

African Journal of Microbiology Research

Volume 8 Number 23, 4 June, 2014

ISSN 1996-0808



*Academic
Journals*

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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant *Staphylococcus aureus* in community-acquired skin infections. *Emerg. Infect. Dis.* 11: 928-930.

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

Microbial properties of Ethiopian dairy products: A review

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Received 24 February, 2014; Accepted 12 May, 2014

Milk is considered as nature's most perfect food known to man. It provides nutrients to all ages of the human race. Similarly, it is a good growth medium for spoilage and pathogenic micro-organisms. The purpose of this review was to synthesize the earlier report on microbial properties of milk and fermented milk products in different parts of Ethiopia. The dairy products reviewed included milk, ergo (naturally fermented milk), Kibe (traditional butter), Arerra (defatted sour milk) and Ayib (Ethiopian cottage cheese). The existing microbial quality of dairy product produced in rural part of the country was substandard when compared from standards set by various bodies. The problem was compounded from limited knowledge on the hygienic handling practices of dairy products coupled with inadequate dairy infra structures. This is good entry point monitoring the handling, processing and transportation of dairy products is to bring safe product to the consumer.

Key word: Milk, dairy product, microbial quality.

INTRODUCTION

Milk and milk products play an important role in human nutrition throughout the world. Consequently, the products must be of high hygienic quality. In less developed areas and especially in hot tropics high quality of safe product is most important but not easily accomplished (Zelalem and Faye, 2006; Asaminew and Eyassu, 2011). In addition to being a nutritious food for humans, milk provides a favorable environment for the growth of microorganisms (Walstra et al., 2006). Food spoilage is an enormous economic problem worldwide.

Through microbial activity alone, approximately one-fourth of the world's food supply is lost. The highly perishable nature of milk and mishandling, the amount produced is subjected to high post-harvest losses in Ethiopia. Losses are up to 20-35% had been reported in Ethiopia for milk and dairy products from milking to consumption (Getachew, 2003).

The microorganisms principally encountered in the dairy industry are bacteria, yeasts, moulds and viruses. Some of the bacteria (lactic acid bacteria) are useful on

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milk processing, causing milk to sour naturally. However, milk can also contain pathogenic bacteria, such as *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Listeria* spp., *Brucella* spp., etc. The presence of these pathogenic bacteria in milk emerged as major public health concerns, especially those individuals who still drink milk (Lingathurai and Vellathurai, 2010). The consumption of raw milk and its derivatives is also common in Ethiopia (Zelalem, 2003), which is not safe from consumer health point of view as it may lead to the transmission of various diseases. On the other hand bacteria can cause spoilage of the milk and spoilage and poor yields of products (Oliver et al., 2005). Keeping fresh milk at an elevated temperature together with unhygienic practices in the milking process may also result in microbiologically inferior quality. Apparently, these are common practices for small-scale Ethiopian who produce milk and milk products and sell it to consumers (Zelalem, 2010). Moreover, an increasing number of people are consuming raw unpasteurized milk (Oliver et al., 2009).

It is a well-established fact that consumers want, wholesome and nutritious food that is produced and processed in a sanitary manner and is free from pathogens. However, inadequate dairy infrastructure coupled with limited knowledge on the hygienic handling of milk and milk products, the quality of smallholder milk and milk products in Ethiopia can generally be characterized to be substandard (Zezelem, 2010). Hence, for fulfilling consumer's demand, the hygienic improvement in milk production and processing is necessary (Khan et al., 2008).

The fragmented and un-compiled works on the microbial properties of milk and milk products in Ethiopia so far have failed to have a positive influence on traditional handling practices of milk. Which is evidenced by, the microbial qualities of the products produced from different part of the country remain substandard (Zezelem, 2010). Hence, understanding the existing situation concerning the microbial properties of milk and milk products produced, marketed and consumed in Ethiopia is critical to be able to make any improvement intervention. Therefore, the aim of this paper is to review the microbial properties of milk and fermented milk products in different chains and parts of the country.

MICROBIAL PROPERTIES OF MAJOR ETHIOPIAN DAIRY PRODUCTS

The microbial load of milk is a major factor in determining its quality (Fatine et al., 2012). It indicates the hygienic level exercised during milking, that is, cleanliness of the milking utensils, condition of storage, manner of transport as well as the cleanliness of the udder of the individual animal (Fatine et al., 2012). Milk from a healthy udder contains few bacteria but it picks up many bacteria from

the time it leaves the teat of the cow until it is used for further processing O'Connor (1994). These microorganisms are indicators of both the manner of handling milk from milking till consumption and the quality of the milk.

Microbial analysis of milk and milk products includes tests such as total bacterial count, yeasts and moulds and coliform estimation. They are useful to measure its sanitary quality and most grading of milk is on the basis of some method for estimating numbers of bacteria (Collins et al., 1995). The presence of defect producing types in butter can be indicated by estimating the presence of lipolytic organisms (O'Connor, 1994).

There are varieties of traditionally fermented dairy products in Ethiopia, in which the exact type of lactic acid bacteria responsible for fermentation is unknown due to uncontrolled and spontaneous fermentation. Most of these products are produced by smallholder producers where access to the required dairy infrastructure is limited. Certain reports of research efforts on the microbial properties of locally produced milk and milk products that have been carried out in different parts of the country are briefly summarized below.

MILK

In Ethiopia milk is produced and marketed to consumer without being pasteurized or subjected to and quality standard. About 98% of the annual milk produced by subsistence farmers who live in rural areas where dairy processing in the country is basically limited to smallholder level and hygienic qualities of products are generally poor (Zelalem and Faye, 2006). The hygienic conditions are different according to the production system, adapted practices, level of awareness, and availability of resources (Zelalem, 2010). In most of the cases under smallholder condition, the common hygienic measures taken during milk production especially during milking are limited to letting the calf to suckle for few minutes and/or washing the udder before milking (Zelalem, 2003) and the quality of the water used for cleaning purpose not secured.

Moreover, in the traditional practice the status of the cleanliness of the milkers, the udder of the cow, the milking environment and the milking equipments could be the chief sources of the initial milk contamination (Haile et al., 2012). The traditional milk equipment are reported to be often porous and therefore a reservoir for many organisms and difficult to clean (O'Connor, 1994). All these reasons might increase the microbial load of milk produced in traditional practices of Ethiopia. Producers need, therefore, to pay particular attention for the type as well as cleanliness of milk equipment. Milking equipment should be easy to clean. Aluminum and stainless steel equipment are mostly preferred (Zelalem, 2003).

Earlier research conducted in different part of the country revealed that the microbial counts of milk and milk

milk products produced and marketed are generally much higher than the acceptable limits (Zelalem, 2010). These were evidenced by milk collected from smallholder producers in Southern Ethiopia. The total bacterial count (TBC) reported by Abebe et al. (2012) in Gurage zone; Haile et al. (2012) in Hawassa town and Asrat et al. (2012) in Wollayta zone were on the range of 9.82 log cfu/ml - 4.57 log cfu/ml. It is comparable to the findings of Tollossa et al. (2012) 7.36-7.88 log cfu/ml of raw cow's milk in Borana, Ethiopia; Asaminew and Eyassu (2010) 7.58 log cfu/ml in Bahir Dar Zuria and Mecha districts, Ethiopia; and Solomon et al. (2013) 7.07 log cfu/ml in Debre Zeit town, Ethiopia.

However, raw milk samples from different part of the country TBC counts greater than 5 log cfu/ml which is higher than the given international standard set for minimum acceptable level of bacterial count in milk (IFCN, 2006). In other words, the above indicated count of milk samples collected from the country were considered to be below the standard set for good quality milk. This implies that the sanitary conditions in which milk has been produced and handled are substandard subjecting the product to microbial contamination and multiplication.

As indicated by Chambers (2002) total bacterial count is a good indicator for monitoring the sanitary conditions practiced during production, collection, and handling of raw milk. Hence training of milk handlers about hygiene can significantly reduce the bacterial load in milk. A good example worth mentioning is a reduced total bacterial count observed in milk sampled from farmers who received training on hygienic milk production and handling, and who used recommended milk containers as compared to that produced by the traditional milk producers (Rahel, 2008).

The milk utensils holding the raw milk and pasteurized milk sample from critical points as indicated by Zelalem and Faye (2006) could also contribute to further contamination. A number of research finding reported similar values of aerobic mesophilic counts milk sampled from udder, milking bucket, collection center, milk vending shops and cafeteria is range between 7.28 and 10.28 log cfu/ml (Godefay and Molla, 2000; Haile et al., 2012; Tollossa et al., 2012; Shunda et al., 2013). In all cases increasing trend of counts as the milk passed through udder, milking bucket, collection centers and upon arrival at the processing plant. This could be due to improper handling, storage and transport time after the milk leaves the dairy farms. Milk produced under hygienic conditions from healthy cows should not contain more than 4.7 log cfu/ml (O' Connor, 1994).

Coliform count, on the other hand, is especially associated with the level of hygiene during production and subsequent handling since they are mainly of fecal origin. Previous workers reported similar values of coliform counts in raw cow milk sampled from different part of the country that range between 4.03 log cfu/ml to

6.57 log cfu/ml (Fekadu, 1994; Alganesh, 2002; Zelalem and Faye, 2006; Asaminew and Eyassu, 2010; Asrat et al., 2012; Abebe et al., 2012). Higher counts of different species of *Enterobacteriaceae* were reported with *E. coli* being the most abundantly isolated species (Zelalem et al., 2007a), which is a good indicator of recent fecal contamination (Bintsis et al., 2008). Similarly, the mean coliform counts of raw milk in different part of Ethiopia are similar with the reports of Rai and Dawvedi (1990) from India (5.89 log cfu/ml); Kurwijila et al. (1992) from Tanzania (5 cfu/ml), and Ombui et al. (1995) from Kenya (4.67 log cfu/ml) and Bonfoh et al. (2003) from Mali (6 log cfu/ml).

Even if, it is not practical to produce milk that is always free of coliforms. Their presence in raw milk may therefore be tolerated. However, if present in large numbers, over 100 coliform organisms per milliliter of raw milk, it means that the milk was produced under improper procedures (Walstra et al., 2006). Hence their presence in large number in dairy products is an indication that the products are potentially hazardous to the consumers' health (Godefay and Molla, 2000). It is not only the bacterial counts, which affect the hygienic quality of milk but also the type of bacteria. Microorganisms like *Staphylococcus* spp.; *Streptococcus* spp. and *Escherichia* spp. and *Bacillus* spp. being the most abundantly isolated species in raw milk samples (Tollossa et al., 2012; Shunda et al., 2013), however, the occurrence of pathogenic strains in milk products, which could be hazardous for consumers.

ERGO (NATURALLY FERMENTED MILK)

Ergo is a traditional fermented milk product, which is made by natural fermentation of milk under ambient temperature, with no defined starter cultures used to initiate the fermentation processes (Assefa et al., 2008). As a result, the microbial load of fermented milk samples, including *Ergo*, could vary from sample to sample based on the microbial load and types of microbes in the original raw milk (Abdulkadir et al., 2011).

Ergo is semi-solid, smooth and uniform appearance and usually has a white milk color with pleasant odour and taste when prepared carefully. It constitutes primary sour milk from which other products may be processed. Depending on the storage temperature, it can be stored for 15-20 days (Almaz et al., 2001). It is popular and is consumed in all parts of Ethiopia by all age categories of people (YONAD, 2009).

Ergo has received extensive microbiological works and it has been found that lactic acid bacteria (LAB) dominated all other microorganisms, followed by yeasts and then moulds. Almaz et al. (2001) reported that Ergo fermentation is carried out by lactic acid bacteria belonging to the genera, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus*. Same

authors also reported that *Micrococcus* sp., coliforms and spore formers were also present in fairly high numbers during the first 12-14 h of fermentation. Their population decreased substantial thereafter, which implies an antimicrobial activity besides low pH in the fermented milk (Savadogeet al., 2004).

The report of Assefa et al. (2008) who indicated 12 LAB isolates from Ergo that include *Lactobacillus plantarum*, *Lactococcus lactis* ssp *cremoris*, *Lactococcus lactis* ssp *lactic*, *Lactobacillus acidophylus*, *Leuconostoc lactis*, *Pediococcus pentosace* and *Pediococcus* ssp. to have antimicrobial activities against different pathogenic microbes including *Shigella flexinery*, *Salmonella typhi*, *Escherichia coli* and *Staphylococcus aureus*. Eyassu et al. (2012) also found diversity of lactic acid bacteria isolated from the traditional fermented camel milk were *Lactobacillus plantarum*, *Lactobacillus delbrueckii subspecies bulgaricus*, *Lactococcuslactis subspecies cremoris*, *Lactococcuslactis subspecies lactis*, *Enterococcus faecalis* in Ethiopia, Savadogo et al. (2004) from Burkina Faso and Abdelgadir et al. (2001) from Sudan which comprised mainly of lactobacilli and lactococci.

According to Ashenafi (2002), in most households of Ethiopia no attempt is made to control the fermentation process of milk and products manufactured under traditional systems generally have poor qualities and do not meet the acceptable quality requirements set by various regulatory agencies. Zelalem (2010) observed the mean yeast and mould counts exceeded 8 log cfu/ml of Ergo sampled from different part of the country. Yeast and mould count of up to 4.6 log cfu/ml of fermented milk sampled from Southern Ethiopia was also reported (Fekadu, 1994). These values are much higher than the acceptable value <10 cfu/ml for yoghurt (Mostert and Jooste, 2002). The presence of different species of yeast in milk and its products may result in the spoilage of the product or conversely could contribute to the enhancement of the flavor of fermented milk, since different yeast species are able to assimilate different milk substrates (Gadaga et al., 1999). Zelalem et al. (2007a) identified *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii* and *Enterobacter sakazakii* were isolated from Ergo samples collected from smallholder producers in the central Ethiopia.

The average total bacterial count during Ergo fermentation in raw milk collected from eight dairy farms in Awassa was greater than 9 log cfu/ml (Ashenafi, 1995). Similarly, TBC of Ergo was generally high ranging from 7.71 log cfu/ml in samples collected from Sheno to about 10 log cfu/ml in samples from Jimma (Zelalem, 2010). Fekadu (1994) also reported 8.6 log cfu/ml total bacterial counts greater than for 75% of fermented milk samples collected from three villages in Southern Ethiopia. Coliform count averaging at 6.57 log cfu/ml was reported for Ergo samples (Zelalem and Faye, 2006).

Lower values were also observed in which 75% of the samples showed coliform count less than 4.4 log cfu/ml (Fekadu, 1994). The average Enterobacteriaceae and coliform counts reported were 4.95 and 4.51 log cfu/ml from Ergo sampled Sheno to Jimma (Zelalem, 2010). The existence of coliform bacteria in milk and milk products is suggestive of fecal contamination and unsanitary practices during production, processing, or storage.

The common traditional milk processing techniques involve smoking of processing utensils using embers of *Olea fricana*. This smoking practice is reported to be beneficial to keep better quality of Ergo through its inhibitory effect on spoilage and pathogenic organisms. For instance, the inhibitory effect of smoking on *Listeria monocytogenes* was reported (Ashenafi and Fekadu, 1994). The effect of lower pH of Ergo in controlling the proliferation of undesirable microorganisms is more effective after 24 h of incubation. However, at this time, the Ergois considered to be too sour for direct consumption since Ergo coagulates within 24 h and preferably consumed at this time for its good flavor (Ashenafi, 2006). Accordingly, the same author recommended that milk should be boiled beforehand and a small amount of three days old Ergo that is normally free from pathogens but contains enough LAB should be inoculated to initiate fermentation.

KIBE (TRADITIONAL BUTTER)

Traditional Ethiopian butter Kibe is always made from Ergo and not from cream (O'Connor et al., 1994). This traditional butter processed and sold by women in every community (YONAD, 2009). Kibe has an attractive appearance with a white to light yellowish colour. Like factory processed butter, it is semi-solid at room temperature. It has a pleasant taste and odour when fresh, but with increased storage, changes occur in odour and taste, unless refrigerated or further processed into NeterKibe (traditional Ghee) by boiling with spices.

Kibe is the most shelf stable of all traditionally processed fermented milk products except for Neter Kibe. Kibe is important role in the diet, both in rural and urban areas, and is utilized also by children of weaning age and the elderly. In addition to direct consumption as a side dish, it is used as oil for food preparation and, after processing into Neter Kibe, it is also used for hairdressing and as a skin cosmetic by both sexes (YONAD, 2009) and it is used for roasting coffee beans in special traditional ceremonies.

The moisture content of the traditional Kibe ranges from 20 to 43% as compared to the international commercial standard for butter of 16%. Spoilage occur when stored at room temperature for a long time is probably mainly by putrefactive microorganisms. Butter is highly stable against microbial spoilage after 2% salt addition, because of its high fat ratio, low moisture and

nitrogen ratio. Microorganisms having lipolytic activity are highly responsible for disorders such as rancidity or loss of flavor. Microbiological information on this product is not fully available in Ethiopia. However, there are some studies published on the microbiological quality of traditional butters from the country (Almaz et al., 2001; Wondu, 2007; Zelalem, 2010).

According to Zelalem (2010) the average total bacterial counts ranged from 6.18 cfu/g in butter samples collected from Selale area to 7.25 cfu/g in samples from Sululta. On another studies the average TBC of 7.49 cfu/g and the presence of high variability among samples depending on the sources were reported (Wondu, 2007). Samples collected from open markets and rural producers, for instance, had higher counts as compared to that obtained from dairy farms and urban producers of southern Ethiopia. In addition to this the TBC of fresh butter sampled from rural and public butter markets in Addis Ababa ranged from 8.27 to 4.7 log cfu/g of butter (ILCA, 1992). These values are higher than the acceptable limit of 4.69 log cfu/g (Mostert and Jooste, 2002). This indicates Kibe produced from different part of the country were substandard handling conditions at all stages in the milk chain.

Coliforms as a hygiene indicator besides moulds and total aerobic mesophilic bacteria as a general hygienic quality factor are important criteria for the determination of the microbiological quality of butter. Zelalem (2010) reported the average Enterobacteriaceae and coliform counts were greater than 4 cfu/g of butter sampled from different parts of the country. Coliform counts ranging from 1.92 to 4.5 log cfu/gm of butter are reported (Wondu, 2007; Asfaw, 2008; Zelalem et al., 2007a). These differences could be attributed to the wide variation in hygienic handling during milking, processing, storage and transport to market. In addition to this Alemu et al. (2013) observed the coliform count of butter samples made from camel and goat milk were 3.07 logcfu/g and 3.02 logcfu/g, respectively. Generally the Enterobacteriaceae and coliform count reported in the country were higher than the acceptable value of <10 cfu/g (Mostert and Jooste, 2002).

The primary spoilage factors in butter are moulds and mostly greenish colored moulds are seen, but red, black and brown colored ones are also seen in butter. The majority of the moulds growing in butter are composed of the species of *Thamnidium*, *Cladosporium* and *Aspergillus*. Through the application of a proper heat treatment, moulds cannot survive in cream even if contamination exists. So, the presence of mould contamination in butter indicates contamination by water or air after production. The protective effect of salt added to butter cannot be underestimated in terms of moulds. The mean yeast and mould counts observed in the Ethiopian highlands were exceeded 8 cfu/g of butter sampled from Addis Ababa to Jimma (Zelalem, 2010). Yeast and mould counts ranging between 4.3 and 6.86

log cfu/g of butter sampled from Wollayta area are reported (Asfaw, 2008). Average yeast and mould count of 7.65 log cfu/g of butter was also reported (Wondu, 2007). Higher values are observed (Zelalem et al., 2005) that varied depending on the type of producer where lower counts were observed for butter sampled from research centers and small-scale producers than that from large-scale producers.

ARERA (DEFATTED SOUR MILK)

Arrera is another byproduct of Ergo obtained after removal of Kibe after churning. It has a similar color to Ergo, but its appearance slightly smoother and its consistency thinner, although thicker than fresh milk and basically contains the casein portion of milk. Its taste and odor are similar to those of Ergo. In contrast to other traditional dairy products, Arrera has less calories. It contains 91.5% moisture, 3.1% protein, 1.4% fat, 3.4% carbohydrate, and 0.6% ash (EHNRI, 1997).

Arrera has a shorter shelf life compared to all other fermented milk products (only 24-48 h), even when smoke is applied to the equipment used for its storage. The product is consumed in all parts of the country where fermented milk is produced and it serves as a beverage either plain or spiced. It is preferred by women for consumption as a side dish or as drink (YONAD, 2009). Surpluses are given to calves, lactating cows and dogs (Almaze et al., 2001). However, it may indirectly serve as additional income for the women by its use as raw material for cottage cheese (Ayib) manufacture, which may be sold in the market. Due to its relatively short shelf life and some traditional taboos or beliefs, Arrera is not sold in the market for direct consumption.

The average counts of total bacteria, Enterobacteriaceae and coliforms were greater than 9, 4.7 and 4.2 cfu/ml, respectively of Arera sampled from Addis Ababa to Jimma (Zelalem, 2010). These values are higher than the acceptable value of <10 cfu/gm (Mostert and Jooste, 2002). Traditionally produced Arera sampled from Wollayta area had total bacterial count of about 9 log cfu/ml (Rahel, 2008). The same author also reported coliform count of 4.86 log cfu/ml. Different species of bacteria were identified in Arera samples collected during both dry and wet seasons, which include: *K. pneumoniae*, *K. oxytoca*, *E. cloacae*, *E. sakazakii*, *E. coli* and some species of Salmonella (Zelalem et al., 2007a).

AYIB (ETHIOPIAN COTTAGE CHEESE)

Ayib is a soft curd-type cheese typical of many regions in Ethiopia, and is made from the defatted sour milk resulting from the churning of sour whole milk by heating the defatted sour milk to coagulate the curd (O'Connor, 1994; Binyam, 2008). The product is white, acidic and it

is mainly consumed locally. Ayib is as important as Kibe and contributes to the overall nutrition of the people and forms part of the staple diet. The resulting Ayib contains 76% water, 14% protein, 7% fat and 2% ash (O'Connor, 1994).

Soft cheeses have higher moisture content when compared to hard cheese and have lower keeping quality due to microbial spoilage (Rhea, 2009). Most soft, unripened cheeses are microbiologically unstable due to metabolic activity of bacteria, yeast or mould contaminants. Rhea (2009) reported the isolation of pathogenic microorganisms such as *L. monocytogenes*, and *S. aureus* from soft cheese. *Escherichia coli* serotype 0157:H7 has been associated with the consumption of French Brie and Camembert soft cheese in the US and Scandinavia. The bacteria species identified in Ethiopian cottage milk samples were *Bacillus cereus* and *S. aureus* (Ashenafi, 2006; Mekonen et al., 2011) in Awassa and Debrezite, respectively. The occurrences of most food toxins caused by *S. aureus* were the result of bad hygienic practices in the household (Seifu et al., 2013). On the other hand *Klebsiella*, *E. coli*, *Enterobacter* and *Klyuvera* species were also reported (Zelalem et al., 2007b; Seifu et al., 2013).

The safety of cheese with respect to food born diseases is a great concern around the world and in developing countries (Ashenafi, 2006). This is especially true in Ethiopia where the consumption of Ethiopian cottage cheese (Ayib) which is typically manufactured in small dairy farms under poor hygienic conditions is common practices (Alehilign, 2004). In spite of the aforementioned prevailing situation and the presence of a number of public health concerns due to food born diseases resulting from the consumption of different food item in Ethiopia there is scarcity of well documented information in general and Ethiopian cottage cheese in particular.

Although the microbial quality of soft, white cheese, high counts of microorganisms were found in the majority of the samples of Ethiopian cottage cheese samples collected from an open market in Awassa had high counts of mesophilic aerobic bacteria, yeasts and enterococci (Ashenafi, 2006). Same author also reported aerobic mesophilic bacterial counts of over 8 log cfu/g. Comparable counts are also reported (Zelalem et al., 2007b; Binyam, 2008; Seifu et al., 2013). The sources of contamination could be from handlers, and utensils, used for packaging and possibly imparting flavor (Ashenafi, 2006). Eyassu (2013) reported total viable bacteria count ranging from 5.4 to 7.8 log cfu/g from Metata Ayib samples which are made from Ethiopian cottage cheese in West Gojjam zone. The maximum limit of aerobic mesophilic bacterial count which is commonly employed to indicate the sanitary quality of food, for raw milk intended for processing is 5 log cfu/ml and intended for direct human consumption is 4.69 log cfu/ml (Council Directive 92/46 EEC 1992).

Earlier research done in different parts of the country the coliform counts of Ayib samples were reported on the range between 2 and 5.70 log cfu/g (Ashenafi, 2002, 2006; Zelalem et al., 2005; Binyam, 2008; Seifu et al., 2013) with differences being a function of source of samples and handling conditions. However, the coliforms were not detected in the MetataAyib samples collected from west Gojjam. The absence of coliforms in the Metata Ayib it is due to the low pH (4.0) of MetataAyib which might have inhibited the growth of coliforms. As indicated by Zelalem et al. (2005) coliform counts varied among samples collected from different producers where Ayib sampled from research centers had lower coliform counts (4.85 log cfu/gm) as compared to that sampled from large-scale (5.68 log cfu/gm) and small-scale (5.48 log cfu/gm) farms showing variations in the hygienic conditions practiced among the different producers. In all cases values are higher than the recommended level of <10 cfu/g (Mostert and Jooste, 2002) indicating the poor hygienic conditions practiced during production, processing and handling of milk and milk products.

According to Seifu et al. (2013) the mean count of Enterobacteriaceae Ayib sample collected from five vendors of open market places at Jimma town was 6.504 log cfu/g, similar values were reported (Ashenafi, 2006; Zelalem et al., 2007b; Tekletsadik and Tsige, 2011). Related literature values show that the count of Enterobacteriaceae in the cheeses made from heat treated milk is 3 log cfu/g (CEC, 2005), and in the ready to eat foods more than 4 log cfu/g is unsatisfactory (Gilbert et al., 2000). Binyam (2008) reported yeast and mould in Ayib sampled in Shashemane with average counts of around 6.35 log cfu/g of the product, which indicated a suboptimal microbial quality of market Ayib in the area. Ashenafi (2006) on the other hand reported lower values (5 log cfu/g) for most Ayib samples in Southern Ethiopia. Generally countries produced soft cheeses including Ethiopian cottage cheese due to poor keeping quality and public health risk to the consumer. Therefore, strict hygienic control measures along the food chain to improve the hygienic conditions during manufacturing, handling, storage and commercialization of cheese in order to guarantee the quality of this highly popular product in the country.

CONCLUSION

Milk and milk products play a crucial role in human nutrition both urban and peri-urban area of the country. However, evidences showed that the microbial properties milk and Ethiopian traditional fermented milk products made from different dairy producer were substandard in quality. This is due to absence standard hygienic condition followed by producers during milk production. The hygienic conditions under smallholder conditions is limited to letting the calf to suckle for few minutes and/or

washing the udder before milking. Moreover, unhygienic cleaning and handling of milk containers and inadequate dairy infrastructure coupled with limited knowledge of the hygienic production and handling of milk and milk products result in the contamination of the milk. Therefore, strict hygienic control measures along the food chain to improve the hygienic conditions of milk from production to consumption are necessary.

Conflict of Interests

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

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Full Length Research Paper

Microbial changes in egg white “curd/cottage cheese” during storage

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Received 10 February, 2014; Accepted 19 May, 2014

Egg white curd cheese is a curdled protein concentrate made from egg whites. Its advantages are: high protein content, a taste similar to cow's milk curd cheese, minimum carbohydrates, and no fat or cholesterol. Thus, it offers an attractive substitute for cow's milk curd cheese to many followers of a “modern lifestyle” as well as those living with health problems (sensitivity to milk products). Both the internal and sensory characteristics of egg white curd cheese were studied. In addition, the level of potentially dangerous microbes in the product was calculated. The fresh product was in excellent microbiological condition, and over a storage period of three weeks, no *Salmonella* or *Staphylococcus aureus* could be detected in it. The levels of *Enterobacteriaceae* and all live aerobic spore counts were completely satisfactory. In summary, it can be stated that we have succeeded in developing a microbiologically stable product which is applicable for numerous diets, has a flavor resembling mild cow's mild curd cheese, lacks enhancers and preservatives, and can even be consumed as it is.

Key words: Eggwhite, curdcheese, *Salmonella*, paleolithic diet.

INTRODUCTION

Egg represents a self-contained, balanced source of nutrition for people of all ages. Its yolk provides essential unsaturated fatty acid (linoleic acid 18:2n6), mono-unsaturated oleic acid, iron, phosphorus and numerous trace elements. Egg white, on the other hand, is rich in protein of high biological value. Egg protein is often taken

as a standard when determining the protein value of many other foods (Layman and Rodriguez, 2009). A further extremely important characteristic of egg white is that it lacks fat and carbohydrate, or rather contains them in negligible amounts (Table 1). In addition, it can be considered cholesterol-free (Cotterill and Glauert, 1979).

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Table 1. Total composition of one fresh chicken egg weighing 58 g (Zoltán, 1997).

Component	Internal contents		Yolk	Egg white	Shell and membrane
	With shell (%)	Without shell (%)			
Whole egg	100.0	-	32.3	56.7	11.0
Water	65.6	73.6	48.7	87.9	2.0
Egg white	12.1	12.8	16.7	10.6	4.5
Fat	10.5	11.8	32.6	-	-
Carbohydrate	0.9	1.0	1.0	0.9	-
Ash	10.9	0.8	1.0	0.6	93.5

Table 2. Chromaticities detected by the average human eye.

dE*ab	Level of discrimination
0 ... 0.5	Undetectable
0.5 ... 1.5	Threshold
1.5 ... 3.0	Detectable
3.0 ... 6.0	Recognizable
6.0 ... 12	Distinctly recognizable

Thanks to these positive qualities, it has become a vital part of many modern diets, including reducing diets, not to mention bodybuilders' nutrition.

Egg processors continually work to bring new products to market. They likewise attempt to develop products which meet customers' demands. One example of these consumers is the one who follow paleolithic diets, which means they do not use dairy products, but they do eat eggs. Arising from this situation is the possibility of developing egg-based products which mimic the taste of milk products. One of such product is 100% egg white cheese curd, which may be useful not only in the paleolithic diets mentioned above, but also in the diets of those sensitive to milk, people on weight-loss regimens and bodybuilders. Herein, we present results obtained in the course of our work with experimental products. Naturally the sensory tests have serious significance for foods, but we plan to publish this data (which shows a very positive picture) at a later date.

MATERIALS AND METHODS

Microbiological experiments

Egg white curdcheese (1 kg product containing the egg white of 60-70 pcs eggs) is made of egg white, with addition of salt and vinegar. Its carbohydrate and salt content are both 0.4%. Its unopened package provides 30 days shelf life (www.capriovus.eu). Samples were stored at 4°C for five weeks. Weekly counts were made for the number of mesophilic organisms which had developed.

Pulp was prepared in glass Petri dishes using sterile utensils in a sterile environment, after which one gram of sample was added to 9 ml peptone water and shaken for 5 min. Each sampling was done 3 times the same way. These samples were diluted ten-fold with sterile peptone water. Plate-pouring was done as follows: in the

case of total spore count, Nutrient agar; for *Salmonella*, XLD and Harlequin Salmonella ABC agar; for *Listeria monocytogenes*, PALCAM agar; for *Escherichia coli*/coliform, Chromocult agar; for *Staphylococcus aureus*, BAIRD-PARKER agar; and for *Enterobacteriaceae*, VRBG agar. Thus, the samples were counted for total aerobic spores, *Salmonella*, *Listeria*, *E. coli*/coliform, *Staphylococcus aureus* and *Enterobacteriaceae*. Plates were incubated for 48 hours at 30 (total spores) and 37°C, after which the number of colonies was determined with a colony counter.

Examination of physical parameters

A Sartorius dry matter moisture meter was applied seven times simultaneously for fast determination of sample contents.

During the 5-week storage period, a Minolta CR-200 tri-stimulus color meter was used to determine color changes in the samples. The measurement is based on the theory of mixing in color additive, by which any color can be produced in three given wavelengths, mixture by mixture. The three-dimensional CIELAB color space was used: L* - lightness (showing what percentage of light is reflected by the object), +a* - red hue, -a* - green hue, +b* - yellow hue, -b* - blue hue. In the color space, the following formula is used to calculate the difference between two points:

$$dE^* = \sqrt{dL^{*2} + da^{*2} + db^{*2}}$$

Where, dE* = color variation, dL* = L*sample 1 - L*sample 2, da* = a*sample 1 - a*sample 2, db* = b*sample 1 - b*sample 2.

Table 2 shows the hues that can be distinguished by the average human eye.

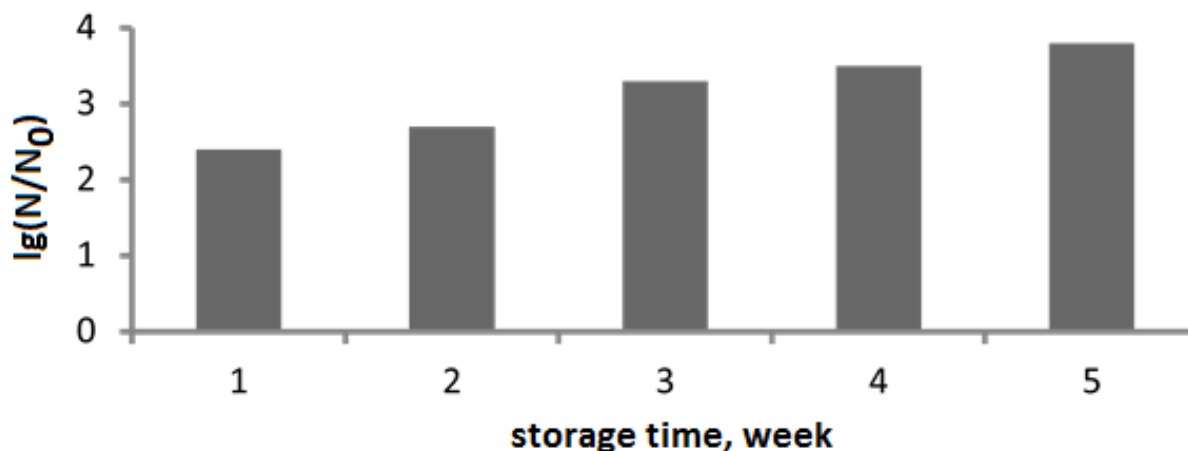
RESULTS

Over the 5-week storage period, no pathogenic microbes were evident in the samples, and the *Enterobacteriaceae* count was below the detectable range. A slight increase

Table 3. Results of pathogen tests, egg white curd cheese under cold storage.

Microbe	Allowed by Hungarian regulations	1st week	2nd week	3rd week	4th week	5th week
<i>Salmonella</i>	M=0/25 g	neg.	neg.	neg.	neg.	neg.
<i>L. monocytogenes</i>	M=0/25 g	neg.	neg.	neg.	neg.	neg.
<i>E. coli</i>	m<1 M<10	<0.3	<0.3	<0.3	<0.3	<0.3
<i>S. aureus</i>	m=10 ² M=10 ³	<10	<10	<10	<10	<10
<i>Enterobacteriaceae</i>	m=10 M=10 ²	<10	<10	<10	<10	<10

neg. = Negative.

**Figure 1.** Changes in viable cell counts, egg white curd cheese, during 4°C storage.

in viable cell count occurred, but aerobic spore counts of the study subject, egg white curd cheese, remained well below the prescribed level ($N \leq 10^5$ CFU/ml) (Table 3).

Unchanged color is important for egg white curd cheese under storage. Our results show that no changes occurred in the samples during the first four weeks in storage, and there was a barely detectable change in color during the fifth week (Figure 2).

Taking both microbiological and sensory studies into account, it is possible to state that for four weeks, the product maintained both its microbiological and color stability.

The dry matter content of the egg white curd cheese samples remained above 20 m/m% throughout the trials. Egg white cottage cheese is a completely new food, so comparing our results with the data of the literature is a complex task. On the one hand, we carried out a comparison with cow's milk cottage cheese, since the goal was to produce a similar material. On the other hand, we searched for similarities with boiled egg products, which, like egg white cottage cheese, are subjected to 90-100°C heat treatment during processing.

The microbiological condition of egg white cottage cheese much more resembles boiled egg products, due

to the relatively high temperature heat treatment. The total mesophilic aerobic live spore count is approximately 10^3 CFU/g or below, after four weeks' cold storage (Figure 1), whereas we were unable to detect any microbes in the cottage cheese which are harmful to health (Ho et al., 1986; Ashenafi, 1990; Kim et al., 2008). Thus, the material was held four weeks in cold storage without special protective gas packaging or vacuum pack, which for cow's milk cottage cheese is only possible for a much shorter time (Mannheim and Soffer, 1996).

Likewise, results of our color measurements were similar to those for boiled egg products or gas packed cow's milk cottage cheese. Changes in color in egg white cottage cheese were much more favourable than in the case of cow's milk cottage cheese. This is due to the tendency of cow's milk cottage cheese to turn sour, which may lead to a more intensive change in color even in the first week (Shellhammer and Singh, 1991). It is also favorable that the dEN* value remained below 0.3 in egg white cottage cheese stored cold for 4 weeks, similar to other foods which may be kept for a long time in refrigeration, such as certain meat products, dairy items and tinned goods (Cheftel and Culioli, 1997; Trezza and Krochta, 2006).

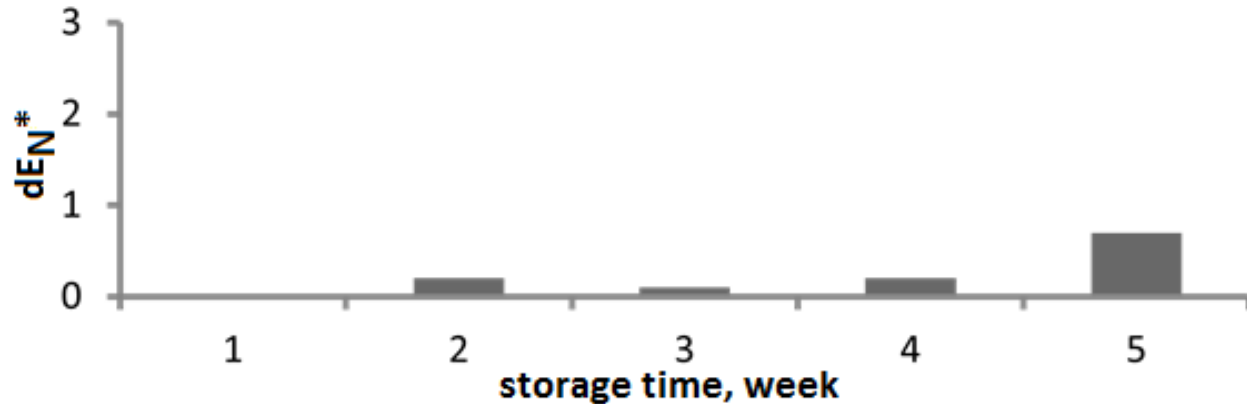


Figure 2. Changes in egg white curd cheese during storage at 4°C.

Conclusions

In summary, it can be stated that this product: applicable for numerous diets, similar in taste to cow's milk curd cheese, free of stabilizers, emulsifiers, and preservatives, and which can be consumed as it is; remained stable both microbiologically and in color throughout the four weeks of storage at 4°C. Therefore, egg white curd cheese, when refrigerated properly, is suitable for sale in the retail market.

ACKNOWLEDGEMENT

Our research materials were produced with generous contributions from the KMR_12-1-2012-0181 research and development project titled "Support of market-oriented development ability in the central Hungary region." We are very grateful for this. This work was supported by the Faculty Research Support-Research Excellence Centre of Excellence 17586-4/2013/TUDPOL project.

Conflict of Interests

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

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Full Length Research Paper

Activity of beta-lactam antibiotics and production of beta-lactamases in bacteria isolated from wound infections in Brazzaville, Congo

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Received 28 July, 2012; Accepted 28 April, 2014

To determine the mechanism of bacterial beta-lactam resistance, 165 bacteria isolated from wounds of hospitalized patients composing of: 42 *Staphylococcus aureus*, 37 *Pseudomonas aeruginosa*, 23 *Escherichia coli*, 22 *Proteus*, 12 *Klebsiella*, 10 coagulase-negative staphylococci (CNS), eight *Enterobacter*, six *Citrobacter*, five *Providencia* were tested for their sensitivity to beta-lactams and their production of beta-lactamases. The antibiotic susceptibility was considered by the method of the standard diffusion on agar Mueller Hinton. The rate of production of β -lactamase in all bacteria was determined using the Strips of nitrocefin. The percentages of resistance to beta-lactams obtained were as follows: *Staphylococcus aureus* (77.90%), *Pseudomonas aeruginosa* (44.14%), *E. coli* (73.8%), *Proteus* (57.4%), *Klebsiella* (63.6%), CNS (57.15%), *Enterobacter* (56.3%), *Citrobacter* (83.3%), *Providencia* (67.5%). The rate of beta-lactamases were as follows: *S. aureus* (7.34%), *P. aeruginosa* (89.19%), *E. coli* (95.65%), *Proteus* (86.36%), *Klebsiella* (91.67%), CNS (90%), *Enterobacter* (87.5%), *Citrobacter* (66.67%), *Providencia* (100%). The studied bacteria produce beta-lactamases which is the primary mechanism of resistance to beta-lactam antibiotics in the majority of the bacteria. Beta-lactamases rates vary from one genus to another. It is extended spectrum beta-lactamase-producing strain.

Key words: Bacteria resistance, beta-lactamases, wounds infections, Brazzaville.

INTRODUCTION

Since the discovery of penicillin 80 years ago, Gram-negative bacteria have become proficient at evading the

lethal activity of β -lactam antibiotics, principally through the production of β -lactamases (Rapp and Urban, 2012).

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Table 1. Activity of beta-lactam antibiotics on staphylococci.

Bêta-lactam tested	<i>Staphylococcus aureus</i>		<i>Negative coagulase Staphylococci</i>	
	S (%)	R (%)	S (%)	R (%)
Amoxicillin+acid clavulanic	10 (23.80)	32 (76.20)	2 (20)	8(80)
Penicillin	0	42 (100)	0 (0.00)	10(100.00)
Céfalotin	3 (7.14)	39(92.86)	1(10.00)	9(90)
Cefotaxime	0	42 (100)	0(0.00)	10(100.00)
Ceftazidime	20 (47.60)	22 (52.40)	8(80.00)	2(20)
oxacillin	3 (7.14)	39 (92.86)	9(90)	1 (10)
Imipénème	29 (60.10)	13 (30.90)	10(100.00)	0(0.00)
Total (%)	65 (22.10)	229 (77.90)	30(42.85)	40 (57.15)

Beta-lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contain a β -lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams and carbapenems (Holten and Onusko, 2000). They are a family of first-line antibiotics both in terms of availability and in terms of the cost. Since their discovery by Flemming in 1929 with penicillin, they are commonly used in the treatment of various infections (Livermore and Brown, 2001).

Their use is of therapeutic importance, in the improvement of the infectious diseases treatment, the reduction of mortality and the eradication of the once formidable diseases. However, since their introduction in medicine, bacteria have developed mechanisms that have resulted in the emergence of the multi resistance. Several biochemical or genetic mechanisms are responsible for this resistance, including the production of enzymes (Andremont et al., 1997; Zogheib and Dupont, 2005). The infections caused by extended-spectrum beta-lactamase (ESBL) producing bacteria, constitute severe problems (Kuzucu et al., 2011).

The purpose of this study was to assess the rate of resistance of these bacteria to beta-lactams and determine the possible mechanism of inhibition of these antibiotics.

MATERIALS AND METHODS

Strains and used antibiotics

Samples were cultured and bacterial isolation was done by conventional methods (Esmaeili et al., 2014). 165 bacteria isolate composing of: 42 strains of *Staphylococcus aureus*, 10 coagulase-negative staphylococci (CNS), 23 *Escherichia coli*, 22 *Proteus*, 12 *Klebsiella*, 8 *Enterobacter*, 6 *Citrobacter* and 5 *Providencia* were tested for their sensitivity to beta-lactam antibiotics.

In staphylococci, seven (7) beta-lactam antibiotics were tested: penicillin G (Oxoid 10 IU), oxacillin (Oxoid 5 μ g), amoxicillin + clavulanic acid (Oxoid 30 μ g), cefalotin (oxid), ceftazidime (bioMérieux), cefotaxime (oxid) and imipeneme (bioMérieux).

In enteric bacteria, eight (8) antibiotics of this family were tested: amoxicillin (oxid) amoxicillin + clavulanic acid (Oxoid 30 μ g),

cefalotin (oxid), ceftazidime (oxid), cefotaxime (oxid), cefuroxime (oxid) and imipeneme (bioMérieux).

For the *Pseudomonas* three (3) antibiotics were tested: carbenicillin (oxid), ceftazidime (bioMérieux) and imipeneme (bioMérieux).

Strains identification

Gram negative bacteria which belong to the enteric bacteria group were identified and used in a leminor galery which comprise urea-indole, Kligler Hajna, citrate Simmons. The identification was confirmed by the tests of ONPG and sugar fermentation.

For pyocyanic bacilli, enhancing pigment production galery was assayed with the King A and B media.

In the genus staphylococcus, Gram positive bacteria were isolated and identified by using cultural and biochemical characters using Chapman media (production or not of yellow pigment). Identification was confirmed by the tests of catalase and coagulase.

Strains susceptibility

Betalactam antibiotics susceptibility was determined by disk diffusion on Mueller Hinton (Sanofi pasteur) medium (Carret et al., 2001; Esmaeili et al., 2014). Discks of beta-lactam antibiotics have been used in natural resistance to each type or kind of bacteria. The method, Etest was also used as described by Saito et al. (2013).

The beta-lactamases were detected using a chromogen substrate, laboratories Oxoid nitrocefin. A colony of a growing strain was put in contact with the substrate on the strip of nitrocefin. After 5 to 10 min of contact, two (2) drops of reagent were added to the nitrocefinase. Hydrolysis by beta-lactamase substrate results in the development of a red color with a blue ring on the disk, which reveals the complex enzyme-substrate (Spicier, 2001). The beta-lactamase distribution was analyzed by excel.

RESULTS

The activities of the beta-lactam antibiotics tested on the staphylococci are given in Table 1 and those of the *Enterobacteriaceae* are given in Table 2. For the *Pseudomonas*, the rate of resistance to beta-lactams obtained is 44.14% with total inhibition of carbenicillin. Ceftazidime and the imipeneme were respectively

Table 2. Activity of beta-lactams in Enterobacteriaceae.

Beta-lactam tested	<i>E.coli</i>			<i>Proteus</i>			<i>Klebsiella</i>			<i>Enterobacter</i>		<i>Citrobacter</i>		<i>Providencia</i>	
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Amoxicillin	0		23 (100)	2 (9.0)		20 (91)	0	2 (16.7)	12 (100)	0	8 (100)	0	6 (100)	0	5 (100)
Amoxicillin + acid clavulanic	2 (8.7)	1 (4.3)	20 (87.0)	1 (4.5)	2 (9)	19 (86.5)	2 (16.7)		8 (66.6)	1 (12.5)	7 (87.5)	0	6 (100)	0	5 (100)
Céfalotin	2 (8.7)	1 (4.3)	20 (87.0)	13 (59)		9 (41)	2 (16.7)		10 (83.3)	2 (25.0)	6 (75.0)	0	6 (100)	0	5 (100)
Ceftazidime	16 (69.7)		7 (30.4)	17 (77.3)		5 (22.7)	10 (83.3)		2 (16.7)	6 (75.0)	2 (25.0)	2 (33.3)	4 (66.7)	4 (80.0)	1 (20.0)
Céfuroxime	1 (4.3)		22 (95.7)	2 (9.0)		20 (91)	1 (8.3)		11 (91.7)	Not tested	-	Not tested	-	Not tested	-
Céfotaxime	3 (13.0)		20 (87.0)	7 (31.8)		15 (68.2)	2 (16.7)		10 (83.3)	Not tested		0	6 (100)	0	5 (100)
Imipénème	22 (95.7)		1 (4.3)	19 (86.4)		3 (13.6)	8 (66.6)	1 (8.4)	3 (25)	7 (87.5)	1 (12.5)	4 (66.7)	2 (33.3)	5 (100)	0
Céftriaxone	6 (26.1)		17 (73.9)	12 (54.5)		10 (45.5)	7 (58.3)		5 (41.7)	5 (62.5)	3 (37.5)	2 (33.3)	4 (66.7)	4 (80.0)	1 (20.0)
Total (%)	25.2	8.6	73.8	41.5	1.1	57.4	33.3	3.1	63.6	43.7	56.3	16.7	83.3	32.5	67.5

inhibited by 21.62 and 10.82% of strains. The numbers of positive strains to the test of nitrocefin for each group of bacteria as well as the rate of beta-lactamases are respectively given in Table 3 and Figure 1.

DISCUSSION

Resistance of Staphylococci to tested beta-lactam antibiotics is not very high. These results are consistent with those given in the literature by several authors such as Aryam et al. (2005) and Tronel et al. (2002). These results can be explained by their use as first-line antibiotic.

The meticillino-observed resistance is due to the

acquisition and integration of the *mecA* gene, which induces the synthesis of the PLP2a which is able to assemble Peptidoglycan with low affinity for oxacillin (methicillin) and other beta-lactam antibiotics (Fatholahzadeh et al., 2008; Benoit et al., 2013).

In enteric bacteria, the imipeneme and ceftazidime have been the most active beta-lactams. These results are consistent with that of Sotto et al. (2001) and Lagacé-Wiens et al. (2014). The sensitivity rates of these strains to ceftazidime, were compared with those obtained by Petra et al. (2007).

The association of amoxicillin with clavulanic acid has been completely inactive in some Enterobacteriaceae, and the other has presented

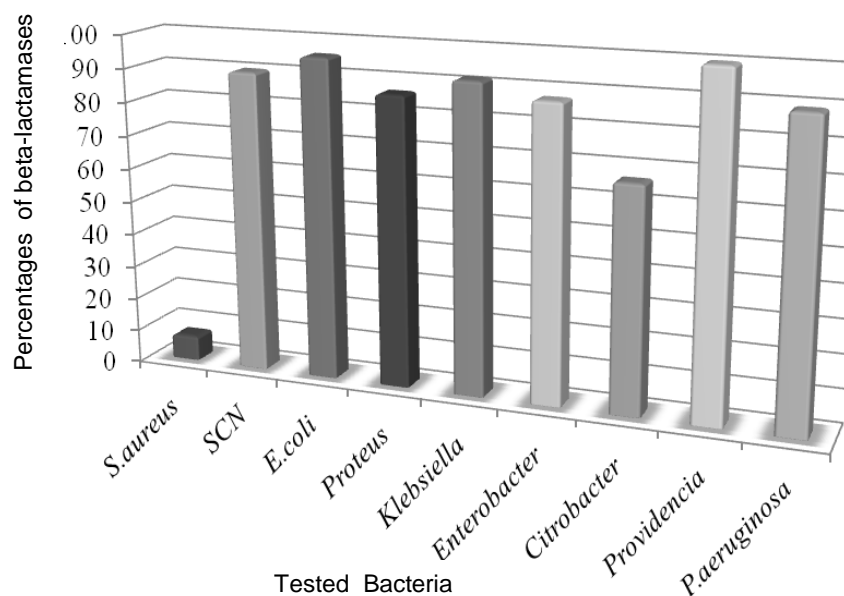
a high resistance. This result can be explained by the modification of penicillin (PLP) binding protein which confers resistance to most of the beta-lactam antibiotics (Sotto et al., 2001). The cephalosporin's resistance may be due to the production of cephalosporinases as suggested by Bertrand and Talon (2001). In most cases, the mechanism of resistance to beta-lactams in Enterobacteriaceae is the production of beta-lactamases (Bedenic, 2004).

With regards to *Pseudomonas*, among the tested beta-lactam antibiotics, total resistance to carbenicillin was observed. This result is different from those obtained by Yu et al., (2006) which demonstrated that carbapenems remain the drugs of choice for serious infections caused by

Table 3. Distribution of positive beta-lactamase strains.

Bacteria tested	Total number of strains	Number of positive strains
<i>S. aureus</i>	42	3
CNS	10	9
<i>E. coli</i>	23	22
<i>Proteus</i>	22	19
<i>Klebsiella</i>	12	9
<i>Enterobacter</i>	8	6
<i>Citrobacter</i>	6	4
<i>Providencia</i>	5	5
<i>P. aeruginosa</i>	37	33

CNS = Coagulase negative Staphylococci.

**Figure 1.** Distribution of beta-lactamases rates.

ESBL-producing organisms.

The ceftazidime was inactivated by 21.62% of strains and two strains of intermediate sensitivity were observed. This level of sensitivity presents a slight difference with the data of Sevillano et al. (2006). The appearance of resistance to ceftazidime in *Pseudomonas* may be a marker of the multi resistance for hyper-producing beta-lactamases strains which represents a factor of risk for the emergence of these strains (Cavallo et al., 2003).

The imipeneme has been the most active antibiotic with only 10.82% of resistant strains. These results are consistent with those obtained by Sinave (2003) and Soussy (2001). The development of resistance to the imipeneme is a risk factor for emergence of strains of *Pseudomonas* multi resistant strain (Rossilini and

Mantengolie, 2005). However, high rates of resistance to the imipeneme and ceftazidime on hospital strains were obtained by Sekowska et al. (2005).

Most of the tested bacteria had shown high levels of beta-lactamases. These results have confirmed the work of Susić (2004), Akujobi (2005) and Bedenic (2004). The low rate obtained in *Staphylococcus aureus*, is explained by the resistance to oxacillin linked to the acquisition of the mec A gene. With regards to the strains of *Enterobacter*, beta-lactamases rates are comparable to those reported by Petra et al. (2007); Koren and Vaculíková (2006). These beta-lactamases can spread more often by the interspecific transfer of resistance between the cocci Gram positive and bacilli Gram negative by conjugation.

Conclusion

The results show that many bacteria present high resistance to the beta-lactam antibiotics. All the studied bacteria are beta-lactamase-producing. There is therefore a concordance between the resistance to these antibiotics and beta-lactamase production. The inhibition of these antibiotics is a major problem in the immediate support of the sick, because of their use as first line antibiotics.

Conflict of Interests

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

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Full Length Research Paper

Etiology of apple tree dieback in Tunisia and abiotic factors associated with the disease

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Received 2 July, 2013, Accepted 19 May, 2014

An apple tree dieback syndrome causing severe tree losses was observed in the main apple producing regions in Tunisia from 2006-2008. This work aimed at identification of the causal agents and the factors that promote apple tree dieback. The causal agents of the syndrome included the following: *Phytophthora* and *Pythium* species: *Phytophthora parasitica* and *Phytophthora inundata*, *Pythium* sp., *Pythium indigoferae*, *Pythium irregulare*, *Pythium rostratiformis*, *Pythium sterilum* and *Pythium undulatum*. Molecular techniques confirmed the results of the morphological identification. Pathogenicity assays showed the involvement of these pathogens in apple tree dieback. Soil salinity was also shown to be an important factor of disease severity in this study.

Key words: Apple, *Malus domestica*, orchard survey, dieback syndrome, sequencing, pathogenicity.

INTRODUCTION

Over 27000 ha of land is been cultivated for apple (*Malus x domestica* Borkh) in Tunisia. Tunisian apple growing-areas are mainly located along the northeast and northwest of the country. It is an economically important crop in Tunisia, mainly in the Kasserine region. Since 1996, a severe dieback of apple trees was observed at Kasserine, Bizerte, Jendouba, Beja and Ben Arous provinces. This dieback caused severe damages and tree losses in numerous apple orchards. Affected trees showed cankers and necrosis in the collar and roots.

Apple tree dieback could be attributed to numerous physiological or parasitical factors (Mazzola et al., 2002; Latorre et al., 2001; Jeffers and Aldwinckle, 1988;

Matheron et al., 1988; Jeffers et al., 1982), among them are Oomycete pathogens that usually play a significant role in the phenomenon (Jeffers and Aldwinckle, 1988; Bolay, 1992a; Latorre et al., 2001; Mazzola et al., 2002). Tsao (1990) indicated that numerous *Pythiaceae* species have been underestimated as pathogens that cause disease on numerous species world-wide. Moreover, numerous root and collar rots are still attributed to attacks by other microorganisms, or to abiotic factors (Jiménez et al., 2008). Recent studies have highlighted the importance of the genera *Pythium* (Romero et al., 2007) and *Phytophthora* (Moralejo et al., 2008; Moralejo et al., 2009) in natural or managed ecosystems. The present

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Table 1. Regions and number of orchards surveyed in each region.

Species	Locations	Isolates numbers
<i>P. irregulare</i>	Foussana	2
	Sbiba	8
<i>Pythium</i> sp.	Sbiba	6
	Mateur	2
<i>P. rostratiformis</i>	Foussana	2
	Utique	2
<i>P. undulatum</i>	Medjez el Beb	1
<i>P. sterilum</i>	Foussana	2
	Mateur	1
<i>P. indigoferae</i>	Morneg	3
	Foussana	2
	Mateur	2
	Utique	1
<i>P. inundata</i>	Mateur	1
	Jendouba	4
	Morneg	3
	Sbiba	4
<i>P. parasitica</i>	Mateur	
	Beni	2
	Khaled (Agrumes)	2
Total		50

work studied the hypothesis that *Pythium* and *Phytophthora* species were involved in the apple decline recorded in Tunisia.

Therefore, the aims of the present study were: 1) to identify the causal agents of the apple tree decline in Tunisia and to establish the pathogenicity of the isolated agents; 2) to study the involvement of irrigation water, irrigation system and soil salinity as factors that could incite apple tree dieback.

MATERIALS AND METHODS

Survey and isolation

The survey included 23 apple orchards (10 trees per orchard) that were sampled during the 2006-2008 growing seasons. The orchards were located in nine regions, which are the leading areas of apple cropping in Tunisia (Table 1). Disease symptoms were recorded from both aerial and below ground parts of the diseased trees. Collar rot symptoms were recorded following bark removal. These orchards were sampled and root, collar and soil samples were collected.

The soil sampling method varied according to the irrigation system used in each orchard. In the flood irrigation system, the samples were taken from 15 to 20 cm depth under soil level in the direction of water circulation. Sampling was performed under the canopy of the diseased trees and the next one in the irrigation water direction. Whereas, in the case of heavily diseased orchards,

samples were randomly taken. In the case of orchards irrigated by drippers, the soil samples were taken from four quadrants under the dripline of the affected trees from a depth of 15-20 cm. In each orchard, the samples of soil were mixed and a small amount of soil sample was placed in two 10 mm diameter x 15 mm depth wells cut into an apple with a corkborer. Two apples per sample were prepared following this method. Apples were incubated at 25°C for 3 days. Infected fruit tissue from the margin of the necrosis was removed and placed on 1.7% corn meal agar (CMA) amended with (per liter) 10 µg pimarinic, 200 µg ampicillin, 10 µg rifampicin, 25 µg pentachloronitrobenzene and 10 µg benomyl (PARB) (Jaffers and Martin, 1986). The antibiotics were added after sterilization.

To recover and isolate the suspected disease causal agents, collar or root tissues were removed from the margin of cankers. The diseased tissues were cut aseptically into approximately 20 small pieces of 3 to 5 mm in length. Then, they were washed in tap water, surface-disinfested by dipping in 70% ethanol for 30 s, rinsed in sterilized de-ionized water and blotted dry on a filter paper. The infected tissues were placed onto PARB selective medium (Jeffers and Martin, 1986) in 90 mm Petri dishes. The samples were incubated for four to five days at 24°C in darkness. To obtain pure cultures of the suspected pathogens a single hyphal tip from the edge of each colony was transferred by micromanipulation onto potato dextrose agar (PDA: 39 g/L), Biokar Diagnostic) and incubated at 25°C for three days. For morphological identification, the cultures were transferred to V8 juice agar (200 mL of V8 Juice; 2 g CaCO₃ and 15 g agar in 800 mL distilled water) and incubated at 24°C in the dark. Stock cultures were maintained on PDA at 12°C in the dark. All cultures were preserved in the culture collections maintained at the Higher Institute of Agronomy located at Chott Mariem, Tunisia.

Diagnosis of suspected causal agents

Morphological identification

Sexual and asexual structures of the different oomycetes were used for identification. *Phytophthora* isolates were identified on the basis of colony morphology, growth rate, cardinal growth temperatures, and production, morphology and dimensions of sporangia, oogonia and antheridia (Waterhouse, 1963; Erwin and Ribeiro, 1996). Identification of the *Pythium* isolates were carried out by microscopic observations from cultures incubated on PDA medium at 24°C in darkness for 4-6 days. Distinctive structures, such as reproductive structures form and size were observed after transfer to a glass microscope slide using the key of Waterhouse (1968), Van der Plaats (1981) and Mugnier and Grosjean (1995).

DNA extraction, ITS sequencing and molecular phylogeny

Mycelial DNA was extracted from pure cultures grown in potato dextrose broth (PDB, Biokar Diagnostic) (Belbahri et al., 2006). Total DNA was extracted using the EZNA Plant Miniprep Kit (Omega Bio-tek) following the manufacturer's instructions. The amplification of the Internal Transcribed Spacer (ITS) of the ribosomal DNA (rDNA) was carried out using the universal primers ITS4 and ITS6 that target conserved regions in the 18S and 28S rDNA genes (White et al., 1990; Cooke et al., 1996). Amplifications were performed according to Chavarriaga et al. (2007). PCR products were purified and DNA sequencing was performed at the analysis technical service of the institute of Plant Molecular and Cellular Biology of the Polytechnic University of Valencia (Spain). Purified PCR products were cycle-sequenced using ABI Prism™ BigDye™ Terminator chemistry with AmpliTaq® DNA polymerase, and sequenced using a Perkin-Elmer, Applied Biosystems Division, 373 A DNA Sequencer. Sequences were edited using the chromas 1.51 software (Technelysium Pty Ltd,



Figure 1. Affected apple tree showing typical symptoms of dieback.

<http://www.technelysium.com.au/chromas.html>). Consensus sequences were aligned using the CLUSTAL W program. The sequences were subjected to an NCBI BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>) for identification and prior to phylogenetic analyses to identify the closest related sequences and building phylogenetic trees from molecular data with MEGA 5. Trees were constructed based on the test Neighbor-Joining tree.

Study of abiotic factors

To study the effect of irrigation method, water source and soil salinity on the disease, three orchards were selected. One was located in Sbiba, a second in Morneg and the third in Utique. These orchards were irrigated by both drip and flood irrigation and the water source was a well or a reservoir. Apple tree dieback severity was investigated and the assessment of the damage on apple trees was estimated by considering the total number of apple trees by orchard, and the number of diseased and dead trees. The effect of soil salinity on the development of dieback was studied by considering the total salt and sodium chloride concentrations and the dieback rate of dieback recorded at some surveyed orchards.

Pathogenicity tests

Phytophthora and *Pythium* isolates were tested to establish their pathogenicity and virulence over the 3-years growing periods. The following apple cultivars were used: Anna, Lorka and Meski and the rootstock MM106. Pathogenicity assays were performed using six species of *Pythium* (two isolates for each species *Pythium* sp. 1 and sp. 2, *Pythium rostratiformis* 1 and 2, and one isolate of *Pythium irregulare*, *Pythium sterilum*, *Pythium indigoferae* and *Pythium undulatum*), two isolates of *Phytophthora nicotianae* Ph 1 and Ph 2 and one isolate of *Phytophthora inundata* were tested separately. The inoculation was used, *in vitro* and *in vivo*. *In vitro*, the method consisted of the use of detached two-years-old shoots (10 cm length and 2 cm diameter) (Krober and Karnatz, 1979). The detached branches were disinfested with 70% ethyl alcohol, rinsed in sterile distilled water and blotted dry. Sets of 36 shoots of each apple cultivar were planted on containers (4920 cm³) filled with peat. A 7 mm in diameter disc of bark was removed from each inoculation court (2 cm approx. above the substrate) and a transverse T-shaped notch was cut through the cambium to a depth of 1.0-1.5 mm into the sapwood. A 7-mm in diameter disc removed from a 4-days-old culture grown on PDA was inserted into the artificial

wound, placing the mycelium in contact with the exposed tissue. The control plants were inoculated with non-inoculated sterile PDA discs. Inoculation sites were covered with parafilm for 1 month. The containers were maintained at 25°C under cool white light from 30-W fluorescent tubes suspended 30 cm above the chambers (12-h photoperiod). Seven days after inoculation court, the area of the lesions was calculated with an analogical planimeter.

Pathogenicity tests were also carried out *in situ* on six-years-old apple trees. Branches of 20 mm in diameter were randomly selected from the canopy of disease free, non symptomatic trees. The branches were disinfested using 95% sodium hypochlorite for 5 min. A 7-mm-diameter disc of bark was removed from each inoculation court and a transverse T-shaped notch was cut through the cambium to a depth of 1.0-1.5 mm into the sapwood. A 7-mm diameter disc from a 4-day-old PDA culture of each strain was inserted with the mycelium in contact with the host tissue. In the control plants, inoculum consisted of sterile PDA discs. Inoculation sites were covered with a distilled water dipped cotton swab and protected by parafilm and aluminium foil for 1 month.

Twenty seven randomly chosen nursery produced Rootstock MM106 plants were twig-inoculated (three replications). Seven years-old Anna, Lorka and Meski apple cultivars trees were twig-inoculated (2 years-old twigs) with random sites inoculation. Controls were included in all pathogenicity assays and re-isolations were made from the infested tissue by plating onto PARB medium to confirm Koch's postulates.

RESULTS

Apple tree decline symptoms description and isolation

The first symptoms of the syndrome appeared during spring months on the apical parts of the trees. Terminal branches on the canopy dried out or wilted and the symptomatology progressed to lower parts of the plant (Figure 1). At the end of the summer, a severe defoliation was observed on the basal parts of young branches. Additional symptoms included small fruits and premature ripening (Figure 2). Lesion expansion toward the lower parts of the tree caused the formation of cankers on the trunk. A visible reddish brown discoloration of the inner bark was observed by cutting away the outer bark layer on the exposed collars; often, it was possible to see a sharp contrast between the infected and healthy tissues (Figure 3). An unpleasant smell was also noticed.

Isolations made from collar, roots or soil collected in the vicinity of dying back trees allowed the recovery of 50 oomycetes isolates. Six species of *Pythium*: *P. indigoferae*, *P. irregulare*, *P. rostratiformis*, *P. sterilum*, *P. undulatum* and *Pythium* sp., and two species of *Phytophthora* (*P. nicotianae* and *P. inundata*) were recovered (Table 2). *Pythium* species were isolated from the roots and soil whereas *Phytophthora* species were isolated from the soil root and collar.

Morphological and molecular identification of pathogenic isolates

The morphological features recorded for homothallic



Figure 2. Affected apple tree showing typical symptoms of collapse.



Figure 3. Typical lesions observed in the collar area of apple trees in affected orchards.

Pythium spp. and the heterothallic *P. nicotianae* isolates are presented in Table 2. The identification of the heterothallic *Pythium* species however was based on the molecular identification given the absence of the reference strains to do complementation assays.

Conductive abiotic factors

The effect of irrigation water source on the severity of the disease was studied and the results proved that reservoir water was associated with disease severity in the three surveyed regions. Figure 4 shows differences of tree infection using the two water origins that can be explained by texture or structure of the soil. Also, the effect of irrigation system showed a clear correlation

between the irrigation system and the severity of the dieback. The percentage of diseased trees was higher in orchards flood irrigated than in drip irrigated ones. The most significant value was found in Morneg region showing the highest difference between the two irrigation systems (Figure 5).

During this study, two variables were also investigated: total salt concentration and sodium chloride concentration. The results revealed that severity of dieback could be correlated with the total salt concentration (Figure 6 and 7). In fact, in Orchard 1 placed in Foussana region only 21% of diseased trees were noticed where the total salt content was only 0.5 g/l. However, in the orchard located in Oued Mliz region, having 1.8 g/l total salt concentration, 60% dieback was noticed. Orchard 2 in Foussana shows 10% dieback despite a total salt concentration of 1.3 g/l.

This result could be explained by the different salts concentrations. While Figure 8 shows a positive correlation between sodium chloride concentrations and tree apple dieback. In Foussana Orchard 2, the lowest level of infestation was registered in the orchard irrigated with the water having the lowest level of sodium chloride. This result confirms the importance of sodium chloride among total salts.

Molecular identification of Pythiaceae isolates

Molecular identification was performed by sequencing the ITS region of rDNA, using the conserved primers ITS4 and ITS6. The ITS sequences of oomycete isolates recovered from collar roots and soil showed the presence of eight *Pythiaceae* species, confirming the results obtained using morphological criteria. Sequences have been blasted and aligned with sequences from GenBank database (Figures 8 and 9).

Pathogenicity tests

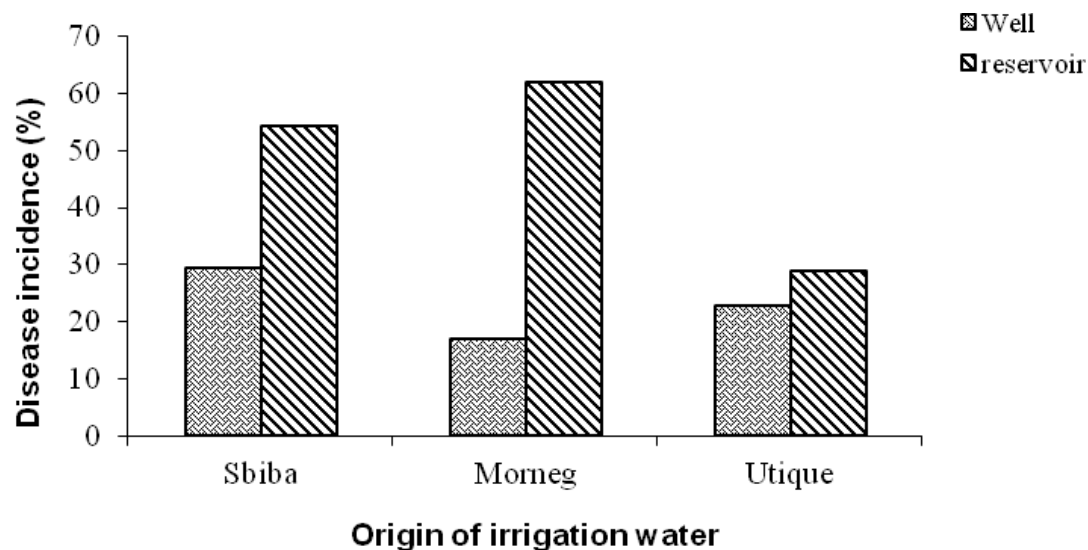
The results of the inoculation tests reproduced the symptoms observed under field conditions. Results showed that the rootstock MM106 was the most susceptible to the different tested isolates. Cultivars Anna and Lorka were the most resistant. The classification of Oomycete isolates differs according to the apple cultivar used. Nevertheless, the most aggressive isolates on the apple cultivars were *Pythium* sp. isolates with *P. undulatum* as the weakest pathogen (Figures 10 and 11). *Pythium* and *Phytophthora* isolates inoculated could be recovered from all the artificially inoculated trees. Control trees did not show any symptoms.

DISCUSSION

The present study show the presence of *P. nicotianae*

Table 2. Morphological characteristic of five Pythiaceae species (sexual structure of *P. inundata* is not determined given the unavailability of reference strains).

Species	Colony patterns (PDA)	Hyphae	Sporangia			Oogonia diameter (μm)	Oospore diameter (μm)	Antheridia	Hyphal swellings	Chlamydo-spores	Cardinal temperature ($^{\circ}\text{C}$)		
			Form	Proliferation	Persistent						T min	T op	T max
<i>P. indigoferae</i>	Petaloid	Homothallic	Filamentous inflated	Absent	Yes	Smooth (19.7 ± 1.7)	Aplerotic (18.9 ± 1.4)	Monoclinous Amphigynous	Absent	Absent	11	25-28	35
<i>P. irregulare</i>	Rosette	Homothallic	Globose	Absent	Yes	Smooth (19 ± 2.6)	Aplerotic (18.7 ± 2.1)	Monoclinous Hypogynous	Spherical	Absent	5	28-32	40
<i>P. rostratifingens</i>	Rosette	Homothallic	Spherical	Absent	Yes	Globose (13.2 ± 1.27)	Plerotic (11.9 ± 1.9)	Monoclinous Hypogynous	Absent	Absent	5-7	16-23	35
<i>Ph. nicotianae</i>	Cotonny	Heterothallic	Spherical Papillate (1 or 2 papilla)	Absent	Yes	Spherical Smooth (20.1 ± 2.22)	Aplerotic (20 ± 2.24)	Diclinous Amphigynous	Spherical	Terminal Intercalary (27.9 ± 4.6)	7-10	26-28	35-39
<i>Ph. inundata</i>	irregular	Heterothallic	Ovoid papillate	Present	yes	-	-	-	Circular	Absent	11	25-29	35

**Figure 4.** Effect of the origin of the water used for irrigation on disease incidence in 3 regions: Sbiba, Morneg and Utique (Average of 6 orchards by region).

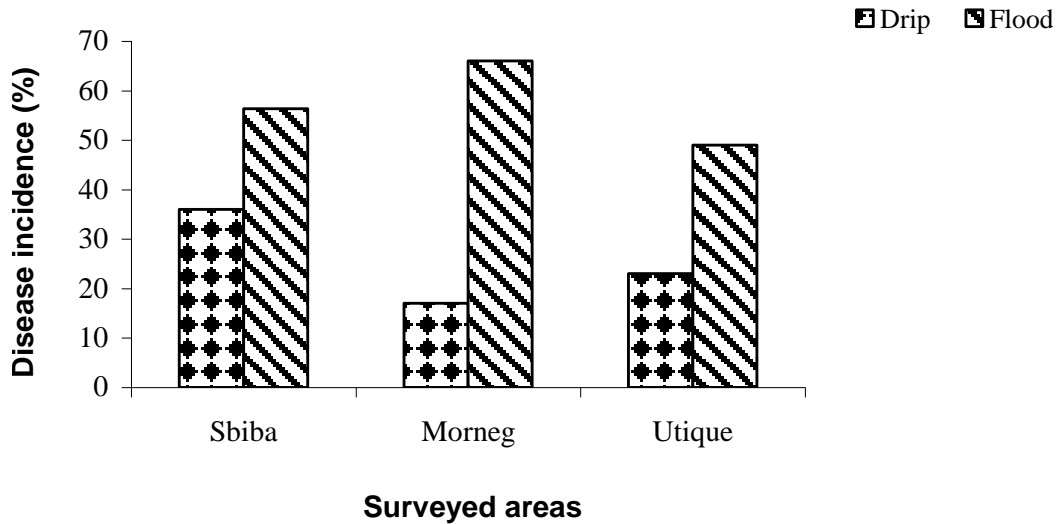


Figure 5. Effect of the irrigation system (drip or flood) on disease incidence in 3 regions: Sbiba, Morneg and Utique (Average of 6 orchards by region).

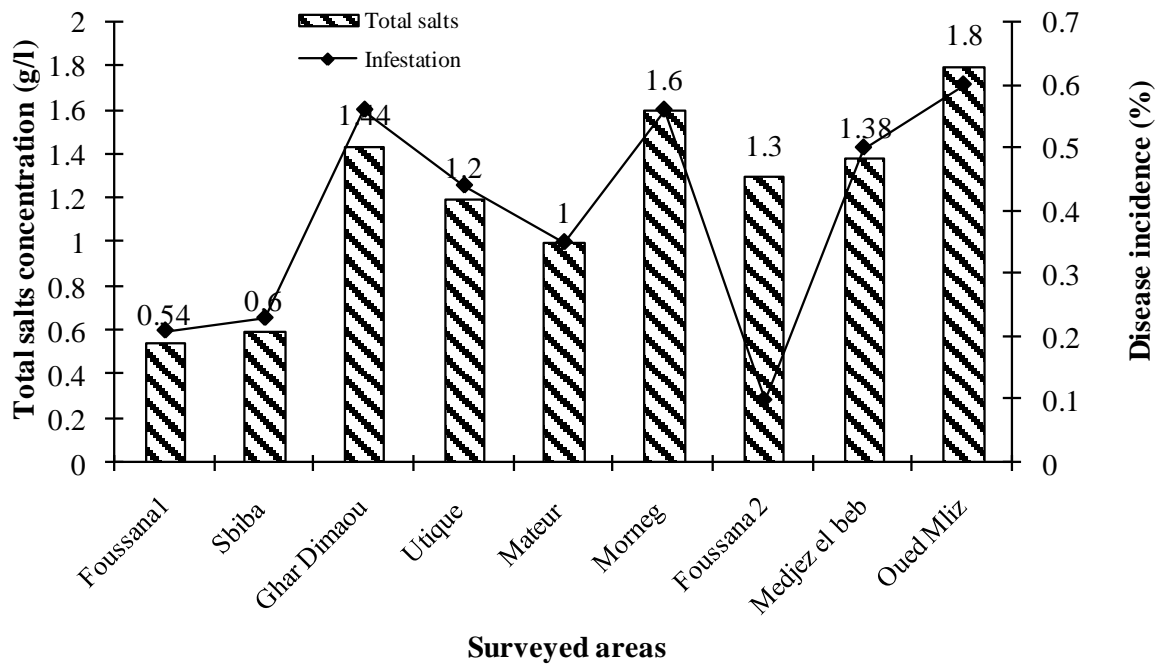


Figure 6. Effect of soil salinity on the incidence of the disease.

associated with apple tree dieback in Tunisia. This pathogen is reported for the first time in Tunisia. In addition, the role of several *Pythium* species on the syndrome was elucidated.

Irrigation increases soil moisture, potentially increasing pathogen activity and promoting development of root, collar rot and other diseases (Ristaino et al., 1993). This is especially the case when irrigation produces patches of

excessive wetness such as in low-lying areas of a field (Larkin et al., 1995) or in areas directly near an irrigation emitter (Café-Filho and Duniway, 1996).

Numerous reports using filtration techniques for recovering fungal spores highlighted the presence of Oomycetes in water. In previous studies, about twelve species in water reservoirs used for irrigation were identified, including *Phytophthora cactorum*, *Ph. cinnamomi*,

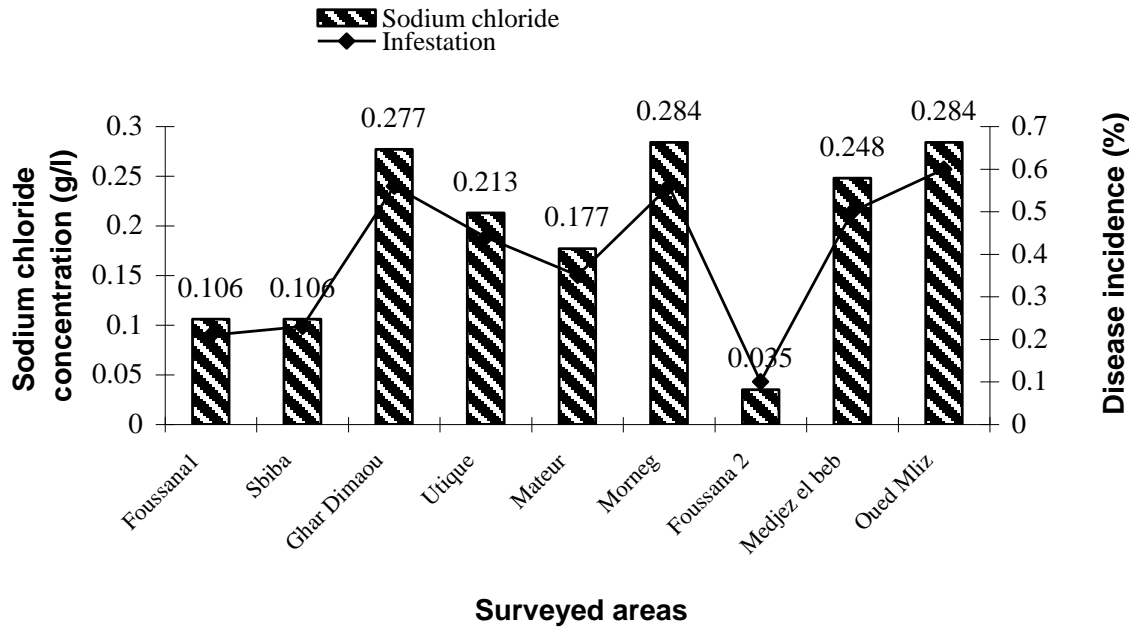


Figure 7. Effect of sodium chloride on the incidence of the disease.

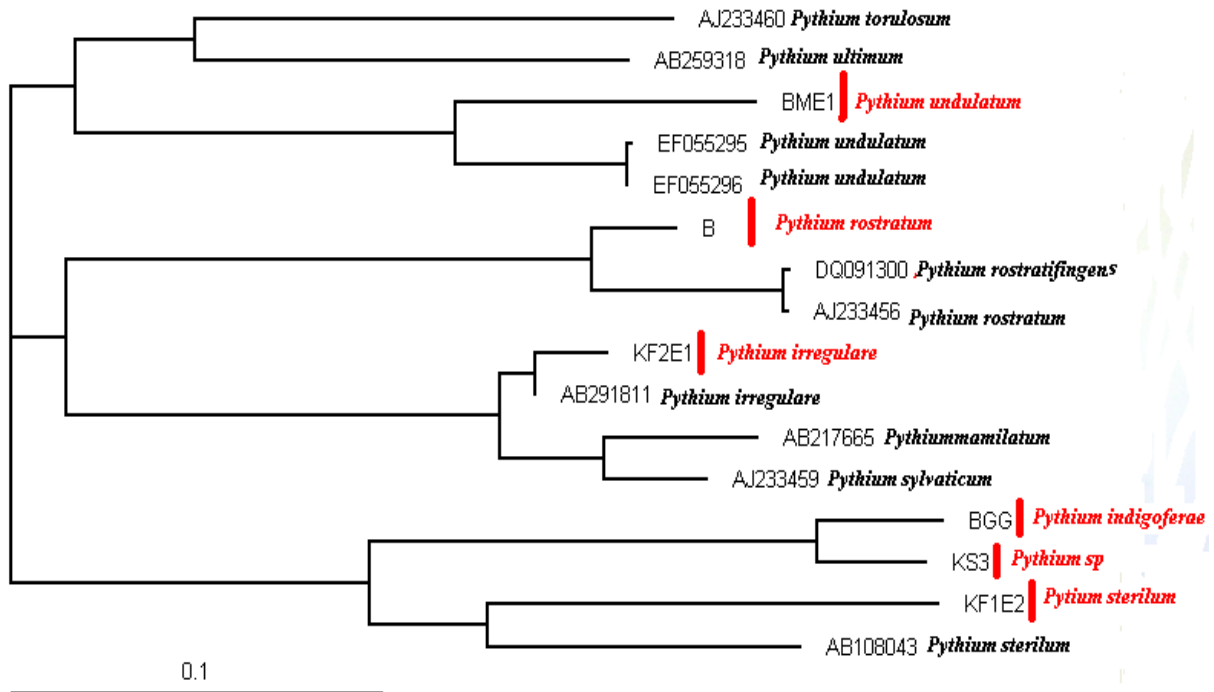


Figure 8. Dendrogram for *Pythium* species based on analysis of ribosomal DNA.

P. citricola, *P. citrophthora*, *P. dreschsleri*, *P. nicotianae*, *P. boehmeriae*, *P. gonapodyides*, *P. hibernalis*, *P. megasperma* and *P. syringae* (Ristaino, 1991). Yamak et al. (2002) reported 149 *Phytophthora* sp. isolates from irrigation waters.

The susceptibility of some plant species to Pythiaceae can be enhanced by specific environmental factors. Bolay (1992b) has estimated that some soils are favorable to disease expansion and the annual loss of apple trees dieback ranges from 1 to 5% of the trees. In some

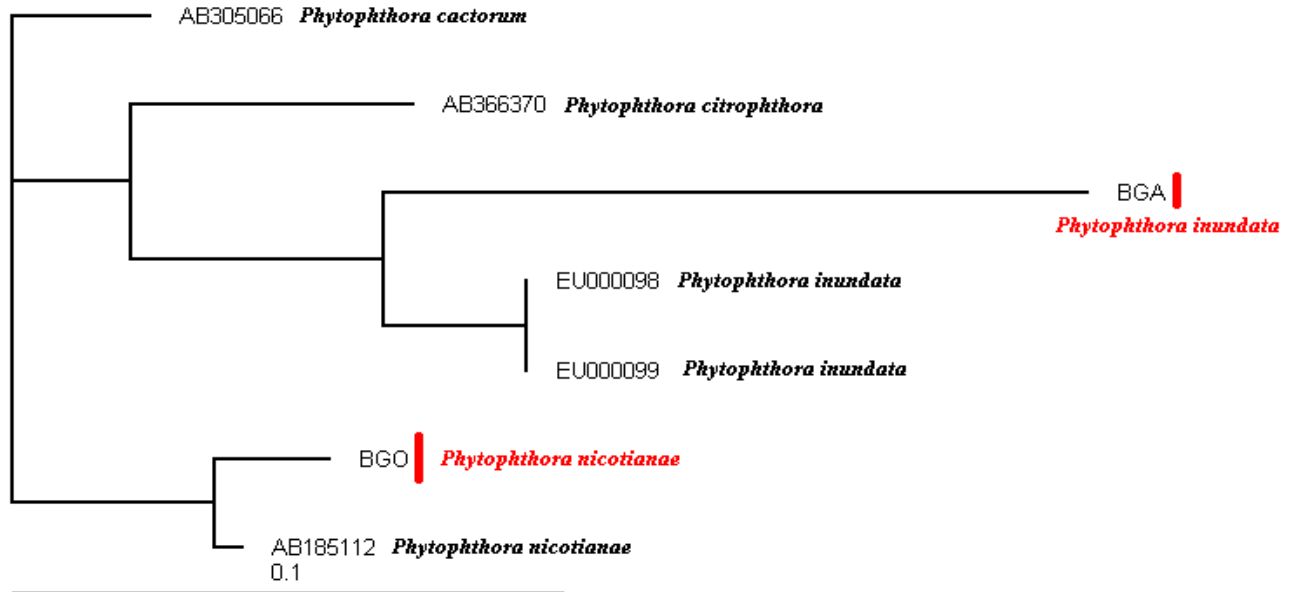


Figure 9. Dendrogram for *Phytophthora* species based on analysis of ribosomal DNA.

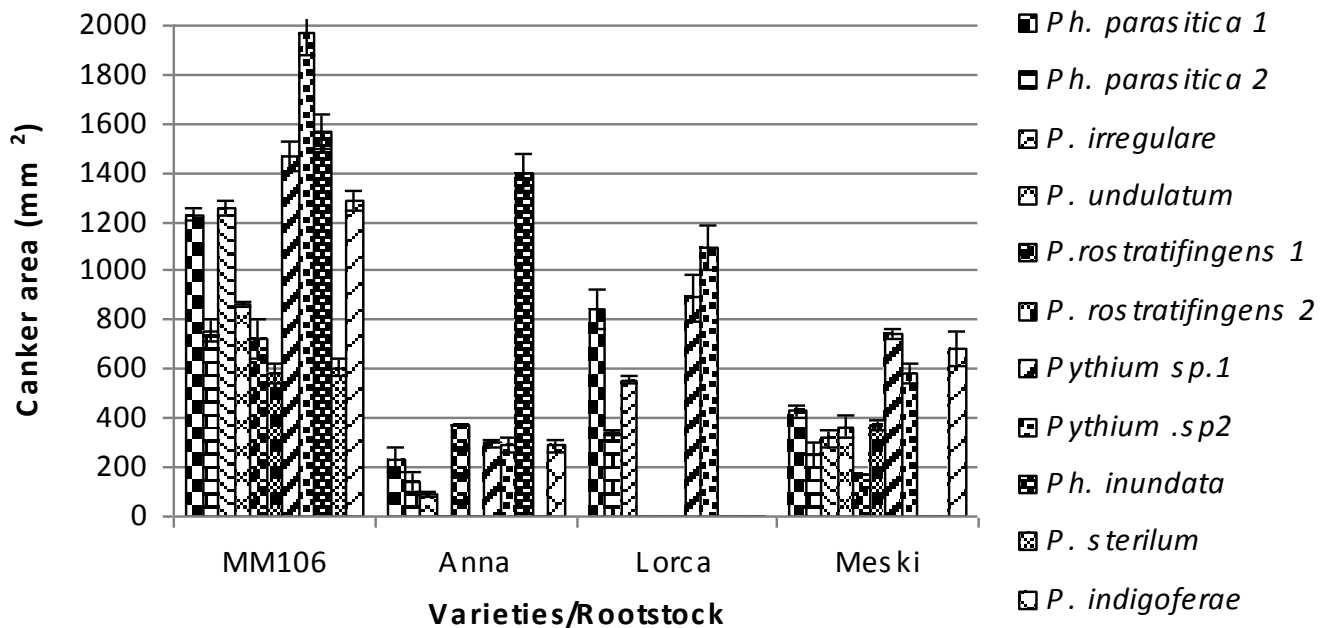


Figure 10. Canker area in 2 years old shoots of apple 7 days after inoculation with 6 *Pythium* species and 2 *Phytophthora* species (average of nine replicates). *Ph.*: *Phytophthora*; *P.*, *Pythium*.

Tunisian apple orchards, the losses could be as severe as 5 to 50%. High soil salinity predisposes crops to infection by Pythiaceae. Soil salinity is normally a problem in arid environments where salt levels fluctuate throughout the year based on water quality and the frequency and duration of irrigation events. During periods of high salinity, diseases caused by Pytiaceae

pathogens can be severe despite high temperature and low relative humidity, which are not favorable for their development. In Morocco, El Guilli et al. (2000) studied the effect of salinity of irrigation water on the severity of *Ph. citrophthora* collar rot on *Citrus* trees and showed a correlation with the severity of infection. Benyahia et al. (2004) studying the effect of salinity on colonization of

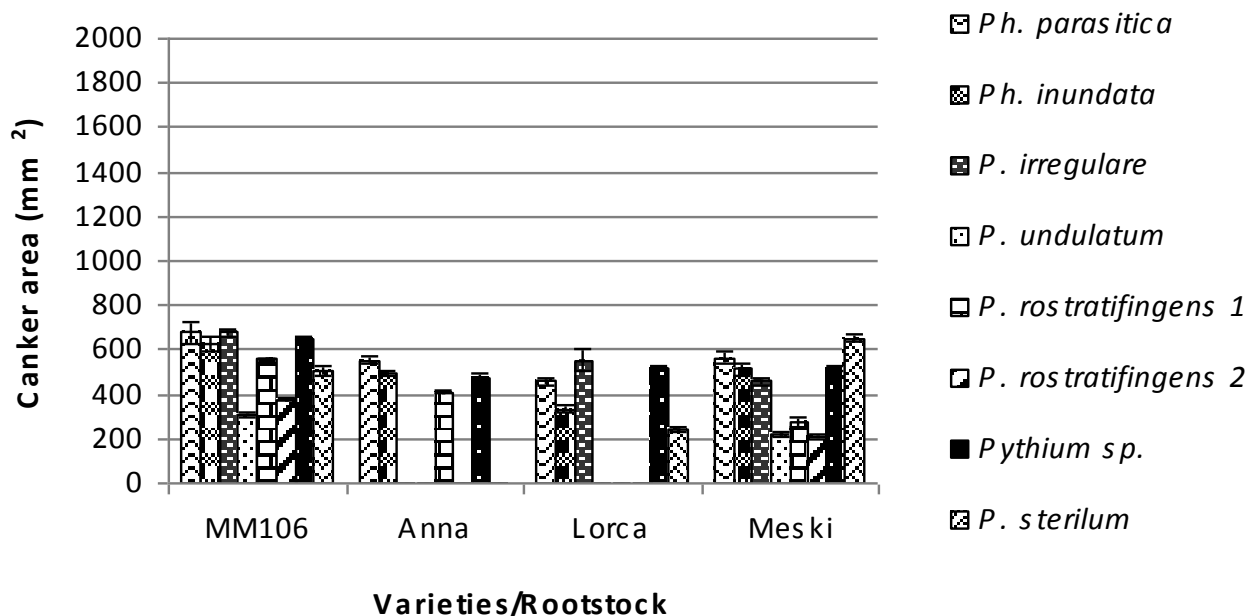


Figure 11. Canker area in 7 years old trees of apple 6 weeks after inoculation with 5 *Pythium* species and 2 *Phytophthora* species (average of six replicates). *Ph.*: *Phytophthora*; *P.*, *Pythium*.

root citrus rootstock (*Citrus aurantium* L.) by *P. nicotianae* showed a clear effect of sodium chloride on disease resistance of the rootstock.

Apple tree dieback has been attributed in many countries such as United States, Canada and Argentina to *Phytophthora* spp., mainly *P. cactorum* (Bolay, 1992a). In Washington State of the USA, apple tree dieback has been mainly attributed to species of the genus *Pythium* spp.: *Pythium* sp., *P. rostratifingens*, *P. indigoferae*, *P. irregulare*, *P. undulatum* and *P. sterilum*. Two *Phytophthora* spp.: *P. nicotianae* and *P. inundata* have also been characterized. This morphological identification was confirmed by sequencing ITS region of rDNA. Such tools proved very useful for identification of Pythiaceus spp.

In vivo inoculation of apple twigs varieties and rootstock MM106 with the *Pythium* and *Phytophthora* spp. Reproduced the disease symptoms observed in the orchards, mainly collar rot symptoms. Using controlled inoculations, it was possible to complete Koch's postulates and demonstrate the association of these fungal-like species in apple tree dieback. *P. undulatum* did not show any pathogenicity towards apple trees on cultivars Anna and Lorca. The oomycetes species in the course of our study have been previously described as causal agents of apple trees dieback (Levésque et al., 2004; Mazzola et al., 2002). *P. irregulare* and *P. rostratum* have been shown to be pathogenic on apples (Mazzola et al., 2002). Lévesque et al. (2004) renamed the isolate of *P. rostratum* used in this study as *P. rostratifingens*. The classification of oomycete isolates and species according

to their virulence differs according to the apple cultivar used. Nevertheless, the most aggressive isolates on the majority of apple cultivars are *Pythium* sp.1 and *Ph. nicotianae*

Conflict of Interests

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

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Full Length Research Paper

Ecosafe bioremediation of dairy industry effluent using *Streptomyces indiaensis* ACT 7 and *Streptomyces hygroscopicus* ACT 14 and application for seed germination of *Vigna radiata*

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Received 7 March, 2012; Accepted 12 May, 2014

Marine actinomycetes strains of *Streptomyces indiaensis* ACT 7 and *Streptomyces hygroscopicus* ACT 14 were efficiently utilized for the bioremediation of dairy industry effluent. The effluent characterization before and after the treatment were observed. The maximum reduction of TS, TDS, TSS, biological oxygen demand (BOD), chemical oxygen demand (COD), chloride, oil and fat content were observed for effluent treatment by mixed consortium. It showed the reduction percent of 92.57 and 90.73 for BOD and COD, respectively. The untreated and treated effluent was used for the germination of *Vigna radiata*. The seed germination of 100 and 70% were observed for treated and untreated effluent studies. The seedling length (27.2 cm) and vigour index (2720) was maximum for *V. radiata* treatment with treated dairy effluent.

Key words: Marine actinomycetes, dairy effluent, bioremediation, mixed consortium, germination, *Vigna radiata*.

INTRODUCTION

There has been a substantial increase in generation of industrial wastewater due to the exponential increase in the number of industries. The waste water is discharged either into open land or nearby aquatic ecosystems that results in the contamination of ground water and causes severe effects to the aquatic life. The release of effluents into our environment, coupled with the growth of Industrialization has resulted in massive and destructive operations in our ecosystems (Manu et al., 2012). Indis-

criminate discharge of untreated or partially treated waste water directly or indirectly into aquatic bodies may render water resources unwholesome and hazardous to man and other living systems (Olorunfemi et al., 2010). The untreated effluent also causes harmful effects to the agricultural field and effect the growth of plants. Dairy effluent has high organic loads as milk is its basic constituent with high levels of biological oxygen demand (BOD), chemical oxygen demand (COD) and oil content

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(Manu et al., 2012). The dairy effluent affects the aesthetic value of the receiving water and causes damage to aquatic life (Hur et al., 2010). Its high BOD depletes the dissolved oxygen content of the aquatic system and in due course, creates anaerobic conditions. Also, the foul smell of the effluent is a nuisance to the public and it becomes the breeding ground for flies and mosquitoes (Piyush and Rathore, 2001). The removal of organic matter from the waste water using chemicals is more expensive and cause severe effects to the environment. Bioremediation is adopted to remove toxicants using novel microbes. Effluents emanating from dairy processing industries can be treated by using effective microbes. A mixed bacterial culture comprising *Pseudomonas aeruginosa* LP602, *Bacillus* sp. B304 and *Acinetobacter calcoaceticus* LP009 were used for the treatment of lipid rich wastewater (Mongkolthanasak and Dharmasasthithi, 2002). Silambarasan et al. (2012) used *Pithophora* sp. for the treatment of dairy industry effluent. In the present study, marine actinomycetes were isolated from the marine sediment samples and exploited for the dairy industry effluent treatment and the treated effluent can be efficiently used for the germination studies of *Vigna radiata*.

MATERIALS AND METHODS

Collection of marine sediment sample and Isolation of Actinomycetes

The marine sediment samples were collected by using a core sampler at a depth of 3 m from two different points of Tiruchendur coastal area (Latitude: 8°29.80 N Longitude: 78°07.73 E), Tuticorin District, Tamil Nadu, East Coast of India. The actinomycetes were isolated from the sediment samples by serial dilution plate method (Ellaiah, 1996) using actinomycetes isolation agar and starch casein nitrate agar medium (Kuster and Williams, 1964).

Effluent sample collection

The untreated dairy effluent sample of 10 L was collected from a dairy industry located in Coimbatore, Tamil Nadu, India. The sample was transferred into a sterile container and transported to the laboratory immediately. The effluent was stored at 4°C for further analysis.

Inoculum preparation

A loopful of cells from the isolated pure cultures were inoculated separately in different flasks with 100 ml of inorganic starch broth (Shirling and Gottlieb, 1996). The flasks were incubated at 40°C in an incubator shaker at 180 rpm for 72 h. A volume of 50 ml of inoculum was used for each 500 ml of effluent treatment.

Effluent treatment by isolates

The dairy effluent was treated by using selective isolates. The effluent was filtered and autoclaved at 121°C for 15 min to make them sterile from other microorganisms. The different parameters

like BOD, COD, chloride, TS, TDS, TSS and oil content (APHA, 1998) of untreated effluent were analyzed. A volume of 500 ml of the effluent sample was taken in 3 sets of the flask. To this, 100 ml of nutrient solution and 50 ml of inoculum was added. Each strain was added as single inoculums (*Streptomyces indiaensis* ACT 7; *Streptomyces hygrosopicus* ACT 14) and mixed *Streptomyces* spp. (*S. indiaensis* ACT 7 and *S. hygrosopicus* ACT 14) to the effluent sample amended with nutrients. The microbial treatment was given for 20 days. The flasks were kept in an incubator shaker with 180 rpm/min. All the experiments were done in triplicates.

Germination of seeds and growth of seedlings using effluent

Seeds were collected from pulses Research Center, Tamil Nadu Agriculture University (TNAU), Coimbatore. The germination of seeds and the growth of seedlings were examined using treated and untreated effluent of dairy industry. The Green gram (*Vigna radiata*) healthy seeds (20) were taken and surface sterilized with 0.1% mercuric chloride and kept in the sterilized Petri dish with filter paper. To this, 5 ml of effluent was added and observed for the germination and growth of seedlings (Dhanam, 2009). The germinated seeds were transferred on to the soil tray with a spacing of about 3 cm each. Seedlings irrigated with distilled water served as control. Seedlings irrigated with treated and untreated effluents were used for experiments. The various parameters like germination percentage, root and shoot length, seedling length, vigour index (Baki and Anderson, 1973), percent of phytotoxicity (Chou et al., 1978), effluent tolerant index (Turner and Marshal, 1972) were examined.

RESULTS AND DISCUSSION

From the marine sediment samples, 30 strains of actinomycetes were isolated. Among 30 isolates, 2 efficient strains were finally selected for bioremediation of dairy effluent. In this investigation, 16 S rRNA gene sequencing was carried out to identify the marine actinomycetes by comparing the similar sequences retrieved from databases using BLAST search. The sequences were submitted in Genbank under the accession numbers of JQ801298.1, JQ801299.1 for *Streptomyces indiaensis* ACT 7 and *Streptomyces hygrosopicus* ACT 14, respectively.

In the present study, the untreated dairy industry effluent was treated by *S. indiaensis* ACT 7, *S. hygrosopicus* ACT 14 and mixed consortium of *Streptomyces* spp. for 20 days. The different parameters like BOD, COD, chloride, TS, TDS, TSS and oil content were analyzed on 10th and 20th day. The impact of the untreated and treated dairy industry effluent was checked with the germination of *V. radiata*. The results are presented in Tables 1 to 4. *S. indiaensis* ACT 7, *S. hygrosopicus* ACT 14 and mixed consortium of *Streptomyces* spp. treatment showed the reduction of the following parameters on 20th day: BOD was reduced to 81.27, 83.72 and 92.57% respectively. Similarly COD was reduced to 87.69, 86.35 and 90.73%. Chloride was reduced to 85.01, 82.84 and 94.14%. Oil content was reduced to 94.50, 95.31 and 98.87%. The higher amount of TS may be due to the maximum concentration of biodegradable organic matter in the

Table 1. Biotreatment of dairy industry effluent using *S. indiaensis* ACT 7.

Parameter	Untreated effluent	Treated effluent			
	(mg/l)	10 th day	Removal (%)	20 th day	Removal (%)
TS	2312.48	1138.26	50.77	324.34	85.97
TSS	472.380	125.860	73.35	38.340	91.88
TDS	1840.10	1012.40	44.98	286.00	84.45
BOD	1280.00	558.340	56.37	239.67	81.27
COD	2320.00	1036.48	55.32	285.55	87.69
Chloride	654.200	312.400	52.24	98.000	85.01
Oil	224.350	86.8400	61.29	12.330	94.50

Values are mean of three replicates.

Table 2. Biotreatment of dairy industry effluent using *S. hygroscopicus* ACT 14.

Parameter	Untreated effluent (mg/l)	Treated effluent			
		10 th day	Removal (%)	20 th day	Removal (%)
TS	2312.48	1025.74	55.64	352.61	84.75
TSS	472.380	151.120	68.00	54.290	88.50
TDS	1840.10	874.620	52.46	298.32	83.78
BOD	1280.00	512.640	59.95	208.33	83.72
COD	2320.00	1124.50	51.53	316.67	86.35
Chloride	654.200	345.800	47.14	112.24	82.84
Oil	224.350	80.4800	64.12	10.52	95.31

Values are mean of three replicates.

Table 3. Biotreatment of dairy industry effluent using mixed consortium.

Parameter	Untreated effluent (mg/l)	Treated effluent			
		10 th day	Removal (%)	20 th day	Removal (%)
TS	2312.48	916.54	60.36	184.15	92.03
TSS	472.380	96.140	79.64	21.840	95.37
TDS	1840.10	820.40	55.41	162.31	91.17
BOD	1280.00	434.50	66.05	95.00	92.57
COD	2320.00	889.34	61.66	215.00	90.73
Chloride	654.200	224.54	65.67	38.300	94.14
Oil	224.350	54.380	75.76	2.5200	98.87

Values are mean of three replicates.

Table 4. Studies of untreated and treated dairy effluent on seed germination of *Vigna radiata*.

Parameter	Control	Untreated dairy effluent	Treated dairy effluent
Concentration of effluent		100.0	100.0
Germination (percentage)	100.0	70.0	100.0
Shoot length (cm)	17.20	11.27	23.0
Root length (cm)	03.40	1.57	4.1
Seedling length (cm)	20.60	12.84	27.2
Seedling vigour index	2060	992.91	2720
Phytotoxicity	-	42.67	-
Effluent tolerant index	-	1.74	-

Values are mean of three replicates.

dairy effluent. The high levels of TDS may be due to the presence of salt content in the effluent. TS were reduced to 73.00, 76.28 and 90.53%. TDS was reduced to 70.32, 72.93 and 90.42%. TSS was reduced to 83.41, 89.30 and 90.97%. Biotreatment of the industrial effluents using microbes showed high reduction of BOD and COD levels. It enhanced the removal of most of the toxicants in the effluent. Oil and fat contents from the effluent can create unsightly floating matter and films on water surfaces. It may also interfere with biological life forms in the surface waters. If the surface water is contaminated with oil and grease, oxygen transfer from the atmosphere is reduced and creates a very high oxygen demand on account of their biodegradability (Piyush and Rathore, 2001). Reduction of BOD and COD levels were due to the removal of dissolved organic compounds from the effluent. According to Silambarasan et al. (2012) *Pithophora* sp. reduced most of the toxicants as follows: TS (39.82%), TSS (92.22%), chloride (38.87%), BOD (64.67%), COD (61.65%). Vida et al. (2007) reported the highest COD reduction obtained by two isolates BP3 and BP4 with 70.7 and 69.5%, respectively.

The phytotoxicity analysis was done by the germination and growth of *V. radiata* using untreated and treated dairy effluent (Table 4). *V. radiata* showed 70 and 100% of germination using untreated and treated effluent. Seedling length by untreated and treated effluent was 12.84 and 27.2 cm. The treated effluent increased the seedling length of *V. radiata* than control (20.60 cm). The seedling vigour index using untreated and treated effluent was 992.91 and 2720. The phytotoxicity was 42.67% for *Vigna* sp. irrigated with untreated effluent and the effluent tolerant index was 1.74. According to Dhanam (2009), the untreated dairy effluent showed 81% of germination and seedling length of 11.1 cm. According to Manu et al. (2012), the untreated effluent showed 82% of germination and seedling length of 15.94 cm. The germination percentage, seedling growth and other growth parameters were decreased at 100% concentration of untreated effluent in the present study. It might be due to high osmotic pressure caused by untreated effluent. Presence of high amounts of toxicants in the effluent inhibits the germination and seedling growth of *V. radiata*. The treated effluent should not contain the toxicants and enhanced the growth of seedling as compared to control.

The studies on bioremediation of dairy effluent by *Streptomyces* sp. showed an efficient method for the prevention of water pollution. The mixed consortium showed the maximum reduction of toxicants from dairy effluent than single isolates for treatment purpose. The treated effluent can be recommended for the agricultural irrigation purpose because it enhanced the germination percentage and seedling growth than control. Bioremediation of effluent by marine *Streptomyces* sp. and utilization of the effluent for the agricultural purpose is an efficient ecosafe method for the treatment of effluent.

Conflict of Interests

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

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Full Length Research Paper

Anti-candidal activity of *Piper betle* (L.), *Vitex negundo* (L.) and *Jasminum grandiflorum* (L.)

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Received 15 February, 2014; Accepted 19 May, 2014

The discovery of antimicrobials from traditional medicinal plants is gaining importance. The objectives of this study were to determine the anti-candidal activity of young and mature leaves of *Piper betle* collected from dry and wet zones of Sri Lanka, leaves and roots of *Vitex negundo* and leaves of *Jasminum grandiflorum* and the determination of minimum inhibitory concentrations (MIC). Water and ethanolic extracts of plant material were tested against standard cultures of *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis*. The MICs of active extracts were determined. Ethanolic extracts of young leaves of *P. betle* showed significantly higher ($p < 0.05$) anti-candidal activity against all *Candida* species bioassayed, while mature leaves showed less activity. MICs of ethanolic extract of young leaves of *P. betle* were within 0.64 - 3.2 mg/mL. There was no significant difference between the activity of leaves of *P. betle* collected from wet zone and dry zone ($p > 0.05$). Water extracts of leaves of *P. betle* and water and ethanolic extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum* did not show a significant anti-candidal activity. Hence, young leaves of *P. betle* can be used as an anti-candidal agent since betel leaves are used in masticatory mixtures.

Key words: Candidiasis, *Piper betle*, young and mature leaves, MIC.

INTRODUCTION

Much focus is being given to traditionally-utilized medicinal plants for their antimicrobial properties, due to the shortcomings in most of the widely used synthetic drugs, viz; the evolution of resistance by microorganisms

to prevailing antimicrobials, potential health hazards, side effects and the loss of public reliance (Livermore, 2004). In the recent past, research has proven the potential of herbal products in the development of novel, safe,

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Abbreviations: MIC, Minimum inhibitory concentration; DMSO, dimethyl sulfoxide; MHA, Mueller Hinton agar; BSAC, British Society for Antimicrobial Chemotherapy.

effective and potent antimicrobials (Rai and Mares, 2003).

Most antimicrobial and medicinal properties of plants can be attributed to secondary metabolites (Cowan, 1999). Young leaves, young stems and reproductive plant parts have been observed to contain higher concentrations of secondary metabolites while mature plant parts have lower concentrations (Taiz and Zeiger, 2006). In addition, more secondary metabolites are produced when the plant is under stress including water stress and pathogen attack (Taiz and Zeiger, 1991). Controversially, it is also reported that water stress leads to low concentrations of secondary metabolites in plants (Gutbrodt et al., 2011).

Piper betle L. (Piperaceae) is a perennial, evergreen vine with stout, semi-woody stems. It has the vernacular names 'bulath' in Sinhala, betel vine or betel pepper in English and 'ilaikkodi', 'nirvalli' in Tamil. The plant grows well in warm, humid climates and is indigenous to and cultivated in Sri Lanka, India, Malay Peninsula, Philippines and East Africa, exhibiting a wide-spread distribution.

The active compounds isolated from the leaves are hydroxychavicol, hydroxychavicol acetate, chavibetol, cadenine, piperbetol, methylpiperbetol, piperol A and piperol B. In addition, the alkaloid arakene, terpenes, sesquiterpenes, cineol, eugenol, methyl ether and caryophyllene also are present in the leaves (Jayaweera, 1982; Datta et al., 2011; Pin et al., 2010). The major components of *P. betle* essential oil are known to be eugenol and acetyleugenol (Prakash et al., 2010). The leaves of *P. betle* are well recognized in Ayurveda for their antimicrobial and antioxidant properties (Datta et al., 2011).

The antifungal activity of hydroxychavicol isolated from the chloroform extraction of aqueous leaf extract of *P. betle* has been investigated by Ali et al. (2010). They have documented the antifungal effect of the leaf extract on yeasts, *Aspergillus* spp. and dermatophytes. Bruised leaves are applied on cuts and wounds as an antiseptic (Jayaweera, 1982). Due to the strong pungent aromatic flavour, betel leaves are used as a masticatory agent by certain Asian communities (Datta et al., 2011).

Vitex negundo L. (Verbenaceae) is a slender, deciduous tree growing up to heights of about 8 m. It is known as 'nika', 'nirgundi' in Sinhala and as 'nir-nochchi', 'nochchi' in Tamil. This plant is native to many countries including Zanzibar, Mozambique, Madagascar and Sri Lanka. The chemical constituents of leaves of *V. negundo* include flavonoids, nishindine, 1-sabinene, 1- α -pinene, viridiflorol, beta-caryophyllene, 4-terpineol, gamma-terpinene, caryophyllene oxide and sesquiterpenes including copaene (Singh et al., 1999; Jayaweera, 1982). Gautam et al. (2008) have carried out a phytochemical investigation of the methanolic extract of leaves of *V. negundo* to identify the major chemical constituents, that is, negundoside, agnuside, vitegnoside, 7,8

dimethyl herbacetin 3-rhamnoside, 5,3'-dihydroxy-7,8,4'-trimethoxy flavanone, 5-hydroxy-3,6,7,3',4'-pentamethoxy flavone, 5,7 dihydroxy- 6,4' dimethoxy flavonone and 5 hydroxy-7,4' dimethoxy flavones. The leaves and roots are known to have antimicrobial properties. In Sri Lanka, *V. negundo* is used in the treatment of toothache, eye diseases and rheumatism (Vishwanathan and Basavaraju, 2010).

Jasminum grandiflorum L. (Oleaceae) is a climbing shrub with green stems. It is known as 'saman pichcha', 'desaman' in Sinhala and as 'kodimalligai', 'pichi' in Tamil. *J. grandiflorum* is a common plant grown in Sri Lankan home gardens and is especially reckoned for its fragrant flowers. The active compounds of the leaves include the alkaloid jasmnine, salicylic acid and an astringent principle.

In addition, Sadhu et al. (2007) have identified secoiridoid glucosides, 2"-epifraxamoside and demethyl-2"-epifraxamoside and the secoiridoid jasminanhydride from aerial parts of *J. grandiflorum*. In traditional Ayurveda, the leaves of the plant are used as a remedy to treat ulcers in the mouth and to relieve toothache. Also, the fresh juice of leaves is used to soften corns (Jayaweera, 1982).

The current study was carried out with the aim of determining the anti-candidal activity of leaves of *P. betle*, leaves and roots of *V. negundo* and leaves of *J. grandiflorum*. Further, the effects of leaf maturity and the dry and wet zone climatic conditions relating to anticandidal activity of *P. betle* were investigated.

MATERIALS AND METHODS

Microbial isolates

Standard cultures of five *Candida* spp., namely *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 13803) were used in the study. Sub culturing was done on sterile Sabouraud's Dextrose Agar (SDA) and the plates were incubated at 37°C for 24 h. The resultant colonies were kept at 4°C for short-term storage.

Identification of plants

Herbarium specimens of the three test plants were prepared with twigs carrying reproductive plant parts and the specimens were identified to be *P. betle*, *V. negundo* and *J. grandiflorum* by comparing with the specimens at the National Herbarium of the Royal Botanic Gardens, Peradeniya, Sri Lanka. The three specimens were deposited at the Herbarium of the Department of Botany, University of Peradeniya, Sri Lanka.

Plant material

Water extracts of leaves of *P. betle* were prepared with leaves collected from Kandy (Central province) and for the preparation of ethanolic extracts, young and mature leaves of *P. betle* were collected from 8 locations each from the dry zone (Central province,

Table 1. Volumes of the stock solution added to obtain the dilution range of the extract by Andrews (2005 and 2001) methods.

Volume of the stock solution (μL)	Concentration of final solution (mg mL^{-1})
a) 2560	12.8
1280	6.4
640	3.2
320	1.6
b) 256	1.28
128	0.64
64	0.32
32	0.16
0	0

a) Andrews (2005); b) Andrews (2001).

North Central province and North Western province) and wet zone (Southern province, Sabaragamuwa province, Western province and Central province) of Sri Lanka. Each second leaf counting from the leaf bud was taken as a young leaf while each fifth leaf counting from the leaf bud was taken as a mature leaf. Each sample comprised 12 leaves collected from 6 betel vines.

For the preparation of water and ethanolic extracts, leaves and roots of *V. negundo* and leaves of *J. grandiflorum* were collected from Kandy (Central province).

The plant material was cleaned to remove adhering soil, dust, debris and other alien material, washed well with distilled water and air-dried at room temperature ($27 \pm 1^\circ\text{C}$).

Preparation of extracts

Water extracts

Leaves of *P. betle* were cut into pieces (0.5 x 0.5 cm). Twenty grams of the leaves were boiled in 80 mL of distilled water, until the final volume reached 10 mL, according to traditional Ayurvedic practice. The extract was filtered using Whatmann No. 1 filter paper and the filtrate was transferred to sterile glass vials.

The same procedure was carried out for the preparation of water extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum*, separately.

Ethanolic extracts

Young and mature leaves of *P. betle* were cut into pieces (0.5 x 0.5 cm) and 20 g of each sample was weighed (SETRA EL - 410s). The sample was soaked separately for 10 min in 200 mL of 99% ethanol at room temperature ($27 \pm 1^\circ\text{C}$). Using the vacuum infiltration technique where soluble compounds are extracted to the solvent under a vacuum, the plant parts were stirred with the same aliquot of alcohol in a magnetic stirrer (STUART CB 161) at a speed of 3 revolutions per second, at room temperature for one hour. Subsequently, the extract obtained was filtered through a sterile Whatmann No. 1 filter paper and the filtrate was collected. Alcohol was removed from the ethanolic extract using a rotary evaporator (STUART RE 300) at a speed of 25 rounds per minute at 40°C (Rangama et al., 2009). The total amount of extract obtained from 20 g of leaves of *P. betle* was dissolved in 6 mL of dimethyl sulfoxide (DMSO) and was subjected to the agar well diffusion bioassay as described below. The extract was freeze-dried and stored at -70°C , for the determination of the minimum inhibitory concentration.

The same procedure was carried out for the preparation of ethanolic extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum*.

Agar well diffusion bioassay

The previously isolated 24 h standard cultures were used to prepare broth cultures (McFarland 0.5 standard). Twenty five milliliters each of sterile Mueller Hinton Agar (MHA) was poured into sterile 90 mm Petri dishes and were left to set followed by allowing to dry at 44°C . From each liquid microbial culture prepared, 2 ml was pipetted onto separate MHA plates and spread evenly by swirling the plate. The excess liquid culture was pipetted out using a micropipette. The plates were dried at 44°C for 10 min. Using a sterile 8 mm cork borer, equidistant wells of 8 mm in diameter and 4 mm in depth were bored on each MHA plate. Each well was sealed at the bottom with 5 μL of molten MHA and was labelled for the purpose of identification. Approximately 200 μL of each water extract was directly loaded into its corresponding well, ensuring that the wells neither overflowed nor were loaded below the top. The ethanolic extracts of the plant material were dissolved in 6 ml of DMSO and 200 μL of each extract was loaded into wells separately. The plates were left at room temperature ($27 \pm 1^\circ\text{C}$) for 30 min to allow the solutions to diffuse into MHA and were subsequently incubated at 37°C for 24 h. After incubation, the zone of inhibition around each well was measured. The above procedure was carried out in quadruplicate for each *Candida* isolate, separately.

Determination of the minimum inhibitory concentration (MIC) of the extracts

MIC of young leaves of *P. betle* was determined using both the Andrews (2001) method and the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2005) method. Since the leaf extract of *P. betle* was saturated at a concentration of 1.28 mg/mL in the Andrews (2001) method, BSAC method was subsequently followed to test for higher concentrations of the extract.

Andrews (2001, 2005) methods

Different volumes of the stock solution were added to 20 mL of sterile molten MHA maintained at a temperature of around 45°C , as indicated in Table 1 and mixed well. Each solution was transferred into a sterile 90 mm Petri dish which was marked into 5 partitions on the reverse side.

Table 2. Attributes of the ethanolic extracts of the plant material.

Extract	Solubility in water	Solubility in DMSO	Form		Colour *
			After rotary evaporation	After freeze drying	
Young leaves of <i>P. betle</i>	Partial	Complete	Sticky semi-solid	Sticky powder	Dark green
Mature leaves of <i>P. betle</i>	Partial	Complete	Sticky semi-solid	nd	Dark green
Leaves of <i>V. negundo</i>	Partial	Complete	Sticky semi-solid	nd	Dark brownish green
Roots of <i>V. negundo</i>	Complete	Complete	Sticky semi-solid	nd	Brownish
Leaves of <i>J. grandiflorum</i>	Partial	Complete	Sticky semi-solid	nd	Green

nd = No data as further experiments were not carried out for these extracts since they did not have a significant anti-candidal activity; *The colour of the extracts after rotary evaporation and after freeze drying was the same.

Table 3. Inhibition by water and ethanolic extracts of the plant material.

Isolate	Average radius of zone of inhibition (mm)							
	<i>P. betle</i> leaves		<i>V. negundo</i> leaves		<i>V. negundo</i> roots		<i>J. grandiflorum</i> leaves	
	WE	EE	WE	EE	WE	EE	WE	EE
<i>C. albicans</i>	0.2±0.61	0	1.8±0.41	0	0.4±0.25	0.2±0.41	0.1±0.20	
<i>C. glabrata</i>	0.2±0.61	0	0.8±0.42	0	0	0	0	
<i>C. krusei</i>	1.7±0.41	0	0.8±0.75	0	0	0	0	
<i>C. parapsilosis</i>	0	0	2.6±0.86	1.8±1.61	0	0	0.8±0.99	
<i>C. tropicalis</i>	0.4±0.55	0	1.7±0.68	0	0	0	0.3±0.82	

n=6, WE - water extract, EE - ethanolic extract, data for EE of leaves of *P. betle* is given in Figure 2.

MHA was allowed to solidify and the plates were subsequently dried at 44°C. Similarly, agar dilution plates were prepared using the negative control DMSO.

Inoculation of the plates

Liquid microbial cultures were prepared and the turbidity was made similar to that of the McFarland 0.5 standard solution. Within 30 min of inoculum preparation, 1 µL each of the inoculum was placed on the corresponding partitions on the MHA set plates prepared as mentioned above. These were allowed to be absorbed into the agar before incubation at 37°C overnight (Andrews, 2001). The above procedure was carried out for the agar dilution plates of the negative control, that is, DMSO in place of the leaf extract. The entire experiment was carried out in quadruplicate.

Statistical analysis

The data was subjected to statistical analysis of variance (ANOVA) using the SAS software (v6.12).

RESULTS

Attributes of the water extracts

The water extract of leaves of *P. betle* was dark green in colour, while the extracts of the leaves and roots of *V. negundo* were dull dark green and brownish, respectively.

The colour of the water extract of the leaves of *J. grandiflorum* was light green.

Attributes of the ethanolic extracts

On the other hand, the ethanolic extracts differed in their solubility, form and colour markedly in comparison to the water extracts. Table 2 illustrates the above differences, viz; solubility of the ethanolic extracts in water, in DMSO and their form and colour.

Agar well diffusion bioassay:

The activity of the water extracts of leaves of *P. betle* and water and ethanolic extracts of the leaves and roots of *V. negundo* and the leaves of *J. grandiflorum*, against *Candida* spp. is depicted in Table 3. The ethanolic extract of young leaves of *P. betle* showed a significant anti-candidal activity, which was greater than that of mature leaves, with a significant difference ($p < 0.05$; Figures 1 and 2). No significant difference could be observed between the anti-candidal activity of *P. betle* leaves collected from the wet and dry zones ($p > 0.05$; Figure 2).

Determination of the MIC

Table 4 gives the MIC and the average radius of zone of

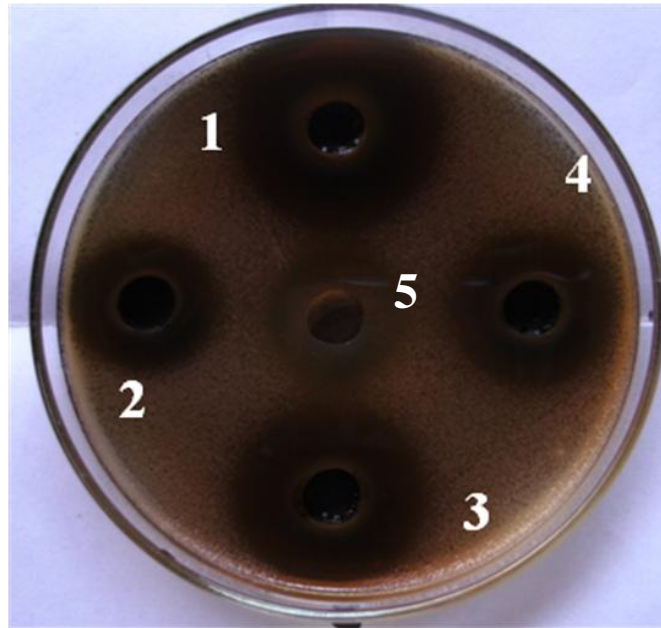


Figure 1. Zones of inhibition given by young leaf extract of *P. betle* from wet zone (1), mature leaf extract of *P. betle* from wet zone (2), young leaf extract of *P. betle* from dry zone (3), mature leaf extract of *P. betle* from dry zone (4) and the negative control DMSO (5).

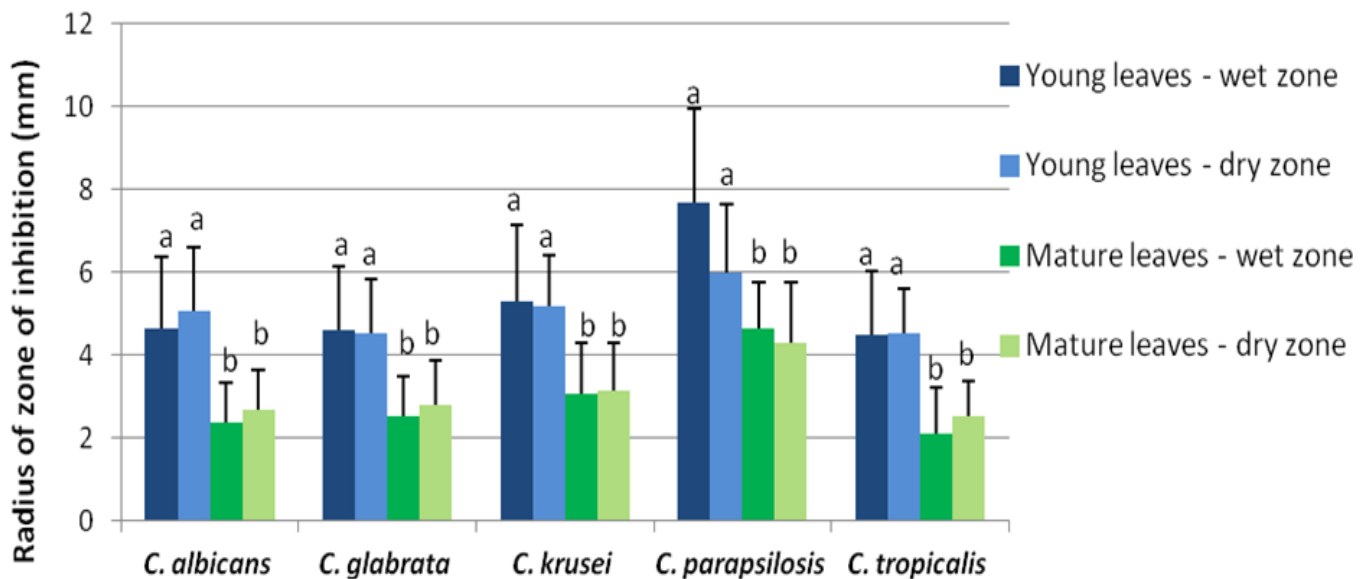


Figure 2. Effect of ethanolic extracts of young and mature leaves of *P. betle* collected from the wet and dry zones against different *Candida* species. $p < 0.05$ between anti-candidal activity of young and mature leaves, $p > 0.05$ between anti-candidal activity of leaves collected from dry and wet zones; $n=4$. Within each isolate, columns with the same letter are not significantly different ($p > 0.05$), by SAS Software (v6.12).

inhibition given by the ethanolic extract of young leaves of *P. betle* against each *Candida* sp. tested, together with the comparison with Fluconazole which is a commercially

available antimicrobial drug. As depicted in Table 4, a negative correlation was observed between the MIC values obtained and the average radius of the zone of

Table 4. Average radius of zone of inhibition and the MICs of the ethanolic extract of leaves of *P. betle*.

Isolate	Average radius of zone of inhibition (mm)	MIC (mg/mL)	MIC for Fluconazole (mg/mL) *
<i>C. albicans</i>	4.6	1.6	0.128
<i>C. glabrata</i>	4.6	0.8	0.064
<i>C. krusei</i>	5.3	1.6	0.064
<i>C. parapsilosis</i>	7.7	0.64	0.016
<i>C. tropicalis</i>	4.5	3.2	> 0.128

*Kanatiwela et al. (2010). Fluconazole is a standard antimycotic drug used against *Candida* spp.

inhibition, viz; when the MIC value was 0.64 mg/mL (the lowest) against *C. parapsilosis* the average radius of zone of inhibition was 7.7 mm (the highest), while when the MIC value was 3.2 mg/mL (the highest) against *C. tropicalis*, the average radius of the zone of inhibition was 4.5 mm (the lowest).

DISCUSSION

Many plants produce antimicrobial compounds mainly as a defense mechanism against stresses, pathogen attack, etc. (Taiz and Zeiger, 1991). As *Candida* spp. evolve resistance towards available antimycotic agents, much focus is being given to the investigation of novel, potent antimicrobial compounds mainly from natural sources including plants (Rai and Mares, 2003). The current study investigated the anti-candidal activity of three plants against standard cultures of 5 *Candida* species. This is the first record where the effects of leaf maturity and climatic conditions (dry and wet zones) have been investigated for anti-candidal activity of plant material.

According to studies carried out in the same laboratory, the ethanolic extract of leaves of *P. betle* proves to be a more potent anti-candidal agent when compared with *Pongamia pinnata*, *Tephrosia purpurea* and *Mimusops elengi* which also are used in traditional oral health care. The leaves, pods and roots of *T. purpurea* and bark of *M. elengi* had not shown a significant anti-candidal activity (Rangama et al., 2009) while the roots of *P. pinnata* has exhibited an MIC of 6.4 mg/mL against *C. albicans* while all other standard cultures were resistant against the extract (Kanatiwela et al., 2010). Hence, comparatively, leaves of *P. betle* prove to be a potential anti-candidal agent.

According to the results obtained in the current study, the ethanolic extract of young leaves of *P. betle* (variety 'val bulath') have a significant anti-candidal activity against all the five *Candida* species investigated: *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. Ethanolic extract of young leaves of *P. betle* showed a higher anti-candidal activity as compared to mature leaves of *P. betle* which indicates that young leaves of *P. betle* have a higher amount of secondary metabolites as compared to mature leaves. This could be

accepted as primarily antimicrobial compounds are secondary metabolites in plants (Taiz and Zeiger, 2006). It is inferred that secondary metabolites accumulate more in tissues with a higher probability of being attacked. Young leaves are more prone to attack by pathogens than mature leaves because of the fragility of their physical defenses such as the lack of a thick waxy cuticle and low cell wall rigidity. In addition, with leaf maturation, an increase in the activity of polyphenol oxydase and peroxidase enzymes occurs through which the phenolic compounds can be converted into quinones. Hence, young leaves may have more antimicrobial phenolics than mature leaves. Also, the rates of cyclization and dehydration of the compounds increase upon leaf maturation. In addition, the differences in the anti-candidal activity of young leaves of *P. betle* over mature leaves can also be attributed to the type of secretory structure in the leaves. Members of family Piperaceae (*P. betle*) contain osmophores which are external secretory structures. Plants with external secretory structures release their secretions upon organ maturation due to trichome cuticle disruption (Figueiredo et al., 2008).

In the current study, no significant difference was observed between the anti-candidal activity of ethanolic extract of leaves of *P. betle* collected from the dry and the wet zones. Therefore, it can be inferred that the dry zone environment has not significantly affected the production and composition of secondary metabolites in the leaves of *P. betle*, during this study. The work of Gutbrodt et al. (2011) also shows that water stress leads to low concentrations of secondary defense compounds in plants which are severely stressed. However, according to Taiz and Zeiger (1991), the production of secondary metabolites is increased when the plant is under stress including water stress and pathogen attack.

A significant anti-candidal activity was not observed in the ethanolic and water extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum*. The low anti-candidal activity of the ethanolic extract of the leaves of *V. negundo* may be improved via the use of a different solvent and a different extraction procedure, considering the polarity of the active compounds. Also, the purification of the active compound may lead to a higher anti-candidal activity.

The MIC values obtained for the ethanolic extract of

young leaves of *P. betle* against the five *Candida* spp. ranged from 0.64 to 3.2 mg/mL. In a previous study done using hydroxychavicol isolated from the chloroform extraction of the aqueous extract of leaves of *P. betle*, the MIC values obtained for *Candida* spp. ranged from 0.015 to 0.5 mg/mL (Ali et al., 2010). The higher MIC values obtained in the current study can be attributed to the fact that in the previous study (Ali et al., 2010) the bioassay was carried out with the purified compound hydroxychavicol, while the current study used the crude extracts of the leaves of *P. betle*. In addition, in the study by Ali et al. (2010), the water extracts were tested while the current study used the ethanolic extracts. Hence, due to differences in the solvent and in the extraction procedure, there may be differences in the types and amounts of the active compounds extracted. Several studies suggest that the phenolics 2-hydroxychavicol (4-allylpyrocatechol) and chavibetol are the major active principles isolated from the ethanolic extract of the leaves of *P. betle* (Jitesh et al., 2006).

It is reported that the ethanolic extract of leaves of *P. betle* shows antimicrobial activity against *C. albicans* (Napisah et al., 2011) whereas literature is minimal on the anti-candidal activity of the ethanolic extract of the leaves of *P. betle* against non-albicans. To obtain precise MIC values, a concentration series within the range obtained in the current study can be further investigated. For more accurate and precise results, the isolation and characterization of the active compounds in the ethanolic extract of the leaves of *P. betle* is required.

The variation of MIC values between different *Candida* species could be attributed to the composition of the cell wall of *Candida* spp. (Odds, 2004). It can be inferred that *C. tropicalis* and *C. albicans* are the most resistant of the isolates while *C. parapsilosis* is the most susceptible to the extract. *C. albicans* is known to be the major human pathogen and its pathogenicity is aided by the virulence features including the production of germ tubes, bio-film formation and protein secretion including phospholipase, protease and esterase activity. Further studies are necessary to explain the high resistance of *C. tropicalis* to the ethanolic extract of leaves of *P. betle*. Non-albicans including *C. tropicalis* too are emerging human pathogens although to a lesser extent as compared to *C. albicans* (Ellepola and Samaranayake, 2000).

It could be concluded that the ethanolic extract of young leaves of *P. betle* has significant anti-candidal activity against all the *Candida* spp. bioassayed. In addition, this was the first study investigating the effect of leaf maturity and climatic conditions on the anticandidal activity of *P. betle*. It was revealed that young leaves possess higher anticandidal activity as compared to mature leaves. Therefore, young leaves are more effective to be used for commercial antimicrobial formulations, as compared to the use of mature leaves. Hence, the use of young leaves of *P. betle* has a potential to replace drugs to which *Candida* spp. have evolved resistance, by incor-

porating the active compounds to tablets, disinfectants, toothpastes, mouth washes, creams, etc. in the pharmaceutical and cosmetic industries.

Conflict of Interests

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

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Full Length Research Paper

Comparative analysis of air, soil and water mycoflora of Samahni Area, District Bhimber Azad Kashmir, Pakistan

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Received 13 February, 2013; Accepted 19 May, 2014

Fungi are potential sources of different diseases for humans, animals and plants. The present research aimed to collect, isolate and identify different fungi with their prevalence ratio in different habitats of Samahni area, Azad Kashmir (Pakistan). Mycoflora prevails in diverse habitats viz: air, water and soil. From sampling sites, air-borne fungal spores were trapped and grown on different culture media viz: potato dextrose agar (PDA) and water agar (WA). The soil and aqua mycoflora was analyzed using PDA and/or WA media using different techniques. A total of 35 different fungal species were investigated to belong to 18 different genera. The density of identified fungal species in each sample was as follows: air mycoflora (AMF), 20 species; soil mycoflora (SMF), 32 species; water mycoflora (WMF), 11 species, respectively. This differential occurrence could be due to variability in geographical location, fungal growth substrates in different prevailing habitats. A considerable significant difference was seen among the fungal colonies of AMF (31.7%), SMF (50.7%) and WMF (17.4%). It was noted that soil mycoflora was more frequently depicted because land provides best environment and nourishment for growth and reproduction of fungi. It was also observed that some fungi were common in water and soil substrates which indicate that some species of soil fungi do use water as mode of distribution and dispersal. The most dominant fungal species were *Cladosporium cladosporioides* (7.1%) in air, *Fusarium oxysporum* (4.9%) in soil and *Fusarium oxysporum* (11.3%) in water samples. The prevalence and their subsequence impacts in forest ecosystem of Samahni are discussed with special reference of their importance to human life.

Key words: Fungi, comparative study, Mycoflora, Azad Kashmir, air pollution, forest.

INTRODUCTION

Fungi are multicellular, achlorophyllous, heterotrophic, eukaryotic and spore bearing organisms surrounded by a well defined cell wall made up of chitin, with or without fungal cellulose, along with many other complex organic

molecules. Fungi are unique organisms due to their morphological, physiological and genetic features; they are ubiquitous, able to colonize all matrices (soil, water, air) in natural environments, in which they play key roles

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in maintaining the ecosystems equilibrium (Anastasi et al., 2009; Valeria et al., 2013). The fungi secrete enzymes which break down solid materials into soluble compounds for absorption through their outer walls. They vary greatly in their ability to utilize different types of substrates. Some species are obligate parasites and are only able to utilize nutrients from living host tissue (Alexopoulos and Mims, 1979; Cullings et al., 2010). Most fungi are able to reproduce asexually and sexually forming spores which serve as units for survival and dispersal in soil, water and air. Fungi are widespread all over the world and high environmental burdens have been shown to be affected by various factors such as wind, moisture, temperature and air pollution leading to variations with respect to species and quantities from one season to another. Fungi thrive better in moist and warm places. However, fungi are also known as non-motile but some species of the oomycetes produce motile spores called zoospores (Letcher and Powell, 2002).

The knowledge of air-spores not only contributes to the understanding of their abundance and seasonal variations but also helpful in forecasting the epidemics of crop plants (Waggoner, 1960; Jadhav et al., 2010). Aerobiologists are mainly concerned with release, dispersion and deposition of spores, interaction of spores with each other, pollution, environmental factors and their impact on plants, animals and human beings (Srivastava, 1991).

Fungi are an important component of the soil micro biota. The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem (Valentien et al., 2009). Many fungi live on dead organic matter saprophytically or in soil where they are regarded as the most important decomposers of plant residues and other organic matter. *Aspergillus fumigatus* is cosmopolitan and thermo tolerant fungus which is isolated primarily from compost, plant material and from soil. Some fungi are cosmopolitan in nature as *Aspergillus nidulans* and *Aspergillus flavus* which are initially isolated from soil (Diana, 1994). However many fungi are also used as bioremediator of soil pollution caused by different environmental factors or man-made activities such as addition of heavy fertilizers or PCBs (Kireeva et al., 2008; Teng et al., 2010).

Water is the most common compound found on earth. Chemically, water is an oxide of hydrogen and it is the only chemical compound that naturally occurs in all three physical states, solid (ice and hail), liquid and vapour. Pure water is colourless, odourless and tasteless. Water has unique physical and chemical properties. Its ubiquitous presence makes it essential for life. The distribution of water is in various compartments of the body, and changes takes place in various disorders. A number of diseases that we suffer are from drinking of contaminated water. Water is used for agricultural, irrigation purposes, hydroelectric power, recreation and navigation. It is a universal solvent and necessary resource

because it has remarkable physical properties (Enger and Smith, 1998; Batko, 1975; Tanveer et al., 2011).

The term pathogen is applied to those organisms that either produce or involved in the production of a disease. Many fungi present in terrestrial or aquatic habitats cause diseases in plants and animals, hence hampering health and socio-economic conditions of the people (Mullenborn et al., 2008). Many fungi including important plant pathogens are able to grow at potentials which cause permanent wilting of crop plants. Slightly acidic conditions are optimum for the growth of most fungi (Abril et al., 2008).

Any soil borne fungal plant pathogens cause diseases of the root or stem disrupting the uptake and translocation of water and nutrients from the soil. Therefore, these commonly cause similar symptoms to drought and nutrient deficiencies; these include wilting, yellowing, stunting and death of plant (Ortonedo et al., 2004). Many fungal species cause "white rot" of wood decay in which the wood becomes a bleached appearance, with a spongy, stringy, or laminated structure, and where lignin as well as cellulose and hemicelluloses is broken down (Anastasi et al., 2009a). Species of Basidiomycetes cause "brown rot" and their number is ca. 6% which cause damage to crops and plants (Hibbett and Donoghue, 2001).

Some areas are mainly populated with pinus forest; and this type of forest species are infected by degrading and decamping taxa of fungi (Pinedo et al., 2009). Some diseases are often very difficult to diagnose accurately and the pathogens may be difficult to grow in culture and identify accurately.

The aims and objective of the study are: 1) to explore the distribution of air, soil and water-borne mycoflora of selected area of Samahni and 2) to check the distribution of pathogenic fungi of the selected areas.

MATERIALS AND METHODS

Mycoflora was studied from different samples of air, soil and water from five different localities of Samahni viz: Dab, Chiti Mitti, Chayee; Chowki and Manaana. It has a territorially mountainous climate. The mycofloral analysis of the selected area was conducted by applying different research methods for different techniques.

Isolation of airborne fungi by culture plate technique

Air spores were trapped directly in sterilized Petri dishes, which were containing already sterilized potato dextrose agar (PDA) and water agar (WA) media. The Petri dishes were brought to the laboratory for incubation. These were examined within a week for mycofloral analysis (Tilak, 1989; Nourian et al., 2007; Ahmed, 2008). The culture plate technique (CPT) was used for the isolation of airborne fungi following by Shah and Bashir (2008). Two types of media viz. potato dextrose agar and water agar were employed to trap the fungal spores from air over the selected area of Samahni. The culture plates were exposed at different locations around the selected area at an altitude of five feet. The exposure time was five to ten minutes. Unexposed plates at each location served as

controls. Each treatment was replicated three times. The plates were sealed after exposure and brought to the laboratory. The plates were incubated at room temperature in the laboratory of the Department of Plant Pathology, Peer Mehr Ali Shah Arid Agriculture University Rawalpindi (PMAS UAAR) for five to seven days or until colonies matured. Fungal colonies were counted and identified after this incubation period. Colonies that failed to sporulate on this medium were routinely sub-cultured onto another new media for further investigation. The procedure was repeated thrice for the detection of air mycoflora. Identification was carried out by following literature of Cook (1963), Domesch et al. (1980) and Hussain et al. (2011).

Isolation of fungi from soil by soil plate method

Soil samples were collected from a depth of 10-15 cm at each site. A sterilized cork borer having 5 mm diameter was used to collect each sample by pushing it into the soil. The cork borer was rinsed briefly after each sampling in 95% ethanol. The cores of soil were pushed out with a sterilized plunger directly into clean unused polythene bags and transported to laboratory. After collection, each sample was examined within a week for the study of microfungi (Irum et al., 2007). The fungal population of the soil samples was determined by soil plate method and serial dilution agar plate method.

Mycoflora was detected by soil plate method with some modifications (Warcup, 1950; Hussain et al., 2011). One gram of soil was scattered on the bottom of each sterile Petri dish that contained already semi-solid sterilized molten cooled (40-45°C) PDA and WA media. Then they were rotated gently to disperse the soil particles in the medium. It was mixed thoroughly and left to solidify for appropriate time. These were incubated at room temperature. Fungi growing on the Petri plates were isolated after incubation period (two to three days) and identified microscopically. The identified fungal colonies were counted and identified by following mycological literature of Domesch et al. (1980) and Hussain et al. (2011).

Isolation of fungi from soil by serial dilution agar plate method

Serial dilution agar plate method detected mycoflora with some alterations (Waksman, 1922; Hussain et al., 2011). The collected soil samples were mixed with sterile distilled water to make a soil suspension. Serial dilutions of soil samples were made in sterile distilled water by adding 1.0 g of soil sample to 9.0 ml of water and were mixed vigorously for uniform distribution to make a soil solution. 1 ml of the soil solution was serially diluted to 10 and 0.5 ml of each of the inoculums used in a pour plate method incorporated with antibiotic mixture. 10 ml sterilized distilled water was added in each set of nine test tubes for dilution of the collected soil samples. 1 ml of soil suspension from the prepared soil suspension was added in a test tube to make 1:10 dilution and 1 ml of suspension from 1:10 dilution transferred to second test tube gave 1:100 dilutions then 1:1000 dilutions was made. Similarly, a series of dilutions were made. 1 ml suspension from each test tube were transferred in sterilized Petri plates and poured with 15 ml of melted cooled PDA and WA media. It was mixed thoroughly and left to solidify for some time. A control was prepared against bacterial growth. All media were steam sterilized at 121°C for 15 min. The medium for the primary isolation of fungi was formulated with streptomycin-penicillin antibiotics added at a final concentration of 0.6 ml/Petri dish of PDA (APHA, 1995). After 2-7 days, fungal colonies were appeared on the PDA and WA media. Fungi were identified by using mycological literature (Raper and Thomas, 1945; Gilman, 1957; Domesch et al., 1980; Ellis, 1971; Nelson et al., 1983; Hussain et al., 2011).

Isolation of fungi from different water samples by direct plate technique

The sterilized conical flasks (250 ml) were used for each water sampling. The flasks were submerged into water in an inverted position to 4-6 cm depth by removing the flask lid. The flasks were allowed to fill with water at this point and the lids were placed over the flask while still under water surface. The collected samples were taken back to the laboratory for fungal study (Nasser, 2004). One ml aliquots from each of the collected water samples were pipetted aseptically into three sterilized Petri plates having semi-solid sterilized molten cooled (40-45°C) PDA and WA media. Total fifteen Petri plates were prepared from five selected sites with the help of sterilized pipette. These were rotated gently to disperse the water in the medium. It was mixed thoroughly and left to solidify for appropriate time. The procedure was replicated three times for better result. The Petri plates were incubated at room temperature. Fungi grown on Petri plates were isolated after two to three days incubation period and identified under 10x objective microscope. The percentage occurrence of each fungus was determined (Warcup, 1950; Hussain et al., 2011).

Isolation of fungi from different water samples by baiting technique

The water samples were taken back to the laboratory in the sterilized flasks, where 40 sterilized hemp seeds were added in each flask (Vanbreuseghem, 1952; Hussain et al., 2011). The flasks were kept in the dark for 24 h at room temperature. The sterilized hemp seeds were colonized after 24 h. The colonized seeds were transferred to 20 ml sterilized mixture of distilled water and tap water in sterilized Petri plates in a 1:1 ratio. Ten seeds were placed in each Petri dish. 10 ml water from above ratio was added in each Petri plates with 2000 unit/L of antibiotic Streptomycin to suppress the bacterial growth. Four Petri plates were prepared from each flask. A total of 20 Petri plates were obtained from five samples. The Petri plates containing colonized hemp seeds were incubated at room temperature. The seeds were observed after two to seven days under 10x objective microscope for the study of fungal growth. Fungal mycelium or hyphae appeared on the hemp seeds as a white tuft after three to five days. Sometime it did not become visible until 5-7 days after setting up the experiment. This process was continued for five weeks. The water was changed in each Petri dish after each examination. The procedure was replicated thrice for good result. Fungi were identified from these primary cultures. The rest of the fungi were identified after sub-culturing (Dick, 1965; Hussain et al., 2011).

RESULTS AND DISCUSSION

This research was aimed to collect different mycoflora from the selected area and to identify them by application of different techniques and find out the prevalence of the mycoflora in different habitats. Its economic importance and pathogenic effects were also considered. In this research, a total of 63 fungal taxa were isolated and described for their importance values in forest ecosystem of Samahni. This research conducted depicts that soil has the highest percentile with 32 species in soil followed by air with 20 species and water with 11 species, respectively (Table 1).

Some species such as *Botritis* sp., *Cladosporium cladosporioides*, *Curvularia lunata*, *Drechslera* sp.,

Table 1. Air-borne, soil-borne and water-borne mycoflora isolated from different samples of selected area of Samahni Azad Kashmir.

Total fungal sp. isolated	Fungi isolated from different samples of		
	Air	Soil	Water
<i>Aspergillus</i> sp.	-	-	+
<i>Aspergillus niger</i>	+	+	-
<i>Aspergillus fumigates</i>	+	+	-
<i>Aspergillus flavus</i>	+	+	-
<i>Aspergillus nidulans</i>	-	+	-
<i>Aspergillus ustus</i>	-	+	-
<i>Alternaria alternate</i>	+	+	-
<i>Alternaria solani</i>	+	+	-
<i>Asteromyces</i> sp.	-	-	+
<i>Botrytis</i> sp.	+	+	+
<i>Cladosporium cladosporioides</i>	+	+	+
<i>Cladosporium herbarum</i>	-	+	-
<i>Curvularia lunata</i>	+	+	+
<i>Curvularia brachyspora</i>	+	+	-
<i>Curvularia clavata</i>	-	+	-
<i>Drechslera</i> sp.	+	+	+
<i>Fusarium chlamydosporum</i>	+	+	-
<i>Fusarium oxysporum</i>	+	+	+
<i>Fusarium incarnatum</i>	-	+	-
<i>Helminthosporium</i> sp.	+	+	-
<i>Helminthosporium nodulosum</i>	-	+	-
<i>Mucor varians</i>	+	+	-
<i>Mucor fragilis</i>	+	+	-
<i>Nigrospora</i> sp.	+	+	+
<i>Penicillium</i> sp.	+	+	+
<i>Penicillium chrysogenum</i>	+	+	-
<i>Penicillium brefeldianum</i>	-	+	-
<i>Pythium debaryanum</i>	-	+	-
<i>Pythium spinosum</i>	-	+	-
<i>Rhizoctonia solani</i>	-	+	-
<i>Stachybotrytis cylindrospora</i>	-	+	-
<i>Trichoderma virens</i>	+	+	-
<i>Thielavia terricola</i>	-	+	-
<i>Verticillium</i> sp.	+	+	+
<i>Verticillium terrestre</i>	-	-	+
Total	20	32	11

Key: + = Present; - = absent.

Fusarium oxysporum, *Nigrospora* sp., *Penicillium* sp. and *Verticillium* sp. were ubiquitously present in all analyzed samples from different sources (Table 1, Figures 4-11). The samples of fungi were isolated and grown on different media such as PDA and WA and their prevalence was found in same occurrence, so the media difference does not affect the process and results in the current research work (Table 2).

Difference was measured by least standard deviations (LSD) for different analytical results as shown in Table 3

and Figure 3; it shows that water-born fungi has highest LSD value (1.5), soil-born has 1.1 and air-born fungi has 0.31, respectively.

The Table 1 shows the presence/absence of fungal species in air, soil and water samples while Table 2 shows the presence/absence of fungal species on different cultural media (PDA and WA). Similarly total number of fungal colonies (%) in air, soil and water samples showed is in Table 3 while total numbers of fungal colonies (%) on different cultural media (PDA and

Table 2. Mycoflora isolated by use of PDA and WA media from different samples (air, soil and water) of selected area of Samahni (Azad Kashmir).

Total isolated fungal spp.	Fungal sp. isolated on PDA and WA media from					
	Air		Soil		Water	
	PDA	WA	PDA	WA	PDA	WA
<i>Aspergillus</i> sp.	-	-	-	-	+	+
<i>Aspergillus niger</i>	+	+	+	+	-	-
<i>Aspergillus fumigates</i>	+	+	+	+	-	-
<i>Aspergillus flavus</i>	+	+	+	+	-	-
<i>Aspergillus nidulans</i>	-	-	+	+	-	-
<i>Aspergillus ustus</i>	-	-	+	+	-	-
<i>Alternaria alternata</i>	+	+	+	+	-	-
<i>Alternaria solani</i>	+	+	+	+	-	-
<i>Asteromyces</i> sp.	-	-	-	-	+	+
<i>Botrytis</i> sp.	+	+	+	+	+	+
<i>Cladosporium cladosporioides</i>	+	+	+	+	+	+
<i>Cladosporium herbarum</i>	-	-	+	+	-	-
<i>Curvularia lunata</i>	+	+	+	+	+	+
<i>Curvularia brachyspora</i>	+	+	+	+	-	-
<i>Curvularia clavata</i>	-	-	+	+	-	-
<i>Drechslera</i> sp.	+	+	+	+	+	+
<i>Fusarium chlamydosporum</i>	+	+	+	+	-	-
<i>Fusarium oxysporum</i>	+	+	+	+	+	+
<i>Fusarium incarnatum</i>	-	-	+	+	-	-
<i>Helminthosporium</i> sp.	+	+	+	+	-	-
<i>Helminthosporium nodulosum</i>	-	-	+	+	-	-
<i>Mucor varians</i>	+	+	+	+	-	-
<i>Mucor fragilis</i>	+	+	+	+	-	-
<i>Nigrospora</i> sp.	+	+	+	+	+	+
<i>Penicillium</i> sp.	+	+	+	+	+	+
<i>Penicillium chrysogenum</i>	+	+	+	+	-	-
<i>Penicillium brefeldianum</i>	-	-	+	+	-	-
<i>Pythium debaryanum</i>	-	-	+	+	-	-
<i>Pythium spinosum</i>	-	-	+	+	-	-
<i>Rhizoctonia solani</i>	-	-	+	+	-	-
<i>Stachybotrytis cylindrospora</i>	-	-	+	+	-	-
<i>Trichoderma virens</i>	+	+	+	+	-	-
<i>Thielavia terricola</i>	-	-	+	+	-	-
<i>Verticillium</i> sp.	+	+	+	+	+	+
<i>Verticillium terrestre</i>	-	-	-	-	+	+
Total	20		32		11	

Key: + = Present; - = absent.

WA) are shown in Table 4.

Plate isolation of airborne fungi by culture technique

A total of twenty different fungal species were isolated by using culture plate technique (CPT) from samples of air (Table 1). The results (Table 2) showed that same fungal species were grown on PDA media and WA media. On

the other hand, the fungal colony (percentage) showed variation among different cultured media. More numbers of fungal colonies (percentage) appeared on PDA as compared to WA due to their nutritional variation (Table 4). The growth of fungi shows quantitative difference on both media. The present study was similar to the study of Moring et al. (1983).

The results (Table 4) showed that the dominant fungal species in air sample was *Cladosporium cladosporioides*

Table 3. Total number of colony (percentage) of air-borne, soil-borne and water-borne mycoflora isolated from different sampling sites of Samahni (Azad Kashmir).

Total fungal sp. isolated	Total no. of colonies (%) of		
	Air-borne fungi	Soil-borne fungi	Water-borne fungi
	Percentage	Percentage	Percentage
<i>Aspergillus</i> sp.	0 ^b	0 ^b	11.1 ^a
<i>Aspergillus niger</i>	5.8 ^a	4.4 ^a	0 ^b
<i>Aspergillus fumigatus</i>	6.1 ^a	4.5 ^b	0 ^c
<i>Aspergillus flavus</i>	5.2 ^a	3.9 ^b	0 ^c
<i>Aspergillus nidulans</i>	0 ^b	3.1 ^a	0 ^b
<i>Aspergillus ustus</i>	0 ^b	2.7 ^a	0 ^b
<i>Alternaria alternata</i>	5.2 ^a	4.5 ^a	0 ^b
<i>Alternaria solani</i>	6.6 ^a	3.4 ^b	0 ^c
<i>Asteromyces</i> sp.	0 ^b	0 ^b	9.4 ^a
<i>Botrytis</i> sp.	5.1 ^b	3.7 ^c	11.0 ^a
<i>Cladosporium cladosporioides</i>	7.1 ^b	2.6 ^c	9.0 ^a
<i>Cladosporium herbarum</i>	0 ^b	3.0 ^a	0 ^b
<i>Curvularia lunata</i>	4.1 ^b	3.9 ^b	7.8 ^a
<i>Curvularia brachyspora</i>	4.8 ^a	2.9 ^b	0 ^c
<i>Curvularia clavata</i>	0 ^b	3.1 ^a	0 ^b
<i>Drechslera</i> sp.	5.6 ^a	2.2 ^b	6.4 ^a
<i>Fusarium chlamydosporum</i>	4.3 ^a	3.6 ^a	0 ^b
<i>Fusarium oxysporum</i>	4.6 ^b	4.9 ^b	11.3 ^a
<i>Fusarium incarnatum</i>	0 ^b	1.9 ^a	0 ^b
<i>Helminthosporium</i> sp.	4.6 ^a	4.0 ^a	0 ^b
<i>Helminthosporium nodulosum</i>	0 ^b	2.1 ^a	0 ^b
<i>Mucor varians</i>	3.2 ^a	2.2 ^b	0 ^c
<i>Mucor fragilis</i>	2.9 ^a	1.4 ^b	0 ^c
<i>Nigrospora</i> sp.	4.4 ^b	3.1 ^b	9.4 ^a
<i>Penicillium</i> sp.	6.8 ^b	4.6 ^c	9.6 ^a
<i>Penicillium chrysogenum</i>	4.5 ^a	3.7 ^a	0 ^b
<i>Penicillium brefeldianum</i>	0 ^b	2.4 ^a	0 ^b
<i>Pythium debaryanum</i>	0 ^b	4.1 ^a	0 ^b
<i>Pythium spinosum</i>	0 ^b	2.4 ^a	0 ^b
<i>Rhizoctonia solani</i>	0 ^b	3.9 ^a	0 ^b
<i>Stachybotrytis cylindrospora</i>	0 ^b	0.9 ^a	0 ^b
<i>Trichoderma virens</i>	4.7 ^a	1.7 ^b	0 ^c
<i>Thielavia terricola</i>	0 ^b	1.6 ^a	0 ^b
<i>Verticillium</i> sp.	4.3 ^b	3.7 ^b	6.2 ^a
<i>Verticillium terrestre</i>	0 ^b	0 ^b	8.8 ^a
LSD value	0.31	1.1	1.5

Values in each row with different letters show significant difference as determined by LSD test at $P \geq 0$.

(7.1%) while minimum airborne fungal species appeared as *Mucor fragilis* (2.9%). The frequencies of airborne fungi were significantly different. These results were correlated with the findings of Nasim et al. (1998), Rao, et al. (2009) and Shah and Bashir (2008).

Out of 35 isolated fungal species, 15 species were absent in air samples (Table 1). *C. cladosporioides* (7.1%) and *Penicillium* sp. (6.8%) are not significantly

different. Therefore, these two species have equal distribution in air samples. The fungal species such as *A. niger* (5.8%), *Drechslera* sp. (5.6%), and *Aspergillus flavus* (5.2%), *Alternaria alternata* (5.6%), were significantly the same in distribution (Table 4). These findings were also similar to the findings of Shah et al. (1995), Shah and Bashir (2008) and Bajwa et al. (1997). They also stated that there were significant similar

Table 4. Total number of fungal colony (%age) of air, soil and water-borne fungi isolated by using PDA and WA media from selected areas of Samahni (Azad Kashmir).

Identified fungal species	Total number of colony					
	Air		Soil		Water	
	PDA (%)	WA (%)	PDA (%)	WA (%)	PDA (%)	WA (%)
<i>Aspergillus</i> sp.	0 ^c	0 ^c	0 ^c	0 ^c	12.64 ^a	8.97 ^b
<i>Aspergillus niger</i>	7.7 ^a	3.0 ^c	4.8 ^b	3.2 ^b	0 ^c	0 ^c
<i>Aspergillus fumigates</i>	7.3 ^a	4.4 ^b	3.8 ^b	5.1 ^b	0 ^c	0 ^c
<i>Aspergillus flavus</i>	5.9 ^a	4.3 ^b	3.2 ^b	4.3 ^b	0 ^c	0 ^c
<i>Aspergillus nidulans</i>	0 ^b	0 ^b	3.0 ^a	2.9 ^a	0 ^b	0 ^b
<i>Aspergillus ustus</i>	0 ^b	0 ^b	3.2 ^a	4.0 ^a	0 ^b	0 ^b
<i>Alternaria alternata</i>	6.4 ^a	3.5 ^b	3.8 ^b	4.9 ^b	0 ^c	0 ^c
<i>Alternaria solani</i>	7.3 ^a	5.6 ^b	3.7 ^c	2.4 ^c	0 ^d	0 ^d
<i>Asteromyces</i> sp.	0 ^c	0 ^c	0 ^c	0 ^c	8.2 ^b	11.1 ^a
<i>Botrytis</i> sp.	4.6 ^b	5.8 ^b	4.3 ^b	2.4 ^b	10.2 ^a	12.0 ^a
<i>Cladosporium cladosporioides</i>	7.7 ^b	6.1 ^b	3.2 ^c	1.4 ^c	8.6 ^a	9.6 ^a
<i>Cladosporium herbarum</i>	0 ^b	0 ^b	3.1 ^a	2.4 ^a	0 ^b	0 ^b
<i>Curvularia lunata</i>	3.9 ^c	4.4 ^c	4.8 ^c	2.1 ^d	7.0 ^b	9.0 ^a
<i>Curvularia brachyspora</i>	4.6 ^a	5.2 ^a	2.5 ^b	3.3 ^b	0 ^c	0 ^c
<i>Curvularia clavata</i>	0 ^c	0 ^c	2.4 ^b	3.7 ^a	0 ^c	0 ^c
<i>Drechslera</i> sp.	4.7 ^c	7.0 ^b	2.6 ^c	12.2 ^a	8.4 ^b	3.6 ^c
<i>Fusarium chlamydosporum</i>	3.9 ^a	4.9 ^a	3.2 ^b	3.7 ^b	0 ^c	0 ^c
<i>Fusarium oxysporum</i>	4.2 ^b	5.2 ^b	4.8 ^b	4.3 ^b	10.9 ^a	12.0 ^a
<i>Fusarium incarnatum</i>	0 ^c	0 ^c	1.6 ^b	2.2 ^a	0 ^c	0 ^c
<i>Helminthosporium</i> sp.	3.8 ^b	5.7 ^a	3.9 ^b	3.5 ^b	0 ^c	0 ^c
<i>Helminthosporium nodulosum</i>	0 ^c	0 ^c	1.7 ^b	2.4 ^a	0 ^c	0 ^c
<i>Mucor varians</i>	3.3 ^a	3.0 ^a	1.8 ^b	2.4 ^b	0 ^c	0 ^c
<i>Mucor fragilis</i>	2.9 ^a	2.9 ^a	1.7 ^b	0.7 ^c	0 ^d	0 ^d
<i>Nigrospora</i> sp.	3.5 ^d	5.7 ^c	2.4 ^d	3.7 ^d	8.6 ^b	10.5 ^a
<i>Penicillium</i> sp.	5.9 ^c	8.1 ^b	5.4 ^c	2.6 ^d	8.4 ^b	11.4 ^a
<i>Penicillium chrysogenum</i>	4.5 ^a	4.7 ^a	3.2 ^b	3.9 ^a	0 ^c	0 ^c
<i>Penicillium brefeldianum</i>	0 ^b	0 ^b	2.1 ^a	2.6 ^a	0 ^b	0 ^b
<i>Pythium debaryanum</i>	0 ^c	0 ^c	4.8 ^a	2.4 ^b	0 ^c	0 ^c
<i>Pythium spinosum</i>	0 ^c	0 ^c	2.0 ^b	2.7 ^a	0 ^c	0 ^c
<i>Rhizoctonia solani</i>	0 ^c	0 ^c	4.8 ^a	2.1 ^b	0 ^c	0 ^c
<i>Stachybotrytis cylindrospora</i>	0 ^c	0 ^c	0.7 ^b	1.0 ^a	0 ^c	0 ^c
<i>Trichoderma virens</i>	4.3 ^a	5.2 ^a	1.6 ^b	1.6 ^b	0 ^c	0 ^c
<i>Thielavia terricola</i>	0 ^b	0 ^b	1.5 ^a	1.5 ^a	0 ^b	0 ^b
<i>Verticillium</i> sp.	3.7 ^c	5.2 ^b	4.6 ^b	2.2 ^d	6.1 ^a	6.4 ^a
<i>Verticillium terrestre</i>	0 ^c	0 ^c	0 ^c	0 ^c	11.1 ^a	5.6 ^b
LSD value	1.6	1.5	0.8	1.2	2.5	2.6

Values in each row with different letters show significant difference as determined by LSD test at $P \geq 0.05$.

distributions among various fungal species.

Isolation of fungi from soil samples by soil plate method

Thirty-two (32) different fungal species were isolated and identified from the selected area by soil plate method while twenty-three fungal species were isolated and

identified from the collected soil samples by using serial dilution agar plate method (DAPM) from the selected area. The results show that same numbers of fungal species were growing on both PDA and WA media. The present study showed that more numbers of fungal species were isolated by applying DPM as compared to DAPM (Table 2). The soils of the selected area have more nutrition and suitable climatic factors for growth and dispersal of fungal spores. Therefore, maximum numbers

of fungi were isolated by soil plate method. This study was correlated with the previous study of Picco and Rodolfi (2000) Fang et al. (2005) and Adhikari et al. (2004). They explained that different environmental factors and more nutrition variation increase the fungal frequency in the soil samples.

The results indicate that these great differences occurred due to the geographical location, fungal growth substrates/media as well as due to applying different sampling methods. Adhikari et al. (2004) and Siham (2007) also investigated that the variation among different fungal species. They also revealed that the variation in results occurred due to variable growth media, different locality and due to implementation of different methods.

Isolation of fungi from soil samples by serial dilution agar plate method

Twenty-three (23) fungal species were isolated and identified from the collected soil samples by using DAPM from the selected area (Table 3). The results show that same numbers of fungal species were growing on both PDA and WA media. The present study also stated that more numbers of fungal species were isolated by applying DPM as compared to DAPM (Table 2).

As indicated in the result, more number of fungal species was isolated by DPM as compared to DAPM. In this method, soil samples were diluted. Therefore, frequency of spores reduced in diluted samples. The results indicated that these great differences occurred due to the geographical location, fungal growth substrates/media as well as due to applying different sampling methods. Adhikari et al. (2004) also investigated the variation among different fungal species. They showed that different sampling methods greatly influenced fungal flora.

Isolation of fungi from water by direct plate method

Nine different fungal species were isolated and identified from the collected water samples by direct plate method of the selected area (Figure 2; Table 3). The results depicts that minimum fungal species were isolated by using DPM. The present study showed that the direct inoculation of water samples on different media was not favorable for better growth of fungal spores.

The results (Table 3) indicated that most dominant fungal species isolated by direct plate method from water samples was *Fusarium oxysporum* (11.1%). The lowest prevalence of fungi in water samples was *Verticillium* sp. (6.1%). The distributions of other isolated waterborne fungal species occurred between these two statuses, that is, 6.1-11.1%. According to statistical analysis, the fungal species of *F. oxysporum* (11.1%), *Aspergillus* sp. (10.8%), *C. cladosporioides* (10.6%), *Asteromyces* sp.

(10.3%) and *Verticillium terrestre* (8.8%) are not significantly different. Therefore, these five species have equal distribution in water samples. Similarly, the occurrence of lowest species *Verticillium* sp. (6.1%), *Curvularia lunata* (6.9%), *Drechslera* sp. (7.5%) and *Nigrospora* sp. (8.5%) were not significantly different as shown in Table 3. The significantly same distribution of fungi appeared in water samples due to similar temperature and nutrient contents. Nourian et al. (2007) obtained similar results.

Isolation of fungi from water by baiting technique

Eleven (11) species were isolated from water samples by baiting technique (BT) from the selected area of Samahni district Bhimber Azad Kashmir (Table 3). The results showed that more number of fungal species was isolated by applying BT on water samples as compared to DPM. It means that low fungal frequency was obtained by using DPM technique while high fungal frequency was obtained by using any bait.

The results (Table 3) indicate that most dominant fungal species isolated from water samples was *F. oxysporum* (11.7%) while the lowest prevalence of fungi in water samples was *Drechslera* sp. (5.4%) The prevalence of other species was seen. According to statistical analysis, the fungal species *F. oxysporum* (11.7%), *Aspergillus* sp. (11.6%), *Botrytis* sp. (11.4%), *Nigrospora* sp. (10.3%) and *Penicillium* sp. (9.4%) are not significantly different. Similarly, the distribution of lowest fungal species, that is, *Drechslera* sp. (5.4%), *Verticillium* sp. (6.4%), and *C. cladosporioides* (7.6%) that occurred in water samples are not significantly different as shown in Table 4. The density of identified fungal species in each sample air mycoflora (AMF), soil mycoflora (SMF), water mycoflora (WMF) and mushrooms (MR) were, 20, 32, 11 respectively (Table 1).

A total of 35 different fungal species were isolated and identified during the present investigations from different samples of air, water and soil from the selected area. The identified species belonged to eighteen different genera namely, *Alternaria*, *Aspergillus*, *Asteromyces*, *Botrytis*, *Cladosporium*, *Curvularia*, *Drechslera*, *Fusarium*, *Helminthosporium*, *Mucor*, *Nigrospora*, *Penicillium*, *Pythium*, *Rhizoctonia*, *Stachybotrytis*, *Trichoderma*, *Thielavia* and *Verticillium* (Table 1). The results (Figure 1) indicated that 17% waterborne fungi, 32% airborne fungi and 51% soil fungi were isolated. The fungal population was very high in the soil sample. The environmental factors, high vegetation coverage, presence of high nutrition in soil and geographical location might increase the fungal frequency in the soil samples (Korkama-Rajala et al., 2008). These observations showed that high percentage of fungal species were isolated from soil samples. The maximum prevalence of fungi in soil samples occurred due to presence of high nutrition



Figure 1. Different fungal species growing on PDA.

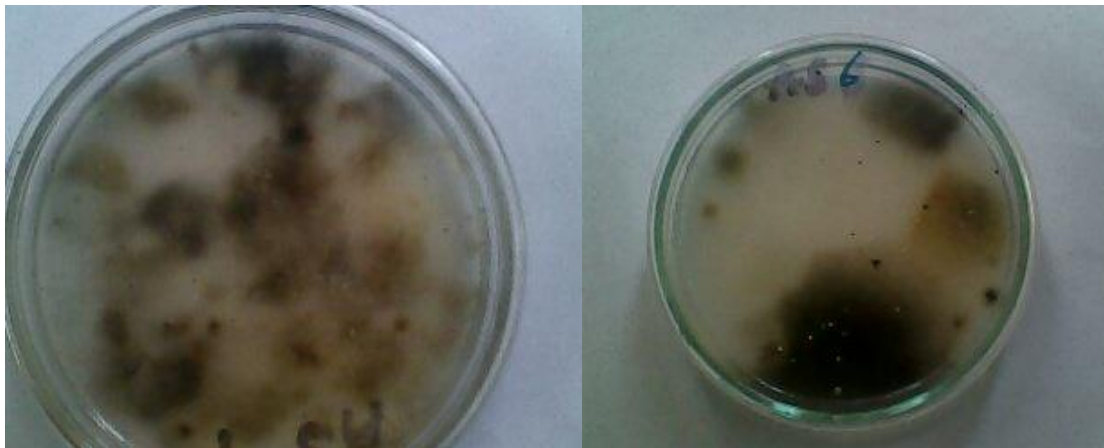


Figure 2. Different fungal species growing on WA.

in soil, variable environmental factors, geographical locations and high vegetation coverage. This study correlated with the previous study of Picco and Rodolfi (2000), Fang et al. (2005), Adhikari et al. (2004) and Soderlund (2009). They explained that different environmental factors, vegetation coverage and more nutrition variation increase the fungal frequency in different habitats.

It was noted that land-habituated mycoflora was more frequently depicted because land provides best environment and nourishment for growth and reproduction. It was also observed that some fungi were common in water and land and it means that some species of soil do use water as mode of distribution and dispersal. *Aspergillus* spp. was found commonly in air and soil habitats but its some species were observed in water. Its presence in aquatic habitat revealed that the contamination of sampling in experiment designing occurred, however, its common occurrence in both habitats showed that it is a terrestrial species. The present result was

similar to the result of Phukan and Baruah (1991) and Soderlund (2009). They isolated some terrestrial fungi from aquatic environment.

The high frequency of *F. oxysporum* and *C. cladosporioides* species are found in all the analyzed habitats. It predicts that these species have good morphological and reproductive mechanism, which makes it cosmopolitan in the environment. Resano et al. (1998) and Renata et al. (2004), supported these results. They showed that *C. cladosporioides* and *F. oxysporum* were commonly found with high frequency in forest vegetation area. According to statistical analysis, significant difference was studied among different fungal species, which were isolated from different habitats and different (PDA and WA) media as shown in Table 4. Nourian et al. (2007), Renata et al. (2004) and Soderlund (2009) applied statistical analysis among different samples of fungi which were significantly different. They explained that the species concentration differed from place to place because of the local environmental changes and

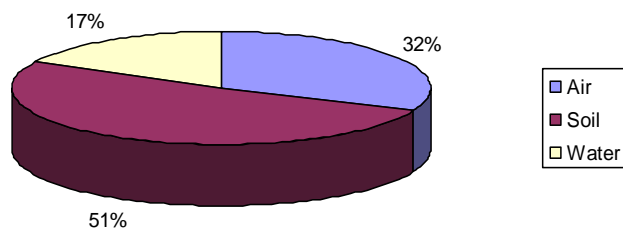


Figure 3. Total number of fungal colonies isolated from air, soil and water samples of Samahni, District Bimber, Azad Kashmir.



Figure 4. *Aspergillus* sp. (40x).



Figure 5. *Aspergillus niger* (40x).



Figure 6. *Aspergillus flavus* (100x).



Figure 7. *Cladosporium cladosporioides* (40x).



Figure 8. *Drechslera* sp. (40x).

due to use of different fungal growth media.

The number of some fungal colonies (percentage) among air and water samples was significantly different. Similarly, statistical analysis between soil and water samples also showed significant difference among isolated species of fungi. Shah et al. (1995), Bajwa et al. (1997) and Nourian et al. (2007), previously indicated

significant variation among different fungal species that appeared due to variable environment of air, soil and water samples.

This mycofloral research demonstrated that *Rhizoctonia*



Figure 9. *Mucar fragilis* (40x).



Figure 11. *Helminthosporium* sp. (40x).



Figure 10. *Alternaria alternata* (40x).

solani produce root rot in various plants of forest which destroy the natural flora. These findings are in accordance with previous results of Renata et al. (2004). The present study indicated that the isolated species of fungi *Cladosporium* and *Alternaria* are involved in spreading of allergy in organisms. These observations were similar to the results of Renata et al. (2004). They showed that *Cladosporium* and *Alternaria* species causes allergy in organisms. They also investigated that *Cladosporium* is a very common fungus that is known and documented as an aeroallergen that is usually associated with plants, wood products and leather goods. *Pythium debaryanum* is a soil pathogen isolated and identified from the investigated area which produces leaf blight and bulb rot symptoms on narcissus plants. These investigations were correlated with the results of Sung et al. (2007). They observed that *P. debaryanum* cause leaf blight and bulb rot symptoms on narcissus plants. The symptoms induced by artificial inoculation were similar to those observed in the selected area.

The study shows that *Verticillium* species are pathoge-

nic to different plants in the selected forest area. It produces *Verticillium* wilt. This study was similar to the previous study of Agrios (2005). He showed that some *Verticillium* species are pathogenic to different plants and cultivated and wild type crop species. He showed that *Verticillium* wilt is a vascular wilt disease of plants caused by *Verticillium* spp. Some species also infect mushrooms, rusts and other fungi in natural flora. Other species of *Verticillium* attack wool and textiles industries and decompose paper. Greatest numbers of these diseases were seen in higher plants by *Verticillium* and *Fusarium* spp. (Green, 1981). Many species of fungi were involved in serious human and animal infections. Some species of fungi also caused serious plant diseases. The present study showed that *A. alternata* is phytopathogenic fungus and show saprophytic symptoms on plants of the selected area. These findings were similar to the findings of Angulo et al. (1996) and Infante et al. (1992).

These findings showed that the fungal species *A. alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, *P. debaryanum*, *Cladosporium* spp. and *Verticillium* spp. affect the local plants and medicinal wealth is being destroyed in area of Samahni.

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Evaluation of antibacterial and antioxidant activity of extracts of eelgrass *Zostera marina* Linnaeus

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Received 23 March, 2014; Accepted 12 May, 2014

Zostera marina L. is one of marine flowering plants, which plays an important role in coastal ecosystems by generating oxygen and organic substance and providing a habitat for other organisms. However, its residues on seashore have caused serious environmental problems in some coasts worldwide. In this paper, crude extract of *Z. marina* L. was prepared using methanol-water (1:1, v/v) and further separated into petroleum ether fraction, ethyl acetate fraction, *n*-butanol fraction and water fraction. The antibacterial activity and antioxidant activity of different fractions from *Z. marina* L. were evaluated in order to provide information for utilizing the marine plant. Antibacterial bioassay showed that *n*-butanol fraction was effective only for *Staphylococcus aureus*, petroleum ether fraction was effective for *S. aureus* and *Bacillus anthracis*, ethyl acetate fraction was effective for *S. aureus*, *B. anthracis*, *Diphtheroid bacilli* and *Staphylococcus epidermidis*, while water fraction had no effect on all the tested strains. Antioxidant assay indicated that all of the four fractions showed α -diphenyl- β -picrylhydrazyl (DPPH) radical-scavenging activity and Fe³⁺ reducing activity, and ethyl acetate fraction exhibited the maximum activity.

Key words: *Zostera marina* L., extracts, antibacterial activity, antioxidant activity.

INTRODUCTION

Antibiotic resistance has become a global concern (Westh et al., 2004), and there has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Therefore, it is essential to search new antimicrobial substances from various sources like plants. Many researches have shown that higher plants represent a

potential source of novel antibiotic prototypes (Afolayan, 2003; Clark et al., 1993; Srinivasan et al., 2001). Some traditional herbs have already been proved to be effective against antibiotic-resistant bacteria (Kone et al., 2004), and further research is necessary to identify the antibacterial compounds (Romero et al., 2005). The pharmacological studies of antibacterial compounds will lead to synthesis of a more potent drug with reduced toxicity (Ebana et al., 1991; Manna and Abalaka,

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2000). Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals, which are continuously produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1984). Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Simic, 1988). However, antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage. Antioxidants are very important not only for the prevention of food oxidation but also for the defense of living cells against oxidative damage (Kim et al., 2003). Antioxidants reduce harmful reactive free radicals and reactive oxygen species (ROS) in cells (Chanwitheesuk et al., 2005), thereby preventing cancer and heart disease (Yan et al., 1998; Qi et al., 2005). Several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are extensively used because of their excellent efficacy and low cost. However, these artificial chemicals have major side-effects including toxicity and DNA damage problems (Choi et al., 1993; Sasaki et al., 2002). Therefore, the identification and isolation of new natural antioxidants from aquatic and terrestrial plants are also essential (Nishida et al., 1996).

Eelgrass (*Zostera marina* L.) is a flowering angiosperm belonging to Potamogetonaceae, which is an important species in coastal ecosystems because it contributes to nutrient cycling and sediment stabilizer, and provides food stuffs and habitat for many marine organisms such as invertebrates and fishes (Harrison, 1982; Moore and Short, 2006). Since halophytes growing in stressful environments make phenolic compounds to suppress the growth of yeast and mold (Bandaranayake, 2002), *Z. marina* L. might produce many secondary metabolites for protecting itself against microorganisms, epiphytes and predation (Harrison and Chan, 1980; Buchsbaum et al., 1990; Vergeer and Develi, 1997). Previous studies indicated that water-soluble extracts of eelgrass leaves could inhibit the growth of micro-algae and bacteria, and grazing by amphipods on dead leaves (Harrison, 1982; Lu and Foo, 1999). These observations indicate that eelgrass is a good resource for screening natural antibiotics.

Kawasaki et al. (1998) analyzed the compounds in the essential oil from eelgrass shoots and found that the major constituents were phytol, hexadecanamide, octadecanamide, pentadecane, heptadecane, nonadecane, (8Z,11Z)-heptadecadienal, (8Z)-heptadecenal, (9Z,12Z,15Z)-octadecatrienal and (9Z,12Z)-octadecadienal. Zosterin, a bioactive pectin from the eelgrass *Z. asiatica* Miki decreases the toxicity of antitumor drugs and purges heavy metals from human

organisms (Loenko et al., 1977). Achamlale et al. (2009) isolated and quantified rosmarinic acid (RosA) in *Z. noltii* Hornem. and *Z. marina* L., and RosA from *Z. marina* L. showed good nematocidal activity and antibacterial activity against pine wood nematode and its associated four bacterial strains (Wang et al., 2012). Antioxidant activity for the polysaccharide zosterin isolated from *Z. marina* L. has been examined by the scavenging of free radical induced peroxide oxidation in mice, and results showed that the low etherified pectin normalizes the level of malonic dialdehyde and the activities of glutathione reductase and glutathione peroxidase in the liver (Khasina et al., 2003).

Eelgrass shows great potential in exploring antibacterial and antioxidant products, but there have been few studies about inhibition effects of extract of *Z. marina* L. on human pathogenic bacteria. In the present study, we prepared four extracts of *Z. marina* L. and evaluated their antioxidant and antimicrobial activities in order to lay a foundation for further utilizing this promising marine plant.

MATERIALS AND METHODS

Eelgrass collection and treatment

Living eelgrass (*Z. marina* L.) was collected at low tide from the sub-tidal beds at Qingdao, China. The eelgrass leaves were washed carefully in fresh water to remove sediment and dried naturally at room temperature, and then crushed in smaller pieces by a disintegrator. Prior to experiment, samples were stored in a refrigerator at 4°C.

Preparation of extracts of *Z. marina* L.

Crushed eelgrass powder 300 g was mixed with 1200 ml methanol:water (1:1, v/v), extracted for 4 h at 40°C and filtrated with filter paper. After the methanol in the extract was removed by vacuum evaporation, the remaining residue was dissolved in 200 ml distilled water followed by subsequent extraction with 200 ml petroleum ether, 200 ml ethyl acetate, and 200 ml *n*-butanol, respectively. Each fraction was also evaporated to dryness, and the residue of each fraction was dissolved in distilled water and stored at 4°C for further use. Each extraction was performed three times.

Assay of antimicrobial activity

The various fractions of *Z. marina* L. were used for assessing their antibacterial activity. The antimicrobial assay was performed by agar well diffusion method (Perez et al., 1990). Six bacteria were used in this study, the four Gram positive bacteria were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus anthracis* and *Diphtheroid bacilli*, while the two Gram negative bacteria used were *Escherichia coli* and *Typhoid bacillus*.

The nutrient broth medium plate was inoculated with the 100 µl bacterial suspension (1×10^8 cfu/ml), and then, wells was punched in the plates with a sterilized borer (6 mm in diameter). After 30 µl sample was introduced into each well, the plates were incubated at 37°C for 24 h in an incubator. 0.1 mg/ml Oxolinic acid was used as positive control and sterilized water was used as negative control. Antibacterial activity was assayed by measuring the diameter of the

Table 1. Inhibitory effect of *n*-butanol fraction on *Staphylococcus aureus*.

Bacteria	Concentration (mg/ml)	Diameter of inhibitory zone (mm) ^a	MIC (mg/ml)
<i>Staphylococcus aureus</i>	20.0	13.67 ± 1.70	20
	25.0	13.67 ± 0.47	
	30.0	14.33 ± 0.47	
	35.0	15.67 ± 0.47	
	40.0	16.67 ± 0.47	

a: Inhibition zones are the mean including cup borer (6 mm) diameter.

inhibition zone. Each experiment was performed three times and the mean values are presented.

The minimum inhibitory concentration (MIC) for each fraction was determined by agar dilution method. Each fraction of *Z. marina* L. was mixed with nutrient agar to final concentrations ranged from 0.156 to 20 mg/ml to prepare the nutrient broth medium plates. The plates were inoculated with the 100 µl bacterial suspension (1×10^8 cfu) respectively and incubated at 37°C for 24 h in an incubator. Oxolinic acid (0.1 mg/ml) was used as positive control and sterilized water was used as negative control. The lowest concentration of each fraction which could inhibit completely the growth of bacteria was defined as MIC.

α-Diphenyl-β-picrylhydrazyl radical-scavenging activity

To test radical scavenging activity, reactions containing α-diphenyl-β-picrylhydrazyl (DPPH) were carried out according to the method of Wangenstein et al. (2004). Each fraction of *Z. marina* L. was diluted with distilled water to various concentrations and mixed with equal volume of DPPH solution, which was prepared at a concentration of 0.2 mmol/L in ethanol. After standing for 30 min at room temperature, absorbance of each reaction system was measured by a UV-VIS spectrophotometer at 517 nm (Persee, TU-1810, China). For control, ethanol was mixed with equal volume of DPPH solution and incubated at room temperature for 30 min. All of the determinations were performed three times. Radical scavenging capacity was calculated as $(A_0 - A_t)/A_0 \times 100\%$, where A_0 is the absorbance of the control and A_t is the absorbance of treatment group. Ascorbic acid was used as the positive control, and the concentration range was 0.04 - 0.20 mg/ml.

Reducing power

Reducing activity of various fractions from *Z. marina* L. was determined according to the methods of Yen and Chen (1995). Briefly, 2.5 ml extract in phosphate buffer (0.2 mol/L, pH 6.6) were added to 2.5 ml potassium ferricyanide (10 g/L) and the mixture was incubated at 50°C for 30 min. After 2.5 ml trichloroacetic acid (100 g/L) was added to the mixture, the solution was centrifuged for 10 min at 3,000 rpm. The upper layer (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml 0.1% ferric trichloride solution, and then absorbance of the resultant solution was recorded at 700 nm. The experiment was replicated for three times. Ascorbic acid was used as the positive control, and the concentration range was 0.02 - 0.10 mg/ml.

RESULTS

Antimicrobial activity

Different fractions isolated from *Z. marina* L. were assayed

for their antibacterial activities. The results indicated that eelgrass extracts could only inhibit the growth of Gram positive bacterial strains, and had no effect on Gram negative bacterial strains such as *E. coli* and *T. bacillus*.

The antibacterial activities on the four Gram positive strains of various fractions of eelgrass also differed greatly. The *n*-butanol fraction was only effective for *S. aureus* (Table 1) with minimum inhibitory concentration (MIC) of 20 mg/ml, petroleum ether fraction was effective for *S. aureus* and *B. anthracis* (Table 2) with MIC of 0.156 and 1.25 mg/ml, respectively. Ethyl acetate fraction showed antibacterial activity against *S. aureus*, *B. anthracis*, *D. bacilli* and *S. epidermidis* (Table 3), with MIC of 0.156, 5, 5 and 10 mg/ml, respectively. Water fraction had no effect on all the tested bacterial strains. The negative control, sterilized water had no effect on all the tested strains, while the positive control, oxolinic acid was effective for all the tested strains at the concentration of 0.1 mg/ml.

Antioxidant activity

The various fractions of eelgrass crude extracts were investigated for their reducing power and DPPH radical scavenging activity. The ethyl acetate fraction showed the strongest antioxidant activity, followed by the *n*-butanol and water fraction (Tables 4 and 5). In the DPPH test, ethyl acetate fraction had the highest radical scavenging activity with EC_{50} of 0.278 mg/ml, and the inhibition rate reached 77.97% at the concentration of 0.5 mg/ml.

The *n*-butanol fraction and water fraction also exhibited DPPH radical scavenging activity to some extents, with EC_{50} of 3.110 and 4.849 mg/ml, respectively (Table 4). There was a significant difference among the EC_{50} of ethyl acetate fraction, *n*-butanol fraction, water fraction and ascorbic acid, and radical scavenging activity of the three fractions of eelgrass were less than that of ascorbic acid.

In the determination of reducing power, the reduction of Fe^{3+} to Fe^{2+} by various fractions of *Z. marina* L. was measured. Results showed that all of the fractions could reduce Fe^{3+} with different efficiency, and the reducing power of all the fractions increased with increase of

Table 2. Inhibitory effects of petroleum ether fraction on *Staphylococcus aureus* and *Bacillus anthracis*.

Bacteria	Concentrations (mg/ml)	Diameter of inhibitory zone (mm) ^a	MIC (mg/ml)
<i>Staphylococcus aureus</i>	0.2	10.67 ± 0.47	0.156
	0.4	11.33 ± 0.47	
	0.8	14.67 ± 0.94	
	1.2	17.33 ± 1.25	
	1.6	23.67 ± 1.25	
<i>Bacillus anthracis</i>	0.2	ND	1.250
	0.4	ND	
	0.8	ND	
	1.2	9.33 ± 0.47	
	1.6	13.00 ± 0.82	

a: Inhibition zones are the mean including cup borer (6 mm) diameter. ND: not detected.

Table 3. Inhibitory effects of ethyl acetate fraction on four sensitive bacterial strains.

Bacteria	Concentration (mg/ml)	Diameter of inhibitory zone (mm) ^a	MIC (mg/ml)
<i>Staphylococcus aureus</i>	0.2	10.33 ± 0.47	0.156
	0.4	11.33 ± 0.47	
	0.6	13.67 ± 0.47	
	0.8	16.67 ± 0.47	
	1.0	19.00 ± 0.82	
<i>Bacillus anthracis</i>	4.0	12.67 ± 0.47	1.250
	8.0	18.67 ± 0.94	
	12.0	20.33 ± 0.47	
	16.0	22.33 ± 0.47	
	20.0	23.00 ± 0.82	
<i>Diphtherid bacillus</i>	4.0	13.33 ± 1.25	5.000
	8.0	17.67 ± 0.47	
	12.0	21.33 ± 0.47	
	16.0	21.67 ± 0.47	
	20.0	21.67 ± 0.47	
<i>Staphylococcus epidermidis</i>	4.0	ND	10.000
	8.0	8.00 ± 0.82	
	12.0	10.67 ± 0.47	
	16.0	12.33 ± 0.47	
	20.0	13.33 ± 0.47	

a: Inhibition zones are the mean including cup borer (6 mm) diameter. ND: not detected.

concentration. The reducing power of the ethyl acetate fraction exhibited highest activity than that of the other fractions (Table 5), and the reducing power was 0.199 at the concentration of 0.1 mg/ml, which was similar to that for ascorbic acid at the concentration of 0.04 mg/ml. However, reducing power was significantly lower compared with that of ascorbic acid.

DISCUSSION

Intact eelgrass leaves decay very slowly (Wang et al., 2012), which indicated they have special chemical constituents preventing microorganisms from utilizing them, and they are widely selected and used as roof materials in some seaside villages in Northern China

Table 4. Dose-dependent DPPH inhibition activity (%; n = 3) of different fractions.

Fraction	Concentration (mg/ml)	DPPH inhibition activity (%)	Regression equation
Petroleum ether fraction	0.20	4.439 ± 0.001	y=0.1291x+0.0096 R ² =0.9517 EC ₅₀ =3.799
	0.40	6.951 ± 0.001	
	0.60	7.454 ± 0.001	
	0.80	12.479 ± 0.001	
	1.00	13.149 ± 0.001	
Ethyl acetate fraction	0.10	26.957 ± 0.002	y=1.5362x+0.0744 R ² =0.9644 EC ₅₀ =0.278
	0.20	40.870 ± 0.000	
	0.30	59.130 ± 0.000	
	0.40	70.145 ± 0.000	
	0.50	77.971 ± 0.000	
<i>n</i> -Butanol fraction	1.00	22.995 ± 0.001	y=0.1415x+0.0600 R ² =0.9701 EC ₅₀ =3.110
	2.00	36.715 ± 0.001	
	3.00	53.527 ± 0.001	
	4.00	63.768 ± 0.000	
	5.00	71.208 ± 0.001	
Water fraction	2.00	23.722 ± 0.001	y=0.0885x+0.0710 R ² =0.9491 EC ₅₀ =4.849
	4.00	48.160 ± 0.000	
	6.00	70.450 ± 0.001	
	8.00	80.061 ± 0.005	
	10.00	85.583 ± 0.000	
Ascorbic acid	0.04	24.932 ± 0.001	y=3.8411x+0.0622 R ² =0.9709 EC ₅₀ =0.114
	0.08	38.571 ± 0.000	
	0.12	57.655 ± 0.001	
	0.16	69.767 ± 0.001	
	0.20	76.832 ± 0.001	

(Yang, 2012). Plants have developed chemical defenses against invasion of microorganisms. Vergeer et al. (1995) reported that the production of phenolic compounds in *Z. marina* L. increased when infected by *Labyrinthula zosterae*, indicating that the production of phenolic compounds is an antimicrobial response. In marine plants, bioactive chemicals such as phenolic compounds play important roles for plant survival and growth, and are now being used to explore new drugs and health foods for human (Baker and Joseph, 1984; Smit, 2004; Katalinic et al., 2006).

In the present study, extracts of *Z. marina* L., especially the ethyl acetate fraction, showed good antibacterial activity against Gram positive bacteria, but had no effect on Gram negative bacteria. The antibacterial activity of ethyl acetate fraction against *S. aureus* is higher than that of ethanol extract of *Ecballium elaterium* (Adwan et al., 2011), but the activity against *S. epidermidis* is little lower than that of water extract of *Aquilaria crassna*

(Kamonwannasit et al., 2013). *B. anthracis* is much more sensitive to ethyl acetate fraction of *Z. marina* L. than that of stem bark crude extract of *Antidesma venosum* (Mwangomo et al., 2012). This result was consistent to the observation that Gram positive bacteria are more susceptible towards plants extracts as compared to Gram negative bacteria (Lin et al., 1999; Parekh and Chanda, 2006). These differences may be attributed to fact that the cell wall in Gram positive bacteria is of a single layer, whereas the Gram negative cell wall is multilayered structure (Yao and Moellering, 1991). Alternatively, the passage of the active compound through the Gram negative cell wall may be inhibited.

It is well known that ROS induces oxidative damage to biomolecules like nucleic acids, lipids, proteins and carbohydrates, which causes cancer and other disease in human bodies (Duan et al., 2006). Plant products such as flavonoids, cumarins, phenolic acids and terpenoids are reported to have DPPH scavenging activity (Puertas-

Table 5. Dose-response of reducing power (mean \pm SE, n = 3) of different fractions.

Fraction	Concentrations(mg/ml)	OD at 700 nm	Regression equation
Petroleum ether fraction	0.20	0.005 \pm 0.000	y=0.0187x+0.0007 R ² =0.9951
	0.40	0.009 \pm 0.000	
	0.60	0.012 \pm 0.001	
	0.80	0.015 \pm 0.000	
	1.00	0.019 \pm 0.001	
Ethyl acetate fraction	0.10	0.199 \pm 0.000	y=1.8486x+0.0162 R ² =0.9974
	0.20	0.417 \pm 0.000	
	0.30	0.577 \pm 0.001	
	0.40	0.739 \pm 0.000	
	0.50	0.938 \pm 0.000	
<i>n</i> -Butanol fraction	1.00	0.183 \pm 0.001	y=0.1466x+0.0191 R ² =0.9969
	2.00	0.325 \pm 0.000	
	3.00	0.464 \pm 0.000	
	4.00	0.587 \pm 0.000	
	5.00	0.756 \pm 0.000	
Water fraction	1.00	0.178 \pm 0.000	y=0.1554x+0.0066 R ² =0.9953
	2.00	0.304 \pm 0.000	
	3.00	0.493 \pm 0.001	
	4.00	0.598 \pm 0.000	
	5.00	0.798 \pm 0.000	
Ascorbic acid	0.02	0.131 \pm 0.000	y=5.1257x+0.0100 R ² =0.9949
	0.04	0.198 \pm 0.000	
	0.06	0.329 \pm 0.000	
	0.08	0.425 \pm 0.001	
	0.10	0.515 \pm 0.000	

Mejía et al., 2002). Jiménez-Escrig et al. (2001) found that DPPH radical scavenging activities are positively correlated with the total polyphenol contents in aqueous and organic solvent extracts of brown and red algae. At the same concentration, the DPPH quenching activity of non-polar solvents (chloroform, ethyl acetate and acetone) extracts was stronger than that of polar solvents (water, ethanol, methanol) extracts of Rhodomelaceae seaweeds (Yan et al., 1998; Yuan et al., 2005). Four solvents were used to prepare the extracts of eelgrass in this study and the resultant fractions were investigated for their antioxidant activity. The ethyl acetate fraction of *Z. marina* L. showed the best DPPH radical scavenging activity and reducing power compared with other solvent fractions.

In conclusion, *Z. marina* L. is an aquatic plant found in wide grasslands that contributes to the coastal ecosystems. However, the residues reaching the coastlines cause an environmental problem with high costs for their disposal. In this study, we found that the ethyl acetate fraction of *Z. marina* L. possessed both antibacterial and antioxidant activities, and this study will lay a solid foundation for utilizing this versatile plant in the future.

Conflict of Interests

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

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Full Length Research Paper

Comparative detection of African swine fever virus by loop-mediated isothermal amplification assay and polymerase chain reaction in domestic pigs in Uganda

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Received 22 April, 2014; Accepted 19 May, 2014

African swine fever (ASF) is a contagious viral disease, which can cause up to 100% mortality among domestic pigs. Pig production is growing rapidly in Uganda among East African countries and is not only a source of food but also an important income for many people living in the rural areas. Field diagnosis of ASF depends only on clinical signs and has to be confirmed in the laboratory since the clinical signs are not pathognomonic. Diagnostic techniques for ASF are focused on serological tests for detection of antigen and antibody, genomic DNA detection by polymerase chain reaction (PCR), and on virus isolation and localization in clinical samples. There have been many recent reports of ASF outbreaks in Uganda yet laboratory diagnosis is limited due to the high cost and expertise required. This work reports the evaluation and application of a loop-mediated isothermal amplification (LAMP) test for detecting African swine fever virus (ASFV) DNA based on the topoisomerase II gene. Thirty (30) tissue samples obtained from suspected ASF outbreaks were collected from different regions of Uganda. The tissue samples were found to have lesions consistent with ASF. One hundred and eighty eight (188) additional blood samples were obtained from the abattoir and field surveillance. Six primers targeting the topoisomerase II gene were used. The sensitivity and specificity of LAMP and OIE recommended diagnostic PCR were compared. The LAMP assay is rapid with results obtained within 1 h (45-60 min). The sensitivity of LAMP for the detection of ASFV was 100% (95% CI: 91.78-100) while the specificity was 44% (95% CI: 36.52-51.69). The Kappa statistic for level of agreement between PCR and LAMP test in the detection of ASFV was 23.7% (95% CI: 16.42-30.91). This Kappa value indicated a fair agreement between the two assays. No cross reaction was observed with *Porcine circovirus type 2* virus and *E. coli* isolated from pigs in Uganda. This is the first study evaluating and applying the LAMP assay in the detection of ASF in domestic pigs in Uganda. The LAMP assay was found to be more sensitive than PCR. Due to its simplicity, sensitivity and specificity, the LAMP assay has the potential for use in the diagnosis and routine surveillance of ASF in Uganda.

Key words: African swine fever virus, loop-mediated isothermal amplification (LAMP), polymerase chain reaction (PCR), sensitivity, specificity, topoisomerase II gene.

INTRODUCTION

The pig farming industry is one of the fastest growing livestock activities in the rural areas of Uganda and has become very attractive throughout the country as a means of increasing food, income and employment but has on several occasions been hampered by African swine fever (ASF) (Atuhaire et al., 2013). According to reports, Uganda has the largest and fastest growing pig production in Eastern Africa with the pig population standing at 3.2 million (Uganda Beaural of Statistics/The Ministry Of Agriculture, 2009). In 2011, Uganda had the highest per capita consumption of pork in sub-Saharan Africa (3.4 kg/person per year). ASF is a highly lethal haemorrhagic disease of domestic swine, with mortality rates approaching 100% (Costard et al., 2009). The causative agent, African swine fever virus (ASFV), is a unique and genetically complex DNA virus. It is the sole member of the Asfarviridae and the only known DNA arbovirus (Dixon et al., 2000). ASFV is a large icosahedral virus which contains a linear double stranded DNA genome (170 to 190 kbp). The virus is endemic in Africa and parts of southern Europe and presents a major economic problem for the development of pig industries in these countries. Depending on the infecting virus isolate, ASFV causes syndromes ranging from peracute to chronic.

Laboratory diagnosis is essential to establish a definitive diagnosis of ASF, provide relevant information about the time of infection and support successfully control and eradication programs (OIE, 2010). Virus isolation (VI) and the haemadsorption test (Malmquist and Hay, 1960) are specific and sensitive but also too laborious and time consuming to be employed for routine or rapid diagnosis in resource poor laboratories. Polymerase chain reaction (PCR) was described as a suitable rapid alternative to VI for the detection of ASFV (Steiger et al., 1992) and may be particularly useful for screening poor quality or degraded samples with non-recoverable virus (King et al., 2003). Several PCR and real-time PCR assays have been described for detection or genotype characterization of ASFV (Agüero et al., 2004; Giammarioli et al., 2008; King et al., 2003; McKillen et al., 2010; Zsak et al., 2005), as well as isothermal amplification assays (Hjertner et al., 2005; James et al., 2010) and Linear-After-The-Exponential PCR (LATE-PCR) assay (Ronish et al., 2011). Also *in-situ* hybridization (ISH) protocols to locate viral genetic material in tissues and cells have been developed (Oura et al., 1998).

In Uganda, diagnosis of ASF by field Veterinarians mainly relies on the clinical signs and post-mortem lesions though they are not pathognomonic for ASF.

Laboratory diagnosis is mainly done only when farmers and Veterinary officers are seeking for help in cases of deaths of pigs at the National Disease Diagnostics and Epidemiology Centre, Entebbe and most recently at the College of Veterinary Medicine, Animal resources and Biosecurity (CoVAB), Makerere University by use of the OIE recommended diagnostic PCR. Routine surveillance of ASF is minimal if any (Rutebarika and Ademun, 2011). Serology has been limited to research only (Atuhaire et al., 2013; Björnheden, 2011; Gallardo et al., 2011; Tejlar, 2012). Some studies have shown no positive antibody response using the OIE-prescribed serological methods in any of the serum samples collected from ASF outbreaks in Uganda (Gallardo et al., 2011). Thus, the immune methods have low specificity and sensitivity. The conventional PCR method is sensitive, accurate but time consuming and requires expensive equipment. Therefore it does not meet the needs of detection in the field setting.

The loop-mediated isothermal amplification (LAMP) assay, since its development (Notomi et al., 2000) has gained popularity in the last decade in the diagnosis of many diseases as an easy to use alternative technique for DNA amplification under isothermal conditions especially in resource poor laboratories. LAMP has been found to be more sensitive and highly specific than PCR in many previous studies; moreover, results can be obtained in 1 h. Recently, a LAMP assay was developed for the detection of ASFV (James et al., 2010). This assay targets the topoisomerase II gene of ASFV and the detection format represents the first step towards developing a practical, simple-to-use and inexpensive molecular assay for ASF diagnosis in the field which is especially relevant to Africa where the disease is endemic in many countries (James et al., 2010).

In this study we report the application of a LAMP assay to the detection of African swine fever virus in suspected ASF outbreaks in Uganda. The study also aimed at using LAMP in establishing the extent ASFV might be circulating in the field. We have evaluated its sensitivity and specificity for the detection of ASFV based on the OIE recommended PCR.

MATERIALS AND METHODS

Samples collected

A total of 30 tissue samples (spleen, lymph nodes, tonsil, and kidney) were collected from domestic pigs after post-mortem in areas reporting suspected ASF outbreaks in Uganda between 2010 and 2013. The tissues were then transported in a cool box

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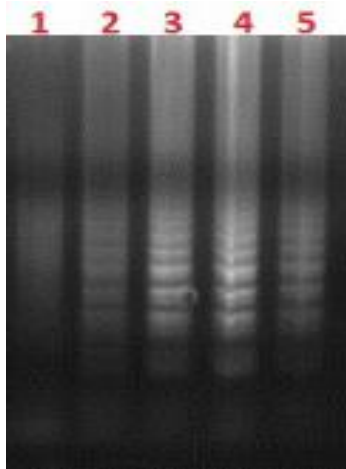


Figure 1. Determination of optimal temperature. 1.5% gel electrophoresis of LAMP products. Lanes 1, 2, 3, 4 and 5, reaction at 60, 63, 64, 65 and 66°C respectively.

containing cooling elements to the Molecular Biology Laboratory at the College of Veterinary Medicine, Animal resources and Biosecurity, Makerere University. Upon arrival, the tissue samples were stored at -80°C until required for DNA extraction. In order to determine the extent ASFV might be circulating in the field, 188 blood samples were collected from apparently healthy domestic pigs in Nalukolongo slaughterhouse, Kampala city.

Extraction of genomic DNA

Viral DNA was extracted directly from 200 µl aliquots of blood collected in EDTA tubes and from tissue samples by using a DNeasy Blood and tissue kit (Qiagen® USA).

Genomic amplification of viral DNA

A 278 bp region corresponding to the central portion of the p72 gene was amplified using the ASF diagnostic primer set recommended by the Office International des Epizooties (OIE) (Paris, France); primer 1 (5'-ATGGATACCGAGGGAATAGC-3') and primer 2 (5'-CTTACCGATGAAAATGATAC-3') (Wilkinson, 2000). Conditions for PCR assays were as previously described (Gallardo et al., 2009) with a modification in the annealing temperature from 50 to 55°C. Amplification products were loaded on a 1.5% agarose gel and run against a 50 bp DNA ladder (BIORON®, Germany). Once sufficient electrophoretic separation was obtained, the products were visualized by UV irradiation and stained with ethidium bromide for gel imaging.

The LAMP assay

A one-step loop-mediated amplification (LAMP) assay targeting the topoisomerase II gene of ASFV was used on the field viruses using primers described recently (James et al., 2010) with modifications. The optimum LAMP reaction mixture (25 µl) contained 50 µM (each) of inner primers FIP and BIP, 5 µM (each) of outer primers F3 and B3, 20 µM of Loop primers, 0.6 mM each deoxynucleoside

triphosphate, 0.4 M betaine, 1 × ThermoPol buffer (20 mM Tris-HCl, 10m M KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100), 3.2U of Bst DNA polymerase (Lucigen®, USA) and 2 µl of template DNA. The mixture was incubated at 65°C for 1 h and then heated at 80°C for 5 min to terminate amplification. The amplification products were viewed using three detection methods namely; 1.5% agarose gel, naked eye against a white background and under UV light after the addition of SYBR green dye.

Comparison of PCR and LAMP assay in ASFV DNA detection

A total of 218 test samples (blood n=188 tissue n=30) were subjected to the two assays. The negative controls constituted blood samples collected from domestic pigs in areas without outbreaks. DNA from *Porcine circovirus type 2* (PCV2) isolate, *E. coli* and *Trypanosoma brucei brucei* were selected for specificity testing.

Data analysis

PCR and LAMP data sets were analysed using the DAG-STAT software program for comparing diagnostic tests and determining the level of agreement between tests (Mckinnon, 2000).

RESULTS

Determination of optimal reaction time and temperature for LAMP assay

The optimal temperature and time for the LAMP reaction for the detection of ASFV were determined prior to testing the entire sample set. Amplicons were formed at 60, 63, 64, 65 and 66°C, but the clearest products were obtained at 65°C (Figure 1). No LAMP products were detected in the reaction mixture at 65°C within 30 min. LAMP products were detected after 45 min although well-formed bands could be detected after 60 min. Therefore, the optimal reaction temperature was 65°C for 60 min.

The ASFV LAMP results after addition of SYBR 1 green

Negative and positive samples were selected and LAMP assay done at 65°C for 60 min. The LAMP reaction products were observed with a naked eye (Figure 2) and by use of a UV illuminator as shown in Figure 3.

The ASFV LAMP assay results for the disease

A total of 188 field blood samples (collected during abattoir and field surveillance) were subjected to PCR assay and the LAMP assay. Representative results are shown in Figure 4. Tissue samples gave similar results.

Comparison of OIE diagnostic PCR and LAMP assay in ASFV detection

A total of 218 samples were subjected to the two assays

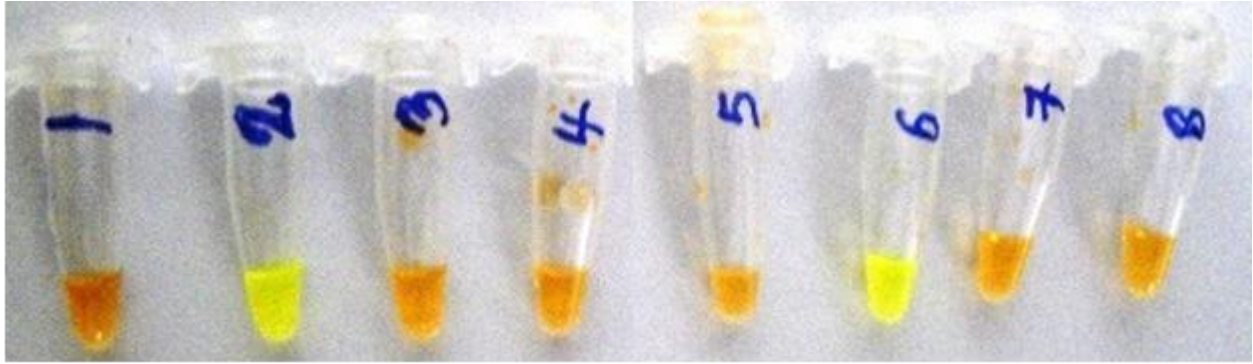


Figure 2. Visualization of ASFV LAMP products with a naked eye on representative blood samples. Tube 1 is negative control (nuclease free water), tube 2 is ASFV positive control, tubes 3, 4, 5, 7 and 8 are negative samples while tube 6 is a positive sample.



Figure 3. Visualization of ASFV LAMP products with UV illuminator on representative blood samples. Tubes 1 and 2 are negative controls, tubes 3, 4, 5 are negative samples, lanes 6-9 are positive samples and tube 10 is ASFV positive control.

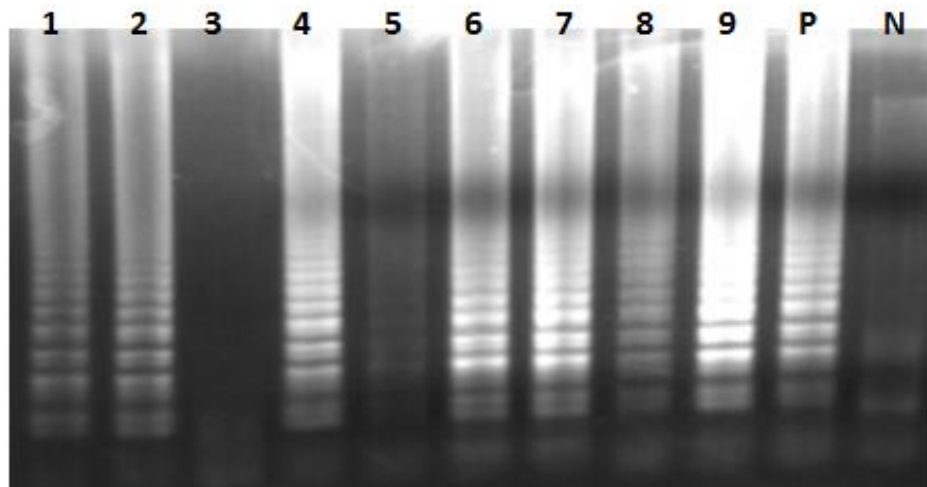


Figure 4. Application of ASFV LAMP assay on representative field blood samples. 1.5% agarose gel electrophoresis of LAMP products showing representative results. Lanes 1, 2, 4, 6, 7, 8, 9, are strong positives, lane 5 is a weak positive, lane 3 is a negative sample, lane P is a positive control (field isolate) and lane N is negative control (nuclease free water).

Table 1. Number of positive and negative sample types by both OIE PCR and LAMP assay in the detection of ASFV in domestic pigs in Uganda.

Sample type	PCR		LAMP		Total
	Positive	Negative	Positive	Negative	
Tissue	21	9	21	9	30
Blood	22	166	120	68	188
Sub total	43	175	141	77	
Total		218		218	218

Table 2. Comparison of PCR and LAMP in the detection of ASFV in domestic pigs in Uganda.

LAMP (test)	PCR (OIE recommended)		
	Positive	Negative	Total
Positive	43	98	141
Negative	0	77	77
Total	43	175	218

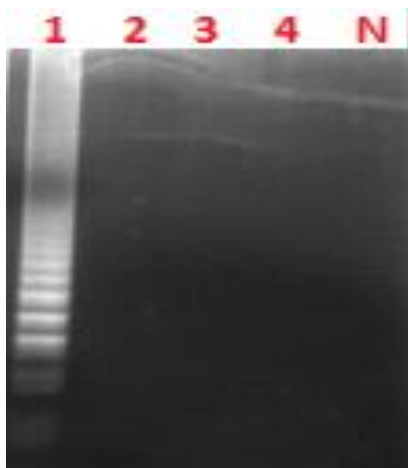


Figure 5. Specificity of the ASFV LAMP assay. 1.5% gel electrophoresis. Lane 1, The ASFV DNA (field isolate); lane 2, PCV2 DNA; lane 3, Porcine *E.coli* DNA; lane 4, *Trypanosoma brucei* DNA; Lane N, negative control (Nuclease free water).

out of which 188 were blood samples and 30 were tissue samples (Table 1).

Forty three samples (19.7%) tested positive with PCR while 141 (64.7%) tested positive with the LAMP assay. Forty three (43) samples were positive by both PCR and LAMP while no sample positive by PCR was negative by LAMP (Table 2).

Using LAMP as the test and the OIE diagnostic PCR as the criterion (reference test), the sensitivity of LAMP for the detection of ASFV was 100% (95% CI: 91.78-100) while the specificity was 44% (95% CI: 36.52-51.69). The

positive predictive value of LAMP test was 30.5% (95% CI: 36.52-51.69). The negative predictive value of LAMP was 100% (95% CI: 95.32-100). The Kappa statistic for level of agreement between PCR and LAMP test in the detection of ASFV was 23.7% (95% CI: 16.42-30.91). This Kappa value indicated a fair agreement between the two assays.

Specificity of LAMP assay for ASFV detection

For the DNA of *Porcine circovirus 2* and *E. coli* isolated from pigs in Uganda, *Trypanosoma brucei brucei* DNA and ASFV DNA (field strain) were subjected to the LAMP assay. The result of agarose gel electrophoresis indicated that only ASFV gave a positive reaction; a ladder-like pattern of bands (Figure 5).

DISCUSSION

The aim of the current study was to evaluate and apply a recently developed ASFV LAMP assay (James et al., 2010) in the detection of ASFV in Uganda as a possible alternative to conventional OIE recommended PCR for future diagnostic purposes. The LAMP assay has been used previously to diagnose infections in humans and animals (Khan et al., 2012; Koizumi et al., 2012; Namangala et al., 2012; Njiru et al., 2011; Zhou et al., 2011). The LAMP assay relies on four specific primers and Bst DNA polymerase which has a helicase function. The target sequences can be amplified with high efficiency, rapidity, and specificity under isothermal conditions (Notomi et al., 2000). Addition of a pair of loop primers accelerates the reaction (Nagamine et al., 2002).

The optimal conditions for ASFV detection by LAMP were determined in this study to be 64-65°C for 45-60 min though amplification was observed as early as 45 min. The best results were obtained at 65°C for 60 min. These findings agree with a previous study by James et al. (2010) that found out an optimal reaction temperature of 64-66°C for 50 min. Since the time required for diagnosis is considered crucial for infections, the fact that results can be obtained within 45 min makes the LAMP assay a good choice for diagnosing ASF.

Furthermore, in this study, the LAMP assay amplification was detected as fluorescence by the naked eye and with a UV illuminator on addition of SYBR 1 green due to the appearance of colour change indicating a positive result eliminating the need for gel electrophoresis and ethidium bromide staining suggesting that this assay can be applied in the field (Njiru et al., 2008).

However, the addition of SYBR 1 green to the reaction mixture was found to be very sensitive to contamination and could give false positive results. Reducing contamination and adding SYBR 1 green from a room different from the template addition and preparation room gave consistent results. A study by James et al. (2010) instead used gel electrophoresis in combination with lateral flow devices for visualisation of a positive LAMP reaction.

The results show that the ASF LAMP assay was highly sensitive for the detection of ASFV compared to the conventional OIE recommended diagnostic PCR. Previous studies have shown a higher sensitivity of the LAMP assay in other diseases than the conventional PCR (Nakao et al., 2010; Zhou et al., 2011) although LAMP and real-time PCR have been shown to have the same sensitivity in the detection of ASFV (James et al., 2010). A previous study established the analytical sensitivity of the ASFV LAMP assay as at least 330 genome copies (James et al., 2010). The sensitivity of LAMP was higher than PCR when the two techniques were applied on field samples obtained from domestic pigs. Twenty one tissues samples positive for ASFV with PCR were also positive with LAMP and nine tissues negative with the two tests. This shows that the two tests were in agreement in the confirmation of ASF outbreaks. However, the two tests gave differing results when compared in ASFV detection using blood samples. The specificity of LAMP was lower than PCR in this study. This finding is not surprising since the reference test (PCR) used for comparison is not the gold standard for detection of ASFV. The kappa statistic indicated a fair agreement between the two tests. A study on evaluation of LAMP and PCR on field samples for detection of *Staphylococcus aureus* in dairy cows suffering from mastitis indicating that LAMP was more sensitive than PCR (Tie et al., 2012).

In this study, no cross-reactivity was observed with PCV2, *E. coli* DNA isolated from pigs in Uganda or *T. brucei brucei* DNA suggesting a high specificity of the

LAMP assay. These findings agree with a study (James et al., 2010) that found out that the ASF LAMP assay was specific since there was no cross reactivity with isolates of the classical swine fever virus. In addition, the specificity of LAMP assay was not affected by non-target genomic DNA in the reaction mixture since DNA was not extracted from cell cultures, which is a highly desirable trait in a diagnostic technique (Notomi et al., 2000). Previous studies show that the LAMP assay involves fewer steps than the PCR assay, and does not require expensive equipment to attain a high level of precision (Yamazaki et al., 2008).

In conclusion, this is the first study evaluating the LAMP assay in the detection of ASF in domestic pigs in Uganda. The assay was optimised and applied on field samples. The LAMP assay was found to be more sensitive than PCR in the detection of ASFV DNA on field samples. Therefore, the ASF LAMP could be an alternative simple, rapid, specific, sensitive, practical, and visualized detection method which is suitable for detection of ASFV. Further studies are required to evaluate the LAMP assay using field samples directly without the need of first extracting DNA. This would further reduce on the diagnosis time for ASF compared to other molecular techniques. The OIE recommended PCR was used as a reference test in this study, therefore there is need to use an established gold standard for detection of ASFV in future evaluation studies of the LAMP assay in the field.

Conflict of Interests

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

ACKNOWLEDGEMENT

This study was funded by the Millennium Science Initiative, under the Uganda National Council of Science and Technology through a grant to Prof. Lonzy Ojok, Dr. William Olaho-Mukani and Dr. Julius B. Okuni of the Appropriate Animal Diagnostic Technologies Project. We are grateful to Ms. Annah Kitibwa and Mr. Alex Boobo of CoVAB for the valuable input during the optimization of the LAMP assay. We thank Dr. Kokas Ikwap and Dr. Eneku Wilfred of CoVAB for the donations of *E.coli* and PCV2 DNA isolated from domestic pigs in Uganda respectively.

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Short Communication

Prevalence of asymptomatic hepatitis B virus surface antigenemia in children in Ilesha, Osun state, South-Western Nigeria

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Received 12 October, 2011; Accepted 26 May, 2014

Hepatitis B virus (HBV) infection is endemic in sub-Saharan Africa with a range of 8 to 20% prevalence of chronic carriers. Most HBV infections occur early in life by perinatal transmission from the mother to her newborn baby, and horizontal transmission from child to child resulting from blood contact. This study was therefore carried out to assess the prevalence of Hepatitis B virus surface antigenemia (HBsAg) in children born in Ilesha, Osun State, Southwestern Nigeria. A total of 144 children at age range 21 days to 13 years were tested for hepatitis B surface antigen (HBsAg) in Ilesha using Bio-Rad Monolisa HBsAg Ultra kit [enzyme-linked immunosorbent assay (ELISA), ELISA method]. Twenty (13.9%) children were seropositive for HBsAg. The age bracket 1-5 years had the highest number of children and the highest number of HBsAg positive cases (15.8%). Female children had a higher HBsAg antigenemia of 15.4% compared to 12.7% for the male children. High prevalence of hepatitis B surface antigen was found among the children. Focus should be re-intensified on childhood vaccination and information dissemination on the risk and mode of transmission of HBV. Women especially, should be educated on HBV to prevent Mother-to-child transmission (MTCT) of the infection.

Key words: Children, enzyme-linked immunosorbent assay (ELISA), Hepatitis B virus (HBV), Hepatitis B virus surface antigenemia (HBsAg), Nigeria.

INTRODUCTION

Currently, about 350 million people worldwide are affected by chronic infection with the hepatitis B virus (HBV), which is the main cause of chronic liver disease (cirrhosis and hepatocellular carcinoma) and liver-related

mortality (Rouet et al., 2008). Hepatitis B virus (HBV) infection is endemic in sub-Saharan Africa (Beasley, 1988; Majori et al., 2008) with an 8 to 20% prevalence of chronic carriers (HBsAg positivity), which is estimated

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Table 1. Prevalence of HBsAg among children in relation to age of the subjects.

Age (years)	Number tested	Number positive (%)
<1	25	3 (12)
1-5	95	15 (15.8)
6-10	17	1 (5.9)
>10	7	1 (14.3)
Total	144	20 (13.9)

Table 2. Prevalence of HBsAg among children in relation to gender (sex) of the subjects.

Sex	Number tested	Number positive (%)
Male	79	10(12.7)
Female	65	10(15.4)
Total	144	20(13.9)

to be approximately 50 million people (Heathcote, 2008).

Most HBV infections occur early in life by perinatal transmission from the mother to her newborn baby (Hollinger and Liang, 2001), or horizontal transmission from playmates or family member resulting from blood contact (Gitlin, 1997). Infected children do not mount an effective immune response and exhibit immune tolerance, so that the risk of chronic infection is high (Beasley, 1988).

HBV is present in the blood, saliva, semen, vaginal secretions, menstrual blood, and, to a lesser extent, perspiration, breast milk, tears, and urine of infected individuals. The virus is resistant to inactivation, can survive outside the body, and is easily transmitted through contact with infected body fluids (Lavanchy, 2004).

Sexual activity, especially heterosexual, and injection-drug use account for the majority of HBV transmission in low-prevalence areas (Seeger and Mason, 2000) while perinatal transmission account for the majority of the transmission in high-prevalence areas (Harry et al., 1994). The global burden of disease attributable to hepatitis B virus remains enormous, and this is due largely to the lack of universal vaccination (Jacob and Kowdley, 2006). This study was therefore carried out to determine the prevalence of asymptomatic HBsAg antigenemia in children in Ilesha, Osun state, Southwestern Nigeria.

MATERIALS AND METHODS

Study population

The study population was made up of 144 apparently healthy children (age bracket 21 days-13 years), attending the welfare clinic of Wesley Guild Hospital, Ilesha, Osun State, Nigeria. The study was carried out from July to October, 2008.

Blood collection and serum separation

Two milliliters (2 ml) of blood sample was collected by venal puncture from each child into sterile vacutainers after informed consents given by the parents/guardians of the children. Sera were collected aseptically after centrifugation at 3000 rpm for 10 min into sterile containers and preserved at -20°C until tested for the presence of HBsAg.

ELISA test

Prior to testing, the sera were allowed to attain room temperature. For the detection of Hepatitis B surface antigen, Bio-Rad Monolisa HBsAg Ultra kit® was used. The kit has a sensitivity of 100% and a specificity of 99.94%. The test and interpretation of the results were carried out according to the manufacturer's specifications.

Statistical analysis

The statistical analysis used was the Chi square test using SPSS15.

RESULTS

In this study, 20 of the 144 children tested for HBsAg were seropositive, indicating a prevalence of 13.9%. Further analysis of the prevalence of HBsAg in relation to the age range of the children showed that children ages 1 to 5 years had the highest prevalence rate of 15.8% compared to the other age groups (Table 1), but there was no significant statistical association ($P = 0.179$) between the HBsAg prevalence and the age of the children in this study.

Table 2 shows the prevalence of HBsAg among children in relation to gender (sex) of the subjects. It shows a higher prevalence among the female children (15.4%) than the male children (12.7%) however, HBsAg prevalence was found not to be associated with sex ($P = 0.319$) in this study.

DISCUSSION

Perinatal infection of infants from infected mothers and horizontal infection early in childhood from exposure to HBsAg-positive family members and playmates are the main routes of HBV transmission in highly endemic areas, such as Southeast Asia, Africa, the Pacific Islands, and the Arctic (Sharma et al., 2005). In this study, the overall prevalence of HBsAg in children was 13.9% ($n=144$), and agrees with reports from other parts of the country showing high HBsAg prevalence rates among children. Nasidi and co-workers (1986) found HBsAg prevalence of 10.3% in children from Lagos and Bauchi states while Akenami et al. (1997), in Calabar detected HBsAg prevalence of 20 and 26% in healthy and malnourished children, respectively. Bukbuk and co-workers (2005) found HBsAg prevalence of 44.7% among pupils in primary school in rural Borno state. Although, the prevalence rates from this study and that of other studies presented here are not the same but they firmly place Nigerian children among the highly endemic group of Hepatitis B virus infected persons (Sharma et al., 2005).

In addition to these reports showing high endemicity of HBV infection among children in Nigeria, there are other studies on HBsAg prevalence rates in different population groups from different parts of the country. Awosere et al. (1999) detected a range of 2.7 to 13.3% among different population groups. Akani et al. (2005) detected a prevalence of 4.3% in pregnant women in Port Harcourt. The prevalence of HBsAg among blood donors was 8.9% in Northern Nigeria (Tribedi, 1994), 5.4% in Benin City (Umolu et al., 2005), and 14.3% in Jos (Uneke et al., 2005).

Additional data from this study on the prevalence of HBsAg among children shows that the prevalence of HBsAg was highest in children age group 1-5 years (15.8%). The reason can be attributed to the fact that children at early childhood are exposed to multiple avenues through which they can be infected with HBV. The mode of early childhood transmission of Hepatitis B virus (HBV) occurs by vertical transmission from mother to child and horizontal transmission via unapparent blood or body fluid exposures from parents, siblings, or playmates that inoculate HBV into cutaneous scratches, abrasions, or other lesions or onto mucosal surfaces (Francis et al., 1981).

In this study, results show that the HBsAg prevalence was higher among female children (15.4%) than their male counterparts (12.7%) but there was no statistical significance. This agrees with the findings of Harry et al. (1994) in Northern Nigeria who showed a prevalence of 22.0 and 11.6% respectively, for females and males. However, more studies on higher HBV prevalence among females compared to males need to be conducted to definitely prove if there is any statistical significance.

The results of this study have emphasized the need for urgent intervention measures. We recommend the administration of hepatitis B vaccine and hepatitis B immune globulin (HBIG) to all healthy infants born to HBsAg positive women and women with unknown HBsAg status at birth or at most during the first year of life. The importance of familial screening for Hepatitis B surface antigen among siblings of HBsAg positive children is highly recommended. Focus should be re-intensified on information dissemination on the risk and mode of transmission of HBV. Women especially, should be educated on HBV to prevent MTCT of the infection and the disease.

In conclusion, this study has shown the prevalence of HBsAg among children in Ilesha, Osun state and contributes to the information on the burden of Hepatitis B infection among children in Nigeria. This result shows Ilesha as an area of high HBV endemicity corresponding to previous reports on hepatitis B prevalence in sub-Saharan Africa.

Conflict of Interests

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

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