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

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Review

Dermatophytes: Diagnosis of dermatophytosis and its treatment

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The enzymatic ability of fungi to decompose keratin has long been interpreted as a key innovation in the evolution of animal dermatology. Dermatophytes are keratinophilic fungi which were originally saprophytic, but have adapted themselves to animal and human parasitism during the course of evolution. Dermatophytes are pathogens, which cause superficial mycosis. The dermatophytes have the capacity to invade keratinized tissue (skin, hair and nails) of humans and other animals to produce an infection. The mycoses caused by fungal infections of the skin and nails is widespread and common amongst all type of mycoses. During last decades, mycotic infections raised to more than 20-25% of the world's population. The review article contains the input of knowledge of various dermatophytes and the diseases caused by them, their identification at the molecular level and treatment strategies.

Key words: Dermatology, dermatophytes, mycoses, keratin.

INTRODUCTION

Earth has been documented as a natural territory for fungi which cover individual kingdom with evolution (Sharma et al., 2015). Dermatophytes are a group of morphologically and physiologically allied molds which have the hazard at the global plane as these are generally causative agent of fungal infections (Smith et al., 1998; Mihali et al., 2012). They hold two imperative properties: they are keratinophilic and keratinolytic agents (Kushwaha et al., 2000). They have an aptitude to digest keratin in their saprophytic circumstances and consume it as a substrate. The World Health Organization estimates universal occurrence of dermatomycoses to be related to 20% (Marques et al., 2000). The infections are universally present in those people who play sports (Maryan, 2009). Infections which are caused by dermatophytes are known as dermatophytosis (Dei and Vernes, 1986). Dermatophytosis is caused by the genera *Microsporum, Trichophyton* and *Epidermophyton*. These organisms are pathogenic members of the keratinophilic (keratin digesting) soil fungi (Witzman and summerbell, 1995). *Microsporum* and *Trichophyton* are human and animal pathogens. *Epidermophyton* is a human pathogen. *Anthropophilic* species are responsible for the majority of human infections. The dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair and nails) of humans (Maraki et al., 2007).

The dermatophytes are significantly varying in diverse level of the world. They increase at exterior temperature of 25-28°C and membrane mycosis is continued by warm

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and humid conditions (Male, 1990; Havlikova et al., 2008). For related reasons, dermatomycoses are moderately generally in tropical and subtropical regions (Nweze, 2010). In a special case, the developing countries as India contain infection by the members of the genus Candida (Rao, 1959). *Trichophyton concentricum* is present in the Far East, India and the pacific's (Ameen, 2010; Lakshmipathy et al., 2010). *Microsporum audouinii*, *Trichophyton violaceum, Trichophyton soudanense* are studied in several parts of Africa (Woldeamanuel et al., 2005). Equally, *Microsporum canis, Trichophyton mentagrophytes, Trichophyton rubrum* survive in southern and central European countries as a universal causative agent of Tinea capitis, Tinea unguium and Tinea pedis (Tao-Xiang et al., 2005). Paracoccidioidomycosis is an exceptional disease worldwide but an ordinary deep presence in Brazil with Latin America (Almeida et al., 2003). The key occurrences of these particular dermatophytes are observed during a study on the sexually transmitted diseases (AIDS). *Trichophyton simii* and *Trichophyton mentagrophytes var. Erinacei* are enclosed to France, Italy and New Zealand (Quaife, 1996). In addition, the occurrence of dermatomycoses is superior in population with little socioeconomic status and also in close nearness of animals (Farzana, 2007; Mikali et al., 2012). However, flooring, clothing, linens, furniture and barber shop instruments, are the vital foundation of dermatophytes.

The dermatophytes have a saprophytic presence. They cause the surface infections through colonization individually of skin, hairs and nails in human beings known as ringworm, jock itch (Khaksari and Bassiri, 2009; Ryan et al., 2010). The colonies are equipped by generating the Arthospores and conidia of the fungus (Lakshmipathy et al., 2010). Indication of dermatophytosis has a distinction basis in the affected region of the body, but one of their priorities is a universal indicator in humans (Nweze, 2010).

Primary infection starts through small skin break. These breaks are coming out through secretion of enzymes that digest keratin (Laham et al., 2011; Achterman and White, 2012; Mikaili et al., 2012). This enzyme is referred to as keratinase (Gupta and Ramnani, 2006). The excreted enzyme plays a vital role in the process of infection and considered as primary virulent factors (Sharma et al., 2012). As it, the *T. mentagrophytes var. Erinacei* present in the hedgehog caused the hedgehog ringworm to the public; especially to children (Quaife, 1996). These dermatophytes also cause the extremities, including Tinea manuum, Tinea corporis, nail infection (Philpot and Brown, 1992; Chang et al., 2009). *T. rubrum* causes infection in nails known as Onychomycosis (Achterman and White, 2012; Ahmad et al., 2010). Currently, Pmycosis infections are mounting at an alarming rate due to the increase in HIV/AIDS occurrence (Fentaw et al., 2010). This is also known as Paracoccidioidomycosis which is produced by *Paracoccidioides brasiliensis* (Almeida et al., 2003). Another infection, ophthalmic Mycoses, is an agent of morbidity and blindness by *Cephaliophora irregulars* (Thomas, 2003). The key component of the present compilation includes study of various dermatogens and diseases caused by them, their diagnosis and treatment strategies.

DERMATOGENS AND DISEASE

Tinea pedis or athlete's foot

Tinea pedis is universal infection observed in one in five adults and the occurrence boost up with age from adolescence (Havlickova et al., 2008). Infections are derived as the itching and shedding of skin scales holding viable infectious agent like arthroconidia of the fungus (Sharma et al., 2012). Swell up and cracked skin has also been exposed to raw tissue, pain and inflammation. This acute inflammation differentiates as the formation of vesicles, pustules. The chronic agents of Tinea pedis are *T. rubrum, T. mentagrophytes var. Interdigitale*, and *E. floccosum* (Weitzman and Summerbell, 1995). Tinea pedis is also known as "one hand two feet syndrome" which means the dermatophyte illness of both feet and one hand and found in patients of lower immunity competence, such as diabetics (Havlickova et al., 2008). Naturally, its influences is observed on the feet, accepting infection or spreading to additional areas of the body (Daniel, 2010).

Tinea cruris

Tinea cruris is also famed as crotch itch, crotch rot, eczema marginatum, gym itch, jock itch, jock rot, and ringworm of the groin (Rapini et al., 2007). Tinea cruris refers to dermatophytosis of the proximal medial thighs and buttocks (Sharma et al., 2012). It takes place frequently in men with exception of auxiliaries infections which are distinguished as a corresponding tiny pattern in the woman (Macura, 1993; Weitzman and Summerbell, 1995; Gupta et al., 2003; Havlickova et al., 2008). The causative beings are attacked on the stratum corneum and the lethal hair of the affected areas (Gupta et al., 2003). The fungus spores are transferred to the groin part by scratching from locating on underclothing or pants. Affected areas display as red, tan, rippling brown, peeling, or cracking skin. The universal causative agents are *T. rubrum, E. floccosum, M. magnum, T. mentagrophytes* and *T. raubitschekii* (Nweze, 2010; Sharma et al., 2012).

Tinea unguium

Tinea unguium is identified as Onychomycosis which is

caused by dermatophytes in nail (Harvey and Stoppler, 2011). Onychomycosis appear as exterior white onychomycosis (pits on outside of the nail) and subungual dermatophytosis (infection beneath the nail plate) (Sharma et al., 2012; Sharma et al., 2015). According to review of diseases of the foot 2003, 16 European countries establish that onychomycosis, is the most frequent fungal infection with prevalence at 27% (Burzykowski et al., 2003). Tinea unguium normally attach to the tip of the toenail and steadily spreads to the nail matrix (Hiroshi, 2007). It is usually observed in men but also in women as lines and ridges on nails during the pregnancy (Harvey and Stoppler, 2011; Nenoff et al., 2007). Trichophyton rubrum is the universal dermatophytes associated with *T. interdigitale, E. floccosum, T. violceum, M. gypseum, T. tonsurans* and *T. Sudanese*. It also contains the Candida subspecies as *Candida albicans, Candida parapsilosis* and *Candida guilliermondii* (Weitzman and Summerbell, 1995; Havlickova et al., 2008; Vorvick et al., 2010). Normally, symptoms of disease are weakness, change in nail shape, breaking of the outside of the nail, lifting up of the nail, loss of shine (Nweze, 2010; Vorvick et al., 2010).

Tinea barbae

Tinea barbae is identified as "Barber's itch, ringworm of the beard, and "Tinea sycosis" (James and Werger, 2006). It is surface disease of the hair, in the beard and mustache region of men (Marcus et al., 2008; Rapini et al., 2007). Tinea barbae starts from the face and neck. It is mostly caused by shaving and abuse of steroids. The main clinical indications are classified into two forms as inflammatory and non-inflammatory. These symptoms depend on kind of fungus and patient's resistant response (Szepietowski et al., 2004). In general ways, the common symptoms of disease are loose and broken off hairs, kerion-like plaques, rash, itching and pimples near a hair follicle in the neck, and genital area (Szepietowski et al., 2008; Vorvick et al., 2010). Reddening and swelling also occur in the entire area with barbeque. Habitually, the zoophilic dermatophytes such as *T. mentagrophytes, T. verrucosum, T. megninii, T. rubrum* and *T. violaceum* are responsible for infection. *M. canis* and *T. mentagrophytes Varerinacei* also cause Tinea barbae, but these are exceptional (Marcus et al., 2008).

Tinea faciei

Tinea faciei is observed mostly on the non-bearded region as a glabrous membrane of the face (Rapini et al., 2007; Lin et al., 2004). Around 19% of all surface fungal infections is caused by Tinea faciei. Forces are in pediatric inhabitants with dermatomycoses (Akhlaghi et al., 2005). Females are more frequently affected than males (Ghilardi et al., 2005). They appear usually as a red hives on the face containing small patches, lifted bumps, the upper lip and chin (Starova et al., 2010). The warning sign are typically pruritic enclosing to itch and burning, which expose to sunlight (Nweze, 2010). Most common agents of T. faciei are *T. tonsurans, T. verrucosum, T. mentagrophytes, M. canis* and *T. rubrum* (Lin et al., 2004; Starova et al., 2010).

Tinea incognito

Tinea incognito is mycosis of skin caused by the immunosuppressive factors such as *T. rubrum, T. mentagrophytes, E. flocosums, M. canis* and *M. gypseum, M. jasiel* (Walikowska et al., 2010). They illuminate personally into iatrogenic skin disease such as suppressed irritation, delay type immune reactions. Specific examples are bacterial infections such as tuberculosis, viral infections such as chicken-pox and fungal diseases such as ringworm, seborrhoeic dermatitis, psoriasis and eczema, but also erythematic migrans (Adrian and Ronald, 1968; Satana et al., 2011). Symptoms are skin atrophy, telangiectasia and florid growth on the feet, ankle, legs or groin (Habif, 1995).

Tinea nigra

Tinea nigra is termed "Tinea nigra palmaris or plantaris (Rapini et al., 2007). This illness is superficial dermatogens that fabricate to dark brown to black effortless patches on the palms of the hands and the soles of the feet (James and Berger, 2006). The infections, is mainly caused by the fungus *E. werneckii, H. werneckii, P. werneckii C. werneckii* (Gupta et al., 2003; Murray et al., 2005). Tinea nigra naturally occurs in children and young, adults female as the stratum corneum reveal abundant dark-colored branching hyphae and round to oval spores with some budding. The colonies are initially found in moist, shiny, black and yeast-like (Palmer et al., 1989).

DERMATOGENS AND DERMATOPHYTOSES

Psoriasis is a chronic cutaneous sickness of unknown causation (Espinoza et al., 1998). According to the hypothesis, various factors construct a "thinning of the walls of the small intestine" exclusively in the jejunum and the lower duodenum (Mcmillin et al., 1999). Photo therapies produce erythema, pruritus, wrinkling, solar elastosis and an increased risk of skin cancer (Pimpinelli et al., 2005). Systemic therapies such as acitretin, methotrexate, cyclosporine, hydroxyurea and thioguanine are associated with significant systemic toxicity and

closely monitored. This thinning allocates toxic product leakage from the intestinal tract into the circulation. These toxic substances ultimately locates their way into the superficial circulation and are eliminated through the skin, which produce the plaques of psoriasis (Mcmillin et al., 1999). The relation between psoriasis and mycosis is accordingly the pattern of lymphoid penetration in typical large-plaque psoriasis, is similar to small-plaque psoriasis but penetrate contain lymphocytes with cerebral form of nuclei called Lutzner cells or Sezary cells. These Sezary cells formation process is also observed in mycosis (Pimpinelli et al., 2005).

DIAGNOSIS OF DERMATOPHYTOSIS AND CAUSAL DERMATOGENS

Dermatophytes are fungi obtaining a mid-transmittable disease which are acquired from infected animals or birds and fomites. Detection of dermatophyte Texas is correlated to epidemiological apprehension. These are important to manage infection and public health issues associated with types of Dermatophytosis (Lin et al., 1992). Traditionally, the dermatophytosis is normally referred to as “tinea” or “ring-worm” infections (Lakshmipathy et al., 2010). Damp foot circumstances lead to irritated symptoms due to mixed infection by dermatophytes and bacteria. Tinea of the extremities, tinea cruris and onychomycosis caused by zoophiles are exceptional (Weitzman and Summerbell, 1995). In humans, pruritus is a widespread symptom. The skin lesion is usually characterized by inflammation with erythema, scaling and occasionally blister formation. The habitual signs of inflammatory reactions such as redness, swelling, heat and alopecia are distinguishing at the infection position (Laksmipathy et al., 2010). The identification of dermatophytes is based on methods that focus on morphological, physiological, ecological and genetic features. Anthropophilic and zoophilic dermatophytes has mostly been recognized via internal transcribed spacer (ITS; sequencing of the rRNA gene) (Sharma and Swati, 2012).

Microscopy

The fungal dermatophytes are inspected by visual microscopy and environmental scanning electron microscopy for morphological and structural studies (Mihali et al., 2012). The sample is assembling from skin scrapings and a fungal culture on Sabouraud’s agar media (Weitzman and Summerbell, 1995; Thomas, 2003). These scrapings and hairs mount in 25% KOH or NAOH with 5% glycerol and heated to emulsify lipids. After, it was observed under 3400 magnification for fungal configuration. Another used microscopy technique is the Congo red fluorescence microscopy technique.

These techniques permit a fast diagnosis of mycotic keratitis in patients. The Giemsa stain also a detectable source of fungal hyphae and yeast cells in tissue. Lacto phenol cotton blue, Gomori methenamine silver (GMS) and periodic acid-Schiff (PAS) are stains for detection of fungi in tissue (Thomas, 2003).

Culture

Culture is a precious accessory of microscopy which is crucial in all infections for treatment with systemic prescription. Identifing characters include colony pigmentation, texture and growth rate and distinctive morphological structures, such as micro conidia, macro conidia, spirals, pectinate branches, pedicels and nodular organs. Some usable media are as follows:

1. Urea agar or broth is used to assist gratitude of ureas-negative species of *Trichophyton* genera. The experiment is utilized with a warning as the occurrence of poorly visible, antibiotic-resistant bacteria in *T. rubrum* colonies which may gain false-positive reactions.
2. BCP-milk solids-glucose agar is used to distinguish dermatophytes as *T. rubrum, T. mentagrophytes, T. soudanense, T. megninii, M. persicolor* and *M. equinum*, on the divergence of releasing ammonium ion from casein and the catabolite domination by glucose (Weitzman and Summerbell, 1995).
3. Potato flake agar or Cycloheximide amended potato glucose use of isolation, identification of *T. rubrum* by quick red pigmentation in germfree, usual isolates and with relatively antibiotic-susceptible contaminants.
4. Littman ox gall agar as restrictive media is preferred to diagnose a non dermatophytes infection.
5. Casamino acids-erythritol- albumin medium is used firstly by Fishcer and Kane. It is an extremely useable medium for isolating dermatophytes from heavily contaminated by bacteria or cycloheximide-tolerant such as *C. albicans*. This medium contains balanced egg albumin, which reduces yeasts such as *C. albicans* (Kunert, 2000)
6. Another isolation medium is Bromcresol purple (BCP)-casein-yeast extract agar which grows all dermatophytes but is designed for the rapid recognition of microcolonies of *T. verrucosum*.

Polymerase chain reaction (PCR)

Since the innovative molecular biology, the technique of PCR occupies enzymatic magnification of even minute quantities of a detailed DNA. It has the vast benefit to culture dermatophytes in easily and typical morphological performance (Sharma et al., 2012). Samples for which DNA can be extracted from include intraocular fluid as tears, any fresh tissue, paraffin-embedded tissue and

even stained or unstained cytology slides or tissue sections. The combination of broad-range PCR intensify to a product like 18S rRNA from all or most common fungi associated with human infection (Yeo and Wong, 2003). Amplification continued by restriction endonuclease analysis, sequencing, or hybridization to a series of genus and species-specific probes that show as possible preference in the effort to diagnose fungal infections (Chemaly and Procop, 2004). PCR is more sensitive than culture as a diagnostic aid in Mycoses (Thomas, 2003). Although PCR is more advantageous, it has extreme sensitivity and specificity that it cannot be used to monitor the patient's response to treatment. Another is the relatively high cost in comparison with the classical method (Sharma et al., 2012; Molyneux, 1959).

TREATMENT OF DERMATOPHYTOSIS

The basic treatments for all Tinea superficial infections excluding hairy regions are suitable use of antifungal agents (Hiroshi, 2007). The utilizable compounds have a molecular mass greater than 500 DA such as Amphotericin B (924.10 DA), Natamycin (665.75 DA) and Ketoconazole (531.44 DA). Amphotericin B and Miconazole are useable cream and sub conjunctive vaccination that extends the contact time between the antifungal and conjunctival tissue (Thomas, 2003). Paracoccidioido mycosis has systemic therapy with Griseofulvin, Terbinafine and Itraconazole, which work on Tinea capitis (Almeida et al., 2003). Tolnaftate is frequently used in uncomplicated cases of Tinea cruris with excellent results (Lakshmapathy et al., 2010). The most utilizable antifungal agents are as follows:

Polyenes

These are significant ocular antifungal armamentarium which attach to ergosterol, a sterol exclusive of the fungal cytoplasmic membrane.

Natamycin

It is used firstly as topical ophthalmic antifungal complex that agreed with the Food and Drug Administration of the United States (Thomas, 2003).

Amphotericin B

Amphotericin B is variably fungi static and occasionally fungicidal, depending on the concentration in serum and the vulnerability of the pathogens. Renal toxicity is estimated by monitoring the blood urea nitrogen and other systemic tests as headaches, chills, fever and anorexia. (Friedrich and Antanikian, 1996).

Ketoconazole

Ketoconazole is orally absorbable antifungal azoles. Oral ketoconazole therapy is effective in Tinea infection. Oral management of ketoconazole lead to side effects such as keratitis-ichthyosis-deafness syndrome (Rippon, 1982).

Traconazole

The synthetic dioxolane triazole itraconazole is well fascinated after oral administration. It contains most universal objection of gastrointestinal disease.

Polyhexamethylene biguanide

PHMB is universal environmental biocides that performs on the cytoplasmic membrane of bacteria, fungi, Acanthamoeba.

Silver sulfadiazine

It liberated and combines to microbial DNA avoiding unzipping of the helix and inhibit the replication of microorganisms without disturbing the epithelial cell regeneration (Ghahfarokhi et al., 2004).

Prevention of dermatophytes infections must be considered in the etiologic area's infection agents through the hairbrush technique. Customs examination of scalps of young children should be performed at the opening of the school term. Good sanitation should be impressed upon those infected and must be instructed not to share headgear, combs and brushes. Barbershop instruments ought to be clean after use (Hoog, 1996). The sources of infection are recognized and treated via shield cloths (gloves, gowns and head covering) for health care (Hussain et al., 2012). Prevention of tinea may be improved by using good foot hygiene, including habitual washing of the feet, systematic drying and use of foot powder for avoiding moisture and occlusion by wearing sandals or other well-ventilated shoes (Veronese et al., 2001). Tinea pedis is controlled by educating infected individuals not to expose others by walking barefoot near swimming pools, locker rooms and public showers and by not sharing foot-gear. The frequent hosing of floors of public baths, swimming pools, etc., and discouraging antifungal foot dips near swimming pools may be helpful as preventive measures.

NATURAL REMEDIES TO FIGHT WITH DERMATOGENS

Nature provides preliminary needs of beings for self-care. Plant remedies have a strong efficacy against several

assorted diseases such as skin disease caused by fungi and moulds. Their essential oils are best candidature in presence of their cytotoxic aptitude against fungus (Sharma et al., 2014) The *Otacanthus azureus* (Linden) *Ronse* essential oil alone or in combination with azoles is a promising antifungal agent in the treatment for human dermatomycoses caused by filamentous fungi (Houel et al., 2013). The combination of ketoconazole and *P. graveolens*'s essential oil for treatment of infections caused by *Trichophyton* species reduce the minimum effective dose of ketoconazole, and thus minimize the side-effects of ketoconazole. (Shin and Lim, 2003) Similarly, A number of plant's essential oils have been search out as plant remedies of plant families, that is, Asteraceae, Liliaceae, Apocynaceae, Solanaceae, Caesalpinaceae, Rutaceae, Piperaceae, Sapotaceae, Caricaceae, Euphorbiaceae, Moraceare, Solaneaceae, Papaveraceae (Natarajan and Natarajan, 2009). For example, antifungal effect of *Hypercom perforatum, Eucalyptus globules* (88%), *Catharanthus roseus* (88%) *Ocimum sanctum* (85.50%), *Azadirachta indica* (84.66%), *Ricinus communis* (75%), *Lawsonia inermis* (74.33%) *Jatropha curcas* (10%) *Eucalyptus intertexta* and *Eucalyptus largiflorens* are determined more active against Epidermophyton, Microsporum and Trichophyton Genera (Ghasemi et al., 2014; Venugopal and Venugopal, 1994; Suklampoo et al., 2012; Scott et al., 2006) In cattles, bergamot oil could be proper candidates as disinfectant agents and could be used as active ingredient for dermatological applications (EL-Ashmawy et al., 2015).

KERATINASE TO TACKLE DERMATOGENS

Keratinase are proteolytic affiliate of decaying of keratin (Dubey and Varma, 1999). Keratin is tremendously strong proteins which have exclusive properties as well as inflexible and hard (Anbu et al., 2004). Keratins contain a high proportion of glycine and alanine where a side group of glycine is a single hydrogen atom where the alanine contains small and no charged methyl group. There are two key shapes of keratin, alpha-keratin and beta-keratin. Alpha-keratin is observed in humans and other mammals while beta-keratin is established in birds and reptiles (Sharma et al., 2012; Sharma, 2015). Few organisms are competent to break it and arise from soil and wastewater habitats which participate as a keratinolytic factor in decaying α-keratins with an incidence of disulphide and hydrogen bonds which are inadequately biodegradable (Babayi et al., 2004). In keratin, the disulphide and hydrogen bonds are occurring through amino acids like cysteine and methionine (Anbu et al., 2004). Molyneux (1959) attempted to isolate firstly keratin degrading bacteria (Ulfig, 2006). The molecular masses of the keratinase enzymes range from 20 to 60 KDa. They are mostly active in alkaline environments, with optimal activity at temperatures up to 50°C (Mahboubi and Mohaddese, 2008). Rippon (1982) illustrates certain strain of *Microsporum* and *Trichophyton* which construct enzymes that dissolve the keratin and associated fibrous proteins established in hair, nails, skin, claws, feather, beak and hooves (Bronson et al., 1983). Ghahfarokhi et al. (2004) deliberated the result of essential oil, which characterizes the synergistic effect on dermatophytes as antifungal agents. Another statement regarding aqueous onion extracts confirm molecular changes such as configuration of resistant forms in *Trichophyton* from its inhibitory effects on the fungal species (Brasch and Graser, 2005).

The majority of keratinolytic and keratinophilic fungi are integrated in two bio-safety level categories: BSL-1 and BSL-2. The BSL-2 fungi belong to the dermatophytes and pose a higher risk to man than the BSL-1 (Fishcer and Kane, 1974). Effect of essential oils of plants against *Epidermophyton floccosum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* which were isolated from patients with dermatomycosis was studied. Among the tested oils, *Mentha viridis* (Mentha) and *Citrus aurantiifolia* (lemon) oils were found to possess complete anti-dermatomycotic activity (Mochizuki et al., 2003). Mint and mint oil yield is modified by biotic and abiotic factors (Bond et al., 1994). Essential oils of *Mentha piperata* and *Citrus maxima* display absolute inhibition of mycelial growth on deramatophtes which explain dominance in fungi toxic potency (Ishibashi, 1983). Keratinophilic fungi are present in the environment with variable patterns that depend on different factors such as human and animal presence (Sharma et al., 2015). Crude methanolic extract of *E. camaldulensis* inhibited the growth of *C. albicans*. It has also shown that an ethanolic leaf extract of *Eucalyptus camaldulensis* had marked fungicidal effect against clinical dermatophytes (Banerjee et al., 2011). Many environmental factors affect keratinolytic and keratinophilic fungi in sewage sludge. (Klemm, 2008). Finally it was investigated that the oil of *Mentha pulegium* L. has a potent antimicrobial activity and the Iranian *Mentha pulegium* L. oil belongs to piperitone/piperitenone type (Ajello and Getz, 1954). There is a possible prospect to search out new therapeutics that plants remedies generate which will be natural drug against mycological disease.

CONCLUSION

From the above collected review literature, it can be concluded that dermatophytes are fungal agents that use keratin to cause infections in birds, animals and human beings. The infections are mostly common in developing countries due to poor hygienic conditions, close proximity to animals and poor socio-economy, and the climatic support the growth of dermatophytes. A variety of diseases are caused by dermatophytes, namely athletes' foot,

ringworm, jock itch, etc. and these usually colonize through skin, hair and nails in human beings. These diseases are usually diagnosed using microscopy, culture techniques, PCR, etc. Treatment involves the use of various antifungal drugs, certain essential oils according to latest research, etc. However, the most important factor for control of tinea infections is maintenance of proper hygienic conditions as it is rightly said that “an ounce of prevention is always better than a cure”.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Alternative technique for culturing sputum for mycobacteria isolation: Feasibility, performance and effect on laboratory quality assessment - A technical note

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Mycobacteria culture remains the cornerstone of tuberculosis diagnosis. Naturally contaminated samples need pre-inoculation processing but some economically challenged medical facilities may benefit from a simpler and cheaper sputum decontamination procedure. The aim of this study was to test a simple decontamination method lacking a centrifugation step to be used in conjunction with the culture on Löwenstein-Jensen medium. A total of 7446 sputum samples collected from 3229 patients were microscopically examined and then cultured on Löwestein-Jensen medium using a simplified Petroff method. All positive cultures were confirmed by direct microscopic examination and biochemical identification. Culture and microscopic status and time to positivity were recorded. Mean and median times to culture and contamination rate were similar as compared to classical Löwenstein-Jensen culture method. Overall results suggest that the described modified of Petroff method may be used with adequate results in resource poor settings as the method does not require an aerosol safe centrifuge and relies on cheap, stable and readily available reagents.

Key words: Culture, mycobacteria, tuberculosis, Löwenstein-Jensen, centrifugation.

INTRODUCTION

Despite the advent of novel molecular techniques for mycobacterial identification, bacteriological examination is still the foundation of a successful tuberculosis control program (Kantor et al., 1998). Although microscopic examination is quick to perform and almost universally available, mycobacterial cultures play a central diagnostic role given their improved sensitivity. Cultures may be even more useful in high prevalence or high resistance

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areas; however in these settings, it is usually less likely to benefit from adequate laboratory facilities due to economic factors.

Culture of spontaneous expectorated sputum, as the most readily available specimen for pulmonary TB diagnosis, generally includes a decontamination step before media inoculation. Various decontamination methods are available, the most widely used being: sodium hydroxide (2-4%) alone, sodium hydroxide N acetyl cysteine, oxalic acid, cetylpyridinium chloride–NaCl and Ogawa Kudoh approaches (Rivas et al., 2010).

The Ogawa medium does not require sample decontamination and it is hypothetically cheaper than Löwenstein-Jensen medium (Soto et al., 2009). However, this is not necessarily true if media are not made on-site (as laboratory requirements are high for media manufacturing); additionally, the Ogawa technique may also be less useful for drug sensitivity testing (DST) (C.D.C. and N.I.H., 2006).

Except for the Ogawa Kudoh approach, which is considered to be particularly suitable for resource limited settings, the decontamination stage is promptly followed by an inoculum concentration step, usually achieved by centrifugation (Yeboah-Manu et al., 2004).

This step is time consuming and requires an aerosol safe centrifuge which is a relative expensive piece of equipment and therefore may represent a strain for low resources settings (Babady and Wengenack, 2012). The use of a standard centrifuge is possible, but since centrifugation carries a significant biohazard risk (mainly by generating aerosols and droplet nuclei, either by careless manipulation or by unfortunate events such as tube breaking or spillage) the use of a safety centrifuge is recommended. Furthermore, biosafety level 3 has been recommended for this type of operations by the Center of Disease Control (C.D.C. and N.I.H., 2006).

The aim of this study was to test a simple decontamination method lacking a centrifugation step to be used with the Löwenstein-Jensen culture media. The rationale behind this approach was to implement a more flexible and cheap technique– a method which allowed for a concentration step if available, or skip it in a low resources setting, and which relies on largely available culture media.

This idea was brought about by necessity, as our laboratory was confronted by a similar problem– an aerosol safe centrifuge was not available for a short period of time.

MATERIALS AND METHODS

The study group included 7446 sputum samples from 3229 patients (suspected for pulmonary tuberculosis or undergoing monitoring during treatment) admitted to our center between March 12th 2009 and June 12th 2009, which were evaluated to be of satisfactory quality as per the WHO criteria (Kantor et al., 1998) (mucoid/mucopurulent, volume more than 3 ml) and processed within 24 h of reception.

The study design and general purpose consent procedure was approved by the "Grigore T. Popa" University of Medicine and Pharmacy Iasi Ethics Committee (the 16th of November 2008 meeting).

Patient records/information was kept anonymous and de-identified prior to analysis by laboratory personnel thus following the relevant local personal data regulations.

Each sample underwent microscopical examination - first using an auramine – rhodamine stain; positive smears were Ziehl Nielsen restained and reexamined. Each sample was subsequently cultured on Löwenstein-Jensen media using a modified Petroff procedure as described below.

From each sputum sample, 3 ml were transferred to a flat bottom flask containing glass beads, using a sterile disposable Pasteur pipette and an equal volume of 4% NaOH solution was added; the sample was gently mixed and set to rest for 30 min at room temperature; the mix was brought to neutral pH using a 8% HCl solution and Brom Thymol blue as indicator. Aliquots of 0.2 ml were then inoculated on Löwenstein-Jensen for a total of three slants per sample. The slants were then incubated at 37°C, horizontally for the first 48 h and then standing-up for up to 60 days. Positive cultures were all confirmed by direct microscopic examination for AFB, and biochemical identification (reductase, catalase and niacin tests).

RESULTS

From a total of 7446 samples, 819 (from 378 individuals patients) were either positive on direct microscopic examination alone (n=84, 10.3%, 42 patients) or associated with at least one non contaminated positive culture followed by positive identification of mycobacteria (n=735, 89.7%, 336 patients) (Figure 1).

There were 84 microscopically positive samples that had associated negative cultures – which all originated from 42 patients undergoing TB treatment at the two months milestone. There were 141 contaminated slants but in only two cases (which both were microscopically positive) no other culture was available as all corresponding slants were contaminated.

As far as culture positive results are concerned, from the total of 735 culture positive samples (from 336 patients), a total of 492 were microscopically positive (66.9% positive) and 243 microscopically negative (33.1%). These figures are similar to those from the regional tuberculosis register which reports a rate of 78.7% bacteriologically confirmed cases (76.6% were both culture and microscopically positive and 23.4% were microscopically negative, but culture positive) for the same time frame.

Mean time to culture positivity was 21 days (interquartile range 21-30 days). Median time to culture positivity (and interquartile ranges) according to the mycobacterial load in the sputum was 30 days (21-30 days) for microscopically negative, 21 days (21-23.25 days) for scarcely positive (less than 1+), and 21 days (21-27.75 days) for samples with 1+ positive sputum smears. All samples with 2+ and 3+ smears had positive culture results by 21 days.

There were 141 contaminated slants with a 1.9% contamination rate, a figure implying adequate decontamination intensity for a Lowenstein-Jensen culture technique.

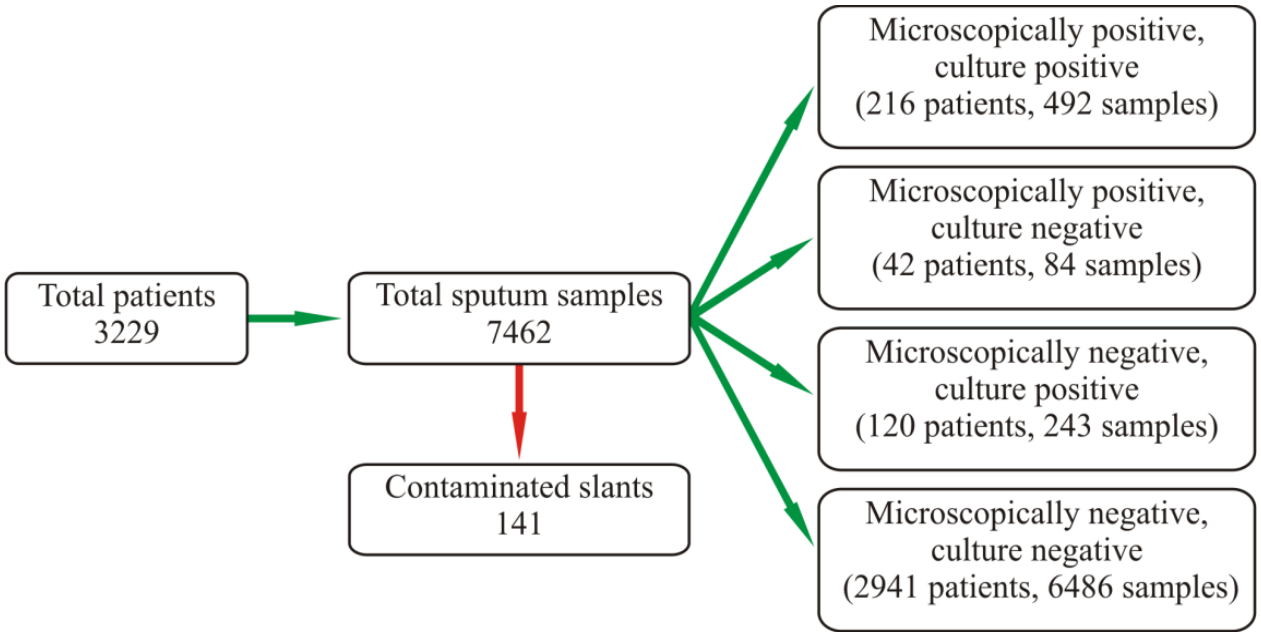

Figure 1. Study group structure – bacteriology status.

There were 6486 microscopically and culture negative samples (from 2941 patients). No definite data concerning the health status of these patients was available. However, we were not able to find any positive result in the local register for any patient with negative culture results in a three months' time window following the initial culture. Although this does not exclude false negative results, it may suggest a low value.

DISCUSSION

Improving the diagnostic output of basic microbiology facilities has been previously explored (Ramos et al., 2010) from an economic point of view, at least as far as liquid media culturing was concerned. Different concentration methodologies have been tested- centrifugation vs. filtration with similar results; however, while filtration may be a cheaper alternative, it still bears an additional cost.

A similar rationale lead to a comparison between standard NaOH decontamination/centrifugation methods and direct inoculation using a MODS approach- significant differences were found in terms of sensitivity, 97 vs. 81% and contamination rate of 3 vs. 18% (Grandjean et al., 2008). This direct inoculation sensitivity loss cannot be ignored and should be evaluated considering the signify-cantly increased contamination rate.

All these reports focused on broth cultures and to our best knowledge, there is little data available on alternative culturing methods involving Lowenstein Jensen medium, which is the main culture media available to resource limited settings (Satti et al., 2010).

Our data show that time to culture positivity is not significantly increased in low bacterial load samples (scant, +1 vs. +2, +3 for microscopy examination) suggesting a minimum sensitivity loss while using the modified culture protocol (Figure 2).

Our results also suggest that the described modified Petroff method may be used in conjunction with Lowenstein Jensen media with adequate results, in order to circumvent the need for an aerosol safe centrifuge, which has been previously considered a potential burden for financially challenged settings (Grandjean et al., 2008).

Sensitivity of this modified technique was not directly assessed in our study as comparism was not available. However, we may indirectly and roughly make an estimate using the proportion of sputum positive smears (confidence on this data is high as microscopy diagnosis was externally audited and considered to be 100% accurate for three years in a row) and considering the time to culture positivity data. Literature data evaluating the quality of direct sputum smear examination estimate that an excellent quality of direct examination generates around 65% microscopically confirmed cases out of all bacteriologically confirmed cases (Tanoue et al., 2002). This figure is close to the 70.3% value for our study group; a major sensitivity loss would have significantly increased the proportion of microscopically confirmed cases.

Forty six (12%) microscopically positive/culture negative cases may also suggest a lower sensitivity (possibly due to lack of concentration of the inoculum) but this data should be interpreted in the context of sample provenance (two months treatment milestone); the

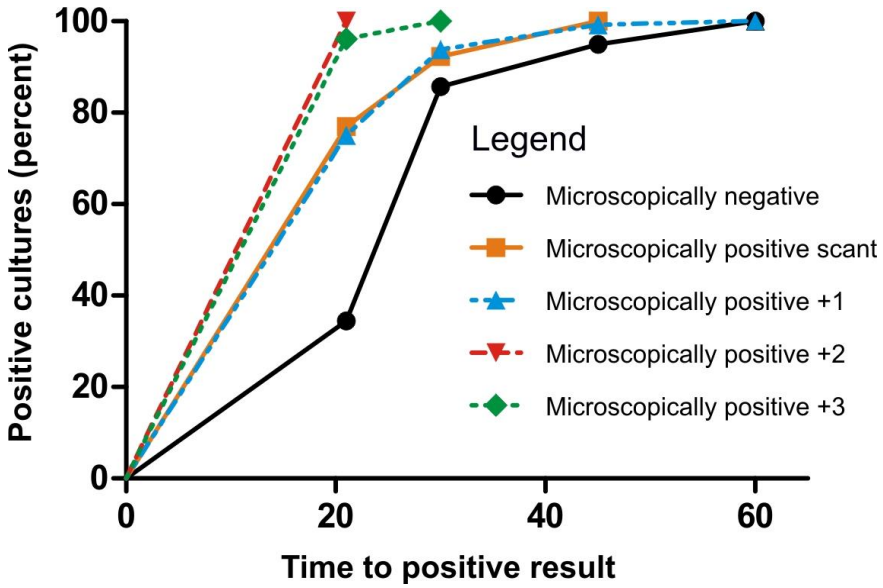

Figure 2. Time to culture positivity stratified by microscopically assessed bacterial load.

possibility of non-viable MT should not be excluded. Along this line, there is somewhat controversial published data on sputum concentration (using the 2% NALC – 2% NaOH method) as having no significant impact on light microscopy results (Cattamanchi et al., 2009).

An extensive literature review article (Steingart et al., 2006) considered NaOH treated and centrifugated sputum to generate an increased microscopic sensitivity, estimating the role of centrifugation at around 7% based on somewhat old data (Cameron and Castles, 1945).

Considering these aspects, we cannot rule out a sensitivity decrease in the order of 10% or less. Therefore, this method may not be useful for low bacterial count samples such as gastric lavage, pleural fluids or samples from HIV infected individuals; these results should not be extrapolated to low quality sputum samples.

Contamination rate for our group was low, less than 2%, thus within the WHO recommended limits (Kantor et al., 1998). While contamination was not an issue for our setting, there are reports on significantly higher rates for alternative culture methods and this is probably to be expected if resources are scarce (Muyoyeta et al., 2009). Using decontaminant laced Lowenstein Jensen media might prove useful for these settings; PANTA, Heptatab and penicillin G proved to be effective as decontaminant additives to an added cost of less than 1 $ per test (Kassaza et al., 2014).

Overall, the described concentration free method has some advantages as it does not require expensive equipment such as an aerosol safe centrifuge, it is less time intensive as compared to classical methods and only relies on cheap, stable and readily available reagents.

This peculiarity may allow for field culturing, thus significantly decreasing time to culture and also contamination rate, even if the slants are later sent to a central facility, thus increasing the access to a referral center (Perkins et al., 2006).

This method makes use of the Lowenstein-Jensen media which is cheap, almost universally available, easily transported and can be used in conjunction with other methods.

Conclusions

Our data suggest that this approach may be viable as a temporary measure in low resource laboratory settings at the putative price of a tolerable sensitivity loss.

Conflict of Interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Effect of different temperature levels and time intervals on germination of uredospores of *Puccinia sorghi*

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In plant disease development, temperature is a critical factor. To determine how specific environmental variables affect corn rust, we determined temperature effects on urediniospore germination. Spore germination was maximum within 24 h of incubation, in the temperature range of 20-30°C. The mean maximum spore germination was recorded at 25°C (66.16% germination) which is statistically on par at 30°C (optimum range). The germination percentage declined gradually at temperatures above and below 25°C. The maximum reduction in germination was recorded at 30°C and 2 h time interval. Thus, the cardinal temperatures for uredospore germination on water agar are 5, 25 and 35°C for 24 h of incubation, respectively. At the maximum and minimum temperature levels, the minimum incubation period extended beyond two hours.

Key words: Effect, temperature, time intervals, *Puccinia sorghi*, maize, common rust.

INTRODUCTION

Maize (*Zea mays* L.) is a graminecious plant domesticated by indigenous people in Mesoamerica in pre-historic times. In Indian agriculture, maize occupies an important place. It is not only utilized as a staple food by the lower income groups, but it is also a crop par excellence for industrial use. Human food accounts for about 35% of the produce, while the balance finds use in industry and poultry feed. Maize is cultivated under diverse environmental conditions. Among the cereals, the maize is the fifth largest in area, third largest in output and yield. Maize is attacked by many diseases in *kharif*, *rabi* and summer seasons which are responsible for severe reduction in yield (Ali and Yan, 2012; Dey et al., 2012). The common rust of maize caused by *Puccinia sorghi* Schw is a severe disease on maize among all foliar diseases. An outbreak of rust was observed in 1966 in breeding nurseries and in fields of corn in the province of Quebec (Brawn, 1966). As in the corn belt in 1950 and 1951, the outbreak in Quebec Ln1966 appeared to be attributable to weather conditions being more favorable than usual. In 1950, rust became epiphytotic, and because of its potential threat to the leading crop in the

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USA, caused considerable alarm there (Semeniuk and Vestal, 1952; Wallin, 1951) and in Canada (McKeen, 1951) that year, again in 1951, and to a lesser extent in 1958 (Ullstrup and Laviolette, 1959). In recent years, common rust caused by *Puccinia sorghi* Schw. was observed in several districts in Northern Karnataka and few other places. According to LeRoux and Dickson (1957) the optimum temperature requirement for spore germination in *P. sorghi* varies with the medium used and the container in which the tests are made. Mederick and Sackston in 1972 noticed that the cardinal temperatures for germination of uredospores of *P. sorghi* on water agar were 2-5, 10-25 and 30-35°C. Losses in grain yields due to common rust disease ranging from 11.2 to 33.6% have been reported from different parts of the country (Gupta, 1981). Common rust of maize caused by *P. sorghi* whch is far more important than *Puccinia polyispora* Underw. Considering the importance of the disease which causes more economic losses to the crop, the present investigation was undertaken.

MATERIALS AND METHODS

An experiment was conducted at College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka, India during *Kharif*, 2010-11 to know the effect of different temperature levels and time intervals on germination of uredospores of *P. sorghi.*

Uredospore germination on water agar

A thin layer of 1.5% agar was applied on a clean microscopic slide. The mature uredospores of *P. sorghi* were collected by placing a drop of water on a pustule and then lifting the floating ones with a needle. A suspension of these uredospores was atomized on the agar film on the glass slides, which were kept in an inverted position in closed Petri dishes lined with moist cotton. A piece of moist cotton was placed on the upper surface of the slide to promote moisture condensation on the lower side. The slides were then incubated at various temperatures for different intervals of time. Care was taken to see that not more than 10-15 min elapsed between the time the spore suspension was made and the slides were brought into the incubator, to minimize the error that may be induced by increased germination. There were three replications for each treatment. Spores with the germ tubes longer than their diameter were considered as germinated, and the percent germination was based on a count of 800 spores for each treatment. The experiment was repeated twice. Percent uredospore germination was calculated by following the formula.

$$\text{Per cent spore germination (PG)} = \frac{A \times 100}{B}$$

Where, A = No. of uredospores germinated; B = No. of uredospores observed.

RESULTS AND DISCUSSION

The results (Table 1) of studies on the effect of temperature on germination of uredospores of *P. sorghi* on water agar indicated that germination varies both in percentage and rate depending on temperature and time interval. Spore germination was maximum within 24 h of incubation, in the temperature range of 20-30°C. The mean maximum spore germination was recorded at 25°C (66.16% germination) which is statistically on par at 30°C (optimum range). No germination occurred below 5°C, at which temperatures there were relatively low percentages of germination only after 8 and 4 h, respectively (Figure 1). Thus, the cardinal temperatures for uredospore germination on water agar are 5, 25 and 35°C, and the minimum incubation period extended beyond two hours only at the maximum and the minimum temperature levels. However, slight increase in germination percentage recorded at all temperature levels as the incubation period extended. Studies indicated that the optimum temperature for uredospore germination is 20-25°C (Plate 1).

The minimum temperature for germination under the conditions used was between 0 and 5°C, the optimum between 10 and 25°C, and the maximum between 30 and 35°C. Essentially, similar values were reported by Weber (1922), who found the minimum for germination to be 4°C, optimum 17°C, and maximum 32°C. Weber (1922) found the minimum temperature for infection of corn by rust to be below 8°C, the optimum about 18°C, and the maximum below 32°C. In *P. graminis*, germination counts made at 2 h gave the best correlation with infection (Sharp et al., 1958). If a similar correlation holds for *P. sorghi*, our results at 2 h may be at least indicative of the effect of temperature on infection.

Our results indicate that, although there is a wide temperature optimum for germination, the speed of germination shows a marked response to relatively small temperature differences. Similar results have been reported for other rusts (Chester, 1964; Shands and Schein, 1962; Sharp et al., 1958). We made our first counts of germination on spores exposed for 2 h. We might have obtained slightly different values if our counts had been made after 3 h, an interval used in various reports on *P. graminis* fsp*. tritici*, but which we used in only one experiment. Germ tubes had emerged from rehydrated urediospores of *P. graminis* fsp*. tritici* after 40-60 min under optimum conditions (Maheshwari and Sussman, 1970). As the usual criterion for germination is the production of a germ tube at least equal in length to the width of the urediospore, we did not think it useful to determine percentage germination after exposures shorter than 2 h. The very low germination rate of urediospores at low temperatures explains the slow rust development observed on plants in inoculated field plots at College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka in the season of 2010, when dew periods only 2 h long coincided with temperatures of 10-13°C (Mederick and Sackston, 1972).

Table 1. Effect of different temperature levels and time intervals on germination of uredospores of *P. sorghi*.

Temperature (°C)	Uredospore germination (%)						
	2 h	4 h	8 h	16 h	20 h	24 h	Mean
0	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00* (00.00)**
5	17.33 (8.88)	20.44 (12.22)	23.94 (16.48)	28.31 (22.51)	32.19 (28.40)	35.93 (34.47)	26.36 (20.49)
10	17.56 (9.12)	30.38 (25.60)	35.85 (34.33)	37.93 (37.82)	41.77 (44.40)	47.15 (53.77)	35.11 (34.17)
15	21.35 (13.27)	32.19 (28.40)	37.18 (36.54)	38.48 (38.74)	47.65 (54.64)	56.09 (68.91)	38.82 (40.08)
20	35.67 (34.02)	47.04 (53.59)	58.84 (73.26)	62.39 (78.55)	66.47 (84.09)	74.38 (92.76)	57.47 (69.38)
25	52.79 (63.47)	60.73 (76.13)	67.47 (85.34)	70.08 (88.42)	73.25 (91.53)	80.39 (97.22)	67.45 (83.69)
30	35.10 (33.09)	46.45 (52.57)	57.94 (71.85)	61.82 (77.73)	64.29 (81.21)	71.95 (90.42)	56.26 (67.81)
35	32.19 (28.40)	42.09 (44.96)	46.73 (53.05)	52.79 (63.47)	56.12 (68.96)	60.93 (76.41)	48.48 (55.88)
40	21.93 (13.96)	27.69 (21.61)	35.46 (33.68)	40.64 (42.45)	46.90 (53.35)	52.44 (62.88)	37.51 (37.99)
45	00.00 (00.00)	14.97 (6.68)	20.44 (12.22)	23.73 (16.21)	26.40 (19.79)	27.04 (20.68)	18.76 (12.60)
50	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)
Mean	21.27 (18.57)	33.25 (29.27)	34.89 (37.89)	37.83 (42.35)	41.37 (47.85)	46.03 (54.32)	35.11 (38.37)
Comparing the means of		SEm±		CD at 1%			
Treatment (A)		0.15		0.54			
Concentration (B)		0.11		0.42			
A X B		0.36		1.32			
CV%				1.53			

* Arcsine transformed values; **Data in parenthesis are original values.

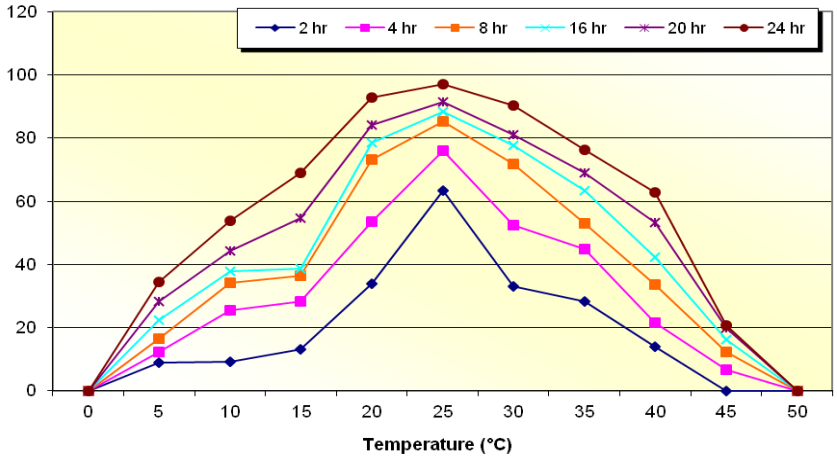

Figure 1. *In vitro* evaluation of different temperature and time intervals on germination of uredospores of *P. sorghi.*

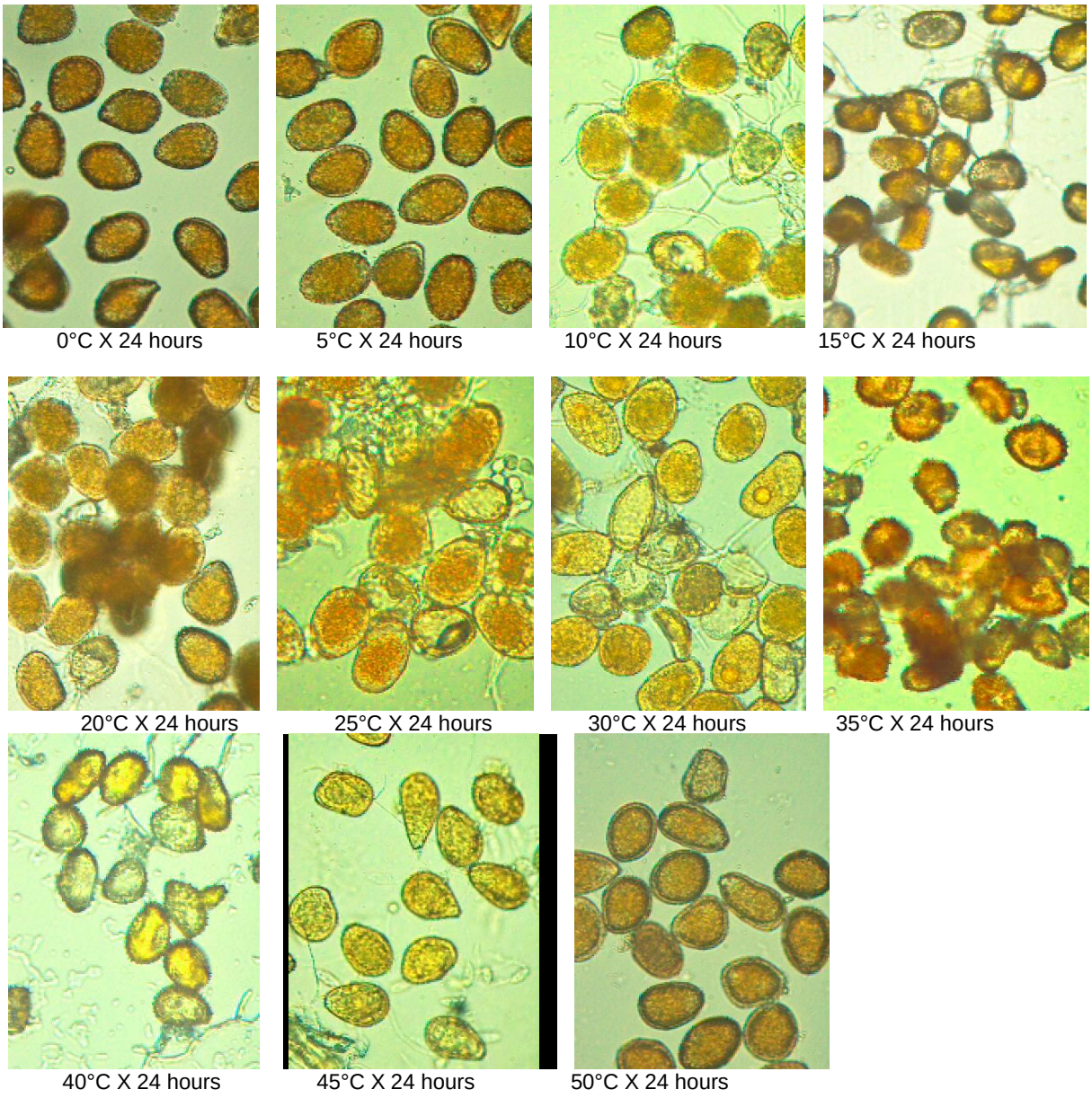

Plate 1. Effect of different temperature and time intervals on germination of uredospores of *P. sorghi.*

The effect of temperature on uredospore germination revealed that, 25°C is the optimum temperature for maximum spore germination (83.70%). The uredospore germination was also influenced by the time interval. At 2-4 h, lower uredospore germination was recorded, intervals between 8-16 h found moderate and 20-24 h recorded maximum germination irrespective of temperature levels. Thus indicates that, a minimum time interval of 20-24 h is required for better uredospore germination and highest being at 25°C. Uredospore germination drastically reduced at 40°C and at above temperature level, indicates unfavorable.

The cardinal temperatures for uredospore germination in the rust fungus vary widely among different species. According to Johnson (1912) the optimum temperature requirement for uredospore germination in the cereal rusts ranges from 7 to 25°C. The cardinal temperature range of 5 to 45°C established for *Puccinia sorghi* is wider than that reported by Weber (1922). On the other hand, it is narrower than that reported by LeRoux and Dickson (1957). The lack of agreement in these reports on cardinal temperatures for uredospore germination in *P. sorghi* may be due to difference in the methods used, conditions under which the spores are produced and

stored (Smith, 1926), difference in the medium and it's container and the time lapse between the preparation of spore suspension and the start of the treatment (LeRoux and Dickson, 1957).

Temperature, humidity, solar irradiation, and other factors can affect spore survival in the atmosphere (Bernd et al., 1987). The first step in infection is urediniospore germination, which is influenced by environmental factors that include temperature (Bonde et al., 2007; Joseph and Hering, 1997; Tapsoba and Wilson, 1997) and duration of leaf surface wetness (Joseph and Hering, 1997). Most spores are extremely sensitive to even a few hours of intense sunlight (Maddison and Manners, 1972). Germination of spores of the common rust fungus may occur over a wide range of relatively cool temperatures (approximately 54 to 82°F) and requires nearly 100% humidity for several hours (Headrick and Pataky, 1987). Although, uredospores in groups were observed to germinate readily, the numbers of spores within groups were not determined. Germ tube elongation of all fungi was negatively affected by increased length of exposure to fluorescent light (Buck et al., 2010). However, the germ tubes from these groups were numerous. Germ tube lengths varied considerably at each temperature and time intervals.

This study will help us to describe and better understand the kinetics of spore germination and how this is influenced by different environmental factors such as temperature and other factors that influence spore germination. Even though spore germination and sporeling growth can occur under a broad set of conditions, optimal responses occur within narrow ranges. Knowledge of the abiotic influence on spore development should improve survival of the early microscopic stages and may help in cultivation trials based on spore shedding methods.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Occurrence and antibiogram profile of *Staphylococcus aureus* isolated from some hospital environment in Zaria, Nigeria

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Staphylococcus aureus **is an opportunistic pathogen and is the most frequent cause of hospital acquired infection. A total sample of 310 was collected for this work; the pathogen was isolated from hands of some healthcare workers/nurses, operation tables, door knobs/door handles, nurses' table tops, bedrails, stretchers, floors, toilets seats, cupboards, and sinks. The total percentage prevalence of the pathogen in Major Ibrahim B. Abdullahi memorial hospital was 16.8% and 20.7% from St. Luke's Anglican hospital. Kirby-Bauer-NCCLS modified single disc diffusion technique was used to determine the antibiogram profile of the pathogen at 0.5 scale MacFarland's standard (1.5 × 10^8 cells/ml). The isolates from the two hospitals were 100% susceptible to vancomycin and 95.7% and 92.6% from Major Ibrahim B. Abdullahi memorial hospital and St Luke's Anlgican hospital respectively were resistant to Ampicillin.**

Key words: *Staphylococcus aureus*, antibiogram profile, pathogen, sensitive, resistant.

INTRODUCTION

Staphylococcus aureus is a Gram positive coccus that occurs in grape-like clusters. It is a eubacterium that is found on the surface of the human skin and mucous membranes (Prescott et al., 2005). They form part of the normal microbial flora of the skin, upper respiratory tract and intestinal tract (Cheesborough, 2005). The pathogen is an opportunistic organism in man and animals and is the most frequent cause of hospital and community infections (Prescott *et al*., 2005). *S. aureus* can cause a range of illness from minor skin infections such as boils, abscesses to life threatening diseases such as pneumonia, meningitis, toxic shock syndrome and sepsis (Lakshmi and Harasreeramulu, 2011).

Drug resistance by the organism is also a major concern (Weinstein, 1998). Both methicillin (oxacillin or cefoxitin) and glycopeptide (vancomycin and teicoplanin) resistance may occur in *S. aureus*. It is found throughout the hospital environment, particularly around patients known to be colonised or infected with the bacterium (Dancer, 2009).

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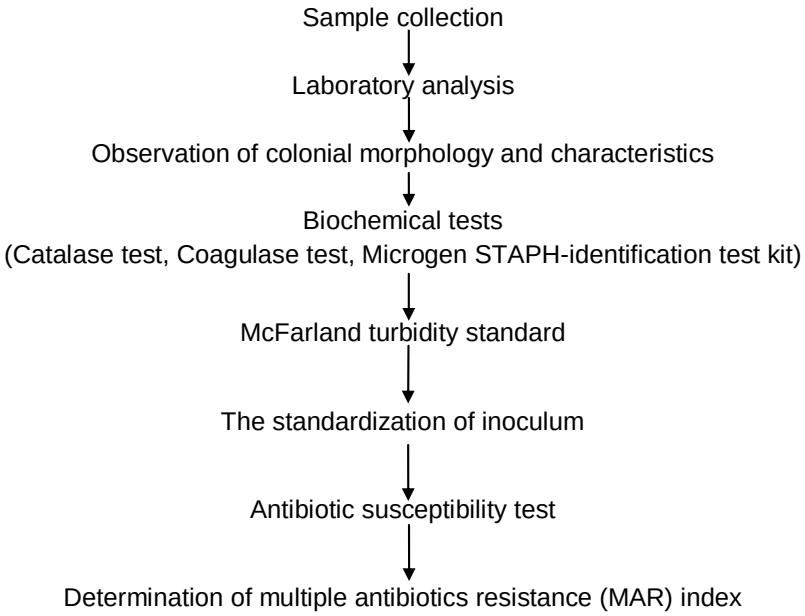

Figure 1. The work flow chart.

Increasingly, nosocomial isolates are resistant to multiple drugs. In the community, *S. aureus* remains an important cause of skin and soft tissue infections, respiratory infections, and (among injection drug users) infective endocarditis (Horst et al., 2011). Scientific evidence suggests that environmental contamination plays an important role in the spread of methicillin-resistant *S. aureus* (MRSA).

The transfer of microorganisms from environmental surfaces to patients is largely through hand contact with the contaminated surfaces (Samuel et al., 2010). It has been estimated that 20 to 40% of nosocomial infections have been attributed to cross infection via the hands of health care personnel (Weinstein, 1991). Contamination of the hands of health care workers (HCWs) could in turn result from either direct patient contact or indirectly from touching contaminated environmental surfaces or patients' skin during routine care activities, sometimes even despite glove use (Kramer et al., 2006, Allegranzi and Pittet, 2009). Many nosocomial infections are caused by pathogens transmitted from one patient to another, by way of healthcare workers who have not washed their hands, or who do not observe simple hospital hygiene measures, and also between patients (Olalekan et al., 2011).

MATERIALS AND METHODS

Study area

The study area of this work encompass some hospitals in Zaria including St Luke Anglican hospital, Wusasa located at 11° 04' N and 007°40' E, then Major Ibrahim B. Abdullahi memorial hospital which is situated at 11° 06' N and 007° 41' E all at Greenwich meridian. These areas were located using Taiwan made Etrex® high-sensitive geographic positioning system (GPS) receiver. A total sample of 310 was collected for this work.

Ethical approval

The ethical approval was obtained from ethical committee of Kaduna state Ministry of Health and was used for sampling. Approval was also obtained from Medical Director, St. Luke's Hospital, Wusasa.

Sampling designing and techniques

Sample collection

The total number of samples collected for this study was 310 and all samples were collected in the morning before commencement of work in each hospital and hand swab of the staff were collected during working hours. Samples for the studies were collected from hands of some of the hospital staff and nurses, floors, toilets seats, operation tables, door knobs/door handles, nurses' table tops, bedrails, stretchers, cupboards, sinks, using sterile swab sticks using sterile cotton swabs wetted with sterile peptone water (Figure 1).

Laboratory analysis

Each sample swab was inoculated into prepared sterile bacteriological peptone water and incubated at 37°C for 24 h for enrichment after which the turbid broth was subcultured unto solid differential media such as Manitol salt agar, Eosin methylene blue agar (EMB), *Pseudomonas centrimide* selective agar and MacConkey agar plates and incubated again at 37°C for 24 h.

Table 1. The prevalence of the pathogens in relation to the two hospital environment.

Hospital	No. of sample screened	*S. aureus*	% prevalence of *S. aureus*
MIBAMH	155	25	16.1
SLAH	155	32	20.6
Total	310	57	18.4

MIBAMH, Major Ibrahim B. Abdullahi Memorial Hospital; SLAH, St. Luke Anglican Hospital.

Discrete colonies were further subcultured onto fresh prepared plates of the selective media and nutrient agar plates to obtain pure cultures. The purified cultures were gram stained and stored on nutrient agar slants for biochemical tests and identification.

Observation of colonial morphology and characteristics

Presumptive morphological identification of the colonies was done by observing their individual appearance on the selective media that were used for the isolation.

Biochemical tests

Catalase test

Three (3) ml of hydrogen peroxide solution was poured into a test tube. With the aid of sterile glass rod, several colonies of the test organism were carefully removed and immersed into 3 ml solution of hydrogen peroxide. Immediate bubbling within few seconds was recorded to be positive test of *Staphylococcus* species.

Coagulase test

A drop of distilled water was added on each end of a slide. A colony of a suspected organism of 24 h culture from blood agar (previously checked by gram staining) was emulsified in each of the drops of the distilled water and made two different suspensions. A loop of the plasma was then added to one of the suspensions and mixed gently. Clumping or agglutinations of the organisms with the plasma within ten (10) seconds indicated a positive result of *S. aureus*; negative result indicates other *Staphylococcus* species.

Microgen STAPH-identification test kit

Other biochemical tests carried out using microgen STAPH-identification kit include sucrose, nitrate, sucrose, trehalose, mannitol, n-acetyl glucoseamine, manose, turanose, alkaline phosphatase, glucosidase, glucuronidase, urease, argenine and l-pyrrolidonyl-α-naphthylamide (www.microgenbioproducts.com, uk).

McFarland turbidity standard

The turbidity standard of the organisms used was 0.5. One percent (1%) v/v solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of distilled water and mixed well. 1% w/v solution of barium chloride was also prepared by dissolving 1 g of the dehydrated salt ($BaCl_2.2H_2O$) in 100 ml of distilled water. Then 0.6 ml of the barium chloride was added to 99.4 ml of the sulphuric acid solution and was mixed well. The small portion of the turbid solution was transferred into a test tube which was used to compare with the inoculated organisms in Mueller-Hinton broth (Cheesbrough, 2004).

Antibiotic susceptibility test

The antimicrobial susceptibility pattern was determined using Kirby-Bauer-NCCLS modified single disc diffusion technique (Cheesbrough, 2004). Disc diffusion technique was performed according to Kirby-Bauer method, as described in the guidelines of Clinical and Laboratory Standards Institute, CLSI, 2012. Single antibiotic disc such as ampicillin (10 µg), vancomycin (30 µg), tetracycline (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), linezolid (10 µg) and gentamicin (10 µg), all the discs were obtained from Oxoid England.

The standardization of inoculums

The concentration of each of the suspension of the test organisms and the standard isolates were prepared by picking a 24 h colony of the organism using sterile wire loop into sterile test tube containing sterile normal saline to form turbidity that match with 0.5 scale of MacFarland's standard (1.5×10^8 cells/ml) (Coyle, 2005). *S. aureus*, ATCC 25923 was obtained from National Institute of Pharmaceutical Research and Development (NIPRD), Abuja. The standard strain was used as the antibiotics susceptible control. The cell suspensions was inoculated by streaking on prepared Mueller-Hinton agar using sterile swab stick, then the antibiotic disc was placed on the inoculated medium aseptically with the help of sterile forceps and incubated at 37°C for 24 h. The zones of inhibition created by each of the antibiotics against the test organisms and the standard strains as positive control were measured and the result was interpreted using guideline from CLSI, 2012. The results were recorded as sensitive, intermediate and resistance.

Determination of multiple antibiotics resistance (MAR) index

The multiple antibiotics resistance index was determined for each of the selected bacterial isolate using a formula MAR = *x/y,* where *x* is the number of antibiotics to which test isolate displayed resistance and *y* is the total number of antibiotics to which the test organism has been evaluated for sensitivity (Olayinka et al., 2004;Tula et al., 2013).

RESULTS AND DISCUSSION

Table 1 shows the prevalence of the pathogen in the two hospitals. The total sample of 155 was collected from each hospital. Table 1 shows that 16.1 and 20.6% of the

Table 2. The occurrence of the isolate in Major Ibrahim B. Abdullahi Memorial Hospital.

Sample source	Sample size	Total positive isolates	Prevalence of *S. aureus* (%)
NHS	11	4	36.4
NTT/ST	9	3	33.3
DK/DH	25	6	24.0
TS	8	-	-
OT	4	-	-
Sink	14	-	-
Stretcher	14	2	14.3
Floor	35	3	8.6
BR	17	5	29.4
CB	15	2	13.3
Total	155	25	16.1

SHS = Nurses' hand swab; NTT/ST = Nurses table top/staff table; DK/DH = Door knob/Door handle; TS= Toilet seat; OT= Operation table; BR = Bedrail; CB = Cup board.

Table 3. The occurrence of the isolate in St. Luke Anglican Hospital.

Sample source	Sample size	Total positive isolates	Prevalence of *S. aureus* (%)
NHS	15	6	40.0
NTT/ST	16	5	31.3
DK/DH	20	4	20.0
TS	7	-	-
OT	6	-	-
Sink	10	-	-
Stretcher	10	2	20.0
Floor	30	5	16.7
BR	22	6	27.3
CB	19	4	21.1
Total	155	32	20.6

SHS = Nurses' hand swab; NTT/ST = Nurses table top/staff table; DK/DH = Door knob/Door handle; TS= Toilet seat; OT= Operation table; BR = Bedrail; CB = Cup board.

pathogens were isolated from Major Ibrahim B. Abdullahi memorial hospital and St. Luke Anglican Hospital respectively. As it is presented in Table 2 the prevalence of the pathogens isolated from surfaces at Major Ibrahim B. Abdullahi memorial hospital showed that 36.4, 33.3, 24.0, 14.3, 8.6, 29.4 and 13.3% of *S. aureus* was isolated from nurses' hand swab, nurses' table top, door knob/handle, stretcher, floor, bedrail and cupboard respectively. Table 3 shows the distribution of the pathogens on surfaces at St. Luke's Anglican hospital. *S. aureus* was isolated from Nurses' hand swab (40.0%), nurses' table tops (31.3%), door knob/handle (20.0%), stretcher (20.0%), bedrail (27.3%), floor (16.7%) and cupboard (21.1%).

The prevalence of *S. aureus* from hands of the nurses from the two hospitals was slightly lower compared to the earlier report of 42.0% of the pathogen from hand swab as reported by Boyce (2007) and 20% as reported by Ekrami et al. (2011). The level of contamination by this pathogen could also be as a result of inadequate decontamination of the microbial load from the surfaces (Addy et al., 2004).

Page et al. (2009) reported that surfaces can act as reservoirs of microbes which could in turn lead to the spread of infection upon being touched, by either healthcare workers, patients or visitors. The presence of the pathogen in the hand swab might be as a result of inadequate hand hygiene and this could be one of the attributing factors of the distribution of the pathogen in the hospital environmental surfaces as reported by Olalekan et al. (2011). A study by Ferreira et al. (2011) revealed that contaminated hands of healthcare workers played important role in transmission of pathogens within the hospital environment and reported that 29% of nurses working in a general hospital had *S. aureus* on their hands and 78% of those working in a hospital for dermatology

Table 4. The antibiotic profile of the isolate from Major Ibrahim B. Abdullahi Memorial Hospital.

Antibiotic	*Staphylococcus aureus* (N = 25)		
	R	I	S
VA (30 µg)	0 (0.0%)	-	25(100%)
AMP (10 µg)	23 (92.0%)	-	2(8.0%)
TE (30 µg)	4(16.0%)	8(32.0%)	13(52.0%)
LZD (10 µg)	0(0.0%)	-	25(100%)
CAZ (30 µg)	2(8.0%)	11(44.0%)	12(48.0%)
FOX (30 µg)	4(16.0%)	-	21(84.0%)
CN (30 µg)	0(0.0%)	11(44.0%)	14(56.0%)

VA = Vancomycin; AMP = Ampicillin; TE = Tetracycline; LZD = Linezolid; CAZ = Ceftazidime; FOX = Cefoxitin; CN = Gentamicin; NT = Not Tested; R = Resistant; I = Intermediate; S = Sensitive.

Table 5. The antibiotic profile of the isolate from St. Luke's Anglican Hospital.

Antibiotic	*Staphylococcus aureus* (N = 32)		
	R	I	S
VA (30 µg)	0(0.0%)	-	32(100%)
AMP (10 µg)	28(87.5%)	-	4(12.5%)
TE (30 µg)	1(3.1%)	15(46.9%)	16(50.0%)
LZD (10 µg)	0(0.0%)	-	32(100%)
CAZ (30 µg)	0(0.0%)	18(56.3%)	14(43.8%)
FOX (30 µg)	5(15.6%)	-	27(84.4%)
CN (30 µg)	0(0.0%)	16(50.0%)	16(50.0%)

VA = Vancomycin; AMP = Ampicillin; TE = Tetracycline; LZD = Linezolid; CAZ = Ceftazidime; FOX = Cefoxitin; CN = Gentamicin; NT = Not Tested; R = Resistant; I = Intermediate; S = Sensitive.

patients had the organism on their hands.

The antibiotic susceptibility profile of isolates from Major Ibrahim B. Abdullahi memorial hospital as in Table 4 shows that *S. aureus* isolates were highly resistant to ampicillin (92.0%), and 16.0 % were resistant to both tetracycline and cefoxitin, very few were resistant to ceftazidime (8.0%). All the isolates of *S. aureus* were susceptible to vancomycin and linezolid; some were susceptible to tetracycline (52.0%), ceftazidime (48.0%), cefoxitin (84.0%) and gentamicin (56.0%). The antibiotic susceptibility profile of pathogens from St. Luke's Anglican hospital as presented in Table 5 shows that all isolates of *S. aureus* were susceptible to vancomycin and linezolid.

This pathogen was also highly resistant to ampicillin (87.5%). Some were resistant to tetracycline (3.1%) and cefoxitin (15.6%). Resistance to cefoxitin by disc diffusion has been used for the detection of MRSA strains in routine testing because cefoxitin is a potential inducer of the system that regulates *mecA* gene (Philip and Shannon, 1993; Madhusudhan et al., 2011). Methicillin-resistant *S. aureus* (MRSA) bacteria are more prevalent in the hospital environment and can be a challenge to infection control practices in most countries. Oie et al. (2002) and Boyce (2007) reported Methicillin-resistant *S. aureus* (MRSA) that frequently contaminated objects including the floor, bed linens, the patient's gown, overbed tables, door knob/door handle and blood pressure cuffs. Of the total number of *S. aureus* isolates from this hospital, 12.5, 50.0, 84.4, 43.8 and 50.0% were susceptible to ampicillin, tetracycline, cefoxitin cetazidime and gentamicin respectively.

High resistance of *S. aureus* to ampicillin; that is 92.0% from Major Ibrahim B. Abdullahi memorial hospital and 92.6% from St. Lukes' hospital is in agreement with 97.0% of *S. aureus* resistance to ampicillin as reported by Terry-Alli et al. (2011) from South Western Nigeria. This research confirms the earlier report of Dudhagara et al. (2011) that a high percentage of *S. aureus* were resistant to ampicillin and other β – lactam drugs, and is also in agreement with research work carried out by Akindele et al. (2010) that of the 100 total number of *S. aureus* isolated

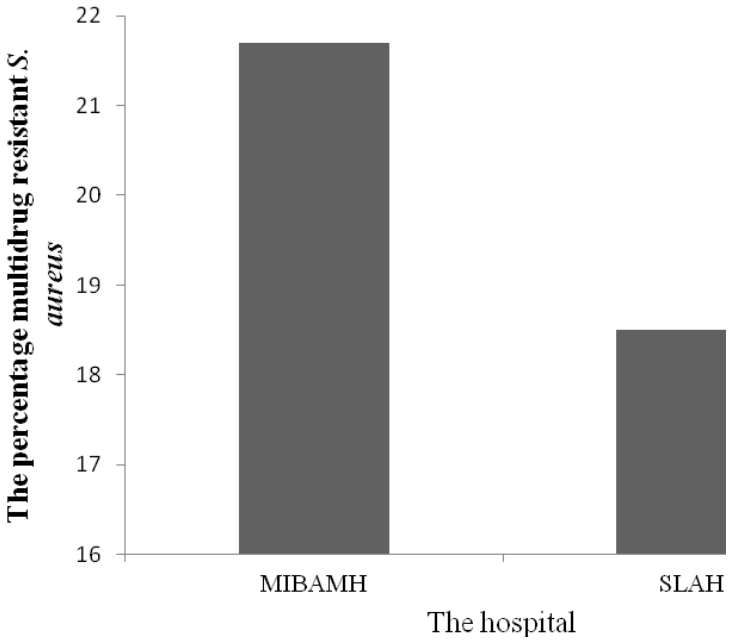

Figure 2. The prevalence of the total multidrug-resistant isolates from the two hospital environment. MIBAMH = Major Ibrahim B. Abdullahi Memorial Hospital; SLAH = St Luke's Anglican Hospital.

Table 6. The Multiple antibiotic resistant (MAR) indexes of the pathogens isolated from the hospitals.

Organism	No. of resistance isolates	MAR Index	Percentage (%) MAR Index
S. aureus	16	0.29	80
	4	0.43	20

from hospital environment 90% of them were resistant to ampicillin. The resistance of *S. aureus* to this antibiotic (AMP) may be as result of the ability of β-lactamase enzyme to break the β-lactam ring of the antibiotic and render it ineffective because *S. aureus* produces β-lactamase in the presence of ampicillin (Oncel et al., 2004). Akindele et al. (2010) reported in their work that β-lactamase production by staphylococci is the recognized mechanism of resistance to β-lactam antibiotics such as ampicillin and penicillin.

The 100% susceptibility of *S. aureus* to vancomycin from the two hospitals in this finding agreed with the findings of Terry-Alli et al. (2011) and the 100% susceptibility to linezolid is in agreement with 100% susceptibility of *S. aureus* to linezolid as reported by Kaleem et al. (2010) that 100% of the isolates of *S. aureus* were susceptible to linezolid and vancomycin and slightly higher than 93% susceptibility pattern as reported by Seza and Fatma (2012). The 0.0% resistance of *S. aureus* to gentamicin in this finding is not similar with report of Akindele *et al*. (2010) that 39% of this pathogen was resistant to gentamicin.

Figure 2 shows the multidrug resistance pattern of the pathogen against the selected. The multidrug resistant *S. aureus* from Major Ibrahim B. Abdullahi memorial hospital (21.7%) and St. Luke's Anglican hospital (18.5%) was not higher than 87.75% multidrug resistant *S. aureus* as reported by Fagade et al. (2010). This finding has corroborated the report of Seza and Fatma (2012) that among the Gram-positive microorganisms, staphylococci are the most frequently resistant pathogen to antibiotics. The surfaces of the two hospital environment can serve as important secondary reservoir for multi-resistant microorganisms, such as the MRSA as reported by Carvhalo et al. (2007); this has to be emphasized because of the apparent ability of these pathogens to survive on dry surfaces. Therefore, the spread of multidrug resistant *S. aureus* in this research can be a great threat to everyone in the two hospital environments and the public.

Table 6 indicates the multiple antibiotic resistant index of the pathogen against the drugs. The multiple antibiotic resistance (MAR) index gives an indirect suggestion of the probable source(s) of the organism. The MAR indices

in this work were greater than 0.20, this indicates this pathogen might have been originated from an environment where antibiotics are often used as reported by Olayinka et al. (2004).

Conclusion and recommendations

The widespread use of antimicrobials, especially over- or inappropriate use of antibiotics, has contributed to an increased incidence of antimicrobial-resistant organisms. Hospital-acquired infections are often caused by antimicrobial-resistant microorganisms. Resistance to antimicrobial agents is a problem in communities as well as health care facilities, but in hospitals, transmission of bacteria is amplified because of the highly susceptible population. Factors that could be associated with transmission of resistant strains of these microorganisms include poor attention to hygiene, overcrowding, lack of an effective infection control program, and shortage of trained infection control providers.

In view of multiple studies indicating the environment to be an important source of bacterial transmission, more stringent routine environmental decontamination practices in healthcare facilities with regular monitoring is necessary in the MDRO containment bundle. Thorough cleaning and disinfection of the environment would remain one of the topmost effective preventive measures intended to provide reassurance that patients as well as staff are not put at unnecessary risks during their stay in the hospital setting. Cleaning remove pathogens from a surface and can be able to reduce residual organic material to a low level.

Most of these infections can be prevented with readily available, relatively inexpensive strategies by: adhering to recommended infection prevention practices, especially good hand hygiene and wearing gloves; paying attention to well-established processes for decontamination and cleaning of soiled surfaces, followed by use of disinfectants should remain the most effective means to reduce transmission of nosocomial pathogens. There is convincing evidence that improved hand hygiene can reduce infection and cross-transmission rates. Therefore, there are local as well as international guidelines for hand-hygiene practices. Healthcare workers (HCWs) should be encouraged to decontaminate (clean) their hands with an antiseptics before and after all patients' contacts.

Conflict of interests

The authors did not declare any conflict of interest.

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Identification, antibiotic resistance and distribution of different classes of integrons among *proteus* species isolated from different sources in Dakahleia and Damietta Egyptian Governorates

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The aim of the present investigation is to trace the epidemiological relatedness between different isolates of *proteus* sp. obtained from Dakahleia governorate as compared with similar sources from the adjacent Damietta governorate in Egypt. A total of 500 bacterial isolates were collected from human and animal sources including urine samples, sputum, wound, throat and ear swabs, endotracheal aspirate, breast swab, bile aspirate, blood samples, human and animal stools. From a total of 70 *Proteus* isolates, 62 were identified as *Proteus mirabilis* while eight as *Proteus vulgaris*. All *Proteus mirabilis* isolates were screened for susceptibility to ten different antimicrobials and were classified into 29 different patterns. Two resistant strains were found to belong to pattern No. 10 (isolates No. 11 and 19). A second pair of isolates namely No. 27&31 was classified into pattern No. 18. A third pair of (isolates No 30 and 16) was found to have great similarity to pattern No. 18 with a minor difference in resistance against one or two antibiotics. For a molecular characterization of the resistance determinants, 15 of the highly resistant *Proteus mirabilis* isolates were screened for the presence of different classes of integrons. Class 1 was the dominantly detected type that confers resistance to trimethoprim and aminoglycosides. One isolate was found to contain class 2. Similar integron components *(dfrA15*) could be proved among strains of different human sources (No. 11 & 19). Another similar type of intergon components *(dfrA17) -(aadA5)* was found in human isolates 31, 27, 30 & 16. In conclusion, three pairs of the studied isolates have been turned out to be epidemiologically related with the possibility of hospital infection and of strain transfer from one governorate to the other.

Key words: *Proteus*, integrons, resistance pattern, swarming.

INTRODUCTION

Bacteria of the genus *Proteus* are part of the normal flora of the intestinal tract of humans and animals and are widespread in the environment. In particular, *Proteus mirabilis* accounts for approximately 3% of nosocomial infections in the United States mainly causing cystitis, pyelonephritis, and prostatitis. In Egypt, *P. mirabilis* constitutes the third most commonly isolated pathogen (after *Escherichia coli* and *Klebsiella pneumoniae*) of urinary tract infections. They are mostly ascending infections, more common among patients with anatomical

or physiological malformations of the urinary tract, as well as among catheterized patients or due to medical care mistakes (Chen et al., 2012). Prevalence of colonization with resistant microorganisms within a hospital can occur through bacterial cross-transmission or contamination originating from an environmental source (Lipsitch and Samore, 2002). In addition, microorganisms can acquire resistance determinants through horizontal gene transfer. Studies on the epidemiology of *proteus* species are of great importance in developing countries. This genus constitutes a great importance due to the wide diversity of infections caused by its member namely *P. mirabilis*. *Proteus* sp. has been proved to be one of the important causative of hospital infection in developing countries (Wasfi et al., 2012). However, comparative studies of *proteus* sp. between hospitals or adjacent governorates are not well studied in Egypt. It is also of great importance to characterize the resistance pattern of *proteus* strains from different sources of the specified regions for future antibiotic treatment policy.

The aim of the present investigation is to trace any relatedness or discriminatory characteristics between *proteus* isolates of the same regional source of Dakahleia governorate and the other adjacent Damietta governorate. Discrimination will be on the basis of antimicrobial resistance pattern, class and molecular structure of integrons as a powerful indicator parameter for discrimination Basis.

METHODOLOGY

Bacterial isolates

A total of 500 bacterial isolates were obtained from Dakahleia and Damietta, governorates in Egypt.Bacterial samples were collected from Dakahleia governorates including: Mansoura University Hospital (MUH), Mansoura Emergency Hospital (MEH), Urology and Nephrology Center, Mansoura University (UNC), Specialized Medical Hospital, Mansoura University (SMH), Pediatric University Hospital (PUH), Automated Slaughter house in Mansoura (MAS) and Private Laboratories Specimens. Damietta isolates were obtained from National Cancer Institute (DNCI), Damietta General Hospital (DGH), Damietta Specialist Hospital (DSH), Damietta Central Joint Laboratory (DCJL), Farms from Damietta. Samples were collected from various sources including (70) urine samples, (30) sputum, (120) wound and (13) throat swabs, (15) ear swabs, (20) endotracheal aspirate, (5) breast swab, (2) bile aspirate, (25) blood samples, (100) human and (100) animal stools Protocol of such studies was approved by Institute IRB/EC before registration of the point as a Master thesis.

Phenotypic identification of *proteus* isolates

Identification of different *proteus* isolates was carried out by the usual phenotypic methods including cultural characteristics with swarming appearance. The bacilli are pleomorphic Gram-negative bacilli. Biochemical reactions including indole test and other biochemical reactions related to different *proteus* sp. were also verified (Crichton, 1996).

Antimicrobial susceptibility testing

All isolates were twice screened for susceptibility to ten antimicrobial discs namely; ampicillin (AMP, 10 µg), ampicillin/sulbactam (SAM, 10/10 µg), cefepime (FEP, 30 µg), cefoperazone (CFP, 75 µg), imipenem (IPM, 10 µg), cefoperazone and sulbactam SCF, 75 µg/30 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), gentamicin (CN, 10 µg), tobramycin (TOP, 10 µg) using the standard disc All discs were supplied by Oxoid USA. The arithmetic mean to the nearest 0.5 mm was calculated for each antibiotic, and the results were categorized for each antibiotic according Clinical Laboratory Standards Institute Recommendation (CLSI, 2010).

Identification of different classes of integron for 15 resistant strains by Multiple PCR amplification

Identification of different classes of integron was carried out by a multiplex polymerase chain reaction (PCR). Amplification of genes representing the constant region of Class 1, 2 and 3 integrons was achieved by using the primer sets listed in Table 1. The template DNA for PCR was prepared as described by Zhang et al. (2004).

Characterization of the variable region of class 1 and class 2 integrons

Amplification of the variable region of class 1 and 2 integrons was performed using four primers 5-CS/3-CS and Ti-F/Ti-B, respectively, as described previously (Zhang et al., 2004). The reaction mixture was prepared as described previously. PCR reactions began with 10 minutes of primary denaturation at 94°C followed by 40 cycles of 94°C for 30 s, annealing temp, 30 s and 72°C for 30 s. Primers 5-CS/3-CS were annealed at 49°C while Ti-F/Ti-B reactions were annealed at 52°C.

Determination of the gene sequence of different classes of integron

Amplified gene fragments were purified using the PCR Purification Kit (MEGA quick-spin fragment DNA purification INTRON biotechnology, Sangdaewon-Dong, Korea) for Sequencing. Purified PCR products were used as a template in sequencing reactions carried out with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Bio-systems, Foster City. USA). The reaction mixtures were analyzed on an ABI 3730 DNA analyzer (Applied Bio-systems, Foster City, USA). Amplicons were sequenced on both strands and predicted peptide sequences analyzed by the online BLAST of the NCBI website software (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide sequences of the antibiotic resistance genes were deposited in Gene Bank under definite accession numbers (Table 1).

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Table 1. Primers used for amplification of class 1, 2 and three integrase genes and their variable regions

Primer	Sequence	Size of amplicon (bp)	Targets	GenBank number	References
intM1-U IntM1-D	5'-ACGAGCGCAAGGTTTCGGT-3' 5'-GAAAGGTCTGGTCATACATG-3'	565	*intI1*	AF550415	Jianyu et al., 2005
IntM2-U IntM2-D	5'-GTGCAACGCATTTTGCAGG-3' 5'-CAACGGAGTCATGCAGATG-3'	403	*intI2*	AP002527	Jianyu et al., 2005
IntM3-U IntM3-D	5'-CATTTGTGTTGTGGACGGC-3' 5'-GACAGATACGTGTTTGGCAA-3'	717	*IntI3*	AY219651	Jianyu et al., 2005
5'-CS 3'-CS	5'-GGCATACAAGCAGCAAGC-3' 5'-AAGCAGACTTGACCTGAT-3'	Variable	Gene cassette(s) of class 1 integron	U12338	Zhang et al., 2004
Ti-F Ti-B	5'- ACCTTTTTGTCGCATATCCGTG -3' 5'- CTAACGCTTGAGTTAAGCC -3'	Variable	Gene cassette(s) of class 2 integron	AJ289189	Jianyu et al., 2005

RESULTS

Phenotypic Identification of *Proteus* species

According to cultural characteristics and biochemical reactions, out of a total of 500 collected isolates of bacteria, seventy solates were identified as *Proteus* species, where 31 isolates were from Dakahleia and 39 isolates were isolated from Damietta. Subsequent identification revealed that 62 isolates were identified as *Proteus mirabilis* (Tables 2 and 3), and eight isolates were identified as *Proteus vulgaris* (Tables 2 and 3).

Antimicrobial susceptibility test

Regarding *P. mirabilis*, and according to Tables 2 and 3, twenty-nine different antibiotic susceptibility patterns were detected among the tested strains. Thirty-seven isolates (59.7%) were resistant to ampicillin, 15 isolates (24%) were resistant to gentamicin, cefoperazone, and ampicillin /sulbactam combination, 14 isolates (22.5%) were resistant to tobramycin and 7 isolates were resistant to ciprofloxacin (11.2%).

In contrast, all isolates (100%) were sensitive to cefoperazone /sulbactam combination. 58 isolates (93.5%) were sensitive to levofloxacin while 57 isolates (92%) were sensitive to imipenem, and 56 isolates (90.3%) were sensitive to cefepime.

Regarding *P. vulgaris*, five different antibiotic susceptibility patterns were detected among the tested isolates (Tables 2 and 3).The majority of the isolates were sensitive to most antimicrobial agents used in this study (Figure 1).

Identification and characterization of gene cassette of class 1, 2 and 3 integrons

Fifteen (15) resistant integron positive *P. mirabilis* were subjected to further investigation of their antibiotic resistance genes present in different classes of integron (Table 4). Only 6 isolates of *P. mirabilis* were found to carry detectable Class 1-related integrons, showing the existence of amplicons of 1665 bp in four, 1496 bp in one and 726-737 bp in three of the class 1 integron positive isolates. Class 2 was identified in only one isolate is having 857 bp amplicon. Class 3 integron could not be detected from any of the integrons studied isolates.

Nucleotide sequences of the antibiotic resistance genes were deposited in Gene Bank under accession numbers: KM386399, KM386400, KM386401, KM386402, KM386403, KM386404, KM386405, KM386406 and KM386407 (Table 4). Table 5 summarizes the main differences between the selected resistant strains including resistance pattern and integron characterization.

DISCUSSION

The aim of the present work is to study the

Table 2. Dakahleia isolates, their origin, sample source, isolation center, and antimicrobial resistance.

Species	Strain No.	Origin	Isolation center	Sample source	Sex	Antibiotyping profiles										Pattern No.
						AMP	CEP	FEP	IPM	SAM	SCF	CN	TOB	CIP	LEV	
P. mirabilis	1	Human	MUH	Wound	F	I	S	S	S	S	S	S	S	S	S	1
	2	Human	MUH	Wound	M	R	I	S	S	S	S	S	S	I	S	2
	3	Human	PUH	Blood	F	R	S	S	S	S	S	S	S	I	S	3
	4	Human	SMH	Ear swab	F	R	R	S	S	R	S	I	I	R	S	4
	5	Human	MEH	Wound	F	R	R	S	S	I	S	R	R	R	I	5
	11	Human	GEC	Endo tracheal aspirate	F	R	R	S	S	S	S	R	R	S	S	10
	12	Human	MCH	Oral swab	F	R	R	S	S	S	S	R	R	S	S	10
	13	Human	MEH	Urine	M	R	I	S	S	S	S	R	S	S	S	11
	14	Human	MCH	Sputum	F	S	S	S	S	S	S	S	S	S	S	12
	15	Human	MEH	Endotracheal tube	M	R	S	S	S	S	S	S	S	S	S	8
	19	Human	MUH	Wound	M	R	R	S	S	S	S	R	R	S	S	10
	20	Human	MEH	Wound	F	S	S	S	S	S	S	S	S	S	S	12
	21	Human	UNC	Urine	M	R	S	R	S	S	S	S	R	R	S	14
	22	Human	MUH	Wound	M	R	I	S	S	S	S	S	S	S	S	15
	24	Human	MUH	Breast swab	F	R	S	S	S	S	S	S	S	S	S	8
	25	Human	PUH	Blood	M	R	S	S	S	I	S	S	S	S	S	16
	27	Human	GEC	Bile aspirate	M	R	R	S	S	R	S	R	R	I	R	18
	29	Human	MEH	Wound	F	R	R	S	S	I	S	R	R	S	S	19
	36	Human	MCH	Sputum	F	I	S	S	S	S	S	S	S	S	S	1
	37	Human	MUH	Oral swab	F	S	S	S	S	S	S	S	S	S	S	12
	53	Human	Private clinic	Stool	F	R	R	S	S	S	S	I	I	I	S	27
	54	Human	Private clinic	Stool	F	I	S	S	I	S	S	S	S	S	S	23
	55	Human	Private clinic	Stool	M	S	S	S	S	S	S	S	S	S	S	12
	56	Human	Private clinic	Stool	F	R	I	I	S	S	S	S	S	I	S	28
	57	Human	Private clinic	Stool	F	S	S	S	S	S	S	S	S	S	S	12
	58	Human	Private clinic	Stool	M	S	S	S	S	S	S	S	S	S	S	12
	62	Animal	MAS	Rectal swab	F	S	S	S	S	S	S	S	S	S	S	12
	64	Animal	MAS	Rectal swab	F	S	S	S	S	S	S	S	S	S	S	12
P. vulgaris	52	Human	Private clinic	Stool	F	S	S	S	S	S	S	S	S	S	S	12
	63	Animal	MAS	Rectal swab	F	I	S	S	S	S	S	S	I	S	S	32
	65	Animal	MAS	Rectal swab	F	R	I	I	S	R	S	I	I	I	S	33

R: Resistant, I : Intermediate, S: Sensitive, AMP : ampicillin (10µg), CEP : cefoperazone (30µg), FEP : cefepime (30µg) , IPM : imipenem (10µg), SAM : ampicillin – sulbactam, SCF : cefoperazone (75µg) – sulbactam (30µg), CN : gentamicin (30µg), TOB : tobramycin (10µg), CIP : ciprofloxacin (5µg), LEV : levofloxacin (5µg). F: female, M : male MUH: Mansoura University Hospital, Mansoura University, MEH : Mansoura emergency hospital, UNC : urology and Nephrology Center, Mansoura University, SMH: specialized medical hospital , Mansoura University, PUH: pediatric university hospital, Mansoura University, GEC : gastroenterology surgical center, Mansoura University, MCH: Mansoura Chest Hospital. MAS: Mansoura automated slaughterhouse.

Table 3. Damietta isolates, their origin, sample source, isolation center, and antimicrobial resistance

Species	Strain No.	Origin	Isolation center	Sample source	Sex	Antibiotyping profiles										Pattern No.
						AMP	CEP	FEP	IPM	SAM	SCF	CN	TOB	CIP	LEV	
P. mirabilis	6	Human	GGHD	Ear swab	M	R	R	R	S	S	S	R	R	S	S	6
	7	Human	GGHD	Wound	M	R	R	S	S	R	S	S	R	S	S	7
	8	Human	DSH	Wound	M	R	S	S	S	S	S	S	S	S	S	8
	9	Human	DSH	Wound	M	R	R	S	S	R	S	S	R	S	S	7
	10	Human	DSH	Wound	F	R	R	R	R	R	S	S	S	S	S	9
	16	Human	GGHD	Urine	F	R	I	S	S	R	S	R	R	I	S	13
	17	Human	DSH	Urine	F	R	S	S	S	S	S	S	S	S	S	8
	18	Human	DSH	Urine	M	R	S	S	S	S	S	S	S	S	S	8
	23	Human	DSH	Wound	M	R	S	S	S	S	S	S	S	S	S	8
	26	Human	GGHD	wound	M	S	S	S	S	S	S	S	I	S	S	17
	30	Human	DSH	Urine	F	R	S	S	S	R	S	R	I	R	I	20
	31	Human	DCJL	Urine	F	R	R	S	S	R	S	R	R	I	R	18
	32	Human	DSH	Urine	M	R	S	S	S	I	S	I	I	S	S	21
	33	Human	DSH	Urine	M	R	S	S	S	S	S	S	S	S	S	8
	34	Human	DSH	Wound	F	R	S	S	S	S	S	S	S	S	S	8
	35	Human	DSH	Urine	M	R	S	S	S	S	S	S	S	S	S	8
	38	Human	DSH	Wound	F	S	S	S	S	S	S	S	S	S	S	12
	39	Human	DSH	Wound	M	S	S	S	S	S	S	S	S	S	S	12
	40	Human	DSH	Wound	F	I	S	S	S	S	S	S	S	S	S	1
	41	Human	GGHD	Stool	M	I	S	S	S	S	S	S	S	S	S	1
	42	Human	GGHD	Stool	F	S	S	S	S	S	S	S	S	S	S	12
	43	Human	Private clinic	Stool	F	R	R	R	R	R	S	R	R	R	R	22
	44	Human	Private clinic	Stool	F	I	S	S	I	S	S	S	S	S	S	23
	46	Human	GGHD	Stool	M	R	I	S	S	S	S	S	S	S	S	15
	47	Human	GGHD	Stool	F	I	I	S	S	S	S	S	S	S	S	24
	49	Human	Private clinic	Stool	M	S	S	S	S	S	S	S	S	S	S	12
	50	Human	GGHD	Stool	M	S	S	S	S	S	S	S	S	S	S	12
	51	Human	Private clinic	Stool	M	R	I	S	S	S	S	R	I	S	S	25
	60	Animal	Private farm	Rectal swab	NI	S	S	S	S	S	S	S	S	S	S	12
	61	Animal	Private farm	Rectal swab	NI	S	S	S	S	S	S	S	S	S	S	12
	66	Animal	Private farm	Rectal swab	F	R	R	I	I	I	S	R	S	R	S	26
	67	Animal	Private farm	Rectal swab	F	R	I	S	S	S	S	S	S	S	S	15
	69	Animal	Private farm	Rectal swab	F	S	S	S	S	S	S	S	S	S	S	12
	70	Animal	Private farm	Rectal swab	F	R	S	S	S	I	S	R	R	R	S	29
P. vulgaris	28	Human	DNCI	Sputum	M	R	I	R	S	I	S	R	S	S	R	30

Table 3. Contd

45	Human	GGHD	Stool	F	S	S	S	S	S	S	S	S	S	S	12
48	Human	Private clinic	Stool	F	R	I	S	I	S	S	S	S	S	S	31
59	Animal	Private farm	Rectal swab	NI	S	S	S	S	S	S	S	S	S	S	12
68	Animal	Private farm	Rectal swab	F	S	S	S	S	S	S	S	S	S	S	12

R: Resistant, I : Intermediate, S: Sensitive, AMP : ampicillin (10 µg), CEP : cefoperazone (30 µg), FEP : cefepime (30 µg) , IPM : imipenem (10 µg), SAM : ampicillin – sulbactam, SCF : cefoperazone (75 µg) – sulbactam (30 µg), CN : gentamicin (30µg), TOP : tobramycin (10 µg), CIP : ciprofloxacin (5 µg), LEV : levofloxacin (5 µg). F: female, M: male, NI: non-identified, DNCI: Damietta national cancer institute, GGHD: general governmental hospital of Damietta, DSH: Damietta specialist hospital, DCJL: Damietta central joint laboratory.

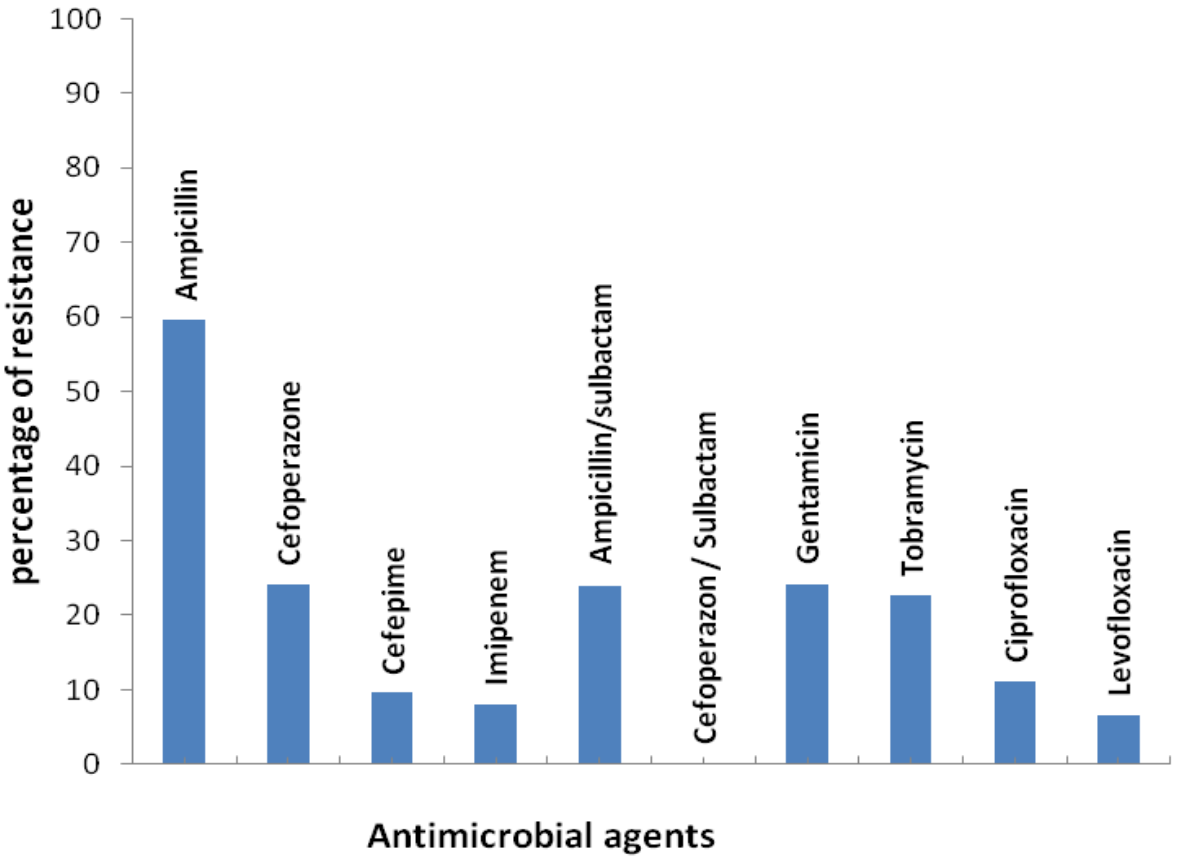

Figure 1. Percentage of Antimicrobial susceptibility pattern of *Proteus.*

Table 4. Integrons and characterization of gene cassettes.

Strain accession number	No of strain and source	*intI1*	*intI2*	*intI3*	size	Gene cassettes
KM386400	19 (Wound M)	+	-	-	726	*dfrA15*
KM386399	11 (endotracheal. M)	+	-	-	737	*dfrA15*
KM386403	31 (Urine D)	+	-	-	1665	*(dfrA17) -(aadA5)*
KM386405	27(Bile M)	+	-	-	1665	*(dfrA17) -(aadA5)*
KM386402	30 (Urine D)	+	-	-	1665	*(dfrA17)- (aadA5)*
KM386404	16 (Urine D)	+	-	-	1665	*(dfrA17)- (aadA5)*
KM386407	43 UP (human St D.)	-	+	-	857	(sat2)
KM386401	66 (cow Stool, D)	+	-	-	726	*dfrA15*
-	4 (Ear M)	-	+	-	156	No gene
-	11 (Endotrach. M)	-	+	-	156	No gene
-	43 (Stool D) down	-	+	-	156	No gene

D: Damietta, M: Mansoura, Strains: 9 and 10 (Wound D), 6(Ear D), 17(Stool D), and 5 (wound M) were devoid of integrons.

Table 5. Relationship between different antibiotic resistance pattern, and integron gene cassette of some related highly resistant isolates of *Proteus mirabilis* and source of isolation.

Strain accession number	Strain No.	Origin	Isolation center	Sample source	Sex	Antibiotyping profiles										Pattern No.	Integron components	Size
						AMP	CEP	FEP	IPM	SAM	SCF	CN	TOB	CIP	LEV			
KM386399	11	Human	GEC	Endo tracheal aspirate	F	R	R	S	S	S	S	R	R	S	S	10	*dfrA15*	737
KM386400	19	Human	MUH	Wound	M	R	R	S	S	S	S	R	R	S	S	10	*dfrA15*	726
KM386403	31	Human	DCJL	Urine	F	R	R	S	S	R	S	R	R	I	R	18	*(dfrA17) -(aadA5)*	1665
KM386405	27	Human	GEC	Bile aspirate	M	R	R	S	S	R	S	R	R	I	R	18	*(dfrA17) -(aadA5)*	1665
KM386402	30	Human	DSH	Urine	F	R	S	S	S	R	S	R	I	R	I	20	*(dfrA17)- (aadA5)*	1665
KM386404	16	Human	GGHD	Urine	F	R	I	S	S	R	S	R	R	I	S	13	*(dfrA17)- (aadA5)*	1665

epidemiological relatedness between different *Proteus* isolated from human animal and regional sources. Two adjacent Egyptian governorates were selected. Out of 500 samples, a total of 70 isolates of *Proteus* species were isolated from patients, stools of healthy humans and animals in Dakahleia and Damietta governments. A similar Egyptian work could not be traced in the available Egyptian literature.

Comparative studies were directed towards conventional methods of typing including biochemical reactions and antibiogram typing methods. It was also of interest to trace out the differences, similarities or relationships between different isolated strains by using integron finger printing of the isolated strains.

The objectives of epidemiological studies are to identify the source of disease, means of transmission, scale of distribution, epidemic and pandemic potential (or extent). Detection of asymptomatic carriers or reservoirs, and other factors associated with the spread of the disease are of great importance. To accomplish such objectives, there must be some means of characterizing the specific strain of the disease agent that is responsible, so that the past, present and future dissemination of the causative strain can be tracked (Bricker, 2011). The present study reveals that 14% of the examined samples were found to contain proteus sp. Out of them,

88.6% were classified as *P. mirabilis* indicating a high percentage as compared with 11.4% of *P. vulgaris.* It appears that the former is more invasive than the latter through the investigated region.

Antimicrobial susceptibility testing is a common practice in the clinical microbiology laboratory. The resultant antibiogram indicates the pattern of *in vitro* resistance or susceptibility of an organism to panel antimicrobial agents. The distribution of antimicrobial resistance among *Proteus* isolates showed that ampicillin was inactive against most of the tested isolates, where forty isolates (57.1%) were resistant to ampicillin. Such finding may be due to its extensive use in the treatment of different infectious diseases, while combination of cefoperazone (75µg) –sulbactam (30 µg) SCF was the most effective antibiotic combination where all the tested isolates were sensitive. The incidence of resistance to extended spectrum β-lactams and cephalosporins was previously observed. A higher percentage of resistance to ampicillin was found in the study of Bahashwan and El Shafey (2013) in Kingdom of Saudi Arabia (KSA) where more than 80 % of *Proteus* isolates were resistant to ampicillin. While similar result of resistance to ampicillin was found by Wong *et al*., 2013. Seventeen isolates (24.3%) were resistant to cephalosporins. A conforming result (26% to cefotaxime) was recorded by Yan-yan et al. (2012). However, a higher percent of resistance (more than 80% to cefpiramide and cephalothin) was reported by Bahashwan and El Shafey (2013). A lower percent of resistance (9.2% to ceftriaxone and 7% to cefepime) was reported by Maraki et al. (2012). β-lactam antibiotics are the most widely used antibiotics in clinical practice. This family of drugs is favored due to their high clinical efficacy, a broad spectrum of activity and safety. Due to frequent use of these antibiotics, bacteria have acquired a number of resistance mechanisms against these drugs (Sandanayaka and Prashad, 2002)

Resistance to aminoglycosides (gentamicin and tobramycin) was also observed. Sixteen isolates were resistant (22.85%). A higher percent of resistance to gentamicin (62%) was reported by Saleh and Hatem (2013). In contrast to the results of Yoon et al. (2011), who conducted their research on acute urinary tract infection in children from Korea. He showed that 100% of the isolates were sensitive to both gentamicin and tobramycin.

Aminoglycosides are commonly used antimicrobial agents in the treatment of infections by both Gram-negative and Gram-positive organisms. Aminoglycosides bind to the ribosomes and thus interfere with protein synthesis. Resistance to these antimicrobial agents is widespread, with more than 50 aminoglycoside-modifying enzymes already described (Shaw et al., 1993). The resistance of *Proteus* isolates to quinolones (ciprofloxacin and levofloxacin) was also observed. Ten samples (14.3%) were resistant, and ten samples were intermediate to either ciprofloxacin or levofloxacin or both. A similar result of resistance was observed by Orhiosefe et al. (2009) and Adamus-Bialek et al. (2013) as reported in their research on *P. mirabilis* strains isolated from clinical samples from Sweden and Poland. In contrast, a higher percent of resistance to ciprofloxacin (40%) was concluded by Kwiecińska-Piróg et al. (2013). Referring to the resistance pattern, it appears that the presence of four imipenem resistant isolates with their susceptibility to cefoperazone-sulbactam, suggests a sort of sulbactam-susceptible carbapenemase. This valuable information may constitute a simple method for their detection like ESBLS. In addition, other types of betalactamases can be easily interpreted from cephalosporin and ampicillin sensitivity tests.

For a more proper discrimination between resistant strains, molecular studies were applied. The term molecular epidemiology was firstly used to describe DNA-based methods to type, or fingerprint, strains of infectious microbes. During the past 15 years, many molecular methods have been adopted for use as typing schemes to assist the course of epidemiological investigations. More recently, molecular techniques have been applied to detect infectious agents in clinical or environmental samples providing greater sensitivity than was possible with conventional culture methods in the laboratory. Therefore, molecular epidemiology includes four applications of molecular techniques in infectious diseases: (1) to demonstrate relatedness between strains for epidemiologic investigations (2) to facilitate diagnosis, (3) to identify the agents of syndromes whose causes are unknown and (4) to identify genes involved in pathogenesis of infection and/or disease (Stout et al., 1992).

Integrons have been identified as a primary source of resistance genes and were suspected to serve as reservoirs of antimicrobial resistance genes within microbial populations (Navia et al., 2004). The role of integrons and gene cassettes in the spread of antibiotic resistance has been well-established (Kaczmarczyk et al., 2011). Class 1 integrons are the dominant type detected in clinical isolates and are most correlated with antibiotic resistance; therefore they have been comprehensively studied. Class 2 integrons are the second major type of integrons obtained from clinical isolates. Integrons, as a natural cloning and expression system, can capture exogenous gene cassettes by site-specific recombination and ensure the expression of the genes within them. Therefore, they play important roles in the acquisition and lateral transfer of antibiotic resistance genes (Wei et al., 2013)

Through the present study, we tried to characterize class 1, 2 and 3 integrons and their gene cassettes conferring resistance to several classes of antibiotics in *Proteus* sp. isolates were collected from clinical patients, human stool, and animals. Sequence analysis of the class 1 integron variable region revealed that the 1665 bp amplicon harbored gene cassettes (*dfrA17*) -(*aadA5*) conferring resistance to trimethoprim and aminoglycosides respectively. The 1496 bp amplicon harbored (*aac(3)-Id*)-

(*aadA7*) encoding aminoglycoside acetyltransferase and aminoglycoside adenyltransferase, respectively. Finally, the 726-737 bp amplicon contained the dfrA15 gene for trimethoprim resistance was found in three isolates. Sequence analysis of the class 2 integron variable region revealed only one isolate is having 857 bp amplicon carried (*sat2*) streptothricin acetyltransferase gene. Wei et al. (2013) reported that in 96 class 1 integron-positive strains, variable regions were successfully amplified in 70 isolates. Eight different gene cassette arrays were detected. The most prevalent gene cassette arrays were *aadB-aadA2* and *dfrA17-aadA5*, which were detected in 37 and 17 isolates, respectively. In the same study, in 101 class 2 integron-positive strains, variable regions were successfully amplified in all of them. Four different gene cassette arrays were detected. The most prevalent of these gene cassette arrays was *dfrA1-sat2-aadA1*, which was detected in their isolates. It worth mentioning that the absence of integrons in 4 of the tested resistant strains indicates that resistance genes can be located apart from integrons.

Comparing the results of integron components with antibiotic resistance pattern number, it appears that class 1 integron components were found in strains No 11&19. Such strains were belonging to antibiotic resistance pattern No10. They were isolated from a wound in Mansoura and bile aspirate from the same governorate. Such data Indicates with absolute confidence that they are one and the same strain running through hospital infection in Mansoura. Two other coincident strains including one isolate obtained from urine samples in Damietta (No31) and one from bile aspiration in Mansoura (No27). They have the same antibiotic sensitivity pattern No18. Such findings undoubtedly confirm the liability of transfer of strains from one governorate to the other. Approaching similarity, Strains No 16 and 30 are strongly related to strains No 30 and 27 with a liability to be transferred through a hospital infection in Damietta. It appears that minor changes in the antibiotic sensitivity pattern may be related to different antibiotic treatment approach even in the same governorate.

Molecular analysis of integron components confirms the presence of identical characteristics between some resistant strains and declares at least two pairs of sporadic strains are running through hospital infection cases or between the two adjacent governorates under investigation.

Such studies conforms with those of Michelin et al. (2008), who worked on 35 strains from different hospitals. The authors concluded that the only epidemiologically related strains were those strain isolated from one and the same patient occurring few days after endogenous infection of the patient himself.

Conclusion

Some of the studied resistant strains of *P. mirabilis* are epidemiologically related. Two pairs of identical strains suggest the possibility of hospital infection and a third pair suggesting the possibility of strain transfer from one adjacent governorate to the other.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Colletotrichum gloeosporioides lipase: Characterization and use in hydrolysis and esterifications

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Colletotrichum gloeosporioides **was evaluated for its capacity to produce extracellular lipase. A crude enzyme preparation obtained after 48 h of fermentation reached 742 U/L of lipolytic activity. Estimated molecular weight of proteins responsible for this activity was about 18 kDa as determined by SDS-PAGE and zymogram analysis. Enzyme preparation showed optimum pH at 10 and stability at optimum temperature (37°C) for 5 h. It showed tolerance to a wide range of salts (NH_4^+, Mg^{+2}, Ca^{+2}, Mn^{+2} and Sn^{+2}) and to some solvents (methanol, ethanol, isopropanol, 1-butanol, acetonitrile *n*-heptane and *n*-hexane) in different concentrations. The crude enzyme preparation was applied in hydrolysis reactions on different substrates (waste cooking soybean oil, cocoa butter and palm kernel oil) reaching high yields (87.6; 80.1; 74.9%, respectively). The preparation was lyophilized and it was applied in the synthesis of pineapple flavor, by esterification with butanol and butyric acid. *C. gloeosporioides* lipolytic enzymes synthesized butyl butyrate with 70% yield, in experiments carried out for 24 h using 1:1 acid/alcohol molar ratio in *n*-heptane medium. The lyophilized preparation was also able to perform transesterification of alcohols and *p*-nitrophenyl palmitate in organic medium (*n*-hexane), showing better activity when propanol was used ($5.4.10^{-3}$ U/kg.min). This study pointed the potential of alkaline lipolytic enzyme produced by *C. gloeosporioides* in biotechnological industry.**

Key words: Lipase, *Colletotrichum gloeosporioides*, hydrolysis, esterification, transesterification.

INTRODUCTION

Lipases are hydrolases (E.C. 3.1.1.3) widely distributed in nature. These enzymes, in association with cutinases and others esterases, play an important role in the biological cycling of lipids (Villeneuve et al., 2000). They are versatile biocatalysts that catalyze the breakdown of triglycerides with release of diacylglycerols, mono-acylglycerols and glycerol. They can catalyze, in non-aqueous media, esterification, interesterification and

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transesterification reactions, and acting on a broad range of substrates as natural oils, synthetic triglycerides and esters of fatty acids (Houde et al., 2004). They usually show good chemoselectivity, regioselectivity and enantio-selectivity, acting over a wide range of temperatures (Joseph et al., 2008).

Lipases have attracted great interest as a group of catalysts with proven potential to be used in biotechnological processes, still under explored, of lipid technology bio-industry (Treichel et al., 2010). Due to its versatility, its catalytic employment covers different areas such as obtaining food and esters used as flavors, production of detergents, application in optically active drugs resolution, production of fine chemicals, agrochemicals, biopolymers, as well as in use as biosensors, in bioremediation, and, even, for cosmetics and perfumes production (Hasan et al., 2006; Joseph et al., 2008).

The demand for new biocatalysts have boosted commercial production of enzymes and this expansion has led to projections of a global market for enzymes of 8 billion dollars in 2015 (Li et al., 2012). However, the industrial use of lipase is still limited by its commercial cost, particularly for applications that require large contingents of biocatalysts and generate low-value product (Ribeiro et al., 2011). Thus, more research is needed to achieve cost reduction and increased catalytic efficiency such as improvement of enzymes (by physical, chemical or genetic properties). Prospecting for microbial groups that form enzymes with new properties regarding specificity and stability to pH and temperature is also a modern trend on this area (Li et al., 2012).

Phytopathogenic fungi produce numerous extracellular enzymes to degrade cell wall polymers from plants in order to obtain nutrients and ensure infection during invasion process of plant tissues (Annis and Goodwin, 1997). The filamentous fungus *Colletotrichum gloeosporioides* is recognized as causing anthracnose (Huang et al., 2014). Especially, in tropical and subtropical fruits, affecting high value crops as mango, strawberry, avocado, citrus, papaya, cashew and passion fruit (Serra et al., 2011; Cannon et al., 2012). *Colletotrichum* is considered the eighth largest phytopathogenic genus in economic impact and scientific interest (Dean et al., 2012). *Colletotrichum* species are reported as producing cutinase, which allows the phytopathogen to penetrate through the cuticular barrier of the hosts (Dickman et al., 1982; Ettinger et al., 1987; Bonnen and Hammerschmidt, 1989). Other enzymes secreted by *Colletotrichum* described in literature are pectate lyase (Drori et al., 2003; Miyara et al., 2008), proteinases (Dunaevsky et al., 2007), lipases (Balaji and Ebenezer, 2008), celulases, amylases and esterases (Venkatesagowda et al., 2012).

In spite of the studies on enzymes produced by this organism, and their pathogenicity importance, there are only a few studies in the literature on biotechnological potential of *C. gloeosporioides* lipolytic enzymes. Thus, the objective of this work was to evaluate *C. gloeosporioides* lipase production by submerged fermentation, characterize the crude enzyme preparation obtained and test enzymatic activities in synthesis (in organic medium) and hydrolysis reactions to produce biotechnologically important products.

MATERIALS AND METHODS

All solvents used were of analytical grade and were obtained from Vetec Química fina LTDA (Rio de Janeiro, Brazil). Cocoa butter used was kindly donated by Cargill Agrícola S.A. Palm oil was obtained in trade from Salvador City, Bahia (Brazil) and the waste cooking soybean oil was donated by the academic community. Aloe and olive oil were obtained in trade from Belo Horizonte City, Minas Gerais (Brazil).

Fungus

A strain of *C. gloeosporioides* with known lipolytic activity from Food Microbiology Laboratory of Universidade Federal de Minas Gerais was used. It was maintained in potato dextrose agar (PDA) plates at 25°C for 5 days and after growth, agar plugs were taken and placed in distilled water at 25°C to long term preservation (Castellani, 1967).

Culture conditions and enzyme production

The isolates were grown on PDA plates for 5 days at 25°C. Spores suspension with concentration of 10^7 spores per milliliter was prepared. For production of lipase, 125 mL Erlenmeyer flasks containing 25 mL of fermentation medium (Colen et al., 2006), $g.L^{-1}$: peptone (10), $MgSO_4$. $7H_2O$ (0.6), KH_2PO_4 (1.0), NH_4NO_3 (1.0) and olive oil (4 mL) at pH 6.0 were inoculated with 1 mL of spore suspension. Cultivation was carried out under agitation (150 rpm) at 30°C for to 96 h. Each 24 h, three flasks were removed and their contents were filtered on Whatman (number 1) membrane, for biomass separation to obtain the crude enzyme preparation. Mycelia retained in the paper filter were used to determine biomass dry weight. In the crude enzyme preparation, were determined pH, total protein content and lipase and protease enzyme activities. Lipase specific activity was calculated (lipase activity/total protein content), each 24 h. Monitoring of enzyme production was carried out to determine higher production time, under conditions employed.

Biomass dry weight

Material retained on the filtration membrane was washed twice with ethanol and dried at 80°C until constant weight (Stone et al., 1992).

Total protein content

It was determined as described by Bradford (1976). Bradford reagent was added in microplates with the crude enzyme preparation (directly or after dilution if necessary). The mixture was gently stirred, left at rest for 15 min before reading absorbance at

595 nm. A bovine serum albumin (Merck) standard curve was built for comparison.

Lipase activity

Methodology was adapted from Winkler and Stuckmann (1979), using the *p*-nitrophenyl palmitate (*p*NPP) as substrate. A 3 mg/mL *p*NPP solution in isopropanol was mixed (1:9) with TRIS-HCl at pH 8.0 (0.09 M), arabic gum (2 $mg.mL^{-1}$) and triton X-100 (14 $mg.mL^{-1}$). The crude enzyme preparation was added to this mixture (directly or after dilution if necessary) and absorbance was determined immediately (at 410 nm) against a blank (without enzyme). Reaction took place for 10 min at 37°C. A standard curve using a *p*NP solution in isopropanol was used to calculate lipase activity. A unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol (*p*NP) per L of crude enzyme preparation added to the test, by minute.

Protease activity

Protease activity was determined according to Charney and Tomarelli (1947). Substrate (1 mL) containing casein 2.5 % (w/v) in bicarbonate buffer (50 $mmol.l^{-1}$), at pH 8.3 was incubated at 37°C. The reaction was initiated with addition of crude enzyme preparation (1 mL) and was paralyzed after 30 min, with 8 mL of trichloroacetic acid 8% (w/v). After filtration, NaOH 0.5 $mol.l^{-1}$ (5 mL) was added to the filtrate (5 mL) and absorbance was read at 445 nm. For each sample, a blank with crude enzyme preparation denatured (boiled for 10 min) was prepared. A unit of protease activity was defined as the amount required to release 1 µmol of azocasein peptides per mL of crude enzyme preparation added to the test, by minute.

Molecular weight estimation

Molecular weight determination was accomplished by submitting the crude enzyme preparation to a SDS-polyacrylamide gel electrophoresis as reported by Laemmli (1970). It was carried out on a 12.5% gel at room temperature. Then, this gel was treated with triton X-100 (2.5%) to remove the SDS obtaining the proteins in native form. A zymogram was carried out by overlaying this gel with a layer of chromogenic substrate solution (Phenol red 0.01%; olive oil 1%; 10 $mmol.L^{-1}$ $CaCl_2$ and 2% agar at pH 7.4). Once synthetic molecular weight markers were used in the same gel used for running the zymogram, degradation of the substrate changed gel color where the catalytic protein was. In this way, molecular weight of lipase was estimated comparing location of the band holding catalytic activity with molecular weight marker bands.

Enzyme storage

Lipolytic activity was monitored by two months at room temperature, the 4 (refrigerator), -18 (freezer) and -80°C (ultrafreezer). Crude enzyme preparation was maintained at -80°C for following tests.

Enzyme stability

Stability tests were conducted according to Castro-Ochoa and collaborators (2005) methodology, modified, using *p*NPP as substrate. All analyses were made in triplicate and lipase activities of the sample without any organic solvent, ion or detergent were taken as the control (100% of activity). Residual activity was measured by *p*-NPP spectrophotometric assay at 37°C, at pH 8.0.

Thermal stability was determined by incubation of crude enzyme preparation (0.5 mL) at different temperatures (30, 40, 50, 60 and 70°C) by 1 h, followed by enzymatic activity quantification. The temperature at which the enzyme was more stable by 1 h was also evaluated after 24 h. For stability in solvents, crude enzyme preparation (0.5 mL) was incubated with three concentrations (6, 30 and 50% v/v) of water miscible solvents (methanol, ethanol, isopropanol, 1-butanol, acetonitrile) and three concentrations (30, 50 and 75% v/v) of water immiscible solvents (toluene, *n*-hexane, *n*-heptane and dichloromethane) at 37°C for 1 h and then enzymatic activity was dosed. For pH stability, crude enzyme preparation was incubated at 37°C for 1 h with different buffers (0.05 mol/L) (citrate-phosphate to pH 4-6; TRIS-HCl to pH 7-9 and glycine-NaOH to pH 10-11) and enzymatic activity was dosed. To study the behavior of the activity and stability of lipase towards different ions, crude enzyme preparation (0.5 mL) was incubated at 37°C for 1 h with aqueous solutions (0.5 mL) containing 1 and 10 $mmol.L^{-1}$ of NH_4^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Sn^{2+} and EDTA and then, enzymatic activity was dosed.

Results were submitted to analysis of variance (ANOVA, single-factor), followed by Tukey test with 95% confidence to evaluate possible statistically significant differences.

Optimum pH and temperature of reaction

Based on lipase thermal stability, optimum temperature was determined by lipolytic assay as described previously, in a 30 - 45°C range of temperature. Optimum pH was determined by lipolytic assay, in a 4-11 range of pH, buffered (50 mM) with same pH solutions.

Applications of lipase

Hydrolytic activity

To evaluate the hydrolytic capacity of lipase in crude enzyme preparation, titrations tests were carried out (Colen et al., 2006), using different oils (olive, palm kernel and aloe oils, waste cooking soybean oil and cocoa butter) as substrates. The enzymatic reactions, prepared in Erlenmeyer flasks were incubated on a reciprocal shaker at 45 cycles/min, at 30°C for 10 min. Each reaction contained 5.0 mL of oil emulsion (25 % v/v) in polyvinyl alcohol (2 % v/v) as substrate, 5 mL of buffer TRIS-HCl 0.1 $mol.L^{-1}$, pH 8.0 and 1 mL of crude enzyme preparation natural or after denaturation process by boiling at 100°C for 10 min (blank reaction). Reaction was stopped by adding 10 mL of acetone/ethanol (1:1). After the reaction, the released fatty acids were determined by titration with NaOH 0.05 N using thymolphthalein (0.2 %) as a pH indicator. Hydrolytic activity was expressed in percentage of hydrolysis (fatty acids released) by min, per mL, under test conditions, and olive oil hydrolysis value was taken as 100%.

Transesterification activity

To assess lipase activity in medium under low water content, a transesterification test was carried out as described by Teng and Xu (2007) and modified. This method is based on transesterification catalyzed by lipase between esters of fatty acids and alcohol, in the absence of water. *p*-nitrophenyl palmitate (*p*NPP) and different alcohols (methanol, ethanol, isopropanol, 1-butanol and dodecanol)

were used as substrate to transesterification. Crude enzyme preparation was lyophilized and weight equivalent to 20 mg of protein was suspended in 0.5 mL of substrate solution (*p*NPP 10 mmol.L^{-1} in *n*-heptane). Reaction was initiated with the addition of alcohol (30 µL) to the mixture, and the reaction was incubated at 37°C for 30 min under agitation (200 rpm). After the mixture settled for 30 s, the clear supernatant (100 µl) was mixed with NaOH 0.1 mol.L^{-1} (1 mL). The *p*NP released, extracted from the alkaline aqueous phase, was dosed at 410 nm against a blank (without enzyme) in a UV-visible spectrophotometer (Varioskan Flash Multimode reader, Thermo Scientific). A standard curve of *p*NP in *n*-heptane was built in order to obtain quantitative results.

Esterification activity

Following Silva and collaborators (2014), modified, a mixture of butanol (250 mmol.L^{-1}) and an acid (butyric, acetic or oleic, at 250 mmol.L^{-1}), diluted with *n*-heptane was used as substrate. This reaction medium (5 mL) was incubated with 1 g of lyophilized crude enzyme preparation containing *C. gloeosporioides* lipase, at 37°C, 200 rpm, during 24 h. The esters yields were quantified by measurements of residual acid concentration in the reaction mixture. An aliquot of 500 µL was taken, diluted with ethanol/acetone 50:50 (v/v), homogenized and titrated with NaOH solution (20 mmol.L^{-1}) using phenolphthalein as indicator. Experiments were carried out in triplicates.

RESULTS

Obtaining crude enzyme preparation

Monitoring the fungus growth, under incubation in liquid substrate fermentation, showed a maximum production of biomass after 48 h, reaching 5 g.L^{-1} of growth medium. Maximum production of lipase, 742 U/L (enzyme units per liter of filtered fermented broth), also occurred in 48 h, with a specific activity of 4.68 (units of lipase per mg of protein per mL of filtrate). Maximum protease activity (2.7 U/L), was detected after 24 h of fermentation, but its activity did not affect lipase activity, that remained virtually unchanged until 96 h of incubation. Then, a period of 48 h of incubation was chosen for crude enzyme production.

Some industrial scale applications of enzymes demand a partial characterization of enzymatic crude extract, in order to suit process conditions to the peculiarities of the enzymes. In this context, an estimation of enzyme molecular weight was conducted, as well as investigation of best storage conditions and evaluation of *C. gloeosporioides* lipolytic enzymes stability towards solvents, ions, pH and temperature. To estimate molecular weight, a SDS-PAGE gel of crude enzyme preparation was compared with a zymogram containing a lipid emulsion at a concentration of 1% and using a pre-stained molecular weight marker. The fraction with enzymatic activity presented approximately 18 kDa (Figure 1), resembling that found by Chen and collaborators (2007) from another lipolytic enzyme of *Colletotrichum.*

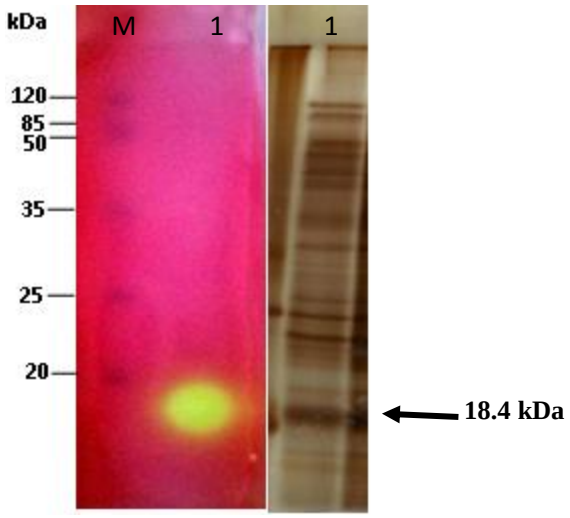

Figure 1. Molecular weight estimation of *C. gloeosporioides* crude enzyme preparation with lipolytic activity by comparison between zymogram (left) and SDS-PAGE analysis (right).

Enzyme storage

Lipolytic activity present in crude enzyme preparation was stored over 60 days at different temperatures. The activity was measured and demonstrated to be very unstable when stored at room temperature (25°C): significant decrease occurred over time (α=0.5), remaining around 50% of initial activity after 60 days. At lower temperatures (4 and -20°C), at the same incubation period, 86.8 and 90% of initial activity were maintained, respectively. Greater stability has been detected when the enzyme preparation was stored on ultrafreezer (-80°C). In this case, enzyme preparation showed greater activity than before being stored, suggesting stability of enzyme activity under freezing conditions. These data corroborate results obtained with carboxilesterases stored at low temperature (Corrêa et al., 2013).

Effect of temperature on enzyme activity and stability

In order to characterize the properties of the crude enzyme preparation some of its characteristics were assayed. Crude enzyme preparation retained 100% of lipolytic activity after 1 h of incubation at 30 and 40°C (Figure 2). Studies with *Fusarium graminearum* lipases, also pointed stability and activity at that temperature range (Nguyen et al., 2010). From 50°C, thermal stability of *C. gloeosporioides* enzyme was drastically reduced,

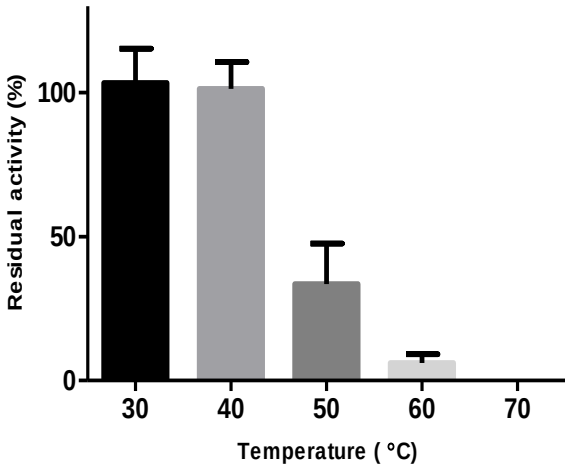

Figure 2. Temperature effect on lipase from *C. gloeosporioides* stability. The lipase was incubated for 60 min at 30, 40, 50, 60 and 70 °C, and then residual activity was measured using pNPP at pH 8.0, 37 °C. Different letters represent statistically significant difference for 95% confidence.

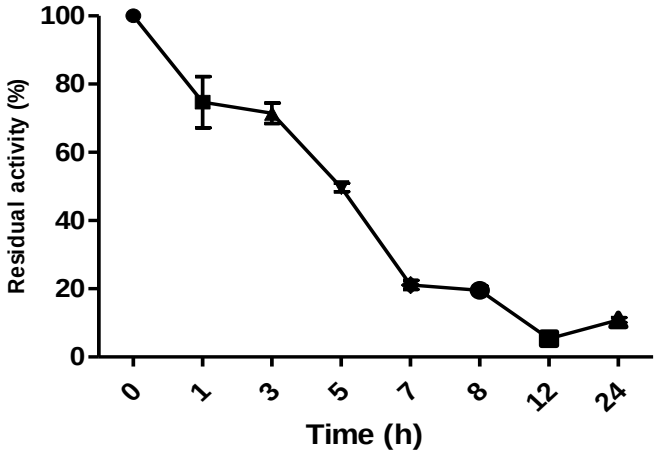

Figure 3. Temperature effect on lipase from *C. gloeosporioides* stability for 24 h. The lipase was incubated for 24 h at 37°C, and then residual activity was measured using *p*NPP at pH 8.0, 37°C.

retaining one-third of its activity, while, at 60°C only 6% of activity was preserved. Total activity loss was verified at 70°C. The lack of activity at higher temperatures suggests modifications in three-dimensional structure and, consequently, loss of function.

Catalytic activity of *C. gloeosporioides* lipase was detected at 37°C. Optimal temperatures close to 37°C have been reported for *Mucor hiemalis f. corticola* lipases (Ulker and Karaoglu, 2012). Then, in the present study, enzyme thermal stability was evaluated at this temperature for 24 h (Figure 3). During the first three hours, lipase activity was maintained, holding 80% of initial activity. Half-life of the enzyme occurred after 5 h of incubation, with sharp decline after this period. These results show a special behavior of *C. gloeosporioides* lipase. Li and collaborators (2013) for instance, evaluating *Stenotrophomonas maltophilia* lipase, observed enzyme half-life as 40 min at 40°C. Therefore, the results are very interesting since long term stability is a required characteristic for enzyme industrial applications.

pH effect on enzyme activity and stability

C. gloeosporioides lipase has remained relatively stable in the pH range 5 to 8 (60% recovery activity or more), with greater stability at pH 6.0, after 1 h at 37°C (Figure 4). It was more unstable at more alkaline pH, keeping about 30% of original activity at pH 9 and 10; retained about 20% of the activity at pH 11. Despite having been shown little resistance to alkaline pH (less than 1 h), the crude enzyme preparation presented optimum pH (higher activity) at 10.

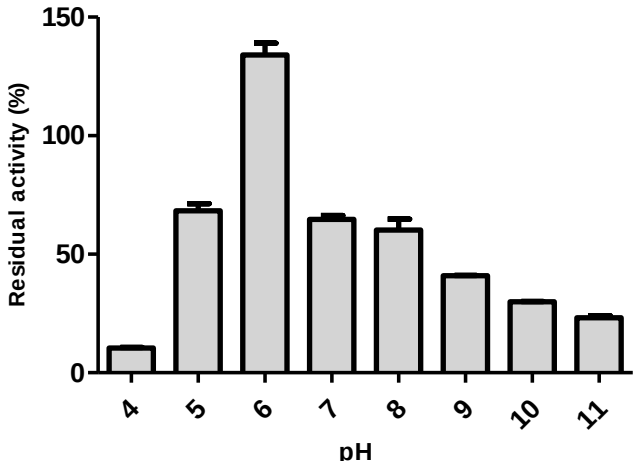

Figure 4. Effects on stability of lipase from *C. gloeosporioides*. The lipase was incubated for 60 min at 37°C in the presence of different buffer (50 mM): citrate-phosphate to pH 4-6; TRIS-HCl to pH 7-9 and glycine-NaOH to pH 10-11, and the residual activity was measured using *p*NPP at pH 8.0, 37°C. Different letters represent statistically significant difference for 95% confidence.

Solvent stability

Stability of lipase in organic medium is important for esterification reactions (Ulker and Karaoglu, 2012).

Table 1. Effects of water miscible and immiscible solvents on stability of *C. gloeosporioides* lipase. The lipase was incubated for 60 min at 37°C in the presence of different solvents in different concentrations (% v/v). Then, the residual activity was measured using *p*NPP at pH 8.0, 37°C.

Concentration (% v/v)	Residual activity (%)			
	6	30	50	75
Methanol[a]	132.5	30.9	13.1	[c]
Ethanol [a]	143.1	28.1	5.6	[c]
Isopropanol [a]	124.9	23.7	3.8	[c]
Butanol [a]	150.6	24.1	18.9	[c]
Acetonitrile[a]	100.8	3.1	0.0	[c]
Toluene [b]	[c]	223.5	5.4	0.0
Heptane [b]	[c]	84.3	118.7	223.3
Dichlorometane [b]	[c]	85.5	80.6	57.1
Hexane [b]	[c]	89.5	122.0	127.8

[a] Miscible solvents; [b]immiscible solvents; [c]not determined.

Enzyme stability was tested in five different water miscible solvents (methanol, ethanol, isopropanol, 1-butanol and acetonitrile) in three concentrations (6, 30 and 50%, v/v). *C. gloeosporioides* lipase exhibited tolerance to low concentrations (6% v/v) of all solvents (Table 1). At higher concentrations, the enzyme was most active in methanol (30% v/v) and butanol (50% v/v). Total loss of activity was observed when acetonitrile concentration was higher than 30% v/v.

This lipase also proved to be tolerant to water immiscible solvents: it was stable in *n*-heptane and *n*-hexane in all concentrations tested, with highest activity with 75% v/v of *n*-heptane (Table 1). In toluene and dichloromethane, the enzyme was more stable at low concentration (30% v/v), with total loss of activity in toluene 50% v/v. Stability of a lipase from *Mucor hiemalis f. hiemalis* in *n*-hexane and *n*-heptane has been previously reported (Hiol et al., 1999).

Effect of ions and EDTA on enzyme stability

Ionic components may interfere with the activity and stability of lipase present on a crude enzyme preparation. *C. gloeosporioides* enzyme was stable against all the tested solutions at 1 mmol.L^{-1} (Figure 5). The ions NH_4^+ and Sn^{2+} stimulated lipolytic activity in both concentrations assayed (1 and 10 mmol.L^{-1}). Yu and collaborators (2013) showed that optimal levels of NH_4^+ lead to increased lipolytic activity in *Rhizopus oryzae*. Stimulation of lipolytic activity in the presence of NH_4^+ has also been demonstrated by lipases from *Nomuraea rileyi MJ* (Supakdamrongkul et al., 2010) and *Aspergillus carneus* (Saxena et al., 2003).

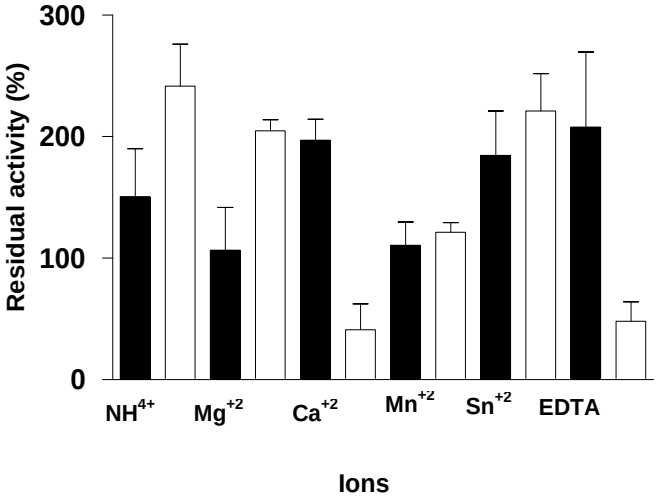

Figure 5. Ions effects on stability of lipase from *C. gloeosporioides*. The lipase was incubated for 60 min at 37°C in the presence of different ions: $NH4^+$, Mg^{2+}, Ca^{2+}, Mn^{2+}, Sn^{2+} and EDTA in two different concentrations: 1 mmol.L^{-1} (columns in black) and 10 mmol.L^{-1} (columns in white) and then the residual activity was measured using *p*NPP at pH 8.0, 37°C.

As well as calcium, chelating agent EDTA affected lipase in different ways, enhancing lipolytic activity at a concentration of 1 mmol.L^{-1} but, at 10 mmol.L^{-1} loss of lipolytic activity by 50% was observed. This effect observed for EDTA suggests that *C. gloeosporioides* lipase may be dependent on divalent ions.

Ions may interfere with enzyme activity, acting directly in enzyme catalytic site (Lee and Rhee, 1993; Supakdamrongkul et al., 2010). They can also act in the substrate, due to formation of complexes between metal ions and ionized fatty acids, increasing their solubility and modifying their behavior on an oil/water interface.

Enzyme applications

C. gloeosporioides lipase showed capacity to catalyze esterification, hydrolysis and transesterification reactions. The hydrolytic activity on different substrates is shown in Figure 6. This enzyme was able to hydrolyze all substrates tested. Considering olive oil hydrolysis as the reference, the highest hydrolysis degree occurred using waste cooking soybean oil (87.6%) and the lowest hydrolysis degree was detected for aloe oil (32.7%). The results showed that *C. gloeosporioides* lipase have broad hydrolytic activity, corroborating with literature for lipases from other sources (Chen et al., 2007; Balaji and Ebenezer, 2008).

In this context, it was observed that *C. gloeosporioides* lipase was able to perform transesterification in organic medium (with *n*-hexane), showing greater activity on short chain alcohols. The major activity of transesterification

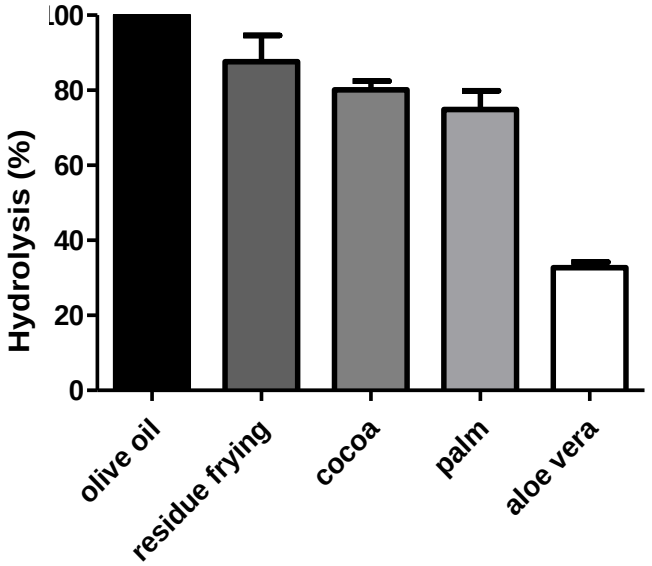

Figure 6. Percentage of hydrolysis of different oils (25 % v/v) using lipase from *C. gloeosporioides* incubated under agitation (45 cycles/min) at 30°C for 10 min. Each reaction contained 5.0 mL of oil emulsion in polyvinyl alcohol (2% v/v), 5 mL of buffer TRIS-HCl 0.1 mol.L^{-1}, pH 8.0 and 1 mL of crude enzyme preparation natural or after denaturation process by boiled to 100°C for 10 min (blank reaction). Olive oil hydrolysis value was taken as 100%.

occurred using propanol ($5.4.10^{-3}$ U/kg.min) and the smallest activities were observed for butanol and dodecanol. According to these results, there is evidence of lipase increased efficiency in transesterification reactions using short chain alcohols (e.g. methanol, ethanol and propanol instead of butanol or dodecanol). This is an interesting feature since other lipases, such as that obtained from *Aspergillus niger* showed better activity using pentanol instead of ethanol and methanol in esterification with lauric acid (Mustranta et al., 1993).

The capacity of *C. gloeosporioides* in esterification was also evaluated in the synthesis of butyl butyrate, butyl acetate (short chain esters used in food industry to produce, for instance, pineapple flavor) and butyl oleate (biodiesel additives). Esterification experiments were carried out for 24 h under 1:1 acid/alcohol molar ratio in *n*-heptane medium and absence of water. The amounts of synthesized butyl butyrate, butyl acetate and butyl oleate were 70, 14 and 40%, respectively. Bayramoglu and collaborators (2011) showed a yield of 42.6% of butyl butyrate synthesis using *Mucor miehei* lipase, in similar conditions.

DISCUSSION

Although lipolytic enzymes from phytopathogenic fungus have been widely studied in order to apply them to infections control, little have been undertaken on the physico-chemical characteristics of *C. gloeosporioides* lipase, to evaluate its potential industrial applications. This study showed individual characteristics of *C. gloeosporioides lipase* that suggests some potential uses.

C. gloeosporioides has recognized lipolytic activity (Colen et al., 2006; Chen et al., 2007; Balaji and Ebenezer, 2008). In this work, this fungus produced a crude enzyme preparation containing lipolytic activity obtained by fermentation in liquid substrate. Despite having detecting proteolytic activity in crude enzyme preparation, activity remained low and virtually unchanged throughout the remaining fermentation time, without generating negative interference on lipolytic activity present in the fermented broth. Composition of the culture medium used, with low protein content, may have influenced the formation of enzyme with negligible proteolytic activity.

C. gloeosporioides lipase presented long thermal stability at its optimum temperature. This long stability is interesting to lipase application once long industrial processing demand stable enzymes in these conditions. This lipase presented better stability at pH around 6, similarly to what was observed for *Mucor griseocyanus* lipase (Coca and Dustet, 2006). However, results found for *C. gloeosporioides* lipase demonstrate a wide range of pH stability and predilection by mild alkaline condition, in agreement with other study, which described other alkaline enzymes produced by this species (Dunaevsky et al., 2007).

The optimal pH alkaline, around 9-10, has been previously described for lipolytic enzymes of *C. gloeosporioides* (Dickman et al., 1982), *Colletotrichum lagenarium* (Bonnen and hammerschmidt, 1989) and *Fusarium solani f. pisi.* (Purdy and Kolattukudy, 1975). Lipases stable below 60°C, which tolerate alkaline pH, are interesting for application in detergent industry (Sangeetha et al., 2010) because enzymes used in this industry need to be active and stable under laundry conditions.

Lipases present in detergent formulations should be compatible with oxidizing and surfactants agents and present wide substrates specificity (Jellouli et al., 2011; Chauhan et al., 2013). Broad substrate specificity of detergent formulations is interesting because it increases their effectiveness on different types of dirt from different sources. On this aspect, the lipase from *C. gloeosporioides* also seems interesting since it was able to act on different fatty materials (olive oil, palm kernel oil, aloe oil, waste cooking soybean oil and cocoa butter).

Other interesting characteristic showed by crude enzyme preparation was a good stability in waste cooking oil, catalyzing oil hydrolysis with great yields. Waste cooking oil is receiving much attention recently because it can be applied as low cost substrate for lipase-mediated biodiesel production (Lam et al., 2010). This application, however, demands lipase stability in the oil, once they have high levels of free fat acids. According to the results

of this study, *C. gloeosporioides* behavior in waste cooking oil associated with its transesterification capacity indicates its applicability in transesterification reactions to produce biodiesel on these low-cost substrates (waste cooking oil).

Other good indicator is the pronounced stability of *C. gloeosporioides* lipase towards immiscible solvents, as well as its tolerance to low concentrations of miscible solvents. Lipases can perform esterification reactions in low water content conditions (Singh and Mukhopadhyay, 2012). Immiscible organic solvents are the preferred reaction medium because they allow greater solubility of substrates. Unlike miscible solvents, they do not contribute to disappearance of the aqueous layer, which is fundamental to folding and stability of the enzyme (Tamalampudi et al., 2007). However, miscible solvents such as short-chain alcohols are common substrates in lipase-mediated esterification reactions (Mustranta et al., 1993; Tan et al., 1996). Thus, stability of these enzymes in front of miscible solvents is also required for an efficient performance of these catalysts.

The stability showed by crude enzyme preparation in water immiscible solvents, associated with its ability in catalyze the synthesis of short chain esters, with particular efficiency in formation of butyl butyrate, when still in free form the enzyme reached high conversion rates, indicating its use in industry to synthesis esters used as food flavoring agents.

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Pseudomonas fluorescens (Pf1) mediated chitinolytic activity in tomato plants against *Fusarium oxysporum* f. sp. *lycopersici*

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Liquid formulation of *Pseudomonas fluorescens* strain Pf1 was found to protect tomato plants from wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*). The lytic enzyme and chitinase activity were assayed in the tomato plants treated with liquid as well as talc based formulations of Pf1 and challenged with the *FOL*. The chitinase activity was significantly increased in tomato plants treated with sequential application of seedling dip + soil drench + foliar spray of liquid formulation followed by the treatment with talc product application. The increased activity was observed up to 5 days after bioformulation treatment and thereafter declined. The enzyme activity of bioformulation treatment was significantly higher than the control treatments. Isoform analysis showed that sequential application of the treatment expressed three chitinase isoforms, whereas in the control plants no isoform was observed. Western blot analysis revealed that two isoforms of chitinase with a molecular weight of 28 and 23 kDa were newly induced by liquid formulation of Pf1 treatment challenged with the pathogen. These results suggest that induction of chitinase enzymes and accumulation of these PR-proteins might have contributed to restriction of *FOL* invasion in tomato roots.

Key words: Tomato *Fusarium* wilt, PR-protein, Chitinase, Western blot analysis, Isoform.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are characterized by a number of activities, which include improvement of plant establishment, increased availability of plant nutrients, enhancement of nutrient uptake, improvement of soil structure and protection against diseases (Glick, 1995 and Lynch, 1990). The soil-borne plant pathogens, *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* Sacc. causes considerable loss in yield. The yield losses due to *Fusarium* wilt disease which was estimated to be 40 to 50%. The disease management in tomato is widely practiced using chemicals. However, indiscriminate use of chemicals led to development of fungicidal resistance by the pathogen, environmental pollution and health hazards. In this, *fluorescent pseudomonads* have received particular attention throughout the global science because of their catabolic versatility, excellent root colonizing abilities and their capacities to produce a wide range of antifungal metabolites. In addition, it is not dose-dependent (Olivain et al., 2004). The use of *fluorescent* for controlling soil

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borne plant diseases has been well documented. The biocontrol agents induce systemic resistance (ISR) through fortifying the physical and mechanical strength of cell wall as well as changing physiological and biochemical reactions of host leading to synthesis of defense chemicals against challenge inoculation of pathogens. Chitinases (EC 3.2.1.14) are PR-proteins which hydrolyze chitin, a major cell wall component (3-10%) of higher fungi. Chitinase cleave a bond between C1 and C4 of two consecutive N-acetyl glucosamine (GlcNAc) either by endolytic or exolytic mechanisms. A large number of plant chitinases have been purified and characterized which are endochitinases with molecular weights ranging from 25 to 36 kDa. Many PR-proteins induced in plants treated with inducing agents have been shown to be chitinases and β-1, 3 glucanases. The production of chitinases in plants has been suggested to be a part of their defense mechanism against fungal pathogens (Schlumbaum et al., 1986). In recent years, several biocontrol agents have been shown to induce systemic resistance in plants. Accumulation of chitinases and peroxidases with the onset of ISR by PGPR has been observed in some plants. Enhanced accumulation of chitinase in tobacco and bean leaves was observed in response to application of *Pseudomonas* spp. to roots (Zdor and Anderson, 1992). Increased chitinase activity in tobacco and maximum activity in cucumber have been observed as a result of systemic resistance by *fluorescent pseudomonads* against *Pseudomonas syringae* pv. *tabaci* (Schneider and Ullrich, 1994). Nandakumar et al. (2001) found early and higher induction of chitinase in *P. fluorescens* Pf1 treated rice plants. Such enhanced induction of chitinase offered protection against *Rhizoctinia solani* in rice. Xue et al. (1998) reported that non-pathogenic treatment of binucleate *Rhizoctonia* elicited a significant and systemic increase in all cellular fractions of chitinase compared to the diseased and control bean plants. Induction of four new chitinase isoforms with molecular weights of 12, 34.5, 53.5 and 63 kDa in *Pseudomonas* treated canes challenge inoculated with *Colletotrichum falcatum* in sugarcane was also observed (Viswanathan and Samiyappan, 2001). Ramamoorthy et al. (2002b) reported the induction of 46 kDa chitinase due to *P. fluorescens* Pf1 treatment when challenge inoculated with *F. oxysporum* f. sp. *lycopersici* in tomato.

Many of the studies are reported for the use of powder or talc based carrier of PGPR, having the shelf life of 3 months. However, only a few reports are available on the development of liquid formulation. Comparing with powder or talc based carrier, liquid based formulation of PGPR has the advantages of higher cell count, zero contamination, longer shelf life, greater protection against environmental stresses, increased field efficacy and convenience of handling. It is possible to add certain chemicals to promote the formation of dormant cyst cells in the liquid formulation. When the organisms are kept in a dormant cyst form and if it is mixed with the soil at the time of use, the dormant form gives rise to active cells, which helps to increase the shelf life to more than one year and tolerance to adverse conditions (Vendan and Thangaraju, 2006). The liquid based formulation having extended shelf life using *Trichoderma asperellum* against *Fusarium* head blight was reported (Kolombet et al., 2008). Manikandan et al. (2010) reported that liquid formulation of strain Pf1 has six month shelf life and enhanced disease resistance in tomato plants against *Fusarium* wilt disease. The present study has been carried out to evaluate effect of *P. fluorescens* Pf1 for the induction of chitinolytic activity involved in the phenylpropanoid pathway for the bioprotection of tomato plants against subsequent infection by *F. oxysporum* f. sp. *lycopersici*.

MATERIALS AND METHODS

Pf1-Liquid formulation and *Fusarium oxysporum* f. sp. *lycopersici* fungus

Pseudomonas fluorescens strain Pf1 was obtained from the Culture Collection Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, India and used for this study. Liquid based Pf1 formulation was prepared in nutrient broth amended with glycerol as per the procedure described by Manikandan et al. (2010). *F. oxysporum* f. sp. *lycopersici* causing agent of wilt disease, was maintained on sterilized sandy loam soil mixed with maize powder at 19:1 (w/w) plates.

Glasshouse study

A glasshouse study was carried out to test the effect of liquid and talc based bioformulations of strain Pf1 on defense activity against *F. oxysporum* f. sp. *lycopersici*. In this study, individual and combined applications of seedling dip, soil drenching and foliar spray were evaluated for liquid based bioformulation and compared with talc based formulation. Tomato seeds were sown in plastic pots (45 × 60 cm) containing pot mixtures (red soil: sand: cow dung manure = 1:1:1 w/w/w sterilized at 121°C, 15 psi for 2 h for two consecutive days). The seedlings were removed 25 days after sowing and dipped in liquid formulation (500 ml ha^{-1} dissolved in 10 L of water) for 30 min. The seedlings were transplanted onto 30 cm diameter pots filled with pot mixture inoculated with 50 ml of conidial suspension of pathogen per pot (Ramamoorthy et al., 2002b) under glass house conditions. At 30 days after planting, 2 ml of the liquid formulation (2×10^9 CFU ml^{-1}) per pot was applied as soil drench. Similarly, seedling dip (200 g dissolved in 10 L of water) and soil application (5 g per pot) was given with talc based bioformulation strain Pf1 as described by Saravanakumar et al. (2009). At the same time foliar application of 0.2% Pf1 liquid as well as talc formulation was applied. Mancozeb (0.2%) was used as chemical control, while plants inoculated with the pathogen alone served as the inoculated control. Totally ten treatments and thirty pots with three seedlings per pot and three replications were maintained for each treatment.

Sample collection

Roots samples were collected from individual treatments to study the induction of defense enzymes such as chitinase in response to

soil borne pathogens in tomato plants under glasshouse conditions. Three roots from treated plants for each treatment were collected starting from 0^{th} day, 1^{st} day upto 13^{th} day at 48 h intervals.

Enzyme extraction

The root tissues were collected from treated and control tomato plants and immediately extracted with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extracts prepared from tomato tissues were used for estimation of chitinase enzyme.

Assay of chitinase

The colorimetric assay of chitinase (EC 3.2.1.14) was carried out according to the procedure developed by Boller and Mauch (1988). One gram of tomato tissue was extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 20,000 g at 4°C and the supernatant was used as enzyme source.

Preparation of reagents

Preparation of colloidal chitin

Colloidal chitin was prepared by treating 1 g of crabshell chitin powder with acetone to form a paste, then slowly adding 20 ml of concentrated hydrochloric acid (HCl) while grinding in a mortar with the temperature maintained at 5°C. After several minutes, the syrupy liquid was filtered through glass wool and poured into vigorously stirred 50% aqueous ethanol to precipitate the chitin in a highly dispersed state. The residue was sedimented and resuspended in distilled water several times to remove excess acid and alcohol and then dialysed against tap water. Chitin content of the suspension was determined by drying a sample *in vacuo* and adjusted with distilled water to a final concentration of 10 mg ml^{-1} (dry weight/volume) and stored at 5°C for further use (Berger and Reynolds, 1958).

Preparation of snail gut enzyme

Six hundred milligrams of the commercial lyophilized snail gut enzyme (Helicase, Sepracor, France) was dissolved in 10 ml of 20 mM potassium chloride (KCl) and chromatographed on a Sephadex G-25 column (38 x 1.5 cm) using a 10 mM KCl solution, containing 1 mM EDTA and adjusted to pH 6.8 for equilibration and elution. The first 20 ml eluted after the void volume was collected (Boller and Mauch, 1988).

Preparation of p-dimethylaminobenzaldehyde (DMAB) reagent

The DMAB reagent was prepared by the procedure described by Reissig et al. (1955). Stock solution of DMAB was prepared by mixing 8 g of DMAB in 70 ml of glacial acetic acid along with 10 ml of concentrated HCl. One volume of stock solution was mixed with 9 volumes of glacial acetic acid immediately before use.

Assay

The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 3,000 *g* for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (w/v) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice-water bath. After addition of 2 ml of DMAB, the mixture was incubated for 20 min at 37°C; immediately thereafter, the absorbance was measured at 585 nm in spectrophotometer (Agilent technologies). *N*-acetylglucosamine (GlcNAc) was used as a standard and the enzyme activity was expressed as n moles GlcNAc equivalents min^{-1} g^{-1} plant tissue.

Preparation of glycol chitin

Glycol chitin was obtained by acetylation of glycol chitosan (Trudel and Asselin, 1989). Five gram of glycol chitosan was dissolved in 100 ml of 10% acetic acid by grinding in a mortar. The viscous solution was allowed to stand overnight at 22ºC. Methanol (450 ml) was slowly added and the solution was vacuum filtered through a Whatman no. 4 filter paper. The filtrate was transferred into a beaker and 7.5 ml of aceticanhydride was added with magnetic stirring. The resulting gel was allowed to stand for 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel pieces was discarded. Gel pieces were transferred to pistel morter and homogenized for 4 min at top speed. This suspension was centrifuged at 12000 rpm for 15 min at 4ºC. The gelatinous pellet was resuspended in about one volume of methanol, homogenized and centrifuged as in the preceding step. The pellet was resuspended in distilled water (500 ml) containing 0.02% (w/v) sodium azide and homogenized for 4 min. This was the final 1% (w/v) stock solution of glycol chitin.

Activity gel electrophoresis

Chitinase activity was detected in the polyacrylamide gel electrophoresis according to Trudel and Asselin (1989) with modification. Gels were incubated in 150 mM sodium acetate buffer at pH 5.0 for 5 min and then in 100 mM sodium acetate buffer at pH 5.0, containing 0.01% glycol chitin for 30 min at 37°C. The gels were finally transferred into a solution containing 0.01% (w/v) Calcofluor white M2R (Fluorescent brightener) in 500 mM Tris HCl (pH 8.9). After 5 min the brightener solution was removed and the gels were rinsed with distilled water for more than 1 h. Lytic zones were visualized and photographed under UV light in gel documentation system (Alpha Innotech Corporation).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One gram of powdered leaf sample was extracted with one millilitre of 0.1M sodium phosphate buffer (pH 7.0) under 4°C. The homogenate was centrifuged for 20 min at 10000 rpm and the supernatant was used for the SDS-PAGE (Laemmli 1970). Hundred microgram of protein from different treatments was taken and mixed with 10 µl of sample buffer in a microfuge tube, boiled for 4 min and incubated at 4°C for 30 min. Then the samples containing equal amount of proteins were loaded into the wells of polyacrylamide gels (Sigma-Aldrich Techware system, Sigma, USA). The medium range molecular weight markers (Bangalore Genei, India) were used and electrophoresis was carried out at constant voltage of 75 volts for 2 h. The gels were stained with 0.2% Coomassie brilliant blue (R250) solution. Based on the R*f* value of each protein band stained, the molecular weight was calculated.

Table 1. Induction of Chitinase activity (nmoles GlcNac equivalents min^{-1} g^{-1} of fresh tissue) in tomato plants treated with Pf1 liquid formulation against *Fusarium oxysporum* f. sp. *lycopersici* under glasshouse condition.

Treatments	0 DAT	1 DAT	3 DAT	5 DAT	7 DAT	9 DAT	11 DAT	13 DAT
T_1 - Seedling dip with liquid formulation	1.64^{ab}	1.65^{ef}	1.67^{e}	1.68^{f}	1.68^{ef}	1.67^{d}	1.65^{de}	1.61^{d}
T_2 - Seedling dip with talc formulation	1.65^{a}	1.67^{e}	1.67^{e}	1.68^{f}	1.68^{ef}	1.67^{d}	1.64^{de}	1.61^{d}
T_3 - Seedling dip with Chemical (Mancozeb)	1.53^{bcd}	1.53^{fg}	1.54^{ef}	1.54^{fg}	1.55^{fg}	1.54^{ef}	1.53^{ef}	1.53^{d}
T_4 - T_1 + soil drenching with liquid formulation	1.60^{abc}	1.98^{c}	2.67^{c}	2.68^{c}	2.32^{c}	1.98^{c}	1.80^{c}	1.75^{b}
T_5 - T_2 + soil drenching with talc formulation	1.62^{abc}	1.81^{d}	2.36^{d}	2.47^{d}	2.11^{d}	1.77^{d}	1.71^{cd}	1.64^{bcd}
T_6 - T_3 + soil drenching with Chemical (Mancozeb)	1.53^{a-d}	1.55^{efg}	1.65^{e}	1.66^{f}	1.65^{ef}	1.64^{de}	1.63^{de}	1.63^{cd}
T_7 - T_4 + foliar spray with liquid formulation	1.63^{abc}	2.42^{a}	3.22^{a}	3.26^{a}	2.86^{a}	2.48^{a}	2.23^{a}	2.21^{a}
T_8 - T_5 + foliar spray with talc formulation	1.62^{abc}	2.21^{b}	2.94^{b}	2.99^{b}	2.64^{b}	2.27^{b}	2.11^{b}	2.19^{a}
T_9 - T_6 + foliar spray with Chemical (Mancozeb)	1.52^{cd}	1.58^{ef}	1.68^{e}	1.88^{e}	1.78^{e}	1.76^{d}	1.75^{cd}	1.74^{bc}
T_{10} - Inoculated Control	1.45^{d}	1.45^{g}	1.47^{f}	1.47^{g}	1.47^{g}	1.46^{f}	1.46^{f}	1.58^{d}

*DAT- Days after treatment. Values are mean of three replications. Means followed by a common letter are not significantly different at 5% level by DMRT.

Western blotting for chitinase detection

Preparation of protein samples and electrophoresis condition were the same as above. After SDS-PAGE electrophoresis, the proteins were electro blotted onto 0.45 µm PVDF membranes (Sigma, USA). The electrophoretic transfer of proteins was carried out from gel to membrane in a Bio Rad semidry transblot apparatus (140 mA, 30 min). The membranes were then stained with Ponceau S stain for 2 min to check the resolution and transfer quality. Ponceau S stain was destained with Tris Buffer Saline Tween 20 (TBST for 2 min and the membrane was blocked for 1.5 h at room temperature (28 ± 2°C) in TBST containing 2.5% (w/v) gelatin. The membrane was then soaked in the diluted primary antibody for overnight in TBST. After incubating with the primary antibody, the membrane was washed with TBST thrice for 10 to 15 min each time to remove the unbound antibody. The membrane was then incubated in secondary antibody for 3 h. Affinity purified goat anti-rabbit immunoglobulin (IgG) alkaline phosphatase conjugate was used as secondary antibody at a dilution of 1:7000. The membrane was then washed thrice with TBST and thrice in TBS for 10 to 15 min each time. Immunological reaction was visualized by soaking the membranes in alkaline phosphatase colour development reagents. Immediately after colour development the membranes were washed in distilled water and dried.

Statistical analyses

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). Prior to statistical analysis of variance (ANOVA) the percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P< 0.05$ and $P< 0.01$) and means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS

Chitinase activity

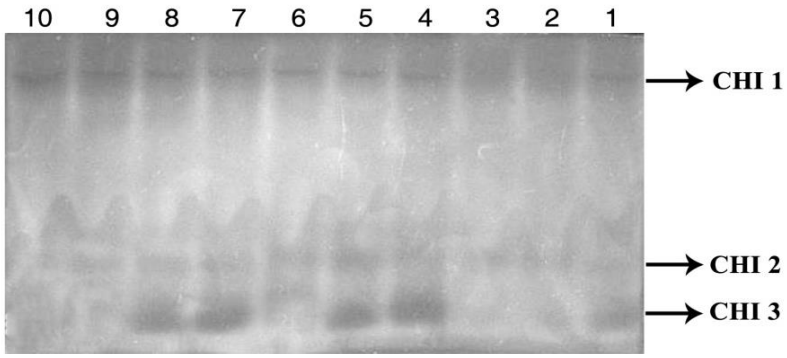
The lytic enzyme, chitinase activity was assayed in the tomato plants treated with the liquid as well as talc based formulations of strain Pf1 and challenge inoculated with the *F. o.* f. sp. *lycopersici.* The chitinase activity has significantly increased in tomato plants treated with combined application of seedling dip + soil drench + foliar spray of liquid formulation and challenge inoculated with *F. o.* f. sp. *lycopersici* followed by the treatment with talc product application of seedling dip + soil drench + foliar spray. The increased activity was observed upto 5 days after final treatment and thereafter declined. The combined application of seedling dip + soil drench with liquid formulation and the same application with the talc product showed the intermediate chitinase activity. The treatment received only seedling dip with liquid and with talc showed the enzyme activity comparatively higher than the untreated and inoculated control (Table 1).

Isoform pattern of chitinase

Treatment of the tomato plants with combination of seedling dip + soil drench and seedling dip + soil drench + foliar spray of Pf1 liquid formulation expressed three chitinase isoforms (Chi1, Chi2 and Chi3), whereas in the control plants no isoform was observed (Figure 1).

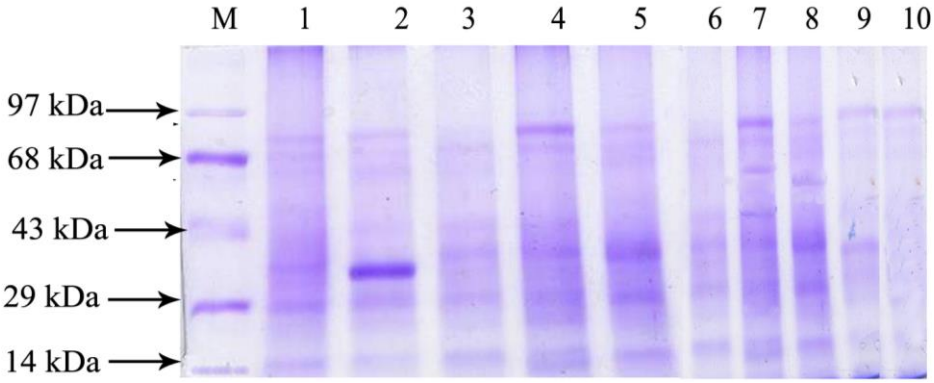
SDS-PAGE analysis of crude protein of Pf1 treated tomato plants challenged with *F. o.* f. sp.*lycopersici*

The protein banding pattern was studied from the tomato plants treated with liquid and talc based formulation of strain of Pf1after challenge inoculation of *F. oxysporum* f. sp*. lycopersici.* The banding pattern of protein was predominant in the treatment with seedling dip + soil drench + foliar spray of Pf1liquid formulation followed by same method of treatment with talc formulation. The proteins of 14, 29, 35 and 68 kDa were expressed in irrespective of the treatments. However, 72 kDa protein appeared in the combined application and also 35 kDa protein had appeared with high intensity (Figure 2).



1. T_1 - Seedling dip with liquid formulation
2. T_2 - Seedling dip with talc formulation
3. T_3 - Seedling dip with Chemical (Mancozeb)
4. T_4 - T_1 + soil drenching with liquid formulation
5. T_5 - T_2 + soil drenching with talc formulation
6. T_6 - T_3 + soil drenching with Chemical (Mancozeb)
7. T_7 - T_4 + foliar spray with liquid formulation
8. T_8 - T_5 + foliar spray with talc formulation
9. T_9 - T_6 + foliar spray with Chemical (Mancozeb)
10. T_{10} - Inoculated Control

Figure 1. Expression of chitinase isoforms in Pf1 liquid formulation treated tomato plants challenge inoculated with *Fusarium oxysporum* f. sp. *lycopersici*

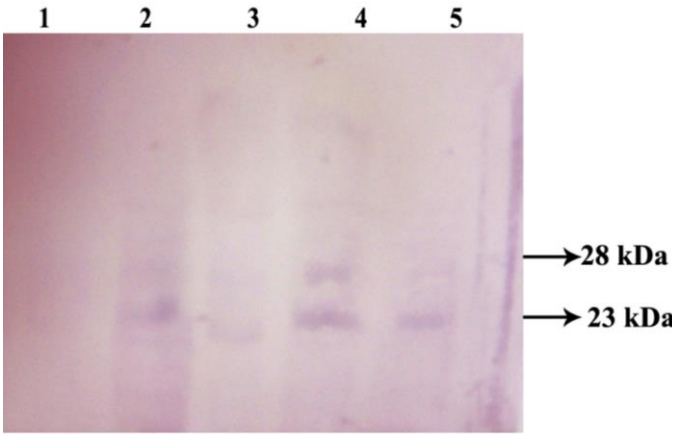


1. T_1 - Seedling dip with liquid formulation
2. T_2 - Seedling dip with talc formulation
3. T_3 - Seedling dip with Chemical (Mancozeb)
4. T_4 - T_1 + soil drenching with liquid formulation
5. T_5 - T_2 + soil drenching with talc formulation
6. T_6 - T_3 + soil drenching with Chemical (Mancozeb)
7. T_7 - T_4 + foliar spray with liquid formulation
8. T_8 - T_5 + foliar spray with talc formulation
9. T_9 - T_6 + foliar spray with Chemical (Mancozeb)
10. T_{10} - Inoculated Control

Figure 2. SDS-PAGE analysis of pathogenesis related proteins induced in tomato plants treated with Pf1 liquid formulation against *Fusarium oxysporum* f. sp. *Lycopersici.*

Western blot analysis of chitinase in Pf1 treated tomato plants against *F. o*. f. sp. *lycopersici*

Western blot analysis of root extracts from tomato plants showed that chitinase antiserum was able to recognize two proteins with sizes of 23 and 28 kDa. No isoforms of chitinase was detected in control treatments in which the tomato plants inoculated with *F. o.* f. sp. *lycopersici*. Also,



1. Untreated control
2. Seedling dip with liquid formulation
3. Seedling dip with talc formulation
4. Seedling dip + soil drenching with liquid formulation
5. Seedling dip+ soil drenching with talc formulation

Figure 3. Western blot analyses of chitinase isoforms in tomato plants induced by Pf1 liquid formulation against *Fusarium oxysporum* f. sp. *Lycopersici.*

the intensity of chitinase isoforms was more in the plants when treated with combined application of seedling dip+ soil drench + foliar spray of liquid based formulation followed by the same method of application with the talc formulation (Figure 3).

DISCUSSION

Synthesis and accumulation of PR proteins have been reported to play an important role in plant defense mechanisms. Chitinases and β-1, 3-glucanases (which are classified under PR-3 and PR-2 groups of PR proteins, respectively) have been reported to associate with resistance in plants against pests and diseases. The enzymatic degradation of the fungal cell wall by hydrolytic enzymes may release non-specific elicitors which in turn elicit various defense reactions. Viswanathan and Samiyappan (1999) reported that ISR induced by *P. fluorescent* is associated with induction of chitinase which appears to be the promising technology for the management of red rot disease of sugarcane. The antifungal nature of induced chitinase in rice plants after treatment with biological control agents has been reported by Nandakumar et al. (2002). Similarly, 57 kDa chitinase showing the antifungal activity against *Pyricularia grisea* in *Pseudomonas* treated finger millet (Radjacommare et al*.,* 2004b). Chitinase isoforms of different relative mobility were induced in response to pathogens after pretreatment with PGPR strains. The induction of chitinase has also been implicated in defense against further invasion of the pathogen in leaves and sheaths of rice against *R*. *solani*. In the present study, treatment of the tomato plants with combination of seedling dip + soil drench and seedling dip + soil drench + foliar spray of Pf1 bioformulation expressed three chitinase isoforms *viz*., Chi1, Chi2 and Chi3 whereas in the control plants no isoform was observed. It indicates the reduced disease incidence might be due to the higher induction of lytic enzymes. This type of findings has been reported by various workers in different crops. The culture filterate of combination of *Pseudomonas* strains (EPB22 + Pf-1) recorded higher chitinolytic activity than the individual strain and also more number of chitinase isoforms detected by the western blot analysis (Harish, 2005).

In conclusion, the present study implies that earlier and higher accumulation of chitinase enzymes involved in phenylpropanoid metabolism and PR-proteins has been found in tomato root tissue treated with *P. fluorescens* Pf1 liquid formulation in response to invasion by *F. o.* f. sp. *lycopersici*. The plant-pathogen interactions have also triggered the activities of defense enzymes initially but later the activities drastically declined when the pathogen colonized the root tissues. Enhanced accumulation and induction of chitnase isoform, by Pf1 liquid formulatin in tomato root tissues might have collectively involved in bioprotection of tomato plants against *F*. *oxysporum* f. sp. *lycopersici*. Thus the combined application of Pf1 bioformulation in liquid *viz.,* seedling dip + soil drench + foliar spray can enhance the disease protection activity and improve the consistency of pathogen suppression

throughout cropping period.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Seroprevalence and risk factors of small ruminant brucellosis in selected districts of Arsi and East Shoa zones, Oromia region, Ethiopia

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A cross-sectional study was conducted in two districts in Arsi zone and one district in East Shoa zone, Ethiopia, to determine seroprevalence and assess the possible risk factors associated with small ruminant brucellosis. A total of 840 blood samples (409 sheep and 431goats) were collected. All sera samples were screened by modified Rose Bengal Test (mRBT) and all positive reactors were further tested by indirect enzyme linked immunosorbent assay (iELISA) test for confirmation. All 39 (4.6%) mRBT positive samples tested positive in iELISA. The individual animal and herd level seroprevalences of small ruminant brucellosis in the study area were 4.6 and 26%, respectively. Individual animal and herd level seroprevalences were highest in Adami Tulu-Jido Kombolcha district in East Shoa and lowest in Dodota Sire district in Arsi zone but differences were not statistically significant. In univariate logistic regression, statistically significant difference in seropositivity were found between different age groups, pregnancy status and parity number but not between flock size, species and sex. Upon multivariate logistic, regression analysis parity and pregnancy status remained significant. A survey among 80 owners revealed general lack of awareness of the disease and showed that they practiced improper handling, disposal of aborted materials and consumption of raw milk as potential risk behaviours. Hence, the study suggests the need for implementing control measures and raising public awareness on prevention methods of the disease.

Key words: Brucellosis, Ethiopia, risk factors, small ruminant, zoonosis.

INTRODUCTION

The small ruminant population of Ethiopia is estimated to be nearly 23.33 million goats and 23.62 million sheep playing an important role in the livelihood of resource poor farmers. They provide their owners with a vast range of products such as meat, milk, skin, hair, horns and manure for cash. Sheep and goats are highly adaptable to broad range of environmental conditions. Moreover, low cost of production, requirement of little land and higher

prolificacy made them attractive asset for development. Investment in sheep and goats avoid losses due to high inflation rates that are found in unstable economies of many developing countries like Ethiopia. There is also a growing export market for sheep and goat meat in the Middle East Gulf states and some African countries. Despite all these, the country fails to optimally utilize this huge resource because of different constraints among which disease stands in the front line. Brucellosis is one of such diseases that hamper the productivity of small ruminants (Yami and Merkel, 2008; Central Statistical Agency, 2012).

Brucellosis is an infectious bacterial disease caused by members of genus *Brucella*. It is a disease of worldwide importance and affects a number of animal species. Brucellosis in small ruminants is mainly caused by *Brucella melitensis,* and rarely by *Brucella abortus* or *Brucella suis* (Hirsh and Zee, 1999; Benkirane, 2006, Verma, 2013). The disease in naturally infected sheep and goats is characterized by abortion, stillbirth and birth of weak offspring in females and acute orchitis and epididymitis in males. Brucellosis is an important zoonosis causing chronic debilitating disease in man. Groups at higher risk for brucellosis are animal health workers, butchers, farmers and those who habitually consume raw milk and come in contact with animals (Radostits et al., 2006; Gupta et al., 2006).

In Ethiopia, few studies have been published so far on small ruminant brucellosis (Tekelye and Kasali, 1990; Teshale et al. 2006; Ashenafi et al., 2007; Ferede et al., 2011; Bekele et al., 2011; Yohannes et al., 2013). Particularly, there is no published data on small ruminant brucellosis in the study area. On the other hand, there is high population of sheep and goat in the study area (CSA, 2012). Therefore, the objectives of this study were to determine the seroprevalence and assess possible risk factors of small ruminant brucellosis in the study area.

MATERIALS AND METHODS

Study area

The study was conducted in Tiyo and Dodota Sire districts of Arsi zone, and Adami Tulu-Jido Kombolcha district of East Shoa zone of Oromia region, Ethiopia. Arsi zone is found at 6°45′N to 8°58′N and 38°32′ E to 40°50′ E. Asella, the capital of the zone is found at 175 km Southeast of Addis Ababa. The animal population of Arsi zone is 2,295,138 cattle, 1,207,182 sheep, 653,327 goats, 202,467 horses, 369,218 donkeys, 21,587 mule and 1,449,583 poultry. The mean annual temperature of the zone is 20-25°c in the low land and 10-15°C in the central high land. On average, the zone gets annual mean rainfall of 1020 mm. The altitude of the zone ranges from 805 m above sea level to 4195 meters at mountain peak of mount Kaka (CSA, 2012; OFEDB, 2007).

Adami Tulu-Jido Kombolcha district is found at 7°9′ N latitude and 38°7′ E longitude. The district is situated in the mid-rift valley, East Showa zone of Oromia region, 167 km South of Addis Ababa. According to CSA (2012) the animal population of East Shoa zone is 973,563 cattle, 299,284 sheep, 488,512 goats, 13,000 horses, 247,399 donkeys, 7,087 mules and 926,465 poultry. The zone is found at an altitude of 1650 m above sea level with a bimodal unevenly distributed rainfall pattern. The average annual rainfall for the last 10 years was 760.9 mm. AdamiTulu-Jido Kombolcha district has a minimum mean temperature of 12.7°C (ALDHA, 2012).

Study animals and study design

A cross-sectional study was conducted from January to June, 2012 to study seroprevalence and associated risk factors of small ruminant brucellosis. The predominant sheep and goat breeds in the study area are Arsi-Bale breeds which are managed under extensive management system. Traditional housing, feeding and milking practices are generally practiced. Vaccination against brucellosis is not practiced in Ethiopia (Figure 1). Blood sample was collected from goats and sheep of above six month age and laboratory tests were done. Questionnaire survey was conducted on randomly selected small ruminant owners.

Sample size and sampling methodology

The sample size was calculated using the formula recommended by Thrusfield (1995) for simple random sampling considering 95% confidence interval level and 5% desired absolute precision. 50% expected prevalence was used as there was no previous study in the area.

$$N = \frac{(CI)^2 \, Pexp \, (1\text{-}Pexp)}{d^2}$$

Where, N-the required sample size, Pexp-expected prevalence rate, CI-confidence interval and d-desired absolute precision
The sample size required as per the above formula is 384 heads for each species. However, the sample size was increased to 840 (409 sheep and 431goats) to increase precision. The zones and districts were selected purposively based on their small ruminant population and accessibility. Simple random sampling technique was used to select peasant associations (PAs) and herds. Nine PAs (three from each district) were selected by lottery method. 131 herds were included in the study. The number of animals and herds tested in each district is indicated in Table 1.

Questionnaire survey

Eighty (80) small ruminant owners were selected randomly by lottery method (out of 131 owners) and interviewed using structured

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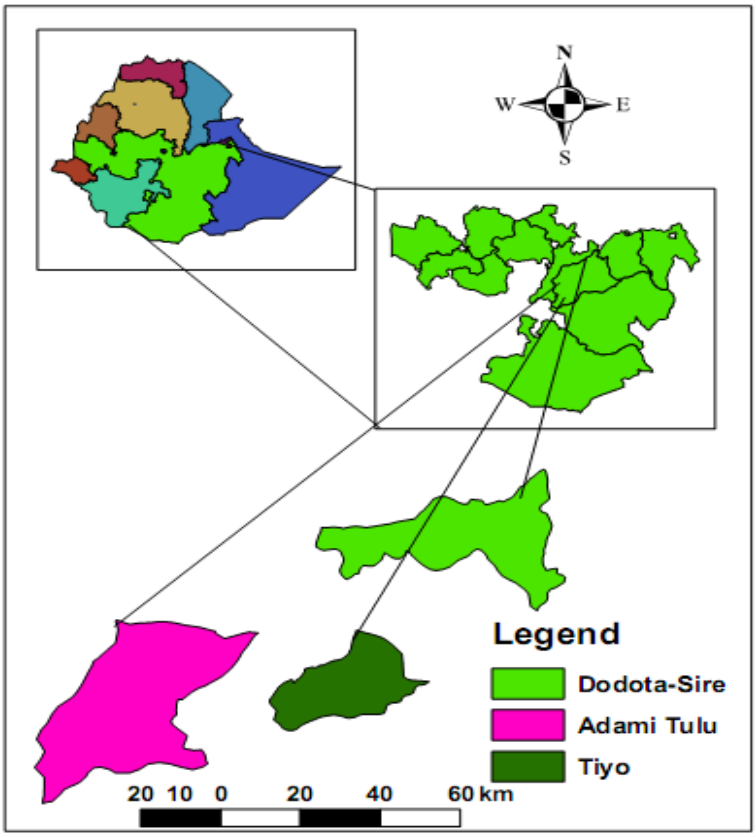

Figure 1. Map of study districts.

Table 1. Individual animal and herd level seroprevalence of small ruminant brucellosis in the three study districts.

Zone	Individual animal seroprevalence				Herd level seroprevalence		
	District	No. animals tested	No. Positive (%)	P-value	No. of tested herds	No. Positive (%)	P-value
Arsi	Tiyo	326	17(5.2)	0.17	44	13(29.5)	0.14
	Dodota Sire	287	8(2.8)	0.14	49	8(16.3)	0.13
E/Shoa	Adami Tulu-Jido kombolcha	227	14(6.2)	0.63	38	13(34.2)	0.65
	Total	840	39(4.6)		131	34(26)	

pre-tasted questionnaire. By doing so, management practices that may predispose the public to infection by brucellosis were accessed.

Blood sample collection and handling

About 7-10 ml of blood samples were collected from jugular vein of each animal using properly labeled plain vacutainer tubes. The individual animal details such as species of animal, sex, age, herd size, source of animal and history of abortion were recorded along with blood sample collection. Samples were allowed to stand tilted overnight at room temperature. Then, serum was separated from clotted blood and transferred to cryogenic vials. Separated sera were stored at -20°C until being tested.

Laboratory tests

The sera samples were removed from the refrigerator and left at room temperature for at least 30 minutes before performing the test. All sera samples were screened by mRBT antigen (Lillidale

diagnostics, UK) according to the modified procedure of Blasco et al. (1994), mixing 75µl of serum and 25 µl of antigen. The interpretation of the results was done according to the degree of agglutination. Samples with no agglutination were recorded as negative while those with agglutination were recorded as positive (Nielsen and Dunkan, 1990). Confirmation of mRBT positive sera was done by iELISA (SERELISA® *Brucella* OCB Ab Mono Indirect kit, SYNBIOTICS EUROPE SAS, France). Indirect ELISA test was performed according to manufacturer's manual. Both mRBT and iELISA were performed at Sebeta National Animal Health Diagnostic and Investigation Center.

Data analysis

Data obtained was stored into Microsoft Excel® spreadsheet. The individual animal level seroprevalence was calculated by dividing RBPT and iELISA positive results by total number of animals tested. Logistic regression analysis using SPSS 16 for windows was used to determine association of risk factors with the disease. All risk factors with P-value <0.2 in the univariable logistic regression analysis were subjected to multivariate logistic regression analysis.

RESULTS

Overall seroprevalence of small ruminant brucellosis

Out of total 840 sera samples collected from the three districts, 39(4.6 %) were positive for mRBT. Upon further testing, all were found to be iELISA positive. The individual animal and herd level seroprevalence of the disease in the study area were 4.6% and 26% respectively. The individual animal level seroprevalence of the disease was found to be higher in Adami Tulu-Jido Kombolcha district (6.2%) than Tiyo (5.2%) and Dodota Sire (2.8%) districts. Herd level seroprevalence was also higher in Adami Tulu-Jido Kombolcha district (34.2%) followed by Tiyo (29.5%) and Dodota Sire was lowest (16.3%). However, there was no statistically significant difference observed between districts both at individual animal and herd level (Table 1).

Association of risk factors with seroprevalence of small ruminant brucellosis

The prevalence of *Brucella* antibodies in goats and sheep was 4.9 and 4.4%, respectively and it was higher in male (6.7%) than females (4.4%). Seroprevalences of 4.1, 4.8 and 6.6% were found for herd sizes of [0-10], [11-20] and >20, respectively. There was higher rate of infection in adult (5.4%) than young age group(1.3%) and in pregnant (6.9%) than non-pregnant(2.3%). The prevalence of the disease was 3.7% and 4.58% in shoats with and without previous abortion respectively. The prevalence of the disease was 1.46, 5.59 and 8.4% for [0-1], [2-4] and>4 parities, respectively. There was a statistically significant difference in seropositivity between different parity groups, age groups and pregnancy status in univariable logistic regression analysis. However, there was no statistically significant difference in seropositivity between different sex, species, herd sizes and abortion status (Table 2). Pregnancy and parity status remained significant in multivariable logistic regression analysis. The odds ratio indicated that pregnant sheep and goats were 3.28 times more likely to be infected with brucellosis than the non pregnant ones. The risk of seropositivity was 6.19 and 3.89 times higher in >4 and [2-4] parity groups respectively in comparison to [0-1] parity group. Multivariable logistic regression analysis of potential risk factors for small ruminant brucellosis is indicated in Table 3.

Questionnaire survey result

Pre-tasted questionnaire was presented to 80 randomly selected farmers to assess association of management risk factors with the disease. Univariable logistic regression analysis of management risk factors obtained through questionnaire survey showed that only seasonal migration of herds was significantly associated with the disease (Table 4). All of the respondents have no knowledge about the disease. Neither did they use any protective material while handling aborted fetus or fetal membranes. Moreover, none of them practiced safe disposal of aborted material. Raw milk consumption was practiced by some of the herd owners interviewed.

DISCUSSION

The seroprevalence obtained in the present study was higher than the report of Tekelye and Kasali (1990) who reported prevalence rates of 1.5% in sheep and 1.3% in goats in Central Ethiopia; and Ferede et al. (2011) who reported prevalence proportions of 0.87% in goats and 0% in sheep in and around Bahir Dar. The report of Ashenafi et al. (2007) with prevalence rate of 5.8% in goats and 3.2% in sheep in pastoral regions of Afar and that of Ashagre et al. (2011) with prevalence of 4.2% in goats in South Omo zone showed fair agreement with this finding. Bekele et al. (2011) reported lower prevalence rate of 1.2% in sheep and 1.9% in goats in Jijiga area. The difference in prevalence might be attributed to the differences in animal husbandry and serological tests employed. Most of the above findings used standard rose bengal plate test (RBPT) and Compliment Fixation Test (CFT) for screening and confirmation of sera samples respectively; but, in this study mRBT and iELISA were used for screening and confirmation respectively.

Table 2. Univariable logistic regression analysis of potential risk factors.

Risk factor	Category	Animals tested	No.of positives	Prevalence (%)	P-value	OR (95% CI)
Species	Goat	431	21	4.9	0.746	1.11 (0.584-2.12)
	Sheep	409	18	4.4		
Sex	Male	88	5	5.68	0.625	1.27 (0.48-3.34)
	Female	752	34	4.5		
Age	(0.6-1year)	160	2	1.3		
	(>1year)	680	37	5.4	0.038	4.55 (1.08-19.06)
Flock size	[1-10]	438	18	4.1	0.59	
	[11-20]	311	15	4.8	0.64	1.18 (0.59-2.38)
	>20	91	6	6.6	0.31	1.65 (0.64-4.27)
Pregnancy	Pregnant	362	2	6.9	0.004	3.14 (1.45-6.82)
	Non-pregnant	390	9	2.3		
Parity	0-1parity	274	4	1.46	0.01	
	2-4parity	358	20	5.59	0.013	3.98 (1.35-11.79)
	>4 parity	119	10	8.4	0.002	6.19 (1.9-20.20
Abortion	No	698	32	4.58	0.77	1.25 (0.3-5.4)
	Yes	54	2	3.7		

*Male goats and sheep aged less than or equal to one year and female animals that had not yet given birth were included in the younger age group.

Table 3. Multivariable logistic regression analysis of potential risk factors.

Risk factor	Level	OR (95%CI for OR)	P-value
Pregnancy	Non-pregnant		
	Pregnant	3.28(1.5-7.2)	0.003
Parity	[0-1]		0.008
	[2-4]	3.89(1.31-11.55)	0.015
	>4	6.58(2.01-21.57)	0.002

The higher prevalence rates recorded by Verma et al. (2012), Bertu et al. (2010), Falade and Hussein (1997) and Waghela (1976) in India, Nigeria, Somalia and Kenya, respectively could be due to differences in agro-ecology and animal husbandry system. Teshale et al. (2006) reported higher prevalence rate of 5.6 and 13.2% in sheep and goats respectively in Afar and Somali areas. Arsi and East Shoa zones are characterized by mixed farming, in which fewer animals are raised in separate herds; however, pastoralists in Afar and Somali regions keep large number of different species of animals.

There was no statistically significant difference in seropositivity between the two species in the study area which agrees with the findings of Bekele et al. (2011) in Jijiga district, Tekleye and Kasali, 1999 in central Ethiopia and Bertu et al. (2010) in plateau state in Nigeria. However, Omer et al. (2002), Teshale et al. (2006) and Ashenafi et al. (2007) reported significantly higher prevalence in goats than in sheep.

No statistically significant difference observed between males and females. However, in support of the present finding, Teshale et al. (2006), Ashenafi et al. (2007),

Table 4. Univariable logistic regression analysis of management risk factors.

Risk factor	Category	Herd owner response (%)	No.of positive herds	Prevalence in herd	P-value	OR (95% CI)
Herd migration	Yes	20 (25)	7	35	0.038	3.5 (1.1-11.4)
	No	60 (75)	8	13.3		
Decision on aborting animal	Sell	62 (77.5)	12	19.5	0.8	1.2 (0.3-4.8)
	Keep	18 (22.5)	3	16.7		
Abortion encountered	Yes	58 (72.5)	14	24.13	0.075	6.7 (0.8-54.3)
	No	22 (27.5)	1	4.5		
Delivery assisted	Yes	33 (41.25)	5	15.2		
	No	47 (58.75)	10	21.3	0.49	0.66 (0.2-2.2)
Knowledge of disease	Yes	0 (0)	0	0		
	No	80 (100)	15	18.75		
Aborted material disposal	Throw into field	58 (100)	14	24.13		
	Burning/burying	0 (0)	0	0		
Handling of aborted fetus/membrane	Bare hand	58 (100)	14	24.13		
	Protective material	0	0	0		
Raw milk consumption	Yes	14 (17.5)	5			
	No	66 (82.5)	10		0.075	6.68 (0.8-54)

Ashagrie et al. (2011) and Bekele et al. (2011) also reported the absence of statistically significant difference between the two sexes. This could be due to the small sample size of males. Males are also kept in the herd for shorter period which decrease their exposure to the disease.

Pregnant sheep and goats showed significantly higher rate of infection than non-pregnant ones. Higher parity was also significantly associated with the disease which agrees with the report of Ashagrie et al. (2011). Age was found to be significantly associated with the disease in univariable logistic regression which agrees with the findings of Bekele et al. (2011) and Ashenafi et al. (2007). Sexually mature and pregnant animals are more susceptible to infection with the organism than sexually immature animals of either of sex, which is due to the fact that sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organism, tend to increase in concentration with age and sexual maturity (Radostits et al. 2006; Quinn et al., 2004). In this finding, seasonal migration of herds showed significant association with the disease.

Conclusion and recommendations

This study reveals that small ruminant brucellosis was distributed at a moderately higher rate in all studied peasant associations and districts. The result of questionnaire survey also showed that the owners of small ruminants lack knowledge about the disease; nor did they practice proper disposal of aborted materials. They assisted delivery with bare hand. Moreover, some of the owners practiced the habit of drinking raw milk. Generally, this finding revealed high risk of transmission of the disease in the small ruminants and people of the studied area. Hence, we recommend the implementation of control measures and raising public awareness on prevention methods of the disease in the area.

Conflict of interests

The authors did not declare any conflict of interest.

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