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Full Length Research Paper

Jatropha seed cake supplementation for improved fungal growth and later use as animal feed

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The use of *Jatropha curcas* as a raw material for biodiesel production results in large amounts of a solid residue called Jatropha seed cake (Jc). This residue is composed of lignin, cellulose, toxic compounds and anti-nutritional factors. Though the monomers that make up lignin and tannins could be used in animal feed, the polymer forms are not easily digestible, which necessitates a processing step. White rot fungi are known to produce enzymes capable of degrading these polymers, as well as anti-nutritional factors and some toxic compounds (for example, phorbol ester). Therefore, we evaluated the degradation of lignocellulosic compounds and biomass production by *Pleurotus ostreatus* in pure Jc or with different levels of added agro-industrial residues. After 45 days incubation, the best performing sample in terms of mycelial growth was pure Jc, followed by those supplemented with 20% eucalypt sawdust (JcEs20), 20% eucalypt bark (JcEb20) and 10% coffee husk (JcCh10). Among them, the JcCh10 presented the lowest lignin content after fungal growth. The content of lignin and cellulose/hemicellulose in this substrate were, respectively, 29.19 and 47.27% lower than pure Jc. Thus, *P. ostreatus* has the potential to degrade lignocellulosic compounds found in Jc, and this degradation increases the possibility of using this residue as animal feed.

Key words: Biofuel, lignocellulosic residues, Jatropha seed cake, *Pleurotus ostreatus*, biodegradation.

INTRODUCTION

The hope of finding a renewable source of energy that causes little environmental damage has led to the use of oilseed plants for oil extraction and biofuels production (Gübitz et al., 1999; Lu et al., 2009; Openshaw, 2000). *Jatropha curcas* Linnaeus (1753) has shown good potential for this purpose due to the high oil content in the seeds (Jongschaap et al., 2007; Openshaw, 2000; Lu et al., 2009). In addition, this species adapts well to climatic variations and different soil compositions (Jongschaap et al., 2007), allowing it to be cultivated in different regions. However, after oil extraction, a large amount of lingo-cellulosic residue remains, which is called Jatropha seed cake (Jc). The disposal and reuse of this residue is a challenge for the biodiesel industry.

Phorbol ester and anti-nutritional factors (Makkar et al. 1997) limit the direct use of Jc for animal feed (Gübitz et

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al.,1999; Raheman and Mondal, 2012). However, these compounds can be degraded by bacteria (Joshi et al., 2011) or white rot fungi, such as *Pleurotus ostreatus* (da Luz et al., 2014; Kasuya et al., 2012). Similarly, fungi have been efficiently used to increase the digestibility and nutritional value of agroindustrial residue, such as cocoa husk (Alemawor et al., 2009), *Brachiaria* sp. (Bisaria et al., 1996) and Jatropha seed cake (Kasuya et al., 2012).

Jc has been used as a substrate for white-rot fungal growth (de Barros et al., 2011; Kasuya et al., 2012; da Luz et al., 2013; Da Luz et al., 2014). However, previous studies focused on the degradation of phorbol ester to improve the usefulness of this material. Here, we focused on improving mycelial growth in the substrate. Recently, fungal growth was associated with the improved chemical composition of Jc (Kasuya et al., 2012). Therefore, better fungal growth might increase, for example, its protein content, *in vivo* digestibility and palatability, all of which are desirable characteristics for further uses in animal feed.

We combined Jc with different agro-industrial residues in order to look for substrate compositions that better supported fungal growth and that reduced lignin and cellulose/hemicellulose content, as lignocellulosic compounds have an adverse effect on digestibility of feed (Woodman and Stewart, 1932). This biological processing by fungi may be an effective alternative means of adding economic value to Jc by transforming it into animal feed.

MATERIALS AND METHODS

Microorganism

The fungus used was *P. ostreatus* isolate PLO 6 (KC782771, GenBank, 2013) from the Laboratory of Mycorrhizal Associations of the Universidade Federal de Viçosa. It was grown in a Petri dish containing 20 mL of potato dextrose agar culture medium (PDA, Merck, Darmstadt, Germany) at pH 5.5 ± 0.3 and incubated at 25°C.

Substrate composition and growth conditions

Pure Jc or Jc supplemented with agro-industrial residues were used for PLO 6 growth. The type and amount of agro-industrial residues added to Jc was chosen based on substrate compositions that are commonly used for white rot fungi growth and lignocellulolytic enzymes production (Nunes et al., 2012; Wang et al., 2009). Forty-five grams of substrate were combined with 25 mL of tap water in a 250 mL glass beaker and autoclaved at 121°C for 20 min. The substrate was then innoculated using fungi grown in rice (de Assunção et al., 2012) and incubated for 45 days at 25°C. This incubation time was determined based on a previous study (da Luz et al., 2013). Three grams sample from each flask were used to determine the pH, as well as ergosterol, lignin and cellulose/hemicellulose levels.

Fungal biomass determination using ergosterol

Ergosterol analysis was used to quantify fungal biomass as described previously (Richardson and Logendra, 1997) but with some modifications. Five grams of substrate was triturated and added to 0.3 g polyvinylpyrrolidone (Sigma) and 15 mL of 95% ethanol. This material was centrifuged for 20 min at 4200 x*g* at 4°C. The supernatant was filtered through a Teflon sieve (200 mm x 53 μm) and stored at 4°C before analysis by high performance liquid chromatography (Shimadzu, CLC-ODS reverse phase and UV detection at 280 nm) using a methanol (Sigma) flow rate of 1.0 mL min^{-1}. A standard curve was prepared using ergostatrien-3β-ol (Sigma) dissolved in 95% ethanol. Fungal biomass was determined by the relationship between the ergosterol content and the dry mass of fungal mycelium cultivated in PDA for 15 days. For dry mass deter-mination, the medium colonized by the fungus was filtered through the Teflon sieve, transferred to a porcelain dish and dried at 60°C until mass was constant (Barajas-Aceves et al., 2002).

Lignin content

For total lignin determination, 1 g sample of each substrate before and after fungal colonization was treated with 10 mL of a mixture of 95% ethanol and 5% toluene and 10 mL hot water (100 ± 10°C) to remove wax and mucilage. This material was filtered, washed and dried at 60°C (Van Soest, 1963; Hatfield et al., 1994). Then, 20 mL of 72% sulfuric acid was added, and the material was autoclaved at 121°C for 1 h. After 12 h at 25°C, the material was filtered (Whatman, GF/D) and washed in hot water until the acid was completely removed.

For insoluble lignin determination, the solid material retained in the filter paper was dried at 105°C until its mass was constant. The soluble lignin content was determined in the acid solution before washing with hot water by measuring the difference between absorbance at wavelengths of 215 and 280 nm (Van Soest, 1963; Hatfield et al., 1994).

Cellulose/hemicellulose content

Cellulose/hemicellulose content was quantified in the same acid solution that was used for lignin content determination (Van Soest, 1963; Hatfield et al., 1994). For this assay, 1 mL of sample was added to 3 mL of sodium hydroxide (2 mol L^{-1}) and 1 mL DNS solution (99.5% dinitrosalicylic acid, 0.4% phenol and 0.14% sodium metabisulfite). This mixture was boiled at 100°C for 5 min. Water was then added to make it 5 mL and the absorbance was measured at 540 nm.

pH determination

The pH of each substrate before and after 45 days of fungal growth was measured as described by Sodré et al. (2001). Five grams of each substrate was placed in Erlenmeyer flasks (125 mL) containing 15 mL water. The flasks were kept in a shaker for 12 h at 220 rpm. The material was then left to stand for 1 h and the pH was measured in the supernatant.

Statistical analysis

This experiment was conducted using a completely randomized design with five replicates for each substrate. The data were subjected to analysis of variance, and averages were compared by Tukey's test ($p < 0.05$) using Saeg software (version 9.1, Universidade Federal de Viçosa).

RESULTS

The percentage of lignocellulosic compounds in the substrate was influenced by the types and amount of

Table 1. Lignin and cellulose/hemicelluloses in substrate before and after growth of *Pleurotus ostreatus*.

Susbtrate	Total lignin (%)		Cellulose/hemicelluloses (%)	
	Before	After	Before	After
Jatropha seed cake	33.14±0.70e	25.73±0.54d	8.63 ± 0.47a	6.94 ± 0.37b
Jatropha seed cake + 10% rice bran	35.88±0.33d	22.28±0.20ef	2.40 ± 0.04c	2.26 ± 0.03f
Jatropha seed cake + 20% rice bran	38.30±0.87c	24.99±0.56d	2.36 ± 0.66c	2.23 ± 0.62f
Jatropha seed cake + 10% eucalypt sawdust	38.74±0.14c	28.84±0.10c	2.83 ± 0.31c	2.64 ± 0.28ef
Jatropha seed cake + 20% eucalypt sawdust	43.72±0.38a	33.9±0.29b	2.60 ± 0.05c	2.44 ± 0.04f
Jatropha seed cake + 10% corn cob	30.33±2.26f	29.13±2.17c	2.92 ± 0.37c	2.66 ± 0.33ef
Jatropha seed cake + 20% corn cob	22.37±0.43h	20.97±0.40f	3.74 ± 0.57bc	3.48 ± 0.53de
Jatropha seed cake + 10% eucalypt bark	38.58±1.95c	29±1.46c	4.44 ± 0.40b	3.85 ± 0.34d
Jatropha seed cake + 20% eucalypt bark	41.75±0.04b	40.27±0.03a	5.68 ± 0.86b	5.09 ± 0.77c
Jatropha seed cake + 10% coffee husk	28.01±0.62g	18.22±0.20g	4.04 ± 0.21b	3.66 ± 0.19d
Jatropha seed cake + 20% coffee husk	29.29±0.11fg	23.96±0.08de	4.06 ± 0.13b	3.68 ± 0.11d
Jatropha seed cake + 0.2% $CaCO_3$+$CaSO_4$	33.14±0.70e	27.57±0.58c	8.63 ± 0.47a	8.05 ± 0.43a

In a column, means with different letters differ by analysis of variance and Tukey test at 5% probability.

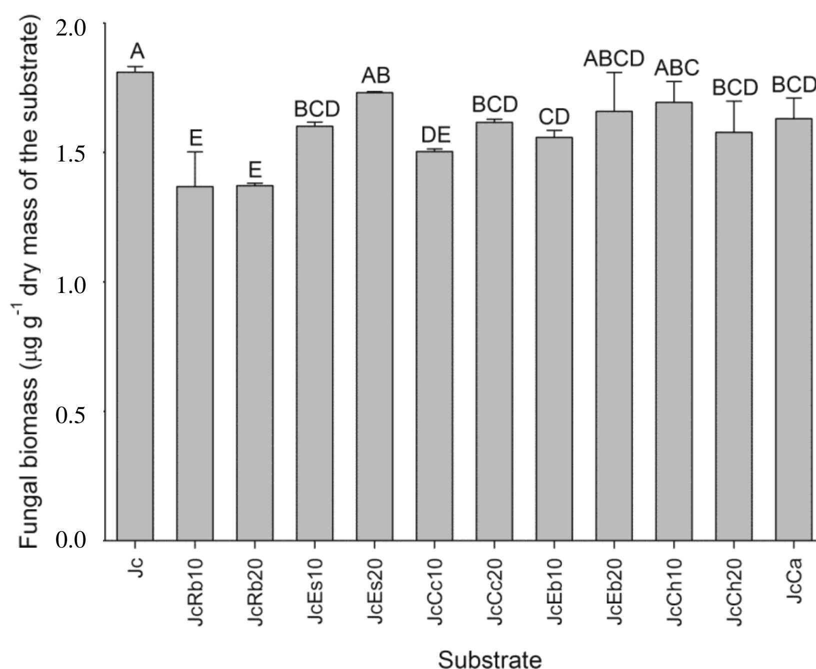

Figure 1. Biomass of *Pleurotus ostreatus* grown at 25°C for 45 days in substrates with different proportions of Jatropha seed cake. Bars followed by the same letters do not differ by Tukey's test (p<0.05). Substrate components are described in Table 1.

agroindustrial residue added in Jc (Table 1; P < 0.05). The cellulose/hemicelluloses content was higher in Jc than in the other substrates (Table 1).

The addition of agroindustrial residues to Jc influenced fungal growth (Figure 1; P < 0.05). Except when rice bran and corncobs were added, any other residue was suitable for fungal growth (Figure 1). The best substrates for *P. ostreatus* growth were Jc-supplemented with 20% eucalypt sawdust, 20% eucalypt bark or 10% coffee husk (Figure 1).

Lignocellulosic compound degradation varied with substrate composition (Figures 2 and 3), leading to differences in the final content of these compounds (Table 1). Coffee husk showed the lowest levels of lignin (P < 0.05), and eucalypt sawdust, the lowest level of cellulose/hemicellulose after fungal growth (P < 0.05).

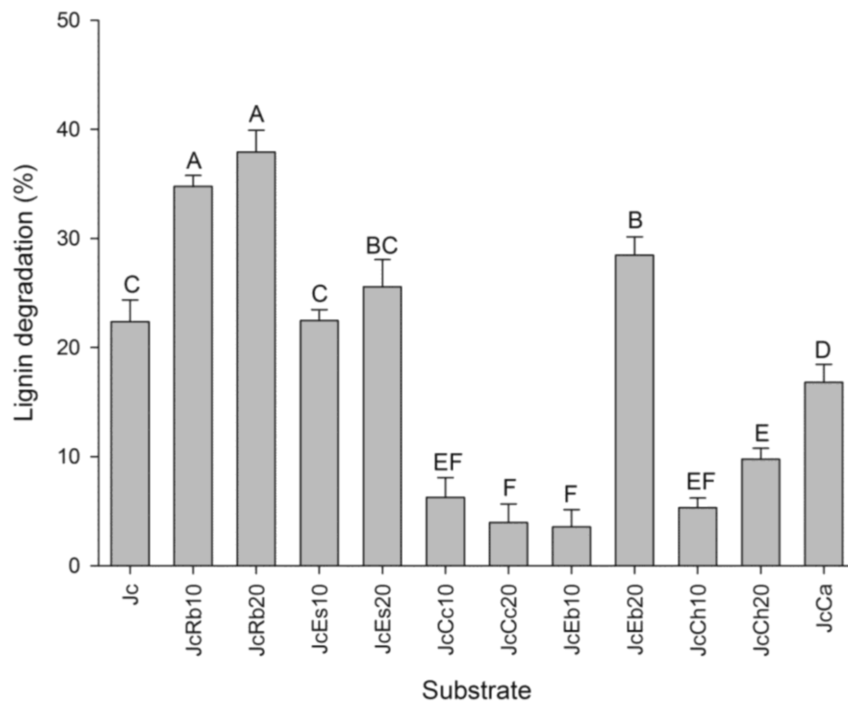

Figure 2. Percentage of lignin degraded by *Pleurotus ostreatus* after growth in substrates with different proportions of Jatropha seed cake at 25°C for 45 days. Bars followed by the same letters do not differ by Tukey's test ($p<0.05$). Substrate components are described in Table 1.

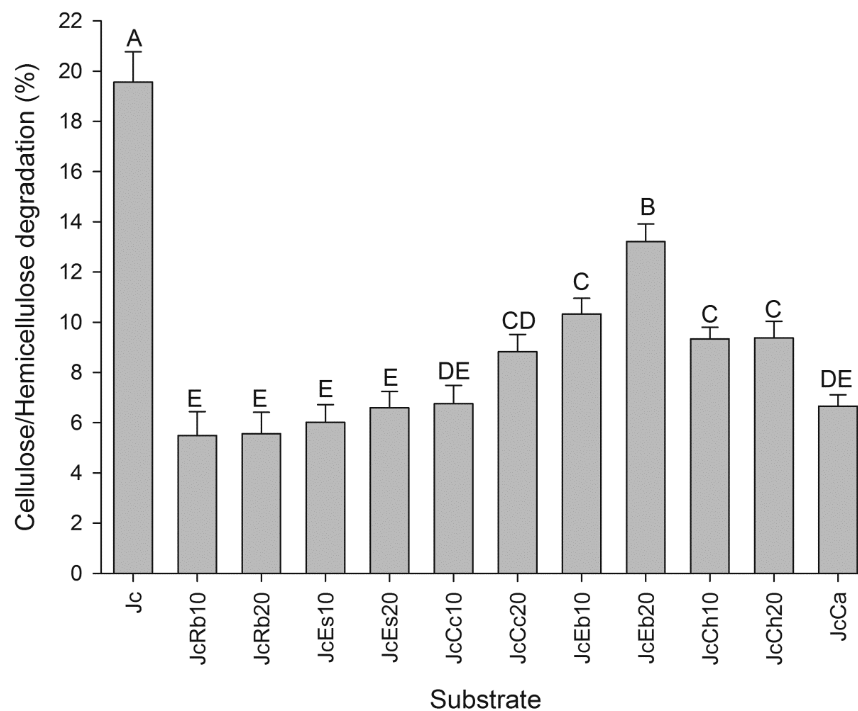

Figure 3. Percentage of cellulose/hemicellulose degraded by *Pleurotus ostreatus* after growth in substrates with different proportions of Jatropha seed cake at 25°C for 45 days. Bars followed by the same letters do not differ by Tukey's test ($p<0.05$). Substrate components are describe in Table 1.

DISCUSSION

In this study, the addition of agroindustrial residues to Jc affected fungal growth (Figure 1), perhaps by altering some physicochemical characteristics of the substrate. The addition of low granulometry substrates, for example, rice bran, likely decreased internal empty spaces available in the substrate (Rossi et al., 2003), reducing gas exchange with the atmosphere and oxygen availability, which is fundamental for aerobic microorganisms. Additionally, the substrate C:N ratio is important for mycelial growth (Mwangi et al., 2012) and alterations in this parameter can significantly change fungal growth rates (Couto et al., 2012; Mwangi et al., 2012). The addition of corncobs, a substrate with lower nitrogen content than Jc, decreased the fungal growth rate (Figure 1). It is worth noting that for better fungal growth, the substrate C:N ratio must be approximately 25:35 (Mwangi et al., 2012). Moreover, the presence of toxic compounds (e.g., caffeine found in the coffee husk) can inhibit or decrease *P. ostreatus* growth (Pandey et al., 2000; Fan et al., 2003). However, this problem seems to be attenuated by performing a prior boil of the substrate (Houdeau et al., 1991; da Silva et al., 2012; de Assunção et al., 2012). Therefore, substrates should be carefully selected to favor fungal growth.

The decreasing lignin and cellulose/hemicellulose degradation seems to influence fungal growth (Figures 2 and 3). Cavallazzi et al. (2004) noted that carbohydrases in *P. ostreatus* can metabolize new carbon resources from polysaccharides, while its lignocellulosic enzymes can break down lignin polymers and phenolic compounds. As lignin and cellulose/hemicellulose are the main source of carbon in Jc (Kasuya et al., 2012), the activity of these enzymes is highly relevant for fungal growth in this substrate. Therefore, it is expected that substrates with more readily available carbon sources (e.g., monosaccharides, cellulose) should support fast and efficient mycelial growth. Indeed, here we observed that *P. ostreatus* grew well in Jc, the substrate with the highest level of cellulose/-hemicellulose (Table 1). However, the addition of agroindustrial residues decreased the level of these compounds (Table 1), which in turn increases the importance of substrate depolymerization by lignocellulolytic enzymes in order to improve availability of carbon for fungal growth (Mata and Savoie, 1998). However, instead of increasing, lignin degradation also decreased when some residues were added (Figure 2), probably reducing carbon availability for mycelial growth, which may explain the reduction in fungal growth (Figure 1). Thus, the addition of substrates with a high level of cellulose/hemicellulose seems to be an interesting strategy to improve fungal growth and expand the potential uses of Jc.

Lignin and cellulose/hemicellulose degradation (Figures 2 and 3) can increase digestible dry mass, given the natural recalcitrance of these compounds (Pérez et al., 2002). Indeed, an increase in digestibility after lignin degradation was shown when *Phanerochaete chrysosporium* was cultivated on cotton stalks (Shi et al., 2009) and *Pleurotus sajor-caju* was cultivated on agroindustrial residues (Bisaria et al., 1996). Here, supplementation with agroindustrial residues was shown to be an effective way to decrease lignin and cellulose/hemicellulose in the substrate before and after fungal growth (Table 1). Among the treatments that did not influence the fungal growth (Figure 1), Jc supplemented with 10% coffee husk had the lowest lignin content (Table 1). Furthermore, the lignin and cellulose/hemicellulose content was lower than pure Jc (Table 1). This difference is important for animal feed, because a negative relationship between digestibility and lignocellulosic compounds content has been observed for more than 70 years (Woodman and Stewart, 1932).

Recently, *P. ostreatus* growth in Jc was observed to increase its *in vitro* digestibility and the amount of non-fiber carbohydrates and crude protein (da Luz et al., 2014), which are all desirable features for animal feed (Montagne et al., 2003). Moreover, Kasuya et al. (2012) showed that Jc after *P. ostreatus* growth was well-accepted by goats, leading to an increase in dry mass intake. The above authors pointed out that consumption and palatability of the feed was not diminished by the inclusion of detoxified Jc. Here, lignin and cellulose/hemicellulose content after fungal growth (Table 1) were lower than that reported by Kasuya et al. (2012). In this study, Jc supplemented with 10% coffee husk and processed via fungal growth showed good potential for use as an animal feed.

However, some considerations about coffee husk should be made, as coffee husk contains antinutritional compounds, such as tannins and caffeine (Pandey et al., 2000). Both compounds were shown to be degraded by *P. ostreatus* (Fan et al., 2003; da Luz et al., 2013), although the degradation of these compounds should be further evaluated, as tannins have an adverse effect on rumen metabolism (Makkar et al., 1995) and caffeine can cause dependence (Griffiths and Woodson, 1988) and, depending on the concentration, can even be toxic to animals (Hosenpud et al., 1995). Indeed, the intake of feed with 20% coffee grounds by rats has been shown to negatively affect dry matter digestibility by depressing feed intake and increasing urinary output and water intake (Campbell et al., 1976). Thus, from this starting point, further studies should evaluate the degradation of tannins and caffeine by fungi in Jc supplemented with 10% coffee husk, and animal assays should be performed using this substrate.

Conflict of interest

The authors have not declared any conflict of interests.

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Review

The immune response of silkworm, *Bombyx mori*

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The silkworm, *Bombyx mori* has been used to study numerous biological phenomena including innate immune response. *B. mori* could be infected by various pathogens such as bacteria, fungi and virus. After infection, these microorganisms induce different types of immune response, including humoral immune responses which secrete antimicrobial peptides (AMPs) into the hemolymph and cellular immune responses which engulfed invading microorganisms by plasmatocytes. Meanwhile, *B. mori* could be also efficiently killed by infection with human pathogens such as *Staphylococcus aureus, Streptoco*ccus pyogenes or Serratia marcescens when these bacterial are injected into the blood. It can now be used as an animal model for bacterial infection study. Here, we will review our current knowledge on molecular mechanisms of antimicrobial peptides production as well as recent progress made in the field of innate immunity in *B.mori*.

Key word: *Bombyx mori*, innate immune response, Toll/IMD pathway, phagocytosis.

INTRODUCTION

The innate immune system is the first-line defense mechanism against various bacteria and viruses infection. Defensive strategies in insect and in mammals are highly conserved at the molecular level, which will help to better understand the molecular mechanisms of innate immunity (Hoffmann et al., 1999; Kimbrell and Beutler, 2001; Muller et al., 2008). It was widely accepted that invertebrates fail to show a high degree of adaptive immune system (Flainik and Du Pasquier, 2004; Loker et al., 2004; Rowley and Powell, 2007). The innate immune responses which include production of antimicrobial substances, phagocytosis, encapsulation, clotting, nodule formation and melanization become the main defensive strategies for insects (Lemaitre and Hoffmann, 2007).

For the completion of the genome sequence of lepidopteran model insect, the silkworm *Bombyx mori,* it has now become the standard model system for pathological and genetic studies in insect (International Silkworm Genome, 2008; Mita et al., 2004; Xia et al., 2004, 2009). For defense against various infections, *B. mori* relies on three types of mechanisms (Ferrandon et al., 2007; Williams, 2007; Yamakawa and Tanaka, 1999). These include humoral immune responses which dependent on antimicrobial peptides (AMPs) secretion into the hemolymph from the fat body (a functional equivalent of the mammalian liver). The second is cellular immune responses, such as the phagocytosis of invading microorganisms by plasmatocytes. The third is an enzymatic cascade leading to melanization. In this review, we summarized recent progress in understanding innate immunity in *B. mori*.

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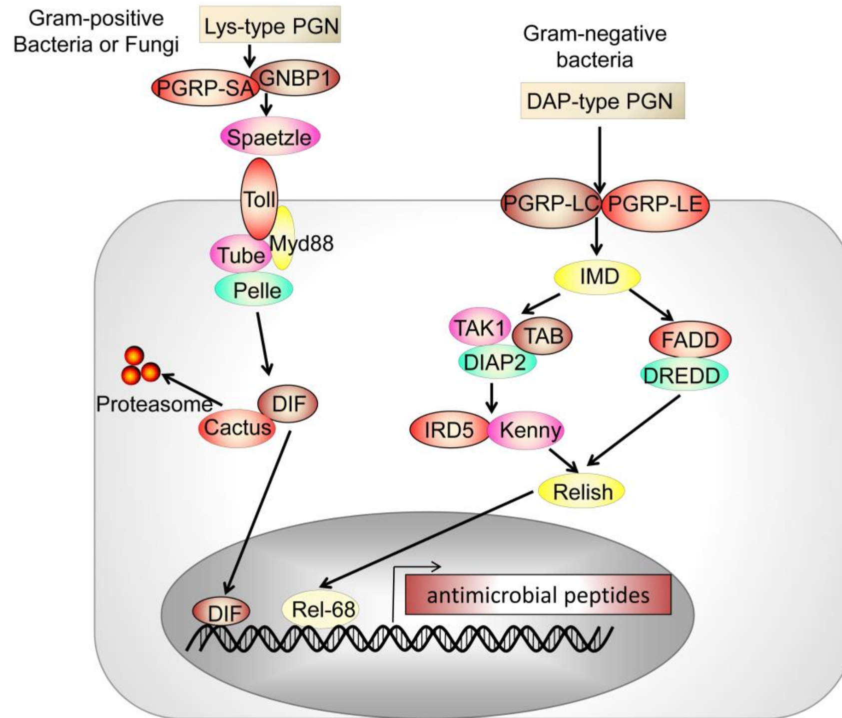

Figure 1. *B. mori* Toll and IMD pathway. The Toll pathway is activated by fungi and Gram-positive bacteria. The Lys-type PGN are recognized by a complex consisting of peptidoglycan recognition protein-SA (PGRP-SA) and Gram-negative binding protein 1 (GNBP1). Recognition of infection triggers a protease cascade that cleaves the precursor of Spatzle into an active form and activates the Toll receptor. Formation of this hetero-trimeric adapter complex leads to nuclear factor-κB (NF-κB)-dependent signaling and antimicrobial peptide (AMP) production. The immune deficiency (IMD) pathway is activated mainly by Gram-negative bacteria. The DAP-type PGN are recognized by a complex consisting of PGRP-LC and PGRP-LE. Recognition of infection induces the formation of a complex comprising of IMD. This complex induces the activation of two cascades and results in the activation of antimicrobial peptide (AMP) production.

HUMORAL IMMUNE RESPONSES

The hallmark of the *B. mori* immune response is the induction of antimicrobial peptides (AMPs) mainly synthesized by the fat body and secreted into the hemolymph (Cheng et al., 2006; Hu et al., 2013; Wu et al., 2010b). Since Steiner et al. (1981) purified and characterized the first AMPs from pupae of the cecropia moth, *Hyalophora cecropia*, more than 200 AMPs have been identified in insects (Bulet et al., 1999). In *B. mori*, 35 AMPs have been found in silkworm genome sequences (Cheng et al., 2006; Kaneko et al., 2008; Wen et al., 2009). These AMPs can be classified into six classes: cecropins, attacins, moricin, lebocin, defensins and gloverines (Bulet et al., 1999; Cheng et al., 2006; Govind, 2008; Imamura et al., 2006).

The expression of the AMPs is regulated by Toll and immune deficiency (IMD) pathways in the fat body (Figure 1). Fungi and Gram-positive bacteria induce the expression of AMP through the Toll signaling pathway, and Gram-negative bacteria induce the expression of AMP through the IMD signaling pathway. Toll and the IMD pathways are reminiscent of mammalian Toll-like receptor (TLR) and tumor necrosis factor-a (TNF-a) pathways (Govind, 2008; Hoffmann, 2003; Lemaitre and Hoffmann, 2007).

B. mori Toll pathway are composed of extracellular cyto-kine Spaetzle (or spätzle), transmembrane receptor Toll, MyD88 adaptors, tubes, Pelle kinase, Dorsal and DIF tran-sac-tivators (Tanaka et al., 2008).

Spaetzle (which is similar to nerve growth factor and coagulogen (Mizuguchi et al., 1998)) was first found as a maternal effect gene that binds to the *Drosophila* Toll receptor. It activates the signal transduction pathways in both embryonic patterning and innate immunity (Morisato and Anderson, 1994). *B. mori* Spaetzle (BmSPZ1) was found to induce the expression of antimicrobial peptide genes when injected with renatured BmSpz1 into the silkworm larvae (Wang et al., 2007). In addition to BmSPZ1, two other Spaetzle homologs BmSPZ2 and BmSPZ3 were recently identified in the *B.mori* genome by

bioinformatic screening (Tanaka et al., 2008). Spaetzle is synthesized and secreted as an inactive precursor and cleaved by a serine protease cascade that are preferentially triggered by Gram-positive or by fungal infection (Valanne et al., 2011). After cleaving with its active C-106, the cleaved form of Spaetzle binds to the Toll receptor and activated Toll receptor. Unlike mammalian Toll-like receptors (TLRs), Toll does not function as a pattern recognition receptor that directly recognized the microbial ligands. Instead Lys-type peptidoglycan (PGN) is recognized either by the PGRP-SA–GNBP1 complex or by PGRP-SD (Lemaitre and Hoffmann, 2007; Valanne et al., 2011; Watanabe et al., 2006). To date, 11 Tolls and 2 Toll analogs genes have been identified in the *B. mori* genome (Cheng et al., 2008; Imamura and Yamakawa, 2002; Tanaka et al., 2008). Expression profiles of Bm Tolls showed that 10 of genes were induced or suppressed with different degrees by different invaders stimulation (Cheng et al., 2008). Bm Toll gene expression was strongly suppressed when lipopolysaccharide (LPS) was injected into silkworm hemocoel (Imamura and Yamakawa, 2002).

Escherichia coli, fungus and *Beauveria bassiana* infection also significantly increased BmToll9 expression in different parts of the gut, suggesting that BmToll9 is probably involved in the local gut immune response (Wu et al., 2010a). The expression of BmToll10-3 gene was significantly increased when infected with *B. mori* nucleopolyhedrovirus (BmNPV) (Sagisaka et al., 2010). The activated Toll receptor recruit a hetero-trimeric complexes composed of MyD88 (homologs of the human Myd88 protein), an adaptor protein Tube and the kinase Pelle (homologs of the human IL1 receptor associated kinase (IRAK)) (Leclerc and Reichhart, 2004; Valanne et al., 2011). *Bacillus bombysepticus* (Bb) infection induced the systemic immune response mainly by the Toll pathway in silkworm. Toll pathway genes including Spz1, Toll1, Toll6, MyD88, Tube and RelA were up-regulated after Bb infection. MyD88 was only expressed after Bb infection (Huang et al., 2009).

Formation of this hetero-trimeric adapter complex leads to rapid phosphorylation and degradation of Cactus (a homologue of the mammalian inhibitor of NF-KB (IKB)) by an uncharacterized mechanism, which is then degraded by the proteasome. As a consequence, the Rel transcription factors DIF are released and move from the cytoplasm to the nucleus (Kawaoka et al., 2008; Lemaitre and Hoffmann, 2007; Tanaka et al., 2005, 2009; Valanne et al., 2011). BmCactus, which constitutively expressed mainly in the fat body and hemocytes, can strongly inhibit activation of the CecB1 gene promoter by either BmRelA or BmRelB (Furukawa et al., 2009). Two BmRelish genes have been identified in the *B. mori*. *E. coli* infection induces the expression of BmRelish1 and BmRelish2 while deletion mutant of BmRelish of the BmRelish gene in transgenic silkworms resulted in failure of the activation of antimicrobial peptide genes (Tanaka et al., 2007).

The canonical components of *B. mori* IMD pathway are composed of IMD, TAK1 (TGF-β activated kinase 1), TAB, DIAP2, two IκB kinase (IKK) complex components, namely IKK-β (IRD5) and IKK-γ (Kenny), which are homologs of mammalian IKK signalosome. In addition, FADD functions as downstream of IMD, controlling the activity of DREDD (a caspase-8 homolog), which acts with the IKK complex to activate Relish (Brennan and Anderson, 2004; Kaneko and Silverman, 2005; Tanaka et al., 2008). Transcriptional profiling of midgut showed that IMD pathway, but not Toll pathway genes were up-regulated during the wandering stage, suggesting that IMD pathway probably regulates the production of antimicrobial peptides in the midgut during the wandering stage (Xu et al., 2012).

PGRP was first purified from hemolymph of the *B. mori* and was found to bind to peptidoglycan and triggered the prophenoloxidase cascade (Ochiai and Ashida, 1999b; Royet and Dziarski, 2007; Yoshida et al., 1996). Insect PGRP genes are divided into two subfamilies (short (S) and long (L) transcripts) based on their structure (Royet and Dziarski, 2007; Royet et al., 2011, 2005). The PGRP family comprises 12 members in *B. mori* with conserved PGRP domains (Figure 2). Six belong to the short (S) subfamily (Tanaka et al., 2008). Comparative proteomic approach identified that PGRP was up-regulated when reared on fresh mulberry leaves when compared with on artificial diet (Zhou et al., 2008). *B. mori* GNBP was found to constitutively express in fat body and rapidly induced following a cuticular or hemoceolien bacterial challenge (Lee et al., 1996). The solution structure of the N-terminal β-1, 3-glucan recognition domain of *B. mori* GNBP3 showed that GNBP3 is a β-1,3-glucan-recognition protein that specifically recognizes a triple-helical structure of β-1,3-glucan (Takahasi et al., 2009).

Cellular response

B. mori larvae contain several thousand hemocytes, which can be divided into the following three cell types on the basis of their structural and functional features: plasmatocytes, crystal cells and lamellocytes (Lemaitre and Hoffmann, 2007; Tan et al., 2013; Wago, 1982; Williams, 2007).

Plasmatocytes are professional phagocytes that function in the phagocytic removal of dead cells and microbial pathogens, which is most similar to the mammalian macrophage lineage and make up 95% of circulating hemocytes. The other 5% of circulating hemocytes consist of crystal cells, which secrete components of the phenol oxidase cascade for the melanization process, as well as for wound repair. A third cell type known as lamellocytes are rarely seen in healthy larvae and primarily function in encapsulation and neutralization of objects too large to be phagocytosed. Phagocytosis and encapsulation are two major mechanisms of the cellular response (Ling et al., 2005; Taniai et al., 1997).

B. mori are efficiently killed by infection with human

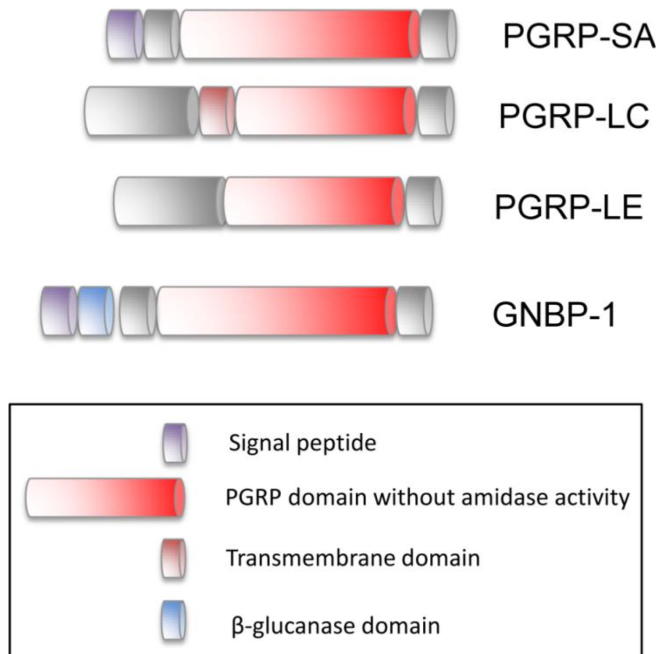

Figure 2. The structure of selected members of the PGRP and GNBP families.

pathogens such as *Staphylococcus aureus, Streptococcus pyogenes* or *Serratia marcescens* when these bacterial are injected into the blood (hemolymph) (Ishii et al., 2012). Silkworm larvae now have been used as an animal model of bacterial infection which is pathogenic to humans due to their low cost and ease of handling (Hamamoto et al., 2004; Ishii et al., 2012; Kaito et al., 2002, 2005; Kaito and Sekimizu, 2007). *B. mori* translationally controlled tumor Protein (BmTCTP) which expresses in intestinal epithet-lial cells and release into the gut lumen can promote the phagocytosis of invading substances by hemocytes (Wang et al., 2013). The phenoloxidase (PO) which exists in the hemocytes cascade regulates the melanization of invading pathogens in *B. mori* (Asano and Ashida, 2001; Diao et al., 2012; Ochiai and Ashida, 1999a; Wang et al., 2013). A high mass complex that contained PO was involved in hemolymph melanization in the *B. mori* (Clark and Strand, 2013). *B. mori* Reeler1 which was strongly induced by *E. coli* K12 and *B. subtilis* in silkworm larval hemocytess is involved in the *B. mori* melanization cascade (Bao et al., 2011).

CONCLUSION

As the completion of the genome sequence of *B. mori,* tremendous progress has been made in the past few years in *B. mori* research especially in *B. mori* innate immunity. However, the molecular mechanisms of *B. mori* response to the infection still need to be further clarified to help in understanding these conserved innate immune response both in insect and mammals.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Xylanase production using fruit waste as cost effective carbon source from thermo-tolerant *Bacillus megaterium*

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Thermotolerant *Bacillus megaterium* BRL-0101 was used to produce xylanase in batch fermentations using various fruit waste as cost effective substrates. A mineral medium based on mango peel (100 g/L) as the carbon source and yeast extract + peptone + $NaNO_3$ (10 g/L) as the nitrogen source proved to be the most effective. A 48-h batch fermentation in this medium with a starting pH of 8.0 produced a xylanase titer of ~2,876 U/mL at the optimal fermentation temperature of 50°C. The optimal temperature, initial pH, the carbon source and its concentration, and the nitrogen sources, size of inoculum, inducer and its concentration, were identified after evaluation of multiple nutrient sources and fermentation conditions.

Key words: Xylanase, *Bacillus megaterium*, fermentation, mango peel.

INTRODUCTION

The cost of enzyme production and low enzyme yields are the major challenges in industrial applications of enzymes. Lignocellulosic biomass is an essentially inexhaustible and renewable carbon source, that consists of cellulose, hemicelluloses and lignin (Harris and Debolt, 2010; Kumar et al., 2008). Xylan is second most abundant polysaccharide after cellulose, the major hemicellulose and accounts for 20-35% of total dry weight in plant biomass. Xylanases (EC. 3.2.1.8) show excellent potential due to their wide industrial applications in pulp bleaching, oligosaccharides production (Kuhad et al., 2010; Ninave et al., 2006; Sanghi et al., 2009), the modification of cereal-based food stuffs, improving the digestibility of animal feed stocks, fruit softening and clarifying juices, texture improvement of bakery products, textile industry, (Nagar et al., 2010; Dhiman et al., 2009; Polizeli et al., 2005), pharmaceutical and chemical applications. Furthermore, xylanases in combination to β-xylosidase are utilized for complete saccharification of lignocellulosic biomass for ethanol production (Rojas et al., 2011; Cavka et al., 2011; Lopez et al., 2011; Abdeshahian et al., 2010; Rajoka and Riaz, 2005; Tengerdy and Szakacs, 2003). Xylanases have an annual worldwide market of about US $ 200 million and the widespread use of xylanase in commercialized industrial applications requires extensive studies to

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optimize their production (Mullai et al., 2010).

Microorganisms are the most common source of industrial enzymes due to their broad biochemical diversity and feasibility of large scale production. Xylanases have been reported mainly from bacteria (Anuradha et al., 2007; Dhiman et al., 2008), fungi (Nair et al., 2008), actinomycetes (Techapun et al., 2002; Ninawe et al., 2006) and yeast (Passoth and Hahn-Hägerdal, 2000). Although several thermophilic micro-organisms have been isolated and exploited for enzymes production and characterization, there is still a need for novel strains capable of producing enhanced levels of enzyme in an economically feasible culture system.

The fermentation performance of microorganism is significantly affected by culture conditions and medium constituents such as: carbon source, nitrogen source, inducer, inoculum size, pH of the media, incubation temperature and agitation rate (Kuhad et al., 1993). For the commercial realization and economic viability of xylanase production, it is necessary to optimize cultural conditions of microorganisms so that higher enzyme production could be obtained. The objectives of present work were to exploit *Bacillus megaterium* for hyper xylanase production from low cost carbon source such as fruit waste and to optimize the fermentation profile of xylanase producing strain.

MATERIALS AND METHODS

Microorganisms

The bacterial strains were collected from Department of Microbiology, University of Baghdad, Baghdad, Iraq and the micro-organisms were grown under laboratory conditions. *Escherichia coli, Bacillus subtilis, Bacillus megaterium* and *Bacillus cereus* capable of xylanase production were used in this study. The microorganisms were maintained on nutrient agar medium containing glucose 20 g/L, peptone 10 g/L and agar 20 g/L (Qureshi et al., 2012).

Fermentation medium

It was composed of (g/L) glucose 20, peptone 10, magnesium sulphate 2, ammonium nitrate 1 and sodium dihydrogen phosphate 2. 50 ml of culture medium was taken in a 250 ml Erlenmeyer flask with an initial pH maintained at 6.0. Flasks were cotton plugged and autoclaved at 1.5 kg/cm^2 for 20 min. 5.0 ml of *Bacillus megaterium* seed culture was inoculated in each flask. The flasks were incubated at 37± 2°C. The samples were collected with regular interval for analyzing growth (OD), residual sugars and xylanase activity. 5 g of fruit wastes were treated with 95 ml (1.0%) diluted H_2SO_4 and kept in autoclave at 115°C for 1 h. After 1 h supernatant was separated and used as source of fermentable sugars for microbial growth and enzyme production.

Optimization of fermentation condition

The parameters that strongly influence the xylanase production such as: incubation time; carbon and nitrogen sources, inducer, pH and temperature were optimized in the present study. Time course of fermentation (12-84 h), 5.0% different carbon sources (orange peel, banana peel mango peel, apple pulp, and oilcake were hydrolyzed with 1.0% H_2SO_4) instead of pure glucose, several organic and inorganic compounds separately and in combination (tryptone, ammonium chloride, potassium nitrate, sodium nitrate, yeast extract and corn steep liquor) as nitrogen source in place of peptone, initial pH (4.5-10) and fermentation temperature (30 to 65°C) were optimized in terms of maximum xylanase production.

Assay of xylanase activity

Xylanase activity was determined by mixing 0.5 ml sample (broth) with 0.5 ml of oat to xylan (Fluka, Germany) (1% w/v) in 50 mM citrate buffer (pH 5.3) at 60°C for 15 min (Bailey et al., 1992). Xylose standard curve was used to calculate the xylanase activity. In the assay, the release of reducing sugars was measured using the dinitrosalicylic acid reagent method (Miller, 1959).

One international unit of enzyme activity was defined as the amount of enzyme, releasing 1 mol of reducing group per minute per mille.

RESULTS

In order to attain maximum xylanase production, fermentation parameters such as nutritional (carbon and nitrogen source and inducer) and physiological (incubation time, size of inoculum, pH, temperature) were optimized. The xylanase production by *B. megaterium* BRL-0101 was highest (2876 IU/mL) under the optimized conditions, that is, peptone 0.25%, yeast extract 0.50%, sodium nitrate 0.25%, mango peel 10.0%, pH 8.0, temperature 50°C, incubation time 48 h, agitation rate 150 rpm and using 10.0% (v/v) inoculum. Figure 1 shows the xylanase production from different bacterial species such as *B. subtilis, E. coli, B. cereus* and *B. megaterium*. *B. megaterium* produced better xylanase titer among the tested organisms. The effect of different fruit waste such as banana peel, mango peel, orange peel and apple peel and oilcake as carbon source were observed on enzyme production. The *B. megaterium* BRL-0101 showed 167 IU/mL xylanase activity with 10% mango peel as carbon source. The enzyme titer in the presence of other carbon sources such as; banana peel, mango peel, apple peel and oilcake was much lower as compared to mango peel (Figure 2). Xylanase production was found to vary with change in the concentration of mango peel as sole carbon source. The enzyme activity was measured in the presence of 1-10.0% (v/v) mango peel. Xylanase production was found to be highest with 10.0% (w/v) mango peel for *B. megaterium* BRL-0101 (Figure 3).

Figure 4 shows the time profile of xylanase production, bacterial growth and residual sugars. The highest xylanase activity of *B. megaterium* BRL-0101 (532 IU/mL) was observed after 48 h of incubation but decreased thereafter (Figure 4). The xylanase production show strong correlation with growth, the maximum growth was observed at 24 h and enzyme titer after 48 h. Effect of inoculum size (1-10%) on xylanase activity of *B. megaterium* was tested. Xylanase production increased

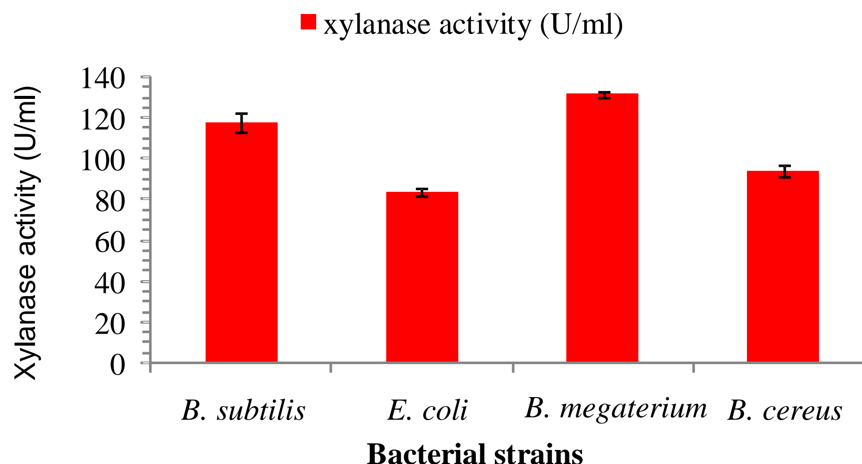

Figure 1. Xylanase production from different bacterial species using glucose as carbon source after 24 h incubation at 37°C, initial pH 6.0.

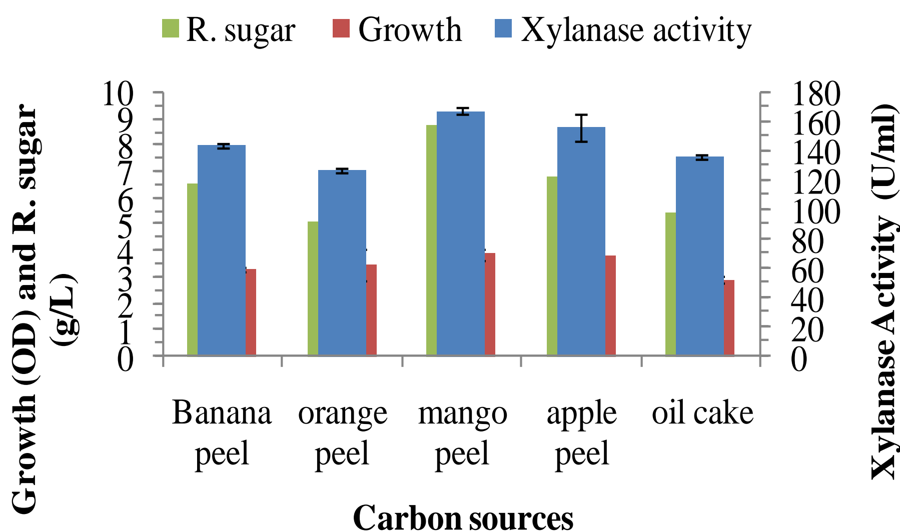

Figure 2. Effect of the carbon source (50 g/L initial concentration) on biomass concentration, xylanase activity and residual reducing sugar level at 24 h of fermentation (37°C, initial pH of 6.0).

with the increasing inoculum size. The highest xylanase activity was noted by using 10% inoculum (743 IU/ml), results are demonstrated in Figure 5. The effects of initial pH of fermentation on enzyme production and microbial growth are shown in Figure 6. The fermentations in Figure 6 were conducted for 48 h in a mineral medium that contained mango peel (10% initial concentration) yeast extract (5 g/L initial concentration) and peptone (2.5 g/L initial concentration). Clearly, the optimal initial pH for xylanase production and growth of *B. megaterium* was at pH 8.0 (Figure 6).

The effect of fermentation temperature on production of biomass and xylanase activity is shown in Figure 7. Clearly, 50°C was the best fermentation temperature for xylanase activity whereas maximum growth was observed at 45°C, further increase in temperature decreased the xylanase activity and growth which might be due to denaturation of enzyme at high temperature. Temperature is one of the important factors, which affects normal functioning of microorganism and enzyme production. The *B. megaterium* BRL-0101 is a commercially available substrates like oat spelt xylan (1887 IU/mL induced xylanase production; Figure 8). Xylan induced enzyme production at large extent, it is well known that xylan from various sources, are excellent inductors for xylanase production. Xylanase production was measured in the

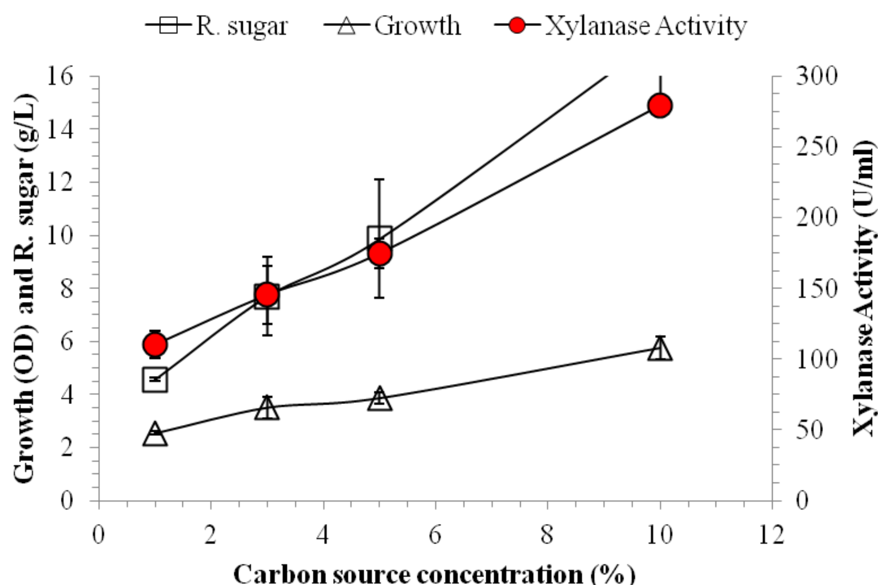

Figure 3. Effect of initial concentration of mango peel on final xylanase activity, biomass concentration and residual sugar concentration at 24 h of fermentation (37°C, initial pH of 6.0).

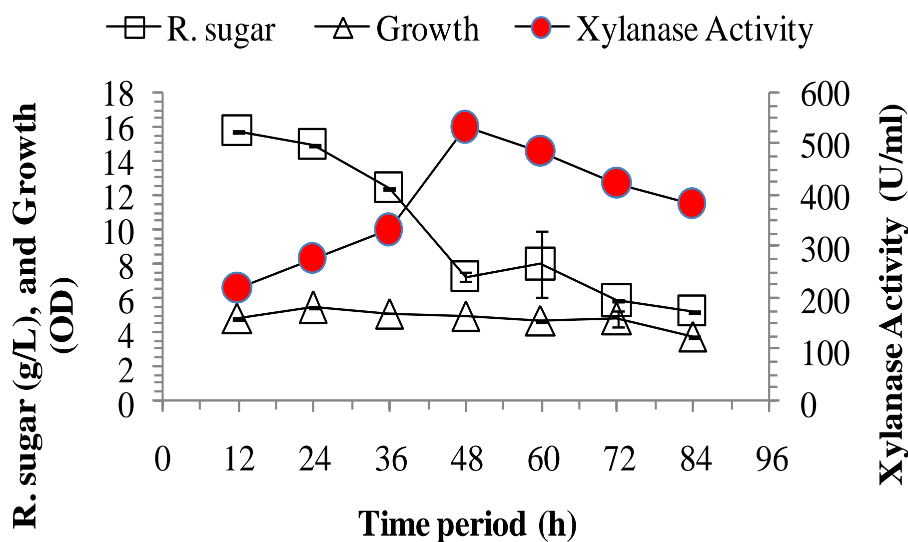

Figure 4. Batch profiles of biomass growth, xylanase activity and reducing sugar concentration during enzyme production (37°C, initial pH of 6.0).

presence of several organic and inorganic nitrogen sources separately and in combination using 10.0% (w/v) mango peel as a carbon source. Figure 9 showing the combination of peptone, yeast extract and sodium nitrate stimulated the highest xylanase production (2876.0 IU/mL) followed by a combination of peptone and yeast extract (2280.0 IU/mL). The combination of yeast extract with $NaNO_3$ and peptone with $NaNO_3$ produced 1955 and 1935 IU/ml, respectively.

DISCUSSION

Xylanases production demands are increasing due to wide applications in paper and pulp, bioethanol production and various pharmaceutical and chemical industries (Kuhad et al., 2010; Nagar et al., 2010; Rojas et al., 2011; Cavka et al., 2011; Lopez et al., 2011; Abdeshahian et al., 2010). This study could be economically feasible for industrial scale xylanases production by using inexpensive

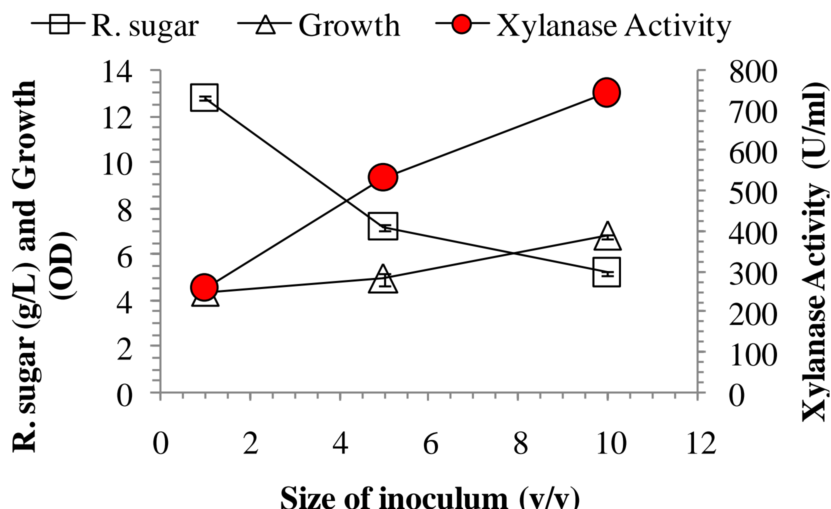

Figure 5. Effect of inoculum size on xylanase production, growth and residual reducing sugar level at 48 h of fermentation (37°C, initial pH of 6.0) in a mango peel (10% initial concentration) mineral medium.

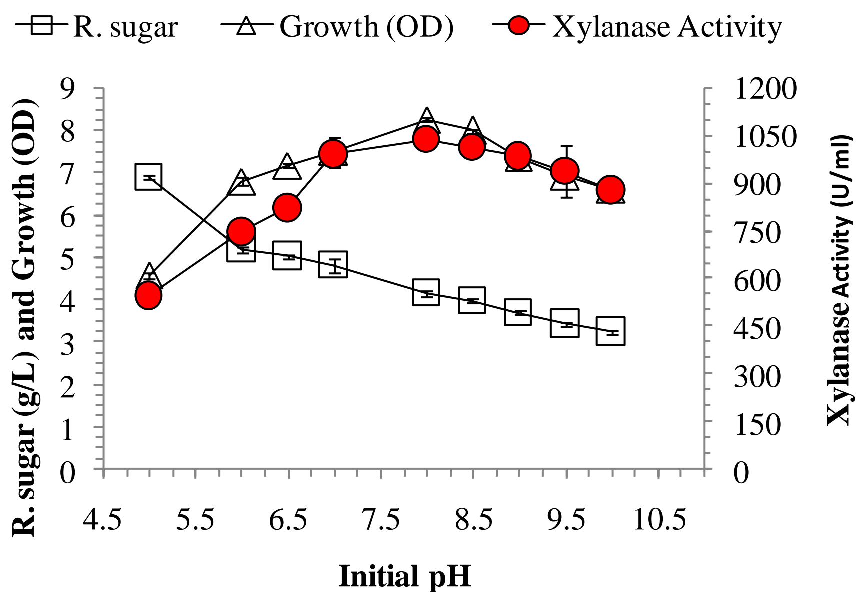

Figure 6. Effect of initial pH on xylanase production, biomass growth and residual sugar concentration at 48 h of fermentation (37°C) in a mineral medium containing mango peel and at initial concentration of 10%.

waste materials as carbon source. Agricultural wastes are abundant, cheap and inexhaustible substrates for value added products formation (Nagar et al., 2012). Carbon source is one of the essential constituents of the microbial growth and fermentation medium which significantly affects the overall cellular growth and metabolism. Mango peel, inexpensive agricultural residue; would affect the cost of the enzyme production directly. The use of pure sugars is uneconomical for xylanase production at large scale, while agricultural wastes are cost effective substrate for xylanase production (Ninawe and Kuhad, 2005). Xylanase activity increased with the passage of time to certain extent and prolonged incubation decreased xylanase titer. The reduction in the xylanase activity with the time of incubation might be due to reduction of nutrients, proteolysis and or change of pH

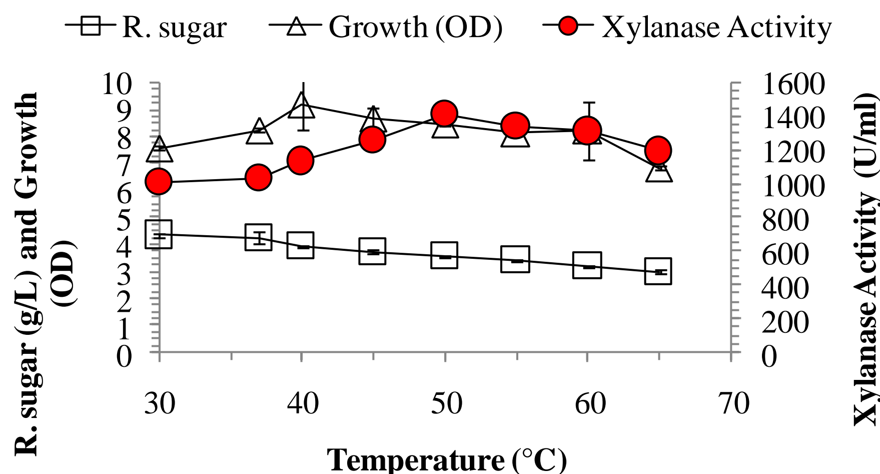

Figure 7. Effect of fermentation temperature on xylanase production, biomass concentration and residual sugar concentration at 48 h. The medium initially contained 10% mango peel. The initial pH was 8.0.

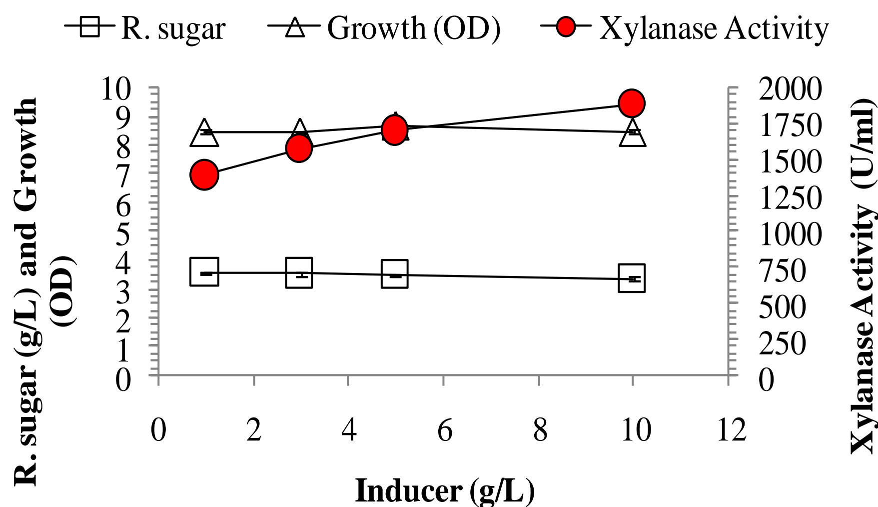

Figure 8. Effect of xylan as inducer on xylanase activity.

because of organic acids formation (Flores et al., 1997). *B. amyloliquefaciens* produced maximum xylanase activity in the culture broth after 48 h of incubation (Lincoln, 1960). *Streptomyces* Sp RCK-2010 secreted highest xylanase activity after 48 h by using wheat bran as carbon source (Kumar et al., 2012). *Bacillus* SSP-34 produced maximum xylanase activity (380 IU/mL) when grown for 96 h (Subramaniyan and Prema, 2000). In contrast to bacteria, fungi take more time such as *Trichoderma reesei* SAF3 produced maximum xylanase after 72 h of growth at 30°C under submerged conditions (Kar et al., 2006). Many researchers have reported hyper xylanases production from 1.0-5.0% (v/v) inoculum (Nagar et al., 2010; Battan et al., 2007; Kar et al., 2006). Low inoculum size is preferred for the synthesis of microbial products at commercial scale due to economical concerns (Lincoln, 1960). Higher inoculum size may increase moisture content and lead to decrease in growth and enzyme production, in the case of reduced inoculum size, desired production will take longer time (Baysal et al., 2003; Kashyap et al., 2002; Farga et al., 2009) an appropriate inoculum and nutrients could produce maximum product. In addition to this, the use of 10 % inoculum size for maximum xylanase production by *Bacillus* sp NCIM 59 has been reported (Kulkarni and Rao, 1996).

The genes involved in the production of certain enzymes in at least some microorganisms are known to

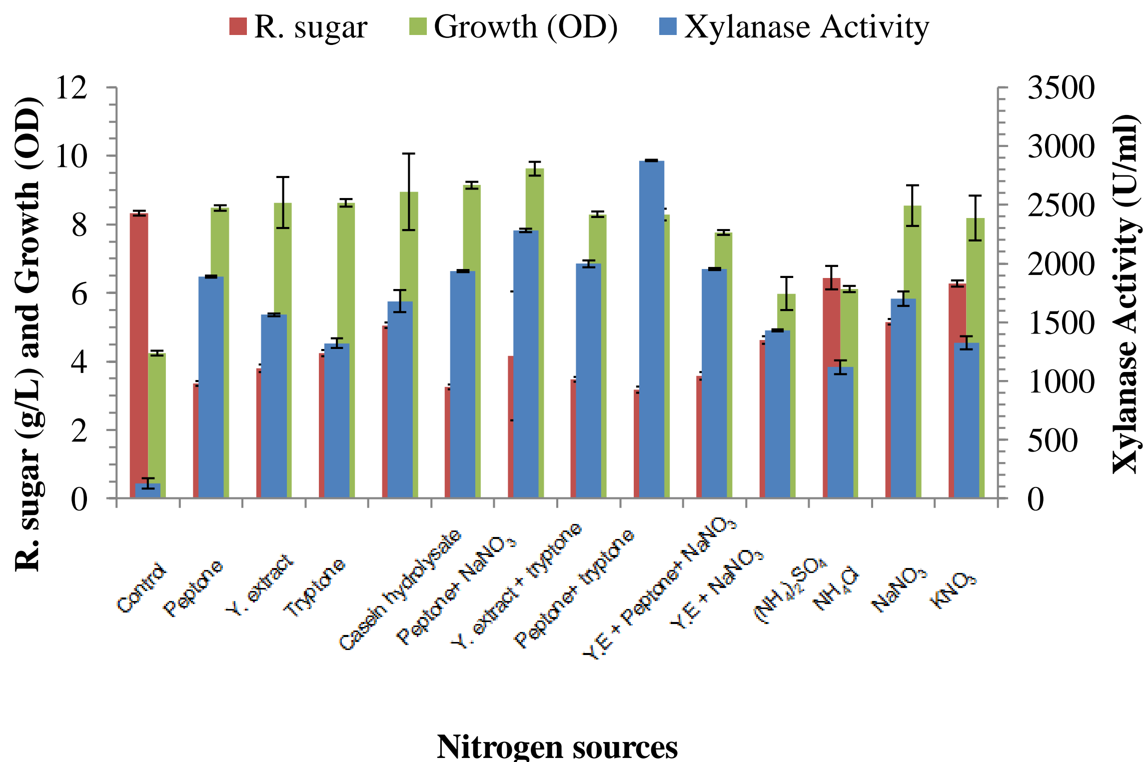

Figure 9. Effect of nitrogen source (10 g/L initial concentration) on xylanase production, biomass concentration and residual reducing sugar level at 48 h of fermentation (50°C, initial pH of 8.0) in a mango peel (10% initial concentration) mineral medium.

be pH regulated (Young et al., 1996). The optimal initial pH value for producing xylanase of course depends on the microorganism. Generally, fungi produce xylanase at acidic pH while bacteria produce xylanase at higher alkaline pH (Dhiman et al., 2009). However, some fungi such as *Aspergillus fischeri* Fxn 1 and *Aspergillus nidulans* KK-99 produced alkaline xylanases (Taneja et al., 2002). Kumar et al. (2012) reported xylanase production from *Streptomyces* sp. RCK-2010 at optimum pH 8.0. Xylanase production is inducible with the nature of substrate used (Nwodo-Chinedu et al., 2008). Highest xylanase production (251 IU/mL) by *Bacillus* SSP-34 was achieved when yeast extract and peptone each at 0.25% was supplemented in the medium as nitrogen source (Subramaniyan and Prema, 2000). However, tryptone was the best nitrogen source for xylanase production by *Bacillus circulans* AB16 (Dhilon and Khana, 2000) and *Geobacillus thermoleovorans* (Sharma et al., 2007). Yeast extract in combination with peptone significantly enhanced xylanase production in *Bacillus* sp. (Ruckmani, 2001). *Streptomyces* sp RCK-2010 produced highest xylanase activity from combination of peptone and beef extract (Kumar et al., 2012). The enhanced xylanase production in the presence of beef extract as well as peptone may be attributed to organic nitrogen source mediated regulation of microbial growth and metabolism (Gupta et al., 2000).

Conclusion

The low-cost process for xylanase production was developed in the present study, agro-industrial residues are interesting for this purpose due to their high availability associated with their low cost. However, these materials usually need treatments to become more susceptible to microbial action, fruit waste were treated with dilute acid to fermentable sugars. The results obtained herein proved mango peel as suitable substrate for xylanase production by *B. megaterium.* Under optimal conditions, xylanase levels as high as 2876 IU/mL was obtained. This study proved practical approach for xylanase production from fruit waste.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

Nocardia seriolae, a causative agent of systematic granuloma in spotted butterfish, *Scatophagus argus*, Linn

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A *Nocardia seriolae* was isolated from diseased spotted butterfish (*Scatophagus argus*, Linn). The cumulative mortality within one month was 30% (660 out of 2200). The diseased fish were two years old with lengths from 18 to 25 cm. Most fish suffered from haemorrhages and ulcers of the skin. The most significant gross pathological change was enlargement of the spleen, kidney and liver. White nodules, varying in size, were found in these organs. The isolated bacteria were either coccoid or filamentous in appearance, with bead-like staining. The identification of NS128 was verified by polymerase chain reaction (PCR) assay for *N. seriolae* that gave the expected specific amplicon of 432 bp of the 16S rDNA sequence with a 100% sequence identity with *N. seriolae* (GenBank accession number AF380937). A partial sequence of the 16S rRNA gene (GenBank accession number EU147501), the RNA polymerase B subunit (rpo B) gene (GenBank accession number DQ119300) and the heat shock protein gene (GenBank accession number DQ431437) of the organism, NS128 and the type strain of *N. seriolae* BCRC 13745 formed a monophyletic clade with a high sequence similarity and a bootstrap of 100%. White nodules that were induced in experimental fish, spotted butterfish and amberjack (*Seriola dumerili*, Risso) were similar to those in naturally infected fish cases and *N. seriolae* were re-isolated using brain heart infusion agar. These finding provides evidence that *N. seriolae* caused systemic granulomas in spotted butterfish. Based on the growth characteristics, and biochemical properties of the bacterium, its histopathological changes, PCR and the phylogenetic analysis, the pathogenic organism was identified as *N. seriolae*. This investigation is the first published on *N. seriolae* infection in spotted butterfish in aquaculture. The results reveal that the *N. seriolae* isolated in the field was pathogenic to spotted butterfish and amberjack.

Key words: Pathogenicity, *Nocardia seriolae*, spotted butterfish, *Scatophagus argus*, polymerase chain reaction (PCR).

INTRODUCTION

Nocardiosis in fish is a disease characterized by systemic nodulation, manifested both on the skin and internal organs. Three species of *Nocardia* have been isolated from diseased fish. They are *Nocardia salmonicida*, *N. seriolae* (formerly *N. kampachi*), and *N. asteroides* (Chen et al., 1989; Isik et al., 1999; Kudo et al., 1988).

N. asteroides in Formosa snakehead, *Channa maculata* and largemouth bass, and *Micropterus salmoides,* in

Taiwan was first described by Chen et al. (1989) and Chen and Tung (1991) in Taiwan. These outbreaks have caused substantial commercial losses. Notably, *N. seriolae* is the causative agent of nocardiosis in cultured yellowtail, *Seriola quinqueradiata* (Kariya et al., 1968; Kumamoto et al., 1985; Kusuda et al., 1974), fingerling or yearling rainbow trout, *Oncorhynchus mykiss*, which develop lesions within one to three months of experimental injection with *N. asteroides* (Snieszko et al., 1964). Elkesh et al. (2013) reported systemic nocardiosis in cultured meagre, *Argyrosomus regius* Asso with a low to variable morbidity and 1–4% total mortality. Recently, Japanese sea-perch, *Lateolabrax japonicum*, striped mullet, *Mugil cephalus*, large yellow croaker, *Larimichthys crocea* (Richardson), three striped tigerfish, *Terapon jarbua,* weakfish, *Cynoscion regalis* (Bloch and Schneider) and yellowtail have all been infected with *N. seriolae* (Chen et al., 2000; Wang et al., 2005; Shimahara et al., 2006; Shimahara et al., 2008; Wang et al., 2009; Cornwell et al., 2011). Although isolation and bacteriological analysis are complex and time-consuming, *N. seriolae* has been identified as one of the major nocardial pathogens in fish. Therefore, molecular detection methods of *Nocardia* species have been developed, including polymerase chain reaction (PCR) (Miyoshi and Suzuki, 2003), real-time PCR (Carrasco et al., 2013) and loop-mediated isothermal amplification (Itano et al., 2006). Miyoshi and Suzuki (2003) developed a PCR assay based on unique regions of the *N. seriolae* 16S rRNA gene that allows specific identification. In the present paper, we describe the first isolation and characterization of pathogenic *Nocardia* species obtained from diseased spotted butterfish, *Scatophagus argus* with systematic granuloma during an outbreak of nocardiosis in the summer of 2005 in Taiwan.

MATERIALS AND METHODS

Collection of fish sample

The farm that was sampled in this investigation has two ponds, one of which was affected. The disease developed between June and July, 2005 in a pond (with salinity 5‰ and water temperature 26°C) that contained 2200 spotted butterfish (*Scatophagus argus*, Linn), that were approximately two year old. The fish had an average body weight of 1.2–1.4 kg and an average length of 21–27 cm. They had been fed with commercial pellets without any trash fish. The moribund eight fish from affected pond were subjected to histopathological and bacteriological examination.

Bacteriology

The inoculating loops were taken from the kidney, heart, spleen, and liver and streaked on tryptic soy agar (TSA), blood agar (BA), brain heart infusion (BHI) agar and Lowenstein–Jensen medium (LJM). Plates were then incubated at 25°C for 30 days.

Histopathology

The kidney, spleen, liver and other internal organs with lesions were fixed in 10% buffered formalin and processed for paraffin sectioning. Sections were stained using haematoxylin and eosin (H&E) and the Ziehl-Neelsen's (ZN) methods.

Bacterial strains

The following bacterial strains were employed as controls for microbiological tests and polymerase chain reaction (PCR) assays: *N. farcinica* (Bioresource Collection & Research Center, Hsinchu, Taiwan, BCRC 13380), *N. seriolae* (BCRC 13745), *N. seriolae* NS127, and *N. salmonicida* (BCRC 12441). The purified bacterial isolate derived from infected fish was labeled NS128. These strains were routinely grown on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Minch.) at 25℃ for five days.

API ZYM test

The API ZYM test was applied to identify the enzymatic profile of NS128 and reference strains. The test kit has 20 cupules: 19 substrates and a control. A representative bacterial colony on BHI agar was suspended in 3 mL of sterile saline (0.85% NaCl) that contained sterile glass beads (2 mm in diameter). The suspension was then shaken using a mixer for 10 min to disperse the bacteria. The turbidity of each suspension was adjusted to a number 6 McFarland standard and 65 µL of bacterial suspension was added to each cupule of the strip following the manufacturer's instructions. Plates were incubated at 25°C for NS128 and reference strains, *N. seriolae* (BCRC 13745), *N. seriolae* (NS127), *N. salmonicida* (BCRC 12441) and at 35°C for reference strain for *N. farcinica* (BCRC 13380). Following incubation for 5 h, ZYM A and B reagents were added and plate reactions were terminated at 5 min.

Specific primer for *N. seriolae* 16S analysis (432 bp)

The DNA sample preparation was modified from the methods developed by Telenti et al. (1993). The PCR amplification was performed by using the oligonucleotide primers NS1: 5'-ACTCACAGCTCAACTGTG-3' and NG1: 5'-ACCGACCACAAGGGGG-3' according to the manufacturer's protocol of Laurent et al. (1999 and 2000) and Miyoshi and Suzuki (2003). The specific primer set NS1-NG1 targets 16S rRNA gene (16S rDNA) of *N. seriolae* and yields the 432 bp amplicons.

16S rDNA analysis (596 bp)

The PCR amplification of the 16S rDNA gene from *Nocardia* spp. was conducted using a purified DNA template with the forward primer NG1 (5'-ACCGACCACAAGGGGG-3') and the reverse primer NG2 (5'-GGTTGTAAACCTCTTTCGA-3') and amplified a 596 bp fragment using a method modified from that of Laurent et al. (1999, 2000).

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Heat shock protein gene analysis (439bp)

The PCR method was performed according to Telenti et al. (1993). Primers Tb11 (5'-ACCAACGATGGTGTGTCCAT-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3') amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence (Schinnick, 1987).

RNA polymerase B subunit (rpo B) gene analysis (342bp)

Kim et al. (1999), adopted a set of primers (MF, 5'-CGACCACTTCGGCAACCG-3'; MR, 5'-TCGATCGGGCACATCCGG-3') which was used to amplify rpo B DNA (342 bp) in this study. The rpo B gene encompasse the Rif^r region, which is associated with rifampicin resistance in *M. tuberculosis*. The PCR products were electrophoresed on a 2% agarose gel.

Phylogenetic analysis

The PCR amplicons from the 16S rRNA gene, the heat shock protein gene, and the RNA polymerase beta subunit (rpo B) gene of *N. seriolae* strain NS127, NS128 and reference strain, *N. seriolae* (BCRC 13745) used a model 377 automatic DNA sequencer (performed by Tri-I Biotech, Taipei,. Taiwan). The DNA sequences thus obtained were aligned with representative sequences from the GenBank, DDBJ, and EMBL databases. To prepare phylogenetic trees, CLUSTAL X software version. 1.81 was utilized with the minimum evolution (Rzhetsky and Nei, 1993), maximum parsimony (Fitch, 1971), and neighbor-joining NJ, (Saitou and Nei, 1987) treeing algorithms. Evolutionary distance matrices for the minimum evolution, MP, and NJ methods were generated using the methods developed by Jukes and Cantor (1969). The MEGA2 computer program (Kumar et al., 2001) was utilized for all analyses. The resulting tree topologies were investigated using bootstrap analyses (Felsenstein, 1985) according to the NJ method with 1000 repetitions.

Sensitivity to drug

For antibiotic sensitivity tests, isolates were spread on Mueller-Hinton agar plates and exposed to antibiotic discs that contained erythromycin (15 µg), doxycycline (30 µg), spiramycin (100 µg), tetracycline (30 µg), oxytetracycline (30 µg), streptomycin (10 µg), and neomycin (30µg). The plates were incubated at 25°C for 18 h and the inhibition of the bacteria by chemotherapeutic agents was evaluated.

Experimental infection

Healthy, spotted butterfish *Scatophagus argus*, with body weights of 50-55 g, and amberjack *Seriola dumerili* with body weights of 100-125 g, were obtained from a fish farm in Pingtung, Taiwan, and maintained in continuously aerated 500 L aquaria that contained 450 L of water at approximately 25°C for seven days until they were acclimatized to the laboratory. The fish were fed twice daily with commercial fish pellets and waste was removed daily. Spotted butterfish and amberjack were assigned to two equal treatment groups (with eight in each group of spotted butterfish and four in each group of amberjack) in separate tanks and fed daily using a commercial diet until they were starved one day before the inoculation to avoid fish stress. *N. seriole* (NS128) was grown on BHI at 25°C for five days and then harvested using normal saline. A bacterial suspension was prepared in saline solution to a final concentration of $4.7x10^7$ CFU mL^{-1}. The 0.1 mL^{-1} ($4.7x10^6$ CFU) bacteria suspension was injected intraperitoneally (IP) into eight spotted butterfish and four amberjack in each treatment group, respectively. The fish in the control group were inoculated with sterile saline. Following the injections, each group was kept separately in a 150 L aquarium under the same conditions as described above for the acclimatization period. The fish were continuously monitored for morbidity and mortality and sampled for histopathological and bacteriological analyses. The experiment was terminated at fifteen days after inoculation. Organ smear and re-isolation were also performed from survivors.

RESULTS

Clinical signs and pathology

The naturally infected cultured spotted butterfish had skin ulcers and necrosis. They had numerous marked yellowish-white nodules, ranging from 0.1 to 0.4 cm in diameter on the serosal surface, and on many internal organs, especially the liver, gills, heart, spleen, and kidney (Figure 1). Cumulative mortality within 30 days was 30% (660 out of 2200). Histopathologically, most nodules were typically granulomas. Some were multifocal, while others were relatively more diffusely distributed. The granulomatous foci, varied in size and comprised necrotic tissue debris with some bacterial clumps in their centres, surrounded by epithelioid cells, particularly in the liver, spleen and kidney (Figure 2). Macrophages and epithelioid cells were present in the young nodules, but no multinucleated giant cells or connective tissue was observed. Acid-fast, filament or bead-like bacteria were identified in the nodules using ZN staining (Figure 3), especially in the centres of the granulomas and within macrophages. Liver, kidney, and spleen were consistently affected.

Bacteriology

The bacteria from lesions of internal organs were Gram and ZN-positive. The bacterial shapes ranged from coccoid to short rods and some appeared as long, filament-tous, multiseptate rods. Bacterial colonies from the spleen, liver and heart appeared three days after culturing on BA, TSA, BHI and LJM at 25°C. The bacteria were Gram and ZN-positive. The bacteria grew at 25°C, but not at 37°C or 41°C; they did not survived for 8 h at 50°C (Table 1). The organisms could neither reduce nitrate nor degrade starch, xanthine, tyrosine or casein. The biochemical characteristics of NS128 were identical to those of reference strain *N. seriolae* BCRC 13745 and *N. seriolae* NS127, but differences were evident among the urea utilizations, temperatures and salinity tolerances between NS128 and other reference strains, BCRC 13364 *N. asteroides,* BCRC 12441 *N. salmonicida* and BCRC 13380 *N. farcinica.*

API ZYM test

Table 2 shows the enzymatic profiles of the *N. seriolae*

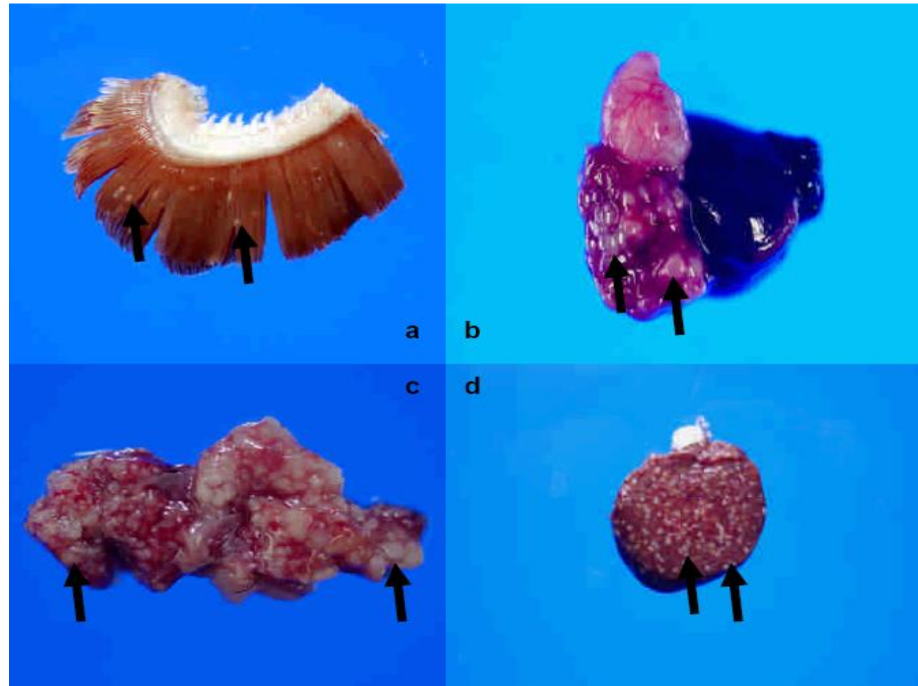

Figure 1. Diseased spotted butterfish *Scatophagus argus* by natural infection showing numerous nodular structures in gill (a), heart (b), kidney (c) and spleen (d) (arrows).

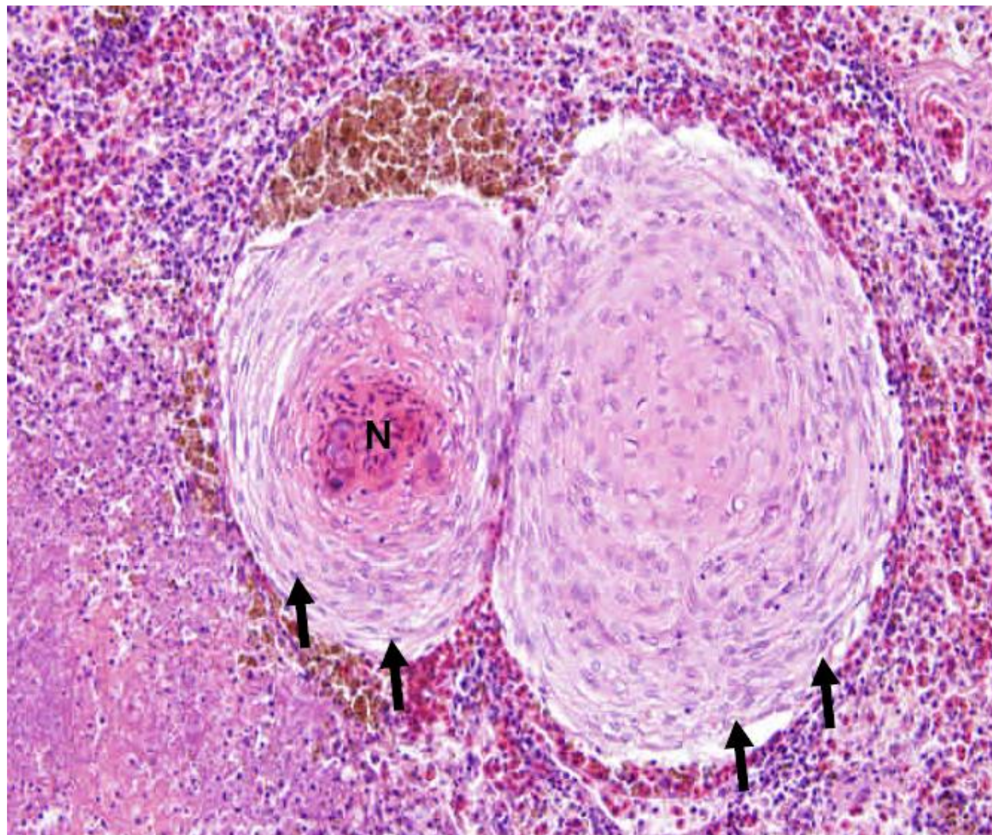

Figure 2. Diseased spotted butterfish *Scatophagus argus* by natural infection. Granuloma (arrows) with marked central necrosis (N) in the spleen of *s*potted butterfish. (H & E stain methods, X 400).

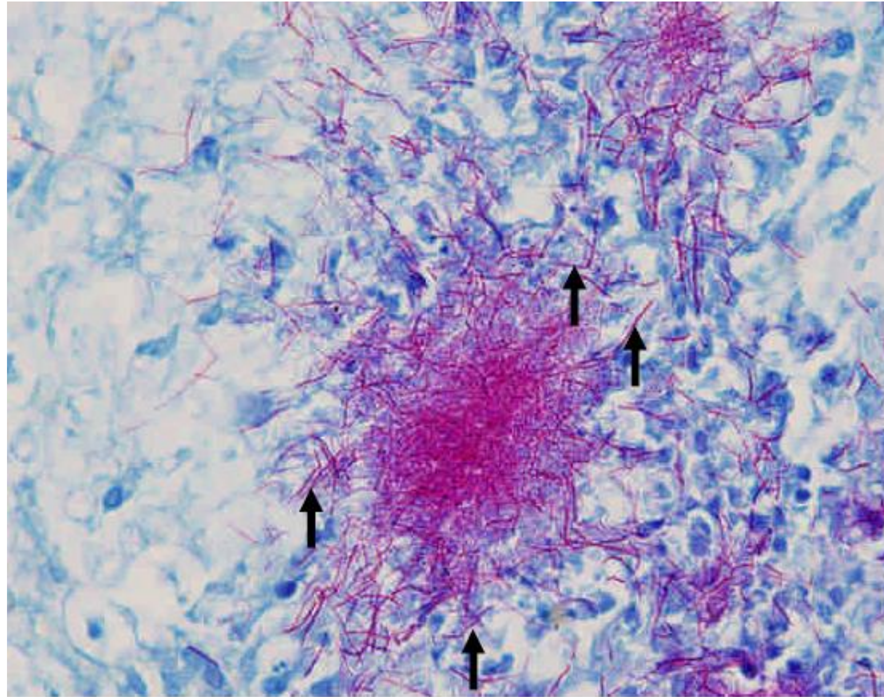

Figure 3. Spleen of the spotted butterfish *Scatophagus argus*, Many beaded filamentous bacteria (arrows) are seen in the necrotic area of granuloma in the spleen (Z-N staining method, X1000).

Table 1. Physiological and biochemical characteristics of NS128 isolates and reference strains of *Nocardia.*

Parameter	NS 128	*Nocardia seriolae* NS127	*Nocardia seriolae* BCRC 13745	*Nocardia asteroides* BCRC 13364	*Nocardia salmonicida* BCRC 12441	*Nocardia farcinica* BCRC 13380
Urea	–	–	–	+	+	+
Starch	–	–	–	–	–	–
Xanthine	–	–	–	–	–	–
Tyrosine	–	–	–	–	–	–
Casein	–	–	–	–	–	–
Gelatin	–	–	–	–	–	–
Growth at						
25°C	+	+	+	+	+	+
37°C	–	–	–	+	–	+
40°C	–	–	–	+	–	–
Survival at 50°C						
2h	–	–	–	+	–	+
4h	–	–	–	+	–	+
8h	–	–	–	+	–	+
Catalase	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–
Acid form						
Mannose	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–
Trehalose	–	–	–	–	–	–

Table 1. Contd

Erythritol	—	—	—	—	—	—
Maltose	—	—	—	—	—	—
Arabinose	—	—	—	—	—	—
PH9.0	—	—	—	+	+	+
Growth with						
1%NaCl	+	+	+	+	+	+
2%NaCl	—	—	—	+	+	+
3%NaCl	—	—	—	—	—	—

isolates and reference strains that were obtained by using the API ZYM test. There were minor differences between NS128 and reference strain *N. seriolae* BCRC 13745 in some assimilation tests, such as with cystine arylamidase, valine arylamidase chymotrypsin, and β-galactosidase. Based on the results of morphological, physiological and biochemical tests, the NS128 that was isolated from diseased spotted butterfish, was classified in the genus *Nocardia*. However, from the growth characteristics, and the biochemical properties of the bacteria, determined by the API ZYM test, determining whether NS128 was conspecific with *N. seriolae* was difficult. The isolate from spotted butterfish was cultured and identified as *N. seriolae* using PCR methods.

Specific primer for *N. seriolae* (432 bp)

The PCR assay amplified a band of 432 bp of 16S rDNA gene from NS128 and the reference strain, *N. seriolae* (BCRC 13745) (Figure 4). These amplicons were not obtained from *N. farcinica,* BCRC 13380, *N. salmonicida* (BCRC 12441), or *Mycobacterium fortuitum* (BCRC 15320).

16S rDNA analysis (596bp)

Isolate NS128 bacteria from infected fish yielded an expected PCR product of 596 bp using NG1 and NG2 primers. The sequence had 100% identity with *N. seriolae* (GenBank accession number AF380937), 97.3% similarity to *N. nova* (GenBank accession number Z36930), 95.8% similarity to *N. asteroides* (GenBank accession number X84851), 94% similarity to *N. salmonicida* Z46750 (GenBank accession number X84851), and 93.6% similarity to *N. otitidiscaviarum* (GenBank accession number X80611).

RNA polymerase gene (rpo B) analysis (342 bp)

The bacterial isolate of NS128 from infected spotted butterfish yielded an expected PCR product of 342 bp using MF and MR primers. The sequence exhibited a 100% identity with *N. seriolae* (GenBank accession number AY017474 and BCRC 13745), 92.9% similarity to *M. fortuitum* AY147156, and 92.2% similarity to *M. mucogenicum* (GenBank accession number AY147174).

Heat shock protein gene (439 bp)

Sequencing the heat shock protein gene yielded an expected PCR product of 439 bp using Tb11 and TB12 primers. The sequence had 100% identity with *N. seriolae* (GenBank accession number AY756533), 95.4% similarity to *N. asteroides* (GenBank accession number AY756513), 94.9% similarity to *N. otitidiscaviarum* (GenBank accession number AY756528), 93.8% similarity to *N. farcinica* (GenBank accession number AY756523), 93.2% similarity to *N. brevicatena* (GenBank accession number Z36930), and 92.7% similarity to *N. nova* (GenBank accession number AY756527).

Phylogenetic analysis

The PCR sequences of the 16S rDNA gene, heat shock protein gene (439 bp), and RNA polymerase B subunit (rpoB) gene analysis (342 bp) of NS128 from diseased spotted butterfish, were deposited in GenBank (Accession Nos.: EU147501 for 16S rDNA, DQ119300 for RNA polymerase B subunit (rpoB) gene, DQ431437 for heat shock protein, respectively). The sequences of 16S rDNA gene, heat shock protein gene, and RNA polymerase B subunit (rpoB) gene of NS128 from diseased spotted butterfish, exhibited identities of 100% with reference strains (*N. seriolae* GenBank accession Nos.: AY380937, AY756553 and BCRC 13745), respectively. CLUSTAL X software version 1.81 was used to determine an evolutionary tree (Figures 5, 6 and 7) that indicates that these strains form a unique clade with the reference strain, *N. seriolae*, at a distance from other *Nocardia* and *Mycobacteria*. This relationship was emphasized by the relatively high nucleotide similarity

Table 2. API-ZYM Kit results of isolates NS128 and reference strains of *Nocardia.*

Characteristics	NS128	*Nocardia seriolae* NS 127	*Nocardia seriolae* BCRC 13745	*Nocardia asteroides* BCRC 13364	*Nocardia salmonicida* BCRC 12441	*Nocardia farcinica* BCRC 13380
Control	—	—	—	—	—	—
Alkaline Phosphatase	+	+	+	+	+	+
Butyrate esterase	—	+	—	—	+	—
Caprylate esterase lipase	+	+	+	+	+	—
Myristate lipase	—	—	—	—	+	—
Leucine arylamidase	+	+	+	+	+	+
Valine arylamidase	+	+	—	—	—	+
Cystine arylamidas	+	—	—	+	—	—
Trypsin	+	+	+	—	+	+
Chymotrypsin	+	—	—	—	—	—
Acid Phosphatase	+	+	+	+	+	+
Naphthol- AS-BI-phosphohydrolase	+	+	+	+	+	+
α-Galactosidase	—	—	—	—	—	—
β- Galactosidase	+	—	—	—	—	—
β- Glucuronidase	—	—	—	—	—	+
α-Glucosidase	+	+	+	+	+	+
β- Glucosidase	+	+	+	+	+	+
N-acrtyl-β-Glucosaminidase	—	—	—	—	—	—
α-Mannosidase	—	—	—	—	—	—
α-Fucosidase	—	—	—	—	—	—

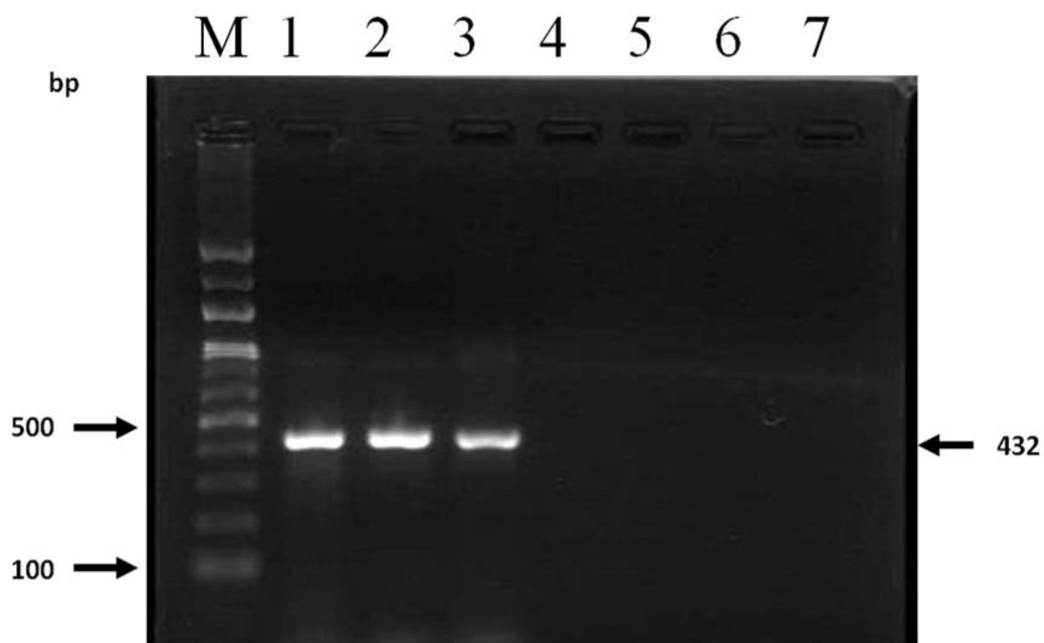

Figure 4. Electrophoretic analysis of 16S rDNA gene of *N. seriolae* isolates by primers NS1 and NG1. M, marker; 1, NS128; 2, *N. seriolae* NS127; *N. seriolae* BCRC13745; 4, *N. salmonicida* BCRC12441; 5, *N. asteroides* BCRC13364; 6, *N. farcinica* BCRC13722; 7, negative control (without template DNA).

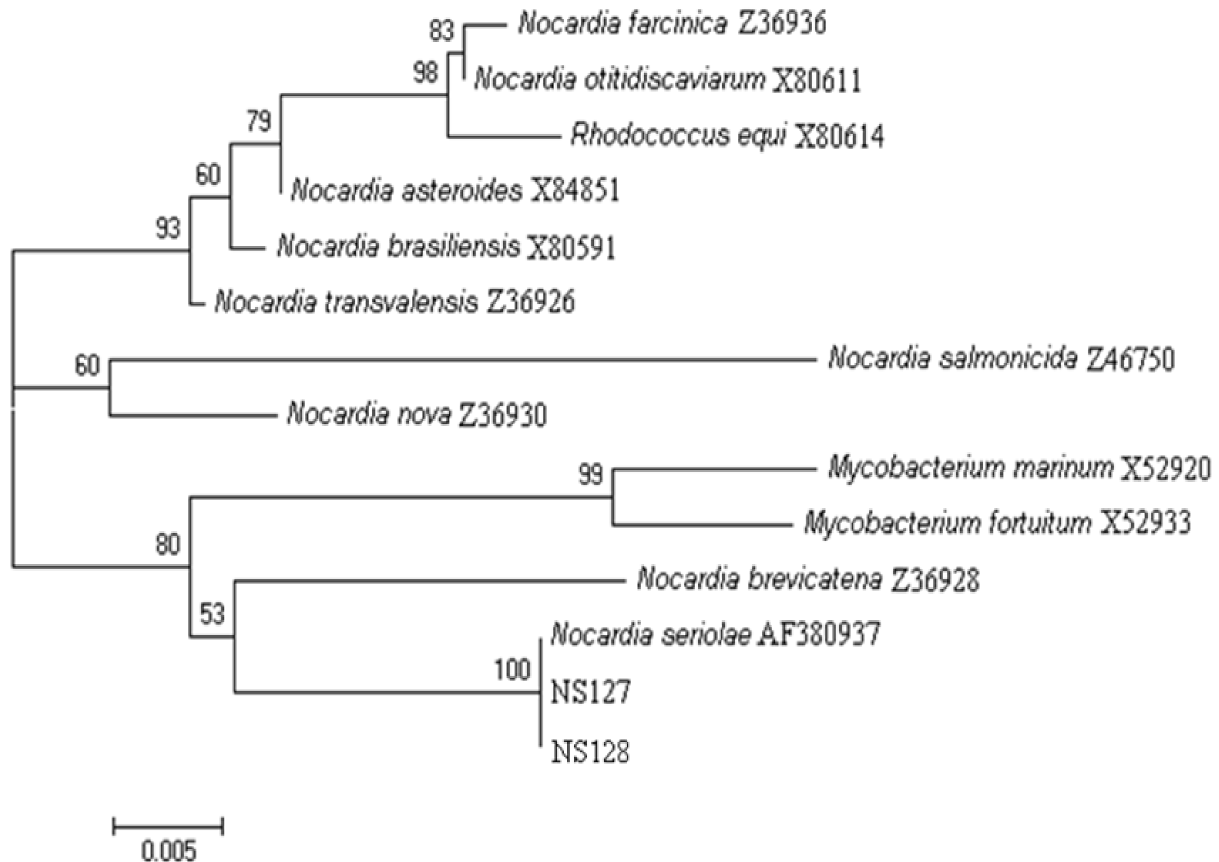

Figure 5. 16S rDNA sequence-based phylogenetic tree of NS128 isolates from our study and from GenBank. Bars indicate genetic distance. Numbers at each node indicate percent bootstrap values. Scale represents 0.005 nucleotide substitutions per position.

value and the high bootstrap-support value (100 % for the 16S rDNA, the heat shock protein gene, and rpoB gene) of NS128 determined by the neighbor-joining, maximum-parsimony and minimum-evolution methods.

Sensitivity to drugs

The isolated and reference strains were sensitive to erythromycin, doxycycline, spiramycin, neomycin and streptomycin. However, they were resistant to tetracycline (Table 3).

Experimental infection

Table 4 presents the cumulative mortalities of both spotted butterfish and amberjack that had been inoculated with 4.7 X 10^6 bacteria of *N. seriolae*. A bacterial concentration of 4.7 x 10^6 CFU per fish caused 100% mortality of the spotted butterfish within ten days of the inoculation. The amberjack showed 100% mortality at 15 days post-inoculation. The bacteria were reisolated from the kidney and liver of all dead fishes. No bacteria were isolated from the liver or kidney of fish selected randomly prior to the experimental infection or from the control group. The infected spotted butterfish had yellowish-white nodules on the serosal surface, mesentery and in many internal organs, especially the kidney, spleen, liver, muscle and skin. The granulomatous nodules found in the spotted butterfish were observed in the muscle, heart, kidney, spleen and liver, and especially in the kidney, and liver. No lesion was formed in either control group. Granulomas and other histopathological changes that resembled those in naturally infected spotted butterfish were exhibited.

DISCUSSION

The isolate, NS128 which is Gram-positive and acid-fast, generated a mycelium that is fragmented into irregular rod-like elements. The phenotypical characteristics of the bacterial isolate, NS128 from diseased spotted butterfish, in Taiwan were almost identical to those of the reference strain, *N. seriolae* BCRC 13745 and *N. seriolae*

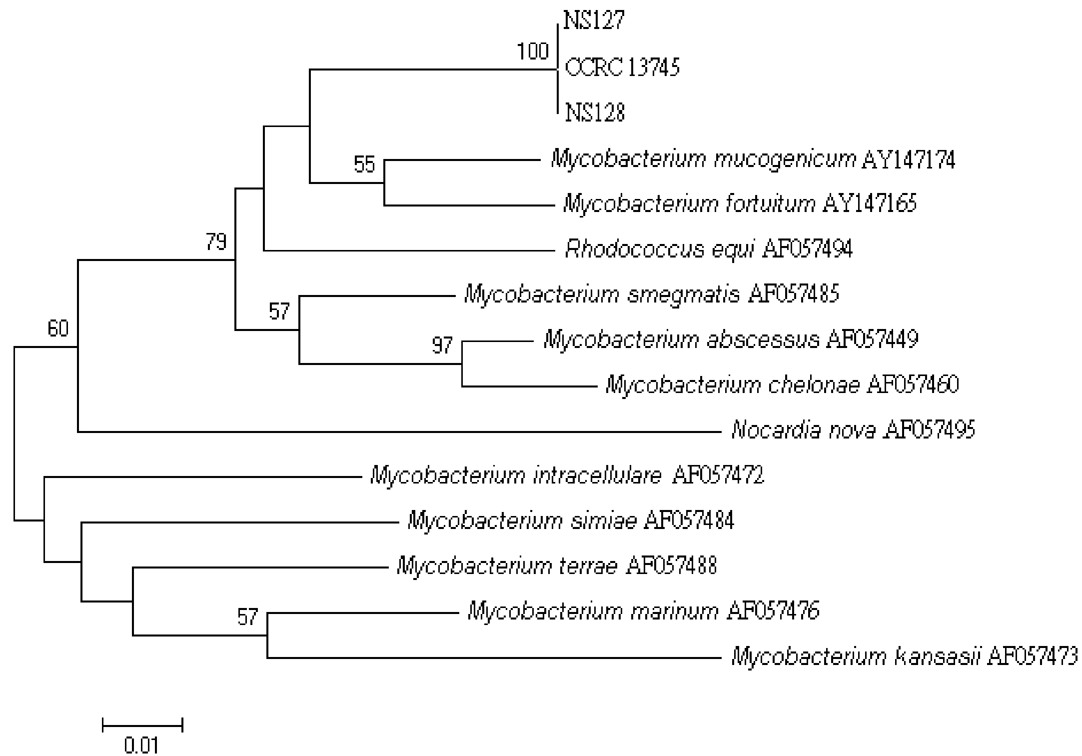

Figure 6. RNA polymerase B subunit gene sequence-based phylogenetic tree of NS128 isolates from our study and from GenBank. Bars indicate genetic distance. Numbers at each node indicate percent bootstrap values. Scale represents 0.01 nucleotide substitutions per position.

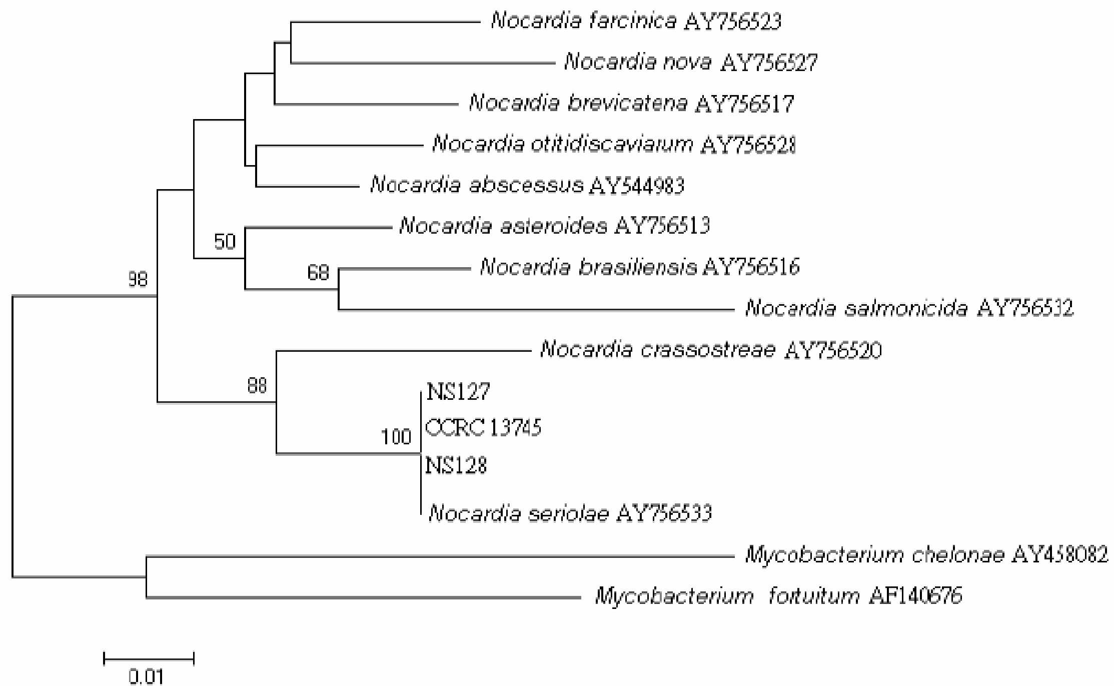

Figure 7. Heat shock protein sequence-based phylogenetic tree of NS128 isolates from our study and from GenBank. Bars indicate genetic distance. Numbers at each node indicate percent bootstrap values. Scale represents 0.01 nucleotide substitutions per position.

Table 3. Sensitivity to antibiotic disks for strains isolated from diseased spotted butterfish *Scatophagus argus* in comparison to the reference strains of the *Nocardia seriolae.*

Antimicrobial class	Disc potency (μg)	Susceptible (mm)	NS128	*Nocardia seriolae* NS127
Erythromycin (E15)	15	≧18	S	S
Doxycycline (DO30)	30	≧16	S	S
Spiramycin (SP100)	100	≧22	S	S
Tetracycline (TE30)	30	≧19	R	R
Oxytetracycline (OT30)	30	≧14	MS	S
Streptomycin (S10)	10	≧15	S	S
Neomycin (N30)	30	≧17	S	S

R, resistance; S, sensitive; MS, moderately.

Table 4. Cumulative mortality (%) of spotted butterfish and amberjack inoculated with *Nocardia seriolae* NS128.

Fish	Group	Route of inoculated	No. of fish used	Cumulative mortality (%) days after injection		
				5	10	15
Spotted butterfish	4.7×10^6	IP	8	12.5	100	
	PBS control	IP	8	0	0	0
Amberjack	4.7×10^6	IP	4	0	25	100
	PBS control	IP	4	0	0	0

NS127. All properties are consistent with the classification of the isolates in the genus *Nocardia* (Chen et al., 2000; Wang et al., 2005). *N. seriolae* which is an emerging pathogen in Taiwan, has been isolated from various fish species, including red snapper *Lutjanus erythropterus*, grey mullet *Mugil cephalus*, and three striped tigerfish *Terapon jarbua* in freshwater (Wang et al., 2009) and brackish water culture. Spotted butterfish when infected with *N. seriolae* exhibit gross lesions and histopathological changes that are similar to those observed in three striped tigerfish (Wang et al., 2009). Notably, *N. seriolae* dominates the nocardia infection of cultured fish in Taiwan.

The experimental investigation in this study demonstrated that NS128 isolated from the field case was pathogenic to spotted butterfish. The lesions from experimentally infected fish were highly similar to those found in naturally infected fish, suggesting that NS128 is probably the pathogen responsible for the losses of cultured spotted butterfish in Taiwan. In this study, the pathogen was reisolated in pure culture only from challenged fish. These experimental results verify that NS128 is the aetiological agent of this disease in cultured spotted butterfish. Most experimental fish exhibited various skin ulcers and granulomas in their internal organs. The multiple focal granulomas caused by nocardiosis have been described as predominant lesions in several fish (Campbell and Mackelvie, 1968; Ribelin and Migaki, 1975; Chen, 1992; Chen et al., 2000). In this investigation, affected fish that had anorexia, were emaciated and had distended abdomens with granulomas that were commonly diffused throughout the visceral organs. More acute lesions, such as those associated with muscle necrosis are characterized by hemorrhage and Zenker's necrosis were associated with a marked inflammatory response at the centre of mass of the organism (Kubota et al., 1968; Chen, 1992).

The experimental results of the IP injection study suggests that NS128 in bacterial suspension (4.7 x 10^6 CFU per fish) caused 100% mortality of spotted butterfish group within ten days of the inoculation and of the amberjack given a same concentration of bacterial suspension also exhibited 100% mortality at fifteen days post-inoculation. In this experiment, the NS128 isolated from the spotted butterfish was pathogenic for spotted butterfish and amberjack. Outbreaks of nocardiosis in largemouth bass in Taiwan have been associated with heavy rainfall, hot weather and handling (Chen et al., 2000). Handling is a major causal factor of the recrudescence of nocardiosis at the fish farms.

Miyoshi and Suzuki (2003) presented the PCR assay results for a 432 bp amplicon from *N. seriolae*, including *N. seriolae* JCM3360, and eight clinical strains. If that PCR assay is regarded as definitive for *N. seriolae*, then the bacterial isolate, NS128 obtained from diseased spotted butterfish must be considered a strain of *N. seriolae*. To support this argument, and increase the probably that NS128 corresponded to *N. seriolae*, the

PCR product from NS128 was sequenced and compared to sequences for *N. seriolae* in public databases.

The PCR method that was employed in investigation can be conducted directly using clinical samples such as infected fish liver, kidney, and muscle (Miyoshi and Suzuki, 2003). This approach was developed to facilitate the rapid identification of isolates from pure cultures and infected fish tissues. The detection of nocardial infections in fish using PCR combined with sequencing has considerable advantages over conventional histopathological and bacteriological diagnosis: speed, specificity and sensitivity for purified *Nocardia* spp. isolated from infected fish (Miyoshi and Suzuki, 2003). The extreme speed, and specificity of PCR may facilitate future work in elucidation of the mode of disease outbreak and propagation of nocardia in fish, whether through contaminated feed, water, or soil distribution of these bacteria in cultured ponds, or from parent to offspring.

Notably, 16S rDNA, heat shock protein gene, and RNA polymerase gene (rpo B) phylogenetic analyses also verified that the organism, NS128 was a member of the genus *Nocardia*. The evolutionary trees (s 5, 6 and 7) reveal that NS128 forms a monophyletic clade with reference strain *N. seriolae* BCRC 13745, and with GenBank accession Nos.: AF380937 and AY756533, respectively. This relationship was verified by the high nucleotide similarity value (100%) and the high bootstrap value (100%). The results together constitute the first verification that *N. seriolae* can cause disease outbreaks in the spotted butterfish.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Recovery of lipophilic *Malassezia* species from two infants with otitis media in Monterrey, Nuevo León, Mexico

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***Malassezia* species are yeasts that can be present on healthy human skin but also associated with external otitis and other skin diseases. The aim of this work was the identification of *Malassezia* -lipophilic- species recovered from infants with otitis media. Microbiological culture, polymerase chain reaction (PCR) and sequencing were performed for *Malassezia* detection in samples obtained from two cases of infants with otitis media. We identified two strains of lipid-dependent *Malassezia* species: *M. furfur* and *M. restricta*. This is the first report regarding the presence of *Malassezia* species in infants with otitis media in Monterrey, México.**

Key words: *Malassezia, Malassezia furfur*, *Malassezia restricta*, otitis media, infants, LSU rDNA D1/D2, yeasts.

INTRODUCTION

Malassezia species are lipophilic and /or lipid-dependent yeasts characterized by a thick cell wall, and are considered as normal components of the skin microbiota of both animals and humans (Crespo et al., 2000; Midgley, 1989; Batra et al., 2005).

Because of the difficult isolation and characterization of *Malassezia* spp. using traditional identification tests, molecular biology probes such as restriction fragment length polymorphism (RFLP) (Guillot et al., 2000; Gaitanis et al., 2002), pulsed-field gel electrophoresis (PFGE) (Gupta et al., 2004), random amplified polymorphic DNA (RAPD) (Castellá et al., 2006), and polymorphism amplified fragment length (AFLP) (Theelen et al., 2001) have been implemented. Actually, pyrosequencing techniques are

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used for a more precise and rapid identification of *Malassezia*, which as a consequence helps to implement more adequate and fast treatments (Kim et al., 2013). Other techniques, such as PCR-RFLP (Jagielski et al., 2014) and multiplex PCR methods (Vuran et al., 2014) have also been proposed and are useful to identify *Malassezia* species directly obtained from patients samples.

Based on molecular characteristics and lipid sources requirements, seven species of the genus *Malassezia* have been described, including *Malassezia furfur* (Robin) Baillon 1889, *M. pachydermatis* (Weidman) Dodge 1935, *M. sympodialis* Simmons & Gueho 1990, *M. globosa* Midgley, Guého and Guillot 1996, *M. obtusa*, *M. restricta* and *M. slooffiae*. *M. pachydermatis* is the only non-lipid-dependent species (Guého et al., 1996). More recently, the following seven new species, *M. dermatis*, *M. japonica*, *M. yamatoensis*, *M. nana*, *M. caprae*, *M. equina* and *M. cuniculi* were recently proposed (Sugita et al., 2002; Sugita et al., 2003; Sugita et al., 2004; Hirai et al., 2004; Cabañes et al., 2007 and 2011).

The presence of *Malassezia* associated with external otitis in carnivores such as dogs and cats is well documented (Eidi et al., 2011; Shokri et al., 2010; Hernández et al., 2012). However, there are few reports of this yeast in human cases of external otitis (Kaneko et al., 2010) and no reports of lipophilic *Malassezia* as causing agent of otitis media in infants and adults. Therefore, the aim of this work was to report the isolation and identification of *Malassezia* by PCR amplification and sequencing of the D1/D2 region of DNA encoding the LSU rRNA in two infants with a clinical diagnosis of otitis media.

MATERIALS AND METHODS

Isolation of *Malassezia* species

The samples were obtained from a 30-day old boy presented with a yellow-green secretion of the middle portion of right ear. The patient demonstrated excessive itching, tympanic hyperemia, and discharge in the ear canal. A diagnosis of otitis media was made based on otoscopic examination. The second sample was obtained from a 7-year old girl presented with bilateral secretion of the middle ear. A diagnosis of chronic otitis media was made. Swab samples of the discharge were taken. Both samples were seeded on potato dextrose agar and modified Dixon medium containing 0.5 g of chloramphenicol and cycloheximide.

Gram stain was performed in the grown colonies. The methodology for species identification was based in the ability to utilize individual Tween test proposed by Guého et al. (1996) and Tween diffusion test proposed by Guillot et al. (1996).

Identification of bacterial species

Bacterial isolation was done on blood culture media and S 110 media. For identification and morphology of microorganisms, Gram stains were performed. Characterization of recovered bacteria was performed by the automated system Vitek® (Laboratory bioMérieux). Briefly, a suspension of each cultured strains was prepared in a test tube with 0.45% of sterile saline solution. Tube suspension MacFarland No.1 ($3x10^8$ cells / ml) was adjusted. Gram negative identification (GNI) was extracted from the individual bag and marked with the strain number with Vitek®marker. The card was then packed in the module, sealed and placed in the reader / incubator module. Vitek® filling procedure was followed until the report was generated. Similarly, the process for Gram positive identification (GPI) card was conducted.

DNA extraction from pure cultures

Reference strains of *M. furfur* (CBS 1878^{NT}) and *M. restricta* (CBS 7878^{T}) along with clinical isolates were grown and maintained on modified Dixon's agar, containing 0.5 g of chloramphenicol and cycloheximide at 32°C for 8 days. DNA extraction was performed as previously described by Ferrer (2001). One hundred ng of genomic DNA was included in the following PCR reaction mixture to achieved final concentrations of 10 mM buffer 10X, 0.1 mM dNTP´s, 1.5 mM MgCl, 0.198 µM each primer (NL1: F- 5´ GCATATCAATAAGCGGAGGAAAAG-3´; NL4-R- 5´-GGTCCGTGTTTCAAGACGG -3´) (O'Donnell, 1993) and 1 U *Taq* DNA polymerase. Amplification was performed with 30 cycles (94°C for 45s, 51°C for 1 min and 72°C for 3 min) using a PTC-100 Pelter Thermol Cycler (MJ Research Inc, Massachussetts, USA). PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide under a UV transiluminator (D&RI Ind. Ltd Transilluminator and Gel-Pro Imager). PCR products were sequenced in an ABI Prism 3130 (Applied Biosystems, Foster City CA, USA). The sequences were submitted to Genbank. The first patient was given oral Amoxicillin every 8 h for 7 days and 1% Bifonazole cream, once daily for 14 days. Patient 2 was given Clindamicyn twice daily and Clotrimazol 2 to 3 times per day for 14 days.

RESULTS

The microscopic observation revealed yeast showing monopolar budding. *M. furfur* was identified in the first patient (JY-1) while *M. restricta* was identified in the second patient (JY-2). In Table 1, a summary of the characteristics of the patient isolates compared with reference strains (*M. furfur* CBS 1878^{NT} and *M. restricta* CBS 7878^{T}) is presented. Bacterial species identified with the Vitek ® automated system (BioMérieux Laboratory, México, DF) were *Klebsiella pneumoniae* and *S. aureus* in patient 1 and *S. aureus* in patient 2. After D1/D2 region amplification and comparison to the reference strain *M. furfur* CBS 1878^{NT}, JY-1 and JY-2 yielded the expected PCR product of 600 bp characteristic of *Malassezia*. Sequence alignments confirmed that the isolate from patient 1 had from 99 to 100% homology to *M. furfur* at the nucleotide level (GenBank accession No. KC415103.1 and AY745725.1, respectively) and sequence from patient 2 had from 98 to 99% homology to *M. restricta* (GenBank accession No. AJ249950.1 and JN651957.1, respectively). The nucleotide sequence data reported from both cases are available in the DDBJ/EMBL/GenBank database under accession Nos. JF323946.1 and JX439915. Children healed after treatment.

DISCUSSION

Despite the fact that *Malassezia* is found as part of the

Table 1. Physiological characteristics of *Malassezia* species studied.

Parameter	Glucose/Peptone Agar 32°C (Guého et al., 1996)	Catalase (Guého et al., 1996;Guillot et al., 1996)	Growth with Tween (Guého et al., 1996)			Tween diffusion Test (Guého et al., 1996)				Esculin (Mayser et al., 1997)	Growth T° (Guého et al., 1996).	
			20	40-60[a]	80	20[b]	40[b]	60[b]	80[b]		37°C	40°C
M. furfur[1]	-	+	+	+	+	+	+	+	+	+/-	+	+
M. restricta[2]	-	-	-	-	-	-	-	-	-	-	+	-
JY-1*	-	+	+	+	+	+	+	+	+	+	+	+
JY-2[+]	-	-	-	-	-	-	-	-	-	-	+	-

[1]*M. furfur* CBS 1887NT; [2]*M. restricta* CBS 7877[T]. *(Genbank accssesion No. JF323946); [+](Genbank accssesion No. JX439915). [a]At least one source of Tween should show growth; [b]Grows throughout the agar surface, but may be slightly inhibited growth around the well.

normal microbiota, infection with the organism is rarely reported. Consistent with our findings, it is important to highlight that the low occurrence of *Malassezia* in otitis media cases may not be due to absence of the organism but rather to the lack of suitable culture media for isolation of the micro-organism in clinical labs. In general, children cases referred to pediatric hospitals are linked to bacterial infections (Olajide et al., 2012; Turner et al., 2002; Parra et al., 2011). In this study, we performed an exhaustive literature review of otitis reports and found few describing the presence of *Malassezia* in external otitis cases in humans. Shiota et al. (2009) reported the presence of this yeast in 5 of 63 patients with otitis (Shiota et al., 2009). The presence of *M. slooffiae* and *M. restricta* in the external auditory canal of clinically healthy people (Kaneko et al., 2010) while presence of *M. sympodialis* in a diabetic patient with external otitis has also been reported (Chai et al., 2000). Sugita et al. (2013) reported the presence of *M. globosa* and *M. restricta* in lesions of the external auditory canal and sole of the foot (Sugita et al., 2013, Zhang et al., 2012). It is known that otitis is primarily caused by bacterial action, however, the presence of *Malassezia* species in infants with otitis media is yet to be reported. While most species of *Malassezia* colonize lipid-rich areas of human skin, these yeasts can be found on any area of the body. Usually *Malassezia* species constitute 53-80% of total skin fungal population (Gao et al., 2010). The presence of yeast with the bacteria found in the present study could indicate the possibility that *Malassezia* spp. may be involved in otitis media in humans. However, a larger epidemiological study is warranted to determine the nature of this relationship (Makimura et al., 2000; Zhang et al., 2012). Findings of this study are clinically significant since these results contribute to further understand the possible effects of fungal presence on human skin.

This investigation is the first report of lipophilic *Malassezia* species in infants with otitis media.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Assessment of *Salmonella* spp. presence among broilers of naked neck Label Rouge lineage in Northwest region of Paraná State, Brazil

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The presence of *Salmonella* serovars in poultry products is a worldwide public health concern. Moreover, salmonellosis is one of the most common foodborne diseases with approximately 10 million cases of human infection occurring per year worldwide. The aim of this study was to assess the presence of *Salmonella* spp. in broilers of the naked neck Label Rouge lineage by cloacal swab technique in a broiler house in the northwestern region of the state of Paraná, Brazil. Cloacal swab samples were collected from 100 broilers raised under the free-range farming system, and the detection of *Salmonella* spp. was carried out using biochemical and serologic techniques. No *Salmonella* spp. was detected on the poultry studied. This result may indicate the free-range farming system as a contributing factor to reduce the presence of foodborne pathogens in poultry production as compared to confinement and intensive farming system. More studies in this area are necessary in order to understand the real benefits of the free-range farming system in relation to foodborne pathogens.

Key words: Salmonellosis, free-range farming system, human health, poultry.

INTRODUCTION

Salmonellosis is one of the most common foodborne diseases with approximately 10 million cases of human infection occurring per year worldwide (Gopinath et al., 2012; Mercado et al., 2012). This disease is caused by different *Salmonella* spp. serovars, and is characterized by an acute onset of fever, abdominal pain, diarrhea, nausea and vomiting in humans. The most common source of foodborne salmonellosis to humans is the consumption of contaminated and inappropriately prepared broiler meat or chicken eggs (Ravel et al., 2009).

The presence of *Salmonella* spp. in poultry products is

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a worldwide public health concern since it causes asymptomatic infection in most poultry cases and does not cause high mortality in most situations. Studies have demonstrated that broilers on the slaughter line may be contaminated with these bacteria, representing risk to human health (Shinohara et al., 2008; Von Rückert et al., 2009; Mercado et al., 2012; Desin et al., 2013). In addition to public health problems, the presence of these bacteria in broilers also causes high economic losses due to a decrease in meat quality, an increase in drug costs and large expenditures in eradication and control strategies (Shinohara et al., 2008).

Currently, Brazil along with other countries is one of the largest broiler producers in the world. This elevated production requires rearing methods that result in products with high technical quality regarding food safety. Therefore, some producers have sought to use free-range farming system in broiler production (Savino et al., 2007; Sun et al., 2013; Sales et al., 2014).

This farming system consists of rearing the broilers in confinement until 28 days of life and after this period, the animals get access to the outdoors. This system allows obtaining better meat quality as compared to the confinement and intensive system, since it preserves the animal welfare, stimulates its immune system, decreases the stress level and reduces or eliminates the use of chemotherapy drugs to prevent infections or being used as growth promoters (Nazareno et al., 2009; Tong et al., 2014). The feed used in this system is preferably prepared with products from vegetable origin, according to the Brazilian Ministry of Agriculture, Livestock and Food Supply legislation (Nazareno et al., 2009; Tong et al., 2014).

The aim of this study was to assess the presence of *Salmonella* spp. isolates in broilers of naked neck Label Rouge lineage by using the cloacal swab technique on a broiler house in the northwestern region of the state of Paraná, Brazil.

MATERIALS AND METHODS

Study setting

The study was approved by the Ethics Committee and Research Involving Animal Experimentation of Paranaense University, Brazil, under protocol 26568/2013. From July 2013 to February 2014, cloacal swabs were collected from broilers of naked neck Label Rouge lineage in a broiler house located in the city of Umuarama, in the northwestern region of Paraná state, Brazil. All the cloacal swabs were collected from animals at 85 days of life raised in the four broiler houses during different periods, comprising four different flocks.

The animals studied were raised under the free-range farming system. The area used for poultry production had approximately 1700 m^2 divided into four different broiler houses, with approximately 1000 animals each. Each broiler house had an enclosed warehouse for water and ration supply, protection from predators, wind, cold and rain. A commercial feed was used to feed the animals during the first 28 days of life. After this period, the animals had access to the outdoor area and their feed was supplemented with banana, cassava flour and foliage.

Collection of biological samples

Fifty cloacal swabs were collected from broilers to detect the presence or absence of Salmonella spp. Each cloacal swab was sampled in two animals, totaling 100 broilers analyzed.

The number of animals studied was chosen using the formula by Barbetta (1999), taking into account a tolerable sampling error of 10% and considering a population of 4000 animals.

$n_o = 1/E_o^2$

$n = N.n_o/(N+n_o)$

Where: N= Population size = 4000; E_o = tolerable sampling error = 10%; n_o = first approximation of the sample = 100; n = birds sampled = 90,90 = 100.

After collection, swabs were stored in transport medium containing 1% buffered peptone water (BPW) and kept at 4°C until processing at the Laboratory of Veterinary Preventive Medicine and Public Health at Paranaense University (UNIPAR).

Microbiological and serological tests

The laboratory techniques used to detect *Salmonella* spp. were performed according to the recommendations of the Brazilian Ministry of Agriculture, Livestock and Supply (Ordinance 126 of 11/03/1995) with modifications.

The swabs were inoculated in sterile tubes containing buffered peptone water (BPW) for 24 h at 36°C. After this period, the bacterial growth was inoculated into both selective media Tetrathionate Broth (TT) and Rappaport-Vassiliadis (RVB) and incubated for 24 h at 36°C. The enriched samples were plated on xylose-lysine deoxycholate agar (XLD agar), brilliant green agar (BGA) and MacConkey agar and incubated overnight at 36°C. Each plate was evaluated for the presence or absence of lactose negative colonies.

Three suspicious colonies that were morphologically similar to *Salmonella* spp. from each plate were sub-cultured for biochemical examinations. Biochemical characteristics were examined on urea broth, triple sugar iron medium (TSI) and lysine iron agar (LIA). When typical *Salmonella* reactions were seen, they were analyzed by serological tests using somatic and flagellar polyvalent antisera (Probac®, Brazil) to confirm the presence of Salmonella (LeMinor, 1988). The positive control used in serological test was *Salmonella* Enteritidis fegotype.

RESULTS

Among the 50 cloacal swabs samples analyzed, 20 (40%) showed colonies with morphological characteristics of *Salmonella* spp. From these, only 6/20 (30%) isolates had the biochemical characteristics confirmed on TSI, LIA and urea broth (Table 1). All these six isolates were tested against *Salmonella* spp. somatic and flagellar polyvalent antisera, but no isolate was considered positive (Table 1).

DISCUSSION

The results indicated absence of *Salmonella* spp. strains in the cloacal swabs analyzed. Similar results were also found in confined and non-confined (free-range) broilers

Table 1. Isolation and characterization of *Salmonella* spp. in the cloacal swabs analyzed.

Test result	Morphological characteristics of culture media	Biochemical characteristics (TSI, LIA and urea broth)*	Serological tests (somatic and flagellar antigens)**
Positive strains	20/50 (40%)	6/20 (30%)	0/6 (0.0%)
Negative strains	30/50 (60%)	14/20 (70%)	6/6 (100%)

*Only strains with morphological characteristics of *Salmonella* spp. were characterized by biochemical tests. **Only strains identified as *Salmonella* spp. in the biochemical tests were submitted for serological tests.

house in other regions of Brazil (Moreira, 2002; Guimarães, 2006; Ravagnani et al., 2012; Pereira and Silva, 2005).

Guimarães (2006) in Brasilia (DF) evaluated 300 cloacal swabs collected from free-range raised broilers, but no *Salmonella* spp. strain was found. According to the author, the results found may indicate the absence or a very low prevalence of *Salmonella* spp. colonization, or the poultry from that broiler house were more resistant to *Salmonella* spp. asymptomatic colonization.

Similar results were found by Pereira and Silva (2005). These authors analyzed 44 cloacal swabs from nine properties in Uberlândia (MG), and found only one positive sample. The absence of *Salmonella* spp. was also observed in chicks and organ fragments from broilers in the states of Ceará and Paraná (Moreira, 2002; Ravagnani et al., 2012).

Despite these data, some studies have reported high prevalence of *Salmonella* spp. in broilers reared under confined and intensive system both in Brazil (Kanashiro et al., 2005; Ribeiro et al., 2007) and other countries (Limawongpranee et al., 1999; Siemon et al., 2007; Alali et al., 2010).

Kanashiro et al. (2005) found prevalence of 84% in broiler flocks and 57.5% in commercial breeders in the following Brazilian States: Bahia, Ceará, Goiás, Paraná, Mato Grosso, Mato Grosso do Sul, Santa Catarina and São Paulo.

Alali et al. (2010) compared the prevalence of Salmonella in organic and conventional broiler poultry farms in the same company in North Carolina and verified that the Salmonella prevalence in fecal samples were 5.6 and 38.8% in the organic and conventional farms, respectively. The authors concluded that the prevalence of fecal *Salmonella* spp. was lower in certified-organic birds than in conventionally raised birds.

Similar results were verified by Siemon et al. (2007). The authors reported that fecal *Salmonella* spp. prevalence in conventional poultry flocks (30%; 125/419) was significantly higher than in pasture flocks (16%; 83/512).

The results shown in this work may indicate the presence of an efficient biosecurity program in broiler production, which protect the animals from contamination (Tessari et al., 2003; Teixeira and Lima, 2008; Van Hoorebeke et al., 2011), reinforcing the evidence that the prevalence of *Salmonella* spp. in free-range raised poultry may be lower than that from poultry raised in feedlots (Guimarães, 2006). Commercial poultry are raised in small compartments where an infected animal can spread the bacteria very quickly, due to the high stocking rate per square meter. Furthermore, the excretas may contaminate the water, feed and litter, turning the broiler house into an environmental source of contamination for the animals.

Another fact to be considered is the use of large quantities of meat and bone meal for animal feed production by the commercial poultry industry. This feed is a rich source of amino acids, minerals and vitamins, but being an organic product, it is highly susceptible to *Salmonella* spp. contamination (Teixeira et al. 2003). The free-range rearing system uses vegetable-derived products in animal feed, which may explain the absence or no isolation of *Salmonella* spp. in this study, since the animals consumed cassava flour, banana and foliage as supplementary feeding.

On the other hand, Bailey and Cosby (2005) evaluated the *Salmonella* spp. prevalence in free-range and certified organic chicken in 135 processed free-range chicken carcasses from four different commercial free-range chicken producers and collected samples from 14 different lots for the presence of *Salmonella* spp. and verified that 9 (64%) from the 14 lots and 42 (31%) from the 135 carcasses were positive for *Salmonella* spp. The authors concluded that the consumers should not assume that free-range or organic conditions would have anything to do with the *Salmonella* spp. status of the chicken.

Furthermore, the age of broilers is considered as a factor that affects the susceptibility for *Salmonella* spp. colonization. Young broilers are more susceptible than adult ones (Bailey, 1987; Andreatt-Fillho et al., 2006). According to Andreatti-Filho (2006), a reduction in *Salmonella* spp. colonization susceptibility is observed with age increase due to intestinal microbiota development and strengthening of the immune system. In the present study, the cloacal swabs were collected from adult animals with 85 days of age.

The prevalence of *Salmonella* spp. in broilers may be influenced by parameters such as flock size, multi-aged placement of complexes or individual farms and a host of other variables (Van Hoorebeke et al., 2011). Therefore, the main form of controlling the presence of *Salmonella* spp. in poultry production is related to biosecurity measures

and vaccination, associated with the right use of antibiotics, prebiotics and probiotics. (Barrow, 2007; Van Immerseel et al., 2005; Picler et al., 2012).

Conclusion

According to the results obtained, *Salmonella* spp. was not isolated in broilers studied. This fact may reflect the correct management and application of biosecurity programs along with the free-range broiler farming system. However, it is important to emphasize that the *Salmonela* spp. isolation by conventional techniques requires a large number of viable cells, which may also explain the absence of positive results in this study. Further studies in this area, including molecular tests, are necessary to understand the real benefits of free-range farming system.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Detection of antibodies to Peste des petits ruminants virus using passive haemagglutination test and cELISA in the White Nile state-Sudan, comparative study

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Peste des petits ruminants (PPR) is an important viral disease of sheep and goats, it causes huge economic losses in susceptible animals. The prevalence of PPR in sheep and goats in White Nile State, Sudan was studied; passive haemagglutinatin test (PHA) and competitive ELISA were used to detect PPR antibodies in 517 serum samples collected from animals that showed symptoms suggestive of PPR disease as well as from clinically healthy ones at White Nile State, Sudan. The serum samples were tested for presence of PPRV antibodies using cELISA and PHA test. The results obtained by PHA were compared with that of ELISA. 314 (60%) of serum samples were positive by cELISA while 291(56.2%) were positive by PHA. By both tests, 262 (83%) out of the tested samples were found positive for PPRV antibodies. The agreement between the two tests was 84.3%, the relative sensitivity was 84% and specificity was 85.2%. Age and localities appears to be risk factor for cELISA and PHA PPR seropositivity. PHA was found to be a useful technique for detecting PPRV antibodies.

Key words: Peste des petits ruminants, passive haemagglutination test, cELISA.

INTRODUCTION

Peste des petits ruminants (PPR) or small ruminant plague is a viral disease primarily affecting goats and sheep (Ezeibe et al., 2004, Ahmed et al., 2010). PPR virus is known to cause fatal disease in camel (Khalafalla et al., 2010) and symptomatic infection of cattle and wildlife (Ahmed et al., 2010; Sibel and Albayrak, 2010). The causative agent of PPR disease is an enveloped, RNA, single stranded, negative sense virus (PPRV) that belongs to the genus morbillivirus in the family paramyxoviridae (Murphy et al., 1999; Waret-Szkuta et al., 2008). There is only one serotype of PPRV (Barrett et al., 1993) and four genetic lineages (lineage 1-4) which are distinguishable by nucleic acid sequencing (Rossiter, 2005). PPR disease is characterized by 3D disease, discharges (nasal, ocular and oral), diarrhoea and death. The other symptoms are high fever, eroded stomatits,

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pneumonia and gastroenteritis (Islam et al., 2003). The disease causes heavy economic losses on the bases of mortality, morbidity, losses through body wastage, poor feed efficiency and loss of meat and milk products (Ogunsanmi et al., 2003). The presence of the disease in a country may limit its trade and export beside the loss of animal protein for human consumption (Khan et al., 2008).

PPR was first described in West Africa in 1942, then the outbreak occurred in many African countries recently in 2008 in Morocco and in 2009 in Tanzania (Kwiatek et al., 2011), also the disease is recognized in the Arabian peninsula, the Middle East and Asia (Banyard et al., 2010, Abubakar et al., 2011). In the Sudan, since PPR was first observed by El Hag Ali at Gadarif state in 1972 continuous outbreaks have occurred in different states (Intisar et al., 2010; Kalafalla et al., 2010).

There are often numbers of risk factors that contribute of transmission of PPR disease, Elsawalhy et al. (2010) stated the amount of movement and exchange of animals between flocks and Abubakar et al. (2011) described the sex, age, species, bread and seasons as risk factor.

Many techniques are routinely used to detect PPR antibodies like, counter immunoelectrophoresis (CIEP), agar gel immunodiffusion (AGID), precipitation inhibition test, indirect fluorescent antibodies test (OIE, 2000), virus neutralization test (VNT) (Rossiter, 1994), competitive ELISA (Libeau et al., 1995), indirect ELISA (Diallo et al., 1995), haemagglutantion test (Wosu, 1991), competitive ELISA based on the reaction between monoclonal antibodies (mAb) and recombinant nucleo protein of PPR virus (Libeau et al., 1992). Passive haemaglutination test is done by coated RBCs with antigen in which RBCs agglutinate in the presence of antibodies. Wosu (1985) was first to demonstrate the haemagglutination test for PPR homogenate antigen using porcine erythrocytes. Haemagglutantion test could be used as reliable alternative in the field condition particularly in places where RP and PPR are co-existing. Wosu (1991) reported that HA is a simple laboratory confirmatory test of PPR disease in sheep and goat.

The aim of this work was to present results of PPR in the survey in the White Nile State, Sudan and evaluate the efficacy of haemagglutination test for detection of PPRV antibodies.

MATERIALS AND METHODS

Sample collection

A survey was conducted in the White Nile State during April 2008 to February 2009, it covered 15 districts. A total of 517 serum samples were collected from sheep (432) and goats (85). The sample collected from animals showed symptoms (ocular and nasal discharge, erosion in mouth and foul smelling diarrhea) suggestive of PPR disease as well as apparently healthy animals.

Detection of antibodies to PPR using competitive ELISA (cELISA)

cELISA kits were manufactured by CIRAD EMVT; Montpellier, France 2005, distributed by BDSL. The test was performed according to manual provided with the kits. The plates were read on ELISA reader using 492 nm filter, percentage inhibition and positive percentage determined by ELISA data information software (FAO/IAEA, Vienna, Austria) loaded on computer connected with ELISA reader. Serum showing percentage inhibition (PI) <40% was considered as negative, 50-80% considered as weak positive, >80% considered as strong positive.

Passive haemagglutination test

Preparation of coated erythrocytes

Preparation of erythrocytes and test procedure was done as described by Vengris and Mare (1971) with some modification.

Sheep blood was collected in equal volume of Alsever's solution and stored for 2-4 days at 4°C. RBCs were centrifuged at 1500 g for 15 min and washed five times with PBS, pH 7.2, and resuspended to 10% in PBS. Formalinization was performed using equal volumes of 3% formalin solution and 10% erythrocyte suspension. The mixture was incubated at 37°C for 20 h after which the cells were centrifuged at 100 g for 20 min and washed three times to remove formalin and then resuspended to 5% suspension in PBS. An equal volume of freshly prepared tannic acid at a concentration of 1:20,000 in PBS was added to 5% RBCs and the mixture was incubated at 37°C for 30 min. Tanned formalized cells were washed once with PBS and coated immediately by adding PPR antigen, stirred at room temperature for 1 h and then washed once with PBS and twice with PBS containing 1% inactivated horse serum. The coated tanned RBCs (sensitized cells) were resuspended to 1% suspension in PBS and stored at 4°C until used. All sera were inactivated at 56°C for 30 min before being tested by PHA.

Test procedure

Passive haemagglutination test was carried out in haemagglutination U shape plate, 25 µl of PBS were added to all wells; two-fold serial dilution of 25 µl tested serum was done. This was followed by adding 25 µl of 1% sensitized RBCs. The plates were covered, shaked carefully for short time to mix the reactants, incubated at room temperature and read after 3 h. A positive agglutination pattern was read as a uniform thin layer of erythrocytes that covers the bottom of the well. The reciprocal of the highest dilution of serum which gave positive agglutination was considered to represent the antibody titer of the serum. A negative pattern consisted of a round button of sediment cells.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 11.5 was used to analyze data. Descriptive statistics of the variables were obtained. For each variable (age, sex, breed and locations), frequencies (number of observations within variable) and prevalence rates by cross-tabbing (number of positive valid samples/number of individuals sampled in the variable) were obtained.

Age, sex and species were first tested in univariable analysis by means of 2-talied chi-square. In second step, logistic regression

Table 1. Results of seroprevalence level of PPR sero-positive using cELISA and the univariate association of risk factors with sero-positive status against PPR (2-tailed chi-squared (P-value < 0.05).

Variable	No tested	No positive	Seroprevalence (%)	95% CI lower-upper	Chi-square	p-value
Localities					45.612	0.000
Aboshateen	12	7	58.3	1.056-1.694		
al Jazera mosran	3	2	66.6	0.697-1.803		
Rabak villages	157	97	61.7	1.256-1.521		
Al kawa	10	9	90	0.714-1.369		
Joda	28	18	64.2	1.152-1.566		
Al Jazera aba	26	10	38.4	1.493-1.892		
Al diwam villages	45	21	46.6	1.281-1.664		
Kosti villages	132	92	69.6	1.002-1.35		
Gafa	54	39	72.2	1.224-1.592		
Al jabaleen	30	5	16.6	1.611-2.122		
Al zeleet	20	14	70	1.052-1.727		
Species					2.158	0.142
Sheep	433	269	62.1	1.357-1.511		
Goat	84	45	53.5	1.287-1.532		
Sex					1.332	0.248
Femal	310	182	58.7	1.326-1.498		
Male	207	132	63.7	1.337-1.552		
Age					28.966	0.000
< 1	46	33	71.7	1.216-1.596		
> 1-2	144	111	77	1.207-1.423		
> 2	327	170	51.9	1.417-1.598		
Seasons					0.107	0.948
Summer	72	43	59.7	1.172-1.478		
Autumn	249	153	61.4	1.313-1.503		
Winter	196	118	60.25	1.393-1.635		
Total	517	314	60.2			

model was used to assess the association between risk factors sex, age and species on PPR seropositivity.

Chi-square was obtained to assess the relationship between cELISA and PHA and the specificity and sensitivity of PHA for diagnostic PPRV antibodies.

RESULTS

The sero-prevalence rate of PPR

314 out of 517 serum samples were found positive for PPRV antibodies tested by cELISA test giving 60.2% prevalence rate (Table 1). 291 serum samples were found positive for PPRV antibodies tested by PHA test giving 56.2% prevalence rate (Table 2), the titer of PPRV antibodies obtained in PHA test ranged between $3\log_2$ - $8\log_2$, (203 serum samples from animals showing PPR symptoms and 87 from clinically appearing healthy) the highest positive titer observed was $6\log_2$ (Table 3).

Risk factor analysis

Using cELISA test, the proportion of PPR sero-positivity differs between variables, the result of univariate analysis are presented in Table 1 showing that sheep were 1.5 times more likely to be sero-positive for PPR than goat and male were 1.1 times more likely to be sero-positive for PPR than female.

The result of logistic regression assessing the relationship between age, sex, seasons and species with sero-positive of PPR in cELISA test is shown in Table 4. The factors found are significantly associated with increased odds of being cELISA positive was age (< 1 and > 1-2).

Using PHA test, the results of univariate analysis are presented in Table 2. Sheep were 1.12 more likely to be sero-positive for PPR than goat and male were 1.1 more likely to be sero-positive for PPR than female.

Table 5 shows the result of logistic regression assessing

Table 2. PPR seroprevalence rate and the univariate association of risk factors with PHA test sero-positive to PPR in sheep and goat (2-tailed chi-squared (P-value < 0.05).

Variable	No test	No +ve	Seroprevalence (%)	95 % CI upper-lower	Chi-square	p-value
Localites					36.619	0.000
aboshateen	12	4	33.3	1.911-1.255		
al Jazera mosran	3	0	0	2.568-1.432		
Rabak villages	157	88	56	1.534-1.262		
Al kawa	10	9	90	1.379-0.705		
Joda	28	16	57.1	1.64-1.214		
Al Jazera aba	26	11	42.3	1.82-1.41		
Al diwam villages	95	15	33.3	1.737-1.343		
Kosti villages	132	89	67.4	1.376-1.018		
Gafa	59	34	57.6	1.536-1.157		
Al jabaleen	30	11	36.6	2.029-1.504		
Al zeleet	20	14	70	1.736-1.043		
Species					8.712	0.003
Sheep	433	256	59.1	1.496-1.337		
Goat	84	36	41.6	1.62-1.369		
Sex					1.836	0.175
Femal	310	167	53.8	1.518-1.341		
Male	207	124	59.9	1.587-1.365		
Age					21.232	0.000
< 1	46	32	69.5	1.581-1.189		
> 1-2	144	100	69.9	1.482-1.259		
> 2	327	159	48.6	1.619-1.433		
Seasons					1.449	0.485
Summer	72	36	50	1.653-1.339		
Autumn	249	141	56.6	1.51-1.314		
Winter	196	114	58.1	1.602-1.353		
Total	517	291	56.2			

Table 3. Titer of PPR antibodies in serum samples collected from sheep and goat in different districts of White Nile State using PHA test.

District	Log_2 titers						Total
	3	4	5	6	7	9	
El Gitana area	9	4	3	5	3	5	29
El Jabaleen area	8	6	7	5	1	-	27
Kosti area	16	10	14	22	15	26	103
Rabak area	9	26	18	26	9	10	98
Gafa area	1	6	13	10	2	2	34
Total	43	52	55	68	30	43	291

the relationship between age, sex, seasons and species with sero-positive of PPR in cELISA. The factors found significantly associated with increased odds of being PHA positive were sheep and age (< 1 and > 1-2).

Comparison between PHA test and cELISA for detection of PPR antibodies

517 serum samples were tested for the presence of PPR antibodies using cELISA and PHA test, 174 were diagnosed negative and 262 were diagnosed positive by both tests (Table 6). The agreement between PHA test and cELISA was 84.3% (262+174/517) and the relative sensitivity of PHA was 84% (262/314) and specificity was 85.2% (173/203)

Measure of the strength of the linear relationship between cELISA and PHA was done by Chi-square, the person correlation, r = 0.681, p = 0.000 (sig (2-taild)), the Kappa value was k = 0.68, which indicate perfect strong correlation between them.

DISCUSSION

PPR is one of the economically important diseases of

Table 4. Results of logistic regression analysis of risk factors (sex, age, seasons and species) with cELISA PPR seropositivity in White Nile state-Sudan.

Variable	OR	95% CI_{OR}	P
Sex			
Male	Ref		
Female	0.789	0.539-1.156	0.224
Species			
Goat	Ref		
Sheep	1.536	0.922-2.558	0.099
Age			
> 2	Ref		
< 1	2.45	1.209-4.783	0.012
> 1-2	3.209	2.046-5.034	0.000
Seasons			
Winter	Ref		
Summer	0.959	0.528-1.741	0.89
Autumn	1.038	0.692-1.555	0.858

Table 5. Results of logistic regression analysis of risk factors (sex, age, seasons and species) with PHA PPR seropositivity in White Nile State, Sudan.

Variable	OR	95% CI_{OR}	P
Sex			
Male	Ref		
Female	0.759	0.522-1.104	0.149
species			
Goat	Ref		
Sheep	2.061	1.239-3.428	0.005
Age			
> 2	Ref		
< 1	2.546	1.290-5.025	0.007
> 1-2	2.586	1.689-3.959	0.000
Seasons			
Winter	Ref		
Summer	0.765	0.426-1.375	0.371
Autumn	0.953	0.640-1.420	0.813

Table 6. Comparison between PHA and cELISA for detection of PPR antibodies in sheep and goats sera collected from different districts in White Nile State during the period of April 2008 to February 2009- result of χ^2 (crosstabs).

Count		cELISA		Total
		Positive	**Negative**	
PHA	Positive	262	29	291
	Negative	52	174	226
Total		314	203	517

small ruminants with variable prevalence in several parts of Asia and Africa. In Sudan, the first outbreak of the disease in sheep and goats was reported in Gedarif (EL Hag, 1973), and then the disease was detected in different parts of the country.

This study was performed in White Nile State which is bordered by Khartoum State in the north, El gezira State, Blue Nile and Sinnar State to the East, Upper Nile State of republic of South Sudan to the South, North and South Kordofan State to the West, PPR disease has been reported in all these areas (Nussieba et al., 2008; Intisar et al., 2010).

Serum samples collected from sheep and goat from different parts from all seasons in White Nile state, some sera were collected from PPR cases, the clinical signs observed were, nasal and ocular discharge, pneumonia, foul smelling diarrhoea, erosion in gum and death as described (Ahmad et al., 2005, Khan et al., 2008).

PPR seroprevalence was detected by a very highly specific (99.4%), sensitive (94.5%) and rapid cELISA kit. The seroprevalence of PPRV antibodies in this study was 60%, this is similar to the previous study by Intisar et al. (2010) who reported seroprevalence of 62% of 90 sera in the White Nile State and it is less than the results obtained by Wifag (2009) where the seroprevalence was 76% in four localities (kosti, El getana, El gabaleen and El doium) at White Nile State. All the previous studies of PPR disease in White Nile state were done on few samples and not covered wide range of localities and seasons. Most of positive tested sera presented very strong positive (high titer) of PPR antibodies, the number of strong positive sera was 257 out of 314 positive samples. Some sera of higher positive results were collected from group of animals some of them showed no clinical signs which are suggested to be probably infected by PPR disease in mild form or those animals were recovered from past recent infection of PPRV. Couacy-Hymann et al. (2007) reported that the Sudan Sinnar strain (lineage 3) produced acute to mild disease.

In this study, higher prevalence rate was observed in the east of the state, which is bordered by Sinnar and El Gezira state, in this area, the movement of animals between El Gezira, Sinnar and White Nile States is a continuous process of sharing pasture and water. Most of positives with higher titre were observed in sera collected during winter and rainy season; this was similar to the observation reported by Sarkar (2006). The high prevalence of PPRV antibodies in sheep and goat in all seasons indicate that the PPRV is continuously circulating in the state. The prevalence of PPR antibodies was higher in sheep than in goats, this result was in agreement with similar study done in White Nile state by Intisar et al. (2010), also Khan et al. (2007) and Abdalla et al (2012) stated that the prevalence of PPR antibodies in goat is greater than in sheep. The number of samples taken from sheep were greater than those taken from

goats and the higher seroprevalence in sheep, could be attributed to the fact that owners keep large numbers of sheep for its economic impact. Continuous movement of sheep in large groups crossing long distance seeking for good pasture and water increased the chance for sheep to be more infected by PPRV than resident goats. Males showed significantly higher seroprevalence rate than females, a similar result observed by Abubakar et al. (2011) and Sarkar and Hemayeatal (2011), and disagree with Khan et al. (2008).

Age appears as to be risk factor for cELISA PPR seropositive, which is similar to the study of Waret-Szkuta et al. (2008), and also localities were identified as risk factor.

Passive haemagglutination test was performed in this study to detect PPR antibodies in 517 serum samples. The titers of PHA test ranged between $3\log_2$ - $8\log_2$. PHA was found to be useful for detecting antobidies to PPR; this is similar to the report by EL Hussein et al. (2005) who studied seroprevalence of Infectious Bovine Rhinotrachitis virus antibodies in Sudanese cattle by PHA test, the titers they reported ranged between $3\log_2$ – 8 $\log_2$. Ali et al. (2004) diagnosed sheep pox virus antibodies in different areas in Sudan using AGID and PHA test, the titers they reported ranged between $1\log_2$ - $10\log_2$.

The comparison between PHA and cELISA was based on testing 517 serum samples, the agreement between PHA test and cELISA was 84.3% and the relative sensitivity of PHA was 84% (262/314) and specificity was 85.2% (173/203). PHA test showed similar results to cELISA in which the seroprevalence of PPRV antibodies is high at some seasons (during winter and autumn), in sheep and males, also age and localities appeared to be risk factor for PHA PPR seropositivity.

This study compared passive PHA and cELISA, the two tests are able to detect PPR antibodies from serum. cELISA is rapid, simple, specific and sensitive to detect PPR antibodies in serum but it is very expensive, need expert technician and not available at regional laboratories. The performance of PHA test is easy and does not require expert technician like cELISA. The long effective life of formalinized sensitized RBCs when stored at refrigeration makes it an ideal test to use in diagnosis of PPR. PHA test is sensitive, specific; low cost does not need any special equipment and it could be used for routine diagnosis of PPR.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Multiple molecular markers for diagnosis of conjunctivitis caused by *Candida* spp. in Iraq

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Candidiasis of the eye and its symptoms may lead to blindness in many patients. This study aimed to survey and diagnose ocular candidiasis by identification of the causative *Candida* spp. from 165 swabs samples collected from patients suffering from conjunctivitis (pink eye infection). A total of 35 clinical isolates of *Candida* spp. were isolated and preliminarily identified by CHROMagar technology. Based on the molecular diagnosis by specific polymerase chain reaction (PCR) primers and *Candida albicans* microsatellite (CAI), 8 of 24 isolates were identified as *C. albicans* and the rest as non-albicans. Ribotyping of 24 isolates was performed for 7 genotypes with a universal primer pair. *C. albicans* was detected with the highest frequency (33%; 8/24), followed by *C. famata* (29%) and C. *rugosa* (4.2%) with the lowest frequency. We concluded that ocular candidiasis is an important disease caused by *Candida* spp. as it was highly prevalent among the sampled swabs (approximately 21.2%; 35/165) from conjunctivitis patients. In addition, the use of molecular markers together with affirmable diagnostic tools for *Candida* isolates can produce coincident results at the molecular level. The elucidation of the results showed existence of type polymorphisms of CAI microsatellite as two bands of 297 and 240 bp.

Key words: Eyes candidiasis, molecular diagnosis, rDNA typing, CAI microsatellite polymorphism, Iraq.

INTRODUCTION

Over the past 10 years, the number of ocular candidiasis has significantly increased, particularly in poor countries (Bharathi et al., 2003; Saha and Das, 2006). Fungal keratitis is an important eyes disease, keratitis is the medical term for inflammation of the cornea, the most frequent cause of keratitis is bacteria, viruses, fungi and parasitic. More than 30 genera of filamentous fungi and yeasts were reported as the causative agents of keratitis. Predominantly saprophytic fungi such as *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria* and yeasts may cause eye infections in animal and humans (Rosa et al., 2003; Srinivasan, 2004; Thomas and Kaliamurthy, 2013).

Saha and Das (2006) implicated *Candida albicans* as a pathogen in 45.8% of keratitis cases. Callanan et al. (2006) mentioned that *Candida* spp. are opportunistic pathogens that cause mucosal infections such as keratitis, exogenous endophthalmitis infections in post-surgical patients, and other systematic mycoses, depending on the strain virulence and host vulnerability.

Candida is a genus of yeasts and is the most common cause of fungal infections worldwide. Many species are harmless commensalism or endosymbionts of hosts

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including humans; however, when mucosal barriers are disrupted or the immune system is compromised they can invade and cause disease. *C. albicans* is representative of the important pathogens causing exogenous fungal endophthalmitis, endogenous endophthalmitis and keratitis (Gregori et al., 2007). Several studies have been performed in the USA and other geographical areas to investigate the suffering of animals from eye keratitis (Moore et al., 1988; De Sousa et al., 2001). Oude Lashof et al. (2011) reported epidemic of ocular candidiasis in 370 patients, of which 49 had consistent findings with the diagnosis of positive ocular fungal infection; some of these fungi continue to inhabit the patient body as normal flora, while others behave as pathogens. Several reports refer to fungal eye infection as critical, as it accounts for 78% of all endophthalmitis infections (Aliyeva et al., 2004).

Although eyes have an inherent multi-defense mechanism against microbial infections, monitoring the increase of fungal eye infection needs to be synchronized with the increase in the number of patients with acquired immune deficiency (Matthews, 1994). Especially, individuals exposed to broad-spectrum antibiotics over a long period and those afflicted with AIDS are more disposed to fungal eye infection (Scalise, 1997).

Despite availability of reliable and rapid methods for yeasts identification, precise diagnosis of yeasts continues to remain controversial based on the phenotypic criteria such as chlamydospores abundance, germ tube formation, and colony colors on the CHROMagar candida medium and the difference in the colony colors between *C. albicans* and *C. dubliniensis* and others *Candida* spp. that show pink to white-pink color colonies, causing misidentification (Abaci et al., 2008). Most of the available biochemical tests are expensive, time consuming and do not always give rapid and precise results; on the other hand, identification of *Candida* isolates at the molecular level is crucial to assist in early diagnosis and for timely prescription of appropriate antifungal drugs (Imran and Al-Shukry, 2014).

The preference for using combinations of identification tools (phenotypic and molecular tools) in the identification processes is still controversial among researchers: Campbell et al. (1998) reported that ApI *Candida* (ApI= carbohydrate assimilation patterns by commercial test kit gave equivocal results) and Auxacolor tests require supplemental biochemical tests and morphological assessment for accurate taxonomic judgment, while Kanbe et al. (2002) preferred molecular assessment combined with conventional tests. Liguori et al. (2010) considered multiplex polymerase chain reaction (PCR) assays as an alternative to the conventional techniques for the identification of *Candida* spp.

The use of molecular markers for diagnosis of *C. albicans* is an efficient tool with high discriminatory power, including typing of PCR products based on the amplification of the target regions with specific primers like CABF59/CABR110, rDNA typing (Kanbe et al.,2002) and *C. albicans* microsatellite (CAI microsatellite) polymorphism. CAI microsatellite (short tandem locus on chromosome 4), CAA separated by trinucleotides (CTG), and the repeat sequence $(CAA)_n$ $CTG(CAA)_n$ have been increasingly used as markers for genetic analysis. Sampaio et al. (2003) reported the PCR product of CAI microsatellite to be 252 bp in size. Costa et al. (2010) and Dalle et al. (2000) reported the importance of microsatellites loci numbers and the variations in microsatellites lengths in the high level of polymorphisms, suggesting microsatellites (known as simple sequence repeats or short tandem repeats, are repeating sequences of 2-5 base pairs of DNA) as reliable markers for typing *Candida* isolates. PCR analysis of microsatellites was found to be a highly efficient molecular tool for rapid and accurate identification of *C. albicans* strains (Hennequin et al., 2001; Sampaio et al., 2005; Spampinato and Leonardi, 2013). Microsatellite length polymorphisms (MLP) analysis has also recently emerged. Botterel et al. (2001) and Sampaio et al. (2003) reported that microsatellite CAI, located on the noncoding region, imparted discriminatory power and was correlated with high polymorphism.

This study aimed to survey and diagnose *Candida* spp. infecting the human eyes by using the CHROMagar medium and multiple molecular markers such as specific PCR primers for *C. albicans*, rDNA typing and CAI microsatellite polymorphisms.

MATERIALS AND METHODS

Patient samples

A total of 165 swabs of infected human eyes were collected from different age (1 month to 70 years) and gender of patients attending the outpatient clinics in the main hospital of Karbala province, Iraq, between June 2012 and May 2013. All samples were collected using sterile swabs. The swabs were directly diluted in 1 mL sterile distilled water, and a loop full of this suspension was streaked on Sabouraud dextrose agar (SDA) medium plate supplemented with chloramphenicol and streptomycin (50:50 µg/mL). The cultures were incubated for 24-48 h at 37°C (Oliveira et al., 2011).

CHROMagar culturing

Purified single colonies from SDA were streaked on CHROMagar and incubated for additional? 24 - 48 h at 30°C. *Candida* isolates were classified according to their colors on the CHROMagar medium based on the color key described by Nadeem et al. (2010).

Extraction of genomic DNA

A total of 24 isolates of *Candida* spp. were subjected to DNA extraction and PCR analysis. Loopful of single colony suspension of each *Candida* isolates was mixed with lyses buffer [200 mM Tris-HCl, 20 mM ethylenediamine-tetraacetic acid (EDTA), 150 mM NaCl and 0.5% sodium dodecyl sulfate (SDS)] for 10 min by

Table 1. The targets, primer sequences, PCR products and references.

Primer or target region	Name of primer	Sequence (5'-3')	Amplified fragment size (bp)	Reference
Specific primer	CABF59	TTGAACATCTCCAGTTTCAAAGGT	515	Kanbe et al., 2002
	CABR110	GTTGGCGTTGGCAATAGCTCTG		
CAI microsatellite	Forward	ATGCCATTGAGTGGAATTGG	252	Sampaio et al., 2005
	Reverse	AGTGGCTTGTGTTGGGTTTT		
ITS1-5.8S-ITS2	ITS1	TCCGTAGGTGAACCTGCGG	450-650	Tamura et al., 2001
	ITS4	TCCTCCGCTTATTGATATGC		

vortexing. Then, 200 µL of phenol:chloroform was added to the suspension and mixed by centrifugation at 7000 rpm for 2 min. The supernatant was transferred into fresh sterile tubes, to which 500 µL isopropanol was added with mixing by centrifugation at 13000 rpm for 12 min for DNA precipitation. Then, isopropanol was decanted and the pellet was washed twice with 70% ethyl alcohol and centrifuged again at 7000 rpm for 2 min. Next, ethanol was decanted and the pellet DNA was dried and re-suspended in 50 µL Tris-EDTA (TE) buffer and preserved at −20°C until use (Iwata et al., 2006).

Primers and PCR assays

Simple PCR method was performed with a specific pair of primers (Table 1) (CABF59 and CABR110) (Kanbe et al., 2002) and CAI microsatellite pair primer for *C. albicans* (Hennequin et al., 2001), ITS1-ITS4 pair primer for ribotyping and diagnosis of *Candida* spp. (Ahmad et al., 2010). In detail, a total of 0.7 µL DNA for each one of the 24 *Candida* isolates was mixed with the PCR mixture consisting of 12.5 µL 20X Master Mix (Promega), 2 µL of each primer (10 pemole), and 8 µL deionized water made up to 25 µL with molecular-grade water reaction volume; each primer pair set was used in a single PCR reaction, using the Thermal Cycler System (Labenat, USA).

The PCR conditions were designated for all primer pairs: first denaturized at 95°C for 5 min, followed by 30 cycles of initial denaturation at 95°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

The PCR products were analyzed by 1.2% agarose gel (Bio Basic Canada Inc.) electrophoresis performed at 100 V using Tris-borate-EDTA (TBE) buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by using the Desktop Gel imager scope 21 ultraviolet transilluminator (Korea Comp.)

RESULTS

The phenotypic tests

A total of 165 swabs were examined from 165 patients; of which, 93/165 (56.4%) were positive for fungal infection. In addition, 53/93 (57%) isolates of filamentous fungi were isolated. The most frequent species were *Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani*, *Acrimonum* sp., and *Blastomyces*. Moreover, 35/93 (37.63%) *Candida* strains were isolated, 8 of which were classified as *C. albicans* (green color colony on CHROMagar) and the others as non-albicans isolates (pink to white-pink color colony on CHROMagar). Due to imprecise distinction between pink and white-pink colors, all non-albicans isolates have been mentioned to show pink color (Table 2).

Molecular diagnosis of *C. albicans* by specific primer pair

A specific primer pair (Table 1) was found to be the best primer for diagnosis of *C. albicans* by successful amplification of the target region for *C. albicans*, producing PCR product of approximately 515-bp size (lanes 1-2, 5, 9-10, 14-15,21), but it did not yield amplification products with non-albican isolates (Figure 1). *C. albicans* was detected with the highest frequency (33%; 8/24), followed by *C. famata* (29%) and C. *rugosa* (4.2%) with the lowest frequency (Table 2).

Ribotyping of ITS1-5.8S-ITS2 region

Amplification of the ITS1-5.8S-ITS2 region of 24 isolates of *Candida* spp. with the universal primer pair ITS1 and ITS4 (standard ITS1+ITS4 primers used by most labs for amplified The ITS region for most widely sequenced DNA region in fungi) yielded PCR products ranging from 419 to 650 bps in length; some PCR products showed size species-specific differences: *C. albicans* was about 530 bp; *Candida famata* 650 bp; *Candida saitoana* 600 bp, and *Candida rugosa* 419 bp (Table 2, Figure 2). The pink color of non-albican isolates on the CHROMagar medium made it difficult to identify these isolates in comparison with molecular diagnosis with universal primer pair ITS1 and ITS4 (Table 2).

The ribotyping rDNA of the ITS1-5.8S-ITS2 region for 24 isolates of *Candida* spp. revealed 7 genotypes: the *C. albicans* showed a high-frequency percentage (33%; 8/24), followed by *C. famata* (29%), and a lower frequency for C. *rugosa* (4.2%) (Table 2). Ribotyping rDNA of *Candida* species elucidate no intraspecies variability among 24 isolates of *Candida* of interest (Figure 2).

Table 2. The colony color on CHROMagar medium, frequency percentage based on ribotyping, and PCR products of the set of specific, universal and microsatellite primers pairs.

Candida sp.	Isolate no.	CHROMagar color	PCR product (bp)			Frequency (%)
			ITS	CABC	CAI	
Candida albicans	1	Green	530	515	240&297	33.3
C. albicans	2	Green	530	515	240&297	33.3
C. famata	3	Pink	650	-	-	29.2
C. rugosa	4	Pink	419	-	-	4.2
C. albicans	5	Green	530	515	240&297	33.3
C. famata	6	Pink	650	-	-	29.2
C. famata	7	Pink	650	-	-	29.2
C. famata	8	Pink	650	-	-	29.2
C. albicans	9	Green	530	515	240&297	33.3
C. albicans	10	Green	530	515	240&297	33.3
C. famata	11	Pink	650	-	-	29.2
C. famata	12	Pink	650	-	-	29.2
C. utilis	13	Pink	560	-	-	12.5
C. albicans	14	Green	530	515	240&297	33.3
C. albicans	15	Green	530	515	240&297	33.3
C. famata	16	Pink	650	-	-	29.2
C. saitoana	17	Pink	600	-	-	12.5
C. saitoana	18	Pink	600	-	-	12.5
C. inconspicua	19	Pink	503	-	-	4.2
C. saitoana	20	Pink	600	-	-	12.5
C. albicans	21	Green	530	515	240&297	33.3
C. *guilliermondii*	22	Pink	603	-	-	4.2
C. utilis	23	Pink	560	-	-	12.5
C. utilis	24	Pink	560	-	-	12.5

Figure 1. Gel electrophoresis of PCR products of amplified target regions for primers CABF59 and CABR110. Bands in lanes (1-2, 5, 9-10, 15-16, 21) are for *C. albicans* (515 bp); others lanes are for non-albicans isolates (no target region). M = molecular marker, 100 bp.

Polymorphism of CAI microsatellite of *C. albicans* isolates

The amplification of repeated CAI microsatellite identified 8 isolates as *C. albicans* from 24 isolates. CAI microsatellite pair primer was successfully amplified by CAI microsatellite for all *C. albicans* and produced two bands of approximately 240 and 297 bp sizes, but it could not amplify for non-albicans isolates (Table 2 and Figure 3).

DISCUSSION

Over the past 10 years, the number of ocular candidiasis has significantly increased, particularly in poor countries (Saha and Das, 2006). The study presented herein showed a high prevalence of ocular candidiasis, 93 (56.4% patients samples) of 165 patients with fungi caused by *Candida* were positive for fungal infection (21.2% patients samples) affecting the human eye health and aesthetics of the patient (Oude Lashof et al., 2011).

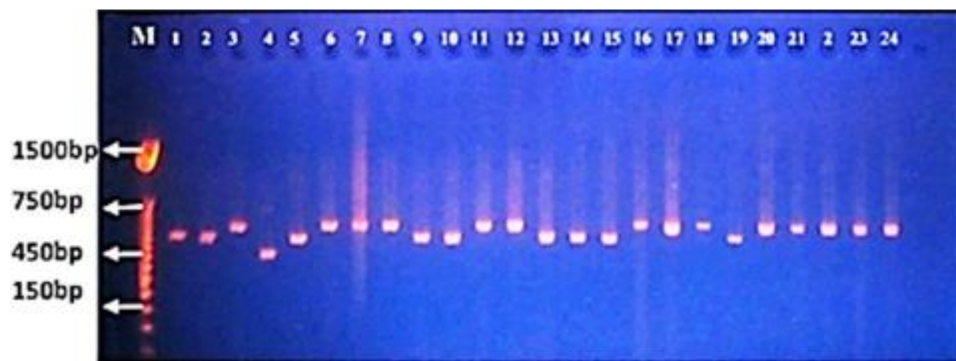

Figure 2. Gel electrophoresis of PCR product of the amplified target region for the primer ITS1-5.8S-ITS2. Bands in lanes 1-2, 5, 9-10, 14-15, and 21 are for *C. albicans* (530 bp); 4 for *C. rugosa* (419 bp); 3,6-8,11-12, and 16 for *C. famata* (650 bp); 22 for C. *guilliermondii* (603 bp); 13, 23-24 for *C. utilis* (560 bp); 17, 18, 20 for *C. saitoana* (600 bp); 19 for *C. inconspicua* (503 bp). M = molecular marker, 100 bp.

Figure 3. Gel electrophoresis of PCR product of the amplified target region for primers in CAI microsatellite. Bands in lanes 1-2, 5, 9-10, 15-16, and 21 are for *C. albicans* (240&297 bp); others lanes are for non-albicans isolate s(no target region). M = molecular marker, 100 bp.

C. albicans was the most frequent cause of ocular candidiasis, followed by *C. famat*, *C. saitoana* and *C. regosa*, respectively. This result is coincident with earlier studies of Durand (2013) and who mentioned a high prevalence of *C. albicans* as the most common cause of ocular candidiasis, accounting for 92% of the cases in this review.

We believe emphasize that the rate of such infection is higher among patients in Iraq who receive lesser care and inadequate and imprecise diagnosis. Fungal keratitis is most common in tropical regions and developing countries, where it constitutes over 50% of keratitis (Galarreta et al., 2007). Our results show that 37.63% of eyes candidiasis caused by *Candida* spp. And in accordance with earlier studies in South India, about 44% of corneal ulcers are caused by fungi. 17% in Nepal, 36% in Bangladesh, 38% in Ghana and 35% in south Florida in the US (Upadhyay et al., 1991; Hagan et al.,1995). For efficient treatment with antifungals, early diagnosis of invasive *Candida* infection and accurate identifications of the causative agents by molecular markers are necessary and also accelerates clinical administration for the better treatment decision and increases the chance of successful treatment and recovery of the patients; moreover, several of the conventional diagnosis methods are not very efficient.

Our results based on use multiple molecular markers for diagnosis of conjunctivitis caused by *Candida* highlight the demerits of the routine conventional assay such as CHROMagar candida medium used, which is in agreement with the report of Ahmed et al. (2010) who asserts that phenotypic tests may not always provide clear identification for most *Candida* spp.; as 16 isolates of *Candida* appear as pink or white-pink color colony, which could be delusive because several *Candida* spp. produce approximately the same reaction color on CHROMagar (Beighoton et al., 1995) (Table 2). Therefore, molecular methods, especially PCR, are being increasingly used for the rapid detection of fungal spp. (Tamura et al., 2001; Alfonso, 2008; Ferrer and Ali, 2011). However, the use of a single molecular marker may not be sufficient for precise diagnosis of all *Candida*

infections because of microevolutionary changes among fungal species (Sampaio et al., 2005).

On the other hand, the use of specific primers for *C. albicans* revealed PCR product to be of approximately 530 bp in length in this study. Our PCR results confirmed that specific primers could not amplify the target DNA of non-albicans isolates (Figure 1), as also reported by Kanbe et al. (2002). The use of specific primers for each *Candida* spp. is costly and often impractical, but use of a simple method such as ribotyping with universal primers allows cheaper and more reliable typing of a large number of isolates.

Ribotyping method showed 7 genotypes of *Candida* spp. based on the use of universal primers ITS1 and ITS4. The ribotyping rDNA of *Candida* sp. elucidated no intraspecies variability among 24 isolates of *Candida* of interest (Figure 2). These results are coincident with that of Fujita et al. (2001) within the range of PCR profile (419 - 650 bp). We thus believe that ribotyping is a rapid, easy and reliable method as it produces specific patterns and can often not require the use of specific primers for individual *Candida* sp. Ribotyping methods was used with numerous clinical and environmental isolates (Isogal et al., 2010); similar results were also observed by Katiraee et al. (2010). Shokohi et al. (2010, 2011) emphasised that factors such as personal hygiene, culture and internal immunity defect may play a role in incidence of eyes infections among the Iranian communities.

Our CAI microsatellite results showed two bands of 240 and 297 bp sizes (Figure 3). This result can be attributed to the existence of two loci of CAI microsatellite in *C. albicans*, leading to the production of two bands or may arise from multiple repetition of CAI microsatellite. These results conflict with those of Sampaio et al. (2005) who obtained PCR product of 252 bp size.

Spampinato and Leonardi (2013) suggested a more complex scenario of 27 repeated units of CAA and one unit of CAG in a secondary structure; the changes in the microsatellite length could be due to the insertion of trinucleotide. Insertion in the first band of 297 bp size could be due to 27-times repeated CAA unit, while the second band of 240 bp size could be due to the low repeated CAA unit.

However, our results were coincident with those of Spampinato and Leonardi (2013), who reported that the PCR analysis of microsatellite is a highly efficient molecular tool for the rapid and accurate identification of *C. albicans*. The length of variation with a high level of polymorphisms of the PCR product makes them reliable markers for the detection of *Candida* sp. (Pupko and Graur, 1999; Sampaio et al., 2010). The analysis of PCR products of *C. albicans* CAI alleles revealed them to be a microsatellite composed of variable units of CAA and CAG. The differences in the products were mainly due to the variable number of CAA repeated units (Katiraee et al., 2010).

We thus conclude that ocular candidiasis is an important disease in Iraq, caused by *C. albicans* and *C. famata* the most common pathogens associated with as first risk, with a prevalence of 21.2% in conjunctivitis patients, making it important to be diagnosed in a timely manner. Multiple molecular markers is recommended for the diagnosis and identification of the causative agents of these infections to achieve a conformable data. CAI microsatellites are a highly efficient polymorphism assay that can be used as a diagnostic and differentiating tool for *Candida* sp. to facilitate identification of *Candida* spp. in clinical samples. The result of this study is very importance to the populations of Iraq because it showed many common fungi associated with conjunctivitis patients in Iraq, Hopefully, this will lead to an increase in research initiatives on this complex problem.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Effect of variation in land use, age of host tree, season and geographic location on the diversity of endophytic fungi in the needles of *Afrocarpus falcatus*

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The aim of the current study was to investigate the effect of variation in land use, age of host tree, season and geographic location on the diversity of culturable endophytic fungi of needles of *Afrocarpus falcatus*. Endophytic fungi represent a genetically diverse group of microorganisms associated with healthy tissues of terrestrial plants. They are believed to be mostly beneficial to their host plants and produce novel antimicrobial compounds; or may be latent pathogens that become active at specific stage of development or under a set of environmental conditions. Wondo Genet and Menagesha Suba were the two geographic locations with differing altitudinal and climatic features selected for sample collection. Needle samples were collected from old and young trees growing in natural forests and open lands during dry and wet seasons. Identification of the isolates to the genus level was performed on the basis of culture characteristics and spore morphology. A total of 687 endophytic fungal isolates were obtained and categorized into 64 morphotaxa. Fifty two (81.25%) of the morphotaxa were identified to 12 genera while 12 morphotaxa were left as unidentified. Thirty two (50%) of the morphotaxa were isolated only from the Wondo Genet site while only 3 (4.7%) were isolated from the Menagesha Suba site. The four most diverse genera were *Cercosporella, Xylaria, Botryosphaeria* and *Pestalotiopsis*. *Phoma* was the most abundantly represented genus followed by *Xylaria* and *Pestalotiopsis* while *Trichotecium* and *Mycosphaerella* were the rarest genera. The Wondo Genet site during the dry season was the most diverse (H'= 3.36649) and many of the morphotaxa were unique to the site; which might be ascribed to the differences in altitudinal, climatic and perhaps additional factors that were not considered in the current study.

Key words: Fungal diversity, morphotaxa*, Afrocarpus falcatus.*

INTRODUCTION

The term endophyte belongs to those groups of microorganisms that inhabit the living internal tissue of green plants without causing immediate or clear effect on the plant (Hirsch and Brauna, 1992) and may cause a clear negative effect when the plant faces some kind of environmental stress. Endophytic fungi are those groups

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of fungi that live in the internal tissue of living plants and are important component of fungal diversity and affect plant community diversity and structure. According to different authors, the number of endophytic fungi inhabiting plants tissues is estimated to be about 1 million (Dreyfuss and Chapela, 1994).

Endophytic fungi serve as a biocontrol of plant pathogen by actively inhibiting pathogens from invading the host plant. This is consistent with the observation of Herre et al. (2005), that in the vast majority of cases, endophytes appear not to harm their host plant, and not to reproduce. This helps the host plant to cope with (withstand) the damage caused by pathogens. The presence of endophytes in plants enhances host defense against pathogenic infection and reduce the damage due to herbivores (Herre et al., 2005) thus improving the existence of the host plant.

According to Arnold and Herre (2003), the presence of endophytes in the host substantially reduces leaf loss and damage due to an oomycete pathogen. In addition to increasing host defense against pathogens and herbivores, it has been also reported that some species of endophytic fungi increases drought and heavy metal tolerance. Furthermore, an endophytic fungus *Theobroma cacao* was reported to contribute to the host stress tolerance from ecosystem by affecting levels of photosynthesis and hydraulic properties (Herre et al., 2005).

Almost all green plants harbor neutral endophytes but because they are symptomless and are difficult to detect and can only be successfully surveyed by plating out carefully prepared surface-sterilized tissues. For example, a study by Fisher and Petrini (1990) on the endophytes isolated from *Alnus* xylem and barks tissues in roots and stems yielded 85 different fungal taxa (Fisher and Petrini, 1990).

Many of the endophytic fungi remain quiescent (inactive) within their hosts until the host is stressed or begins to undergo senescence. An extreme example of this type of endophyte is the fungi occurring in conifer needles (Dix and Webster, 1995).

Given the many ecological benefits of endophytic fungi to their host plants and to the socio-economic development of nations, there were no enough studies on endophytic fungi in Ethiopia in general and on forest trees in particular. Not only their diversity but also their distribution within and among host plants under different ecological situations needs to be explored.

The current study attempted to examine the diversity of endophytic fungal assemblages of *A. falcatus* trees growing in different geographical locations having different altitudinal ranges and land use systems. If there occur any variation between the aforementioned parameters it was meant to show if they have some impacts on the diversity of endophytic fungi in the needles.

The objective of this study was to assess the diversity of endophytic fungi associated with needles of *A. falcatus* in relation with season, land use, age of host tree and geographic variation. Simultaneously, this research work has an aim to isolate and morphologically categorize culturable endophytic fungi inhabiting needles of *A. falcatus* collected from natural forests and open lands at Wondo Genet and Menagesha Suba. Finally, the research aims to assess the impact of age of host, seasonal, altitudinal and land use variations on the diversity of endophytic fungi inhabiting the needles of *A. falcatus*.

METHODOLOGY

Description of the study area

The study was conducted at two geographic locations, namely Wondo Genet and Menagesha Suba that are over 300 km apart. Moreover, they are believed to have, among others, different altitudinal ranges and climatic factors.

Wondo Genet

Geographical location: Wondo Genet is situated 263 km South of Addis Ababa and 13 km southeast from the nearest town, Shashemene, West Arsi Zone in Oromia Regional State and about 38 km from Hawassa town. It is located between 38°37'-38° 42'E longitude and 7°02'-7°07'N latitude and an altitude of between 1600 and 2500 masl. The study was conducted in the plantations and natural forests found in the premises of Wondo Genet College of Forestry and Natural Resources (http://www.wgcf-nr.org/resource/land.html).

Climatic condition of Wondo Genet is traditionally categorized under Weyna dega. Wondo Genet has a bimodal rainfall pattern. The 'short' rains generally arrive in mid- to late February, while the 'long' rains begin late June and continue to the end of September. Occasional, sporadic rain occurs during the dry seasons, but it has little impact on the growth patterns of the plants. Annual rainfall ranges from approximately 700 to 1400 mm. The mean annual temperature at the College campus is 19°C. The area has a high population density of 588 people per km^2 and has an estimated total population of 5,792 consisting of 2,857 men and 2,935 women (CSA, 2005).

The forest of Wondo Genet constitutes both the natural and plantation forests. The natural forest constitutes an important pocket that is disturbed but still in good condition with its plant and animal species protected from the impacts of human beings in relation to many of the remaining natural forests in the country. Some of the major tree species in the natural forest include *Albizia gummifera*, *Celtis africana*, *Cordia africana*, *Croton macrostachyus*, *Prunus africana* and *Afrocarpus falcatus* (*Podocarpus falcatus* syn.), *Aningeria adolfi-friederici*, scattered *Acacia* trees and rank tall grasses are found in this area (http://www.wgcf-nr.org/resource/land.html).

Menagesha Suba Forest

Menagesha Suba forest is located 50 km south-west of Addis Ababa at 38°31'-38035'E longitude and 8°56'-9^{0}4'N latitude (Teketay, 1996) with an altitude ranging between 2200 to 3000 masl. The area consists of an isolated mountain.

The natural forest communities of Menagesha Suba consisted of Hypericum belts, Hagenia-juniperus forest, Juniperus forest, Juniperus-Afrocarpus forest and Afrocarpus forest in descending order of elevation (from 3500 m down to 2000 m) (Feyera and Demel, 2001).

Climatic condition of Menagesha Suba is traditionally categorized as 'Dega' to 'Weyna Dega'. The annual temperature of the area is between 15-17°C and has an annual rainfall of 1100 mm (Fetene, 2006).

Sample collection

Sample collection was carried out during the period from June to August 2010 to cover the wet season and from November to January, 2010 to represent the dry season. In both sites (Wondo Genet and Menagesha Suba), two land use types (natural forest and open lands such as parks and farm lands) were selected. In both land use types, three young (having dbh 13.5 - 16.5 cm) and three old (dbh 68-90cm) trees of *A. falcatus* were randomly selected for needle sampling.

From each sample tree, 4 healthy looking (asymptomatic) needles were randomly picked from bottom, middle and top parts of the crown of a tree. Thus, from each site, a total of 12 needles per age group and 24 per land use type and season and 192 needles in total were collected for isolation.

The needle samples were collected in paper bags, labeled and transported to the laboratory immediately upon collection for isolation (Barik et al., 2010). Samples from Menagesha Suba were transported to the laboratory within 24 h after collection with intermittent wetting with water to protect the needles from drying.

Nutrient media preparation

A 2% malt extract agar (MEA) was used to isolate fungi. For plate culture, the sterilized MEA was poured onto Petri dishes for both primary and pure culture.

After the needle samples were cut into 10 mm fragments, the fragments were briefly sprayed with 70% ethanol before being transferred to 3% sodium hypochlorite (NaOCl) for surface sterilization (Guo et al., 2001). The ethanol acts as a surfactant and the NaOCl is the actual sterilizing agent (Bills, 1996).

They were then washed five times consecutively. The surface sterilized needles were then dried by blotting on sterile filter paper under aseptic condition. Four surface sterilized needle fragments per plate were randomly picked and plated on MEA plates and incubated at 25°C for 4-10 days to allow growth of fungal colonies on the medium. Pure cultures were obtained by sub-culturing them on MEA medium and maintained on slants tubes containing the same medium.

Identification of fungi

Identification was based upon cultural and microscopic (conidial) characteristics. The cultural characteristics included the color of the upper and reverse sides of the cultures, mycelial color formation such as dark, brown, grey, yellow or other colors, colony diameter, shape of colony margin, mycelial growth patterns such as fluffy aerial hyphae, appresssed or submerged hyphae, formation of aerial hair-like tufts of hyphae were used to characterize the fungal endophytes.

Based on culture characteristics and spore morphology, the isolated fungi were categorized into morphotaxa identified to the genus level, while those which could be separated into distinct groups based on culture characteristics but could not be identified to any of the known genera were recognized as unidentified taxa. Lactophenol cotton blue staining solution was used for staining of non-pigmented fungal spore for microscopic examination purpose. Conidial morphological characteristics including shape and color of the spores, separation, presence or absence of specialized appendages on the spores were used to characterize the fungal structure.

In addition, different standard identification manuals were used to provisionally identify the fungal isolates to the genus level. Whenever more than one morphological group occurs within a genus, they were designated as sp.1, 2, 3, etc.

Data analysis and presentations

Data obtained from the research work was presented using tables as percentages. Colonization rate (CR, expressed as a percentage) was calculated as the total number of needle segments infected by fungi divided by the total number of needle segments incubated and it was used to compare the degree of infection by endophytic fungi of needle tissues collected from host trees under different land use, age group and geographic location (Lv et al., 2010).

$$CR = \frac{\text{Total number of needle segment incubated}}{\text{The number of needle segments infected by fungi}} \times 100$$

The isolation rate (IR, is a quotient calculated by dividing the number of isolates obtained from needle segments by the total number of needle segments incubated. This allows for the measurement of fungal species richness in a needle (Lv et al., 2010). IR was calculated according to the equation below:

$$IR = \frac{\text{Number of isolates obtained from plant segment}}{\text{Total number of plant segmented incubated}}$$

The relative frequency (RF, expressed as a percentage) was calculated as the total number of isolate from a single taxa divided by the total number of isolates from taxa obtained from all tissue incubated (Lv et al., 2010). It was used to determine the most frequently isolated taxa among the rest of taxa.

$$RF = \frac{\text{Number of isolates of a taxon}}{\text{Total number of isolates of all taxa}}$$

Additionally, both the Shannon diversity index and Sorensen Similarity index was calculated for the two sites. Shannon diversity index (*H'*) was used to characterize species diversity in a community.

Shannon index of diversity was calculated for all factors which were assumed to have effect on the diversity of endophytic fungi. To check the significance level of the variations ANOVA was employed using SPSS version 20 used at α =0.05.

RESULTS AND DISCUSSION

A total of 687 endophytic fungal isolates were recovered from 192 needles (960 needle fragments) collected from all sampling points (land uses, age of host trees, geographic location and season). The distribution of endophytic fungal isolates between the two sampling sites showed that those trees from Wondo Genet were found to harbor the highest number (53.6%); whereas

Table 1. Total number of endophytic fungal isolates recovered from needles of *A. falcatus* trees from different geographic locations, land uses and seasons.

Land use	Wondo Genet		Menagesha Suba		Total
	Wet season	Dry season	Wet season	Dry season	
Open Land	90	79	100	52	321
Natural forest	86	113	94	73	366
Total	176	192	194	125	687
	368		319		

Table 2. Total number of endophytic fungal isolates recovered from needles of *A. falcatus* trees from different geographic locations, land uses and different host tree age group.

Land use	Wondo Genet		Menagesha Suba		Total
	Young	Old	Young	Old	
Open Land	90	79	82	70	321
Natural forest	103	96	80	87	366
Total	193	175	162	157	**687**

those from Menagesha Suba samples harbor the lower number of isolates (46.4%) of isolates (Table 1). However, the data is not statistically significant at P>0.05. Within each geographic location, varying number of endophytic fungal isolates were recovered from the two land uses (open land and natural forest) during wet and dry seasons (Table 1), this was because that higher diversity of plant life in natural forest and fungal abundance decreases as altitude increases (Brosi et al., 2011; Kodsueb et al., 2008).

The highest number of endophytic fungal isolates (113) was recovered from needles collected from natural forest at Wondo Genet during the dry season while the lowest number of isolates (52) was recovered from needles collected from open land trees at Menagesha Suba during the dry season (Table 1). The previous study by Talley et al. (2002), Gamboa and Bayman (2001) and Rojas and Stephenson (2008) strengthen our finding that the abundance of endophytic fungi is higher in undisturbed forest community, it is due to the high inoculums by endophytic fungal spore arising from the diverse array of plant life.

The difference between open land and natural forest trees in the number of endophytic fungal isolates recovered was higher during the dry season both at Wondo Genet and Menagesha Suba, although the difference was not statistically significant at P>0.05. This might be due to the high water stress in wet season that leads to the lowest number of endophytic fungi in both sampling sites.

It also appears from the results of the current study that young trees generally harbor more endophytic fungal isolates than old ones but the data was not statistically significant at P>0.05 in Wondo Genet and in Menagesha Suba (Table 2). The current observation concurs with those of Helander et al. (2006) stating that younger plants harbor higher number of endophytic fungi than older ones. This might be due to the soft leaf chemistry of younger plants. Furthermore, it appears that a combination of seasonal variation, age of host tree and land use type influences the abundance of endophytic fungal communities associated with the needles of *A. falcatus* (Table 2).

Isolation attempts from open lands trees generated more number of fungal isolates during the wet season than during the dry season at both Wondo Genet (90) and Menagesha Suba (100), whereas higher number of isolates were recovered from natural forest trees during the dry season at Wondo Genet (113) and from natural forest during the wet season at Menagesha Suba (94) (Table 1).

The decrease in the number of isolates from natural forests during the wet season and the higher number of isolates during wet season from open land trees might be explained by the excess moisture in the natural forests that could be a stress for the endophytic fungi associated with the needles of *A. falcatus* (Talley et al., 2002). This could also explain the higher number of isolates from natural forest trees but lower number of isolates from the open lands during the dry season in both geographic locations (Table 1).

The general comparison of land uses against age of host trees indicated that more endophytic fungal isolates were recovered from natural forest (366) than open land (321) considering both the geographic locations together. This may be related to the higher plant community diver-

Table 3. Distribution of the morphotaxa in all the sampling points in both seasons and sites.

Season	Sampling site and land use	Morphotaxa	RF (%)
Wet	Wondo Genet Open land	33	26%
	Wondo Genet Natural forest	47	36.5
	Menagesha Suba open land	10	8
	Menagesha Suba Natural forest	13	10
Dry	Wondo Genet Open land	37	29
	Wondo Genet natural forest	54	42
	Menagesha Suba Open land	22	17.5
	Menagesha Suba natural forest	28	21.5

RF: Relative frequency.

Table 4. Distribution of morphotaxa of endophytic fungal isolates of Wondo Genet and Menagesha Suba sites in both seasons.

Season	Sampling sites	Morphotaxa recovered	RF (%)
Wet	Wondo Genet	45	70
	Menagesha Suba	9	14
Dry	Wondo Genet	47	73
	Menagesha Suba	30	47

RF: Relative frequency.

sity of natural forest than that of open land habitat (Tsui et al., 1998; Aung et al., 2008; Gamboa and Bayman, 2001; Rodrigues, 1994), which may serve as a potential source of inoculums. In addition, natural forests at both study sites are relatively well protected and richer in plant communities and thus more diverse in endophytic fungal communities than the isolated trees in the open lands. The vegetation that surrounds it is believed to be an important source of inoculums for endophytes (Gamboa and Bayman, 2001; Rodrigues, 1994).

Likewise, isolation attempts from young trees generally recovered more endophytic fungal isolates than from old trees (Table 2). Moreover, needles of young trees from natural forest at Wondo Genet yielded more endophytic fungal isolates than those from open land trees. However, young trees from natural forest at Menagesha Suba yielded slightly lower number of isolates than those from open land (Table 2); but the difference was not statistically significant at $P>0.05$. Additionally, young trees, except from natural forest at Menagesha Suba, yielded more number of isolates than old trees. The result of the current study was in agreement with the finding of Helander et al. (2006).

The endophytic fungal isolates were grouped into 64 morphotaxa. From the geographic locations, isolations from Wondo Genet during the dry season recovered more morphotaxa (73%), followed by wet season Wondo Genet (70%) of the morphotaxa (Table 4). The lower number of morphotaxa was recovered in both the dry and wet season in Menagesha Suba as compared to Wondo Genet.

The results of the current study were in agreement with the findings of Osono and Hirose (2009) and Hashizume et al. (2010) which stated that diversity of endophytic fungi decreases with increase with altitude and vice versa.

The largest number of morphotaxa (48.43%) was isolated from needles from young trees in the natural forest at Wondo Genet during the dry season followed by trees from the same site but during the wet season (42.19%) (Table 3). Similarly, at Menagesha Suba, the highest number of morphotaxa was recovered from young trees from natural forest during both the dry and wet seasons. Generally, young trees in the natural forest yielded the highest number of morphotaxa. Helander et al. (2006) also observed similar pattern of association of endophytic fungi with young trees than in the old trees and in the undisturbed forest than in disturbed forest. This pattern of distribution was consistent with the abundance of the endophytic fungal isolates (Tables 1 and 2).

Out of the 64 morphotaxa, 32 (50%) were unique to

Table 5. Genera encompassing the morphotaxa of endophytic fungi isolated from needles of *A. falcatus* from Wondo Genet and Menagesha Suba sites.

Genera	No. of morphotaxa	No of isolates	Origin				RF (%)
			WG		MS		
			Wet	Dry	Wet	Dry	
Botryosphaeria spp.	7	50	√	√	√	√	10.93
Pestalotiopsis spp.	5	65	√	√	√	√	7.81
Xylaria spp.	9	70	√	√	√	√	14.06
Phoma spp.	4	219	√	√	√	√	6.25
Cercosporella spp.	10	53	√	√	√	√	15.62
Fusarium spp.	3	13	√	X	√	√	4.65
Microsphaeropsis spp.	2	5	√	√	X	√	3.12
Chaetomium spp.	3	7	√	√	X	√	4.68
Aspergillus spp.	5	53	√	√	√	√	7.81
Trichotecium sp.	1	1	√	X	X	X	1.56
Penicillium spp.	2	54	√	√	X	√	3.12
Mycosphaerella sp.	1	2	X	√	X	X	1.56
Unidentified groups	12	95	√	√	X	√	18.75
Total	**64**	**687**					

√-Present, X- absent, WG-Wondo Genet, MS-Menagesha Suba, RF- Relative frequency.

Wondo Genet while only 3 (4.7%) were unique to Menagesha Suba. Moreover, 61 of the 64 morphotaxa were recorded at Wondo Genet while only 50% (32 of the 64) morphotaxa were recorded from Menagesha Suba. This clearly indicates that the two geographic locations are significantly different not only in terms of species richness and abundance but also due to the proportion of unique taxa they harbor. This observation may be due to several factors that may have been involved, most notably the geographic and climatic variations between the two sites. Furthermore, genetic variations of the host trees and the floristic composition of the sites may have contributed to the observed differences. However, such speculations need to be verified with further studies.

Comparing the seasons, dry season was better for recovering higher number of endophytic fungal morphotaxa than the wet season at both study sites, that is, 47 of the 64 in dry season at Wondo Genet and Menagesha Suba 30 of the 64 (Table 4). The highest proportion of morphotaxa were recovered during the dry season at Wondo Genet from young trees in the natural forest (48%) followed by the same site but during the wet season (42%) (Table 3).

Likewise, the lowest proportion of morphotaxa were recovered during the wet season from trees in open land (8%) (Table 3). This may indicate that differences in endophytic fungal diversity may be influenced by altitudinal and seasonal variations. The findings of the current study were in agreement with those of Brosi et al. (2011), Talley et al. (2002) and Kodsueb et al. (2008). The decrease in temperature and high water stress leads to decrease in endophytic fungal diversity in wet season (Krishnamurthy et al., 2009). Rojas and Stephenson (2008) also concluded that endophytic fungal distribution decreases as altitude increases and vice versa; and natural forests harbor higher fungal diversity than the open land (Tsui et al., 1998).

Identification of the morphotaxa

Among the identified morphotaxa, the four most diverse (species rich) genera were *Cercosporella, Xylaria*, *Botryosphaeria* and *Pestalotiopsis*, represented by 10, 9, 7 and 5 morphotaxa, respectively. Similarly, the three most abundant genera were *Phoma*, *Xylaria* and *Pestalotiopsis*, represented by 219, 70 and 65 isolates, respectively while *Trichotecium* and *Mycosphaerella* were the least diverse and the rarest genera among the endophytic fungal communities encountered during the current study (Table 5). Furthermore, *Mycosphaerella* and *Trichotecium* were not isolated from Menagesha Suba site during both seasons. Moreover, the unidentified genera were not isolated from Menagesha Suba site during the wet season. The rest of the morphotaxa were encountered at both the study sites. However, 12 morphotaxa could not be identified to the genus level as they did not sporulate under the laboratory conditions of the current study. They accounted for about 18% (95 isolates) of the collection (Table 5).

In general, comparison, higher degree of colonization rate (CR) by fungal endophyte was found at Wondo

Table 6. Number of needle segment that yielded one or more fungal isolates, total no. of needle segment that incubated the colonization rate and isolation rate in both seasons at both sampling sites.

Sample points	No. of needle segments that yielded one or more fungal isolates	CR (%)	IR
WWOLY	52	86.67	0.8
WWOLO	43	71.67	0.7
WWNFY	49	81.67	0.6834
WWNFO	45	75	0.75
DWOLY	49	81.67	0.7
DWOLO	40	66.67	0.6167
DWNFY	53	88.34	1.034
DWNFO	56	93.34	0.85
WMOLY	48	80	0.967
WMOLO	40	66.67	0.7
WMNFY	60	100	0.7834
WMNFO	55	91.67	0.7834
DMOLY	21	35	0.4
DMOLO	29	48.34	0.467
DMNFY	20	33.34	0.55
DMNFO	30	50	0.667

DMNFO, Dry season Menagesha Suba natural forest older; DMNFY, Dry season Menagesha Suba natural forest younger; DMOLO, Dry season Menagesha Suba open land older; DMOLY, Dry season Menagesha Suba open land younger; DWNFY, Dry season Wondo Genet natural forest younger; DWNFO, Dry season Wondo Genet natural forest older; DWOLY, Dry season Wondo Genet open land younger; DWOLO, Dry season Wondo Genet open land older; WMNFO, Wet season Menagesha Suba natural forest older; WMNFY, Wet season Menagesha Suba natural forest younger; WMOLO, Wet season Menagesha Suba open land older; WMOLY, Wet season Menagesha Suba open land younger; WWNFO, Wet season Wondo Genet natural forest older; WWNFY, Wet season Wondo Genet natural forest younger; WWOLO, Wet season Wondo Genet open land older; WWOLY, Wet season Wondo Genet open land younger.

Genet than Menagesha Suba, natural forest than open land and on young trees than old trees (Table 6). Similarly, in general, higher isolation rate (IR) by fungal endophytes was recovered at Wondo Genet than Menagesha Suba and from natural forest than open land (Table 6). This result is also in agreement with abundance of endophytic fungi in Tables 1 and 2. The previous studies by Granath et al. (2007) and Raviraja et al. (1998) have indicated that species richness is greater during the dry season than the wet season and also claimed that species richness decreases with increase in altitude.

The highest diversity index (*H'*=3.01856) of endophytic fungi was calculated for DWNFY followed by DWOLY (*H'*=3.0055), while the lowest diversity index was obtained for WMOLO (H'=1.004) (Figure 1). The results of the current study are in agreement with findings of Rojas and Stephenson (2008), Hashizume et al. (2008) and Arnold and Lutzoni (2007) strengthening the claim that diversity of endophytic fungal species decreases with the increasing altitude.

This result is also in accordance with the previous observations on the species richness and abundance in the same study. The highest diversity of endophytic fungi was recovered during the dry season than wet season both at Wondo Genet and Menagesha Suba. It is also in accordance with the findings of Brosi et al. (2011) where it indicated that diversity of endophytic fungi was higher during the dry season. The lower *H'* index for Menagesha Suba was influenced by the altitude (Hashizume et al., 2008; Higgins et al., 2007).

The dominant morphogenera at Wondo Genet during the wet season from old trees was *Botryosphaeria* spp. (35.7%); but the dominant morphogenera from samples in open land at old trees at Wondo Genet during the wet season was *Xylaria* spp. (22.58%). However, the dominant morphogenera from open land older trees at Menagesha Suba during the wet season was *Phoma* spp. (85.7%).

The dominantly isolated morphotaxon among the rest of taxa at Wondo Genet from open land young trees during the wet season was *Penicillium* sp. 2 (37.5%)

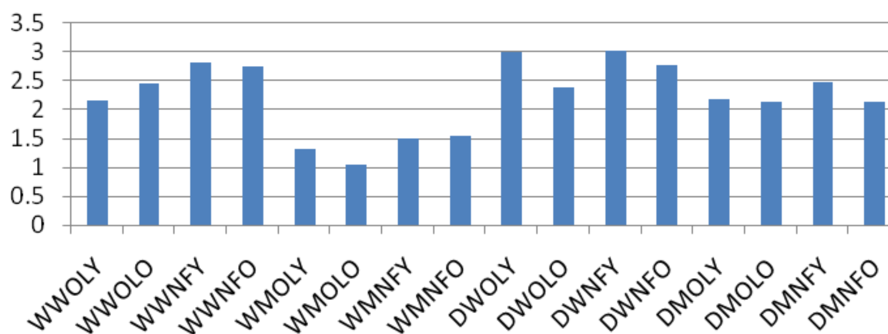

Figure 1. Shannon diversity index for all the sampling points in both sampling sites. Full explanation of the abbreviation is shown in the footnote of Table 6.

followed by *Phoma* sp. 4 (14.58%). However, in the same season and site but from older trees the dominantly isolated morphotaxon during the wet season was *Botryosphaeria* sp. 7 (28.57%) followed by *Xylaria* sp. 9 (11.9%). Indicating that in the same season, site and land use but different ages, there is difference in the type of dominant species.

Similarly, in wet season at Wondo Genet, the dominantly isolated morphotaxa from natural forest younger *A. falcatus* was *Aspergillus* sp. 4 (19.51%); however in the same season and site, the dominantly isolated morphotaxon from natural forest older *A. falcatus* was *Cercosporella* sp. 5 (20%) followed by *Phoma* sp. 4 (11.11%).

The dominantly isolated morphotaxon in dry season from open land at Wondo Genet from both younger and older *A .falcatus* was different, that is, from younger *A. falcatus* was *Pestalotiopsis* sp. 4 (11.9%) and that of older *A. falcatus* was *Aspergillus* sp. 2 (27.03%), indicating that there occur difference dominancy of taxa between age. However, the dominantly isolated morphotaxon in dry season from natural forest at Wondo Genet was *Pestalotiopsis* sp. 4 (19.35% and 15.68%) from younger and older *A. falcatus*, respectively.

The dominant morphotaxon at wet season in Menagesha Suba from open land younger and older *A. falcatus* was *Phoma* sp. 1 (55.17 and 61.9% respectively). However, in wet season, the dominant morphotaxon from natural forest younger *A. falcatus* was *Phoma* sp. 1 (42.55%) but the dominant taxa in the same season at Menagesha Suba from natural forest older *A. falcatus* was *Phoma* sp. 3 (42.55%).

In dry season at Menagesha Suba site in both open land and natural forest, there was difference in the type of dominant morphotaxon. Generally, in the result obtained from Table 6, there is difference in dominant morphotaxon between ages of host, season of sampling and altitude (Suryanarayanan et al., 2002).

Species diversity

The Shannon diversity index for seasonal variation on endophytic fungi shows that higher diversity of endophytic fungi was recovered at Wondo Genet site during the dry season (*H'* = 3.36649) than during the wet season (*H'*= 3.35275) but it was statistically not significant at P>0.05. This is because the high water stress and low temperature in wet season decrease the diversity of endophytic fungi (Gonthier et al., 2006; Talley et al., 2002). This result was also in accordance with the previous study by Hashizume et al. (2008) that is, the diversity endophytic fungi increases with increasing temperature during the dry season.

Similarly, at Menagesha Suba site higher endophytic fungi diversity was recovered during the dry season (*H'*= 2.65877) than wet season (*H'*= 1.47950), this is because the diversity of endophytic fungi is higher in dry season (higher in temperature) (Brosi et al., (2011; Vacher et al., 2008 and Compant et al., 2010) than wet season due to high water stress present (Gonthier et al., 2006), which is also statistically not significant at P>0.05.

In accordance with the work of Osono and Hirose (2009) and Hashizume et al. (2008) higher diversity of endophytic fungi diversity was found higher at Wondo Genet site than Menagesha Suba forest in the same season.

The Shannon diversity index for geographic variation between sampling sites indicates that the diversity of endophytic fungi was numerically higher at Wondo Genet site (*H'*= 3.35275) than from trees at Menagesha Suba site (*H'*= 1.47950) during the wet season, but the data was statistically not significant at P>0.05, indicating that the diversity of endophytic fungi decreases as altitude increases (Rojas and Stephenson, 2008; Talley et al., 2002).

Similarly, the diversity of endophytic fungi at Wondo Genet site (*H'*=3.36649) was higher than samples at

Table 7. Shannon diversity index for endophytic fungi isolated from *A. falcatus* tree of different age group.

Sampling site and season	*H'*
Wondo Genet at wet season (young trees)	2.85575
Wondo Genet at wet season (old trees)	3.16488
Wondo Genet at dry season(young trees)	3.29748
Wondo Genet at dry season (old trees)	3.01591
Menagesha Suba at wet season (young trees)	1.44382
Menagesha Suba at wet season (old trees)	1.41303
Menagesha Suba at dry season (young trees)	2.55959
Menagesha Suba at dry season (old tree)	2.29078

Table 8. Shannon diversity index for different land uses of each sampling site and season.

Sampling site and season	*H'*
Wondo Genet at wet season (from open land)	2.82897
Wondo Genet at wet season (from natural forest)	3.20512
Wondo Genet at dry season (from open land)	3.11148
Wondo Genet at dry season (from natural forest)	3.09124
Menagesha Suba at wet season (from open land)	1.23645
Menagesha Suba at wet season (from natural forest)	1.61325
Menagesha Suba at dry season (from open land)	2.50492
Menagesha Suba at dry season (from natural forest)	2.50277

Menagesha Suba site (*H'*=2.65877) during the dry season, but it is statistically not significant at P>0.05. The previous study by Rojas and Stephenson (2008) and Arnold and Lutzoni (2007) also indicates that as altitude increases the diversity of endophytic fungi decreases and vice versa.

At Wondo Genet site during both dry and wet seasons, the diversity of endophytic fungi was nume-rically higher than what was observed during both seasons at Menagesha Suba site, indicating that species richness and diversity increases as a function of decreasing latitude (Arnold and Lutzoni, 2007; Rojas and Stephenson, 2008; Hawksworth, 1991) but the data was not statistically significant at P>0.05.

In accordance with the finding of Wang and Guo (2007) the diversity of endophytic fungi was numerically higher than older trees at Wondo Genet during the wet season (*H'*=3.16488) than younger trees (*H'*= 2.85575) (Table 7), but it was statistically not significant at P>0.05.

However, the Shannon diversity index for endophytic fungi isolated from young trees at Wondo Genet during the dry season was higher on young tree than that of samples from old trees at the same site (Table 8). This result was in agreement with the finding of Helander et al. (2006) indicating that young plants harbor higher number of fungi than old ones.

The diversity of endophytic fungi was higher for young tress than old trees at Menagesha Suba during both the dry and wet seasons, but the data is statistically not significant at P>0.05. The results of the current study are also in agreement with those of Helander et al. (2006).

In accordance with the finding of Tsui et al. (1998), the diversity of endophytic fungi in both sampling sites during the wet season was higher than samples collected from natural forest trees (Table 8). This is because natural forest have higher plant diversity (Helander et al., (2006) that is, surrounding vegetation is an important source of inoculums for endophytes (Gamboa and Bayman, 2001; Rodrigues, 1994) but the data is not statistically significant.

However, the diversity of endophytic fungi in dry season both at Wondo Genet and Menagesha Suba site was found to be higher than samples collected from open land *A. falcatus* and that of natural forest (Table 8). This finding was in accordance with the finding of Aung et al. (2008), indicating that the diversity of endophytic fungi is higher in disturbed forest. This might be due to the high disturbance; dust formation and wind movement in dry season serve trees in open land to get more inoculants than natural forest.

Table 9. Sorensen similarity index between seasons in the same land use.

Sampling site	Season	Total number of isolates	No. of commonly shared isolates	*QS*
WG	Wet	176	25	0.1358696
	Dry	192		
MS	Wet	194	3	0.0188088
	Dry	125		

QS: Sorensen similarity index, WG- Wondo Genet, MS- Managesha Suba.

Table 10. Number of isolates of each land use systems, number of commonly shared isolates and Sorensen similarity index.

Site and season	Land use	Total no. of isolates	No. of commonly shared isolates	*QS*
WG at Wet season	Open land	90	15	0.1704545
	Natural Forest	86		
WG at Dry season	Open land	79	20	0.2083333
	Natural Forest	113		
MS at Wet season	Open land	100	6	0.0618557
	Natural Forest	94		
MS at Dry season	Open land	52	10	0.16
	Natural Forest	47		

WG-Wondo Genet, MS-Managesha Suba.

Species similarity

Species similarity between sampling units was calculated using Sorensen similarity index. The highest similarity between sampling seasons was found at Wondo Genet (*QS*=0.1358696) than the similarity index value observed at Menagesha Suba site (*QS*= 0.0188088) (Table 9), which is in contrast with the previous finding by Wang and Guo (2007) stating that composition of endophyte assemblages is not greatly influenced by geographical or climatic factors. In addition, the similarity between seasons at Wondo Genet site was higher than Menagesha Suba site. The result, also, suggests that the similarity between sampling seasons decreases as altitude increases (Table 9).

The highest similarity between land use systems was found at Wondo Genet site during the dry season (*QS*= 0.2083333) than during the wet season (*QS*=0.1704545) (Table 10). Similarly, the similarity between land use systems at Menagesha Suba site was numerically higher during the dry season (*QS*=0.16) than during the wet season (*QS*=0.0618557) (Table 10). Indicating that the similarity between land use systems during both seasons at Wondo Genet site was higher than what was observed during both seasons at Menagesha Suba site (Table 10). The lowest number of commonly shared isolates (6) between seasons was recovered at Menagesha Suba and indicating that higher moisture difference between the two sampling seasons at Menagesha Suba. This result is in agreement with the finding of Stanwood (2009).

The Sorensen similarity index for age of host tree indicates that at Wondo Genet site the similarity between age groups was numerically higher during the dry season than (*QS*= 0.1979167) than during the wet season (0.1704545) (Table 11), this is also in contrast with the previous finding by Wang and Guo (2007). Similarly, the Sorensen similarity index between age groups at Menagesha Suba site was found to be numerically higher in dry season (*QS*=0.144) than wet season (0.039278). These results suggest that as altitude increases the similarity between age groups decreases. As altitude increases, the similarity between age groups in the same season was found to be higher in Wondo Genet than Menagesha Suba site.

In agreement with the work of Osono and Hirose (2009) the similarity between land use systems decreases as altitude increases.

The similarity between the two sites (Wondo Genet and Menagesha Suba) was higher during the dry season (*QS*= 0.126183) than during the wet season (*QS*=0.0324524) (Table 12). This result makes us to

Table 11. Showing total number of isolates from young and old host tree, commonly shared number of isolates and Sorensen similarity index.

Site and season	Age of sampling units	Total no. of isolates	No. of commonly shared isolates	*QS*
WG, Wet	Young	89	15	0.1704545
	Old	87		
WG, Dry	Young	104	19	0.1979167
	Old	88		
MS, Wet	Young	105	3	0.039278
	Old	89		
MS, Dry	Young	57	9	0.144
	Old	68		

QS: Sorensen Similarity index, WG- Wondo Genet, MS- Managesha Suba.

Table 12. number of commonly shared isolates between geographic variations and Sorensen similarity index.

Season	Sampling sites	Total no. of isolates	No. of commonly shared isolates	*QS*
Wet	Wondo Genet	176	6	0.0324324
	Menagesha Suba	194		
Dry	Wondo Genet	192	20	0.126183
	Menagesha Suba	125		

conclude that the similarity between two different geographic locations (Wondo Genet site and Menagesha Suba site) was higher during the dry season than during the wet season. The finding of this study shows that geographic location affects distribution of endophytic fungal diversity (Gore and Bucak, 2007).

Conclusion and recommendations

Variation in the type of dominant morphotaxa between ages of host within the same site, season and land use system was observed. It suggests that there occur variation in the type of dominant morphotaxa between seasons, geographic locations, age of host trees and land use systems. The diversity of endophytic fungi was numerically higher in Wondo Genet site (1600 and 2500 masl) than Menagesha Suba (2200 to 3000 masl) but statistically same.

The diversity of endophytic fungi during the wet season at both sampling sites (Wondo Genet and Menagesha) was numerically higher in trees in natural forest than trees from open land. During the dry season, numerically higher endophytic fungi diversity was recovered in open land. Seasonal variation in the diversity of endophytic fungi was recorded that is, the diversity of endophytic fungi was higher in dry season than wet season but the data is not statistically significant. Lower altitude favors higher number, unique and rarest morphotaxa than higher altitude.

Since the diversity of endophytic fungi was numerically higher in dry seasons of the year, lower altitude (Wondo Genet as in our case), and concerned bodies should act accordingly to utilize the economic significance of tree endophytic fungi. To have full information on the diversity of endophytic fungi enough samples per tree should be collected. Further researches should focus on investigation of endophytic fungi for several agro-climatic zones, at different slopes and seasons. A full-fledged taxonomic study should be pursued at a species level through polyphasic approach. Further research should be conducted on a different plant species and different organ of the plant (leaf, root, stem and seed etc). Work on antimicrobial and biocontrol properties of the endophytic fungi should be undertaken.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Antimicrobial activities of six plants used in Traditional Arabic Palestinian Herbal Medicine

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Ethanolic extracts of six plants: *Arum palaestinum* Bioss, *Urtica pilulifera* L., *Thymbra capitata* (L.) *Cav.*, *Origanum syriacum* L., *Teucrium creticum* L., and *Teucrium polium* L., used in Traditional Arabic Palestinian Herbal Medicine were evaluated for their antibacterial, anti-candida, and antidermatophyte activities using well diffusion, micro-dilution and food poisoned techniques. The extracts were tested against: six bacterial strains including *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli* and *Klebsiella pneumoniae*; five *Candida albicans* isolates, and two dermatophytes: *Microsporum canis*, and *Trichophyton rubrum*. The most active plants extracts were *T. capitata* and *O. syriacum* against the tested bacteria, while the remaining plant extracts did not express any activity or exhibited only very low activity against tested bacteria species and candida isolates. O. syriacum was also the most active plant against all *Candida* strains with inhibition zones that ranged from 22.5 to 29.5 mm. On the other hand, *T. capitata* extract showed the highest activity against the test dermatophytes (producing a complete inhibition at ≤ 45 µg/mL).

Key words: Medicinal plants, antibacterial activity, antifungal activity, *Arum palaestinum*, *Urtica pilulifera*, *Thymbra capitata*, *Origanum syriacum*, *Teucrium creticum*, *Tecrium polium*.

INTRODUCTION

In Palestine, the screening of flora for pharmacological active compounds started in the late nineties (Ali-Shtayeh et al., 1998) and continue to provide useful means for treating ailments (Ali-Shtayeh and Jamous, 2008). Little scientific research has been carried out to investigate the plants used in Traditional Arabic Palestinian Herbal Medicine (TAPHM) (Ali-Shtayeh et al., 1998, 2003). In the course of our investigations we found that several plants of the Palestinian ethno- medicine possess interesting biological activities, which could be of interest for all parts of the world. The activities have been selected because of their strong medicinal relevance (Saad et al., 2005; Abu-Lafi et al., 2007).

Arum palaestinum Bioss (Araceae) is considered edible

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after being soaked in salty water or dried. The plant is also used in folk medicine to treat several diseases such as stomach acidity, atherosclerosis, cancer, diabetes and food toxicity (Ali-Shtayeh et al., 1998). Phytochemical and biological investigations carried out on the plant have shown that it possesses an inhibitory effect on smooth muscle contraction in rats (Afifi et al., 1999), anticancer activity against lymphoblastic leukemia due to the presence of a pyrrole alkaloid (El- Desouky et al., 2007), and antioxidant activity (Husein et al., 2014).

Urtica pilulifera L. (Urticaceae), has long been used in traditional medicine in many countries around the world to treat various ailments including: sore joints, rheumatism, hemorrhage, renal stones and inflammation of the bladder, diabetes mellitus and other ailments (Kavalali et al., 2003; Lopatkin et al., 2005; Irshid and Mansi, 2009).

Thymbra capitata (L.) Cav. (Lamiaceae) is an aromatic plant found in Palestine and locally known under the common name Zaa'tman. The essential oil of the plant was reported to have antimicrobial activities most of which are mediated by thymol and carvacrol (Bhaskara et al., 1998). The plant is also important as a source of perfume, cosmetics, flavoring and pharmaceutical industries (Tabata et al, 1988). In traditional food the plant has been used for its flavors as refreshing drink or in cooking. It is used in folk medicine against cold, influenza and throat infection (Ali-Shtayeh and Jamous, 2008). Later on the plant was found to contain antiseptic and antimicrobial agents (Bremnes, 2002).

Origanum syriacum L. (Lamiaceae) is one of the most popular herbs among Palestinians (Ali-Shtayeh and Jamous, 2008). The green leaves of the herb are rich in essential oil, which is responsible for its characteristics of flavor and fragrance. Oil of cultivated *O. syriacum* is an important commercial product and is obtained mainly by steam distillation of the fresh leaves. Plant extract is also found to have strong biological activity, and this may be due to the presence of phenols, thymol and carvacrol as major constituents of thyme oil in the plant (Abu-Lafi et al., 2007).

Teucrium creticum L. (Lamiaceae) is found in Palestine and locally known under the common name Ja'adh. The plant is used traditionally to cure diabetes in Palestine (Ali-Shtayeh et al., 2012). No phytochemical studies were found on this plant, and this may be attributed to its limited distribution in Palestine (Saad et al., 2005).

Teucrium polium L. (Lamiaceae) is found in Palestine and locally known under the common name Ja'adhat al-sibian. The plant is well known for its diuretic, antipyretic, diaphoretic, antispasmodic, tonic, anti-inflammatory, antihypertensive, anorexic, analgesic (Saad et al., 2005; Tariq et al., 1989) antibacterial (Mansouri et al., 1999) and antidiabetic effects (Esmaeili and Yazdanparast, 2004). Hot infusion of tender parts of plant is taken for stomach and intestinal troubles (Panovska and Kulevanova, 2005). The plant shows antimicrobial activity against *Bacillus subtitles* and *Staphylococcus aureus* (Lemordant et al., 1997). Plants belonging to the genus Teucrium have been shown to contain different classes of compounds such as fatty acid, esters, terpenes, flavonoids and polyphenolics (Harborne et al., 1986; Rizq et al., 1986).

In this study, the ethanolic extracts prepared from six Palestinian medicinal plants have been investigated for their antibacterial and antifungal activities by means of the agar diffusion, micro-dilution and food-poisoned methods. In addition, the current study reports values of minimum inhibitory concentration (MIC) for active plants extracts.

MATERIALS AND METHODS

Chemicals

The chemicals used included chloramphenicol, peptone, agar, dextrose, ethanol, Muller-Hinton (Fluka), Sabouraud dextrose agar (Difco), gentamicin, ampicilline, amphotericin B, econazole, ethanol, and DMSO. All chemicals and reagents were of analytical grade.

Plant material

Six medicinal plant species A. *palaestinum* (Voucher number BERC-C0064), U. *pilulifera* (BERC-C0066), T. *capitata* (BERC-C0245), O. *syriacum* (BERC-C0026), T. *creticum* (BERC-C0173), and T. *polium* (BERC-C0167), screened in this study were collected from April-June 2013 from Nablus region and were identified by Prof. M. S. Ali-Shtayeh from the Biodiversity and Environmental Research Center, BERC, Til Village, Nablus. Voucher specimens are deposited in the Herbarium of BERC.

Plants extracts preparation

Fifty grams of each dried plant was ground using a Molenix (Mooele Depose type 241) for a minute and the resulting powder was extracted by continuous stirring with 200 ml 70% ethanol at 24°C for 72 h. Extracts were filtered through Whatman No. 4 filter paper, and the residue was then washed with additional 50 ml ethanol. The combined ethanol extracts were dried using rotary evaporator followed by freeze drying and stored at -20°C for future use.

Microorganisms used and growth conditions

Six bacterial strains from the American Type Culture Collection (ATCC; Rockville, MD, USA) were employed. They included Gram-positive (G +ve) bacterium: *Staphylococcus aureus* (ATCC 25923), and the following Gram-negative (G -ve) bacteria: *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 14028), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 13883).

Five *Candida albicans* strains were used, three of which were clinical isolates from cutaneous candidiasis patients (BERC-N43, BERC-N72, and BERC-N66), and the other two from CBS type cultures: CBS6589, and CBS9120.

Two dermatophytes strains: *Microsporum canis* (CBS132.88), and *Trichophyton rubrum* (BERC-EH-TR9, clinical strain from a tinea pedis patient) were also used.

Disk diffusion method

Disc diffusion method was made according to Zongo et al. (2009).

Dried plant extracts were dissolved in DMSO to a final concentration (100 mg/mL) and sterilized by filtration through a 0.45 µm membrane filter. Inoculums (10^6bacterial cells/mL) were spread on Muller-Hinton agar plates (1 mL inoculum/ plate). Filter paper discs (6 mm in diameter) were individually impregnated with 40 µL of each plant extract, and controls, and placed onto the surface of inoculated Petri dishes. Before incubation, all Petri dishes were kept in the refrigerator (4°C) for 2 h and incubated after at 37°C for 24 h for bacteria growth. After incubation, the diameters (mm) of inhibition zones were measured including diameter of discs. The antimicrobial potentials were estimated according to the index reported by Rodriguez et al. (2007). All the experiments were done in triplicates. Gentamicin (10 mg/mL) and DMSO served as a positive and negative control, respectively.

Well diffusion method

Anti-*Candida* susceptibility testing was done using well-diffusion method to detect the antifungal activities of plant samples (Perez et al., 1990). A sterile swab was used to evenly distribute fungal culture over Muller-Hinton agar plates supplemented with glucose- methylene blue. The plates were allowed to dry for 15 min before use in the test. Wells were then created and 50 µL of the crude extract (100 mg/mL) of each plant extract were pippeted into each well. The same extract was used in each plate; with a total of two plates used for each extract including two wells for the positive and negative controls. The plates were incubated at 37ºC for 24 h after which they were examined for inhibition zones (Delahaye et al., 2009). All the experiments were done in triplicates. Amphotericin B (32 µg/mL) and DMSO were used as positive and negative controls, respectively.

Micro-dilution Test

Broth micro-dilution was performed following the CLSI M27-A2 method (NCCLS, 2002) with little modifications. Muller-Hinton media (pH 7.2) was used for bacteria, while Muller-Hinton supplemented with glucose-methylene blue was used for *Candida*. Plant extracts were dissolved in DMSO and the correct volume was pippeted in the first micro-well plate with Muller-Hinton, for the concentration of each plant extract to be 25 mg/mL in that well. The cell suspension was prepared in 0.85% saline, with an optical density equivalent to 0.5 McFarland standards, and diluted 1:100 in the media to obtain a final concentration of 1×10^4 to 5×10^4 colony-forming units per milliliter (CFU/mL). This suspension was inoculated in each well of a micro-dilution plate previously prepared with the plant extracts to give concentrations from 25 mg/mL down to 0.012 mg/mL (Scorzoni et al., 2007). The plates were incubated with agitation at 37°C for 24 h for all species. The control drugs were gentamicin for bacteria strains, and amphotericin B for *Candida,* respectively. Concentrations of controls were ranged from 250-1 µg/mL for gentamicin, and from 16.0-0.125 µg/mL for amphotericin B. Value of minimum inhibitory concentration (MIC), determined by broth macro- dilution, and defined as the lowest concentration of the drug completely inhibited the growth of the isolate. For plant extracts, MIC value was defined as the lowest concentration able to inhibit any visible bacterial or candidal growth. Results were read visually and spectrophotometerically.

Antidermatophytes testing

Plants extracts were tested at different concentrations for their antidermatophytes activity against the test pathogens using a modified poisoned food technique (Dikshit and Husain, 1984). Differentamounts of each extract were incorporatedin pre-sterilized SDA medium to prepare a series of concentrations of the extract (15, 30, 45, and 60 µg/mL). A mycelial agar disk of 5 mm diameter was cut out of 12 days old culture of the test dermatophytes and inoculated onto the freshly prepared agar plates. In controls, sterile distilled water was used in place of the tested sample as a negative control, while econazole (5 µg/mL) was used as the positive control. Three replicate plates were used for each treatment (concentration). The inoculated plates were incubated in the dark at 24°C and the results were recorded after 10 days.

Percentage of mycelial inhibition was calculated using the following formula:

% mycelial inhibition = (dc- ds / dc) x100%

Where, dc is the colony diameter of the negative control and ds is colony diameter of the sample.

RESULTS

Six medicinal plant species belonging to 3 families were selected based on their uses in Traditional Arabic Palestinian Herbal Medicine (TAPHM) for the treatment of various ailments. The antibacterial, anti-*Candida* and antidermatophytes activities of ethanolic extracts of the selected plants were screened in this study.

Antibacterial activity

The ethanolic extracts of six Palestinian plants were subjected to a preliminary screening for antimicrobial activity against six human pathogenic bacteria *S. aureus*, *E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa* and *S. typhi*. The inhibition zones of the plant extracts against different types of bacteria strains and MIC values of plants extracts are presented in Table 1. Gentamicin was used as a positive control. Two plant extracts (*T. capitata*, and *O. syriacum*) inhibited the growth of all tested bacteria strains. The MIC values for *T. capitata* were 390 µg/mL for *Pr. vulgaris*,*E coli*, and *K. pneumonia* while for *O. syriacum* it was 390 µg/mL for *K. pneumonia*. Other plants extracts revealed very low or no activity against different types of bacteria (Table 1).

Anti-*candida* activity

The anticandida activity of tested plants extracts was evaluated according to their mean of inhibition zone (100 mg/mL) against various candida isolates, and the MIC values of active plants extracts against the five isolates of *Candida*. Results of inhibition zone were compared with the activity of standard amphotericin B (32 µg/mL). *Origanum syriacum* and *T. capitata* exhibited strong and moderate inhibition activity against candida isolates, while the other 4 plant extracts show no activity (Table 2). The MIC value for O. syriacum is 150 µg/mL

Table 1. Inhibition zones (mm) of plant extract against different types of bacteria strains using the disc diffusion method.

Microorganism	Inhibition zone (mm+SD)			MIC (µg/mL)		
	T. capitata	*O. syriacum*	Positive control	*T. capitata*	*O. syriacum*	Positive control
P. vulgaris	13.38± 0.7*	13.8± 0.7	23.3± 0.7	390	780	7.8
S. aureus	14.3± 0.9	15.0± 1.3	23.7± 0.5	780	780	3.9
E. coli	15.2± 0.7	14.5± 0.5	23.8± 0.7	390	780	7.8
P. aeruginosa	15.2± 1.6	14.7± 1.5	24.5± 0.5	780	780	7.8
S. typhi	14.5± 0.8	12.8± 0.7	23.2± 0.7	780	780	7.8
K. pneumoniae	15.8± 1.1	15.5± 1.0	24.8± 1.6	390	390	3.9

*Values of inhibition zone diameter in mm ± SD.

Table 2. Inhibition zone (mm) and MIC values of tested extracts against *Candida albicans* isolates compared to amphotericin B.

Candida albicans isolates	Inhibition zone (mm)			MIC (µg/ml)		
	T. capitata	*O. syriacum*	Amphotericin B	*T. capitata*	*O. syriacum*	Amphotericin B
BERC-N43	13.5± 1.5*	22.5± 2.5	31.0± 1.7	310	310	8
BERC-N72	17.0± 0.0	22.5± 0.5	30.5± 0.5	310	625	8
BERC-N66	20.0± 0.0	29.5± 0.5	31.5± 0.5	310	310	8
CBS6589	19.0± 2.0	25.0± 0.5	33.5± 0.5	625	310	2
CBS9120	17.5±0.5	24.5±0.5	29.5± 0.5	310	150	2

*Values of inhibition zone diameter in mm ± SD.

Table 3. Mean of % inhibition ±SD of fungi at 45 µg/mL concentration.

Plant	% Inhibition	
	Dermatophyte	
	Trichophyton rubrum	*Microsporum canis*
Arum palaestinum	49.6± 1.4	62.6± 3.5
Urtica pilulifera	81.3± 1.3	88.6± 1.1
Thymbra capitata	100± 0.0	100± 0.0
Origanum syriacum	93.6± 0.4	84.3± 1.7
Teucrium creticum	71.6± 0.5	66.3± 0.8
Teucrium polium	89.6± 1.3	100± 0.0
Econazole (5 µg/ml)	100± 0.0	100± 0.0

against the type culture isolate, while it was 390 µg/mL against the clinical isolates.

Antidermatophyte activity

The antidermatophyte activity of the tested plants extracts was expressed as the means of % inhibition values of dermatophytes growth. Four different concentrations of plants extracts concentrations were tested. The percent of inhibition of the two dermatophytes ranged between 49.6-100%, with *T. capitata* exhibiting a significant % of inhibition (100%) against the two dermatophyte species at a concentration of 45 µg/mL comparable to the positive control (Table 3).

DISCUSSION

Substances derived from plants have recently attracted much attention with regard to human health, due to their low cost, broad availability and limited toxicity. Thus, plant based antimicrobial compounds have vast therapeutically potential as they can serve the purpose

without any side effects that are often associated with synthetic drugs.

Antimicrobial activity

The primary results of bioassays for plants extracts open the possibility of finding new clinically effective antimicrobial compounds, such as the extract of *Micromeria nervosa* which is found in different locations of Palestine and are well known plant used for various medical purposes (Ali-Shtayeh et al., 1997).

Antimicrobial activity of plant materials can be classified according to their MIC values. MIC values lower than 500 indicate the strong antimicrobial activity. Furthermore, MIC values equal to 600-1500 µg/mL and higher than 1600 µg/mL indicate intermediate and weak antimicrobial activities respectively (Durate et al., 2007; Fabri et al., 2009; Michielin et al., 2009). Therefore, it was clear from the results of inhibition zone that the most pronounced activity against all test bacteria with inhibition zones ≥12 mm at a concentration of 100 mg/mL was shown by the ethanolic extracts of *O. syriacum* and *T. capitata*. The majority of the remaining plant extracts did not express any activity (Table 1). This tends to show that the biological activities of the plant extracts are different from one plant to another. The absence of antibacterial activity of ethanolic extracts of those plants indicates either the absence of active chemical compounds or the insolubility of the active ingredients in the solvent used.

The activity of *T. capitata* and *O. syriacum* might be attributed to the active compounds in their essential oils. *O. syriacum* is an aromatic, perenial, herbaceous plant. The plant has been reported to pose antibacterial activity (Leja and Thopil, 2007). The major volatiles and semi-volatiles of Palestinian wild *O. syriacum* are α-phelandrene, α -pinene, b-myrecene, o-cymene, p-cymene, c-terpinene, thymol, and carvacrol (Abu-Lafi et al., 2008). The plant has a long history of medicinal use among Palestinian (Ali-Shtayeh and Jamous, 2008) as well as by the Creks as an antidote to poisoning and snake venom, by the Romans for stomach disorders and more recently for digestive, antispasmodic and sedative properties (Evans, 2002).

T. capitata essential oil is traditionally considered to exhibit powerful antiseptic properties, thus being used to treat cutaneous infections (Palmeira-de-Oliveira et al., 2012). Essential oils of *T. capitata* were analyzed using gas chromatography (GC) in combination with retention indices (RI), gas chromatography-mass spectrometry (GC-SM) and 13C NMR spectroscopy. Carvacrol (68.2%-85.9%) was the major component of the plant, while the content of thymol (0.1-0.3%) was very low. Other components present in appreciable amounts were gamma-terpinene (up to 8.9%), p-cymene (up to 7.1%), linalool (up to 4.4%) and (E)-beta-caryophyllene (up to 4.1%) (Bakhy et al., 2013). Thus, the antimicrobial activity of *T. capitata* can be attributed mainly to the presence of carvacrol in the plant (Ali-Shtayeh et al., 1997) such as carvacrol, thymol, *p*-cymene and terpinene.

Continued further exploration of plant-derived antimicrobials is needed today. Further research is necessary to determine the identity of antibacterial compounds from these plants and also to determine their full spectrum of efficacy. However the present study of *in vitro* antibacterial evaluation of some extracts forms a primary platform for further phytochemical and pharmacological studies to discover new antibiotic drugs.

Anti-Candida activity

The results reveal that extracts of *T. capitata* and *O. syriacum* are potent antimicrobials against all the microorganisms studied. *O. syriacum* showed inhibition zone of 29.5±0.5 mm against BERC N66, (94% of amphotericin B activity) and *T. capitata* (inhibition zone 20.0 mm) against the same genotype of *Candida*. Other extracts did not show significant activity against the studied microorganisms (Table 3). It is noteworthy to mention that plants belonging to the same family sometimes exhibited comparable anticandidal activity since these plants are expected to possess similar active constituents (*T. capitata*, and *O. syriacum* of the Family Labiateae), while some plants of the same family exhibited different effects (*T. creticum* and *T. polium* of the Family Labiateae) indicating that there might be some constituents found in one member of the family but not in the other.

Antidermatophyte activity

Only a few antifungal substances are known or available in the market as compared to antibacterial substances. Antimycotic substances are also relatively unsatisfactory in the control of dermatophytes. The discovery of active components exhibiting a broad spectrum antifungal activity may prove useful for the development of antifungal agents. Laboratory assessment showed the nature of fungi static activity encountered in plant extracts. Table 3 shows percentage of inhibition due to plant type at (45 µg/mL) concentration. Values of % inhibition increase with increasing concentration of each plant extract (Figure 1).

All tested plants revealed antimycotic activity against all two tested dermatophytes, with *T. capitata* exhibiting the highest activity; a complete inhibition by the ethanolic extract of this plant was at a concentration lower than 45 µg/mL. *O. syriacum* extract have also showed considerable antimycotic activity against the same tested dermatophytes, complete inhibition was at a concentration <60 µg/mL. The oil of *T. capitata* have been shown to exhibit antidermatophytic activity, with MIC values ranging from 0.08 to 0.32 µL/mL (Salgueiro

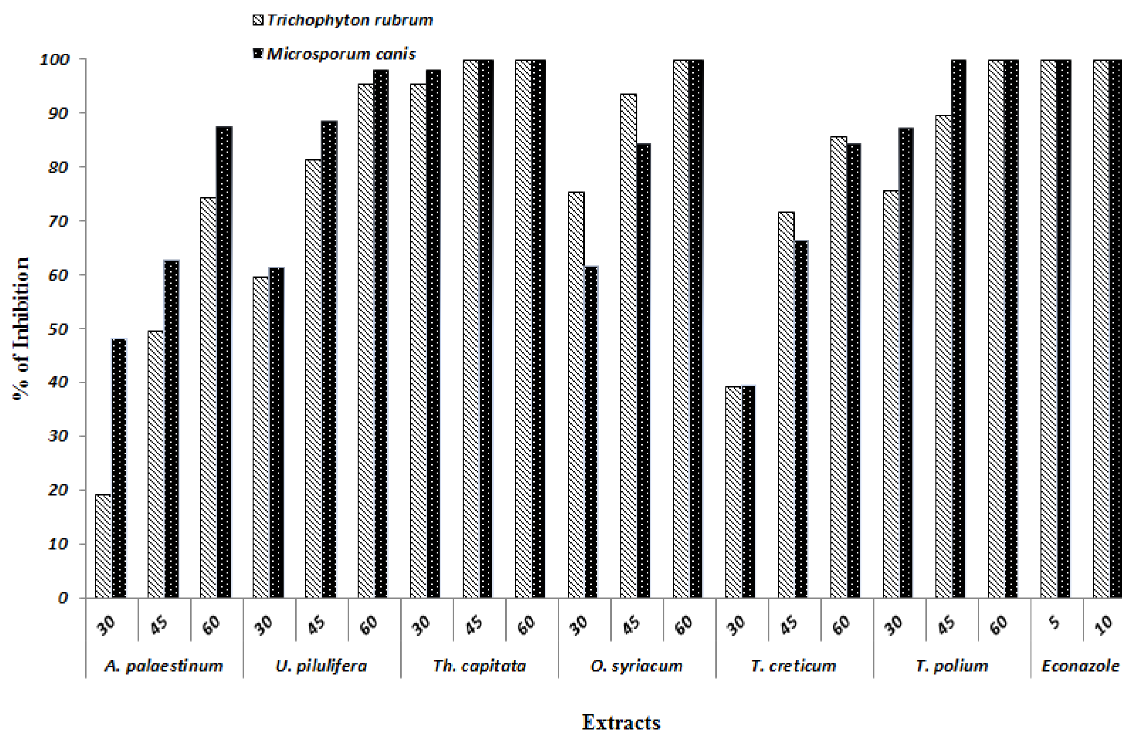

Figure 1. Percent of inhibition of plants extracts against *T. rubrum* and *M. canis* at different concentrations (30, 45, and 60 µg/mL).

et al., 2004). In addition to these plants, *A. palaestinum* showed 50% inhibition against *M. canis* at a concentrationof ~45 µg/mL.

The present work has shown that most of the studied plants are potentially good source of antidermatophytes and demonstrates the importance of such plants in medicine and in assisting primary health care. However, the screened plants are among the wild edible plants consumed by Palestinian (Ali-Shtayeh et al., 2008). From those results we can suggest that plants extracts investigated here could find practical application in the introduction of highly active and safe antimicrobial agents including antidermatophytes. These results justify the usage of those plants in folk medicine (Ali-Shtayeh et al., 2008, Ali-Shtayeh and Jamous, 2008).

Conclusion

Overall, some crude extracts prepared from plants commonly grown in Palestine or commonly used by Palestinian were found to exert, *in-vitro* some antimicrobial effect. However, further work is needed to isolate and identify the active compound(s) from active plant species.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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