catalytic activity

Aliquots (1 ml each) of the enzyme had their pH values adjusted to 6, 7, 8, 9 and 10 using McIlvaine or glycine-NaOH buffer. Then, they were incubated with HHL (10 μM) at 28°c. After 8 h the reaction was terminated using 2% SDS and the procedure was completed as previously described. The reaction rates after 8 h were determined and activity at different pH was compared to activity at pH 7.

Determination of kinetic constants; Vmax and Km

Aliquots of the enzyme extract (0.5 ml) were incubated with different concentrations of HHL; 5, 10, 15, and 20 μM at 28°C with shaking at 160 rpm. Then, the reaction was terminated after 8 h by 2% SDS and the residual amount of HHL measured by well diffusion assay as previously described. The initial reaction velocity was calculated from the following equation:

$$\text{Rate of HHL degradation} = \frac{-\,d[A]}{d[t]}$$

Where A is the concentration of HHL and t is the time in min. The data were plot as a Lineweaver-Burk curve from which kinetic constants, Vmax (the maximum velocity of reaction) and Km (Michaelis–Menten constant) were calculated.

Enzyme substrate specificity

The ability of the enzyme to hydrolyze AHLs was assessed using standard signals, butanoyl (C4), heptanoyl (C7) and octanoyl (C8) homoserine lactones. This was done by measuring the catalytic activity rate of the enzyme as described above for hexanoyl homoserine lactone. The reaction was carried out for 8 h at 28°C and pH7. Units of enzyme activity to total protein concentration was determined in terms of C4, C7 and C8 HSL and compared to that previously measured for HHL standard.

Identification of the selected *Streptomyces* isolate

This was done by 16S ribosomal RNA gene analysis where DNA extraction, PCR and PCR clean up took place at Sigma Scientific Services Company, Egypt. Sequencing was done at GATC Company, by the use of ABI 3730xl DNA Sequencer. Afterwards, the obtained 16S ribosomal RNA gene sequence files of the selected isolate were assembled using Staden package program version 3 (Staden, 1996) and the obtained sequence was compared with those in GenBank database using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the identification of the selected isolate and phylogenetic tree was constructed.

RESULTS

Primary screening using the synthetic signal molecule (HHL)

Results show that out of the 63 screened isolates, only eight isolates (12.69% of the total *Streptomyces* isolates) were found to have quorum sensing inhibitory activity while the rest of isolates (55 isolates) showed negative results and failed to degrade HHL. The eight positive isolates with HHL degrading activity resulting from primary screening (St11, St13, St14, St35, St38, St57, St61 and St62) were subjected to secondary screening.

Secondary screening of the positive isolates using *P. aeruginosa* extracts

From the results of Table 1, the degradation of AHL signals contained in 6 of the tested *P. aeruginosa* extracts was demonstrated by the supernatant of *Streptomyces* isolate St62 and for 4 extracts by supernatants of *Streptomyces* isolates St11, St14, St35, St38 and St61 and for 2 extracts by supernatants of isolates, St13 and St57. It was also found that none of the isolates could completely degrade AHL signals of the extract of *P. aeruginosa* isolate P13.

Testing the activity of tested isolates at different levels of substrate concentration

Results from Figure 1, shows that only supernatants of *Streptomyces* isolates St11, St61 and St62 could degrade 2 μM HHL while the rest of isolates supernatants showed no ability to degrade HHL in concentrations higher than 0.5 μM. The supernatants of two isolates, St13 and St38 showed faint purple color at concentration 2 μM of the HHL but it was considered and represented as a negative result.

Measuring the activity of the crude enzyme extracts of positive isolates using well diffusion method

The extracts of five tested isolates (St11, St13, St38, St61 and St62) exhibited degrading activity against HHL,

Table 1. Degrading activity profile of tested *Streptomyces* isolates' supernatants against AHL signals contained in the extracts of seven *P. aeruginosa* isolates.

Tested *Streptomyces* supernatants code no.	Degrading activity of AHL signals contained in tested extracts of *P. aeruginosa* isolates							Total no. of *P. aeruginosa* extracts showed degradation*
	P13	P14	P16	P17	P18	P19	P27	
St11	-	++	+	+	++	++	++	4
St13	-	++	-	-	+	++	-	2
St14	-	++	+	-	++	++	++	4
St35	-	++	-	-	++	++	++	4
St38	-	++	+	+	++	++	++	4
St57	-	++	+	+	+	++	+	2
St61	+	++	+	+	++	++	++	4
St62	+	++	++	++	++	++	++	6

++, Complete degradation of target AHL signals; +, Partial degradation of target AHL signals. -, No degradation of target AHL signals; Shaded row refers to the isolate selected for further studies; *refers to degradation in their signal contents.

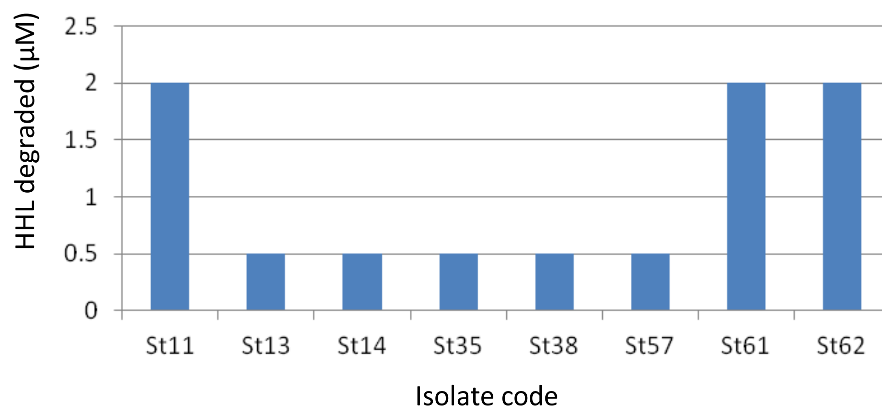

Figure 1. AHL degrading activity of tested *Streptomyces* isolates at different HHL concentrations.

demonstrated by the development of colorless zones by the biosensor around extracts containing wells. On the other hand, the crude enzyme extracts of the other three tested isolates (coded St14, St35 and St57) failed to degrade AHL around the wells evidenced by the absence of inhibition zones. The diameters of colorless zones developed are recorded in Table 2.

Comparing the HHL degrading activity of the tested *Streptomyces* isolates and their activity spectrum against *P. aeruginosa* extracts, isolate coded St62 which showed activity against signals contained in six *P. aeruginosa* extracts, was selected for further studies.

Physical parameters that affect the enzyme stability of the isolate St62

Thermal stability

When incubated at 50°C for either 30 or 60 min, the enzyme of isolate St62 retained more than 98% of its activity. When incubated at 80°C for 30 min, it retained about 92% of the activity and when incubated for 60 min, it retained about 83% of the activity. When the enzyme was incubated at 90°C for 30 min the activity was lost completely evidenced by the absence of inhibition zone in comparison to the control (Figure 2).

Stability at different pH

As shown from the results in Figure 3, the enzyme of isolate St62 retained more than 90% of the activity when pre-incubated at pH 8, pH 9 and pH 10 while the activity was lost completely after pre-incubation at pH 4 and pH 5. When pre-incubated at pH 6 for 30 min, the enzyme retained more than 80% of the activity relative to the control.

Effect of some divalent metals and EDTA on activity

At the two test concentrations of Ca^{++}, Mg^{++}, Zn^{++}, Fe^{++}

Table 2. AHL[(a)] degrading activity of the tested *Streptomyces* isolates as determined by well diffusion method.

***Streptomyces* isolate code**	**AHL degrading activity expressed as inhibition zone diameter[(b)] (mm)**
St11	22
St13	18
St38	14
St61	19
St62	21

[a]HHL was used as a substrate; [b] The listed values were the average of replicate readings and inhibition zone refers to zone showing no purple color development by the grown biosensor cells.

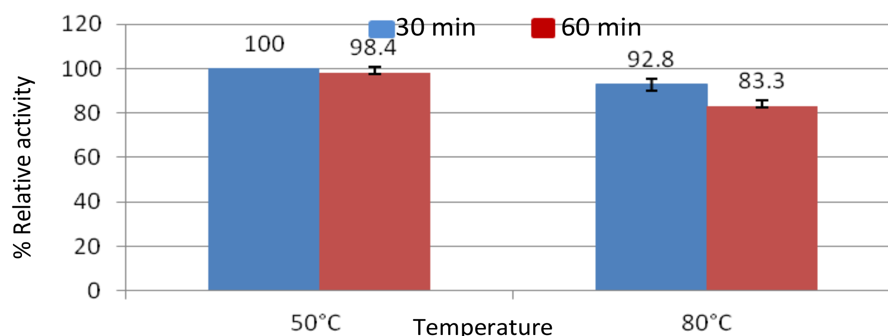

Figure 2. Residual activity after pre-incubation at different temperatures. % relative activity was calculated with respect to control (sample kept at room temperature).

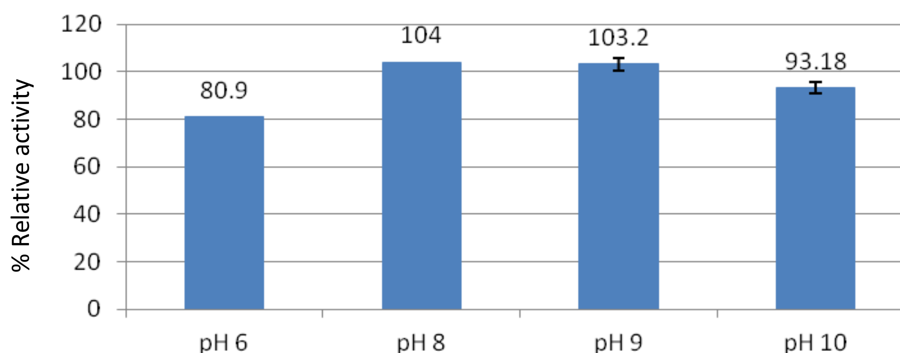

Figure 3. Residual activity after pre-incubation at different pH. % relative activity was calculated with respect to control (sample at pH 7).

and EDTA, the recorded enzyme activity was ~100%. Activity was neither stimulated nor inhibited. However, a concentration of 1 mM Cu^{++} reduced the activity to 90.5% and a concentration of 2 mM Cu^{++} to 81.8% (Figure 4).

Catalytic parameters of crude enzyme of isolate St62

Catalytic activity rate

The time course of HHL degradation by the enzyme of the tested S*treptomyces* isolate St62 is shown in Figure 5. The catalytic activity rate after 8 h was 7.8 $nM.min^{-1}$ then it decreased to 6.04 $nM.min^{-1}$ after 12 h. The activity in terms of acylase activity units to total protein concentrations was found to be equal to 5.2 U/mg total protein. One acylase activity unit is defined as the amount of enzyme that hydrolyzed 1 nM of the substrate in 1 min. amount of enzyme that hydrolyzed 1 nM of the substrate in 1 min.

Effect of temperature on catalytic activity

The activity was almost not affected between 20 and

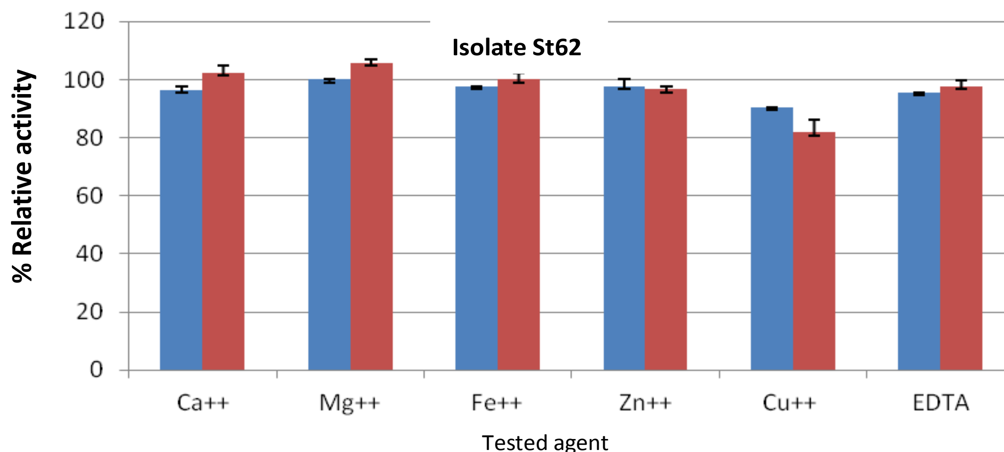

Figure 4. Effect of some divalent metals and EDTA on activity of isolate St62. % relative activity was calculated with respect to control (sample without an additive). Blue: 1 mM concentration, red: 10 mM concentration except for Cu^{++} and Zn^{++} (2 mM).

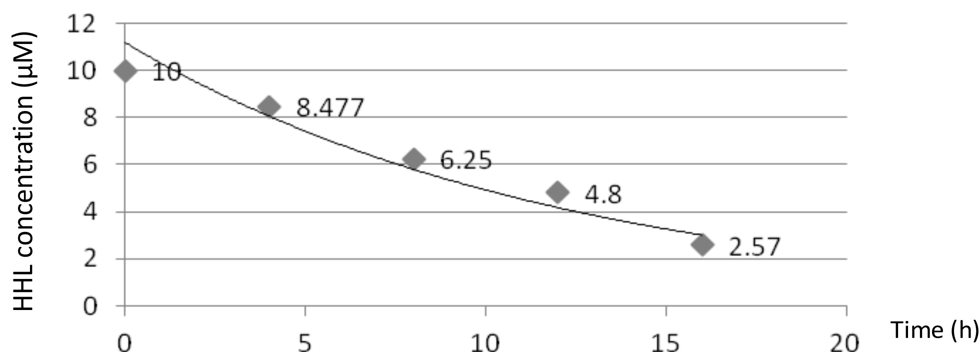

Figure 5. Time course of HHL degradation by the enzyme of *Streptomyces* isolate St62

Table 3. Effect of temperature on catalytic activity of crude enzyme of isolate St62.

Temperature (°C)	% relative activity
5	84.40
20	98.5
40	111
50	100
70	ND*

*ND= not determined

Table 4. Effect of pH on catalytic activity of crude enzyme of isolate St62.

pH	% Relative activity
pH 6	84.85
pH 8	126
pH 9	ND*
pH 10	ND*

*ND= not determined.

50°C. At 5°C, relative activity was reduced to 84.4% while at 70°C the HHL was partially degraded so results could not be assessed (Table 3).

Effect of pH on catalytic activity

At pH 6, relative activity decreased to 84.85% while it increased at pH 8 to 126% (Table 4). However, at pH 9 and 10, activity could not be measured accurately due to the partial degradation of the synthetic signal used.

Determination of Kinetic constants; Vmax and Km

From Lineweaver-Burk plot (Figure 6) Vmax and Km were calculated and they were found to be equal to 19.92 nM.min^{-}1 per mg protein and 23.05 nM, respectively.

Enzyme substrate specificity

Results from Table 5, show that the tested enzyme has a

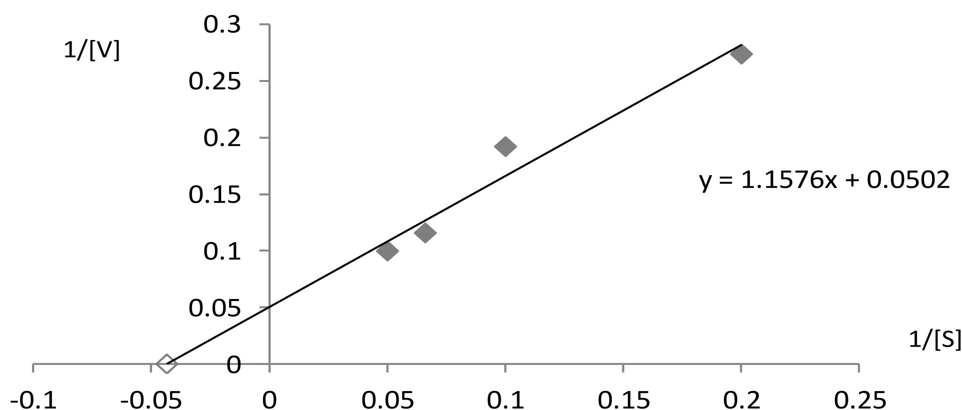

Figure 6. Lineweaver-Burk plot for AHL degrading enzyme of *Streptomyces* isolate St62**.**

broad substrate spectrum evidenced by its ability to degrade the 4 tested AHL compounds used in the study. Maximum activity was observed against C7-HSL and C8-HSL with very slight difference between them while least degrading activity was observed against C4-HSL (almost one third the activity against C7-HSL).

Table 5. Enzyme substrate specificity of the AHL degrading enzyme of *Streptomyces* isolate St62 against different AHL standards expressed as activity units per mg total protein.

Substrate	Activity (U/mg)
C4-HSL	2.5
C6-HSL	5.2
C7-HSL	7.2
C8-HSL	7.08

Identification of the selected *Streptomyces* isolate St62

According to the 16S ribosomal RNA sequencing, the selected *Streptomyces* isolate St62 was found to have 97% similarity with *Streptomyces minutiscleroticus* strain NRRL B-12202 (accession code=NR_044149.1) and was identified as *Streptomyces minutiscleroticus.* It is worth noting that although other *Streptomyces* isolates were reported to exert quorum quenching activity, this is a newly identified quorum quenching *Streptomyces* species. Figure 7 shows the phylogenetic tree for this isolate.

DISCUSSION

In this study, we screened soil *Streptomyces* for quorum quenching activity and we studied the activity of enzyme of the isolate St62. Screening showed that out of the 63 recovered isolates, only eight isolates were found to have quorum quenching activity with a prevalence of quorum quenching enzymes of 12.69% in the recovered *Streptomyces* isolates. Secondary screening results showed the extract of only one *Streptomyces* isolate-St62- could degrade the signal molecules contained in 6 of the *P. aeruginosa* extracts. It was also found that none of the isolates could completely degrade AHL signals of the extract of *P. aeruginosa* isolate P13. The explanation behind this weak activity of *Streptomyces* supernatants on the naturally produced *P. aeruginosa* signals might lie in the substrate specificity of the acylase enzymes believed to be responsible for quorum quenching activity. In 2005, Park and his coworkers stated that the acylase enzyme in their study exhibited a relatively low activity of short acyl side chain AHLs, C6-HSL and 3-oxo-C6-HSL and did not degrade detectable amounts of C4- HSL (Park et al., 2005). It is also observed here that the *P. aeruginosa* isolate P13 whose signals were not degraded by any of the *Streptomyces* supernatants produces two signals detected by CV026. These two signals are namely C4-HSL and C6-HSL. This isolate also displays high AHL productivity (0.283 µM). This directs us to believe that the reason behind the inability of the *Streptomyces* supernatants to degrade the signals of this *P. aeruginosa* isolate is due to its production of high amounts of C4-HSL which exceed the low capability of the acylase of *Streptomyces* isolates to degrade C4-HSL. The same reasons could also be used to explain why only one *Streptomyces* isolate could degrade the signals of *P. aeruginosa* isolates P16 and P17, both are also BHL producers. Signals of isolate P18, degraded by 6 out of the 8 *Streptomyces* isolates, seem to contain a concen-tration of C4-HSL less than those produced by other *P. aeruginosa* isolates.

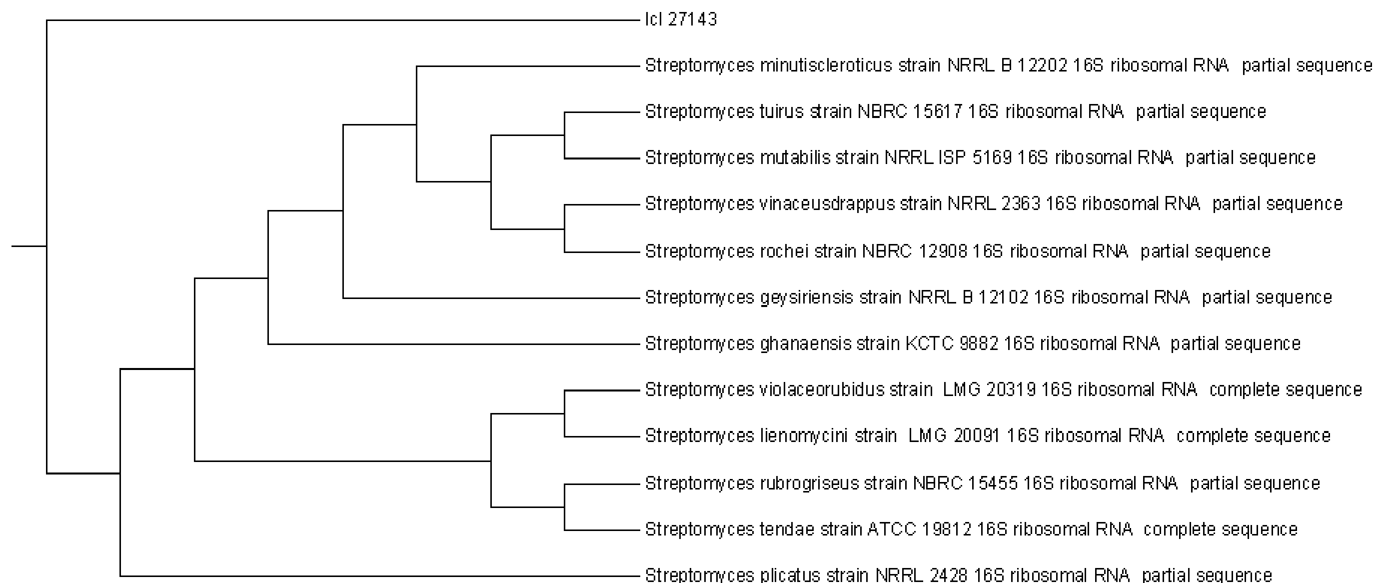

Figure 7. The phylogenetic tree for *Streptomyces minutiscleroticus*

Signals of isolates P14 and P19 were degraded by the supernatants of the eight tested *Streptomyces* isolates. This may be attributed to their production of C6-HSL only as displayed using TLC (Sakr et al., 2014). This was not the case for *P. aeruginosa* isolate P27 although it was proved by TLC to produce C6-HSL only. This may be due to the increased production of C6-HSL signal by this isolate (with AHL productivity of 0.395 µM).

Comparing the activity of the positive isolate by well diffusion method showed that isolates St11 and St62 showed the largest inhibition zones, three isolates, that coded St14, St 35 and St 57 did not show any inhibition zones. This might be attributed to the slow reaction rate of degrading enzymes and their low yield by the test isolates which might have been slower than the ability of the biosensor, CV026, to sense HHL in the area around the wells. In other words, CV026 sensed HHL and started synthesizing violacein before the enzyme started to degrade HHL in considerable amounts. Having the broadest spectrum of activity on signals in the clinically isolated *P. aeruginosa* extracts and a relatively high activity on different HHL concentrations (2 µM), isolate St62 was chosen for conducting further characterization of its enzyme.

Studying the physical stability of the enzyme of isolate St62 showed that the enzyme has high thermal stability. *Streptomyces* isolates are believed to exhibit AHL degrading activity through acylase enzyme (Park et al., 2005), accordingly, results in this study are consistent with two previous studies on an amino-acylase from a Streptomyces mobaraensis isolate (Koreishi et al., 2005), and a penicillin acylase (Torres-Bacete et al., 2007). In their study, Torres-Bacete and his coworkers stated that the enzyme was found to have a thermostable structure, showing a midpoint transition temperature of 81.5°C where unfolding was observed at high temperatures (Torres-Bacete et al., 2007).

The enzyme also displayed high stability over a wide pH range where it retained more than 90% of the activity when pre-incubated at pH 8, 9 10 and when pre-incubated at pH 6 for 30 min, the enzyme retained more than 80% of its activity. In a previous study on an amino-acylase, Koreishi and his coworkers stated that the enzyme was stable between pH 5.5-8 when pre-incubated for 1h at 37°C while at pH 10, the enzyme retained about 45% of its activity (Koreishi et al., 2005).

As for the effect of some metals and EDTA on activity, the enzyme in the present study proved to maintain its activity in the presence of all tested metal ions and EDTA except for partial reduction in activity by Cu++. In their study, Koreishi and his coworkers stated that a concentration of 0.1 mM Cu++decreased the activity of amino-acylase to about 20% (Koreishi et al., 2005). The same study reported a reduction in the relative activity of the enzyme by the addition of 0.1 mM Mg++, Fe++ and Ca++ to a range of 25-56% and an increase in activity when 0.1 mM Zn++ was added to 142% which wasn't the case in the present study. According to the same study, EDTA did not have an effect on activity as was the case here. The study also stated that amino-acylase is a metalloenzyme evidenced by the reduction in activity achieved by phenanthroline(Koreishi et al., 2005). On the other hand, in their study on a Penicillin acylase, Torres-Bacete and coworkers stated that the enzyme does not require any external cofactor, metal ion, or reducing agent for maximal activity (Torres-Bacete et al., 2007).

Studying the catalytic activity, the crude enzyme of isolate St62 with total protein concentration of 3 mg/ml

could effectively degrade HHL with a rate of 7.8 nM.min^{-1}. The specific activity in terms of AHL acylase activity units per mg total protein was found to be equal to 5.2 U/mg.

Catalytic activity was almost not affected between 20 and 50°C with maximum activity displayed at 40 °C which is different from the penicillin acylase in the study Torres-Bacete and coworkers where the highest hydrolytic activity was achieved at 75°C (Torres-Bacete et al., 2007). Maximum catalytic activity was observed at pH 8 where it reached 126%.

Results also show that the studied enzyme has a broad substrate spectrum evidenced by its ability to degrade the 4 tested AHL compounds in its crude form. However, a notable difference was observed in the catalytic spectrum of the enzyme towards tested AHLs. Maximum activity was observed against C7-HSL and C8-HSL and was almost three times the activity against C4-HSL. Activity against C6-HSL was almost double the activity against C4-HSL. The findings in this study are consistent with several other studies on different acylases that reported high deacylation activity against AHLs with long acyl chains and weak activity on short chained AHLs with sometimes no activity at all detected with C4- HSL (Huang et al., 2003; Park et al., 2005; Romero et al., 2008). An acylase from a *Ralostonia* isolate however, has been reported to rapidly degrade C4-HSL and 3-oxo-C12-HSL with equal efficiencies while showing a significantly low activity against 3-oxo-C6-HSL (Lin et al., 2003).

By conducting the 16S ribosomal RNA gene sequence analysis, alignment of the obtained sequence against the respective nucleotides sequences of the 16S rRNA genes present in the database could be carried out. Displaying an identity percentage of 97%, the selected *Streptomyces* isolate St62 was identified as *Streptomyces minutiscleroticus.* Some previously studied *Streptomyces* isolates were reported to have enzymes with quorum quenching abilities like the study carried out by Park and coworkers (Park et al., 2005)and *Streptomyces mobaraensis* in the study of Koreishi and coworkers (Koreishi et al., 2005), however, the isolate in this study appears to be the first identified quorum quenching *Streptomyces* of this type.

Conclusion

AHL degrading enzyme of *S. minutiscleroticus* isolate in this study has proved to establish high thermal stability and high stability over a wide pH range. The enzyme also proved to be resistant to the effect of heavy metals and metal chelating reagent, EDTA, and retained maximum activity in the presence of all the tested metal ions and EDTA except for partial reduction in activity when Cu^{++} was added. Maximum catalytic activity was maintained over a wide range of temperature of 20-50°C and maximum catalytic activity was observed at pH 8. The enzyme displayed preference in hydrolyzing AHLs with long acyl chains. In conclusion, AHL degrading enzyme of *S. minutiscleroticus* appears to be a good candidate for further development into an antipathogenic drug.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Cao Y, He S, Zhou Z, Zhang M, Mao W, Zhang H and Yaoa B (2012). Orally Administered Thermostable N-Acyl Homoserine Lactonase from *Bacillus* sp. Strain AI96 Attenuates *Aeromonas hydrophila* Infection in Zebrafish. Appl. Environ. Microbiol. 78: 1899–1908.

Carlier A, Uroz S, Smadja B, Fray R, Latour X and Dessaux Y (2003). The Ti plasmid of *Agrobacterium tumefaciens* harbors an attM-paralogous gene, aiiB, also encoding N-acyl homoserine lactonase activity. Appl. Environ. Microbiol. 69:4989–4993.

Diggle SP, Crusz SA and Cámara M (2007). Quorum sensing. Curr. Biol. 17:907-910.

Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF and Zhang LH (2001). Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. Nature 411: 813–817.

Fuqua C, Parsek MR and Greenberg EP (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Genet. 35: 439-468.

Fuqua WC, Winans SC and Greenberg EP (1994). Quorum Sensing in Bacteria: the LuxR-LuxI Family of Cell Density-Responsive Transcriptional Regulators. J. Bacteriol. 176(2): 269-275.

Heidemarie J, Krieger-Brauer and Braun V (1980). Functions related to the receptor protein specified by the *tsx* gene of *Escherichia coli*. Archiv. Microbiol. 124: 233-242.

Hentzer M, Givskov M (2003). Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J. Clin. Invest. 112:1300-1307.

Huang JJ, Han JI, Zhang LH, Leadbetter JR (2003). Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. Appl. Environ. Microbiol. 69:5941–5949.

Jafra S, van-der-Wolf JM (2004). Fast screening method for detection of acyl-HSL-degrading soil isolates. J. Microbiol. Methods 57(3):415-420.

Khmel IA, Metlitskaya AZ (2006). Quorum Sensing Regulation of Gene Expression: A Promising Target for Drugs against Bacterial Pathogenicity. Mol. Biol. 40(2): 169–182.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000). Practical Streptomyces Genetics. Int. Microbiol. 3(4): 260-261.

Koreishi M, Asayama F, Imanaka H, Imamura K, Kadota M, Tsuno T, Nakanishi K (2005). Purification and Characterization of a Novel Aminoacylase from *Streptomyces mobaraensis*. Biosci. Biotechnol Biochem. 69(10):1914-1922.

Lee SJ, Park SY, Lee JJ, Yum DY, Koo BT, Lee JK (2002). Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis.* Appl. Environ. Microbiol. 68:3919-3924.

Lin YH, Xu JL, Hu J, Wang LH, Ong SL, Leadbetter JR, Zhang LH (2003). Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. Mol. Microbiol. 47(3):849-860.

Lowry H, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

McClean KH, Winson MK, Leigh F, Adrian T, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GSAB, Williams P (1997). Quorum sensing and *Chromobacterium violaceum* : exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 143:3703-3711.

Park SY, Kang HO, Jang HS, Lee JK, Koo BT, Yum DY (2005). Identification of extracellular N-acylhomoserine lactone acylase from a Streptomyces sp. and its application to quorum quenching. Appl. Environ. Microbiol. 71: 2632–2641.

Park SY, Lee SJ, Oh TK, Oh JW, Koo BT, Yum DY, Lee JK (2003). AhlD, an N-acylhomoserine lactonase in *Arthrobacter sp*., and predicted homologues in other bacteria. Microbiology 149:1541-1550.

Parsek MR, Greenberg EP (2000). Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. USA. 97: 8789–8793.

Pearson JP, Feldman M, Iglewski BH, Prince A (2000). *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infect. Immun. 68: 4331-4334.

Pirhonen M, Flego D, Heinkinheirno R, Palva ET (1993). A small diffusable molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwjnia carotovora*. EMBO J. 12:2467-2476.

Rasmussen TB, Givskov M (2006). Quorum-sensing inhibitors as anti-pathogenic drugs. Int. J. Med. Microbiol. 296: 149–161.

Ravn L, Christensen AB, Molin S, Givskov M and Gram L (2001). Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics. J. Microbiol. Methods 44(3): 239-251.

Romero M, Diggle SP, Heeb S, Camara M, Otero A (2008). Quorum quenching activity in *Anabaena* sp. PCC7120: identification of AiiC, a novel AHL-acylase. FEMS Microbiol. Lett. 280: 73–80.

Sakr MM, Aboulwafa MM, Aboshanab KM, Hassouna NA (2014). Screening and preliminary characterization of the quorum quenching activity of some *Bacillus* isolates from soil against clinically isolated *Pseudomonas aeruginosa* homoserine lactones. Malays. J. Microbiol. 10(2):80-91.

Shepherd MD, Kharel MK, Bosserman MA, Rohr J (2010). Laboratory Maintenance of *Streptomyces* Species. Curr. Protoc Microbiol. John Wiley & Sons, Inc.: 1-10.

Staden R (1996). The Staden sequence analysis package. Mol. Biotechnol. 5: 233-241.

Sun-Yang P, Hye-Ok K, Hak-Sun J, Jung-Kee L, Bon-Tag k and Do-Young Y (2005). Identification of Extracellular N-Acylhomoserine Lactone Acylase from a *Streptomyces sp.* and Its Application to Quorum Quenching. Appl. Environ. Microbiol. 71: 2632–2641.

Torres-Bacete J, Hormigo D, Stuart M, Arroyo M, Torres P, Castillón MP, Acebal C, García JL, Mata Idl (2007). Newly Discovered Penicillin Acylase Activity of Aculeacin A Acylase from *Actinoplanes utahensis*. Appl. Environ. Microbiol. 73(16): 5378-5381.

Waksman SA (1959). The Actinomycetes, nature, occurrence and activities. Williams and Wilkins Co. Baltimore. 1: 573.

Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, Goldner M, Dessaux Y, Ca´mara M, Smith H, Williams P (2002). N-Acylhomoserine Lactones Undergo Lactonolysis in a pH-, Temperature-, and Acyl Chain Length-Dependent Mannerduring Growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect. Immun. 70:5635-5646.

Zhang HB, Wang LH, Zhang LH (2002). Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA. 99:4638-4643.

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Full Length Research Paper

AmpC β-lactamase production in enterobacteria associated with bovine mastitis in Brazil

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Enterobacteriaceae are the main etiological agents of environmental bovine mastitis and are often resistant to antimicrobials, especially β-lactam class due to the production of β-lactamases. These can be classified into several groups according to the molecular structure and substrate activity. The present study evaluated the profile of pheno-genotypic resistance to β-lactams, with emphasis on the functional detection of β-lactamases. The isolates were obtained from milk samples from farms located in the interior region of the state of Rio de Janeiro. 47.6% (20/42) of the bacterial isolates producing enzymes classified as Group 1 (AmpC type) were detected, and in disk approximation test, 30% were classified as the inducible type and 70% as not inducible. To the best of the authors' knowledge, this is the first report on AmpC β-lactamase production in enterobacteria involved in mastitis etiology in Brazil.

Key words: Raw milk, environmental mastitis, dairy cattle, antimicrobial resistance.

INTRODUCTION

Mastitis is an inflammation of mammary gland and udder tissue and it is described as the most common and costly disease of dairy cattle and presents clinically or subclinically (Langoni et al., 2009; Brasil, 2012). This inflammation is caused by stress, physical injury, metabolism and infection (Freitas et al., 2005; Tozzetti et al., 2008).

Enterobacteria are opportunistic pathogens often infecting the udder skin and teat during milking and via contact of the animal with contaminated environments. They are the most important agents in the etiology of environmental mastitis (Moreira et al., 2008).

Causative agents are very often resistant to several antimicrobials and also have developed resistance due to intensive selective pressure caused by use of other antimicrobials (Srinivasan et al., 2007; Langoni et al., 2009; Brasil, 2012).

The most common mechanism of resistance for β-lactam antimicrobials in clinically important Gram-negative bacteria is hydrolysis of these antimicrobial agents by β-lactamases (Bush and Jacoby, 2010). The

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broad-spectrum β-lactamases (ESBL) are enzymes that hydrolysis the penicilins and cephalosporins but are susceptible when β-lactam is associated with inhibitor but others cephalosporinases are resistant to β-lactamases inhibitors and can hydrolyze carbapenem as AmpC and carbapenemases (Dalmarco, 2006; Jacoby, 2009; Thomson, 2010). The molecular and functional classify-cation of the β-lactamases was proposed by Ambler (1980) and Bush and Jacoby (2010), respectively. AmpC β-lactamases are only produced by Gram negative bacteria and belong to the functional group 1 by Bush and Group C classification by Ambler (Aguilar, 2009).

These enzymes can be detected by phenotypic antimicrobial markers and confirmed by specific tests. The research is done by searching for *bla* genes, which may be present in the bacterial chromosome or plasmid. *Bla* gene encode enzyme production and its expression can be induced by the presence of β-lactam or can be continuously activated (Livermore, 1995).

The group of β-lactamases is increasing due to the discovery of new enzymes (ANVISA, 2013; Deshpande et al., 2013). Bacteria producing β-lactamases already widely studied in clinical microbiology can be retrieved from cow's milk, beef, pork and chicken, along with animal faeces (Li et al., 2007; Locatelli et al., 2009; Bush and Jacoby, 2010; Geser et al., 2012; Silva and Lincopan, 2012; Dahmen et al., 2013). β-Lactamases-producing Enterobacteriaceae in raw milk can pose a risk to public health in countries even developed ones, where raw cheese is commonly consumed (Dahmen et al., 2013). ESBL and transferable AmpC-producing *Escherichia coli* and *Salmonella* was isolated by broiler meat in Europe and may be considered a public health problem (Egervärn et al., 2014).

To date, there is lack of information on the distribution of β-lactamases in animal isolates in South America.

The impact of healthy animals as a potential reservoir for genes coding β-lactamases should be monitored to prevent the spread of resistance to β-lactam antibiotics and to choose suitable prophylactic and therapeutic measures. The present study evaluated specifically production of AmpC β-lactamases in enterobacteria isolated from dairy herds in the interior region of the state of Rio de Janeiro, Brazil.

MATERIALS AND METHODS

Milk samples, isolation and identification of Enterobacteriaceae

The milk samples were collected from nine dairy farms in interior region of Rio de Janeiro. All animals were evaluated by clinical examination and no animal were considered clinical mastitis. These animals were submitted to the California Mastitis Test (CMT) to detect subclinical mastitis and 20% of the positive cows were included in the study. A total of 381 milk samples were collected according to the method described by Sampaio (1998). The characteristics enterobacteria colonies on blood agar (Columbia Blood Agar Base – HiMedia – with 5% defibrinated sheep blood) were picked in selective medium and all Gram negative glucose-fermenting, oxidase-negative and catalase-positive strains were identified by phenotypic laboratory tests in accordance with Koneman et al. (2010). These identification of enterobacteria were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (MALDI-TOF LT Microflex Bruker; MALDI Biotyper 2.0 program, Bruker) (Welham et al., 1998; Pribil and Fenselau, 2005; Suarez et al., 2014) and all isolates identified in the laboratory as *E. coli* were confirmed by phenotypic tests by the Oswaldo Cruz Foundation - FIOCRUZ (report number: 384/12).

Antimicrobials

To detect the AmpC β-lactamase producing β-lactams, the following were used: amoxicillin with clavulanic acid (AMC) (30 µg), cefoxitin (CFO) (30 µg), ceftazidime (CAZ) (30 µg), ceftriaxone (CRO) (30 µg), ertapenem (ERT) (10 µg), imipenem (IPM) (10 µg) (Sensidisc DME).

Susceptibility and confirmatory testing

The inoculum for these tests was obtained from overnight BHI broth (Merck) cultures and adjusted to achieve turbidity equivalent to a 0.5 McFarland standard and the standard strains used as control were *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 (CLSI, 2012).

Susceptibility was determined by agar diffusion techniques by using Muller-Hinton agar plates (HiMedia) and by following the current recommendations of the National Committee for Clinical Laboratory Standards (CLSI, 2008; 2012). Isolates that showed resistant to cefotaxime, ceftazidime and clavulanate associated with a β-lactam, were suspected of producing AmpC type beta-lactamases.

These isolates were submitted to the three-dimensional extract test (Shahid et al., 2004, 2010). The characteristic of AmpC expression was revealed by disk approximation for classification as inducible or not (Martínez-Rojas, 2009).

Modified Hodge test to confirm carbapenemase-producing isolates

An overnight culture of indicator organism *E. coli* ATCC 25922 was adjusted to a turbidity 0.5 of Mc Farland scale and these were used to swab inoculate the surface of the Muller-Hinton agar plates. After drying the surface, test organisms were heavily streaked from the center to the periphery of the plate using an inoculating loop and a 10 µg ertapenem disk was placed at the center, and incubated for 18 h. The test is interpreted as positive by the presence of distortion of the inhibition zone (CLSI, 2012).

Genotypic resistance evaluation

The DNA extraction was performed according to the method of Chapman et al. (2001) for the PCR technique, using the bla_{ampC} was amplified with primers 5’ CCC CGC TTA TAG AGC AAC AA and 5’ TCA ATG GTC GAC TTC ACA CC to produce a 634 pb product (Sobia et al., 2011).

A typical 20 µl PCR reaction mixture for every primer set consisted of 10X Buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl and

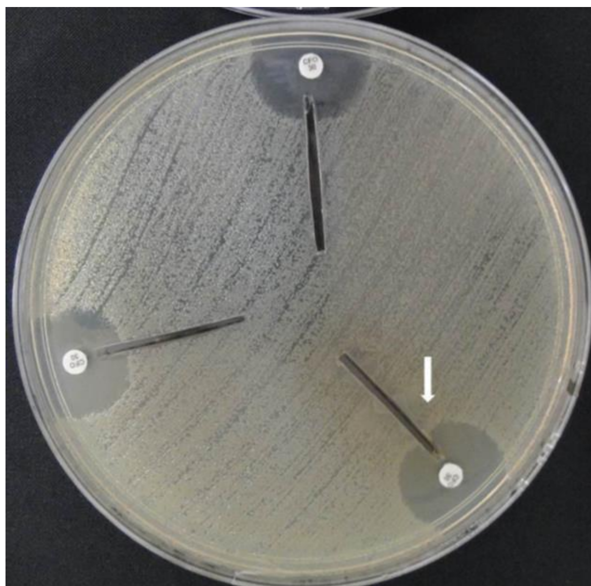

Figure 1. Three-dimensional extract test (TET) was used to confirm the production of AmpC β-lactamase. The arrow showing the distortion on the inhibition zone, confirm the AmpC production for this isolate. The other two isolates show no distortion in the inhibition zone being considered negative in this test.

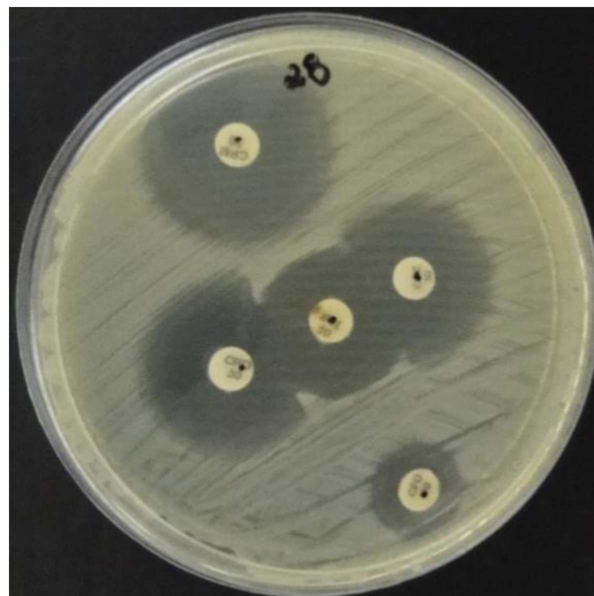

Figure 2. Induction of resistance by the presence of imipenem disc by "D Zone" formed. This isolate is shown to produce AmpC enzyme in the presence of inducer antimicrobial and it is classified as chromosomal gene carrier.

0.1% Triton X-100), 1.25 mM $MgCl_2$, 5 pmol of each primer (Invitrogen), 0.2 mM dNTP (Thermo Scientific), and 2 U Taq polymerase (Fermentas) and 2 µl of DNA extract. The cycling conditions were: initial denaturation at 95°C for 15 min; 35 cycles of 94°C for 60 s; 58°C for 2 min; 72°C for 3 min; and a final elongation at 72°C for 10 min.

The PCR products were analyzed by electrophoresis on gel with 1.5% agarose and revealed with diluted Sybr Green (Invitrogen). The gel was observed under UV gel documentation (L-PIX EX) using 100 pb DNA ladder (Fermentas) to confirm the product molecular weight.

RESULTS

Milk samples, isolation and identification of Enterobacteriaceae

A total of 381 milk samples were collected from 339 subclinical mastitic cows and 356 bacteria samples were isolated, with the presence of 88.2% Gram-positive (*Staphylococcus* sp.) and 11.8% Gram-negative bacteria (n=42/356). In 78% of cases (33/42) was isolated the enterobacteria in combination with other bacterial groups, like *Staphylococcus* spp., *Streptococcus* spp., *Listeria* spp. and *Corynebacterium* spp., indicating a mixed infection.

The most enterobacteria isolated were *Proteus mirabilis* (45.3%) and *E. coli* (40.5%), the other enterobacteria isolated were *Citrobacter freundii* (4.8%), *Serratia marcescens* (4.8%), *Serratia rubidae* (2.4%) and *Enterobacter aerogenes* (2.4%). The MALDI-TOF confirmed 92.86% of phenotypical identification. All *E. coli* were confirmed by FIOCRUZ.

Susceptibility and confirmatory test for AmpC-β-lactamase production

The antimicrobial susceptibility showed 35.7% resistance to CRO, 38.1% resistance to CAZ, 47.6% resistance to CFO and 83.3% resistance to AMC. The β-lactamase study showed that Group 1 β-lactamase (AmpC type) was predominant, being detected in 47.6% of the species, these isolates also submitted to TET (Figure 1) confirmed the production of the enzyme AmpC in 25%. Resistance to ceftazidime divided Group 1 into two subgroups: 1 (21.4%) and 1e (26.2%). These isolates were tested by disc approximation with imipenem and revealed 30% as the inducible type (*S. marcescens, S. rubidae, E. coli, P. mirabilis* and *E. aerogenes*) and 70% as not inducible type (Figure 2).

Modified Hodge test to confirm carbapenemase-producing isolates

Three isolates belonging to Group 1 were suspected to be carbapenemase producers due to their resistance to imipenem. These isolates were tested phenotypically by disk diffusion with ertapenem and by the modified Hodge test (THM) and none of the isolates were found to

express phenotypic carbapenemase production in both tests.

Genotypic resistance evaluation

The gene bla_{ampC} were detected in 23.8% of the isolates. Only 15% (3/20) of the isolates of the group 1 (1 and 1e) had the bla_{ampC} gene.

DISCUSSION

Enterobacteria were not the main cause of mastitis agents in these farms despite being present in samples. Thus, it was observed that low environmental hygiene contributed to these microorganisms which were present in the udders of these animals and affected by subclinical mastitis. In a study conducted by Santos (2006), enterobacteria were individually isolated in only 2.44% (2/82) and mixed infections with *Staphylococcus* spp. were more frequent (12/18), especially for the enterobacteria in association with coagulase-negative staphylococci.

The MALDI-TOF is widely used to identify microbial species and enzymes produced by these organisms (Pribil and Fenselau, 2005; Seng et al., 2010; Wang et al., 2013). For bacterial identification, some differences were observed when comparing MALDI identification with conventional identifications. Risch et al. (2010) observed 86.8% correct species identification by MALDI-TOF and Veen et al. (2010) observed the correct identification in 97.7% of Enterobacteriaceae.

E. coli was not the predominant species as expected. *E. coli* are considered one of the main agents of bovine mastitis and infection is characterized by environmental origin. Infections are related to opportunistic behavior of the agent, which infects animals mainly through contact of faeces with the teats. Poor sanitary conditions of farms available contribute to *E. coli* infections (Moreira et al., 2008).

The involvement of *P. mirabilis* in the etiology of bovine mastitis is underestimated and few reports in the literature point to its importance (Hogan and Smith, 2003). *Proteus* spp. commonly contaminates drop hoses used to wash udders before milking. These species were found in higher frequency in farms where drop hoses were used to wash the udder before milking.

These bacteria are present in the environment and are susceptible to a wide genetic exchange. Antimicrobials have often been detected in hospital effluents, municipal water supply treated wastewater, surface water and in some cases in groundwater (Meireles, 2008). According to Srinivasan et al. (2007), *E. coli* isolated from cows with mastitis were multiresistant and even carried several resistance genes.

The β-lactamase were classified based on resistance to cephalosporins, with cefoxitin the score for enzyme production type AmpC. The AmpC-type enzymes have activity on oxyimino-cephalosporin and on penicillins and monobactams. Some of these enzymes are weak hydrolyzing imipenem when expressed in large quantities and are weakly inhibited by clavulanic acid (Bush et al., 1995; Kao et al., 2010). This group belonging to molecular class C is mainly encoded for chromosomes in many enterobacteria and other microorganisms (Bush and Jacoby, 2010).

For phenotypical confirmation, the TET was not able to detect the most enzymes, but this may be linked to other resistance mechanisms (Shahid et al., 2004; Sobia et al., 2011). Villar et al. (1997) described bacterial isolates belonging to the Enterobacteriaceae that had absence or modification of porins. Recently, Hernández et al. (2010) studied the relationship between changes in porins expression and decreased sensitivity to β-lactams. These authors found isolated deficient in porins less sensitive to β-lactam regardless of β-lactamase production, and those isolated producers of β-lactamases were also resistant to carbapenems. Thus, this explanation is relevant as phenotypic resistance with low percentage of isolates confirmed production of the enzyme.

Many species including *C. freundii, E. cloacae* and *S. marcescens,* are natural producers of AmpC and its expression is low but inducible, unlike the *Proteus* spp., which normally have plasmid genes (Martínez-Rojas, 2009; Bush and Jacoby, 2010).

The imipenem resistance in three bacteria may be due to a variant of the AmpC enzyme which was recently described with hydrolytic activity against this antimicrobial (Bush and Jacoby, 2010; Kao et al., 2010).

Seven isolates not classified in Group 1 presented gene bla_{ampC} but probably did not express these traits phenotypically. The low detection of gene in Group 1 isolates can be explained by presence of other AmpC families genes or another mechanism resistance that express this resistance. The dissemination of genes conferring resistance to β-lactams can be broad in interior region of Rio de Janeiro, confirming the ease of genetic transmission, either by contamination of the environment or by human-animal contact.

The distinction between a β-lactamase AmpC chromosomal and plasmid by the gene is not always easy, but is of interest in terms of epidemiological surveillance, it has important consequences because of the easy spread of these plasmids. So in the future, other genes to detect AmpC enzymes will be studied, along with determination of gene origin by bacterial transformation.

Conflict of interests

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of

this article.

REFERENCES

Aguilar MAP (2009). Molecular characterization of carbapenem resistance in Enterobacteriaceae isolated in Brazilian hospitals. Dissertation. São Paulo University.

Ambler RP (1980). The structure of β-lactamases. Phil. Trans. R. Soc. B 289: 321-331.

ANVISA (2013). National Health Surveillance Agency. Technical note Nº 01/2013. Prevention measures and Infection Control for multiresistant Enterobacteriaceae. <http://portal.anvisa.gov.br/wps/wcm/connect/ea4d4c004f4ec3b9892 5d9d785749fbd/Microsoft+Word++NOTA+T%C3%89CNICA+ENTER OBACTERIAS+17+04+2013(1).pdf?MOD=AJPERES>

Brasil (2012). Bovine Masitis: control and prevention. *In:* Technical Report. UFLA, Lavras, MG.

Bush K, Jacoby GA (2010). Updated functional classification of β-lactamases. Antimicrob Agents Chemother 54 (3): 969-976.

Bush K, Jacoby GA, Medeiros AA (1995). A funcional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 39(6):1211-1233.

Chapman PA, Ellinn M, Ashton R, Shafique W (2001). Comparison of culture, PCR and immunoassays for detecting *Escherichia coli* O157 following enrichment culture and immunomagnetic separation performed on naturally contaminated raw meat products. Intl. J. Food Microbiol. 68:11-20.

CLSI (2008). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. Clinical and Laboratory Standards Institute**,** Wayne, PA.

CLSI (2012). Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute**,**Wayne, PA.

Dahmen S, Métayer V, Gay E, Madec JY, Haenni M (2013). Characterization of extended-spectrum β-lactamase (ESBL)-carrying plasmids and clones of Enterobacteriaceae causing cattle mastitis in France. Vet. Microbiol. 162:793-799.

Dalmarco EM, Blatt SL, Córdova CMM (2006). Laboratory Identification of extended spectrum β-lactamases – A review. RBAC 38: 171-177.

Deshpande LM, Davies TA, Blandino G, Nicoletti G, Jones RN, Castanheira M (2013). IMP-33, a New IMP Variant Detected in Pseudomonas aeruginosa from Sicily. Antimicrobial Agents and Chemotherapy 57: 6401-6403.

Egervärn M, Börjesson S, Byfors S, Finn M, Kaipe C, Englund S, Lindblad M (2014). Escherichia coli with extended-spectrum β-lactamases or transferable AmpC β-lactamases and Salmonella on meat imported into Sweden. Intl. J. Food Microbiol., 171: 8-14.

Freitas MFL, Pinheiro Jr JW, Stamford TLM, Rabelo SSA, Silva DR, Silveira FVM, Santos FGB, Sena MJ, Mota RA (2005). Antimicrobial susceptibility *in vitro* profile of *Staphylococcus* positive coagulase isolated from dairy cows with mastitis in the Arid Zone of Pernambuco. Arquivos do Instituto Biológico, 72(2):171-177.

Geser N, Stephan R, Hächler H (2012). Occurrence and characteristics of extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. BMC Vet. Res. 8:1-9.

Hernández JR, Conejo MC, Pascual A (2010). Comparative activity of ertapenem against *Klebsiella pneumoniae* producing extended-spectrum β-lactamases or plasmid AmpC β-lactamase: inoculum effect and role of loss porins. Enfermedades Infecciosas y Microbiología Clínic 28(1):27-30.

Hogan J, Smith KL (2003). Coliform mastitis. Vet. Res. 34(5): 507–519.

Jacoby GA (2009). AmpC β-lactamases. Clinical Microbiology Reviews 22(1):161–182.

Kao CC, Liu MF, Lin CF, Huang YC, Liu PY, Chang CW, Shi ZY (2010). Antimicrobial susceptibility and multiplex PCR screening of AmpC genes from isolates of *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens.* J. Microbiol. Imunol. Infect. 43 (3):180-187.

Koneman EW, Allen SD, Janda WM, Schreckenbergere PC, Winn WC (2010). Microbiologic Diagnostic. MEDS, Rio de Janeiro, RJ.

Langoni H, Laurino F, Faccioli PY, Silva AV, Menozzi BD (2009). Microbiologic Cultive and the Sensibility in Pathogenic Isolates from Bovine Mastites. Vet. Zootec. 16:708-715.

Li XZ, Mehrotra M, Ghimire S, Adewoye L (2007). Review: β-lactam resistance and β-lactamases in bacteria of animal origin. Vet. Microbiol. 121(3-4):197-214.

Livermore DM (1995). β-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. 8(4):557-584. PMid: 8665470.

Locatelli C, Caronte I, Scaccabarozzi L, Migliavacca R, Pagani L, Moroni P (2009). Extended-spectrum β-lactamase production in *E. coli* strains isolated from clinical bovine mastitis. Vet. Res. Commun. 33:S141-S144.

Martínez-Rojas DDD (2009). AmpC β-lactamases: generalities and phenotypic methods for detection. Rev. Soc. Ven. Microbiol. 29:78-83.

Meireles MAOM (2008). Antimicrobial use and bacterial resistance: socioeconomic and behavioral aspects and their clinical and ecological impact. Belo Horizonte, Brasil, 47p. (Monography. Biological Science Institute. Federal University of Minas Gerais).

Moreira MAS, Ferreira AB, Trindade TFSL, Reis ALO, Moraes CA (2008). Antimicrobial resistance dependent on the multidrug efflux in *Escherichia coli* isolated from mastitic milk. Arq. Bras. Med. Vet. Zoote.c 60(6):1307-1314.

Pribil P, Fenselau C (2005). Characterization of Enterobacteria Using MALDI-TOF Mass Spectrometry. Anal. Chem. 77: 6092-6095.

Risch M, Radjenovic D, Han JN, Wydler M, Nydegger U, Rich L (2010). Comparison of MALDI TOF with conventional identification of clinically relevant bactéria. Swiss Med Wkly 140:w13095.

Santos CDM (2006). Staphylococcus sp and Enterobacteriaceae Isolated from Mastitis Recurrent Eight Herds of Uberlandia Region - MG: antimicrobial susceptibility profile. Dissertation (Veterinary Science). Federal University of Uberlandia.

Shahid M (2010). *Citrobacter* spp. simultaneously harboring $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{ampC}, and insertion sequences IS26 and orf513: an evolutionary phenomenon of recent concern for antibiotic resistance. J. Clin. Microbiol. 48:1833-1838.

Shahid M, Malik A, Agrawal M,Singhal S (2004). Phenotypic detection of extended-spectrum and AmpC β-lactamases by a new spot-inoculation method and modified three-dimennsional extract test: comparison with the convenctional three-dimensional extract test. J Antimicrob. Chemother. 54(3):684-687.

Sampaio IBM (1998). Statistic aplies to animal experimetation. Statistics applied to animal experimentation. Foundation for Education and Research in Veterinary Medicine e Zootecnia, Belo Horizonte.

Seng P, Rolain JM, Fournier PE, La Scola B, Drancourt M, Raoult D (2010). MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol. 5 (11):1734-1754.

Silva KC, Lincopan N (2012). Epidemiology of extended spectrum β-lactamases in Brazil: clinical impact and implications for agribusiness. J. Bras. Patol. Med. Lab. 48 (2):91-99.

Sobia F, Mohammad S, Singh A, Khan HM, Shukla I, Malik A (2011). Occurrence of bla_{ampC} in cefoxitin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from a North Indian tertiary care hospital. New Zealand Inst Med Lab Sci 65 (1):5-9.

Srinivasan V, Gillespie BE., Lewis MJ, Nguyen LT, Headrick SI, Schukken YH and Oliver SP (2007). Phenotypic and genotypic antimicrobial resistance patterns of *Escherichia coli* isolated from dairy cows with mastitis. Vet. Microbiol. 124 (3-4):319–328.

Suarez S, Nassif X, Ferroni A (2014). Applications of MALDI-TOF technology in clinical microbiology. Pathol. Biol. (Paris).

Thomson KS (2010). Extended-Spectrum-β-lactamase, AmpC, and Carbapenemase Issues. J. Clin. Microbiol. 48(4):1019-1025.

Tozzetti DS, Bataier, MBN, Almeida LR (2008). Prevention, control and treatment of bovine mastitis – Literature review. Revista Científica Eletrônica de Medicina Veterinária anoVI(10).

Villar HE, Danel F, Livermore DM (1997). Permeability to carbapenems of *Proteus mirabilis* mutants selected for resistence to imipenem or other β-lactams. J. Antimicro. Chemother. 40:365-370.

van Veen SQ, Claas ECJ, Kuijper EJ (2010). High-Throughput

Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. J. Clinical Microbiology 48(3):900-907.

Wang L, Han C, Sui W, Wang M, Lu X (2013). MALDI-TOF MS applied to indirect carbapenemase detection: a validated procedure to clearly distinguish between carbapenemase-positive and carbapenemase-negative bacterial strains. Anal. Bioanal. Chem. 405(15): 5259-5266.

Welham KJ, Domin MA, Scannell DE, Cohen E, Ashton DS (1998). The Characterization of Micro-organisms by Matrix assited Laser Desorption/Ionization Time-of-flight Mass Spectrometry. Rapid Communications in Mass Spectrometry 12:176-180.

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Public health significance of food borne pathogens in edible flours

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Foodborne pathogens cause a considerable public health burden and challenge. They cause illnesses particularly in groups at risk, such as children, elderly and immuno- compromised persons. The microbiological quality of locally produced and industrially processed wheat, unripe plantain, yam and cassava flours were investigated for the presence of foodborne pathogens that could pose a risks to individuals that consume them. Proximate analysis of the flours showed significant statistical differences in the moisture content, crude fibre, lipid, ash, carbohydrate and protein composition of the various flour samples. Bacteria isolated included *Bacillus* sp., *Staphylococcus* sp., *Escherichia* sp, *Salmonella* sp, *Klebsiella* sp, *Enterobacter* sp, *Lactobacillus* sp, *Proteus* sp., *Psuedomonas* sp., *Clostridium* sp. and *Corynecbacterium* sp. The frequencies of occurrence of *Staphylococcus* sp., *Escherichia* sp., *Salmonella* sp. and *Klebsiella* sp. in locally produced flours were found to be relatively higher than those isolated from industrially processed and controlled flour samples. Fungi isolated included *Aspergillus flavus, A. niger, Rhizopus stolonifer, Saccharomyces* sp., *Penicillium* sp., *Fusarium* sp, *Mucor* sp, *Candida* sp. and *Geotrichum* sp. In locally produced flours, the frequencies of occurrence of *A. flavus*, *A. niger* and *Rhizopus stolonifer* were relatively higher than those isolated from industrially processed and control samples. Results from this study show that the frequency of occurrence of the potentially harmful organisms such as *Salmonella* sp. and *A. flavus* in locally produced flours exceeds the WHO recommended standards. This may be detrimental to the health of the individuals that consume them. Emphasizing the need for routine medical and laboratory examination of commercially available flour, there should be planning of health education programs for local producers, strict application and implementation of quality control and good manufacturing practice to prevent food borne diseases and ensure the safety of edible flour products.

Key words: Flour, food borne pathogens, microbial quality, proximate analysis, food borne illness.

INTRODUCTION

Flour meals of plantain, wheat, cassava and yam constitute a large part of the daily diet in both rural and urban population of Southern Nigeria. Individuals may develop diabetes and obesity due to high consumption of foods with a high Glycemic index (Oboh and Erema, 2010). Plantain flour is sometimes used by traditional and

some orthodox medical practitioners in Nigeria in the dietary management of diabetes mellitus and some disease conditions (Eleazu et al., 2011). Unripe plantain meal is usually consumed by Nigerian diabetic patients to reduce post-prandial glucose level (Oboh and Erema, 2010). Unripe plantain flour can be added to other types of flour to produce a highly nutritive product that is beneficial to human diets (Egbebi and Bademosi, 2011).

Wheat flour is a clean, soft and dry whole grain product derived from milling or grinding of fully cleaned moisturized wheat (*Triticum* species) grains; it provides more nourishment for humans than any other food source (Ndife et al., 2011). Whole grains such as wheat are rich in antioxidants which have known health benefits and prevent disease (Slavin, 2004). Semolina is also used to designate coarse middlings from other varieties of wheat, and from other grains such as rice and corn; its consumption is increasing daily among Nigerians because of its smooth fine texture and easy preparation. Whole grains have high concentrations of dietary fiber, resistant starch. Oligosaccharides and epidemiological studies have shown that whole-grain intake prevents cancer, cardiovascular diseases, diabetes, and obesity (Slavin, 2004).

Yam (*Discorea* sp.) is an important source of carbohydrate for many people of the Sub Saharan region, especially in West Africa (Ojokoh and Gabriel, 2010). Yams are processed into dried chips to overcome its high perishability (Hounhouigan et al., 2003). The traditionally processed parboiled dried yam is milled into flour and stirred in boiling water to make a thick paste known as “Amala”; it is eaten with soup by consumers (Akissoe et al., 2001). Yam flour has been fortified with plantain and cassava flour in order to improve its viscosity and texture (Abulude and Ojediran, 2006). Enriching yam flour with soybean can reduce the problem of malnutrition in places where yam is consumed as a staple food (Malomo et al., 2012).

Cassava (*Manihot esculenta* Crantz) is a perennial woody shrub producing edible roots that can be processed into various forms of important food items such as *fufu*, *garri* and *lafun* (Ogiehor et al., 2007; Padonou et al., 2009). *Lafun* is fermented cassava flour, popularly consumed in Southern Nigeria. Cassava shoots contain cyanogenic glycosides that break down to produce hydrogen cyanide, which can cause both acute and chronic toxicity in humans (Ekwu et al., 2005). By adequate processing, cyanogenic glycosides and hydrogen cyanide can be removed or reduced prior to consumption, thus significantly reducing the potential health risk. Continued efforts to improve its nutritional value are important because cassava is a staple food for many people in developing countries (Murtaugh et al., 2003).

The quality of the flour and storage condition after milling is very important in the shelf life and hygienic quality of the flour. Although flour is generally regarded as a safe product due to its low water activity, a variety of pathogenic and non-pathogenic microorganisms contaminate it during processing (Berghofer et al., 2003). Low-moisture foods and ingredients have not been discussed traditionally in terms of food safety, primarily because these products do not offer welcoming environments for microorganism growth (Akissoe et al., 2001). Yet, flour has also been associated with food borne outbreaks. While most flour-based products undergo a validated kill step at the point of production, such as baking or cooking, many other products may be at risk (Ndife et al., 2011). Improvements in the microbiological safety of foods have been largely driven by public demand in response to disease outbreaks; these improvements have been implemented by international standards and legislation, and are considered to have had an impact on diarrheal incidence as reflected in trends reported worldwide (Nawal et al., 2013).

Diarrheal diseases, almost all of which are caused by foodborne or waterborne microbial pathogens, are leading causes of illness and death in less developed countries, killing an estimated 1.9 million people annually at the global level (Nawal et al., 2013). The presence of aflatoxins in food products such as yam flour, plantain flour, corn flour and others destined for consumption in Nigeria has been reported in previous studies (Ekwu et al., 2005; Ogiehor et al., 2007). This study investigates the microbial quality and proximate chemical composition of unripe plantain, yam, wheat and cassava flours sold for consumption in Port Harcourt metropolis.

MATERIALS AND METHODS

Sample collection

A total of 52 flour samples were collected for analysis. Two (2) locally processed yam, unripe plantain, cassava and wheat flour samples were collected for analysis, from each of the following markets: Choba, Oil Mill, Mile I, Mile III and Oyigbo markets in Port Harcourt metropolis. Two samples of different industrially processed flours were purchased at the Everyday Supermarket, Rumuola and labeled appropriately. Dried unripe plantain, wheat, yam and cassava were ground into fine flour, observing HACCP protocols as described by ISO (2005); it was labeled as control. The samples were packaged in polythene bags and transported to the Microbiology Laboratory of the University of Port Harcourt for analysis.

Proximate analysis

Moisture and ash were determined by the air oven method, crude protein was determined by the micro-Kjeldahl method and the conversion factor from nitrogen to protein was 6.25 (AOAC, 2012).

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Table 1. Mean proximate composition of the industrial, local and control samples of different flours.

Flour	Source	Moisture (%)	Lipid (g/100 g)	Ash (g/100 g)	Protein (g/100 g)	Carbohydrate (g/100 g)	Crude fiber (g/100 g)
Wheat	Industrial	29.1[a]	1.0[a]	5.6[a]	7.7[a]	56.6[a]	1.8[a]
	Local	34.7[b]	1.2[b]	2.1[b]	5.9[b]	53.4[b]	1.7[a]
	Control	30.2[c]	1.2[b]	2.4[c]	6.2[c]	54.3[b]	1.8[a]
Unripe Plantain	Industrial	5.7[a]	2.5[b]	1.6[a]	3.5[a]	82.5[a]	1.5[a]
	Local	5.8[a]	2.6[b]	1.8[b]	3.2[b]	82.6[a]	1.6[b]
	Control	5.8[a]	2.6[b]	1.9[b]	3.6[a]	85.5[b]	1.7[b]
Yam	Industrial	9.5[a]	0.6[a]	5.2[a]	7.3[a]	83.3[a]	2.3[a]
	Local	10.3[a]	0.4[b]	3.2[b]	6.2[b]	87.8[b]	2.4[a]
	Control	8.5[b]	0.5[b]	4.2[b]	5.9[c]	87.9[b]	2.4[a]
Cassava	Industrial	15.1[a]	0.3[a]	1.4[a]	1.0[a]	75.5[a]	3.6[a]
	Local	13.8[b]	0.5[b]	1.0[b]	0.9[a]	77.0[b]	2.5[b]
	Control	13.5[b]	0.6[b]	1.2[c]	1.0[a]	77.2[b]	2.7[b]

Table 2. Mean heterotrophic bacteria count of the industrial, local and control samples recorded in the different flours.

Source	Plantain (10^5 Cfu/g)		Wheat (10^5 Cfu/g)		Yam (10^5 Cfu/g)		Cassava (10^5 Cfu/g)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Industrial	3.0 - 3.6	3.2	2.3 - 2.6	2.5	3.2 - 4.2	3.6	3.0 - 7.0	4.4
Local	3.1 - 4.9	3.8	3.0 - 4.0	3.4	3.8 - 4.3	4.0	2.5 - 8.6	4.2
Control	3.0	3.0	2.7	2.7	3.2	3.2	3.1	3.1

Crude lipids were determined by the soxhlet extraction method of Egan et al. (1981). Total carbohydrate content was determined by using the Anthrone method (Egan et al., 1981). The crude fiber content was calculated by difference.

Enumeration of microorganisms

Enumeration of microorganisms was carried out basically following the approach described by Amoa-Awua and Jakobsen (1995). Enumeration of total viable count was done using plate count agar (Oxoid, CM325, UK). Yeast and mould counts were done on Sabouraud dextrose agar (Oxoid). All cultures were incubated at 37°C for 24 h while yeasts and mould counts were incubated at 25°C for 72 h.

Identification of isolates

Pure isolates of the different distinctive bacterial colonies formed were stored on nutrient agar slants at 4°C for further confirmatory tests which included IMVIC test, carbohydrate utilization, and reaction on TSI, gelatin liquefaction, nitrate reduction, urease production and motility. Wet mount of the fungal isolates was prepared in lactophenol cotton blue, examined under low power binocular microscope and compared to the published morphological characteristics of fungi (Watanabe, 2010).

Statistical analysis

One way analysis of variance (ANOVA) was used to determine significant differences ($p < 0.05$) within the groups measured at 95% confidence level. The data were entered and analyzed using SPSS (Statistical Packages of Social Sciences) version 16.0.

RESULTS AND DISCUSSION

The moisture content, crude fiber, lipid, ash, carbohydrate and protein content of the industrial, local and control samples of the various flours are presented in Table 1. Significant statistical differences were recorded in the moisture content, crude fiber, lipid, ash, carbohydrate and protein content of the industrial, local and control samples of the various flours within the groups ($p < 0.05$).

Table 2 shows that the mean heterotrophic count is recorded in the flour samples. Locally produced unripe plantain flour has a mean heterotrophic count of 3.8 X 10^5 CFU/g. Locally produced wheat flour has a heterotrophic count of 3.4 x 10^5 CFU/g; well within the range for safe consumption. Compared to findings of similar studies by Oboh and Erema (2010); Ndife et al. (2011) indicated that wheat flour with heterotrophic count below 3.5 x 10^5 is

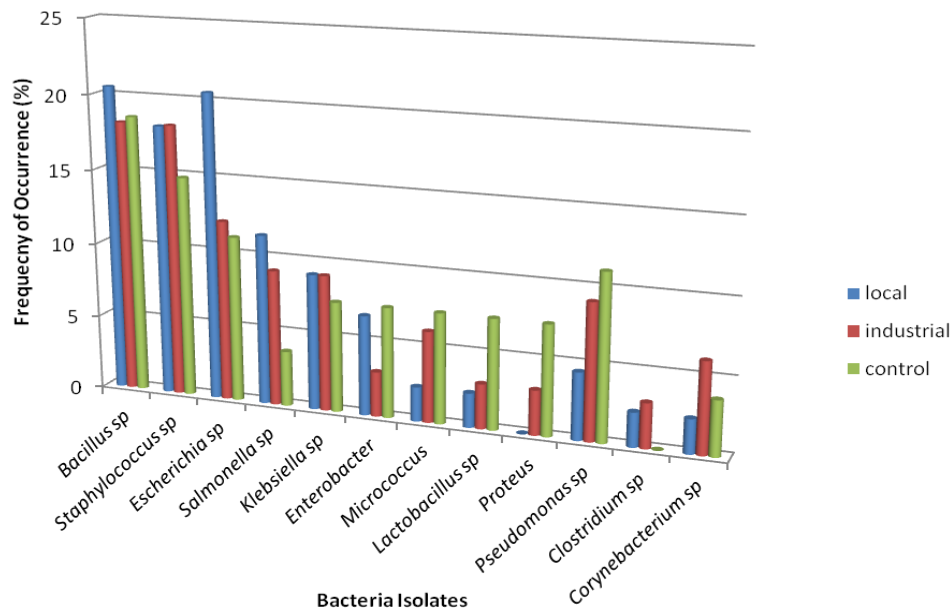

Figure 1. Comparison of bacteria occurrence isolated from locally produced, industrially processed and control flour samples.

Table 3. Mean fungi count of the industrial, local and control samples recorded in the different flours.

Source	Plantain (10^3 Cfu/g)		Wheat (10^3 Cfu/g)		Yam (10^3 Cfu/g)		Cassava (10^3 Cfu/g)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Industrial	2.8 - 3.2	3.0	2.3 - 2.6	2.5	2.8 - 3.2	2.7	2.9 - 3.2	3.0
Local	3.1 - 4.9	3.6	3.3 - 3.8	3.5	3.7 - 4.2	3.8	3.4 - 4.2	3.8
Control	3.0	3.0	2.7	2.7	3.2	3.2	3.1	3.1

ideal for human consumption and poses little risk of causing food borne illnesses. Heterotrophic counts of locally and industrially processed yam and cassava flours ranged from 3.6 x 10^5 – 4.4 x 10^5 CFU/g. This may be due to the relatively high moisture content of yam and cassava flour (Ojokoh and Gabriel, 2010; Eleazu et al., 2011).

Bacteria isolated included *Bacillus* sp., *Staphylococcus* sp., *Escherichia* sp., *Salmonella* sp., *Klebsiella* sp., *Enterobacter* sp., *Lactobacillus* sp., *Proteus* sp., *Psuedomonas* sp., *Clostridium* sp. and *Corynecbacterium* sp. as shown in Figure 1. These bacteria were also isolated from flour samples in similar studies (Ojokoh and Gabriel, 2010; Eleazu et al., 2011). The frequencies of occurrence of *Staphylococcus* sp., *Escherichia* sp., *Salmonella* sp. and *Klebsiella* sp. in locally produced flours were higher than those isolated from industrially processed and control flour samples, except for *Staphylococcus* and *Klebsiella* sp. which had the same frequency of occurrence in locally and industrially produced flours. The presence of these potentially pathogenic bacteria in relatively high frequencies in the locally produced flour may be attributed to the unhygienic processing environment of locally produced flours (Ogiehor et al., 2007; Nawal et al., 2013). The presence of *Staphylococcus, Escherichia* and *Salmonella* in high frequencies is associated with food spoilage, food borne illnesses and food poisoning leading to diarrhea, fever and other health complications in individuals (Adeleke, 2009; Nawal et al., 2013)

Table 3 shows the fungal count of the flour samples. Unripe plantain flour, wheat and cassava flours processed locally had fungal counts ranging from 3.5 – 3.8 x $10^{3.}$ This may be due to the high moisture content of these flours (Abulude and Ojediran, 2006; Ojokoh and Gabriel, 2010). The mean fungal counts of the industrially and locally processed yam flour were 3.6 x 10^3 and 3.8 x 10^3 respectively. This could be attributed to the high carbo-hydrate and moisture content of the flours (Padonou et al., 2009; Akpe et al., 2010).

Fungi isolated included *A. flavus, A. niger, R. stolonifer, Saccharomyces, Penicillium, Fusarium, Mucor, Candida* and *Geotrichum* sp. as shown in Figure 2. These fungi were also isolated from flour samples in similar studies

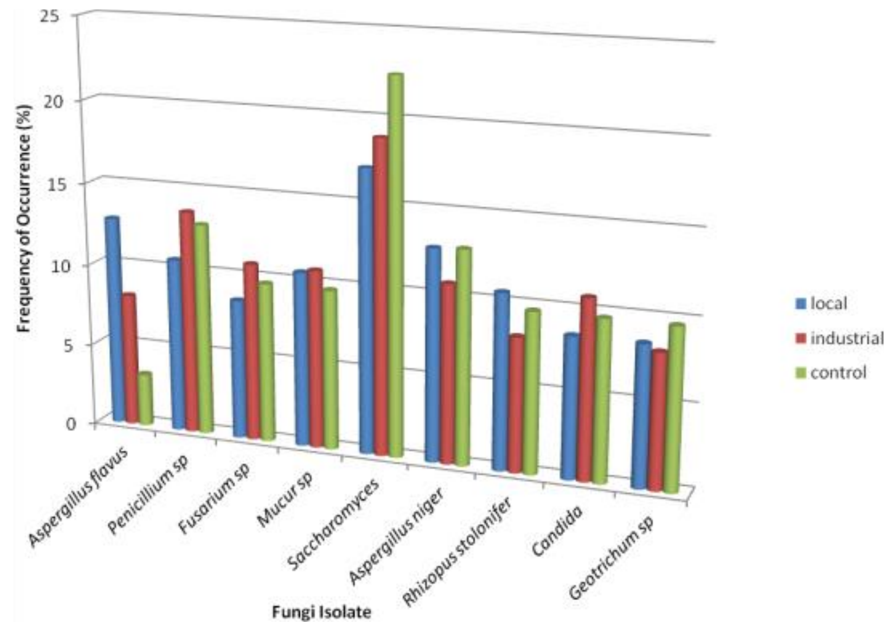

Figure 2. Comparison of fungi occurrence isolated from locally produced, industrially processed and control flour samples.

(Abulude and Ojediran, 2006; Padonou et al., 2009). The frequencies of occurrence of *A. flavus*, *A. niger* and *R. stolonifer* in locally produced flours were higher than those isolated from industrially processed and control samples. Similar studies suggested that high occurrence of *A. flavus* will most likely lead to the high occurrence of aflatoxins in the flour products which will cause complications of food poisoning and related illnesses in the individuals that consume these products (Adeleke, 2009; Gbolagade et al., 2011; Esho et al., 2013)

The relatively low occurrence of potentially harmful bacteria and fungi in industrially processed flours is attributed to the chemical treatments of the flours in a sterile environment during production (Oboh and Erema, 2010; Gbolagade et al., 2011; Esho et al., 2013). This treatment will ultimately reduce the occurrence of these potentially harmful organisms and increase the shelf life of the products.

Conclusion

The bacterial and fungal counts recorded in most of the flours sampled meet the FAO/WHO, 2012, required microbial limits of not more than 10^5 CFU/g. However, the locally made flours had a relatively higher CFU range compared to the industrially made flours. The bacterial and fungal counts recorded in locally made flours were relatively higher compared to the industrially made and control flour samples. This may be due to poor handling during processing and storage. Results from this study show that the occurrence of organisms such as *Aspergillus* sp., *Salmonella* sp. and *Escherichia* sp. was relatively higher in locally processed flour samples. The locally made flours are readily available and cheaper than industrially processed flours, making them the popular choices among the residents of Port Harcourt. It is therefore important that local producers be educated on the need to observe Hazard analysis and critical control point (HACCP) principles in their environment during production and storage to reduce the rate of occurrence of these potentially pathogenic organisms. Routine inspection of commercially available flours should be carried out by appropriate health institutions to ascertain the quality of these products and ensure their safety for consumption by all individuals.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Abulude FO, Ojediran VA (2006). Development and Quality Evaluation of Fortified ‘Amala’. Acta Sci. Pol. Technol. Aliment. 5(2): 127 - 134.

Adeleke SI (2009). Food Poisoning Due to Yam Flour Consumption in Kano (Northwest) Nigeria. Online J. Health Allied Sci. 8(2):10

Akissoe NH, Hounhouigan JD, Bricas N, Vernier P, Nago MC, Olorunda OA (2001). Physical, Chemical and sensory evaluation of dried yam (*Discorea rotundata*) tubers, Flour and Amala a Flour Derived Product. Trop. Sci. 41:151-156.

Akpe AR, Usuoge POA, Enabulele OI (2010). Bacteriological and Physico-Chemical Quality of Wheaten White Bread Flour Made for Nigerian Market. Pak. J. Nutr. 9(11):1078-1083.

Amoa-Awua WKA, Jakobsen M (1995). The role of Bacillus species in the fermentation of cassava. J. Appl. Bacteriol. 79:250-256.

AOAC (2012). Official Methods of Analysis of AOAC International, 19th

Ed. Association of Official Analytical Chemists International. 481 North Frederick Avenue.

Berghofer LK, Hocking AD, Miskelly D, Jansson E (2003) Microbiology of wheat and Flour Milling in Australia. Int. J. Food Microbiol. 85:137-49.

Egan H, Kirk RS, Sawyer R (1981). Pearson's Chemical Analysis of Foods. 8th Edition, Churchill Livingstone.

Egbebi AO, Bademosi TA (2011). Chemical Compositions of Ripe and Unripe Banana and Plantain. Int. J. Trop. Med. Public Health 1(1):1-5.

Ekwu FC, Ozo NO, Ikegwu OJ (2005). Quality of Fufu Flour from White Yam Varieties (*Dioscorea* spp). Nig. Food J. 23:107-113.

Eleazu CO, Okafor PN, Amajor J, Awa E, Ikpeama AI, Eleazu KC (2011). Chemical Composition, antioxidant activity, functional properties and inhibitory action of unripe plantain (M. *Paradisiacae*) flour. Afr. J. Biotechnol. 10(74):16948-16952.

Esho FK, Budbazar E, Akiko K, Keiko K (2013). Microbial Assessment and Prevalence of Foodborne Pathogens in Natural Cheeses in Japan. BioMed. Res. Int. 3:101-105.

FAO/WHO (2012). Food Standards Programme Codex Alimentarius Commission Forty-first Session Rome.

Gbolagade J, Ibironke A, Omitade Y (2011). Nutritional Compositions, Fungi and Aflatoxins Detection in Stored 'Gbodo' (fermented *Dioscorea rotundata*) and 'elubo ogede' (fermented *Musa parasidiaca*) from South western Nigeria. Afr. J. Food Sci. 5(2):105-110.

Hounhouigan DJ, Kayode AP, Bricas N, Nago CM (2003). Desirable Culinary and Sensory Characteristics of Yams in Urban Benin. Benin J. Agric. Sci. 21: 2815-2820.

ISO (2005). Food Safety Management System. International Organization for Standardization. ISBN 978-92-67-10454-6.

Malomo O, Ogunmoyela OAB, Adekoyeni OO, Jimoh O, Oluwajoba SO, Sobanwa MO (2012). Rheological and Functional Properties of Soy-Poundo Yam Flour. Int. J. Food Sci. Nutr. Eng. 2(6):101-107

Murtaugh MA, Jacobs DR, Jacob B, Steffen LM, Marquart L (2003). Epidemiological support for the protection of whole grains against diabetes. Proc. Nutr. Soc. 62:143-149.

Nawal A, Hassanain MA, Hassanain WM (2013). Public Health Importance of Foodborne Pathogens. World J. Med. Sci. 9(4):208-222.

Ndife J, Abdulraheem LO, Zakari UM (2011). Evaluation of the nutritional and sensory quality of functional breads produced from whole wheat and soya bean flour blends. Afr. J. Food Sci. 5(8):466-472.

Oboh HA, Erema VG (2010). Glycemic Indices of Processed Unripe Plantain (*Musa paradisiaca*) Meals. Afr. J. Food Sci. 4(8):514 – 521.

Ogiehor IS, Ikenebomeh MJ, Ekundayo AO (2007). The Bioload and Aflatoxin Content of Market Garri From Some Selected States in Southern Nigeria. Afr. Health Sci. 7(4):223-227.

Ojokoh AO, Gabriel RAO (2010). A Comparative Study on the Storage of Yam Chips (Gbodo) and Yam Flour (Elubo). Afr. J. Biotechnol. 9(21): 3175-3177.

Padonou SW, Hounhouigan JD, Nago MC (2009). Physical, Chemical and Microbiological Characteristics of Lafun Produced in Benin. Afr. J. Biotechnol. 8(14): 3320-3325.

Slavin J (2004). Whole Grains and Human Health. Nutr. Res. Rev. 17: 1-12.

Watanabe T (2010). Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and key Species 3rd ed. Boca Raton; CRC press.

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Full Length Research Paper

Observations of mycotal species growth on the rainbow trout eggs (*Oncorhynchus mykiss*)

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The authors investigated the growth of hydromycobiota on the eggs of rainbow trout (*Oncorhynchus mykiss*) in waters of different trophicity. Of the *O. mykiss* eggs that were investigated, 16.6% were found to be infected by mycotal species. Thirty mycotal species were found on eggs obtained from adult female representatives. *Achlya polyandra*, *A. radiosa*, *Aphanomyces laevis*, *Leptomitus lacteus*, *Saprolegnia ferax* and *Saprolegnia parasitica* belonged to the species that were most commonly encountered. *Aphanomyces frigidophilus*, *Candida albicans* and *Zoopage phanera* were rarely found in salmonid fishes. Amino acid, carbohydrate and urease tests were used, and all analyses of species from the *Achlya*, *Aphanomyces*, *Leptolegnia*, *Pythium* and *Saprolegnia* genera showed that they assimilate glucose and starch. However, they did not assimilate glycine, leucine, lysine, ornithine, and arabinose. Urease was assimilated only by species from the *Leptolegnia*, *Pythium* and *Saprolegnia* genera.

Key words: *Oncorhynchus mykiss*, rainbow trout, eggs, mycotal species, infection, hydrochemistry.

INTRODUCTION

Rainbow trout were classified as part of the *Salmo* genus until 1988 when the use of the generic name *Oncorhynchus* was adopted for all Pacific trout and salmon, to distinguish them as different (Smith and Stearley, 1989). Analyses of mitochondrial DNA showed that the mtDNA of rainbow trout had more similarity to Pacific salmon than to brown trout and Atlantic salmon (Berg and Farris, 1984; Thomas et al., 1986; Gyllensten and Wilson, 1987). This was confirmed through osteological analysis (Smith and Stearley, 1989). Today, all forms of rainbow trout belong to the *Oncorhynchus* genus, as the *O. mykiss* species (derived from the Kamchatkan name "mikizha" or "mykiz") (Ethier and Starnes, 1993).

Both marine and freshwater fish species deliver not only protein and fat, but also biologically active substances that are important for human organisms. Therefore, the consumption of fish is increasing from year to year (Food and Agricultural Organization of the United Nations (FAO), 2012). However, in recent years freshwater fishing has increased much more than fishing in seas and oceans. The growing number of freshwater fish farms may account for this. An important factor to consider in investigating what limits the fish populations in some cultures is the mycotal pathogens that can result in reduced breeding, sometimes affecting from 50% (Hatai and Hoshiai, 1992)

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to 75% (Lartzeva, 1986) of the incubated eggs. For example, Chien (1981) has described the mass death of the rainbow trout that occurred off the coast of Taiwan during their reproduction period and which were caused by the *Aphanomyces laevis*. As a result of this, we became interested in the extent to which mycotal species affect the eggs of the rainbow trout in waters of different trophicity.

MATERIALS AND METHODS

Occurrence of rainbow trout

Oncorhynchus mykiss (Walbaum, 1792) (syn. *Salmo gairdneri* Richardson, 1836; *Salmo irideus* Gibbons, 1855) (English name: rainbow trout).

O. mykiss is native to the Pacific Slope, extending from the Kuskokwin River, in Alaska, through Rio Santo Domingo, in Baja, California, to rivers in Mexico, to the upper Mackenzie River (Arctic Basin), in Alberta and British Columbia, Canada, and to the endorheic basins of southern Oregon, U.S.A. This species has also been widely introduced to coastal waters elsewhere in North America and in rest of the world, including South Africa (Page and Burr, 1991; ITIS, 2010).

In the Eastern Pacific, *O. mykiss* occurs on the Kamchatkan Peninsula and has been recorded on the Commander Islands (east of Kamchatka) and sporadically in the far south of the Okhotsk Sea where the mouth of the Amur River flows along the mainland (ITIS, 2010).

This species inhabits cold headwaters, creeks, small-to-large rivers and lakes (usually not stocked in water that reaches summer temperatures above 25°C) and ponds with very low oxygen concentrations. It feeds on a variety of aquatic invertebrates and on small fishes. *O. mykiss* is cultured in many countries (including some in the south of Africa) and is often hatched and stocked in rivers and lakes, especially to attract recreational fish species (Frimodt, 1995; ITIS, 2010). With the exception of the common carp, the rainbow trout is one of the oldest species that is being cultured (Gall and Crandell, 1992). According to Wales (1939), the first transfer of eggs took place from McCloud River (northern California) to a hatchery in Caledonia, New York, in 1874. Rainbow trout eggs were also shipped from North America to Japan in 1877 and to England in 1885. The European rainbow trout farming industry began in Denmark (Laird and Needham, 1988; Gall and Crandell, 1992).

Characteristics of water bodies

Three water bodies located in the north-eastern region of Poland (53°07'N, 23°10'E to 53°13'N, 23°20'E) were chosen for this study: Pond Fosa: an area of 2.5 hectares (ha); maximum depth of 1.75 m; breeding site of wild ducks; a culture of crucian carp intended for anglers; Pond Komosa: an area of 12.1 ha; maximum depth of 2.25 m; surrounded by coniferous trees of the dense Knyszynska Forest; River Suprasl: a length of 106.6 kilometers (km); a right-bank tributary of the middle part of the Narew River, flowing through the Knyszynska Forest.

Nineteen (19) parameters of those water samples were measured (Table 1) according to generally accepted methods (APHA, 2005).

Isolating and identifying mycotal species

Eggs were collected (after fertilization) at the end of April from the hatchery at the Gawrych Ruda Farm. 150 - 450 eggs for each of the water bodie were investigated.

Table 1. Chemical and physical properties of water in particular water bodies (in mg l^{-1}).

Specification	River	Pond	
	Supraśl	Fosa	Komosa
Temperature (°C)	17.0	18.0	17.4
pH	7.8	7.1	7.6
DO	11.2	6.4	12.8
BOD_5	2.8	12.8	7.4
Oxidability(COD)	6.60	20.2	13.20
CO_2	6.6	22.4	8.3
Alkalinity in $CaCO_3$ (mval l^{-1})	4.5	5.8	3.9
$N\text{-}NH_3$	0.142	0.864	0.161
$N\text{-}NO_2$	0.006	0.114	0.009
$N\text{-}NO_3$	0.014	0.552	0.034
$P\text{-}PO_4$	0.158	3.598	0.255
Sulphates (SO_4)	32.38	85.1	42.75
Chlorides (Cl)	17.12	79.3	23.51
Total hardness in Ca	73.42	24.2	68.40
Total hardness in Mg	11.58	20.6	28.81
Fe	0.48	1.06	1.54
Dry residue	197.0	429.0	375.0
Dissolved solids	179.0	370.0	312.0
Suspended solids	18.0	59.0	63.0

Water samples from specific water bodies 800 ml each were placed into 1000-ml vessels and 50 eggs were transferred to each vessel in accordance with the general principles of culture (Watanabe, 2000). The vessels were stored at temperature of 7± 0.5°C, with access to daylight that resembled natural conditions and following the recommended instructions (Seymour and Fuller, 1987). The pH of the water was analysed separately for every vessel (Peterson and Brindge, 1994). The water analysis and experiments were done in three parallel repetitions. Eggs were taken for each vessel, and the eggs that were covered with fungal mycelia were observed every 3 - 4 days under a light-microscope. The presence of any morphological structures, such as zoospores, antheridia and oogonia, belonging to aquatic fungi were recorded. Fungal species were identified using the keys of Johnson et al. (2005), Pystina (1998) and Petrini and Petrini (2013). The systematics of straminipiles species according to Dick (2001) were used in this experiment. The experiments were carried out for one month, and the results were then tested for significance using ANOVA and evaluated by the Scheffe test (Winer, 1997).

Determination of the amino acid, carbohydrate and urease assimilation tests

Amino acid, carbohydrate and urease tests were performed on the *Achlya*, *Aphanomyces*, *Leptolegnia*, *Pythium* and *Saprolegnia* genera, based on Yuasa and Hatai (1996). For the carbohydrate utilization test, Yeast Nitrogen Base agar (Difco) was the medium used for the cultures of the fungal isolates. GY agar (Difco) was used for the urease test. The basal medium used in the amino acid assimilation test was the same as that used for the carbohydrate assimilation test. Bromo thymol blue and phenol red that was added to the yeast nitrogen-based broth and the GY broth, respectively, were used as indicators. These methods are described in detail in

our previous paper (Czeczuga et al., 2011b).

RESULTS

Table 1 shows the hydrochemical parameters of the water that was used in the experiment. The water from Pond Fosa was the most eutrophic. It showed heightened oxidability and alkalinity and higher levels of CO_2, N (NO_3), phosphates and other parameters. The lowest indices of the parameters that have been mentioned, as well as the lowest amounts of chlorides and iron, were found in water from Pond Komosa and River Supraśl.

Thirty mycotal species, including twenty four (24) belonging to the *Saprolegniales*, two to the Zoopagales, two to the Pythiales and one each to the Leptomitales, and Saccharomycetales, were found to be growing on the eggs of the *O. mykiss* (Table 2). The highest number of species was growing in water samples from Pond Fosa (the most eutropic), while the lowest number occurred in water from River Supraśl and Pond Komosa (the lowest trophicity). It is worth making a special note that *Aphanomyces frigidophilus*, *Candida albicans* and *Zoopage phanera* have rarely been found in fish living in Polish waters. Table 3 shows the assimilation tests for species from the *Achlya*, *Aphanomyces*, *Leptolegnia*, *Pythium* and *Saprolegnia* genera. All analysed species from the *Achlya*, *Aphanomyces*, *Leptolegnia*, *Pythium* and *Saprolegnia* genera showed that they assimilated glucose and starch. However, they did not assimilate glycine, leucine, lysine, ornithine or arabinose. The urease was assimilated by species from the genera of *Leptolegnia*, *Pythium* and *Saprolegnia* only.

DISCUSSION

The present study has proven that the growth of aquatic mycotic species on the eggs of rainbow trout depends on the chemical characteristic of the water bodies from which the water samples are obtained for the experiment. Chemical analyses of the water samples that were collected enabled water differentiation with respect to the content of chemical compounds. Water from Pond Fosa contained more biogenic compounds, mainly phosphorus. This confirms once again our earlier assumptions (Czeczuga and Woronowicz, 1993) that the degree of infection of the fish eggs in hatcheries depends largely on the state of cleanliness and trophicity of the water that supplies the hatchery.

As shown in Table 2, the most commonly encountered species included *Saprolegnia parasitica*, *S. ferax*, *Achlya polyandra*, *Laptomitus lacteus* and *Aphanomyces laevis*. All these species belong to the group of opportunistic sapro- and necrotrophic pathogens (Bruno and Wood, 1999). *S. parasitica*, which has been described as a pathogen in the eggs of various fish species (Hatai et al., 1990) and in the fish fry in Pacific salmon breeding farms, causes the death of almost the entire population (Neitzel et al., 2004; van West, 2006). It is also responsible for considerable losses in fish populations in lakes. *S. ferax* kills the eggs of sterlet (Lartzeva, 1986) and cyprinids (Czeczuga and Muszynska, 1999). *A. polyandra* was observed quite frequently on the eggs of four lampreys that were examined (Czeczuga, 1997) and on Atlantic salmon (Czeczuga et al., 2011a), and *L. lacteus* infects many fish species in different water bodies. *Aphanomyces frigidophilus* was described as occurring on the eggs of the Japanese char *Salvelinus leucomaenis* (Kitancharoen and Hatai, 1997) and, for the first time in Europe, on the eggs of *Coregonus lavaretus* (Czeczuga et al., 2004). *Aphanomyces frigidophilus* also grows on some species from the *Salmo* genus (Czeczuga et al., 2011a), including sturgeonid fishes (Czeczuga et al., 2012b), Chinook salmon (Czeczuga et al., 2012a) and African catfish (Czeczuga et al., 2013), and on the alevins of the Nile tilapia (Czeczuga et al., 2014b), and on the eggs of *Stenodus* species (Czeczuga et al., 2014a).

The immune response of rainbow trout to *Aphanomyces invadans* has also been examined (Thompson et al., 1999), and according to Khan et al. (1998) and Oidtmann et al. (2008), the rainbow trout is moderately susceptible to *Aphanomyces invadans* through intramuscular infection.

Rainbow trout is one of the main species that is bred on a large scale in fish farms, not only in Europe (Backiel, 1964; Goryczko, 2000) but also on other continents (MacCrimmon, 1972), including Africa (FAO, 2012). Therefore, from the 1930s to the present, mycosis has caused huge losses in the populations of this species and has been studied intensely. Members of this species can be infected by *A. laevis*, *L. lacteus*, *Saprolegnia delica, S. ferax, S. monoica* and *S. parasitica* (Tiffney, 1939a, b; Scott and O'Bier, 1962; Scott, 1964; Chien, 1981; Noland-Tintigner, 1970; Hatai et al., 1990). In addition, *A. laevis*, *Aphanomyces* sp., *L. lacteus*, *S. delica*, *S. monoica*, *S. parasitica* and *S. asterophora* have been observed in rainbow trout eggs (Scott and O'Bier, 1962; Scott, 1964; Florinskaja, 1971; Czeczuga and Woronowicz, 1993). Czeczuga and Muszynska (1996) also revealed the presence of such straminipiles as *Achlya polyandra* and *Achlya radiosa*. Experimental infection with *Saprolegnia* spp. in the eggs of rainbow trout has been investigated by Kitancharoen and Hatai (1996), Kitancharoen et al. (1997), Fregeneda-Grandes et al. (2001), Hussein et al. (2001) and Hussein and Hatai (2002).

It is also worth noting that the fungi *Zoopage phanera* that was found on eggs that were examined from water in the Biala River have been described as predacious fungus-catching soil amoebae (Drechsler, 1935). The growth of *Z. phanera* in fish has been reported on peled eggs (Czeczuga and Woronowicz, 1993). *Candida albicans* yeast has also been seen very rarely as a fish parasite, although it was found on eggs from water samples taken from Pond Komosa. The growth of yeast-like fungi has now been found on coregonid and salmonid fry. Bauer et al. (1973) reported yeast infections on salmon

Table 2. Fungi and straminipiles from the reservoirs analysed (number of investigated eggs – 450, infected – 75, 16.6%).

Taxa	**Pond Fosa**	**Pond Komosa**	**River Suprasl**
Fungi			
Ascomycota			
Saccharomycetales			
1.*Candida albicans* (Robin) Berrk.		x	
Zygomycota			
Zoopagales			
2. *Zoopage phanera* Dreschsler			x
3. *Zoopagus insidians* Sommerst	x	x	x
Straminipila			
Peronosporomycota			
Leptomitales			
4. *Leptomitus lacteus* (Roth) Agardh			
Pythiales			
5. *Pythium artotrogus* de Bary	x		
6. *P. ultimum* Trow	x	x	
Saprolegniales			
7. *Achlya americana* Humphrey			x
8. *A. bisexualis* Coker et Couch		x	
9. *A. colorata* Pringsh	x		
10. *A. hypogyna* Coker et Pemb	x		
11. *A. klebsiana* Pieters		x	
12. *A. orion* Coker et Couch	x		
13. *A. polyandra* Hildebr			x
14. *A. radiosa* Maurizio			x
15. *A. treleaseana* (Humphr.) Kauf.		x	x
16. *Aphanomyces frigidophilus* Kitauch. et Hatai		x	
17. *Ap. laevis* de Bary	x	x	x
18. *Ap. stellatus* de Bary	x		x
19. *Dictyuchus anomalus* Nagai	x		
20. *D. monosporus* Leitgeb		x	
21. *D. sterilis* Coker	x		
22. *Isoachlya monilifera* (de Bary) Kauf.	x		
23. *Leptolegnia caudata* de Bary	x		x
24. *Protoachlya polyandra* (Lindst.) Apinis	x		
25. *Saprolegnia anisospora* de Bary	x		
26. *S. ferax* (Gruith) Thurnet	x	x	x
27. *S. hypogyna* (Pringsh.) de Bary		x	
28. *S. mixta* de Bary		x	
29. *S. parasitica* Coker	x	x	x
30. *Scoliolegnia asterophora* (de Bary) M. W. Dick	x		
Total species (the different letters indicate difference of statistical significance (≤ 0.05)	17a	13b	11b
Number of infected eggs	48a 10.6%	15b 3.3%	12b 2.7%

fry, while Nagornaya et al. (1996) observed the growth of several species of the genus *Candida* on the eggs of rainbow trout. *C. albicans* growth was observed on the eggs of *Coregonus albula* in a hatchery (Czeczuga and Woronowicz, 1993), and species of fungi belonging to *Candida*, *Rhodotorula* and *Torulopsis* have been isolated

Table 3. Amino acid, carbohydrate and urease assimilation by straminipiles isolated from eggs of *Oncorhynchus mykiss*.

Species of genus	Amino acid	Carbohydrate	Urease
Achlya	Asp, Glu, Arg, Ala	Fru, Glu, Man, Raf, Suc, Mal, Lac, Mel, Cel, Tre, Sta, Dex, Rha, Gly	-
Aphanomyces	Glu, Ala, Cys, Phe	Glu, Sta	-
Leptolegnia	Asp, Glu,	Fru, Glu, Man, Mal, Mel, Cel, Tre, Sta, Dex, Gly	+
Pythium	Ala, His, Orn, Phe, Cys	Fru, Glu, Man, Gal, Raf, Suc, Mal, Lac, Mel, Cel, Tre, Sta, Dex, Rha, Gly, Sal	+

in fish from the African continent (Refai et al., 2010).

Finally, the amino-acid, carbohydrate and urease assimilation by straminipiles on the eggs of rainbow trout in the water bodies that have been mentioned differ from the assimilation by straminipiles in the waters of Japan (Yuasa and Hatai, 1996; Kitancharoen and Hatai, 1998). Perhaps it is related to huge biological variety of straminipiles species.

Conclusion

Examination of the growth of fungi and straminipiles organisms on the eggs of rainbow trout (*O. mykiss* Walbaum), in three trophically different water bodies was performed. Thirty species of mycotal organisms, developing and growing on the eggs of rainbow trout (24 belonging to the Saprolegniales, 2 to the *Pythiales*, 2 to the *Zoopagales*, 1 to the *Leptomitales* and 1 to the *Saccharomycetales*), were found. The greatest number of mycotal organisms was found in water from the most eutrophic Pond Fosa (17 species) and the lowest was identified in water from the less eutrophic River Supraśl (11) and Pond Komosa (13). Also, the greatest number of infected was found in water from Pond Fosa (48 eggs – 10.6% out of 450 investigated) and the lowest in water from River Supraśl (12 – 2.7%) and Pond Komosa (15 – 3.3%). *Achlya* and *Saprolegnia* were the most prevalent genera. The most commonly encountered species were: *Z. insidians*, *A. laevis*, *S. ferax* and *S. parasitica*. *C. albicans*, *Z. phanera* and *A. frigidophilus* were rarely found.

Species of *Achlya*, *Aphanomyces*, *Leptolegnia*, *Pythium* and *Saprolegnia* genera did not assimilate methionine, lysine, ornithine, leucine and glycine. All species of *Achlya*, *Aphanomyces*, *Leptolegnia*, *Pythium* and *Saprolegnia* genera assimilated glucose and starch, but did not assimilate arabinose. Urease was only assimilated by species from the *Leptolegnia*, *Pythium* and *Saprolegnia* genera.

The investigations showed that different trophicity of respective water bodies increases the prevalence of mycotal infections of the eggs of the rainbow trout.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

APHA (American Public Health Association) (2005). Standard Methods for the Examination of Water and Wastewater. APHA, Washington, DC.

Backiel T (1964). Pstrągi [Trout]. PWRiL, Warszawa. (In Polish).

Bauer ON, Trilenko WL, Semenova NW (1973). Candidamycosis of the juvenile salmon. Ryb. Choz. 10:23-25.

Berg WT, Farris SD (1984). Restriction endonuclease analysis of salmonid mitochondrial DNA. Can. J. Fish. Aquat. Sci. 41:1041-1047.

Bruno DW, Wood BP (1999). *Saprolegnia* and other Oomycetes. In: Woo PTK, Bruno DW (Eds.) Fish Diseases and Disorders. Viral, Bacterial and Fungal Infections, vol. 3. CABI Publishing, Wallingford, UK. pp. 599-659.

Chien CY (1981). Observations on the growth and morphology of saprolegniaceaus fungi isolated from rainbow trout (*Salmo gairdneri*). Fish Pathol. 15: 241-247.

Czeczuga B (1997). Aquatic fungi growing on lamprey eggs (Petromyzontidae). Bull. Lampetra 3: 7-19.

Czeczuga B, Bartel R, Semeniuk A, Czeczuga-Semeniuk E, Muszyńska E, Godlewska A, Mazalska B, Grochowski A (2011a). Straminipilous organisms (Mycota) growing on the eggs of Atlantic salmon (*Salmo salar* L.) entering Polish rivers for spawning or reared in fresh water. Trends Comp. Biochem. Physiol. 15:73-81.

Czeczuga B, Czeczuga-Semeniuk E, Semeniuk A (2011b). Microfungi – like organisms developing on the eggs of pink salmon *Oncorhynchus gorbusha*. Curr. Trends Microbiol. 7: 21-29.

Czeczuga B, Czeczuga-Semeniuk E, Semeniuk A (2012a). Aquatic fungi developing on eggs of Chinook salmon *Oncorhynchus tshawytscha* and some their biochemical characteristics. Trends Comp. Biochem. Physiol. 16: 85-92.

Czeczuga B, Czeczuga-Semeniuk E, Semeniuk A, Semeniuk J (2013). Straminipiles (Oomycota) developing on the eggs of an African catfish *Clarias gariepinus* Burchell in water bodies of Poland. Afr. J. Microbiol. Res. 7(20):2378-2384.

Czeczuga B, Kiziewicz B, Godlewska A (2004). Zoosporic fungi growing on eggs *Coregonus lavaretus holsatus* Thienemann, 1916 from Lake Wdzydze in Kaszuby. Pol. J. Environ. Stud. 13: 355-359.

Czeczuga B, Muszyńska E (1996). Growth of zoosporic fungi on the eggs of North Pacific salmon of the genus *Oncorhynchus* in laboratory conditions. Acta Ichthyol. Piscat. 26: 25-37.

Czeczuga B, Muszyńska E (1999). Aquatic fungi growing on the eggs of fish representing 33 cyprinid taxa (Cyprinidae) in laboratory condition. Acta Ichthyol. Piscat. 29:53-72.

Czeczuga B, Semeniuk A, Czeczuga-Semeniuk E (2012b). Aquatic fungi developing on eggs of six sturgeon species from Far East. Curr. Trends Microbiol. 8:51-59.

Czeczuga B, Semeniuk A, Czeczuga-Semeniuk E (2014b). Straminipiles fungi growing on the olevins on the Nile tilapia in limnologically and trophically different water bodies. Afr. J. Agric. Res. 9(18):1346-1353.

Czeczuga B, Semeniuk A, Czeczuga-Semeniuk E (2014a). Effect of the different thropically water bodies on the straminipiles fungal infection of *Stenodus* species (Coregonidae) eggs. Afr. J. Microbiol. Res. 8(6): 503-510.

Czeczuga B, Woronowicz L (1993). Aquatic fungi developing on the eggs of certain freshwater fish species and their environments. Acta. Ichthyol. Piscat. 23: 39-57.

Dick MW (2001). Straminipilous Fungi: Systematics of the

Peronosporomycetes Including Amounts of the Marine Straminipilous Protists, the Plasmodiophorids and Similar Organisms. Kluwer, Dordrecht. NL.

Drechsler C (1935). Some conidial Phycomycetes destructive to terricolous amoebae. Mycologia 27:6-40.

Ethier DA, Starnes WC (1993). The fishes of Tennessee. The University of Tennessee Press, Knoxville, Tennessee, USA.

FAO (2012). The State of World Fisheries and Aquaculture. FAO Fisheries and Aquaculture Department Food and Agriculture Organization of the United Nations, Rome.

Florinskaja AA (1971). On finding *Saprolegnia* on eggs and fish during artificial cultivation in the region of Leningrad. Tr. UNTTPRCh 18: 222-226.

Fregeneda-Grandes JM, Fernandez-Diez M, Aller Gancedo JM (2001). Experimental pathogenicity in rainbow trout *Oncorhynchus mykiss* (Walbuum), of two distinct morphotypes of long – spined *Saprolegnia* isolates obtained from wild broun trout *Salmo trutta* L., and river water. J. Fish Dis. 24:351-359.

Frimodt C (1995). Multilingual illustrated guide to the world's commercial warmwater fish. Fishing News Books, Osney Mead, Oxford, England.

Gall GAE, Crandell PA (1992). The rainbow trout. Aquaculture 100:1-10.

Goryczko K (2000). Pstrąg tęczowy [The rainbow trout *Oncorhynchus mykiss* Walbaum 1792]. In: Brylińska M. (Eds.) Freshwater Fishes of Poland. Wyd. Nauk. PWN, Warszawa, pp. 428-431.[In Polish].

Gyllensten U, Wilson AC (1987). Mitochondrial DNA of salmonids. In: Ryman N & Utter F. (Eds.) Populations Genetics and Fishery Management. University of Washington Press, Seattle, WA. pp. 301-318.

Hatai K, Hoshiai G (1992). Mass mortality in cultured coho salmon (*Oncorhynchus kisutch*) due to *Saprolegnia parasitica* Coker. J. Wild Dis. 28: 532-536.

Hatai K, Willoughby LG, Beakes GW (1990). Some characteristics of *Saprolegnia* obtained from fish hatheries in Japan. Mycol. Res. 94:182-190.

Hussein MMA, Hatai K (2002). Pathogenicity of *Saprolegnia* species associated with outbreaks of salmonid saprolegniosis in Japan. Fish. Sci. 68:1067-1072.

Hussein MMA, Hatai K, Nomura T (2001). Saprolegniosis in salmonids and their eggs in Japan. J. Wildl. Dis. 37:204-207.

ITIS – Integrated Taxonomic Information System (2010). Report. Retrieved from http://www.fishegase.org./Summary/speciesSummary/php?ID=2692&genusname

Johnson TW Jr, Seymour LR, Patgett DE (2005). Systematics of Saprolegniaceae: New combination. Mycotaxon 92:11-32.

Khan MH, Marshall L, Thompson KD, Campbell PA, Lilley JH (1998). Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the oomycete fish pathogen, *Aphanomyces invadans*. Bull. Eur. Assoc. Fish Pathol. 18:192-197.

Kitancharoen N, Hatai K (1996). Experimental infection of *Saprolegnia* spp. in rainbow trout eggs. Fish Pathol. 31:49-50.

Kitancharoen N, Hatai K (1997). *Aphanomyces frigidophilus* sp. nov. from eggs of Japanese char, *Salvelinus leucomaensis*. Mycoscience 38:135-140.

Kitancharoen N, Hatai K (1998). Some biochemical characteristics of fungi isolated from salmonid eggs. Mycoscience 39:249-255.

Kitancharoen N, Hatai K, Yamamoto A (1997). Aquatic fungi developing on eggs of salmonids. J. Aquat. Anim. Health 9:314-316.

Laird LM, Needham T (1988). The farmed salmonids. In: Laird LM & Needham T (Eds.) Salmon and Trout Farming. Ellis Horwood, Chichester. pp. 15-31.

Lartzeva LV (1986). *Saprolegnia* on the spawn of sturgeons and salmon. Hydrobiol. J. 22:103-107.

MacCrimmon HR (1972). World distribution of rainbow trout (*Salmo gairdneri*): further observations. J. Fish. Res. Board. Can. 29:1788-1791.

Nagornaya SS, Ignatova EA, Isaeva NM, Dovydov ON, Podgorsky VS (1996). Yeast, contaminating salmon eggs. Mikrobiol. Jurn. 58:9-12.

Neitzel DA, Elston RA, Abernethy CS (2004). DOE Report. Prevention of Prespawning Mortality: Cause of Salmon Headburns and Cranial lesions. pp. 1-326.

Noland-Tintinner N (1970). Deux epidemies de Saprolegniose des poisons par *Saprolegnia ferax* (Smith) et par *Saprolegnia declina* (Humphrey). A Parasit. (Paris) 45: 761-770.

Oidtmann B, Steinbauer P, Geiger S, Hoffmann RW (2008). Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel. Dis. Aquat. Org. 82: 195-207.

Page LM, Burr BM (1991). A field guide to freshwater fishes of Notrh America, north of Mexico. Houghton Mifflin Company, Boston.

Peterson RRM, Bridge PD (1994). Biochemical Techniques for Filamentous Fungi. CAB International, UK.

Petrini LE, Petrini O (2013). Identifying Moulds. A Practical Guide. J. Cramer, Stuttgart.

Pystina KA (1998). Genus *Pythium* Pringsh. Nauka, Sankt Petersburg.

Refai MK, Mohamed LA, Kenawy ANM, Shimaa El-SMA (2010). The assessment of mycotic settlement of freshwater fishes in Egypt. J. Am. Sci. 6: 594-602.

Scott WW (1964). Fungi associated with fish diseases. Dev. Ind. Microbiol. 5: 109-123.

Scott WW, O'Bier AH (1962). Aquatic fungi associated with diseased fish and fish eggs. Prog. Fish Cult. 24: 3-15.

Seymour RL, Fuller MS (1987). Collection and isolation of water molds (Saprolegniaceae) from water and soil. In: Fuller MS, Jaworski A (Eds.) Zoosporic Fungi in Teaching and Research. Southeastern Publishing Athens. pp. 125-127.

Smith GR, Stearley RF (1989). The classification and scientific names of rainbow and cutthroat trout. Fisheries 14: 4-10.

Thomas WK, Withler RE, Reckenback AT (1986). Mitochondrial DNA analysis of Pacific salmonid evolution. Can. J. Zool. 64: 1058-1064.

Thompson KD, Lilley JH, Chen SC, Adams A, Richards RH (1999). The immune response of rainbow trout (*Oncorhynchus mykiss*) against *Aphanomyces invadans*. Fish Shellfish Immun. 9:195-210.

Tiffney WN (1939a). The identity of certain species of the Saprolegniaceae parasitic to fish. J. Elisha Mitchell Sci. Soc. 55:134-151.

Tiffney WN (1939b). The host range of *Saprolegnia parasitica*. Mycologia 31: 310-321.

van West P (2006). *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. Mycologist 20: 99-104.

Wales JH (1939). General report of investigations of the McCloud River drainage in 1938. Calif. Fish Game 25:272-309.

Watanabe T (2002). Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species. CRL Press, Boca Raton, Florida.

Winer BJ (1997). Statistical Principles in Experimental Design. McGraw Hill, New York.

Yuasa K, Hatai K (1996). Some biochemical characteristics of the genera *Saprolegnia*, *Achlya* and *Aphanomyces* isolated from fishes with fungal infection. Mycoscience 37:477-479.

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Full Length Research Paper

Effect of environmental factors on toxin production of *Drechslera bicolor,* a causal agent of leaf blight in bell pepper

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Drechslera bicolor **(Mitra.) Subram. and Jain, cause of leaf blight in bell pepper [*Capsicum annuum var. grossum*** **(L.) Sendt.], leads to necrotic lesions, early leaf senescence and yield losses. Detailed physiological analysis can contribute to an improved understanding of bell pepper disease interaction and cultivar improvement. *D. bicolor*** **produced maximum toxin in Richards' medium and toxin was found most active at 25±2°C, pH 6.0 and 100% relative humidity. Different hosts were screened for disease resistance and it was observed that the partial purified toxin showed some degree of host specificity. Fungal toxin was able to produce symptoms in all the tested hosts with the main infected host being bell pepper. Prominent symptoms of chlorosis and wilting were observed on chilli followed by tomato, brinjal and lady finger. The detached leaf dip method followed by carborundum abrasion method was found to be the best inoculation methods in the production of wilting and chlorosis in bell pepper. These results indicated that the maintenance of physiological function during leaf blight infection could result in improved bell pepper yields under diseased conditions.**

Key words: *Capsicum annuum var. grossum*, leaf blight, physiology, phytotoxin, bioassay.

INTRODUCTION

Sweet or bell pepper (*Capsicum annuum* var. *grossum*, L.), a member of the family Solanaceae is regarded as one of the most popular and nutritious vegetable crops. During cultivation, the crop is affected by a large number of diseases caused by fungi, bacteria, viruses and mycoplasmas, which reduce yield drastically. These pathogens also attack during transit and storage (Chadha, 2003). Phytotoxins are important metabolic products produced by fungi, which partially or fully express characteristic disease symptoms (Samaddar and Scheffer, 1971). The earliest effects of phytotoxins has been reported to cause lesions on cell membranes hampering the cell permeability thereby triggering increased leakage of electrolytes from the susceptible tissues in low concentration dosages (Thatcher, 1939). Partially purified toxins from *Verticillium dahliae,* a wilt

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pathogen, were used to detect the losses of cell permeability in the susceptible tissues of cotton hosts (Gour and Dube, 1985). Interestingly, Gour and Agarwal (1988) has successfully demonstrated that partially purified toxins from wilt pathogen of *Fusarium oxysporum* f. sp. *cumini* did not induce wilt symptoms in resistant varieties, whereas the susceptible cultivars showed the development of characteristic symptoms, establishing the fact that the toxins can be used in screening the varieties for disease resistance. Considering this basic phenomenon, fungal toxins have been widely exploited for disease screening. The present experiment was undertaken for isolation of toxins and their bioassay on seed and intact plants to demonstrate the toxic efficacies.

MATERIALS AND METHODS

Isolation, identification and pathogenicity

Isolation was done from diseased leaves collected from Hi-tech Horticultural Farm, MPUAT, Udaipur and monoconidial culture maintained on potato dextrose agar slants for further studies. Identification of the fungus was confirmed by ITCC, New Delhi (Identification No. 279/6513-07). Koch's postulates were proved on cv. Bombay red plants before the fungus was tested for its potential to secrete the toxic metabolite.

Fungus characterization and host range

The number of conidia in the resultant suspension was determined using a haemocytometer, and expressed as number of conidia mm^2 of medium 1-20 spores per microscopic field- poor (+), 21-40 spores- good (++) and 41 and above spores abundant (+++). Spore size (length and width) measurements were taken by measuring 50 spores of each isolate using stage and ocular micrometer and septa were also counted in 50 spores of each isolate. Conidial development and variation in conidial morphology of isolates were studied by "slide culture" method. The blight development on different host leaves and fruits were recorded 5 and 6 days post inoculations, respectively.

Suitable medium, temperature, pH and relative humidity for growth, sporulation and toxin production

Twelve different natural, synthetic and semi-synthetic sterilized liquid media were examined under similar conditions of pH, temperature and incubation period, to find out the most suitable medium for all further studies. The amount of sporulation was determined on the basis of number of spores per microscopic field at 100x magnification. The effect of six different temperatures viz., 15, 20, 25, 30, 35 and 40 ± 2°C on the growth, sporulation and production of toxin was examined. Observations of sporulation and weight of mycelial growth were also recorded. Evaluation of suitable pH for maximum growth and toxin production was done using flasks containing 25 ml Richard's medium adjusted to six different pH levels. The growth of fungus at different pH was measured by determining mycelial dry weight and sporulation was measured under low microscopic field. The different humidity levels were first prepared by adding equal volume of concentrated H_2SO_4 and distilled water and further dilutions were made as par Buxton and Mellanby (1934). Observations were recorded for mycelial weight and sporulation.

Toxin study

Extraction of toxins

250 ml Erlenmeyer flasks each containing 30 ml of Czapek-Dox medium were inoculated with 4 mm diameter fungal plugs of 7-day old cultures of *D. bicolor.* After 15 days of growth under stationary conditions at room temperature (25±2°C), a cell free clear culture filtrate was obtained by filtration of fungal growth through Whatman filter paper no 42 (Gour and Agrawal, 1988).

Purification of toxin(s)

The culture filtrate was centrifuged at 3000 rpm for 20 min. The clear supernatant solution was collected in a clean sterilized conical flask and pellet sedimented at the bottom of the centrifuge tube was discarded. The clear supernatant solution served as a sample of crude toxin preparation produced by *D. bicolor in vitro.* The crude toxins were partially purified for its active factors using ethyl alcohol (1:2), ammonium sulphate (1:2) and acetone (1:2) fractionation (Gour et al., 1992). Chilled solvents were added to each of the 50 ml of culture filtrates and kept at 4°C. The precipitate collected was dissolved in double-distilled water. Since the solution obtained from ammonium sulphate solvent was comparatively found more toxic, all further steps of purification of toxins were carried out with only ammonium sulphate fractionated solutions following the method described by Kumar et al. (2013).

Bioassay tests

Different methods viz., detached twig dip, pin prick, carborundum abrasion, toxin(s) spray and injection were used for bioassay of toxin. All the methods were run in four replications under similar conditions. The leaves were observed for symptom development after 24 h of treatment. A control of sterile distilled water was kept for each treatment (Kumar et al., 2013). The disease symptoms were rated on a disease rating scale: For chlorosis (0-5): 0 = no chlorosis; 1 = slight chlorosis occurs and covers >/= 1% leaf area; 2 = slight chlorosis with slight vein clearing; 3 = chlorosis increase and primary lesions formed; 4 = light brown patches formed; 5 = chlorosis increase and covers > 50% leaf area. For wilting (0-5): 0 = no wilting; 1 = slight wilting occurs; 2 = wilting increased but leaves contained some water; 3 = leaves curled by wilting; 4 = leaves dried and completely curled; 5 = leaves completely dried and dead.

RESULTS

Initial symptoms appeared as yellowing of leaves near the tip of young leaves. This was followed by a rapid increase in leaf diseased area. Large straw or brown blight patches were formed which covered the whole leaf area leading to leaf coalescence and drop. The apical portion of sweet pepper fruit was found rotten with rapid discolouration and ultimate progression of internal decay. Later on, the rotted fruits became completely deformed.

Fungus characterization

The fungal colony was bottle green to whitish grey black in colour, hyphae with olivaceous dark green septate (width 10.73 µm); conidiophores were single or in groups,

Table 1. Host range studies of *D. bicolor*.

Plant	Scientific name	Plant part	Reaction (blight/wilt)	Incubation period (Days)
Bell pepper	*Capsicum annum var.grossum* L.	Fruit	+	6
Chilli (Morphological types)				
(a) Thin	*Capsicum annum var. frutescens* L.	Fruit	+	6
(b) Thick	*Capsicum annum var. chinense* L.	Fruit	+	6
(e) Yellow thick	*Capsicum annum var. annum* L.	Fruit	+	6
Lady finger	*Abelmoschus esculentus* (L.) Moch.	Fruit	+	8
Brinjal	*Solanum melongena* L.	Fruit	+	8
Tomato	*Solanum lycopersicum* (L.) Mill.	Fruit	+	8
Datura	*Datura stramonium*	Fruit	+	9
Chilli	*Capsicum annum* L.	Leaf	+	5 – 7
Bell pepper	*Capsium annum var.grossum* L.	Leaf	+	5 – 7
Brinjal	*Solanum melongena* L.	Leaf	+	5 – 7
Tomato	*Solanum lycopersicum* (L.) Mill.	Leaf	+	5 – 7
Lady finger	*Abelmoschus esculentus* (L.) Moch.	Leaf	+	8-10
Groundnut	*Arachis hypogaca* L.	Leaf	+	8-10
Cluster bean	*Cyamopsis tetragonoloba* L.	Leaf	+	8-10
Sorghum	*Sorghum bicolor* (L). Monech	Leaf	+	10-12
Maize	*Zea mays* L.	Leaf	+	10-12

+ = Visible symptoms.

sometimes swollen at the base up to (400 x 5-10 µm) and conidia were light brown to olive brown, straight, cylindrical, rounded with 5-12 septa, (104.03 X 13.43 µm); central cells of mature conidia were dark brown or smoky brown. On 15 different plant species, host range was studied and the fungus produced visible symptoms on all hosts within 5-12 days post inoculation (Table 1).

Media, temperature, pH and relative humidity for growth, sporulation and toxin production

Maximum toxic metabolites were present in the cultural filtrate obtained from inoculated Richard's liquid medium, where the susceptible twig not only expressed maximum chlorosis (3.1) but also maximum wilting (5.0). Similarly, malt extract liquid medium was also found better for the production of the maximum chlorosis and wilting. However, it is evident from this study that the toxic metabolites were present in all the culture filtrates tested from different synthetic media (Table 2). Maximum fungal growth, chlorosis, wilting and toxin production were found at 25±2°C followed by 30±2°C. Nevertheless, the quantity of toxin production correlated to the growth of the pathogen, that is, least chlorosis and wilting was obtained at 40±2°C (Table 3). The production of toxin was also influenced by different pH levels. Maximum toxic metabolites were produced at pH 6.0 as indicated by twigs dip in different treatment solutions after 72 h of toxin treatment and least activities of toxin was noticed at pH 4.0 and 9.0 (Table 4). Optimum relative humidity for the growth and sporulation of *D. bicolor* was between 90 and 100%. Maximum growth of the fungus was found at 100% RH as well as maximum chlorosis and wilting at this level, indicating maximum production of toxin which was followed by 90% RH (Table 5).

Toxin study

Fungal pathogens are known to synthesize various kinds of secondary metabolites, which play a key role during pathogenesis. Toxins are important metabolites to express symptoms of diseases after the post infection stages.

Toxin bioassay

D. bicolor produced metabolites on Czapek-Dox medium which by partial purification with ammonium sulphate, induced characteristic disease symptoms. Toxin-treated plant cuttings indicated yellowing of older leaves within 12 h, there was general yellowing after 25 h and the leaves dried completely after 48 h of treatment. Since the ammonium sulphate extracted toxin solutions caused severe chlorosis and wilt in bell pepper plant cuttings, ammonium sulphate was the most suitable solvent for isolation of toxins from culture filtrates. However, fractionation with acetone and ethyl alcohol was partly successful (Table 6).

Table 2. Effect of different media on growth, sporulation and toxin production of *D. bicolor.*

Media	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
Sach's	84.75	+	0.00	0.50	0.75	0.00	0.25	0.50
Asthana & Hawkar's	107.50	+	0.25	0.50	0.75	0.25	0.50	1.00
Sabourd's	95.25	++	0.50	0.50	0.75	0.25	0.50	1.00
Czpek Dox	134.25	++	0.50	0.75	1.00	0.50	1.00	2.00
Richard's	361.25	++	1.00	2.00	3.10	1.75	3.00	5.00
Malt extract	288.00	+++	1.25	2.05	3.00	2.00	3.05	5.00
Yeast extract	144.00	++	0.50	0.50	0.75	0.50	1.00	2.00
Potato dextrose	207.50	+++	0.75	0.75	1.25	0.50	1.25	2.25
Bell pepper leaf	253.25	++	0.50	1.00	1.50	0.75	1.50	2.50
Basal medium (modified)	193.75	++	0.50	0.75	1.25	0.50	1.25	2.25
Elliot's' medium	144.50	+	0.50	0.50	1.00	0.50	1.00	2.00
Oat meal	146.00	++	0.50	0.75	1.00	0.50	1.00	2.00
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	2.51							
CD 0.05	7.19							

*Average symptom rating of five replications (0-5 rating scale); - = Nil, + = poor, ++ = good and +++ = abundant.

Table 3. Effect of different temperatures on growth, sporulation and toxin production of *D. bicolor.*

Temperature (± 2°C)	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
15	131.50	++	0.75	1.00	1.25	1.00	1.50	2.75
20	164.75	++	1.00	1.25	1.50	1.00	2.00	3.00
25	208.75	+++	1.00	1.50	2.00	2.00	3.75	5.00
30	187.50	+++	1.00	1.25	1.75	2.00	3.25	3.75
35	107.50	+	0.75	1.00	1.00	0.50	1.00	1.25
40	82.75	-	0.00	0.75	1.00	0.00	0.75	1.00
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	2.78							
CD 0.05	8.25							

*Average symptoms rating of four replications (0-5 rating scale); - = Nil, + = poor, ++ = good and +++ = abundant.

Table 4. Effect of different pH on growth, sporulation and toxin production of *D. bicolor.*

pH	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
4	85.75	++	0.50	1.25	1.50	1.00	1.25	1.75
5	169.75	++	0.75	1.50	1.75	1.50	3.00	3.25
6	183.75	+++	1.00	2.00	3.00	2.00	3.75	5.00
7	176.25	+++	1.00	1.75	2.00	1.75	3.50	4.50
8	99.50	++	0.50	1.00	1.25	1.25	1.75	2.00
9	80.25	+	0.00	0.00	0.50	0.75	1.00	1.50
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	1.821							
CD	5.410							

*Average symptoms rating of four replications (0-5 rating scale); - = nil, + = poor, ++ = good and +++ = abundant.

Table 5. Effect of different relative humidity on growth, sporulation and toxin production of *D. bicolor.*

Humidity (%)	Dry mycelial weight (mg)	Sporulation	Symptoms expressed by toxin (S)*					
			Chlorosis			Wilting		
			24 h	48 h	72 h	24 h	48 h	72 h
40	144.75	-	0.25	0.50	1.00	0.75	1.00	1.50
50	165.50	+	0.75	1.00	1.50	1.00	1.25	1.75
60	174.25	++	1.00	1.50	1.75	1.25	1.50	2.00
70	182.75	++	1.00	1.75	2.00	1.50	1.75	2.50
80	199.50	++	1.25	2.00	2.50	1.75	2.00	2.75
90	285.75	+++	1.50	2.25	3.00	2.00	2.25	3.00
100	317.50	+++	1.75	2.50	3.75	2.25	3.50	5.00
Control	-	-	0.00	0.00	0.00	0.00	0.00	0.00
SEm ±	2.127							
CD	6.255							

*Average symptoms rating of four replications (0-5 rating scale); - = nil, + = poor, ++ = good and +++ = abundant.

Table 6. Partially purification of toxin (s) from culture filtrates of *D. bicolor* with different solvents.

Solvents	Plant cuttings*	
	Wilting	Chlorosis
Ammonium sulphate	5.0	5.0
Ethyl alcohol	2.0	3.5
Acetone	2.2	3.0
Sterilized uninoculated Czapek-Dox medium	0.0	0.0
Distilled water	0.0	0.0

*Average symptom rating of five replications (0-5 rating scale).

Table 7. Effect of different bioassay methods on production of symptoms by partially purified toxin (s) of *D. bicolor.*

Methods of toxin treatments	Detached		Intact	
	Wilting	Chlorosis	Wilting	Chlorosis
Detached leaf dip method **	5.00	4.00	-	-
Pin prick method	2.50	3.80	2.50	3.00
Carborundum abrasion method	2.75	3.90	3.00	4.00
Injection method	2.00	3.80	2.75	3.50
Spray method	1.00	1.00	2.00	2.00

* Average symptom rating of five replications (0-5 rating scale); ** only *in vitro* treatment.

Bioassay tests

Detached leaf dip method and carborundum abrasion method were the best inoculation method in the production of typical disease symptoms (Table 7). In the detached leaf twig method leaf chlorosis was followed by wilting and drooping.

DISCUSSION

Fungus characterization

Similar descriptions was reported by Pandey and Shukla (1978) who studied the host range of *Helminthosporium* spp. causing leaf spot diseases of sorghum.

Media, temperatures, pH and relative humidity for growth, sporulation and toxin production

Various workers studied different media, temperatures, pH and relative humidity levels against different plant pathogenic fungi in the laboratory and came with interesting results. Shukla and Husain (1987) reported Fries' medium, pH 5.0 and 21 days of incubation for production of toxic metabolites of *Drechslera maydis* causing severe leaf blight of *Costus speciosus*. The toxin was stable in acidic conditions (pH 3.5-7.0) but unstable under alkaline conditions. Raut and Wangikar (1974) reported Czpek's medium, temperature 25°C, 90-100% RH and pH 6 as best for *Drechslera tetramera,* leaf spot of barley. Kumar and Mishra (1993) found PDA with pH 6.8- 7.0 as best for *Drechslera oryzae* and Nagaraja et al. (1992) reported Richard's medium, 25°C temperature, pH 5.8 as best for *Drechslera sorokiniana* leaf spot of *Dioscorea* sp.

Toxin bioassay

Janardhanan et al. (1981) isolated toxin from the culture filtrate of *D. maydis* and induced necrosis and chlorosis on *Costus speciosus* leaves followed by severe yellowing and defoliation. It also produced symptoms on mono and dicot plants. It showed strong growth inhibiting activity, causing total inhibition of root elongation in germinating wheat seeds. Gour and Dube (1985) isolated partially purified toxins from *Verticillium dahliae,* a wilt pathogen, and detected the loss of cell permeability in the susceptible tissue of cotton hosts. Similarly, Gour and Agarwal (1988) isolated partially purified toxins from *Fusarium oxysporum* f. sp. *cumini* and demonstrated use of toxins in screening the varieties for diseases resistance. Kramer et al. (1989) in their study found that partially purified toxin preparation from culture filtrate of the pathogen *D. teres* caused concentrated specific necrotic lesions on leaf tips and margins of susceptible genotypes of barley.

Bioassay tests

Kumar et al. (2013) found that the plants which were spray inoculated with different dilutions of *A. alternata* partially purified toxin developed chlorosis initially followed by scattered black dotted spots on leaves sprayed with solution of 2:8 dilutions under pot conditions. Fungal pathogens are known to synthesize various kinds of secondary metabolites which play a key role in pathogenesis. Several workers have documented the properties and specificity of fungal toxins in host plants. Gilchrist and Grogean (1976) and Kramer et al. (1989) observed similar results with the toxins produced by *A. alternata* and *Drechslera teres*, respectively. Effects of phytotoxic metabolites of *A. solani* resulted in marginal and inter veinal leaf necrosis and subsequent wilting of tomato seedlings (Maiero et al., 1991).

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Buxton PA, Mallanby K (1934). The measurement and control of humidity. Bull. Ent. Res. 25:171-175.

Chadha KL (2003). Hand Book of Horticulture, Directorate of Information and Publications Agriculture, Indian Council of Agricultural Research, New Delhi, India. pp. 368-371.

Gilchrist DG, Grogan RG (1976). Production and nature of a host specific toxin from *Alternaria alternata* f.sp. *lycopersici.* Physiol. Biochem. 66:165–171.

Gour HN, Dube HC (1985). Effects of ouabain and phytotoxic metabolites from *Verticillium dahliae* on cell membranes of cotton plants. Physiol. Plant Pathol. 27:109-118.

Gour HN, Agarwal S (1988). A wilt toxin from *Fusarium oxysporum* f. sp. *cumini* Patel and Prasad. Curr. Sci. 57:849-851.

Gour HN, Nitharwal PD, Sanjeev A (1992). Partial purification of toxin from *Curvularia lunata* (Wakker) Boedijin. Biochem. Physiol. *Pflanzen.* 188:128-135.

Janardhanan KW, Gupta ML, Hussain A (1981). Isolation of a phytotoxic metabolite produced by *Drechslera maydis* (Nis) Subra causing leaf blight of *Costus speciosus* (Koen.) Sm. Phytopathol Mediterr. 20:13-16.

Kramer RM, Opel M, Siebert A (1989). Response of detached barley leaves to partially purified toxin preparation of *Drechslera teres* (Sacc.) shoem. Archiv. Phytopathol. Pflanz. 25: 277-283.

Kumar A, Pankaj S, Gour HN (2013). Patho-biochemical investigations of blight and wilt diseases of tomato (*Lycopersicon esculentum* Mill). Proc. Natl. Acad. Sci. India Sect. B Biol. Sci. 83:479-483.

Kumar RN, Mishra RR (1993). Effect of various factors on spore germination and growth of brown spot pathogen *Drechslera oryzae.* Indian Phytopathol. 46:405-406.

Maiero M, Bean GA, Nag TJ (1991). Toxin production by Alternaria solani and its related phytotoxicity to tomato breeding lines. Phytopathology 81:1030-1033.

Nagaraja SV, Kulkarni S, Hegde RK (1992). Physiological studies *Drechslera sorokiniana* (Sacc.) Subram. and Jain, a causal agent of leaf spot of *Dioscorea* sp. Mysore J. Agric. Sci. 26:261-264.

Pandey SC, Shukla TN (1978). Host range studies of *Helminthosporium* spp. causing leaf spot diseases of sorghum. Indian J. Mycol. Plant Pathol. 8:207-208.

Raut DB, Wangikar PD (1974). Physiological studies on *H.* [*Drechslera*] Mc Kinney causing leaf spot of hybrid jowar CSH-1. PKV Res. J. 3: 60-67.

Samaddar KR, Scheffer RP (1971). Early effects of Helminthosporium victoriae toxin on plasma membranes and counteraction by chemical treatments. Physiol Plant Pathol. 1: 319-328.

Shukla RS, Husain A (1987). Influence of various factors on toxicity of culture filtrate of a *Drechslera maydis* strain from *Costus Speciosus*. J. Phytopathol. 118: 187-192.

Thatcher FS (1939). Osmotic and permeability relations in the nutrition of the fungus parasites. Am. J. Bot. 26:449-458.

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Full Length Research Paper

Antifungal effect of *Coriolopsis polyzona* (Pers) on fungi isolated from remnant foods and wastewater from restaurants in Akure metropolis, Nigeria

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The present study assessed the antifungal properties of extracts of *Coriolopsis polyzona* against fungi isolated from remnant foods and wastewater that are discharged into the environment without treatment. Species of fungi isolated from remnant foods and wastewater in Akure metropolis are *Aspergillus fumigatus*, *Aspergillus niger*, *Mucor mucedo*, *Penicillium chrysogenum*, *Apsergillus flavus*, *Fusarium oxysporum*, *Penicillium italicium*, *Triscelophorus monosporus* and *Rhizopous stolonifer*. Phytochemical constituents in the extracts of *C. polyzona* ranged from 0.75 to 14.20 mg/g for saponins, flavonoids, alkaloids, tannins and steroids. The percentage inhibition of fungal mycelia by *C. polyzona* extracts against isolated fungi at concentration of 50 to150 mg/ml ranged from 2.7 to 37.7%. Purified extracts exhibited better mycelia inhibition of 42.2% on *Penicillium chrysogenum* and 34.1% on *Aspergillus fumigatus* that were resistance to fluconazole and ketoconazole respectively. The presence of phytochemicals and functional groups such as hydroxyl (OH), alkyl (C-H) carbonyl (C=O) and aromatic ring (C=C) in the extracts of *C. polyzona* may be responsible for the antifungal effect of the extracts. Compounds associated with these functional groups if isolated and purified may help in combating the present phenomenon of resistance of fungi to commercial antifungal agents.

Key words: Restaurant wastes, *Coriolopsis polyzona*, mycelia inhibition, functional groups, environment.

INTRODUCTION

Mushrooms are medicinal foods used extensively in traditional medicine for combating diet-related diseases, treating chronic diseases, delay ageing and increase life expectancy (Khatun et al., 2012). Mushrooms' medicinal properties are owing to the possession of wide range of secondary metabolites that are of high therapeutic values. Some of these medicinal properties are antibacterial, antifungal, antiviral, anticancer, anti-inflammatory and antioxidants (Wasser, 2002; Ameri et al., 2011; Keles et al., 2011). Moreover, the combination of vitamins, essential mineral salts, protein, polysaccharides, fibres and low lipid content in mushrooms are dietary supplements to strengthen the immune system (Wani et al., 2010). Species of *Termitomyces*, *Ganoderma, Pleurotus, Cordyceps sinensis, Inonotus obliquus, Polyporus umbellatus, Shizophyllum commune* and others

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are medicinal macrofungi with nutraceutical properties (Barros et al., 2008; Jiang and Sliva, 2010; and Hesham et al., 2013).

The nutraceutical activities of medicinal macrofungi had been traced to the presence of phytochemicals. The biological and metabolic diversity of mushrooms are due to their ability to utilize several substrates; decomposing dead organic matters and thus explore different habitat for colonization (Thatoi and Singdevsachan, 2014). This bioconversion of organic waste materials has not only brought about biodegradation but plays a major role in nutrient cycles that are of great benefits to man and nature. *Coriolopsis polyzona* is a white rot fungus commonly found on decaying woods and known to mineralize lignin and phenolic compounds by their multienzyme systems (Alaoui et al., 2008). The bio-assimilation of higher molecular compounds by macrofungi has led to the secretion of biological active compounds that are of great medicinal importance. However, the continuous searching for novel bioactive compounds in medicinal mushrooms will yield greater success in pharmaceuticals and food science. This will definitely provide perpetual solution to the significant increase in antibiotics resistance by pathogenic organisms and continuous failure witnessed in some chemotherapy agents.

Restaurants in Akure metropolis dispose their wastewater and remnant foods to the environment without treatment. These wastes are laden with pathogenic organisms that are resistant to antibiotics (Ogidi and Oyetayo, 2013). The indiscriminate dumping of refuse containing pathogenic organisms into the environment had contributed to the transfer of resistance genes in the ecosystem, contamination of watercourses and causes of eutrophication (Akpor and Muchie, 2011).

Fungi spores are widely dispersed into air and these spores are known to contribute to allergic diseases (Hageskal et al., 2009). Brakhage et al. (2010) reported that fungi produce volatile organic compounds that contribute to ill health. Therefore the emerging and transfer of resistance genes by pathogenic fungi containing spores in food chain cannot be overemphasized. This situation has contributed to the ineffectiveness of antibiotics. The search for effective antifungal agents had increased recently. Attention had been turned to mushrooms because they are known to contain bioactive compounds that are effective against microbial entities (Lindequist et al., 2005). The present study is therefore aimed at assessing the antifungal property of extracts of *Coriolopsis polyzona*, a wild macrofungus, against fungi isolated from wastewater and remnant foods.

MATERIALS AND METHODS

Collection of macrofungus

Samples of macrofungus were collected from farmland and nearby forest at the Federal University of Technology Akure, Nigeria (Lat 07° 14ᴵN Long 05° 11ᴵE). Sample of fruiting body was morphologically identified and confirmed by molecular methods.

Preparation of extracts from *C. polyzona*

Collected samples of *C. polyzona* were dried and ground to powder using mill machine (Retsch GmbH 5657 HAAN). The fine powder of *C. polyzona,* 100 g each, was soaked in acetone and methanol for 48 h. The filtrates obtained were dried using rotary evaporator (RE-52A, UNION Laboratories, England) and labelled as ACE and MCE for acetone extract and methanol extract respectively.

Isolation and Identification of fungi

Samples of wastewater and remnant foods were examined microbiologically using standard methods. The microscopic identification was done according to Chander (2002) and Barnett et al. (1983). The identified fungi were maintained on Potato dextrose agar (PDA Lab M) slants at 4°Cin refrigerator for subsequent used.

Quantitative determination of phytochemical contents in extracts of *C. polyzona*

The flavonoid content was determined by aluminum chloride colorimetric method described by Singleton et al. (1999). The methods described by Harborne (1998) were adopted to quantify alkaloid content of the extracts while tannins, saponins, tepernoids and steroid amount was quantified using the method described by AOAC (2003).

Antifungal Activities of the crude extracts and purified fractions

The antifungal activities of extracts were determined by Poison food technique (Parajuli et al., 2005). Volume of 1.0 ml each concentration (50, 100 150 mg/ml) of acetone extract (ACE) and methanol extract (MCE) were aseptically poured into Petri dish followed by the addition of equal amount of PDA. The Petri dish was agitated while adding PDA so as to get even mixture of the contents. PDA plates with dimethyl sulfoxide (DMSO) serves as control. Seven days old culture of the test fungi were used to prepare inoculums discs (6mm diameter) using cork borer. Fungal inoculum was aseptically placed upside down in the centre of each plate containing MCE and ACE. The experiment was performed in three replicates. The average diameter of fungal colonies was measured on the 7th day after inoculation and percentage of mycelia growth inhibition was calculated. The Percentage growth inhibitions in different concentrations were calculated as:

Percentage growth inhibition= $\frac{g_c - g_t}{g_c}\ x\ 100$

Where, g_c = Growth of mycelia colony after incubation period in control set subtracting the diameter of inoculums disc; g_t = Growth of mycelia colony after incubation period in treatment set subtracting the diameter of inoculums disc.

The antifungal effect of partially purified fractions was also carried out according to the method above.

Partial purification of extracts

The separation of extracts was done according to the method of Owolabi and Olarinoye (2008). The column chromatography was

Table 1 .Fungal isolates from sampled remnant foods and wastewater from restaurants in Akure metropolis.

Fungi Isolates	RMF	WWS
Aspergillus fumigatus	+	+
Aspergillus niger	+	+
Mucor mucedo	+	–
Penicillium chrysogenum	+	+
Apsergillus flavus	+	–
Fusarium oxysporum	+	+
Penicillium italicium	+	+
Triscelophorus monosporus	–	+
Rhizopous stolonifer	+	–

RMF= Remnant foods; WWS= wastewater; + = present; – = absent.

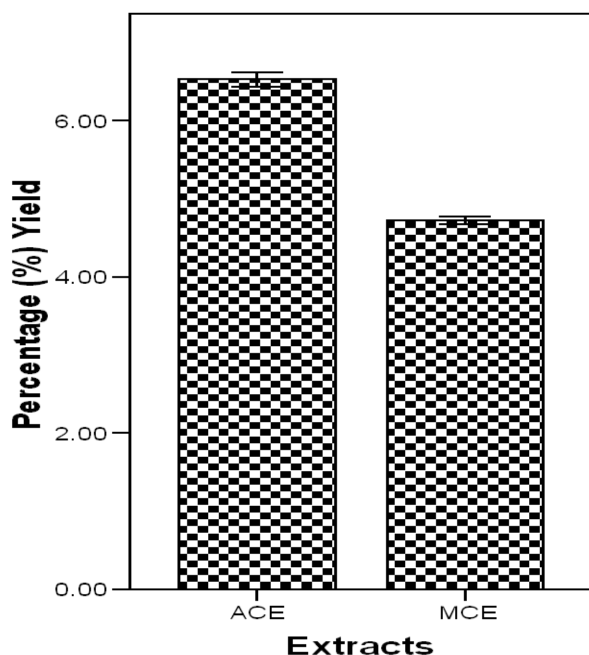

Figure 1. Percentage yield of acetone and methanol extracts of *Coriolopsis polyzona.*

Table 2. Quantitative constituents of phytochemicals in extracts (mg/g) of *Coriolopsis polyzona.*

Phytochemicals	ACE	MCE
Saponins	2.10±0.00	1.50±0.11
Flavonoids	6.80±0.04	4.55±0.02
Alkaloids	14.20±0.10	11.60±0.04
Tannins	5.20±0.04	7.70±0.20
Steroid	0.75±0.01	–

Values are mean±SD of replicates. ACE= acetone extract; MCE= methanol extract.

filled with petroleum ether (500ml), 60 grams of silica gel (60-120mesh) was tamped into column chromatography. The sample of extracts was mixed with silica gel and ground together to homogenize. This was transferred with spatula into the packed column chromatography.The column was opened and eluants were collected in 100ml aliquot portion. The polarity of mobile phase was varied by introducing chloroform and methanol. Thin layer chromatography (TLC) plate was saturated in chromatographic tank containing chloroform: methanol (10: 5) solvent systems.

Infra-red spectroscopic analysis

The presence of functional groups in *Coriolopsis polyzona* extracts was assessed using FT Infra-Red spectrophotometer (Spectrum BX).The FT IR wave number (cm^{-1}) obtained was interpreted according to Willams (1982).

Statistical analysis

The experiment was carried out in triplicates. Data obtained were analyzed using one way analysis of variance and means were compared by Duncan's Multiple Range Test using (SPSS 17.0 version).

RESULTS

Species of fungi isolated from remnant foods and wastewater collected from restaurants are shown in Table 1. The fungi isolated are *Aspergillus fumigatus*, *Aspergillus niger*, *Mucor mucedo*, *Penicillium chrysogenum*, *Apsergillus flavus*, *Fusarium oxysporum*, and *Penicillium italicium.* Figure 1 shows the percentage yield of methanol and acetone extracts. Acetone has the highest percentage yield (6.5%) than methanol (4.7%). Table 2 shows the phytochemical contents of *C. polyzona* extracts. Acetone extract (ACE) had alkaloid content of 14.20 mg/g while methanol extract (MCE) had 11.6 mg/g. Flavonoids content of the extracts ranged from 4.55 to 6.80 mg/g and tannins is 5.2 and 7.7mg/g for ACE and MCE respectively.

The mycelia inhibition of *C. polyzona* extracts ranged from 4.7 to 37.7% against isolated fungi from remnant foods and 2.7 to 37.3% against isolated fungi from wastewater samples at different concentration of 50, 100 and 150 mg/ml (Tables 3 and 4). Acetone and methanol extracts of *C. polyzona* exhibited highest mycelia inhibition against *Penicillium chrysogenum* and *A. niger* than mycelia inhibition caused by fluconazole (Table 3). Acetone extract shows mycelia inhibition of 37.7% against *P. chrysogenum* that was found to show resistance against Ketoconazole (Table 4). Plate 1a shows the mycelia inhibition of *C. polyzona* against isolated fungi while Plate 1b containing DMSO (control) shows no mycelia inhibition against tested fungi. The percentage of mycelia inhibition observed in purified extracts is closely related to the commercial antibiotics used as positive control (Tables 3, 4 and 5). The purified fractions of *C. polyzona* also exhibited more potent mycelia inhibition against *P. chrysogenum*, *A. fumigatus*,

Table 3. Percentage of mycelia inhibition of fungi isolates from remnant foodsby*Coriolopsis polyzona* extractsat different concentrations (mg/ml).

Tested isolates	MCE 150	ACE 150	MCE 100	ACE 100	MCE 50	ACE 50	KET 20	FLU 20
Penicillium italicium	17.3±0.5	21.6±0.4	14.8±0.1	10.3±0.1	5.9±0.0	6.4±0.1	56.2±0.1	0.0±0.0
A. niger	27.9±0.9	24.8±0.2	20.8±0.0	17.0±0.0	0.0±0.0	11.8±0.0	41.0±0.0	29.0±0.0
Fusarium. oxysporum	12.4±0.5	26.4±0.4	10.6±0.5	12.3±0.0	9.1±0.0	8.4±0.0	61.6±0.0	75.6±0.0
Mucor mucedo	26.6±0.4	22.4±0.7	13.6±1.1	20.9±0.0	9.0±0.0	8.8±0.0	42.8±0.2	62.4±0.1
Rhizopous stolonifer	0.0±0.0	8.9±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	68.2±0.1	38.0±1.0
Penicilliumchrysogenum	28.5±0.9	37.7±0.1	15.8±0.1	24.5±0.0	10.2±0.0	12.4±0.1	50.5±0.0	50.6±0.5
A. fumigatus	20.3±0.3	26.6±0.5	10.3±0.6	21.2±0.0	0.0±0.0	10.0±0.0	59.7±0.0	0.0±0.0
A. flavus	0.0±0.0	10.7±0.2	0.0±0.0	7.6±0.0	0.0±0.0	4.7±0.1	68.0±0.0	53.7±0.3

Values are mean of replicates (n = 3). 0.0 = no mycelia inhibition; ACE= acetone extract; MCE= methanol extract. FLU= fluconazole; KET=ketoconazole.

Table 4. Percentage of mycelia inhibition of fungi isolates from wastewater by *Coriolopsis polyzona* extractsat different concentrations (mg/ml).

Tested isolates	MCE 150	ACE 150	MCE 100	ACE 100	MCE 50	ACE 50	FLU 20	KET 20
Fusarium oxysporum	5.6±0.0	8.0±0.0	0.0±0.0	4.0±0.0	0.0±0.0	4.0±0.3	56.6±0.5	68.0±0.0
Penicullium italicium	2.6±0.0	10.7±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	64.0±0.0	63.6±0.1
Asperillus fumigatus	10.3±0.2	24.2±0.0	5.0±0.0	8.3±0.0	2.7±0.0	2.7±0.0	0.0±0.0	40.8±0.1
Triscelophorus monosporus	10.5±0.1	21.2±0.3	5.9±0.1	10.4±0.0	4.7±0.0	4.0±0.0	61.5±0.0	59.4±0.1
Penicillium chyrsogenum	12.6±0.1	37.3±0.2	5.0±0.0	20.1±0.0	5.4±0.0	8.0±0.0	32.5±0.0	0.0±0.0
Aspergillus niger	10.3±0.0	7.9±0.0	.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	21.7±0.1	41.7±0.1

Values are mean of replicates (n = 3). 0.0 = no mycelia inhibition; ACE= acetone extract; MCE= methanol extract. FLU= fluconazole; KET=ketoconazole.

Plate 1a. Mycelia inhibitionof *Aspergillus niger* by*Coriolopsis polyzona* extract.

Plate 1b. Negative control.

F. oxysporum and *A. niger* as shown in Table 5. Table 6 shows the functional groups of bioactive compounds in *C. polyzona* extracts. The results of FTIR analysis revealed the presence of the following functional groups; hydroxyl (OH), alkyl (C-H), carbonyl (C=O) and aromatic ring (C=C).

Table 5. Mycelia Inhibition (%) of purified fractions of *Coriolopsis polyzona* against isolated fungi at 5.0 mg/ml.

Parameter	AC	AP	MC
Isolated fungi from remnant foods			
Penicillium italicium	25.0	22.4	ND
A. niger	30.3	0.0	ND
Fusarium. oxysporum	32.2	31.2	ND
Mucor mucedo	27.8	23.5	ND
Rhizopous stolonifer	17.5	20.5	0.0
Penicilliumchrysogenum	40.6	42.2	38.3
A. fumigatus	34.1	0.0	23.8
A. flavus	15.6	0.0	0.0
Isolated fungi from wastewater			
Fusarium oxysporum	13.3	15.3	ND
Penicullium italicium	8.8	11.2	ND
Asperillus fumigatus	19.4	20.7	ND
Triscelophorus monosporus	22.5	ND	ND
Penicillium chyrsogenum	15.2	ND	ND
Aspergillus niger	14.3	14.1	ND

Values are mean of replicates (n = 3). AC= purified fraction from acetone extract; AP= purified fraction from acetone extract; MC= purified fraction from methanol extract; ND= Not detected.

Table 6. Functional groups obtained from purified fractions of *Coriolopsis polyzona* at different wave number (cm^{-1}).

IRWave Number (cm^{-1})	**Functional groups**
AC	
3377.3	OH $_{stretch}$
2945.15	CH_3/CH $_{stretch}$
2834.78	CH_3/CH $_{stretch}$
1652.27	C = C $_{stretch}$
1410.30	C=O $_{stretch}$
1026.12	C – O $_{stretch}$
AP	
3428.33	OH $_{stretch}$
2932.99	CH $_{stretch}$
1637.26	C=C $_{stretch}$
1405.11	C=O $_{stretch}$
1030.85	C – O $_{stretch}$
MC	
3426.00	OH $_{stretch}$
1642.12	C=C $_{stretch}$
1399.65	CH $_{stretch}$
1021.65	C –O $_{stretch}$

AC= purified fraction from acetone extract;AP= purified fraction from acetone extract;MC= purified fraction from methanol extract.

DISCUSSION

The practice of disposing wastes from restaurants without treatment had been in existence without check in most communities in Nigeria. This has led to build up of chemical and biological pollutants in the environment. The accumulation of biological pollutants such as microorganisms in the environment beyond self-purification will be a threat to public health (Akpor and Muchie, 2011). Build-up of microorganisms in the environment has also contributed to the problem of resistance of microorganisms to commercial antibiotics as a result of exchange of resistant genes (Frost et al., 2005). This research work therefore assessed the antifungal effect of extracts obtained from a wild macrofungus, *C. polyzona,* against fungi isolated from restaurant wastes.

The genera of fungi isolated from remnant foods and wastewater from restaurants in Akure metropolis includes *Aspergillus*, *Peniculliumand* *Fusarium.* The occurrence of different species of fungicould be due to the exposure time of food during processing and after processing to airborne fungi, the microbiological quality of water used for food preparation, sanitary quality of the equipment and health status of employees. The importance of these fungi to endanger human health had been highlighted (Warris et al., 2001). Hedayati et al. (2011) had earlier isolated these groups of fungi from hospital drinking water in Iran. The findings of Oranusi et al. (2011) revealed that some of these fungi are of public health importance. Makun et al. (2009) also reported that many of these fungi species produce toxic metabolites in Nigerian staples and ascribed the mycotoxins to species of *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor*.

The phytochemical contents in extracts of *C. polyzona* are flavonoids, alkaloids, tannins, saponins and steroids.

These phytochemicals had been reported to possess antifungal effects (De Silva et al., 2013). Therefore, the inhibitory potentials of extracts obtained from *C. polyzona* were well pronounced against isolated fungi from wastewater and remnant foods (Tables 2, 3 and 4). *Coriolopsis* species had earlier been reported to possess antimicrobial properties (Oyetayo et al., 2010). Gbolagade et al. (2007) had also earlier reported the antifungal properties of some Nigerian higher fungi on species of pathogenic fungi.

The percentage inhibition of mycelial growth by extracts of *C. polyzona* was similar to the antifungal activities of some wild macrofungi reported by Imtiaj and Lee (2007).The mycelia inhibition (2.7 to 37.3%) caused by extracts of *C. polyzona* on the isolated fungi conformed to results obtained by Ayodele and Idoko (2011) who reported mycelia inhibition of 13.82 to 32.10% against *A. niger* and *Penicillium notatum* by culture filtrate of *Lentinus squarrosulus, Psathyrella atroumbonata, Volvorella volvacea* and *Coprinellus micaceus*.

The mycelia inhibition of these isolated fungi by *C. polyzona* extracts could be attributed to the presence of secondary metabolites, which comprises of different functional groups such as ketones, hydroxyl, methyl, carboxylic acid and aromatic ring. Compounds possessing these functional groups are known to be potentially useful phytochemicals that can exhibit antimicrobial activities (Cowan, 1999). Barros et al. (2008) and Rai et al. (2005) had associated the medicinal values of macrofungi to diversities of secondary metabolites that contained different functional groups of biologically active compounds.

The possession of antifungal properties by extracts of *C. polyzona* show that it contains bioactive compounds that could be extracted and explored as new drugs against the continuous failure of chemotherapies and prevalent resistance of microorganisms to commonly used antibiotics.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Akpor OB,Muchie M (2011). Environmental and public health implications of wastewater quality. Afr. J. Biotechnol. 10(13): 2379-2387.

Alaoui SM., Merzouki M, Penninckx MJ, Benlemlih M (2008). Relationship between cultivation mode of white rot fungi and their efficiency for olive oil mill wastewaters treatment. Electronic J. Biotechnol. 11(4):1-8.

Ameri A, Ghadge C, Vaidya JG, Deokule SS (2011). Anti-Staphylococcus aureus activity of *Pisolithus albus* from Pune, India. J. Med. Plants Res. 5(4): 527-532.

Association of Official Analytical Chemists (AOAC) (2003). Official Methods of Analysis of the Association of Official Analytical Chemists 17th ed. AOAC International: Gaithersburg,USA.

Ayodele SM, Idoko ME (2011). Antimicrobial activities of four wild edible mushrooms in Nigeria. Int. J. Sci. Nat. 2(1): 55-58

Barnett JA, Payme RW, YawoD (1983). Yeast characteristics and identification, 2nd edition Cambridge University Press, New York.

Barros L, Cruz T, Baptista P, Estevinho LM, Ferreira ICFR (2008). Wild and commercial mushrooms as source of nutrients and nutraceuticals. Food Chem. Toxicol. 46:2742-2747

Brakhage AA, Bruns S, Thywissen A, ZipfelPF, Behnsen J (2010). Interaction of phagocytes with filamentous fungi. Curr. Opin. Microbiol. 13:409-415

Chander J (2002). Routine mycological techniques. Medical Mycology. 2nd Edition, Mehta Publishers, New Delhi. pp. 286-398.

Cowan MM (1999). Plant products as antimicrobial agent. Clin. Microbiol. Rev. 12(4):564-582.

De Silva DD,Rapior S, Sudarman E, Stadler M,Xu J,Alias SA, Hyde KD (2013). Bioactive metabolites from macrofungi: ethnopharmacology, biological activities and chemistry. Fungal Diver. 62:1-40

Frost LS, Leplae R, Summer AO, Tonssaint A (2005). Mobile genetic elements: The agent of open source evolution. Nat. Rev. Microbiol. 3:722- 732.

Gbolagade J, Kigigha L, Ohimain E (2007). Antagonistic effect of extracts of some Nigerian higher fungi against selected pathogenic microorganisms. Am. Eurasian J. Agric. Environ. Sci. 2 (4): 364-368.

Hageskal G, Lima N, Skaar I (2009). The study of fungi in drinking water. Mycol. Res. 113:165-172.

Harborne JB (1998). Phytochemical methods. 3rd edition., Chapman and Hall Ltd., London, pp. 135- 203.

Hedayati MT, Mayahi S, Movahedi M, Shokohi T (2011). Study on fungal flora of tap water as a potential reservoir of fungi in hospitals in Sari city, Iran. J. Med. Mycol. 21:10-14.

Hesham EE, Elsayed EA, Aziz R, Wadaan MA (2013). Mushrooms and truffles: historical biofactories for complementary medicine in Africa and in the middle east. Evid. Based Complement. Alternat. Med. 2013:620451.

Imtiaj A, Lee TS (2007). Screening of antibacterial and antifungal activities from Korean wild mushroom. World J. Agric. Sci. 3(3):316-321.

Jiang J, Sliva D (2010). Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells. Int. J. Oncol. 37(6):1529-1536.

Keles A, Koca I, Gencelep H (2011). Antioxidant properties of wild edible mushrooms. J. Food Prot. Technol. 2 (6): 130-135.

Khatun S, Islam A, Cakilcioglu U, Chatterjee NC (2012). Research on mushroom as a potential source of nutraceuticals: A review on Indian perspective. Am. J. Exp. Agric. 2(1):47-73.

Lindequist U, Niedermeyer TNJ, Julich WD (2005). The pharmacological potential of mushrooms. Evid. Based Complement. Alternat. Med. 2(3): 285-299.

Makun HA, Gbodi TA, Akanya OH, Saloko AE, Ogbadu GH (2009). Health implication of toxigenic fungi found in two Nigerian staples: guinea corn and rice. Afr. J. Food Sci. 3(9):250-256.

Ogidi CO,Oyetayo VO (2013). Antibiotic sensitivity pattern of microorganisms isolated from remnant foods and wastewater from restaurants. FUTA J. Res. Sci. 9 (2):209-216.

Oranusi S, Omagbemi F, Eni, AO (2011). Microbiological safety evaluation of snacks sold in fast food shops in Ota, Ogun state, Nigeria. Int. J. Agric. Food Sci. 1(4): 75-79.

Owolabi BJ, Olarinoye NO (2008). Organic chemistry; The fundamentals. Bohan press, Ibadan. pp. 72-76.

Oyetayo VO, Okuo MO, Olatunji EK (2010). Inhibitory potential of ethanolic extract of two wild polyporales macrofungi on aerobic microbial isolates of meat. Nig. J. Microbiol. 24(1):2033-2037.

Parajuli RR, Tiwari RD, Chaudhary RP, Gupta VN (2005). Fungitoxicity of the essential oils and some aromatic plants of manang against *Alternaria brassicicola*. Sci. World 3(3):39-43.

Rai M, Tidke G, Wasser SP (2005). Therapeutic potential of mushrooms. Nat. Prod. Rad. 4(4): 246-257.

Singleton VL, Orthofer R, Lamuela-Raventos RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Cioalteau Reagents. Methods Enzymol. 299: 152-178.

Thatoi HN,Singdevsachan SK (2014). Diversity, nutritional composition and medicinal potential of Indian mushrooms: A review. Afr. J. Biotechnol. 13(4):523-545.

Wani BA, Bodha, RH, Wani AH (2010). Nutritional and medicinal importance of mushrooms. J. Med. Plants Res. 4(24):2598-2604

Warris A, Gaustad P, Meis JF, Voss A, Verweij PE, Brahamsen TG (2001) Recovery of filamentous fungi from water in a paediatric bone marrow transplantation unit. J. Hosp. Infect. 47:143-148.

Wasser SP (2002). Medicinal mushrooms as a source of antitumor and immunodulating polysaccharides. Appl. Microbiol. Biotechnol. 60:258-274.

Willams DHA (1982). Spectroscopic method in organic chemistry,Willey and Son, London.

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Full Length Research Paper

Molecular ecological typing of wild type *Aspergillus terreus* from arid soils and screening of lovastatin production

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Arid soils are complex ecosystem that maintains topographically distinct mycoflora populations. A total of 45 soil samples collected from the arid soils in Iraqi desert were cultured by dilution plate method and screened for *Aspergillus terreus*. The aim of this study was to enhance ecological knowledge of cultured colonies variation of *A. terreus* as well as environmental interactions in arid soils. An attempt is made to comprehensively screen desert soil for the wild type *A. terreus* producing lovastatin. The results show that the most frequent *Aspergillus* spp. included: *A. niger* (159 isolates), *A. terreus* (143), *A. flavus* (115) and *A. fumigates* (42) and other fungi. Genetically, the diagnoses of 19 isolates of *A. terreus* were in the scope of our interest. The specific primer pair had monomorphic bands of approximately PCR product of 450 bp. Ribotyping isolates of *A. terreus* with primer pairs (ITS1/ITS4 and ITS1/ITS2) were 19 isolates, with a single PCR product of 550-600 bp and 280-380 bp, respectively. RAPD-PCR was also used to distinguish between ecological patterns generated and allowed a distinction of very closely related environmental isolates. Lovastatin production was carried out with thin layer chromatography. Results suggest that phenotypic variations in *A. terreus* isolates were not useful for identifying them, and showed no significance in the identification in all the sites studied. However, using discriminatory molecular methods, such as amplification of the targeted regions by specific and universal characterization of the isolates could be pivotal in understanding ecological variation. Wild type soil isolates of *A. terreus* have the ability to produce lovastatin.

Key words: Molecular ecological typing, *Aspergillus terreus,* arid soils, lovastatin, Iraq.

INTRODUCTION

Aspergillus terreus is a common fungus, which has an important role in desert soils (Amin et al., 2010). Arid soil is complex ecosystem with topographically distinct myco floras. Its microbial communities are subjected to surface heating, cycles of rainfall and extreme desiccation, changing water table, CO_2 enrichment and UV light supplementation and attenuation; it is directly exposed to sun light due to the absence or abundance of vegetation variation (Lipson et al., 1999).

A. terreus is a widely dispersed species among the soil

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mycoflora; it belongs to the genus *Aspergillus*, and of the sub-genus *Terrei Nidulantes* (Raper and Fennell, 1965; Varga et al., 2005). Many isolates of this fungus show morphological variation in growth criteria (Rath et al., 1999). Most of the *A. terreus* isolates produce a variety of secondary metabolites that are of pharmaceutically significance; they are used for producing lovastatin drug (Casas L´opez et al., 2003). Morphological characters include making weak classification at the species and genus levels, while molecular methods are powerful tools in species identification (Hong et al., 2008).

In advanced fungal molecular studies, ribosomal marker genes have been used, such as universal genes found in all fungi. Fungal communities are commonly genotyped by the multiple genes of rDNA (ITS, IGS, 18S and 25SrRNA, 25S and their introns) (Imran and Al-Asadi, 2014). These genes allow for phylogenetic analysis, and assist in taxonomic classification (Chase and Fay, 2009).

Various genotypic methods have been used to successfully do fingerprinting in several fungi such as *A. fumigatus*, *Fusarium solani* and *Candida* spp. (Crowhurst et al., 1991; Loudon et al., 1993; Nariasimhan and Asokam 2010, Imran and Al-Shukry 2014). Ribotyping analysis of the ITS region revealed detailed information about molecular analysis and showed reproducible polymorphism in several studies (Chase and Fay, 2009). Random Amplification of Polymorphic DNA (RAPD) assay revealed polymorphic DNA patterns of *Aspergillus* spp. (Anderson et al., 1995). Furthermore, RAPD-PCR assay is adapted for doing fingerprinting of fungi, as it reveals an accurate and simple method for differentiation among fungal isolates (Aubin et al., 1991; Symones et al., 2000; Nariasimhan and Asokan, 2010).

It is not clear whether the variations in the characteristics of the colony of *A. terreus* isolates are due to environmental response or genetic variation. As a result, there is the need for a reliable molecular ecolo-gical typing of *A. terreus* isolates in arid soils. Furthermore, the important roles played by these fungi in the arid regions include biodegradation, drug biosynthesis, recycling of materials and microbial activities and are potential source of novel pharmaceuticals (Amin et al., 2010). Few comparable data in the literature are available for *A. terreus* in the world (Lass-Florl et al., 2007).

The role of genetic variants on phenotypic traits often depends upon environmental and physiological conditions, but such gene-environment interactions are poorly understood. The beneficial role of soil mycoflora is the focus of this study.

Several studies have been attempted to evaluate the ability of *A. terreus* isolates to produce lovastatin drug (Alberts et al., 1980; Juzlova et al., 1996; Casas L´opez et al., 2003; Miyake et al., 2006). The wild and mutant strain of *A. terreus* appears to be the most commonly producer of lovastatin, although it uses various ways to produce biological and significant product (Novak et al., 1997; Hajjaj et al., 2001).

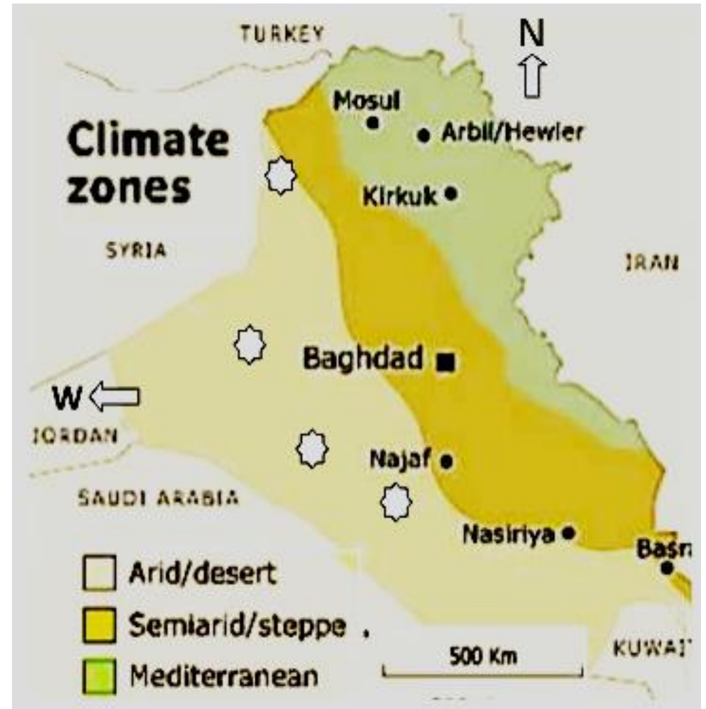

Figure 1. Climatic zones in the map of Iraq, ( ) sampling sites of the arid soil for the survey of A. terreus.

Unfortunately, there is no information on the molecular ecology of fungal communities in the desert of Iraq and also there are no previous studies on the role of genetic variants on phenotypic traits of *A. terreus* based on environmental changes. The aim of our study was to evaluate the phenotypic and genotypic variations based on ITS typing region, using RAPD-PCR of the environmental isolates of *A. terreus;* it aimed to perform a phylogenic analysis, explains their variations by molecular ecological diagnosis and how this understanding enables fungal diagnosis and screening of lovastatin produced by the wild type *A. terreus* cultures.

MATERIALS AND METHODS

Site descriptions and samples collection

The study area is located around the geographical coordinates of 33° 20' N. 44° 23' E. in Iraq. Western and southern Iraq cover a vast desert area of about 168.000 square km: south-west (Najaf and Kerbela provinces) and north-west (Ramadi and South Mosul provinces) (Figure 1). The common vegetation observed in the area includes: *Alhaji*, *Tamarix* and *Salsols*. Soil pH ranges from 6.8 to 8.8 (Guest and Al-Rawi, 1966).

A total of 45 soil samples (150-200 g each) were collected from October to April 2011-2012, in different localities in the desert regions. Soil temperatures were approximately around 5°C in January, 56°C in August and 37°C in October at the time the collections were made. Soil samples were taken from a depth of 5 cm and stored in polythene bags at 4°C.

Culturing and isolating of *A. terreus*

The pure cultures of *A. terreus* were isolated by the serial dilution technique using Potato Dextrose Agar (PDA) medium. Suspected yellow isolates of *A. terreus* were sub cultured on the PDA medium in separate triplicate plates for each isolate and incubated at 28°C for 7 days (Suhail et al., 2007). Microscopic examination was performed using mounted hyphal inoculums from the colony margins. This was done by using adhesive transparent tape placed on a slide with a drop of lacto phenol cotton blue stain. *A. terreus* isolates were identified phenotypically using the taxonomic key created by Raper and Fennel (1965). They were maintained on PDA slants at 28°C for four days and were kept in refrigerator at 4°C until use; they were sub cultured every two weeks. The frequency of a fungus is denoted by the number of samplings in which it is recorded against the total: Frequency (%) = No. of observation in which colony appears / total number of observation recorded x 100 (Adhikari et al., 2004).

Genomic DNA extraction

The culture media for each of the 19 *A. terreus* isolates were frozen for 1 h and tiny portions of the mycelia mat were harvested into 1.5 ml tube. The harvested mats were suspended in 400 µl of lysis extraction buffer (400 mM Tris-HCl, 20 mM EDTA,150 mM NaCl , and 0.5% SDS adjusted 8.5 pH) then vortexed for 5 min and added to 10 µL protinase K. Tubes were incubated in 65°C water bath overnight. A mixture of phenol: chloroform: Isoamyl alcohol (25:24:1) was added to the tubes. Tubes were centrifuged at 5000 rpm for 10 min. The aqueous supernatant was transferred to a new tube. An equal volume of cool isopropanol was added and agitated many times; it was centrifuged at 1000 rpm for 10 min. The supernatant was poured out. The pellet containing DNA was rinsed with 70% ethanol. It was air dried; pellets were re-suspended with 100 µL TE and placed in 70°C water bath. 6 µL of RNase A was added, and incubated at 37°C for 30 min. The tubes were centrifuged at 5000 rpm for 2 min. The supernatant was transferred to a new tube and frozen at -20°C until use (Saghai-Maroof et al., 1984; Edwards et al., 1991).

Genotyping of *A. terreus*

The 19 *A. terreus* isolates were confirmed by amplification, using specific pair primer: ATE1:CTA TTG TAC CTT GTT GCT GGCG; ATE2 :AGT TGC AAA TAA ATG CGT CGG CGG (Logotheti et al., 2009). Ribotyping of the targeted rDNA (ITS1 -5.8S-ITS2 region) of the 19 isolates was done with primer pairs of ITS1/ITS4 and ITS1/ITS2 that amplified ITS1-5.8S-ITS2 and ITS1 regions. The reaction was performed in a thermal cycler (LABENAT, USA) with 1.2 µL of each primer (20 pmole) and 0.8 µL of genomic DNA; the water was adjusted to a final volume of 25 µL. PCR protocol consisted of the initial denaturation at 95°C for 5 min; followed by 35 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min; and a final elongation of 72°C for 5 min. Finally, 10 µL of PCR products was loaded onto 1.2% agarose gel that was premixed with ethidium bromide stain (0.5 µg/ml) and TBE running buffer for 45 min at 100 V at room temperature. The products were visualized under a UV transilluminator and then photographed.

RAPD-PCR ecological typing

The primer R108 (5´-GTATTGCCCT-3´), described by Aufauvre-Brown et al. (1992), was used for RAPD-PCR typing of the 19 environmental isolates of *A. terreus*. Amplification reactions were done in a final volume of 20 µL containing 0.5 µL of genomic DNA, 1 µL Primer (50 pmole) , and 12.5 µL master mix PCR buffer. Water was adjusted to the final volume reaction. PCR was performed in a thermal cycler (LABENAT, USA) with the following temperature profile: 1 cycle of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 36°C and 1 min at 72°C and a final extension step at 72°C for 10 min. 10 µL of amplification products were loaded onto 2% agarose gel, which was premixed with ethidium bromide stain (0.5 µg/ml) and TBE running buffer for 1.30 h at 80 V at room temperature. The products were photographed using a UV transilluminator. (Lass-Florl et al., 2007).

Phylogenetic analysis

A phylogenetic tree dendrogram (UPGMA) of the 19 environmental isolates of *A. terreus* was constructed by using UVI band software, and the similarity coefficient factor was evaluated according to Ute et al. (1994).

Cultivation of the *A. terreus* isolates for production lovastatin

Three *A. terreus* isolates were selected to produce lovastatin. They were selected based on their color variation on agar plate. Production was performed in two complex media: The first culture was prepared in 250-ml Erlenmeyer flask containing 40 ml of medium A (10 g of glucose, 5 g of corn steep liquor, 40 g of tomato paste, 10 g of oatmeal, and 10 ml of trace elements, 1 g of $FeSO_4 \cdot 7H_2O$, 1 g of $MnSO_4 \cdot 4H_2O$, 200 mg of $ZnSO_4 \cdot 7H_2O$, 100 mg of $CaCl_2 \cdot 2H_2O$, 25 mg of $CuCl_2 \cdot 2H_2O$, 56 mg of H_3BO_3, and 19 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per liter of solution (Alberts et al., 1980); it was inoculated with $1 \cdot 10^7$ conidiospores. The flasks were shaken at 200 rpm for a day at 28°C. The second culture was prepared by inoculating 200 ml of medium B (containing [per liter] 45 g of glucose, 24 g of peptone, 2.5 g of yeast extract) (Alberts, 1990) with 6 ml of the previous culture in a 1-liter Erlenmeyer flask. The flasks were shaken at 200 rpm and incubated at 28°C for 12 days. For cultivations in fermentors, a 1-litre Erlenmeyer flask containing 200 ml of medium A was inoculated with 4 ml (10^7) of conidiospore suspension. The flask was shaken at 200 rpm for 1 day and then transferred to the fermentor (Alberts, 1990).

RESULTS

Cultural and microscopic characteristics of *A. terreus*

A. terreus grew fast on PDA medium. Macroscopic characters of the colonies included difference in their colors, from pale-yellow to dark yellow. There was reverse pigmentation, from yellow to dark gray color. The texture of the colonies showed raised surfaces which are velvety, yet tough, yellowish and powdery (Figure 2a). Microscopic characters did not show more variations among the isolates; conidiophores were typically long, hyaline and smooth giving rise to sub-spherical vesicles that were biseriate. Conidia had smooth wall, and were slightly elliptical) Figure 2b).

Isolation and Identification

A total of 143 desert *A. terreus* isolated from South-west Najef Province (n=28 isolates), Ramadi Province (n=40), South Mosul Province (n=46 isolates), and Western Kerbela province (n=25 isolates) (Table 1) were studied.

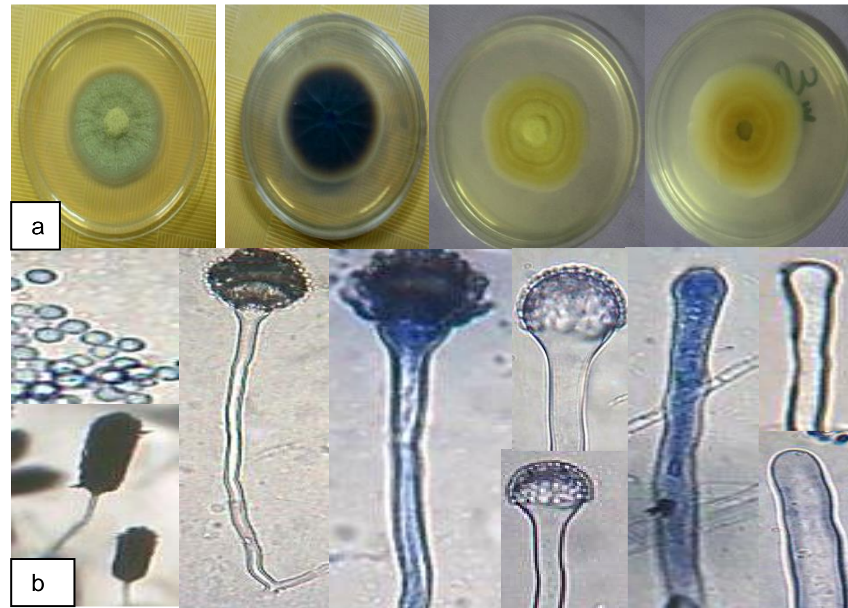

Figure 2. The appearance of the characteristics of A. terreus on the PDA medium incubate at 28 ºC for 7 days: a-colony characters superficial and reverse view , b-microscopic characters (conidia, conidial heads, conidiophores development stages).

Table 1. Fungal species and their frequency. Occurrence percentage and number of isolates drawn from the arid soils of Iraq.

Fungal specie	Number of isolates	Occurrence (%)	Frequency (%)
A. niger	159	13.81	24.68
A. terreus	143	13.25	20.8
A. fumigates	42	9.39	6.52
A. flavus	115	11.04	17.31
P. chrysogenum	52	6.35	8.15
P. digitatium	53	6.36	8,15
A. alternate	5	4.41	0.7
Cladosporium spp.	4	1.14	0.62
Monilia spp.	16	4.97	2.48
Trichoderma spp.	10	3.31	1.55
Rhizopus spp.	28	12.7	4.34
Rhodoterla spp.	3	1.65	0.46
Candida spp.	8	3.31	1.24
White mycelium	6	8.83	0.9
Total isolates	644		

Molecular ecological typing

Molecular diagnosis of A. terreus isolates

The specific pair of primers for *A. terreus* ATE1 and ATE2 was successfully annealed, and the targeted regions of the 19 environmental isolates of *A. terreus* were amplified. The PCR product showed monomorphic bands of 450 bp in length (primer included) (Figure 3a). These results confirmed the diagnosis that all the isolates used in this study belonged to *A. terreus*.

Ribotyping the ITS region for environmental isolates of A. terreus

The targeted rDNA (ITS1 -5.8S-ITS2 region) of the 19 isolates was amplified with primer pairs: ITS1/ITS4 that

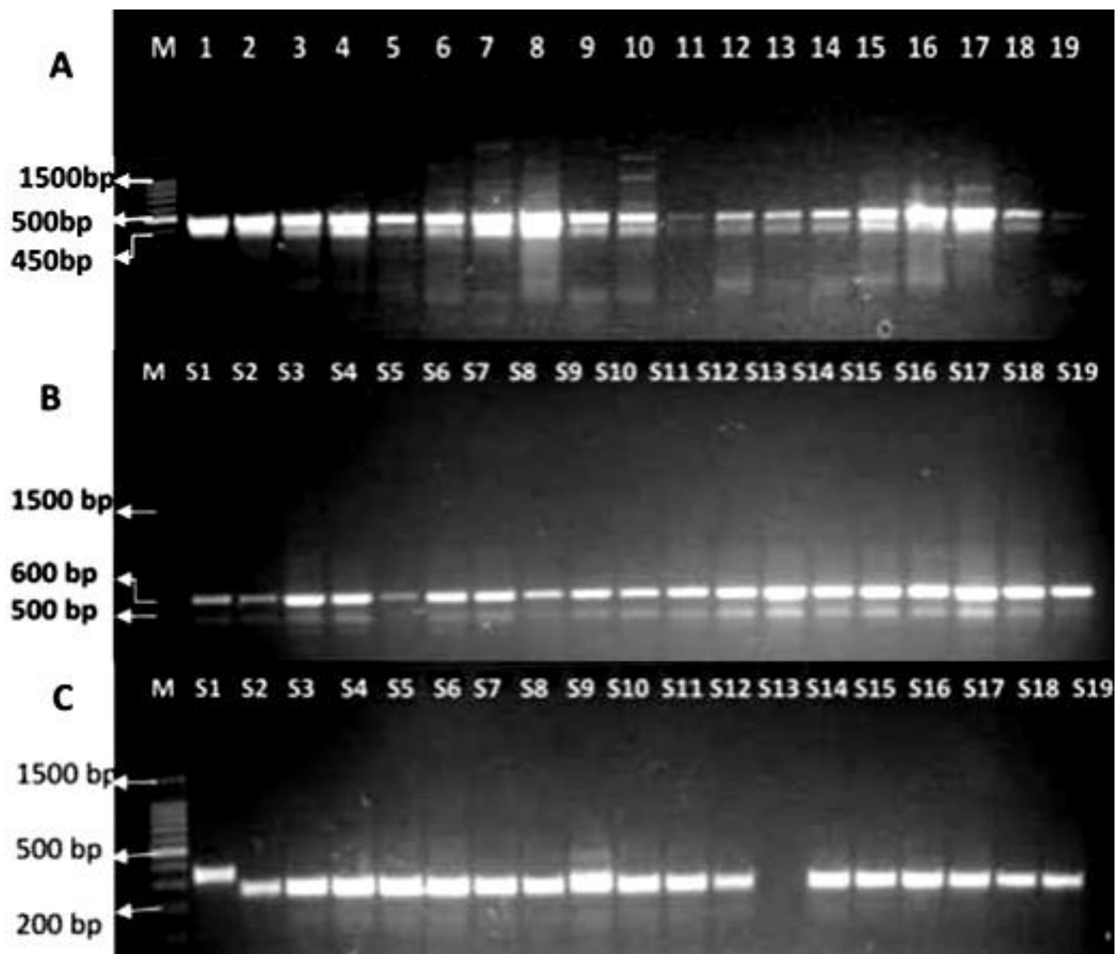

Figure 3. Agarose gel electrophoresis of the PCR products for *A. terreus* isolates amplified by: A-pair primers ATE1 and ATE2; B, amplified ITS1-5.8S-ITS2 region by pair primers 1TS1 - ITS4; C, amplified ITS1-ITS2 region by pair primers 1TS1 -ITS2. Lane M= Molecular marker 100 bp; lanes 1- 19 *A. terreus* isolates.

produced an amplicon length of approximately 550 -600 bp, which was obtained for all the tested isolates of *A. terreus* (Figure 3b); and ITS1/ITS2 primer that amplified the ITS1 region, which produced an amplicon length of approximately 280-380 bp (Figure 3c).

RAPD-PCR ecological typing

Several bands (1 to 5 bands) in various sizes ranging from about 100 to 1000 base pairs were obtained by using primer R108. This primer generated by RAPD-PCR patterns can discriminate between very closely related environmental isolates, but incidental similarities among the typing of distantly related isolates may also occur. We highlighted that similar typing patterns of bands correspond to the same ecological genotype having the same locus or loci and generating similar or different patterns with identical band sizes in the different environmental isolates of *A. terreus* (Figure 4).

Phylogenetic tree of the *A. terreus* isolates based on RAPD-PCR

The results show that there was a wide range of degrees of similarity among the 19 isolates of *A. terreus*: J and L isolates showed 100% similarity coefficient value (0% distance coefficient), C and D showed 80% similarity, both S, T and N, O showed 68% similarity; H,I isolates showed 50% similarity coefficient value, while the R isolate showed a distinct ecological genotype with coefficient value of 0% similarity. Figure 5 shows all the similarity coefficient values.

Screening of fungal cultures for lovastatin production

The fungal cultures were grown under submerged fermentation conditions to assess their potential to produce lovastatin. From the results (data not shown), it is clear that all the three *A. terreus* cultures were able to

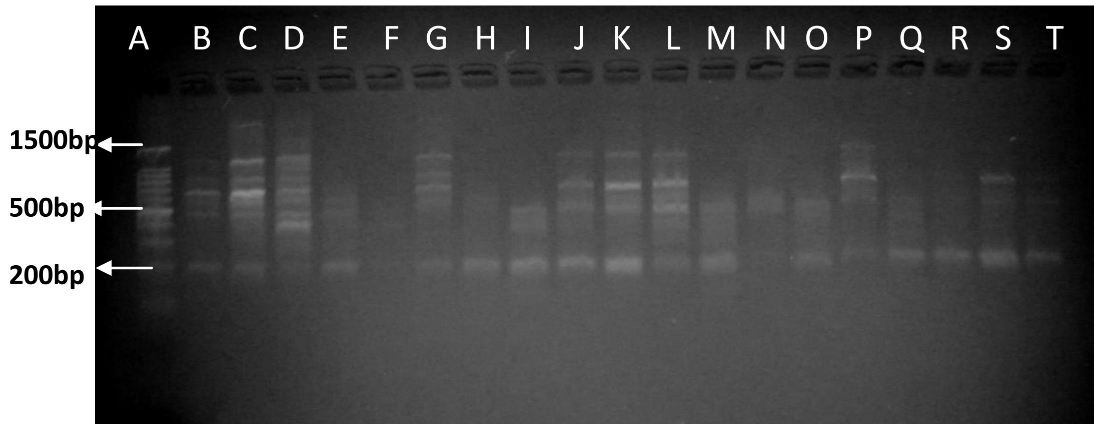

Figure 4. Agarose gel electrophoresis of the RAPD-PCR products for environmental isolates of *A.terreus* by primer (R108). The PCR products of the isolates were analysed in 2% agarose gel. A=Molecular marker 100bp, ecological genotypes were J-R.

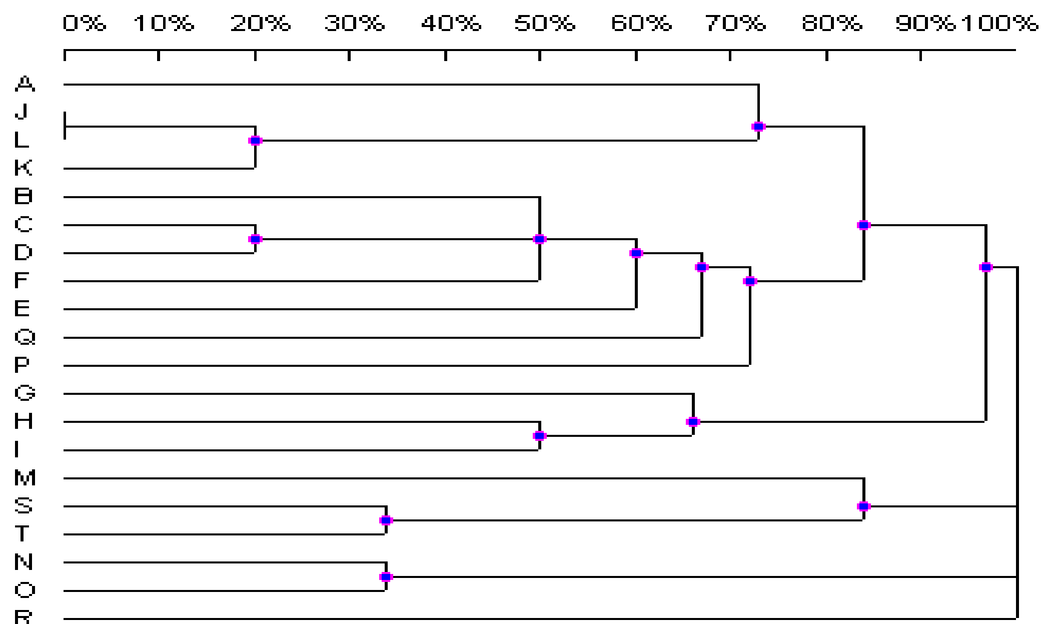

Figure 5. Phylogenetic tree dendrogram for 19 environmental isolates of *A. terreus* constructed using UVI band software based on the RAPD-PCR bands. A=Molecular marker; J-R= isolates of *A .terreus*. Scale 0%-100% =distance coefficient (UPGMA).

produce lovastatin. Lovastatin production was confirmed by using thin layer chromatography. It was observed that, both commercial lovastatin and the sample spots had approximately same R_f value = 0.32, with light brown in both treatments and standard drug.

DISCUSSION

Our study enhances understanding of the impact of extreme environmental factors and shows high variation in the characteristics of the colonies of the *A. terreus* isolate in each site of study. We also concluded that variations in the colonies in all the soil samples were governed by dominant environmental characteristics of the desert soil such as soil texture (sandy – gravel texture), dry areas, and sites containing varied densities and low diversity of vegetation as well as scarcity of rain (Guest and Al-Rawi, 1966). The results of this study described the taxonomic criteria that allow rapid typing of

A. terreus isolate from the arid soils, including the cultural and microscopic features used for the identification of *A. terreus* isolates. These phenotypic variations correlated with the ecological effects and genetic interactions. In the desert environment, intensive mutagenic actions are expected from UV radiation.

The results of our study shows that no much genetic variations were observed in the ITS region typing of the19 isolates of *A. terreus*. These markers were designed to identify defined strains of *A. terreus* in arid soils. They also showed that the monomorphic PCR bands of *A. terreus* can be used to explain why phenotypic variations do not correlate with the intron region but may correlate with the dynamics of the genomes at the exon regions in *A. terreus* populations. Our results do not agree with those of Varga et al. (2005), who elucidated some species: *A. alabamensis, A. terreus* var. *floccosus*, *A. terreus* var. *africanus*, *A. terreus* var. *aureus* as well as *A. aureoterreus,* according to Balajee et al. (2009); they presented that seven lineages were observed among isolates that have previously been treated as *A. terreus* based on ITS region (Chase and Fay, 2009) and parts of the β-*tubulin* and *calmodulin* genes.

Our results agree with the explanation of Sniegowski et al. (1997) and Wilke et al. (2001), that most mutations are deleterious. Mutation rates are generally low and reduce individual fitness, so increasing the probability of an adaptive mutation to appear.

The results based on RAPD-PCR marker showed the suitable values for identifying genotypes and described the differentiation of *A. terreus* populations (Figures 4 and 5). These results agree with those of Crowhurst et al. (1991). Unfortunately, previous studies on Iraqi soil fungi used only microscopic and culture based methods (Ismail and Abdullah, 1977; Haleem et al., 2013); so there are no molecular results to compare our work with.

Based on the phenotypic variations in the colonies' character, we postulate that unique *A. terreus* genotypes may occupy particular environmental habitats of desert soils in Iraq. Results of this study showed a great diversity of genotypes among isolates of *A. terreus* by using rDNA and RAPD data, which explored the genotypes of the isolates recovered from the four collection stations under study.

Our results show wide range of variability in phenotypic characters of all the isolates. It was clear that they all belonged to *A. terreus,* as the PCR products from the genotype confirmed the diagnosis by specific primer pairs (ATE1 and ATE2). They distinctively removed any delusion that could lead to confusing these isolates with other species, by revealing the yellow colour of the colonies. This result coincides with the results of Logotheti et al. (2009). Variations in phenotype characters are due to the concept of gene-environment correlation (Smith and Kruglyak, 2008), which can occur through multi-mechanisms. Many of these microbes live in extreme environments, for example; high temperatures, high salt concentrations, low pH, and high radiation. Some physical factors also influence fungal growth and metabolite production (Gautam et al., 2009). The biotechnological potential of microorganisms to produce is based on their special adaptations to their environment (Gautam et al., 2010). Sunlight, salinity and soil ecology by natural selection, genetic drift and gene flow and gene mutation are representatives of the domain sources of genetic variations (Kurtzman, 1985; Carlile et al., 2001; Fe´ral, 2002; Terry et al., 2004; Lass-Florl et al., 2007; Smith and Kruglyak, 2008).

Molecular typing based on RAPD-PCR patterns used for the 19 isolates of *A. terreus* showed distinctive patterns. This result agrees with that of Lass-Florl et al. (2007) who classified clinical isolates of *A. terreus*, using RAPD-PCR patterns. These variations in the pattern may help explain the sources of variation; provide solution to several phenotypic variations in *A. terreus* colony and explain the difference in colors of reverse pigmentations. A phylogeny tree based on RAPD-PCR profile was sufficient in genotyping *A. terreus* isolates collected from the arid regions of Iraq; it showed variable degrees of similarity among the 19 isolates of *A. terreus* and divided them into many genotypes. Only two isolates showed 100% similarity coefficient values. Other isolates showed 0-80% similarity coefficient values (Figure 5). These results agree with those of Lasker (2002) whose genotyped *A. fumigatus* isolates, and also the results of Raclasky et al. (2006) and Nariasimhan and Asokan (2010).

The RAPD-PCR patterns used for *A. terreus* isolates were more effective than monomorphic ribotyping patterns used for ecological genotyping (Loudon et al., 1993; Symones et al., 2000). Finally, ecological genotyping find minor differences among isolates at the species to genus level (Birch et al., 1995).

Our results concur with that of Lewington et al. (2007), in which the wavelength of statin produced by fungal isolates ranges between 200-400 nm. On the other hand, this result conflicts with some earlier studies in which pH, medium and choice of wild type or mutant govern lovastatin production. We found that no specific pH or media induce lovastatin production. This result is in line with that of Kumar et al. (2000) who reported that lovastatin is generally produced by batch fermentation in complex media. *A. terreus* fermentations are typically carried out at 28°C and pHs of 5.8–6.3

Conclusion

This study may encourage future research of ecological genotyping of closely related environmental isolates. It showed highly discriminatory profiles of RAPD –PCR. RAPD–PCR could identify genetic diversity among closely related isolates in the same species. The molecular genotyping of *A. terreus* based on ITS region was reliable, but not as discriminating as RAPD – PCR.

These methods are useful tools in taxonomical studies, give precise, rapid results with low cost and no time consuming. This study confirmed genotyping as an important method to find solution to fungal ecological diversity problems.

Ethical approval

Both authors hereby declare that all actions have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Adhikari A, Sen MM, Gupta - Bhattacharya S, Chanda S (2004). Airborne viable, non-viable and allergenic fungi in a rural agricultural area of India: a 2- year study at five outdoor sampling stations. Sci. Total Environ. 326:123-141.

Alberts AW (1990). Lovastatin and Simvastatin—inhibitors of HMG CoA reductase and cholesterol-biosynthesis. Cardiology 77:14-21.

Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Patchett A, Monaghan R, Currie S, Stapley E, Albers-Schonberg G, Hensens O, Hirshfield J, Hoogsteen K, Liesch J, Springer J (1980) .Mevinolin: A Highly Potent Competitive Inhibitor Of Hydroxy Methylglutaryl Coenzyme A Reductase And A Cholesterol-Lowering Agent. Proc. Natl. Acad. Sci. USA 77:3957-3961.

Amin HA, El-Menoufy HA, El-Mehalawy AA, Mostafa EE (2010). Microbial production of glycyrrhetic acid 3-O-mono-β-D-glucuronide from glycyrrhizin by *Aspergillus terreus*. Malay. J. Microbiol. 6 (2): 209-216.

Anderson MJ, Gull K, Denning DW (1995). Molecular typing by Random Amplification of Polymorphic DNA and M13 Southern Hybridization of Related Paired Isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. 34 (1): 87-93.

Aubin JT, Collandre D, Candotti D, Ingrand C, Rouzioux, Muraux JM (1991). Several groups among human Herpes virus, six strains can be distinguished by Southern blotting and PCR. J. Clin. Microbiol. 29(2): 367-372.

Aufauvre-Brown A, Cohen J, Holden DW (1992). Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. 30:2991 -2993.

Balajee SA, Baddley JW, Peterson SW, Nickle D, Varga J, Boey A, Lass-Florl C, Frisvad JC, Samson RA (2009). *Aspergillus alabamensis*, a new clinically relevant species in the section Terrei. Eukaryot. Cell 8 (5):713-722.

Birch M, Anderson MJ, Denning DW (1995). Molecular typing of *Aspergillus* species by random amplification of polymorphic DNA. Eur. J. Clin. Microbiol.18(11): 838-841.

Carlile MJ, Watkinson SC, Gooday GW (2001). Genetic Variation and Evolution in the fungi. 2ed Academic press, pp. 245-295.

Casas Lṕeza JL, SՃnchez Péreza JA, FernՃndez Sevillaa JM, Acién FernՃndeza FG, Molina Grimaa E, Chistib Y (2003). Production of ovastatin by *Aspergillus terreus*: effects of the C:N ratio and the principal nutrients on growth and metabolite production. Enzyme Microb. Technol. 33:270-277.

Chase MW, Fay MF (2009). Barcoding of plants and fungi. Science 325:682-683.

Crowhurst RN, Hawthorne BT, Rikkerink EHA, Templeton MD (1991). Differentiation of *Fusarium solani* f.sp. *Cucurbitae* races 1 and 2 by Random amplification of polymorphic DNA. Curr. Genet. 20: 391-396.

Edwards K, Johnstone C, Thompson C (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nuclei Acids Res. 19:1349.

Fe´ral JP (2002). How useful are the genetic markers in attempts to understand and manage marine biodiversity? J. Exp. Mar. Biol. Ecol. 268: 121-145.

Gautam SP, Bundela PS, Pandey AK, Awasthi MK, Sarsaiya S (2009). Prevalence of fungi in Municipal solid waste of Jabalpur city (M.P.). J. Basic Appl. Mycol. 8 (1-2): 80-81.

Gautam SP, Bundela PS, Pandey AK, Awasthi MK, Sarsaiya S (2010). Composting of municipal solid waste of Jabalpur City. Glob. J. Environ. Res. 4:43-46.

Guest E, Al Rawi A (1966). Introduction to the Flora of Iraq, Ministry of Agriculture of the Republic of Iraq Vol.1.

Hajjaj H, Niederberger P, Duboc P (2001). Lovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. Appl. Environ. Microbiol. 67:2596-602.

Haleem RA, Abdullah SK, Jubrael JMS (2013). Occurrence and distribution of fungi associated with grapevine decline in Kurdistan region-Iraq. Agric. Biol. J. N. Am. 4(3):336-348.

Hong SB, Shin HD, Nong J (2008). New taxa of Neosatorya and Aspergillus in Aspergillus section Fumigatic. Antonie Van Leeuwen hoek 93(1-2): 87-98.

Imran ZK, Al-Asadi YF (2014). Multiple molecular markers for diagnosis of conjunctivitis caused by *Candida* spp. in Iraq. Afr. J. Microbiol. Res. 8(38):3482-3488.

Imran ZK, Al-Shukry HN (2014). Molecular diagnosis of vaginal candidiasis by polymerase chain reaction (PCR) and random amplification polymorphism DNA (RAPD-PCR) in Babylon Province, Iraq. Afr. J. Microbiol. Res. 8(6):496-502.

Ismail ALS, Abdullah SK (1977). Studies on the soil fungi of Iraq. Proc. Indian Acad. Sci. 86(3):151-154.

Juzlova P, Martinkova L, Kren V (1996). Secondary metabolites of the fungus *Monascus*: a review. J. Ind. Microbiol.16:163-170.

Kumar MS, Jana SK, Senthil V, Shashanka V, Kumar SV, Sadhukhan AK (2000).Repeated fed-batch process for improving lovastatin production. Process Biochem. 36(4):363–368.

Kurtzman CP (1985). Molecular taxonomy of the fungi, In: Bennett, I.W. and Lasuse, L.L. Eds., Gene Manipulation in Fungi, Academic press, Orlando. pp. 35-63.

Lasker BA (2002). Evaluation of Performance of Four Genotypic Methods for Studying the Genetic Epidemiology of *Aspergillus fumigatus* Isolates. J. Clin. Microbiol. 40 (8):2886-2892.

Lass-Florl C, Grif K, Kontoyiannis DP (2007). Molecular typing of *Aspergillus terreus* isolates in Houston, Texas, and Innsbruck, Austria: evidence of great genetic diversity. J. Clin. Microbiol. 45: 2686-2690.

Lewington S, Whitlock G, Clarke R, Sherliker P, Emberson J, Halsey J, Qizilbash N, Peto R, Collins R (2007).Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. Lancet 370:9602:1829-1839.

Lipson DA, Schmidt SK, Monson RK (1999). Links between microbial popula-tion dynamics and nitrogen availability in an alpine ecosystem. Ecology 80:1623-1631.

Logotheti M, Kotsovili-Tseleni A, Arsenis G, Legakis NI (2009). Multiplex PCR for the discrimination of *A. fumigatus, A. flavus, A. niger and A. terreus.* J. Microbiol. Methods 76:209-211.

Loudon KW, Burnie JP, Coke AP , Matthews RC (1993). Application of polymerase chain reaction to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. J. Clin. Microbiol. 31:1117-1121.

Miyake T, Uchitomi K, Zhang MY, Kono I, Nozaki N, Sammoto H, Inagaki K (2006). Effects of the principal nutrients on lovastatin production by *Monascus pilosus*. Biosci. Biotechnol. Biochem. 70(5):1154-1159.

Nariasimhan B, Asokan M (2010). Genetic variability of *Aspergillus terreus* from dried grapes using RAPD-PCR. Adv. Biosci. Biotechnol. 1:345-353.

Novak N, Gerdin S, Berovic M (1997). Increased lovastatin formation by *Aspergillus terreus* using repeated fed-batch process. Biotechnol. Lett. 19:947-948.

Raclaský V, Trtková J, Rusková L, Buchta V, Bolehovská R, Vacková M, Hamal P (2006). Primer R108 performs best in the RAPD strain typing of three *Aspergillus* species frequently isolated from patients. Folia Microbiol. (Praha) 51 (2):136-140.

Raper KB, Fennel DI (1965). *Aspergillus terreus* group. In: Raper KB, Fennel DI, eds, The genus *Aspergillus.* Baltimore, MD: The Williams & Wilkins Co., pp. 293-344.

Rath PM, Kamphoff S, Ansorg R (1999). Value of different methods for the characterization of *Aspergillus terreus* strains. J. Med. Microbiol. 48: 161-166.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984). Ribosomal spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA. 81:8014-8019.

Smith EN, Kruglyak L (2008). Gene-Environment Interaction in Yeast Gene Expression. PLoS Biol. 6(4):0810-0824.

Sniegowski PD, Gerrish PJ, Lenski RE (1997). Evolution of high mutationrates in experimental populations of Escherichia coli. Nature 387:703-705.

Suhail M, Irum F, Jatt T, Korejo F, Abro H (2007) . *Aspergillus* Mycoflora isolated from soil of Kotri Barrage Sindh, Pakistan. Pak. J. Bot. 39(3): 981-984.

Symones F, Boucher JP, Heinemann S, Norland N (2000). Molecular typing of *Aspergillus terreus* isolates by Random amplification of polymorphic DNA. J. Hosp. Infect. 44(4):273-280.

Terry VC, Lars O, Yuri C, Terry C, Torben RC, Brian H, Rolf AI, Margareta J, Dyanna J, Sven J, Nadya M, Nicolai P, et al., (2004). Responses to Projected Changes in Climateand UV-B at the Species Level. Ambio 33:7.

Ute M, Kim L, Peter B, Alan J (1994). Phylogenetic relationships of *Babesia divergens* as determined from comparison of small subunit ribosomal RNA gene sequences. Mol. Biochem. Parasitol. 68:161-165.

Varga J, Toth B, Kocscube S, Rigo K, Teren J , Kozakiewicz Z (2005). Evolutionary relationships among *Aspergillus terreus* isolates and their relatives. Antonie van Leewenhoek 88(2):141-150.

Wilke CO, Wang J, Ofria C, Lenski RE, Adami C (2001). Evolution of digital organisms at high mutation rate leads to survival of the flattest. Nature 412:331-333

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Full Length Research Paper

Frequent carriage of invasive *Salmonellae* amongst patients infected with schistosomiasis in Sudan

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Bacteria-parasite association has been documented as a factor that is responsible for continued and prolonged bacterial infection, such as typhoid and paratyphoid fever in schistosomiasis patients. This work aimed to determine the presence of typhoid and paratyphoid *Salmonella* among schistosomiasis patients and to evaluate the efficacy of Widal test on such population. A cross sectional descriptive study was conducted between November 2005 and May 2006 in Managil region, Gezira State, Sudan. A total of 203 males participated in the study. Urine, stool and blood samples were collected and processed for the investigation of schistosomiasis and *Salmonella* infection based on standard methods. Widal test was performed to estimate diagnostic cut-off value of enteric fever. Of the 203 studied subjects, 42 (20.7%) were diagnosed with *Schistosoma haematobium*, whereas eight (3.9%) had *Schistosoma mansoni* infection. Of these, *Salmonella* species were detected in 30 (60%) cases, of which *Salmonella typhi* represented 63.3%, followed by *Salmonella paratyphi A* and *B* (16.7%, each) and *Salmonella paratyphi C* (3.3%). Based on the culture results (n=30) as a diagnostic method used for enteric fever, Widal test was positive in 12 cases, with a sensitivity of 40% and specificity of 75%. Of the Widal positive cases, titers of 1:160, 1:320, 1:640 were detected in 58.3, 33.3 and 8.3% of samples, respectively. In schistosomiasis endemic regions, enteric fever was associated with schistosomiasis, which requires investigation of both infections concomitantly. Regardless of the low sensitivity of Widal test, titer of ≥1/160 is a diagnostic value for enteric fever in this study group.

Key words: Schistosomiasis, typhoid and paratyphoid *Salmonella*, detection, Widal test, Sudan.

INTRODUCTION

Typhoid and paratyphoid fever (enteric fever) is an acute systemic infection caused mainly by the bacterium, *Salmonella enteric* serotype *typhi* and other serotypes of *Salmonella paratyphi A, B*, and *C* (Chart et al., 2007; Buckle et al., 2012). It continues to be a global health problem, especially in the tropics and sub tropic countries; over 27 million persons suffer from this disease annually (Buckle et al., 2012). Schistosomiasis is a

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tropical parasitic disease caused by blood fluke worms of the genus *Schistosoma* such as *S. haematobium* and *S. mansoni* (Dabo et al., 2011). *Schistosoma* infection is endemic in many sub-Saharan African countries where the introduction of river regulation and irrigated agriculture commonly results in increasing distribution and prevalence of schistosomiasis (King et al., 2005). The association between bacteria-parasite has been observed as a factor that results in prolonged bacterial infection, such as typhoid and paratyphoid fever in schistosomiasis patients (Lambertucci et al., 1998; Bouree et al., 2002). Concurrent *Schistosoma-Salmonella* infections appear when *Salmonella* species enter the systemic circulation and adhere to the tegument of adult *Schistosoma* through the fimbriae. This interaction can lead to a massive release of occult *Salmonella* (Barnhill et al., 2011). Examinations with the scanning electron microscope showed that pili function by joining *Salmonella* to the surface tegument of *S. mansoni* and *S. haematobium* (LoVerde et al., 1980). Typhoid and paratyphoid *Salmonella* is easily recovered from the blood, feces or urine samples of schistosomiasis patients (Lambertucci et al., 1998). Moreover, enteric fever can be diagnosed by different laboratory methods, including serological tests such as Widal agglutinations or ELISA, culture of clinical specimens of stool, blood and urine (Chart et al., 2007). The Widal test, which detects agglutinating antibodies to somatic lipopolysaccharide O antigens and flagella H antigens was introduced over a century ago and remains a widely used tool for the serological diagnosis of enteric fever (el-Shafie, 1991; House et al., 2001). In Sudan, despite the high endemicity of both schistosomiasis and enteric fever (el-Shafie, 1991; Ahmed et al., 2012; Ibrahim and Ibrahim, 2014), there is little available data on *Schistosoma-Salmonella* infections (Salih et al., 1977). Therefore, the present study aimed to determine the presence of typhoid and paratyphoid *Salmonella* among schistosomiasis patients in Managil region, Central Sudan and to detect the most common *Salmonella* serotypes that cause enteric fever. In addition, it aimed to evaluate the efficacy of the Widal agglutination test used for the diagnosis of enteric fever comparable to cultural methods.

MATERIALS AND METHODS

Study area and population

This is a descriptive cross sectional study conducted between November, 2005 and May, 2006 in Managil Region (156 km South of Khartoum Capital), Gezira State, Central Sudan. The state is an endemic area for schistosomiasis due to the agricultural activities of the populations in the Gezira-Managil irrigation schemes (Hilali et al., 1995). A total of 203 males between 10 to 55 years old participated in the study. The studied subjects were students of the Quran school (n = 148), employees (n = 28) and farmers (n = 27). Those who were previously infected with the infection or under treatment were excluded from the study. Each participant accepted and agreed to participate in the study after informing his parents about the importance of the study. The study was approved by the Committee of Research Council of Faculty of Medical Laboratory Sciences, University of Khartoum.

Samples processing

Clinical samples of urine, stool and blood were collected from each individual and processed for the investigation of schistosomiasis and typhoid and paratyphoid *Salmonella* infection. About 20 ml of urine was collected in sterile plastic container from each subject suspected to have urinary schistosomiasis. To obtain the stool samples, each individual was given a dry and clean container to provide at least 10 g of sample. Stool and urine samples were obtained from each individual, between 10 am and 2 pm, when highest egg excretion occurs (Cheesbrough, 2000b). The diagnosis of *Schistosoma* infection was carried out in the study field by applying direct microscopic examination of the samples. Two smears were prepared from each stool sample and examined for the presence of *S. mansoni* eggs using standard Kato-Katz method (Katz et al., 1972). The urine centrifugation technique was used to detect the presence *S. haematobium* eggs as previously described (Cheesbrough, 2000b). Then, about 5 ml of venous blood was collected from each subject, having schistosomiasis in a clean, dry sterile plain tube, and allowed to clot at room temperature. The sera were separated by centrifugation at 13,000 rpm for 5 min, transferred into clean, sterile plain tubes, and stored at -20°C for further Widal agglutination test.

Each sample of urine or stool yielded positive result; schistosomiasis was cultured immediately in 5 ml of sterile selenite F broth (SFB) (Oxoid, Basingstoke, England) for further isolation and identification of possible pathogens of typhoid and paratyphoid *Salmonella* at the Research Laboratory of Faculty of Medical Laboratory, University of Khartoum.

Isolation and identification of *Salmonella* species

Isolation of *Salmonella* species from urine and stool samples was done by following the standard laboratory methods (Cheesbrough, 2000a). All the samples containing SFB were sub-cultured on xylose lysine deoxycholate (XLD) (Oxoid, Basingstoke, England) and deoxycholate citrate agar (DCA) (Oxoid, Basingstoke, England). They were incubated overnight at 37°C. The plates were then examined for the presence of non-lactose fermenting colonies. Suspected colonies of *Salmonella* isolates were identified on the bases of colonial morphology, gram staining, biochemical tests, and they were confirmed serologically using monovalent and polyvalent antisera (Cheesbrough, 2000a).

Widal test for investigating enteric fever

Widal agglutination test was performed to examine *Salmonella* serotypes using O and H antigens of *Salmonella typhi* and *Salmonella paratyphi A, B* and *C* antigens as described by House et al. (2001). Before carrying out the test, the serum samples (n=50) were divided into two categories: group A collected from culture proven cases and group B from culture negative cases. Widal agglutination reagent kits (Plasmetec, UK) test was performed in both groups according to the manufacturer's instruction. Briefly, each serum sample was diluted serially starting from 1:80 to 1:1280 with 0.85 NaCl in two rows of test tubes for the detection of O and H agglutination. Single drops of O and H antigens were added to corresponding tubes and were incubated at 37°C in a water bath for 18-24 h. The tubes were examined macroscopically and microscopically for the presence of agglutination. Partial or complete agglutination with variable

Table 1. Distribution of *Salmonella* serotypes among Schistosomiasis patients in an endemic area in Sudan.

Type of infection	Number of *Salmonella* isolates	*Salmonella* serotype			
		S. typhi	*S. typhi A*	*S. paratyphi B*	*S. paratyphi C*
S. haematobium (n=42)	23	16	3.0	4.0	0.0
S. mansoni (n=8)	7.0	3.0	2.0	1.0	1.0
Total	**30**	**19 (63.3%)**	**5.0 (16.7%)**	**5.0 (16.7%)**	**1.0 (3.3%)**

Table 2. Comparison between culture method and Widal test in diagnosis of typhoid fever.

Number of cases	Culture	Widal test
15	-	-
18	+	-
12	+	+
5	-	+
Total (n = 50)	**60 % (30/50)**	**34%(17/50)**

degrees of clearing the supernatant fluid was recorded as a positive result.

Statistical analysis

Data were analyzed using SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL, USA). The prevalence and descriptive analysis was calculated. Considering culture results as the standard method, the sensitivity and specificity of the Widal test results were interpreted and calculated using the following formulas:

Sensitivity is $a/(a+c)$, specificity is $d/(d+b)$,

Where, a is test positive and true culture positive, b is test positive and true culture negative, c is test negative and true culture positive, and d is test negative and true culture negative.

RESULTS

Of the 203 subjects whose urine and stool samples were screened for the presence of *Schistosoma* eggs, 50 (24.6%) were found to be infected with schistosomiasis. The majority of the positive cases were students (n = 46), followed by farmers (n = 3) and the employee (n = 1). Out of the 203-screened subjects, 42 (20.7%) cases were caused by S. *haematobium*, and 8 (3.9%) cases were due to *S. mansoni* infection.

Distribution of *Salmonella* serotypes among schistosomiasis patients

A total of 50 urine and stool samples were cultured for the presence of *Salmonella* organisms. Of these, 30 (60%) samples yielded positive results for different serotypes of *Salmonella* and were considered as a true positive for the presence of enteric fever. The most common *Salmonella* serotypes isolated from schistosomiasis patients were *S. typhi* (63.3%; 19/30), followed by *S. paratyphi A* and *B* (16.7%; 5/30, each) and *S. paratyphi C* (3.3%; 1/30) (Table 1).

Evaluation of Widal agglutination test

Table 2 summarizes the cultural and serological results obtained from the schistosomiasis patients. Based on the culture results (n = 30) as a diagnostic method for detecting the presence of enteric fever, Widal test was found to be positive in 12 cases (group 1), with a sensitivity of 40% (12/30) and specificity of 75% (15/20). Of the 12 Widal positive cases, titer of 1:160 was detected in seven (58.3%) samples, titer of 1/320 was detected in four (33.3%) samples and titer of 1:640 was detected in one (8.3%) sample. Among the 20 culture negative cases (group 2), four (20%) samples were given anti *Salmonella* antibody titer of 1:80, whereas titer of 1:160 was detected in one (5%) sample (Figure 1). These findings indicated that titer of equal or more than 1:160 value for both O and H agglutinins is a diagnostic titer for detecting the presence of enteric fever.

DISCUSSION

In our setting, we found that 60% of schistosomiasis patients carried typhoid and paratyphoid *Salmonella*. The presence of *Salmonella* organisms in schistosomiasis patients has been reported in other studies (Tuazon et al., 1985; Barnhill et al., 2011). Furthermore, *Schistosoma*-*Salmonella* interactions are seen in all species of *Schistosoma*, notably *S. haematobium*, *S. mansoni*, *S. intercalatum* and *S. japonicum* (Gendrel, 1993). This association may play an important role in the persistent or delayed *Salmonella* infections (Bouree et al., 2002). In an earlier study, Gendrel et al. (1986) reported that Salmonella infection was clinically prolonged by bilharziasis in 1 out of 3 patients. This could be explained by a decreased host immune response following schistosomiasis (Bouree et al., 2002). Therefore, bacterium-host-parasite interaction may in part explain why *Salmonella* infection and schistosomiasis clinically occur frequently together and present difficult therapeutic problem (Young et al., 1973). However, such

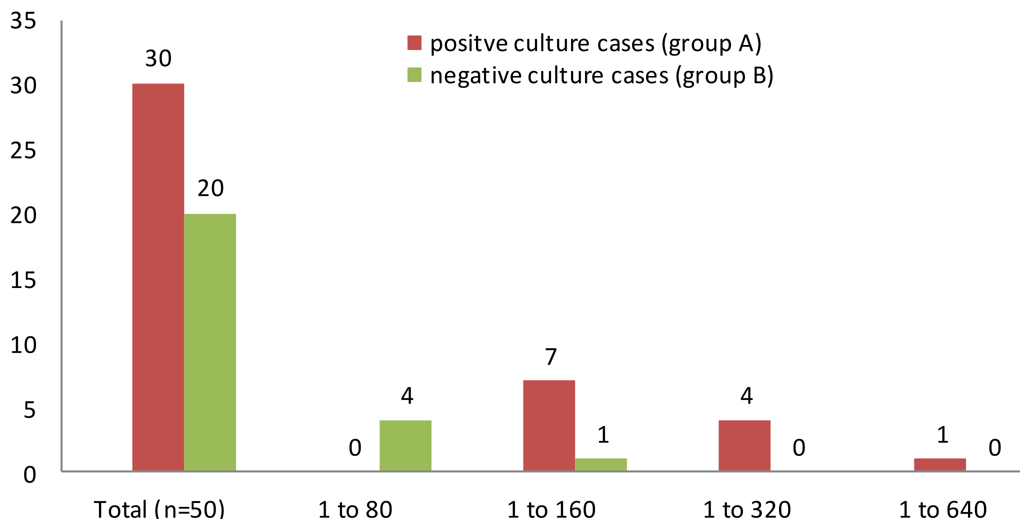

Figure 1. Widal agglutination titers determined from culture positive and negative results of enteric fever among schistosomiasis patients (n=50) in Sudan.

infections need to be treated concomitantly (Gendrel et al., 1986).

Culture methods of clinical specimens remain the most accurate diagnostic procedure for isolating the causative organisms of suspected enteric fever (Chart et al., 2007; Wain and Hosoglu, 2008). In our setting, among the 50 schistosomiasis patients, positive culture results in different types of typhoid, and paratyphoid *Salmonella* was recorded in 60% (30/50) cases (Table 1). In this study, we found that *S. typhi* was the most frequent isolate that represented 63.3% of the isolates. Equal isolation rate was recorded for *S. paratyphi A* and *B* (16.7%, each), and one (3.3%) isolate was found to be *S. paratyphi C*. These findings indicate that the incidence of typhoid fever in schistosomiasis patients is more frequent than paratyphoid fever. Similar findings have been reported earlier among Sudanese patients (Salih et al., 1977). Other studies have reported different serotypes of *Salmonella* among the general population instead of schistosomiasis patients. Shetty et al. (2012) have reported that out of 103 *Salmonella* isolates, 85 (82.52%) were *S. typhi*, 16 (15.53%) were *Salmonella paratyphi A* and two (1.94%) were *Salmonella paratyphi B*. On the contrary, the isolation rate of *S. paratyphi* A was 1.5 times higher than that of *S. typhi, as* reported by others (Palit et al., 2006).

In the present study, among the 30 culture proven cases, 40% yielded significant Widal agglutination reactions. This level is similar to that recorded in Turkey (Hosoglu et al., 2008), but lower than that reported in Pakistan, where the Widal test was positive in 73.68% culture positive cases of enteric fever (Khoharo, 2011). Nevertheless, the Widal agglutination test has been widely used in many developing countries for diagnosing enteric fever, but it has a low sensitivity, specificity, which varies between the geographical areas (House et al., 2001; Omuse et al., 2010). In considering the cultural methods as a gold standard test for the diagnosis of enteric fever, we determined the reliability of the Widal test. We found that its sensitivity was 40%, with a specificity of 75%. This is in line with the results obtained in Bangladesh, where the Widal agglutination test yielded a sensitivity of 42.85% and a specificity of 85.0% (Begum et al., 2009). Likewise, many studies have evaluated the efficacy of the Widal agglutination test (Wain et al., 2008; Ley et al., 2010). Sharing of O and H antigens by other *Salmonella* serotypes and members of *Enterobacteriaceae* makes the role of Widal test even more controversial in diagnosing typhoid fever (Hosoglu et al., 2008). In this study, our findings indicated that Widal test has a low sensitivity and specificity; hence the need for alternative methods in order to improve laboratory diagnosis of enteric fever.

The interpretation of the Widal agglutination test becomes problematic, with a great number of articles reporting different diagnostic cut-off values (Wain and Hosoglu, 2008). Since there are no current data available regarding baseline titers of Widal test among schistosomiasis patients in the Sudan, this study was undertaken to compile the baseline titers for these specific populations. Widal agglutination titer of equal or more than 1:160 was represented among all the culture proven cases. These findings confirmed that the titer of equal or more than 1:160 is a diagnostic titer of enteric fever among schistosomiasis patients. In a previous study among healthy population in Sudan, el-Shafie et al. (1991) reported that a titer above 1:320 suggests the diagnosis of *S. typhi*; 1:160 for both *S. paratyphi B* and *S. paratyphi A.* Regardless of schistosomal infections, different cut-off values of Widal test have been recorded

as a diagnostic titer for typhoid and paratyphoid fever in other studies (Ley et al., 2010; Omuse et al., 2010). Therefore, in order to use the Widal test effectively, each endemic area should determine the appropriate titer for the diagnosis of typhoid and paratyphoid *Salmonella* (Willke et al., 2002).

Conclusion

The study concludes that in schistosomiasis endemic areas, there is a direct relationship between *Schistosoma* - *Salmonella* infection that needs routine screening for the presence of typhoid and paratyphoid fever among schistosomiasis patients. In our setting, *S. typyi* was found to be the most *Salmonella* organisms causing this syndrome (63.3%). Bacteriological techniques are more sensitive and accurate than the serological test in the diagnosis of *Schistosoma* -*Salmonella* relationship. Regardless of the low sensitivity of Widal test, titer of equal or more than 1:160 is a diagnostic cut-off value for enteric fever in this study group.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Ahmed AM, El Tash LA, Mohamed EY, Adam I (2012). High levels of Schistosoma mansoni infections among schoolchildren in central Sudan one year after treatment with praziquantel. J. Helminthol. 86(2):228-232.

Barnhill AE, Novozhilova E, Day TA, Carlson SA (2011). *Schistosoma*-associated *Salmonella* resist antibiotics via specific fimbrial attachments to the flatworm. Parasit. Vectors 4:123.

Begum Z, Hossain MA, Musa AK, Shamsuzzaman AK, Mahmud MC, Ahsan MM, Sumona AA, Ahmed S, Jahan NA, Alam M, Begum A (2009) Comparison between DOT EIA IgM and Widal Test as early diagnosis of typhoid fever. Mymensingh Med. J. 18:13-17.

Bouree P, Botterel F, Romand S (2002). Delayed Salmonella bacteriuria in a patient infected with Schistosoma haematobium. J. Egypt Soc. Parasitol. 32(2):355-360.

Buckle GC, Walker CL, Black RE (2012). Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. J. Glob. Health 2(1):010401.

Chart H, Cheasty T, de Pinna E, Siorvanes L, Wain J, Alam D, Nizami Q, Bhutta Z, Threlfall EJ (2007). Serodiagnosis of Salmonella enterica serovar Typhi and S. enterica serovars Paratyphi A, B and C human infections. J. Med. Microbiol. 56(9):1161-1166.

Cheesbrough M (2000a) Microbiological tests. In: *District Laboratory Practice in Tropical Countries* Part II. Cambridge University Press, Cambridge.

Cheesbrough M (2000b) Parasitological tests. In: District Laboratory Practice in Tropical Countries Part I. Cambridge University Press, Cambridge.

Dabo A, Badawi HM, Bary B, Doumbo OK (2011). Urinary schistosomiasis among preschool-aged children in Sahelian rural unities in Mali. Parasit. Vectors 4:21.

el-Shafie S (1991). The Widal test in a normal healthy population in the Sudan. East. Afr. Med. J. 68(4):266-269.

Gendrel D (1993). [Salmonella-Schistosoma interactions]. Rev. Prat. 43(4):450-452.

Gendrel D, Richard-Lenoble D, Nardou M, Moreno JL, Kombila M, Engohan E, Moussavou A, Galliot A, Toure R (1986). Interaction between Salmonella and Schistosoma intercalatum. Presse. Med. 15(15):689-692.

Hilali AH, Madsen H, Daffalla AA, Wassila M, Christensen NO (1995). Infection and transmission pattern of Schistosoma mansoni in the Managil irrigation scheme, Sudan. Ann. Trop. Med. Parasitol. 89(3):279-286.

Hosoglu S, Boşnak V, Akalin S, Geyik MF, Ayaz C (2008). Evaluation of false negativity of the Widal test among culture proven typhoid fever cases. J. Infect. Dev. Ctries. 2(6):475-478.

House D, Wain J, Ho VA, Diep TS, Chinh NT, Bay PV, Vinh H, Duc M, Parry ChM, Dougan G, White NJ, Hien TT, Farrar JJ (2001). Serology of typhoid fever in an area of endemicity and its relevance to diagnosis. J. Clin. Microbiol. 39(3):1002-1007.

Ibrahim AM, Ibrahim ME (2014). Evaluation of microscopical and serological techniques in the diagnosis of *Schistosoma mansoni* infection at Sennar State, central Sudan, Asian Pac. J. Trop. Dis. 4:8-13.

Katz N, Chaves A, Pellegrino J (1972). A simple device for quantitative stool thicksmear technique in schistosomiasis mansoni. Rev. Inst. Med. Trop. Sao Paulo 14(6):397-400.

Khoharo HK (2011). A comparative study of the typhidot (Dot-EIA) and Widal tests in blood culture positive cases of typhoid fever. Trop. Doct. 41(3):136-138.

King CH, Dickman K, Tisch DJ (2005). Reassessment of the cost of chronic helmintic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. Lancet. 365(9470):1561-1569.

Lambertucci JR, Rayes AAM, Serufo JC, Gerspacher-Lara R, Brasileiro-Filho G, Teixeira R, Antunes CMF, Goes AM, PCoelho PMZ (1998). Schistosomiasis and associated infections. Mem. Inst. Oswaldo Cruz. 93 (Suppl 1): 135-139.

Ley B, Mtove G, Thriemer K, Amos B, von Seidlein L, Hendriksen I, Mwambuli A, Shoo A, Malahiyo R, Ame SM, Kim DR, Ochiai LR, Clemens JD, Reyburn H, Wilfing H, Magesa S, Deen JL (2010). B Evaluation of the Widal tube agglutination test for the diagnosis of typhoid fever among children admitted to a rural hdospital in Tanzania and a comparison with previous studies.MC Infect. Dis. 10:180.

LoVerde PT, Amento C, Higashi GI (1980). Parasite-parasite interaction of *Salmonella typhimurium* and *Schistosoma*. J. Infect. Dis. 141(2):177-185.

Omuse G, Kohli R, Revathi G (2010). Diagnostic utility of a single Widal test in the diagnosis of typhoid fever at Aga Khan University Hospital (AKUH), Nairobi, Kenya. Trop. Doct. 40(1): 43-44.

Palit A, Ghosh S, Dutta S, Sur D, Bhattacharya MK, Bhattacharya SK (2006). Increasing prevalence of Salmonella enterica serotype Paratyphi-A in patients with enteric fever in a periurban slum setting of Kolkata, India. Int. J. Environ. Health Res. 16(6):455-459.

Salih SY, Subaa HA, Asha HA, Satir AA (1977). Salmonellosis complicating schistosomiasis in the Sudan. J. Trop. Med. Hyg. 80(1):14-18.

Shetty AK, Shetty IN, Furtado ZV, Antony B, Boloor R (2012). Antibiogram of salmonella isolates from blood with an emphasis on nalidixic Acid and chloramphenicol susceptibility in a tertiary care hospital in coastal karnataka: a prospective study. J. Lab. Physicians. 4(2): 74-77.

Tuazon CU, Nash T, Cheever A, Neva F (1985). Interaction of Schistosoma japonicum with Salmonellae and other gram-negative bacteria. J. Infect. Dis. 152(4): 722-726.

Wain J, Diep TS, Bay PV, Walsh AL, Vinh H, Duong NM, Ho VA, Hien TT, Farrar J, White NJ, Parry CM, Day NP (2008). Specimens and culture media for the laboratory diagnosis of typhoid fever. J. Infect. Dev. Ctries. 2(6):469-474.

Wain J, Hosoglu S (2008). The laboratory diagnosis of enteric fever. J. Infect. Dev. Ctries. 2(6):421-425.
Willke A, Ergonul O, Bayar B (2002). Widal test in diagnosis of typhoid fever in Turkey. Clin. Diagn. Lab. Immunol. 9(4):938-941.
Young SW, Higashi G, Kamel R, el-Abdin AZ, Mikhail IA (1973). Interaction of salmonellae and schistosomes in host-parasite relations. Trans. R. Soc. Trop. Med. Hyg. 67:797-802.

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Characterisation of yeasts isolated from traditional opaque beer beverages brewed in Zimbabwean households

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Variability exists in raw materials and processing methods used to produce household traditional opaque beers in Zimbabwe, resulting in beers of variable quality, depending on fermenting micro-organisms involved. Yeasts are important in determining the alcohol content, nutrition and organoleptic properties of the beers. This study aimed at determining the diversity and characteristics of the predominant yeasts isolated from a variety of beers collected from rural households in different geographical localizations. Predominant yeasts from 13 beer samples were characterized using morphological, biochemical and physiological tests. A total of 14 morphologically different yeasts were isolated. Yeast counts in the beer samples ranged from 7.87 to 9.56 log colony forming units/ml. From the 14 yeast isolates, a total of 11 yeasts were identified to species level. *Saccharomyces cerevisiae* was the predominant species identified in the beers. Other yeast species identified in the beers were *Issatchenkia occidentalis, Kluyveromyces marxianus, Candida glabrata* and *Sporobolomyces holsaticus.* Two yeast isolates were identified as belonging to the genus *Rhodotorula*. Ten of the isolates were able to ferment at least one of the fermentation substrates D-glucose, D-galactose, maltose, sucrose and raffinose, while three isolates were incapable of fermenting any of the fermentation substrates used. None of the isolates were able to ferment lactose. Five of the *S. cerevisiae* isolates were able to grow at 40°C while *K. marxianus* was the only isolate capable of growing at 45°C.

Key words: Yeasts, characterisation, traditional opaque beer.

INTRODUCTION

Traditional opaque beers in Zimbabwe are of socio-cultural and nutritional value and are marketed for income generation. A variety of cereals are used in the production of traditional opaque beers at household and village levels in Zimbabwe (Sanni, 1993; Mugocha et al., 2000). Most beers are prepared mainly from bulrush millet (*Pennisetum typhoideum*) and finger millet (*Eleusine coracana*) malts, although sorghum (*Sorghum*

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bicolor) malt and sprouted maize are occasionally used (Gadaga et al., 1999). Preparation of traditional opaque beer varies in the different regions of Zimbabwe and various methods have been cited by several reviewers (Benhura and Chingombe, 1989; Chamunorwa et al., 2002; Lyumugabe et al., 2012). The method used to prepare opaque beer is a tradition preserved by the brewers and passed down to the next generation (Liyumugabe et al., 2012). Generally, the preparation of traditional opaque beer includes the cooking of a cereal meal, souring, mashing, straining and alcoholic fermentation for seven days (Beta et al., 1997; Bvochora et al., 2001; Chamunorwa et al., 2002). The beer is consumed in an active state of fermentation and has a short shelf life.

Traditional opaque beer is produced through an uncontrolled, spontaneous fermentation process and the micro-organisms responsible for the fermentation are believed to include yeasts and bacteria from the malt, and from fermentation pots passed from previous brews (Gadaga et al., 1999). Based on the use of various raw materials and chance inoculation, varied yeasts and bacteria are likely to be involved in the production of the traditional beer, resulting in beers with varied organoleptic properties, shelf life and safety. Microbiological and biochemical characteristics of traditional sorghum beers have been studied in many African countries, targeting strain isolation and identification, development of starter cultures and improvement of quality and safety (Maoura et al., 2005; Lyumugabe et al., 2012). Very varied yeasts and bacteria have been found in some African sorghum beers, with *Saccharomyces cerevisiae* and *Lactobacillus* sp. usually predominating (Maoura et al., 2005; Lyumugabe et al., 2010; Kayode et al., 2011). Chamunorwa and co-workers (2002) identified a variety of lactic acid bacteria from sorghum opaque beer brewed in Zimbabwe.

While traditional opaque beer forms a very important part of the Zimbabwean culture, there are no published reports of studies of the varied yeasts involved in the fermentations. Yeasts play an important role in determining the alcoholic content of the beers as well as overall product quality and nutrition. Isolation and characterisation of the diverse yeast microflora is therefore important in potential improvement of the efficiency of fermentation and production of consistent quality and safe beers.

The present study therefore involved isolating, characterising and identifying, where possible, the yeasts responsible for the fermentation to produce traditional opaque beers prepared from various raw materials in various households and villages.

MATERIALS AND METHODS

Collection of traditional opaque beer samples

Thirteen (13) samples of traditional opaque beer were randomly collected from 12 rural households in various regions of Zimbabwe, namely Domboshava, Musana, Chihota, Chipinge and Masvingo. All the beer samples were collected at a stage when the beer was ready for consumption, in an actively fermenting state. Beer samples were collected from the traditional earthenware beer pots and placed in plastic screw-capped bottles. The samples were analyzed within 3 h after sampling.

Microbial analysis of traditional opaque beer samples

Aliquots of the beers (1 ml) were serially diluted using sterile peptone water (Oxoid) and 0.1 ml quantities were plated out on Wort agar (Oxoid) plates to determine yeast counts, respectively. Plates were incubated at room temperature (approximately 26-27°C) for five days then colony counts were carried out using a colony counter (Stuart Scientific). Microbial load was expressed as log colony forming units per millilitre (log cfu / ml) of opaque beer.

Isolation and identification of yeasts

Predominant yeast colonies with distinct morphological differences were picked and purified by streaking three times on Wort agar. Cellular morphology was examined using a Zeiss phase contrast microscope. The sources of the isolates used in the study are shown in Table 2.

The formation of ascospores was examined according to the method described by Van der Walt and Yarrow (1984). The Dalmau plate technique (Kurtzman and Fell, 1998; Barnett et al., 2000) was used to characterise the formation of pseudo- and true hyphae.

Yeast isolates were identified using the conventional methods described by Van der Walt and Yarrow (1984), Kurtzman and Fell (1998) and Barnett et al. (2000). Characterisation of the yeasts was carried out by subjecting the isolates to various physiological and biochemical tests which included fermentation of sugars, liquid assimilation of carbon compounds, assimilation of nitrogen compounds, growth at 25, 30, 37, 40, 42 and 45°C, growth in vitamin free media, cycloheximide resistance, urease test and growth at high sugar concentrations. All tests were carried out in duplicate.

Determination of yeast lipid profiles

Lipids in the yeast cells were extracted using chloroform/methanol (2:1) following the method described by Mpofu et al. (2008). Fatty acids in the lipids were determined by an HP5890 Series II Gas Chromatograph equipped with a Supelcowax 10 column (30 m x 0.55 mm) (Mpofu et al., 2008). Fatty acid profiles detected for the yeast strains were compared with known fatty acid profiles of yeast strains for their identification.

RESULTS AND DISCUSSION

Yeast and lactic acid bacteria counts

Table 1 shows the yeast counts obtained for the opaque beer samples obtained from various Zimbabwean rural households. Yeast counts ranged from 7.87 to 9.56 log cfu/ml.

The range for yeast counts is similar to the range reported by other workers for the Bulgarian cereal-based beverage, *boza* (Gotcheva et al., 2000). Yeast counts in

Table 1. Yeast counts of opaque beer samples from rural Zimbabwean households.

Beer sample number	Site of collection	Raw materials used in beer preparation	Yeast count (log cfu/ml)
1	Domboshava	Maize meal and bulrush millet malt	7.87
2	Domboshava	Maize meal and finger millet malt	8.71
3	Musana	Maize meal and bulrush millet malt	8.91
4	Chihota	Maize meal and sorghum malt	9.22
5	Chipinge	Maize meal, bulrush millet malt and sorghum malt	8.76
6	Chipinge	Maize meal and bulrush millet malt	8.11
7	Chipinge	Maize meal and bulrush millet malt	9.11
8	Chipinge	Maize meal, bulrush millet malt and sorghum malt	9.22
9	Chipinge	Maize meal and sorghum malt	9.38
10	Chipinge	Maize meal and sorghum malt	8.78
11	Masvingo	Finger millet meal and malt	9.25
12	Masvingo	Finger millet meal and malt	9.44
13	Masvingo	Finger millet meal and malt	9.56

Results represent the mean of duplicate sample determinations.

Table 2. Table showing the opaque beer samples from which the characterized yeasts were isolated and the yeast identies.

Yeast reference Number	Beer sample number	Raw materials used in preparation of beer	Site of collection	Yeast isolate identity
1	1	Maize meal and bulrush millet malt	Domboshava	*Saccharomyces cerevisiae*
2	11	Finger millet meal and finger millet malt	Masvingo	*Saccharomyces cerevisiae*
3	2	Maize meal and bulrush millet malt	Domboshava	*Saccharomyces cerevisiae*
4	5	Maize meal and sorghum malt	Chipinge	*Saccharomyces cerevisiae*
5	6	Maize meal, bulrush millet malt and sorghum malt	Chipinge	*Saccharomyces cerevisiae*
6	2	Maize meal and bulrush millet malt	Domboshava	Unidentified
7	2	Maize meal and bulrush millet malt	Domboshava	*Issatchenkia occidentalis*
8	7	Maize meal, bulrush millet malt and sorghum malt	Chipinge	*Kluyveromyces marxianus*
9	6	Maize meal and sorghum malt	Chipinge	*Sporobolomyces holsaticus*
10	8	Maize meal and sorghum malt	Chipinge	*Saccharomyces cerevisiae*
11	11	Finger millet meal and malt	Masvingo	*Rhodotorula*
12	8	Finger millet meal and malt	Masvingo	*Rhodotorula*
13	12	Finger millet meal and malt	Masvingo	*Issatchenkia occidentalis*
14	13	Maize meal, finger millet malt and bulrush millet malt	Masvingo	*Candida glabrata*

the range of 7.41 to 7.50 log cfu/ml were reported for *boza*. The range of yeast counts of 7.78 to 9.56 log cfu/ml recorded for the beers was higher than the range of 4.9 to 6.79 log cfu/g, recorded in Nigerian traditional fermented beverages (Sanni and Lonner, 1993). Microflora involved in many cereal based fermentations is mainly a mixture of lactic acid bacteria and yeasts (Chamunorwa et al., 2002; Kayode et al., 2011; Luymugabe et al., 2012).

Identification of yeasts

Fourteen morphologically different yeasts were isolated from the 13 traditional opaque beer samples. The isolates were coded 1 to 14. Table 2 shows the yeast isolates and the opaque beer samples from which the studied yeasts were isolated.

From the 14 yeasts isolated, 11 were identified to species level, 2 were identified to the genus level and 1

Table 3. Fermentation of different sugars by yeasts isolated from opaque beer.

Sugar	Yeast reference number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
D-Glucose	+	+	+	+	+	+	+	+	-	+	-	-	+	+
D-Galactose	+	+	+	+	+	-	-	+	-	+	-	-	-	-
Maltose	+	+	+	+	+	+	-	+	-	+	-	-	-	-
Sucrose	+	+	+	+	+	-	-	+	-	+	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	+	+	+	+	+	-	-	+	-	+	-	-	-	-

isolate was not identified. Yeast isolates identified were *S. cerevisiae*, *Issatchenkia occidentalis*, *Kluyveromyces marxianus*, *Sporobolomyces holsaticus* and *Candida glabrata.* Isolates 11 and 12 were identified as belonging to the genus *Rhodotorula*, the nearest species being *Rhodotorula mucilaginosa* and *Rhodotorula minuta*. *K. marxianus* species was previously isolated from sorghum grain and sorghum beer in South Africa and from *pozol*, fermented maize dough in Mexico (Barnett et al., 2000). In this study, isolate *K. marxianus* was isolated from traditional opaque beer brewed using maize meal, bulrush millet malt and sorghum malt. The *S. holsaticus* species is commonly found in leaves (Kurtzman and Fell, 1998) and may be originating from leaves used for sieving the beer in some beer-brewing households. Isolates 11 and 12 were identified up to genus level as *Rhodotorula.* The nearest species may be *R. mucilaginosa* or *R. minuta*, according to the identification keys used (Barnett et al., 2000). *Rhodotorula* yeasts were isolated from traditional opaque beer brewed using finger millet meal and finger millet malt in Masvingo. *R. mucilaginosa* is reported to have been previously isolated from pasteurized beer in Germany and from malt syrup (Kurtzman and Fell, 1998). Isolate 14 was identified as belonging to the genus *Candida*. Isolate 14 was identified as *C. glabrata*, which was previously isolated from sorghum malt (Kurtzman and Fell, 1998) and was isolated in a brew using finger millet malt and bulrush millet malt in this study.

Morphological characteristics of yeasts

S. cerevisiae isolates were characterized by cream to pale brown colonies, round to ovoid cells, multipolar budding and ascospore formation. *I. occidentalis* isolates had pale-cream, butyrous colonies with margins fringed with pseudohyphae and ovoid ascospores. *K. marxianus* was characterized by cream, flat butyrous colonies, cylindrical, highly branched cells and cylindrical asci. Yeast cells of the species *S. holsaticus* were characterized by pink, mucoid colonies, blastospores in chains and kidney-shaped ballistoconidia borne on stalks. Yeasts belonging to the genus *Rhodotorula* had pink, butyrous colonies and spheroidal cells, often in chains. The morphological and cultural characteristics of the *Candida* species showed that the species had smooth, flat, cream colonies, round to ovoid cells and no ascospores were observed in this species.

Fermentation of sugars

None of the *S. cerevisiae* isolates could ferment lactose while the majority of the *S. cerevisiae* isolates were able to ferment D-glucose, D-galactose, sucrose and raffinose (Table 3). Due to its ability to ferment a wide range of sugars, *S. cerevisiae* has been found to predominate in most traditional opaque beers and other fermented beverages (Mugula et al., 2003; Lyumugabe et al., 2010) and is used in commercial production of beer, wine and bread. Most of the yeast isolates were able to ferment D-glucose, D-galactose, sucrose and raffinose. *K. marxianus* was capable of fermenting glucose, galactose, sucrose and raffinose. *K. marxianus* and its anamorph, *Candida kefyr* are important yeast species in sorghum fermented beverages because of their ability to ferment a wide range of sugars. *C. glabrata* could ferment glucose only out of the sugars studied. *I. occidentalis* species is capable of fermenting glucose and has been isolated before from bread and bakers' yeast (Barnett et al., 2000).

Assimilation of carbon compounds

Table 4 shows the carbon assimilation pattern of the various yeast isolates. The majority of *S. cerevisiae* yeast isolates were capable of assimilating D-glucose, D-galactose, sucrose, maltose, α, α-trehalose and raffinose. Assimilation of lactate is a variable characteristic in *S. cerevisiae* (Kurtzman and Fell, 1998) and isolates 4 and 5 were incapable of assimilating lactate. Isolate 6, which was not identified in this study, had most physiological characteristics similar to *S. cerevisiae* but differed in its ability to assimilate L-arabinose, L-arabinitol and D-mannitol. *I. occidentalis* yeast species were able to assimilate D-glucose, glycerol, lactate, succinate and

Table 4. Assimilation of carbon compounds.

Carbon compound	Yeast reference number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	-	-
L-Sorbose	-	-	-	-	-	-	-	-	+	-	+	+	-	-
D-Glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-	-	+	-	+	+	-	-
D-Xylose	-	-	-	-	-	-	-	+	-	+	+	-	-	-
L-Arabinose	-	-	-	-	-	+	-	-	+	-	+	+	-	-
D-Arabinose	-	-	-	-	-	-	-	-	+	-	+	+	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Maltose	+	+	+	+	+	+	-	-	-	+	+	+	-	-
α,α,Trehalose	+	+	+	+	+	+	-	-	-	-	+	+	-	+
Methyl α Glucoside	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Soluble starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	+	+	+	-	-	-	+	-
Meso erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	+	-	+	+	-	-
Xylitol	-	-	-	-	-	-	-	+	-	-	+	+	-	-
L-Arabinitol	-	-	-	-	-	+	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 keto D-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Gluconate	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DL-Lactate	-	+	+	-	-	-	+	+	-	-	-	-	+	-
Succinate	-	-	-	-	-	-	+	+	+	-	+	+	+	-
Citrate	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propan 1,2 diol	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Butan 2,3 diol	-	-	-	-	-	-	-	+	-	-	-	-	-	-

ethanol as sole sources of carbon. *K. marxianus*, *S. holsaticus* and *Rhodotorula* isolates were able to assimilate most of the carbon compounds tested while *C. glabrata* managed to assimilate D-glucose; α,α,trehalose; D-gluconate and ethanol only, of the carbon sources tested. Further studies need to be carried out to determine the role of *C. glabrata* in beer.

Assimilation of nitrogen compounds

All the yeast isolates were able to assimilate ammonium sulphate (Table 5). *S. cerevisiae* and *C. glabra*ta species were unable to use nitrate, nitrite, ethylamine, L-lysine, creatine and creatinine as sole nitrogen sources. *I. occidentalis* and *K. marxianus* were able to use ethylamine and L-lysine as nitrogen sources while *S. holsaticus* and the *Rhodotorula* genus yeast isolates were able to assimilate ethylamine.

Growth at different temperatures

All the *S. cerevisiae* yeast strains and *I. occidentalis* were

Table 5. Assimilation of nitrogen compounds.

Nitrogen compound	Yeast reference number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Ammonium sulphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrite	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethylamine	-	-	-	-	-	-	+	+	+	-	+	+	+	-
L-lysine	-	-	-	-	-	-	+	+	+	-	+	+	+	-
Creatine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Creatinine	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6. Growth at different temperatures.

Temperature	Yeast reference number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
25°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	-	+	-	-	+	+
40°C	-	-	+	+	+	+	+	+	-	+	-	-	+	+
42°C	-	-	-	-	-	-	-	+	-	-	-	-	-	-
45°C	-	-	-	-	-	-	-	+	-	-	-	-	-	-

Table 7a. Fatty acid ratios (%) of *Saccharomyces cerevisiae* yeasts and isolate 6 from opaque beer.

Fatty acid	Yeast reference number						
	1	2	3	4	5	6	10
16:0	16.90	9.18	16.16	16.13	14.71	15.78	15.65
16:1	34.18	47.95	39.17	34.70	39.33	34.15	35.55
18:0	11.82	6.95	12.06	13.44	10.66	12.36	12.22
18:1	31.71	34.45	28.90	31.86	33.28	35.81	34.03
18:2	5.38	1.46	3.72	3.87	2.06	1.90	2.54
18:3ω3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18:3ω6	0.00	0.00	0.00	0.00	0.00	0.00	0.00

capable of growing at temperatures from 25 to 37°C (Table 6). *S. cerevisiae* isolates 3, 4, 5 and 10 and *C. glabrata* were able to grow at 40°C. Thermotolerant yeast isolates are very useful in industrial fermentation plants where high fermentation temperatures are used. There are many advantages associated with producing ethanol at higher than conventional temperatures (25-30°C) and these include reduced running costs with respect to maintaining growth temperatures in large scale systems, reduced risk of contamination and increased productivity (Singh et al., 1998). There are several reports on thermotolerant *Kluyveromyces* yeast strains (Banat et al., 1995; Singh et al., 1998), but few reports describe *S. cerevisiae* isolates capable of growth and ethanol production at elevated temperatures (Banat et al., 1995; Abdel-Fattah et al., 2000). The *K. marxianus* species are also thermotolerant, being able to grow at temperatures as high as 45°C, thus making them important for industrial production of fuel ethanol.

Fatty acid ratios

Fatty acid ratios of 16:1 and 18:1 fatty acids were characteristically high in *S. cerevisiae* (Table 7a). Table 7b shows the fatty acid ratios of isolates 7 and 13 and other yeasts besides the *S. cerevisiae* species. The potential use of lipid profiles for yeast identification is under current investigation.

Additional tests

All the *S. cerevisiae* isolates were incapable of growth in

Table 7b. Fatty acid ratios (%) of yeasts isolated from opaque beer.

Fatty acid	Yeast reference number						
	7	8	9	11	12	13	14
16:0	18.50	21.64	18.62	11.74	13.03	20.16	16.26
16:1	19.80	25.13	2.60	2.99	2.16	11.55	31.78
18:0	8.84	5.35	4.64	2.33	3.42	9.96	11.75
18:1	39.42	34.09	39.05	71.80	64.31	49.71	36.53
18:2	10.05	12.64	30.51	9.98	15.48	8.61	3.68
18:3ω3	3.38	1.05	4.57	1.15	1.60	0.00	0.00
18:3ω6	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 8. Results of additional characterisation tests carried out on yeasts isolated from opaque beer.

Growth in/on	Yeast reference number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.01 % Cycloheximide	-	-	-	-	-	-	-	+	+	-	+	+	+	+
0.1% Cycloheximide	-	-	-	-	-	-	-	+	+	-	+	+	-	-
1% Acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
60% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutin	-	-	-	-	-	-	-	+	-	+	+	-	-	-
Vitamin free medium	-	-	-	-	-	-	+	-	+	-	+	+	+	-
Test														
Diazonium Blue B test	-	-	-	-	-	-	-	-	+	-	+	+	-	-
Starch test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease test	-	-	-	-	-	-	-	-	+	-	+	+	-	-
Ascospore/Basisdiospore	+	+	+	+	+	+	+	+	-	+	-	-	+	-
Pseudo/true hyphae	-	-	-	-	-	-	+	-	-	-	-	-	+	-

vitamin free media and in 0.01 and 0.1% cycloheximide concentrations (Table 8). None of the *S. cerevisiae* isolates displayed urease activity or were able to split arbutin. *R. mucilaginosa* isolates displayed urease activity and revealed ß glucosidase activity by their ability split arbutin.

Conclusion

A wide diversity of yeasts with different characteristics was isolated and identified in traditional opaque beer samples, *S. cerevisiae* being predominant. All the yeast isolates were capable of using glucose as a carbon source. The non-fermenting yeast isolates may contribute to the flavor characteristics of the beer or may be spoilage microbes. Further studies will involve molecular characterization of the yeast isolates in an attempt to better characterize them and determine the role of the various yeast species in determining the overall product quality of the beers. Studies are underway to determine the biochemical characteristics of traditional opaque beers. Several thermo tolerant yeasts were isolated and it is necessary to further research on their fermentation characteristics at the elevated temperatures for potential use in industrial ethanol production.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Abdel-Fattah WR, Fadil M, Nigam P, Banat, IM (2000). Isolation of thermotolerant ethanologenic yeasts and use of selected strains in industrial scale fermentation in an Egyptian distillery. Biotechnol. Bioeng. 68:531-535.

Banat IM, Marchant R (1995). Characterization and potential industrial applications of five novel, thermotolerant, fermentative yeast strains. World J. Microbiol. Biotechnol. 11:304-306.

Barnett JA, Payne RW, Yarrow D (2000). Yeasts: characteristics and identification, Cambridge University Press, Cambridge.

Benhura MA, Chingombe A (1989). Traditional brewing methods in Zimbabwe. Zimbabwe Science News. 23:69-70.

Beta T, Dzama K (1997). Sorghum processing technologies in Southern Africa. In: Cereals: Novel uses and processes. Campbell, GM, Webb, C, Mckee, SL (Ed-s). Plenum Press. New York. pp. 265-271.

Bvochora, JM, Zvauya R (2001). Biochemical changes occurring during the application of high gravity fermentation technology to the brewing of Zimbabwean traditional opaque beer. Proc. Biochem. 37: 365-370.

Chamunorwa AT, Feresu SB, Mutukumira AN (2002). Identification of lactic acid bacteria isolated from opaque beer (*Chibuku*) for potential use as a starter culture. J. Food Technol. Afr. 7:93-97.

Gadaga TH, Mutukumira AN, Narvhus JA, Feresu SB (1999). A review of traditional fermented foods and beverages of Zimbabwe. Int. J. Food. Microbiol. 53:1-11.

Gotcheva V, Pandiella SS, Angelov A, Roshkova ZG, Webb C (2000). Microflora identification of the Bulgarian cereal-based fermented *boza*. Proc. Biochem. 36:127-130.

Kayode APP, Vieira-Dalode G, Linnemann AR, Kotchoni SO, Hounhouigan AJD, van Boekel MAJS., Nout MJR (2011). Diversity of yeasts involved in the fermentation of tchoukoutou, an opques sorghum beer from Benin. Afr. J. Microbiol. Res. 5(18):2737-2742.

Kurtzman CP, Fell JW (1998). The Yeasts - a taxonomic study. Elsevier Science Publishers, Amsterdam.

Lyumugabe F, Gros J, Nzungize J, Bajyana E, Thonart P (2012). Characteristics of African traditional beers brewed with sorghum malt: a review. Biotechnol. Agron. Soc. Environ. 16(4): 509-530.

Maoura N (2005). Identification and typing of the yeast strains isolated from *bili bili*, a traditional sorghum beer of Chad. Afr. J. Biotechnol. 4:646-656.

Mpofu A, Kock JLF, Pretorius EE, Pohl CH, Zvauya R (2008). Identification of yeasts isolated from mukumbi, a Zimbabwean traditional wine. J. Sustainable Dev. Afr. 10(3):88-102.

Mugocha PT, Taylor JRN, Bester BH (2000). Fermentation of a composite finger millet-dairy beverage. World J. Microbiol. Biotechnol. 16:341-344.

Mugula JK, Nnkoa SAM, Narvhus JA, Sorhaug T (2003). Microbiological and fermentation characteristics of *togwa*, a Tanzanian fermented food. Int. J. Food Microbiol. 80:187-199.

Sanni AI (1993). The need for process optimisation of African fermented foods and beverages. Int. J. Food. Microbiol. 18:85-95.

Sanni AI, Lönner C (1993). Identification of yeasts isolated from Nigerian traditional alcoholic beverages. Food Microbiol. 10: 517-523.

Singh D, Banat IM, Nigam P, Marchant R (1998). Industrial scale ethanol production using the thermotolerant yeast *Kluyveromyces marxianus* IMB3 in an Indian distillery. Biotechnol. Lett. 20: 753-755.

Van der Walt JP, Yarrow D (1984). Methods for the isolation, maintenance, classification and identification of yeasts. In: The Yeasts: A taxonomic study. Kreger-van Rij, NJW (Ed). Elsevier Science Publishers, Amsterdam. pp. 45-105.

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