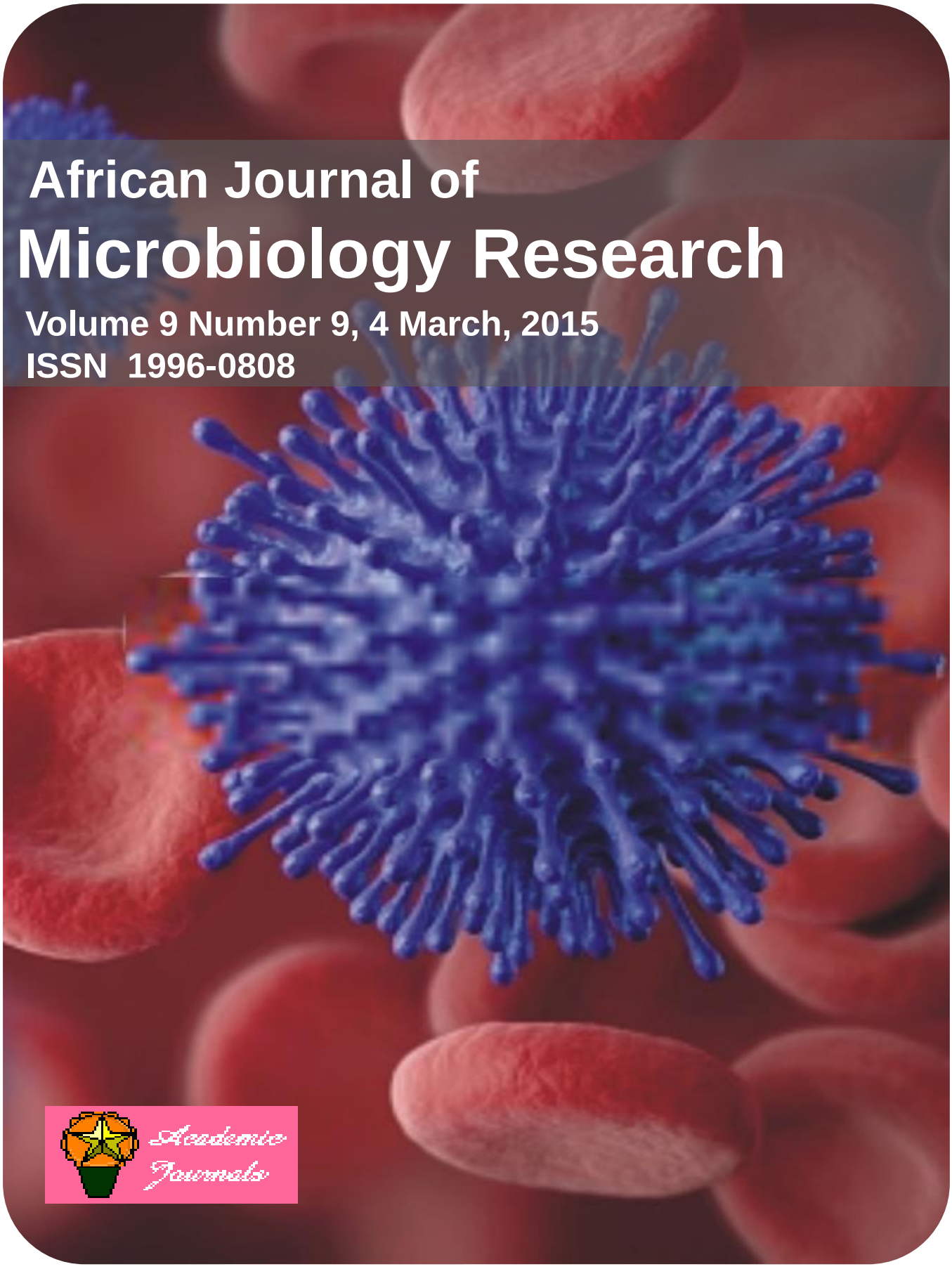

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: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

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Review

A review of perception and myth on causes of cholera infection in endemic areas of Nigeria

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Cholera epidemic is a recurrent disease in developing countries with poor environmental sanitation and inadequate supply of potable water. In endemic areas of West Africa, opinions and myths surrounding the real causes of cholera diseases have given rise to varying methods of control. This review gives an insight into these various opinions and myths. Some of these are that magico-religious factors, witchcraft, eating soil, god's will and evil air in the community are responsible for cholera outbreaks. Wrong perception and myth of cholera hinders acceptance and accessibility to launch effective operational response to affected communities during an outbreak. This also lead to delay in providing intervention and treatment during an outbreak.

Key words: Myths, aetiology, *Vibrio cholerae*, epidemic, perception.

INTRODUCTION

Cholera is a natural disease of epidemic proportion. It is caused by a comma-shaped, rod like, motile, Gram negative bacterium called *Vibrio cholerae*, with the characteristic acute watery diarrhoea, vomiting, muscle cramps and severe dehydration (Kaper et al., 1995, Sack et al., 2004). It is an ancient disease reported globally and is associated with high mortality and morbidity rates. This life threatening infection has an estimated annual burden of 2-4 million cases in endemic areas (Ali et al., 2012). The world has experienced seven major pandemics of this disease since the early 19th century (Faruque et al., 1998). The first six were caused by toxigenic strains of classical (CL) biotype, serogroup 01, which was reported to have originated from India, while the current 7th is caused by *V. cholerae* 01 of the El Tor (ET) biotype (Zhang et al., 2014).

In Africa, majority of cases between early 1990 and 2013 occurred in Angola, Democratic Republic of the Congo, Mozambique, Nigeria, Somalia, Tanzania and South Africa. In Nigeria, the first recorded cases of cholera were in a village near Lagos, on 26th December, 1970 with 22,931 cases and 2945 deaths (WHO, 2012a).

Biological and cultural factors of life combine to drive the principles of achieving optimal health. In countries where cholera is endemic, there are myths and divergent perceptions on the real cause(s) of the disease. Consequently, there are variations in approach towards the control and containment of the epidemic. In Africa,

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while some people believe that cholera is as a result of "miasma" (bad air) as postulated in the 19th century, or "anger of the gods", others are of the opinion that it is due to the presence of witchcraft and "black magic" (Erinosho and Oke, 1994; Tesh, 1995; HRC, 2010; Mertem et al., 2013). These wrong perceptions have their consequences on disease management. Like in other countries of the world, recurrent episodes of cholera in Nigeria could be stopped if the predisposing factors and aetiology are well understood. This has not been the case because at each outbreak, efforts at ameliorating and controlling the epidemic are reactive, unfortunately evidence of sustainable practices is lacking. As such, there are recurrences of the disease both in urban and rural areas especially during the rainy seasons. Changing our myth on the cause of cholera and understanding the fact that cholera causes dehydration and kills people and can be properly eradicated following standard infection prevention and control measures will help in the containment of this disease. Similarly, understanding the facts about cholera is a key factor in developing and maintaining long lasting solution to continuous outbreaks. Stopping cholera epidemic should be focused on interventions aimed at blocking all means by which the human population gets in contact with strains of *V. cholerae* responsible for continuous outbreaks in Nigeria. This paper reviews the perception and myths surrounding the aetiology of cholera in endemic areas, with focus on Nigeria.

AETIOLOGY OF CHOLERA

V. cholerae bacterium is implicated in the diarrhoea illness called cholera. More than 200 *V. cholerae* serogroups are recognized worldwide, however most epidemics of cholera are usually caused by 01 and 0139 serotypes. These serotypes are known to produce cholera toxins *ctx*AB and toxin-coregulated pilus (*tcp*) which are the important virulent factors (Waturangi et al., 2012). Gastroenteritis caused by non-01 and non-139 is well documents (Albert et al., 1993; Dutta et al., 2013; Marin et a., 2013). The symptoms of infection varies, ranging from mild to severe watery diarrhea but there are no fever and bloody diarrhea typically seen in gastroenteritis due to non-O1, non-O139.

In Nigeria, there is little information on the circulating strains. Recently however, two biotypes were reported by some workers. Marin et al. (2013) described multidrug resistant, atypical ET and non- 01/ non-0139 as the agent responsible for cholera/diarrhoea outbreaks of 2009 and 2010 in Borno and Osun states, Oyedeji et al. (2013) reported enterotoxin (*ctx*A) carrying classic biotype 01 in the Borno, Bauchi and Gombe states within the same period, while Akinsinde et al. (2014) went ahead to demonstrate that these epidemic strains were of multiple phage types.

Pathophysiology of cholera

Cholera infection is a classic example of enterotoxin mediated gastroenteritis (Lambert, 1984). The cholera organism expresses toxins that grossly derange the gastro-intestinal tract (GIT) fluid balance such that the daily fluid output in form of faeces far exceeds the normal yield of 0.1 to 0.2 L without invasion of the GIT (Goossens and Op de Beeck, 1981).

The site of infection is the upper small intestine and to reach there, the inocula of the ingested *V. cholerae* have to be large enough so that sufficient infective dose survives and traverses the acid defense of the stomach (Handa et al., 2014). Further adaptation such as motility and secretion of protease enzymes facilitates the organisms to breach the mucus layer of the intestine while the elaboration of the toxin-coregulated pilus (tcp) enables the organisms to bind to the small gut wall (Keusch et al., 1998, Muanprasat et al., 2013).

Once established mostly in the duodenum and upper jejunum, a B pentameric moiety of cholera toxin (CT) ensures the attachment of the *Vibrio* to intestinal epithelial cells. Another component of CT, the A1 monomeric subunit is then released to activate a cascade of complicated enzymo-chemical reactions. These culminate in persistent stimulation of adenylate cyclase enzyme with attendant increase in intracellular cyclic adenosine monophosphate (cAMP).

The cAMP in turn, promotes marked fluid secretion from the proximal parts of the small intestine (Handa et al., 2014; Muanprasat, 2013). It is this resultant enormous ingress of near isotonic fluid into the GIT which overwhelms the absorptive capacity of the GIT yielding a net output in stool that may reach 1 to 2 L hourly in severe infection (Mhalu, 1983). The final sequel is a dangerous compromise in total body fluid homeostasis associated with this infection.

EPIDEMIOLOGY OF CHOLERA IN NIGERIA

Cholera is believed to have originated from Ganges Delta of the Indian Subcontinent before spreading across to the other continents of the world. Cholera infections in African countries dated as far back as 1960 (WHO, 2012a, 2013a). Although Nigeria recorded some cases in 1961, its first epidemic occurred in 1970 (WHO, 2012a, 2013). This was followed over the years by sporadic episodes up till 1997 (Lawoyin et al., 1999) and then the severe outbreak in 2010. Cholera infection is endemic, occurring in all geographic regions, in Nigeria (Shittu et al., 2010). This infection affects both male and female with severity of infection recorded in children who are exposed to the organism for the first time and in the elderly, who have lower gastric acid production and diminishing immunity.

Over the years, greater percentage of outbreaks has

Table 1. Number of cholera cases in Nigeria, deaths and case-fatality rate (CFR) between 2007-2013.

Year	No of States affected	No of cases	No of Deaths	Case Fatality Rate (%)
2007	-	1661	48	10.4
2008	-	5410	247	4.8
2009	-	13691	431	3.1
2010	18	41787	1716	4.1
2011	25	23377	742	3.2
2012	11	589	18	2.6
2013	19	4576	156	3.4
Total		**91090**	**3358**	**3.7**

Sources: (WHO, 2008, 2009, 2012a, 2012b, 2013; FMoH, 2012, 2013a, 2013b; Dalhat et al., 2014).

been caused by *V. cholera* 01 ogawa El Tor biotype. Recent outbreaks resulting into high cases of deaths in Papua New Guinea, Africa, and the Caribbean Sea were attributed to new atypical El Tor strains with a classical cholera toxin gene believed to have originated from the Bay of Bengal and similar to the strain peculiar to Orissa, India. This species in association with non-O1/non-O139 *V. cholerae* strains was implicated as the major strain responsible for the 2010 cholera outbreak that affected over 40,000 people with a case fatality rate (CFR) of 3.75% in Nigeria (Oyedeji et al., 2013; Oladele et al., 2012). Majority of the new atypical El Tor strains are multi-drug resistant (Marin et al., 2013). Since, 2007 cholera infection has become an annual occurrence in the six geographic zones especially after flooding experience with over 91,090 recorded cases and 3358 deaths (Table 1).

Risk factors for cholera

All age groups are affected but the risk is reduced in breast-feeding infants partly as a result of reduced exposure and partly from maternal antibodies to *V. cholerae*. The presence of certain factors can increase the incidence of cholera in a community leading to an epidemic by increasing the susceptibility of individuals to experiencing severe signs and symptoms. Globally, documented risk factors for cholera hinged mainly on factors that promote faecal-oral transmission of *V. cholerae*.

The single most important factor, particularly in developing world is poor sanitary conditions. Cholera is more likely to flourish in situations where there is poor personal and environmental hygiene often resulting from lack of safe water supply and poor disposal of human waste. Contamination of river water supply by human waste was said to be responsible for the 2010 cholera outbreak in Ghana (Opare et al., 2012) and open land/river defeacation increased the odds of cholera risk in Papau New Guinea (Rosewell et al., 2012). Drinking and domestic use of contaminated water was described as the cholera risk factor in India between 2004 and 2010 (Datta et al., 2012; Mukherjee et al., 2011). These challenges with water supply and sanitation are common to refugee camps, impoverished countries, and areas devastated by famine, war or natural disasters as in Haiti cholera outbreak (Dunkle et al., 2011).

Reduced or nonexistent stomach acid (hypochlorhydria or achlorhydria) is another documented predisposing factor to cholera. This is because *V. cholerae* cannot survive in an acidic environment, and ordinary stomach acid often serves as a first-line defense against infection. Therefore, people with low levels of stomach acid as in children, older adults and people who take antacids, H-2 blockers or proton pump inhibitors do not have this protection and are at greater risk of developing severe cholera symptoms (Kaper et al., 1995).

Exposure to an individual infected with cholera in a household setting is another risk factor. People are at significantly increased risk of cholera if they live with someone who has the disease. For example, contact with exudates and secretions from people infected with cholera was a significant factor reported in 2008 Harare cholera outbreak in Zimbabwe (Kone- Coulibaly et al., 2010).

Host genetic factors and climatic factors were documented to significantly increase cholera risk in some populations. Individual with type O blood for reasons that are not entirely clear are reported to be twice as likely to develop cholera as are people with other blood types (Chaudhuri and De, 1977; Harris and Khan, 2005). Also, the incidence of cholera was found to be significantly related to higher temperature and humidity as well as lower precipitation in the Middle East just as lower cholera risk was reported in the highest elevation suburbs of Harare, Zimbabwe in 2010 (Luque et al., 2012).

Consumption of raw or undercooked shellfish is associated with increased risk of *V. cholerae* infection. Although large-scale cholera outbreaks no longer occur

Table 2. Reported risk factors associated with cholera outbreaks in Nigeria.

S/N	Publication	Reference	Study type	Location	Risk factors
1	Recurrent cholera epidemics in Kano--northern Nigeria	Usman et al., 2005	Descriptive	Kano, North west	Contaminated water
2	A large cholera outbreak in Kano City, Nigeria: the importance of hand washing with soap and the danger of street-vended water	Hutin et al., 2003	Case control study	Kano, Northwestern	Drinking of street vended water Lack of tap water Poor hand washing practice
3	Outbreak of cholera in Ibadan, Nigeria	Lawoyin et al., 1999	Descriptive	Ibadan, South western	Overcrowding, Raining season, Contamination of water source
4	Epidemiology and spectrum of vibrio diarrhoeas in the lower cross river basin of Nigeria	Eko et al., 1994	Descriptive	Calabar, Uyo, South-south	Poor sewage disposal systems, Contact with sea water, Consumption of fishery products and leftover foods
5	Features of cholera and *Vibrio parahaemolyticus* diarrhoea endemicity in Calabar, Nigeria	Utsalo et al., 1992	Case control	Calabar, South-south	Poor water supply Poor sewage disposal systems
6	Epidemiological features of an outbreak of gastroenteritis/cholera in Katsina, Northern Nigeria	Umoh et al., 1983	Descriptive	Kastina, North-West	Water vendors

in industrialized nations, eating shellfish from waters known to harbor the bacteria greatly increases the risk. A study from coastal towns of Côte d'Ivoire reported incidence of cholera from consumption of crustaceans (Traore et al., 2012).

In Nigeria, several risk factors have been reported to be associated with cholera outbreaks in the last four decades (Table 2). Massive cholera outbreak in Kano, northwestern Nigeria resulted from drinking contaminated water usually from water vendors, lack of tap water and poor hand washing practice (Umoh et al., 1983; Usman et al., 2005; Hutin et al., 2003). As at 2008, 42% of the entire population lacked access to improved water source and 68% to proper sanitation facilities (WHO, 2008). Another outbreak at Ibadan, southwestern Nigeria was said to be predicated on overcrowding, onset of rainy season and contamination of water source (WHO, 2012a; Oguntoke et al., 2009). In Calabar, South-south Nigeria, poor sewage disposal systems, lack of potable water supply, contact with sea water and consumption of fishery products and leftover foods were reported as risk factors for *Vibrio* diseases (Eko et al., 1994; Utsalo et al., 1992).

Prevention of cholera through vaccination

Cholera infection can be controlled by the administration of cholera vaccine targeted at both Inaba and Ogawa serotypes, classical and El Tor biotype as well as against *V. cholerae* O139 and the cholera toxin (WHO, 2004). For over 20 years, the oral cholera vaccine Dukoral (SBL Vaccin AB, Sweden) with short term (4-6 months) protection recommended for travelers to endemic areas has been in use. The use of this vaccine was limited due to the global concern over the side effects experienced by vaccinated individuals and cost; thus creating the need for a more effective, safe and affordable cholera vaccine for international use. Presently, Dukoral has been modified to a new vaccine Shanchol (Shanchol, Shantha Biotechnics, India), approved by WHO and the clinical trials successfully completed (Bhattacharya et al., 2013). This new vaccine has a long lasting protection with 65% efficacy over a five year period. Comparatively, it confers 42% cumulative efficacy in children aged 1-5 years who are at greatest risk of disease (Bhattacharya et al., 2013; Sridhar and Arora, 2013). Acceptance of these vaccines has been a challenge in some countries due to cultural beliefs. Based on previous experiences and massive deaths, countries are making efforts to promote effective vaccine programs and policies to control cholera epidemic (Vicari et al., 2013; Ivers et al., 2013). WHO recommends the use of vaccines in combination with health services that provide rapid detection and treatment of cholera cases with appropriate agents,

while making provisions for accessibility to safe water, good sanitation, promotion of personal hygiene, improvement in health education and community mobilization (Vicari et al., 2013; Lucas et al., 2005; WHO 2012b). While this is going on, some reports have taken time to analyze the impact of myths and perception on the acceptability of cholera vaccines. Willingness to participate is high in populations that have received awareness on cholera (Vicari et al., 2013; Ivers et al., 2013).

THE SOCIO-CULTURAL FACTORS IN CHOLERA CONTROL

Taking cognizance of the principle that man is both a biological and cultural being, health professionals need to be aware of this human duality in order to achieve their goal of optimising human health (Oke, 2002). Health is both a medical and socio-behavioural concept just as medicine itself is a natural as well as a socio-behavioural science (Oke, 2002; Otite, 1987). Using the schematic framework developed by Maclachlam (1958) and expatiated by Fabrega (1971), the concept of disease implies germ theory (Koch, 1884) which is a fundamental foundation of modern medicine developed by biomedical science on one hand and a broad cultural definition on the other hand. There is therefore, the need to distinguish between scientific and culturally defined state of health and illness especially as it relates to cholera (Oke, 2002; Armstrong, 1971). The germ theory of disease is derived from the assumptions that every disease has a specific cause which its treatment could best be accomplished by removing or controlling the cause with a biomedical framework, while cultural belief is attributable to the role of socio-cultural factors in the aetiology and prognosis of disease. In Nigeria, some diseases are believed to be caused by natural (such as malnutrition), preternatural (belief in sorcery and witchcraft) and mystical (cosmic or supernatural powers) factors (Armstrong, 1971; Maclean, 1971; Snow, 1936). However, there is a paucity of data on perception, myths and socio-cultural belief on causes of the variety of recurrent endemic infectious diseases.

Myths and perceptions on the causes of cholera epidemic vary considerably. During the outbreak in Haiti in 2010 following the powerful earthquake which devastated the country, people believed that cholera was brought by foreigners in order to use or to harm their citizens (HRC, 2010). This undermined the credibility of foreign organisations carrying out information campaigns in communities. Some were of the opinion that cholera was a deliberate infection spread through a magic '*kolera* powder' ("*kolera poud*") prepared by Voodoo priests and transmitted by Voodoo worshippers. There were protests against efforts to establish cholera treatment centres during the cholera outbreak. In the absence of public health information indicating otherwise, many people believed the small, localized treatment centres will increase the spread of cholera in their community. Due to the contagious nature of the disease, they preferred the centres being located far away from their residence so that they would not be infected. This is contrary to the reality that the closer an infected person is to a treatment centre, the better the chances of survival.

A cross-sectional study on local perceptions of cholera and anticipated acceptance of oral cholera vaccines in Katanga province, Democratic Republic of Congo (DRC) showed that the majority of respondents considered insufficient hygiene and sanitation levels as the key cause of cholera. Ingestion of contaminated water and food were spontaneously mentioned by 63.0 and 61.0%, respectively, as main transmission routes of cholera (Merten et al., 2013). Other common explanations were contact with contaminated water, or flies, a dirty environment, lack of latrines and poor hand washing practice. In contrast, magico-religious explanations were mentioned spontaneously by less than 10.0% of respondents. However, after probing through a survey, about 59.0% of respondents confirmed sorcery and witchcraft as possible source of cholera thus proffering information of the reality of what the respondents believed in. Similarly, eating soil, and God's will were deduced as possible origins of cholera by 48.0 and 41.0% of respondents, respectively (Merten et al., 2013). Within the contents of the abstract reviewed, no study so far has given a scientific proof on ambiguous religious belief, witchcraft and eating soil as causes of cholera infection). Scientific evidence have shown that the disease is highly contagious and one gets infected by faecal-oral transmission of toxin-producing serogroups (Marin et al., 2013; Oyedeji et al., 2013; Zhang et al., 2014).

Irrespective of various opinions and perception on the causes of cholera epidemic, the fact remains that cholera is a water borne preventable and treatable disease, transmitted through ingestion of *V. cholerae* in contaminated food and water (Marin et al., 2013; Smith, 2002; Ghose, 2011; Farmer et al., 2003). This disease is more likely to flourish in situations where there are poor personal and environmental hygiene resulting from lack of safe water supply and poor disposal of human waste. During outbreaks, infected individuals experience uncontrollable vomiting and diarrhea with profuse "rice water stool" due to the production of poisonous cholera enterotoxins which in turn leads to dehydration. The victims experience electrolyte imbalance, painful muscle cramps, watery eye, and loss of skin elasticity and absence of urine excretion (Bentivoglio et al., 1995). In mismanaged, undiagnosed and untreated cases, death occurs within few days due to dehydration. An entire community can be affected at the same time limiting the possibilities of adequate attention from care givers (Oladele et al., 2012). Cholera, like any other epidemic condition, is primarily addressed through prevention campaigns and administration of medical treatment.

Impacts of perception and myths of the aetiology of cholera on its control, management and prevention

During the outbreaks in the early nineties, people believed that cholera was caused by “miasma” (bad air) due to poisonous odors of decayed matter (Tesh, 1995). As such, the early work of Fillipo Pacini who proposed the germ-theory of cholera and identified the comma-shaped organism as the cause of the disease as far back as 1854 was apparently ignored. In continents ravaged by cholera, efforts were made to find out the real cause(s) of the associated massive death. By the time the aetiology and risk factors became obvious, the perception was transformed and this was followed by visible changes in sanitation, infrastructural development, provision of appropriate drugs and invention of oral rehydration therapy and related drugs for treatment. There were improvements in providing clean water supply, as well as vaccination. This was strengthened by the great awareness and orientation on the importance of public health and the need for maintaining a healthy environment.

Individuals in Democratic Republic of Congo (DRC) believed that cholera was due to the continuing presence of witchcraft (Merten et al., 2013). They visited the witch doctors for cure until they found them dropping dead due to cholera. Certain population had their perception on Christian religious faith-based practices centered on praying for healing and the belief that God was responsible for cholera outbreaks (Merten et al., 2013). The past experiences with cholera outbreaks created high awareness in DRC on the source, aetiology, control and containment of cholera (Bompangue et al., 2008) and recent report shows that 93% visit health facilities during out breaks (Merten et al., 2013).

Cholera is one of the main factors that brought about development of sanitation and environmental hygiene infrastructure in America and Europe. According to Hamlin (2009) who corroborated the opinion of Tauxe and colleagues (1994), cholera spurred the sanitary reform movement and led to development of the field of public health in 19th-century in Europe and North America. This trend is gradually extending to some Asian countries. The seventh cholera pandemic has affected many countries in Africa, some in Asia and in Latin America signaling that sanitation; infrastructure and public health facilities are inadequate. In African countries, the recurrent epidemic of cholera should be a driving force for constructive change both in our social life, health and environment. This is not so as some countries still lack basic infrastructure and safe water coupled with inadequate hygiene practice. Recurrent cholera infection is documented yearly in Nigeria since 2007 (Table 1). Although reports have shown that provision of adequate infrastructure, improved sanitation, supply of safe water and hygiene education is important in the prevention of cholera, nothing seems to change. Access to an improved water source was stagnated at 47% from 1990 to 2006 (WHO/UNICEF, 2010). This increased to 54% in 2010 but is still below the 75% mark by WHO (WHO/UNICEF, 2010). This is faced with challenges in water production facilities which rarely operate to capacity due to broken down equipment, or lack of power or fuel for pumping (WHO/UNICEF, 2010). Assessment of World Bank development indicator (World Bank, 2014), showed that the percentage of Nigerians with improved water source stood at 63% for a period of 2000-2004. This was the same (63%) for 2005-2009 and just 1% increase for a period of 2012-2014. Similarly, 35% of the population has access to improved sanitation as at 2010 (WHO/UNICEF, 2010). Whether people's perception and myths is dictating social investment by the government is unknown or yet to be determined.

Massive cholera outbreak in Kano, northwestern Nigeria resulted from drinking contaminated water usually from water vendors, lack of tap water and poor hand washing practice (Hutin et al., 2003). As at 2008, 42% of the entire population lacked access to improved water source and 68% to proper sanitation facilities (WHO, 2008).

Another outbreak at Ibadan, southwestern Nigeria was said to be predicated on overcrowding, onset of rainy season and contamination of water source (WHO, 2012; Oguntoke et al., 2009).

In spite of the provision of adequate infrastructure, improved sanitation, supply of safe water and hygiene education for the prevention of cholera, cultural perception and myths on cholera also has an impact on occurrence, duration of epidemics, increase in number of cases and subsequent deaths. When there is a negative rumour on the causes of an illness, the ability to launch an effective operational response is impaired. This may lead to delay in providing intervention and treatment by health worker. Infected patients might refuse to visit treatment centre. In cases were wrong impressions are not corrected, the predisposing factors may not be identified and addressed which might lead to reoccurrence. Furthermore, distrust and suspicion affects the acceptance and work of humanitarian agencies. Consequently, the safety of health workers, non governmental agencies and volunteers are not guaranteed. Such reactions were experienced during the Haiti outbreak where there was distrust to humanitarian agencies and violent reactions like the killing of 45 Voodoo priest for producing the magic “poud kolera” that brought cholera (HRC, 2010; IFRC, 2014). That reaction resulted to the training of Voodoo worshipers by Haiti Red Cross on early detection of cholera, prevention and treatment. That community consequently became active in combating cholera in the communities (HRC, 2010; IFRC, 2014).

According to Ayeni (2014), the government of Nigeria needs to collaborate with its citizen to address issues of urbanization and environmental health problems associated with cholera especially by encouraging participating

in government-backed campaigns. However, such involvement largely depends on the ability of the community members to understand the causes and control measures of this endemic disease. When wrong beliefs on infectious agents and disease outbreaks is identified and addressed, it would likely be easy to promote accurate information and community participation through proper education.

CONCLUSION

Wrong perception and myth of cholera hinders acceptance and accessibility to launch effective operational response to affected communities during an outbreak. It also leads to delay in providing intervention and treatment. Reports on the perception and myths of cholera in Nigeria are lacking, suggesting the urgent need for more research into cultural belief of cholera and other infectious diseases.

Competing interest

The authors declare that there are no competing interests whatsoever.

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Full Length Research Paper

Impact of purified human milk oligosaccharides as a sole carbon source on the growth of lactobacilli in *in vitro* model

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Recently, there is a growing interest in the use of oligosaccharides as prebiotics in order to modulate the growth of beneficial gut microbiota. It is known that human milk is a rich source of complex oligosaccharides. This paper reports the *in vitro* growth of six strains of lactobacilli in media containing purified human milk oligosaccharides (HMOs) obtained from breast milk. Based on the evaluation of bacterial densities in the growth media, together with the evaluation of pH values and bacterial metabolite detection, we concluded that the lactobacilli tested did not appear to be active HMO consumers. In the case of four strains (*Lbc*. *fermentum*, *Lbc. animalis* and two strains of *Lbc. delbrueckii* subsp. *bulgaricus*), no increase in bacterial density was detected. Two strains (*Lbc. acidophilus* and *Lbc. casei* subsp. *paracasei*) showed a slight, but insignificant increase in bacterial densities during 24 h of incubation.

Key words: Bifidobacteria, human milk oligosaccharides, lactobacilli, utilization.

INTRODUCTION

Human milk is a dynamic biological system (Bertino et al., 2009) containing nutrients such as proteins, lactose, fatty acids, and others, as well as biomolecules having prebiotic, immunomodulatory, or antimicrobial effects. From this group, human milk oligosaccharides (HMOs) are thought to have an important role, especially in infant nutrition. HMOs represent the third most abundant component in human milk (Casado et al., 2009), after lactose and lipids. The content of HMOs is estimated to make between 5 to 23 g/l (Ninonuevo and Lebrilla, 2009) depending on the lactation phase, genetic factors, dietary, geographical factors, and individual determinants (German et al., 2008).

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Maximum concentrations are present in colostrums, while in mature milk, contents of approximately 12 to 14 g/l are detected (Coppa et al., 2006). HMOs are composed by the following monosaccharides: glucose, galactose, sialic acid, fucose and N-acetylglucosamine (Garrido et al., 2012). Many diverse combinations and compositions of these monosaccharides, as well as several combinations of glycosidic bonds, contribute to the complexity of HMO structures (Ninonuevo and Lebrilla, 2009).

Various functions of HMOs are described in literature. They seem to have important functions in the development of the intestinal epithelium of infants (Lara-Villoslada et al., 2006), in establishing a healthy microbiota (Ninonuevo and Lebrilla, 2009), in acting as pathogen receptors (Barile and Rastall, 2013), and in having immunomodulatory properties (Venema, 2012). They are also an important source of monosaccharides, – as they provide glucose as an energy source (Venema, 2012), and sialic acid for neural tissue and brain development. One of the most important functions of HMOs is the prebiotic (bifidogenic) effect. They seem to play a key role in promoting a bifidobacteria-dominant microbiota in newborns (Coppa et al., 2006). Prebiotics influence the host by stimulating the growth and/or activity of beneficial microbiota already established in the colon (Roberfroid, 2007). The potential bifidogenic effect of breast milk was already observed and published by György et al., in 1954 (Ward et al., 2007). Since then, many other works have supported this hypothesis, and further specified that this bifidogenic effect is linked especially to oligosaccharides present in human milk (Han et al., 2012). HMOs have been proved to selectively stimulate the growth of specific bifidobacterial strains, preferentially *Bif. longum* biovar *infantis* and *Bif. bifidum,* which grew successfully on purified HMOs as the sole carbon source (Ward et al., 2006, 2007; LoCascio et al., 2007; Marcobal et al., 2010; Rockova et al., 2011a,b). It is generally accepted that HMOs have prebiotic effects, selectively serving as a source of energy for desired bacteria in the infant intestine (Bode, 2009). However, research on the capability of utilizing HMOs is mainly focused on bifidobacteria – as the predominant bacterial group in the infants' gut. Data on the utilization of HMOs by other intestinal microorganisms, among others also lactobacilli, as beneficial bacteria is scarce. As demonstrated by Marcobal et al. (2010), aside from bifidobacteria, some other intestinal bacteria are able to metabolize HMOs, including *Bacteroides fragilis* and *Bacteroides vulgatus*. These strains were proved to metabolize HMOs with high efficiency in *in vitro* conditions.

From the genus *Lactobacillus*, only strains *Lbc. gasseri* ATCC33323 (Ward et al., 2006) and *Lbc. acidophilus* NCFM (Marcobal et al., 2010) were tested for their ability to grow on HMOs. In the case of *Lbc. gasseri,* no growth was observed, whereas *Lbc. acidophilus* showed weak, but noticeable growth. No more information on the ability of lactobacilli to utilize HMOs is available according to our knowledge. The aim of this study was to investigate the ability of several strains of lactobacilli to ferment HMOs as a sole carbon source in *in vitro* conditions, thus furthering our knowledge regarding the selectivity of HMOs.

MATERIALS AND METHODS

Bacterial strains

The list of strains (six strains of lactobacilli and one strain of bifidobacteria) used in this work is shown in Tables 1 and 2. The strains were procured from the Culture Collection of Dairy Microorganisms Laktoflora® - CCDM (Prague, Czech Republic), from the Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences in Prague. Human isolates of lactobacilli were obtained from biopsy samples (Dairy Research Institute Tábor, Czech Republic).

Isolation and purification of HMOs

Human milk samples obtained from three different donors, kindly provided by the Gynecology and Obstetrics Clinic of Charles University and the General Faculty Hospital in Prague, were used for the isolation and purification of HMOs. Oligosaccharides were extracted according to the methodology described by Gnoth et al. (2000), with a few modifications. In the first step, milk (100 ml) was centrifuged at 1800 *g* for 30 min at 4°C, thus, lipids, proteins and cells were partially removed. Subsequently, proteins were precipitated by the addition of ethanol (2:1, v/v). The solution was stored at 4°C for 24 h. After centrifugation (under above mentioned conditions), the solvent was removed by rotatory evaporation, and the remainder of the solution was dissolved in deionized water. The whole process of precipitation was repeated twice. Gel filtration chromatography on a column filled with Toyopearl HW40F in 1% acetic acid (flow rate 0.1 ml/min) was used. The eluate was collected in 2.5 ml fractions and screened for the presence of oligosaccharides by thin-layer chromatogramy using isopropanol : water : 25% ammonia solution (5:1:2, by volume) as a mobile phase, and was then visualized by spraying with 10% sulphuric acid in ethanol and heating. Carbohydrate containing fractions (a total volume of 50 ml) were dispensed into vials and cooled at a temperature of 4-8°C for 30 min., and frozen at – 70°C for 90 min. Samples were subsequently lyophilized using Cryodos device (Telstar, Spain). The yield from 100 ml of milk made 0.5 g of purified oligosaccharides.

Bacterial growth on HMOs

Basal medium (tryptone, 10 g; peptone, 10 g; yeast extract, 5 g; Tween 80® 1 ml, distilled water 1 L) was autoclaved (121°C, 15 min). Purified oligosaccharides (1 % w/w) were added as a sole carbon source to the cooled medium after sterile filtration (Puradisc FP 30 filter 0.2 µm, Whatman, Germany). As a negative control, a medium devoid of carbohydrate was used. As a positive control, Wilkins Chalgren broth (Oxoid, Basingstoke, UK) was used. Overnight bacterial cultures were centrifuged (5000 *g*, 7 min) and re-suspended in saline. Bacterial suspensions were inoculated into

Table 1. Utilization of human milk oligosaccharides.

Strain	Density of lactobacilli (change in A_{540})		
	HMO	BM	WCH
Lbc. fermentum RL 25	0.13 ± 0.06^{ab}	0.17 ± 0.12^{b}	5.20 ± 0.20^{c}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 66	0.07 ± 0.06^{a}	0.03 ± 0.06^{a}	5.03 ± 0.21^{bc}
Lbc. acidophilus CCDM 151	0.53 ± 0.06^{c}	0.07 ± 0.06^{ab}	4.87 ± 0.06^{b}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 767	0.03 ± 0.06^{a}	0.03 ± 0.06^{a}	4.77 ± 0.21^{b}
Lbc. casei subsp. *paracasei* PE1TB-P	0.43 ± 0.06^{bc}	0.17 ± 0.06^{b}	4.97 ± 0.15^{bc}
Lbc. animalis CCDM 382	0.17 ± 0.06^{ab}	0.07 ± 0.06^{ab}	5.17 ± 0.15^{c}
Average	$0.23 \pm 0.20^{\beta}$	$0.09 \pm 0.08^{\alpha}$	$5.00 \pm 0.21^{\gamma}$
Bif. bifidum JKM	2.01 ± 0.47^{d}	0.07 ± 0.02^{ab}	3.01 ± 0.11^{a}

Data are expressed as increase in turbidity of bacterial suspension estimated from increase in A540 during 24 h of incubation; values are means from triplicate determination ± standard deviation (SD). HMO, medium containing purified human milk oligosaccharides as a carbon source; WCH, Wilkins Chalgren broth (control medium); BM, basal medium without carbohydrate source (negative control). a-d data in columns with different superscripts differ ($P < 0.05$). αβγ data in lines with different superscripts differ ($P < 0.05$).

Table 2. pH values of media.

Strain	Origin	Final pH values after 24 h of incubation	
		HMO	WCH
Lbc. fermentum RL 25	human faeces	6.20 ± 0.03^{b}	4.86 ± 0.04^{f}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 66	yogurt, Turkey	6.50 ± 0.04^{cd}	4.56 ± 0.04^{b}
Lbc. acidophilus CCDM 151	pill Biolacta	6.14 ± 0.02^{b}	4.66 ± 0.05^{d}
Lbc. delbrueckii subsp. *bulgaricus* CCDM 767	yogurt, Switzerland	6.55 ± 0.03^{d}	4.86 ± 0.04^{f}
Lbc. casei subsp. *paracasei* PE1TB-P	biopsy sample (colon)	6.20 ± 0.05^{b}	4.54 ± 0.05^{a}
Lbc. animalis CCDM 382	raw goat milk	6.45 ± 0.05^{c}	4.68 ± 0.05^{e}
Average		$6.34 \pm 0.17^{\alpha}$	$4.69 \pm 0.14^{\beta}$
Bif. bifidum JKM	infant faeces	5.00 ± 0.20^{a}	4.65 ± 0.15^{c}

Values are means ± standard deviation (SD) of three measurements. $^{a-f}$ data in columns with different superscripts differ ($P < 0.05$). $^{\alpha\beta}$ data in lines with different superscripts differ ($P < 0.05$). HMO – medium containing purified human milk oligosaccharides as a carbon source. WCH, Wilkins Chalgren broth (control medium). Initial pH values of HMO and WCH media were 6.60 and 6.40, respectively.

a medium containing HMOs and then incubated at 37°C for 24 h under anaerobic conditions. All strains were grown in triplicate. The growth of lactobacilli was evaluated as the change in absorbance A_{540} during 24 h of incubation by measuring transmitted light using densitometer DEN-1 (Dynex, Czech Republic). Results were expressed as increase in turbidity of the bacterial suspension estimated from increase in A_{540}. For the determination of pH values, pH meter HACH sension 1 (HACH, USA) was used. The results were evaluated using MS Excel 2007 (Microsoft, Redmond, USA).

Determination of bacterial metabolites

To determine organic acids concentration, the isotachophoretic (ITP) method was used. The samples after fermentation by lactobacilli were subjected to isotachophoretic separations using IONOSEP 2003 device (Recman, Czech Republic). The change in the content of lactic acid as the major metabolite of lactobacilli as well as the content of acetic, butyric, propionic, formic and succinic acids was monitored. Prior to analysis, the samples were diluted with 150 volumes of deionized water, and then purified using the Puradisc FP 30 filter with a pore size of 0.2 µm (Whatman, Germany). Solution containing 10 mM HCl, 22 mM ε-aminocaproic acid and 0.1 % 2-hydroxy-ethylcellulose (pH 4.5) as leading electrolyte (LE) was used. As trailing electrolyte (TE), 5 mM caproic acid was used. All chemicals were obtained from Sigma-Aldrich (Czech Republic). The values of the initial and final stream used were 80 and 30 µA, respectively.

Statistical analyses

For evaluation of the results Statgraphics® Centurion XV (StatPoint, Inc., Warrenton, USA), the multiple range comparison - LSD test was used. A significant difference was statistically considered at the level of $P < 0.05$.

RESULTS AND DISCUSSION

In this work, 6 strains of lactobacilli of different origin were tested for their ability to ferment HMOs as a sole

carbohydrate source. The growth of strains tested is summarised in Table 1. In the case of the four strains (*Lbc. fermentum* RL25, *Lbc. animalis* CCDM 382 and two strains of *Lbc. delbrueckii* subsp. *bulgaricus* CCDM 66 and CCDM 767), no increase in bacterial density in the medium with HMOs was observed. The change in the absorbance A_{540} after 24 h of incubation in these groups of strains ranged from 0.03 to 0.17. In the rest of the strains tested (*Lbc. acidophilus* CCDM 151 and *Lbc. casei* subsp. *paracasei* PE1TB-P), a slight increase in bacterial densities in HMO-containing medium was observed (0.53 for *Lbc. acidophilus*, 0.43 for *Lbc. casei* subsp. *paracasei*). As a positive control, Wilkins Chalgren (WCH) broth was used. In this medium, high cell densities (from 4.77 to 5.20) in all strains were obtained (Table 1). The strain *Bif. bifidum* JKM was used as a positive control, too. This strain is able to effectively utilize HMOs, as demonstrated previously (Rockova et al., 2011a). As a negative control, a basal medium without any added sugar was used. A marginal increase in absorbance $A_{540,}$ even in the absence of sugar, was seen (Table 1). Increased cell numbers for bacterial species like *Lactobacillus, Enterococcus, Enterobacteriaceae* or *Staphylococcus* in media without carbohydrate supplementation were also observed by other authors (Marcobal et al., 2010; Satoh et al., 2013).

The strain PE1TB-P began to grow in WCH broth after the first hour of incubation (Figure 1), while growth in the HMO-containing medium was noticeable after three hours. Instead of exponential growth, a slight steady growth during 24 h of incubation was observed. A very similar trend was noticed for the strain *Lbc. acidophlilus* (data not shown).

To precisely evaluate the fermentation ability, besides measuring the bacterial density, it is important to analyse the changes in pH of growth media, and possibly to analyse metabolite concentration produced by bacteria. Final pH values (Table 2) are consistent with the change in A_{540} measured after 24 h of incubation. The pH of the medium containing purified HMOs decreased from the initial value of 6.60 to 6.34 on average, while in the control medium (WCH), the pH decrease was much more apparent (from 6.40 to 4.69 on average). Anaerobic intestinal microbiota convert carbohydrates to lactic acid and short-chain fatty acids (Loo et al., 1999) such as acetic, propionic and butyric acids. Lactic acid has a role in maintaining lower intestinal pH (Satoh et al., 2013), while butyric acid, sometimes produced by heterofermentative lactic acid bacteria, provides nutrition of the colonic epithelium and has an important role in gut maintenance (Venema, 2012). The results of bacterial metabolite analysis are presented in Figures 2 and 3. The medium with HMOs produced significantly lower concentrations of lactic acid compared to the control medium (WCH broth) after 24 h of fermentation. The production of lactic acid in WCH broth rose to 225 mg/100 ml (in the strain PE1TB-P), while the maximum concentration of lactic acid detected in the medium with HMOs made no more than 40 mg/100 ml (in the strain CCDM 151). To a somewhat lower extent also in the strain PE1TB-P a slight increase in lactic and acetic acids was visible, which indicates some bacterial growth. Concentrations of succinic and formic acids rose marginally (up to 16 and 11 mg/100 ml, respectively), and in the case of propionic and butyric acids, non-detectable concentrations, even lower than 2 mg/100 ml (data not shown), were obtained.

The strain *Bif. bifidum* JKM, used as a positive control, showed very good growth in the medium with HMOs compared to the growth of lactobacilli. The increase in the absorbance A_{540} made 2.01 (Table 1). The growth was accompanied by a decrease in pH values (Table 2) and by an increase of acids produced (Figure 2).

Direct fermentation of HMOs by intestinal microbiota has not yet been well described and there is a lack of information regarding their utilization by specific bacterial species (lactobacilli). The majority of information, that exists on HMO fermentation refers to bifidobacteria as the predominant bacterial group in a healthy infants's gut. Many *in vitro* studies were conducted on the capability of bifidobacteria to ferment HMOs with positive results (Ward et al., 2006, 2007; LoCascio et al., 2007; Marcobal et al., 2010; Satoh et al., 2013), but growth in the presence of HMOs is not a property of all representatives of the genus *Bifidobacterium*. Preferential growth of *Bif. longum* subsp. *infantis,* a species often occurring in infants, was noticed in the aforementioned studies. This strain preferentially utilized oligosaccharides with a degree of polymerization (DP) ≤ 7. These oligosaccharides form a significant part of breastmilk (LoCascio et al., 2007). In the study conducted by Rockova et al. (2011a), bifidobacterial strains of human origin (*Bif. bifidum* and *Bif. longum*) were proved to utilize HMOs with high efficiency in comparison with bifidobacteria of animal origin (*Bif. animalis).* Utilization capability is closely related to the enzymatic equipment that specific bacteria possess. Enzyme lacto-N-biose I phosphorylase was recently proved to be responsible for the cleavage of lacto-N-biose I, which is an important component of HMOs (Satoh et al., 2013). The presence of this enzyme was detected in species *Bifidobacterium bifidum* and *Bifidobacterium longum* occuring in infants' gut (Wada et al., 2008). Conversely, in other bacterial groups like lactobacilli, clostridia or bacteroides, this enzyme was not observed (Wada et al., 2008). The strain *Bif. longum* subsp. *infantis* also possesses other enzymes involved in the cleavage of HMOs, such as fucosidase or sialidase (LoCacio et al., 2007). Additionally, between certain bifidobacterial strains, commensal activities were described, where strains able to cleave long-chain HMOs (*Bif. bifidum*) can provide monosaccharides for other strains

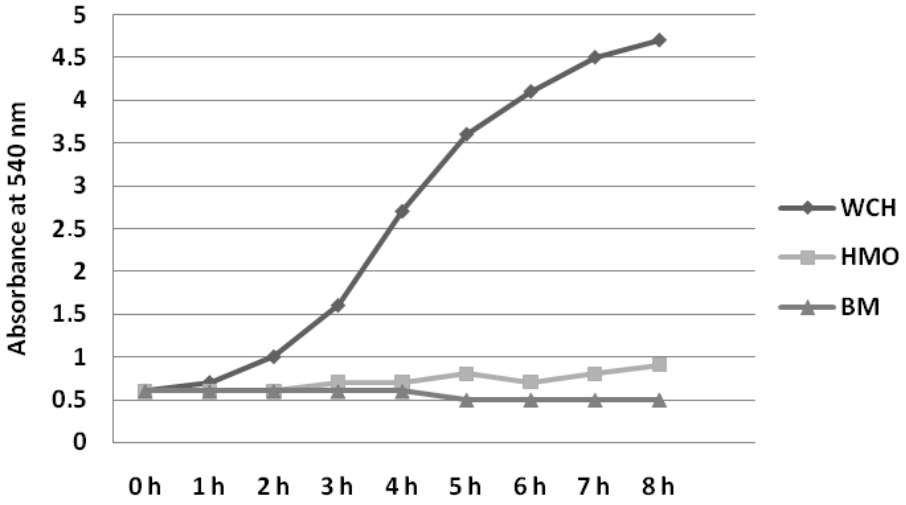

Figure 1. Growth of *Lbc. casei* subsp. *paracasei* PE1TB-P in the medium containing HMOs as a sole carbon source. WCH, Wilkins Chalgren medium as a positive control; BM, basal medium without any carbohydrate as a negative control.

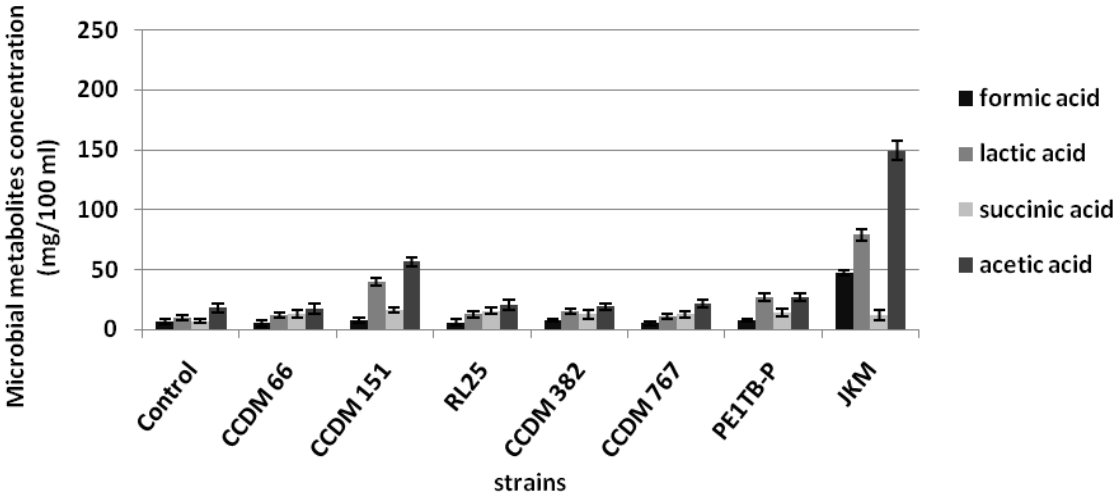

Figure 2. Concentrations of microbial metabolites in the medium containing purified human milk oligosaccharides after 24-h fermentation by lactobacilli. Concentrations of butyric acid and propionic acid made less than 2 mg/100 mL of the media (data not shown). Bars present mean ± SD.

(*Bif. breve*, Ward et al., 2007). Marcobal et al. (2010) demonstrated that HMO fermentation is not an exclusive property of specific strains of bifidobacteria. In the study conducted by this group, apart from *Bif. longum* subsp. *infantis*, for the first time, *Bacteroides fragilis* and *Bacteroides vulgatus* were proved to be able to metabolize HMOs with high efficiency. Either weak or no fermentation was exhibited by genera *Clostridium, Eubacterium, Enterococcus, Streptococcus*, *Veillonella* and *E. coli* strains. From the group of lactobacilli, a strain *Lbc. acidophilus* NCFM was tested which showed some growth ability on this substrate (Marcobal et al., 2010). In another *in vitro* study (Ward et al., 2006), a strain *Lbc. gasseri* ATCC33323 was tested in which the ability to ferment HMOs was not proved.

The major part of HMOs reach the colon in unhydrolyzed form, where they may be utilized by intestinal microbiota into short chain fatty acids (Lasrado and Gudipati, 2013) and thus serve as nutrients – prebiotics (Loo et al., 1999; Ninonuevo and Lebrilla, 2009). A prebiotic effect is proven when the growth of beneficial bacteria is stimulated, while potentially harmful bacteria

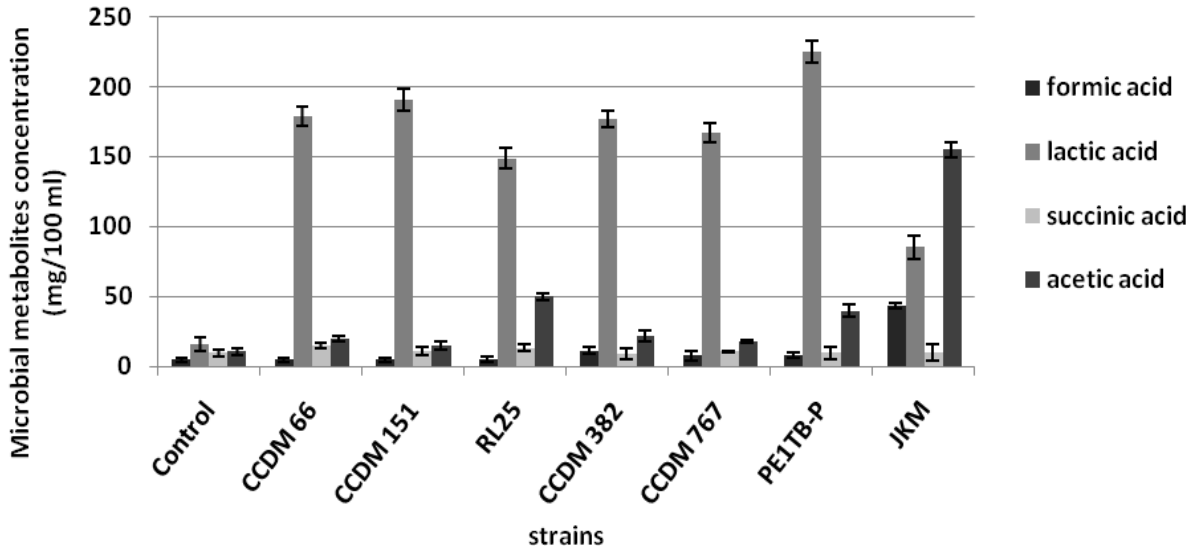

Figure 3. Concentrations of microbial metabolites in the medium containing Wilkins Chalgren broth after 24-h fermentation by lactobacilli. Concentrations of butyric acid and propionic acid made less than 2 mg/100 mL of the media (data not shown). Bars present mean ± SD

are inhibited (Boehm et al., 2004). In this study we conducted an *in vitro* testing on direct fermentation of purified HMOs by lactobacilli. The results of this work support the hypothesis that utilisation of HMOs may be species- and strain specific. Based on the evaluation of the results obtained by absorbance $A_{540,}$ measured together with bacterial metabolite detection and the evaluation of pH values, we concluded that the lactobacilli tested did not appear to be active HMO consumers. This fact supports the hypothesis that HMOs may selectively enhance the growth of specific bacterial groups (particularly bifidobacteria) present in the colon of newborns.

Conflict of Interests

The authors did not declare any conflict of interests.

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Full Length Research Paper

Microbiota of two species of commercially important fish in the Amazon region (Belém-Pará-Brazil): Butterfly peacock bass (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii*)

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The Amazon offers great potential for fishery activities but the fish fauna's specific microbiota is not yet known. This paper identified the bacterial flora composition and the influence of this process on the microbiological spoilage in economically important fish species in the Amazon region: butterfly peacock bass (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii*). To this end, microbiological characterization was performed: counts of total mesophilic aerobic bacteria, psychrotrophic bacteria and coliforms at 35 and 45°C. Bacteria were also isolated through seeding in agar surface using violet red bile glucose (VRBG) for enterobacteria strains and Baird-Parker Agar with egg-yolk tellurite for *Staphylococcus* species, both with incubation at 36°C for 48 h. The bacteria isolated were identified using the API 20E kit (Enterobacteria), and Gram-positive with API Staph (Staphylococci). Finally, the limit temperature for strain growth was tested using spectrophotometry readings at 554 nm at 10 and 15°C at three different times: 0, 3 and 6 h. The mesophilic aerobic bacteria counts for fresh fish samples ranged from 6.03 - 8.23 log CFU/g for piramutaba and 4.52 - 7.24 log CFU/g for butterfly peacock bass. The count ranges of psychrotrophic aerobic bacteria found were 6.14 - 8.56 log CFU/g and 4.52 - 7.24 log CFU/g for piramutaba and butterfly peacock bass, respectively. They also had an average score above 10^3 MPN/g for total coliforms. Sixteen different strains were isolated. The most predominant were *Staphylococcus hominis*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and *Enterobacter intermedius*. When subjected to temperatures of 10 and 15°C, the strains did not achieve growth for 6 h at a 95% significance level.

Key words: Fish, *Cichla ocellaris*, *Brachyplatystoma vailantii*, microflora.

INTRODUCTION

The coast of the state of Pará (Brazil) offers great potential for fishery activities due to the numerous rivers and estuaries that empty into the Atlantic Ocean, forming a complex aquatic environment with high biological productivity. The substantial biomass of fish species in this region is exploited by both artisanal and industrial

fleets (Isaac et al., 2009). Butterfly peacock bass (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii*) feature among these species and are two of the favorite targets of fishing in the region given their considerable importance from both an economic and nutritional perspective. However, as marine fish, freshwater fish are extremely perishable food commodities. Enzymatic and chemical reactions are usually responsible for the initial loss of freshness whereas microbial activity is responsible for the overt spoilage and thereby establishes product shelf life (Gram, 1995; Gram and Huss, 1996). In some cases, chemical changes such as auto-oxidation or enzymatic hydrolysis of the lipid fraction may result in off-flavors, while, in other cases, tissue enzyme activity can lead to unacceptable softening of the fish. The spoilage of fresh fish by microbial activity is usually due to its microbiota located mainly in the outer surfaces (skin and gills) and in the intestines of live and newly caught fish. It can also be the consequence of fish cross-contamination associated with inappropriate handling and storage (Cruz-Romero et al., 2008). The poikilotherm nature of fish allows bacteria to grow in a broad temperature range. Thus, the microbiota of temperate-water fish is dominated by psychrotrophic, aerobic or facultative anaerobic Gram-negative, rod-shaped bacteria, and in particular, by *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella putrefaciens*, *Flavobacterium*, *Cytophaga*, *Vibrio*, *Photobacterium* and *Aeromonas* (Lalitha and Surendran, 2006; Pantazi et al., 2008). The microbiota in tropical freshwater fish is dominated by Gram positive bacteria such as *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Flavobacterium*, *Flexibacter*, *Pseudomonas*, *Psychrobacter*, *Citrobacter*, *Micrococcus*, *Staphylococcus*, *Streptococcus* and *Moraxella* spp. (Gram and Huss, 1996; Apun et al., 1999; Austin, 2002; ICMSF, 2005). The specific microbiota of butterfly peacock bass and piramutaba is not yet known. During the processing of the two species, the microorganisms present in the gut and on the skin can spread to the processing equipment, the workers and sterile flesh fillets. The objective of this paper was to identify the microbiota and study the growth temperature of isolated microorganisms to improve the refrigeration temperature conditions in two fish species from the Amazon region: butterfly peacock bass (*C. ocellaris*) and piramutaba (*B. vailantii)*.

MATERIALS AND METHODS

Fish samples

Four successive commercial-sized fish samples (n=4) (butterfly peacock bass and piramutaba) were collected between March and April 2013 at Ver-o-Peso Market (Belém, Pará, Brazil). At the time of collection, the samples were placed in sterile bags kept under refrigeration (around 10°C) and transported to the Laboratory of Food Microbiology (Federal University of Pará - UFPA) for further analysis.

Microbiological characterization

Twenty five grams of each sample (ventral part of the filet) were aseptically collected and added with 225 mL of 0.1% sterile peptone water (SPW), thus obtaining 1:10 dilution, which were homogenized in a stomacher (STOMACHER 400 CIRCULATOR SEWARD) at 2,300 rpm for 30 s. Next, counts of total mesophilic aerobic bacteria, psychrotrophic bacteria and coliform at 35 and 45°C were performed according to Brazil (2003). The total mesophilic aerobic and psychrotrophic bacteria counts were carried out in pour plate using plate count agar followed by incubation at 35°C/48 h for mesophilic and 7°C/10 day for psychrotrophic bacteria. Coliforms at 35 and 45°C were counted through the most probable number (MPN), with three sets of three tubes. Lauryl sulfate tryptose broth (LST) was used as a presumptive medium and incubated at 35°C for 24-48 h. After reading, the positive tubes were transferred to brilliant green bile broth 2% (GB) and EC broth. The former was incubated at 35°C/24-48 h for confirmation of total coliforms and EC broth tubes were incubated in a water bath at 45.5°C/24 h for confirmation of thermotolerant coliforms.

Bacterial isolation

The homogenized matter used for microbiological characterization was subsequently used for bacteria isolation. The isolation to obtain pure cultures was carried out through seeding in agar surface using violet red bile glucose (VRBG) for enterobacteria strains and Baird-Parker with egg-yolk tellurite for *Staphylococcus* species, both with incubation at 36°C/48 h. Next, one plate was selected for each medium and 5-10 colonies per plate were randomly chosen. The selected colonies were striated in VRBG or Baird-Parker agar plates to obtain a pure culture. After incubation at 36°C/48 h, a colony was transferred from each plate to brain heart infusion (BHI) with 10% glycerol and stored in a freezer to be used in further tests.

Bacterial strain identification

The bacteria isolated were previously identified with Gram stain tests. Next, Gram negative strains were identified using the API 20E kit (Enterobacteria), and Gram positive strains with API Staph (Staphylococci). The procedure was in accordance with the manufacturer's recommended procedures (Biomérieux, France) (Harrigan, 1998).

Limit temperature for growth (adapted from Bordignon-Junior et al., 2012)

Strains were reactivated in BHI broth for 24 h at 36°C. After that, the isolates identified were transferred to a new BHI broth (1:15 mL)

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Table 1. Mean values of microbiological characterization in fresh fish.

Samples	Mesophilic aerobic bacteria(log CFU/g)	Psychrotrophic bacteria (log CFU/g)	Total coliforms (MPN/g)	Thermotolerant Coliforms(MPN /g)
Piramutaba 01	6.23±0.03^{bcd}	6.14±0.13^{c}	1,100^{a}	15^{c}
Piramutaba 02	8.23±0.01^{a}	8.56±0.02^{c}	1,100^{a}	1,100^{a}
Piramutaba 03	6.03±0.01^{cd}	6.46±0.00^{c}	1,100^{a}	1,100^{a}
Piramutaba 04	6.61±0.65^{bc}	7.97±0.03^{a}	1,100^{a}	240^{b}
B. peacock bass 01	4.52±0.74^{e}	6.40±0.06^{d}	1,100^{a}	1,100^{a}
B. peacock bass 02	5.35±0.07^{de}	6.66±0.06^{a}	1,100^{a}	1,100^{a}
B. peacock bass 03	6.16±0.01^{cd}	6.52±0.008^{c}	1,100^{a}	240^{b}
B. peacock bass 04	7.24±0.03^{ab}	8.33±0.04^{b}	1,100^{a}	1,100^{a}

*Different letters in each column means difference at 95% level of significance.

and maintained at different temperatures: 10 and 15°C during three different times: 0, 3 and 6 h. Spectrophotometric readings were performed (Spectrophotometer Model Nova 2000 UV) at 554 nm.

Statistical analysis

Tukey's test was applied to evaluate the difference of means among microorganism groups (mesophilic and psychrotrophic bacteria, total and thermotolerant coliforms) found in different fishes. The optical density data were subjected to ANOVA considering different groups of isolated microorganisms. The software Statistica 8.0 was employed considering a 95% level of significance.

RESULTS AND DISCUSSION

Microbiological characterization

The mesophilic aerobic bacteria counts for fresh fish samples ranged from 6.03 - 8.23 log CFU/g for piramutaba and 4.52 - 7.24 log CFU/g for butterfly peacock bass (Table 1). Brazil (2001) does not establish micro-biological standards for mesophilic bacteria count in fresh fish. However, the International Commission on Microbiological Specifications for Foods (ICMSF, 1986) recommends the limits for mesophilic aerobic should not exceed values of 10^7 CFU/g or cm^2 in chilled fish samples for human consumption.

Aerobic mesophilic bacteria, when present in large numbers, indicate unsanitary conditions. The high count of this microorganism in food may result from unsatisfactory storage conditions, with potential danger to health (Morton, 2001; Coelho et al., 2010). The fish samples analyzed had a mean score of 10^6 and 10^5 for piramutaba and butterfly peacock bass, respectively. A similar result was observed by Fernandez and Barbosa (2010), who reported counts of 10^4 – 10^5 CFU/g for mesophilic bacteria in sardines. Li et al. (2013) found values around 10^3 CFU/g in large yellow croaker (*Pseudosciaena crocea*) from China. Vishwanath et al. (1998) observed a total bacteria plate count range of 10^6 – 10^7 CFU/g for *Muscodor albus* (Manipur, India). Oku and Amakoromo (2013) found bacteria count values of 10^8 – 10^{10} CFU/g for fresh fish (Yenagoa metropolis, Nigeria). Thong Thi *et al*. (2013) found total mesophilic counts on raw pangasius fish around 5.1 log CFU/g.

Previous studies by Shewan (1977), Guizani et al. (2005) and Ercolini et al. (2009) have indicated that mesophilic microorganisms are dominant in tropical fish species. The count of psychrotrophic aerobic bacteria found ranged from 6.14 to 8.56 log CFU/g for piramutaba and from 4.52 to 7.24 log CFU/g for butterfly peacock bass (Table 1).

The Brazilian legislation establishes no maximum limit for psychrotrophic microorganisms in fish for human consumption. But considering the ICMSF (1986), piramutaba and butterfly peacock bass species showed values above the maximum limit (10^7 CFU/g).

Britto et al. (2007) evaluated the bacteriological deterioration of whole jaraqui (*Semaprochilodus* spp.) captured in the Amazon region and found a count of psychrotrophic aerobic bacteria ranging from 2 – 3 log CFU/g. Similar results were found by Hanninen et al. (1997), who found freshwater fish samples contaminated by *Aeromonas* spp. Sallam et al. (2007) found psychrotrophic bacteria count in raw Pacific saury around 3.95 log CFU/g, while Thong Thi et al. (2013) found total psychrotrophic count to be around 4.3 log CFU/g in raw pangasius fish (*Pangasius hypophthalmus).*

The growth of psychrotrophics also indicates poor sanitary conditions. Psychrotrophic bacteria grow in foods refrigerated between 0 – 7°C, with optimum growth temperature around 20°C. Some psychrotrophics may be pathogens, such as *Aeromonas hydrophila*, some strains of *Bacillus cereus*, *Clostridium botulinum* type E, B and F, *Listeria monocytogenes*, *Vibrio cholerae*, *Yersinia enterocolitica*, some strains of enteropathogenic *Escherichia coli*, and other pathogens such as *Salmonella* spp. and *C. perfringens* type C. Some strains of *Bacillus*

Table 2. Identification of *Staphylococcus* strains isolated from piramutaba.

Strains isolated	Number of strains isolated	Strains isolated (%)	ID (%)
Staphylococcus lentus	01	4.76	99.5
Staphylococcus epidermis	01	4.76	97.9
Staphylococcus aureus	04	19.04	88.4-99.6
Staphylococcus hominis	05	23.80	81.3-94.9

Table 3. Identification of *Staphylococcus* strains isolated from butterfly peacock bass.

Strains isolated	Number of strains isolated	Strains isolated (%)	ID (%)
Staphylococcus cohnii ssp. *cohnii*	01	4.76	98.3
Staphylococcus saprophyticus	01	4.76	80.2
Staphylococcus lentus	01	4.76	99.9
Staphylococcus xylosus	01	4.76	97.9
Staphylococcus hominis	06	28.57	81.3-92.3

cereus and *Staphylococcus aureus*, which have lower development temperatures between 7 – 15°C can grow if temperature abuse occurs during storage (Cousin et al., 2001).

In refrigerated fish, the psychrophilic and psychrotrophic bacteria play direct roles in fish deterioration because they multiply well in these conditions (Franco et al., 1996). The butterfly peacock bass and piramutaba collected had an average score above 10^3 MPN/g for total coliforms (Table 1). Although this does not indicate the presence of pathogens, total coliforms are important indicators of potential product deterioration and its mean shelf life (Agnese et al., 2001).

The total and thermotolerant coliforms are indicators of hygienic quality, not representing direct contact of the product with human or animal feces, but reporting on the degree of microbial pollution to which the food has been exposed. This score thus indirectly reflects the quality of production practices.

Brazil (1997, 2001) set the value of 10^2 MPN/g as the maximum acceptable standard for thermotolerant coliforms in fish and fishery products. However, it is observed that the average of the samples collected from piramutaba and butterfly peacock bass are above the limits established by the Brazilian legislation, indicating the possibility of water contamination as well as the existence of some source of organic matter containing animal or even human feces, which compromises the quality of the fish.

Bacterial strain identification

Among the 36 isolates, it was observed that 58.33% were Gram positive and the other 41.67% were Gram negative. From the results of the Gram stain test, the kits were selected to identify the strains.

The data show that Gram negative bacteria belonged to seven different species described in Tables 2 and 3. The most predominant were *Staphylococcus hominis* for butterfly peacock bass (28.57%) and piramutaba (23.81%) and *Staphylococcus aureus* (19.05%), found only in piramutaba.

Staphylococci are not part of the normal fish microbiota (Huss, 1988; Van den Broek et al., 1984). This indicates that if *S. aureus* is found in fish, it most likely originates from human sources (Bulushi et al., 2010). Moreover, the presence of staphylococci in fish is an indication of (a) postharvest contamination due to poor personnel hygiene, or (b) disease in fish (Austin and Austin, 2007; Huss, 1988). In Japan, fish-borne microbes, including *S. aureus*, are a major cause of food poisoning (Cato, 1998) both because of the very high consumption of fish and because of the common practice of eating raw fish (Huss, 1988). Other authors reported food poisoning by *S. aureus* due to fish consumption (Cato, 1998; CDC, 2011; Huss, 1988; Iwamoto et al., 2010, Sokari, 1991, Ayulo et al., 1994, Rodma et al., 1991).

S. hominis is rarely implicated in food poisoning, because it does not multiply quickly in this environment. However, it may contaminate food since humans are carriers of microorganisms and some of these may be related to certain human infections (Pereira and Pereira, 2005; Cunha et al., 2006). The presence of micro-organisms in fish products may also indicate the occurrence of food contamination due to poor hygiene in handling and lack of preservation techniques, since *S. hominis* is not part of microbiota of these aquatic organisms.

The Gram negative bacteria found belong to nine

Table 4. Identification of *Enterobacteriaceae* strains isolated from piramutaba.

Strains isolated	Number of strains isolated	Strains isolated (%)	ID (%)
Stenotrophomonas maltophilia	01	6.67	65.6
Enterobacter intermedius	02	13.34	72.4-92.5
Klebsiella pneumoniae ssp. pneumoniae	01	6.67	94.4
Serratia marcensces	01	6.67	71.9
Buttiauxella agrestis	01	6.67	93.6
Pantoea spp. 1	01	6.67	87.8

Table 5. Identification of *Enterobacteriaceae* strains isolated from butterfly peacock bass.

Strains isolated	Number of strains isolated	Strains isolated (%)	ID (%)
Stenotrophomonas maltophilia	03	20.00	80.4-92.4
Enterobacter intermedius	01	6.67	93.3
Enterobacter amnigenus 2	01	6.67	82.2
Klebsiella pneumoniae ssp. pneumoniae	01	6.67	90.0
Serratia liquefasciens	01	6.67	82.6
Serratia ficaria	01	6.67	98.4

% ID indicates the profile similarity of isolates with the standards established by the kits, according to the manufacturer.

different species of enterobacteria as shown in Tables 4 and 5. The most prevalent were *Stenotrophomonas maltophilia* (20.0%) and *Enterobacter intermedius* (13.34%) in butterfly peacock bass and piramutaba, respectively.

Both *S. maltophilia* and *E. intermedius* have not been found to cause food poisoning. However, the presence of these bacteria is worrying since they may be associated with fish deterioration. Oku and Amakoromo (2013) obtained twelve bacterial isolates from raw tropical freshwater fish samples. The bacteria belonged to five genera identified as: *B. subtilis, Corynebacterium, Lactobacillus*, *Pseudomonas* and *S. aureus.*

The specific microbiota of fresh butterfly peacock bass and piramutaba is not yet known. However, it is known that the flora in tropical fish often carries a slightly higher load of Gram-positive and enteric bacteria than fish from temperate water, but it is otherwise similar to that flora dominated by psychrotrophic Gram negative, rod-shaped bacteria belonging to the genera *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella*, *Flavobacterium*, *Vibrionaceae*, *Aeroemonadaceae* and, to a lesser degree, *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillus* and *Corynebacterium* (Liston, 1980; Apun et al., 1999; Austin, 2002; ICMSF, 2005). Enterobacteriaceae genera have been frequently isolated from the digestive tracts and flesh of freshwater fish (Austin, 2002; Yagoub, 2009; Gonzalez-Rodriguez et al., 2002; Paludan-Müller et al., 1998). Apun et al. (1999) show some Enterobacteriaceae strains such as *K. pneumoniae*, *E. aerogenes*, and *E. coli* have been isolated from the intestines of tropical freshwater fish. *Serratia* spp. have also been found in *Pangasius* fillets (Thong et al., 2013). At ambient temperature (25°C), the microbiota is dominated by mesophilic *Vibrionaceae* (Gorczyca and Pek, 1985; Gram et al., 1990) and, particularly if the fish are caught in polluted waters, mesophilic Enterobacteriaceae become dominant (Gram, 1992).

Limit temperature for growth

The 16 strains (Figures 1 and 2; Tables 6 and 7) from butterfly peacock bass and piramutaba, when subjected to temperatures of 10 and 15°C, did not achieve growth for 6 h at a 95% significance level.

Denton and Kerr (1998) stated that *S. maltophilia* growth does not occur at temperatures lower than 5°C. However, Margesin and Schinner (1991) reported isolation of an *S. maltophilia* strain from an alpine environment capable of growth at 10°C. According to Schmitt et al. (1990), *S. aureus* is capable of growing in a wide range of temperatures, from 7 - 48.5°C with an optimum from 30 to 37°C. Valero et al. (2009) reported *S. aureus* growth can be inhibited at refrigeration temperatures (around 8°C). Schmitt, Schuler-Schmid and Schmidt-Lorenz (1990) stated the lowest temperature limit for growth was about 7°C for seven days. Raw fish should be kept at 10°C throughout processing to inhibit the growth and toxin production of pathogenic bacteria (FDA, 2011).

However, it is important to remember that the fish

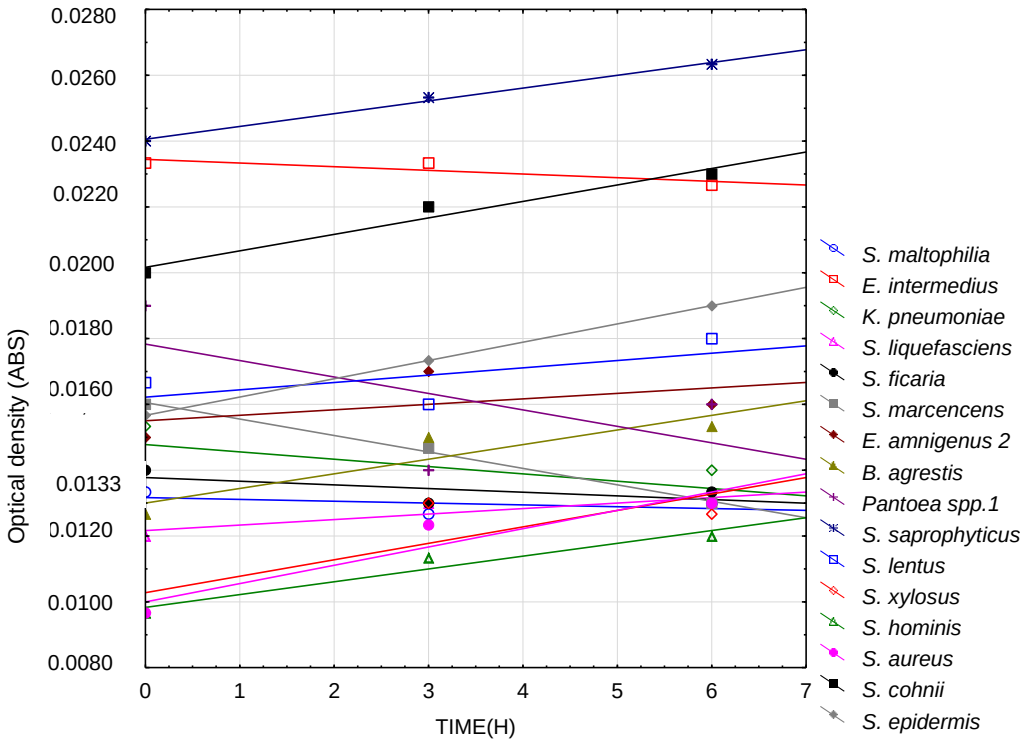

Figure 1. Optical density (absorbance) measured through spectrophotometry (554 nm) at 10°C after 6 h.

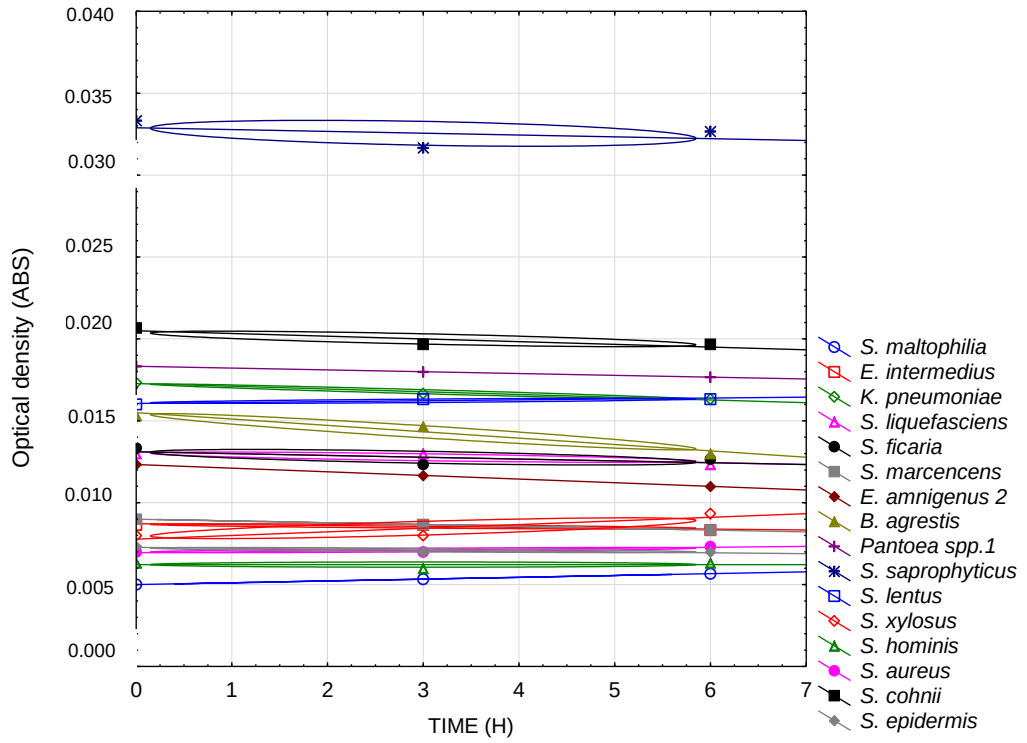

Figure 2. Optical density (absorbance) measured through spectrophotometry (554 nm) at 15°C after 6 h.

Table 6. ANOVA for optical density measured through spectrophotometry (554 nm) at 10°C after 6 h.

Bacterial strains/temperature	F	p
Staphylococcus cohnii ssp. *cohnii*	1.2353	0.3554
Staphylococcus saprophyticus	0.1878	0.8335
Staphylococcus lentus	0.4179	0.6762
Staphylococcus epidermis	0.9740	0.4302
Staphylococcus xylosus	3.9565	0.0802
Staphylococcus aureus	1.7931	0.2452
Staphylococcus hominis	3.5455	0.0962
Stenotrophomonas maltophilia	0.6000	0.5787
Enterobacter intermedius	0.0125	0.9876
Enterobacter amnigenus 2	0.8182	0.4851
Klebsiella pneumoniae ssp. *pneumoniae*	0.6727	0.5450
Serratia liquefaciens	1.0000	0.4219
Serratia ficaria	0.1207	0.8884
Serratia marcensces	2.4400	0.1677
Buttiauxella agrestis	2.4400	0.1677
Pantoea sp.	4.0714	0.0763

Table 7. ANOVA for optical density measured through spectrophotometry (554 nm) at 15°C after 6 h.

Bacterial strains/temperature	F	P
Staphylococcus cohnii ssp. *cohnii*	0.0234	0.9769
Staphylococcus saprophyticus	0.4220	0.6737
Staphylococcus lentus	0.0910	0.9143
Staphylococcus epidermis	0.0357	0.9651
Staphylococcus xylosus	0.6400	0.5598
Staphylococcus aureus	0.0232	0.9771
Staphylococcus hominis	0.0588	0.9434
Stenotrophomonas maltophilia	0.6000	0.5787
Enterobacter intermedius	0.3330	0.7290
Enterobacter amnigenus 2	0.3429	0.7228
Klebsiella pneumoniae ssp. *pneumoniae*	0.3330	0.7290
Serratia liquefaciens	0.0597	0.9426
Serratia ficaria	0.1111	0.8966
Serratia marcensces	0.6000	0.5787
Buttiauxella agrestis	0.1069	0.9003
Pantoea sp.	0.2730	0.7702

studied are sourced from tropical regions, that is, from waters with elevated temperatures. It is suggested that the strains found have adapted to moderate temperatures. Thus, refrigeration conditions are adverse for the multiplication of such microorganisms. Hence, butterfly peacock bass and piramutaba from the Amazon region could maintain their quality considering the microbiological aspects under refrigerated conditions (time and temperature) considering the bacteria isolated in this study. However, due to the high psychrotrophic count (4.52 – 8.56 log CFU/g), greater than the 7 log CFU/g established by the ICMSF (1986), other conservation measures (besides refrigeration) are required to prevent the multiplication of these microorganisms and fish deterioration.

Conclusion

The microbiological evaluation suggests Amazonian fish species marketed at the port of Ver-o-Peso Market have high counts of total mesophilic and psychrotrophic bacteria. Furthermore, it was observed that some isolated mesophilic microorganisms did not grow under refrigeration temperatures of over 6 h. However, due to the high concentrations of psychrotrophic bacteria, these fish require other conservation methods to ensure the microbiological quality.

Conflict of interests

The authors declare there is no conflict of interests.

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Full Length Research Paper

Secondary metabolites from the culture broth of *Lactarius quieticolor* growing in a bioreactor

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In this work, the main secondary metabolites in *Lactarius quieticolor* cultivated in a bioreactor were determined to find a possible use for its broths, which are eliminated as waste products after fermentation. The broths were extracted for adsorption in solid phase using amberlite XAD-2 and solid-phase extraction (SPE), and the extracts were analyzed by gas chromatography-mass spectrometry. The main metabolites extracted with XAD-2 were propanedioic acid (3-oxo-1-cyclopentane-1-yl) (37%) and hydroquinone derivatives (17%), whereas with SPE the main metabolite was decanoic acid (93%), suggesting that *L. quieticolor* broths could be used to obtain secondary metabolites of industrial interest, like decanoic acid.

Key words: Decanoic acid, ectomycorrhizal fungus, edible mushrooms, organic acids.

INTRODUCTION

Edible ectomycorrhizal fungi are a source of essential nutrients: proteins, carbohydrates, vitamins and dietetic fiber, essential for the normal functioning of the body (Cheung 2010). Accordingly, several techniques have been developed for its axenic cultivation and the subsequent inoculation in plants in nurseries and their establishment in the field, in order to obtain the fruiting bodies and improve the plant quality, concurrently. Among the methods used for the production of the fungal inoculum, both liquid culture in bioreactors and solid culture using vermiculite are the most frequent procedures to produce mycelia in large amounts (Rossi et al., 2007). The most common bioreactors used in studies, the stirred tank bioreactor, should be mentioned (Rossi 2006). Only some species of ectomycorrhizal fungi have been used to generate mycelial biomass in bioreactors for the controlled mycorrhization of plants of forest interest. Such species include *Hebeloma cylindrosporum* Romagn (Le Tacon et al*.,* 1985), *Laccaria laccata* Scop. Cooke (Sasek, 1990; Kuek, 1996), *Suillus luteus* (L.) Roussel (Sasek, 1990), *Pisolitus tinctorius* Pers. Coker & Couch (Pradella et al., 1990) and *Cenococcum geophilum* Fr. (Job, 1996). One aspect not considered in most cultures in a bioreactor is that along with increase in mycelial biomass yield, large amounts of

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culture broth are obtained, though they are frequently discharged as a waste by-product at the end of fermentation. These culture broths could contain extracellular enzymes or secondary metabolites of great interest and commercial value (for instance, organic acids, antibiotics, exopolysaccharides, etc.) (Wang et al., 2012; Zárate-Chaves et al., 2013). Secondary metabolite production has been studied in depth in the fruiting bodies of edible mycorrhizal and saprobe fungi, with the aim of finding added nutritional value in the carpophores for the people who consume them. Jikay (2007) did a review on the compounds produced by more than 100 mycorrhizal and saprobe fungi (basidiomycetes and ascomycetes) revealing that more than 300 compounds were isolated, including 150 new terpenes, phenols and nitrogen compounds from fruiting bodies. Muszyńska et al. (2011) identified and quantified for the first time several indole compounds from fruiting bodies of four edible commercial fungi (*Agaricus bisporus* (J.E. Lange), *Cantharellus cibarius* Fr., *Lactarius deliciosus* and *Leccinum rufum* (Schaeff.) Kreisel). These authors concluded that serotonin and melatonin were the only compounds common to all species and that the low content of L-thryptophan, tryptamine and 5-hydroxythryptophan confirmed the safe use of these species for human consumption (Bedry et al., 2001; Wurst et al., 2002).

The genus *Lactarius* is well documented regarding the presence of metabolites in the fruiting bodies, principally of the sesquiterpene type (Sterner et al*.,* 1985), lactaranes (Zhang and Feng, 1996) and pigments (Yang et al., 2006). Ayer and Trifonov (1994), working on *L. deliciosus* cultured in liquid medium, found anofinic acid, a new chroman-4-one, 3-hidroxyacetylindole, cyclic dipeptides, ergosterol and a mixture of fatty acids, despite working in a culture medium not specific to ectomycorrhizal fungi (malt extract plus yeast extract) and at 18°C. The *Lactarius quieticolor* species, less known in the literature than the *L. deliciosus*, has shown an important antioxidant activity due to the presence of ascorbic acid in their fruiting bodies, along with a high protein content (19%) and a series of amino acids (Agrahar-Murugkar and Subbulakshmi, 2005). However, the production of metabolites by submerged mycelial culture has been much less investigated in edible mushrooms. In our laboratory, *L. quieticolor* is being cultivated in a bioreactor, in order to produce large quantities of mycelial biomass for the controlled inoculation of *Pinus radiata* plants for reforestation. During this process, large volumes of broth are produced and discarded. Therefore, it is necessary to study the secondary metabolites produced and secreted by *L. quieticolor* so as to discover a use for the broth discarded as a by-product from the fungal growth in bioreactor. In this study, the secondary metabolites in the culture broths of *L. quieticolor,* cultivated in a bioreactor and flasks, were characterized, and the antifungal activity of the extracts was also verified against entomopathogenic fungi.

MATERIALS AND METHODS

Fungal isolate

The ectomycorrhizal fungus *L. quieticolor*, isolate LBH-Lq-C2, belonging to the culture collection of Herbarium of University of Concepcion (CONC-F 0814) was used in this study. This fungus was isolated from adult plantations of *P. radiata* D. Don at the Biobío Region (36°49'38.51" S and 73°02'06.27" W), at 40 m.a.s.l. The isolate was maintained in the solid media biotin-aneurin-folic acid-agar (BAF) (Moser, 1960) and modified Melin-Norkrans (MMN) (Marx 1969) at pH 5.5. The cultures were stored at 24±1°C in darkness, and re-inoculated on fresh medium every two months.

Culture of *L. quieticolor* in bioreactor

Fermentation was carried out in a LIFLUS GX bioreactor with 5 L total capacity. After each of the accessories of the bioreactor had been sterilized, the calibration of pH electrode, peristaltic pumps for dispensing acid (HCl 1 M) and base (NaOH 1 M) and temperature sensor was carried out. The oxygen electrode was calibrated after the sterilization stage of the bioreactor jar. The bioreactor jar containing 2.0 L of the MMN medium, 10 g/L glucose and pH 5.5 was sterilized for 15 min. After the sterilization and once the culture medium had reached a temperature of approximately 40°C, streptomycin (0.05 g/L) was added in aseptic conditions to avoid bacterial contamination. The starting inoculum for bioreactor consisted of 500 mL of a mycelial suspension in MMN broth, which was previously cultivated during two weeks under agitation (120 rpm) (0.23 g dry biomass). The final volume inside the bioreactor jar after the addition of the inoculum was 2.5 L. Variables such as pH, temperature, dissolved oxygen and agitation velocity were adjusted to 5.5, 24°C, 60% and 120 rpm, respectively, and maintained constant in the bioreactor throughout the entire growth period. The incubation period was 20 days and at the end of the culture the amount of mycelial biomass was determined as mycelium dry weight (g).

Determination of secondary metabolites produced by *L. quieticolor* in bioreactor

After 20 days of fermentation under controlled conditions, 2.3 L of the culture broth were separated from the mycelial biomass by means of filtration in Whatman paper (No.3) and then extracted by adsorption using XAD-2 amberlite (Sigma- Aldrich) and solid phase extraction (SPE) (Żwir-Ferenc and Biziuk, 2006). Part of the culture broth (2 L) was mixed with 40 g amberlite and allowed to stir for 1 h at 150 rpm. Subsequently, the mixture was conditioned in a glass column (70 × 5 cm) and then eluted with 150 mL acetone. For the SPE, 300 mL of the culture broth were filtered in AccuBOND II ODS-C_{18} cartridges (Agilent) and later eluted with 20 mL methanol. Liquid MMN medium, with no fungal inoculation, was used as a control.

Both acetonic and methanolic extracts were characterized by means of gas chromatography–mass spectrometry (GC-MS) in an Agilent 7890A chromatographer with a 5975 mass detector, equipped with a HP5-MS fused silica capillary column of 30 m, 0.25

Table 1. Metabolites detected using GC-MS in the culture broth of *L. quieticolor* growing in a bioreactor LIFLUS GX (5 L).

SPE methanolic extraction	Abundance (%)	Acetonic extraction with XAD-2 amberlite	Abundance (%)
Decanoic acid	93	Propanedioic acid (3-oxo-1-cyclopentane-1-yl)	37
3,4-2-h-Coumarin, 4,4,5,6,8-pentamethyl	1.73	Hydroquinone derivatives	17
Derivative from hydroxynaphthalene	<1	Ergosterol	10
Hexadecanoic acid, methyl ester	<1	2 Metil 1,3 cyclohexanedione	3.6
		Benzoic acid	2.3
		Sesquiterpenes (5 hidroxy 7 -oxbicyclo [4.1.0] hept 3 en 2 one)	1.5

Percent abundances of metabolites from the total obtained in the dry extracts were standardized to 10 mg/mL by GC-MS analysis.

Table 2. Metabolites detected using GC-MS in the culture broth of *L. quieticolor* growing in Erlenmeyer flasks.

SPE methanolic extraction	Abundance (%)	Ethyl acetate extraction	Abundance (%)
2,4-Dimethyl-5,6-dimetoxi-8-aminoquinoline	11.9	2-(2-butoxyethoxy)-Ethanol acetate	32.19
Cubenol	5.28	2,4-bis(1-phenylethyl) phenol	8.5
Squalene	3.37	1,4-dimethyl-7-(1-methylethyl)-Azulene	2.38
Chromone, 2,2,6,8-tetramethyl	2.83		
10-octadecenoic acid, methyl ester	2.4		
Stearic acid	1.6		

Percent abundances of metabolites from the total obtained in the dry extracts were standardized to 10 mg/mL by GC-MS analysis.

mm inside diameter and 0.25 µm film thickness, under the following characteristics: temperature injector 250°C, initial oven temperature 100°C for 5 min, increased by 8°C/min until 250°C and maintained for 15 min. Detector was used in scan mode between 50 and 500 amu. Characterization was carried out by means of comparison with NIST ® database. The presence of low molecular weight organic acids was determined directly in the culture broth by means of high efficiency liquid chromatography (HPLC) in an Agilent 1100 Series diode array detector, using a BP-OA 2000-0 Benson Polymeric column. Phosphoric acid 0.2% (v/v) with a flow of 0.5 mL/min was used as the mobile phase. Detection was carried out at 210 nm.

Determination of secondary metabolites produced by *L. quieticolor* in Erlenmeyer flasks

Erlenmeyer flasks (500 mL) with 250 mL of MMN medium, with 10 g/L glucose and pH 5.5, were sterilized at 121°C/1 atm for 15 min. Later, the flasks were inoculated under aseptic conditions with four agar-mycelium disks (5 mm diameter) of *L. quieticolor* obtained from stock cultures and incubated in darkness at 24±1°C for eight weeks in agitation (120 rpm). Then, the mycelial biomass was separated from the culture broth by paper filtration. Culture broth was extracted only by means of the SPE methanolic (Lq_1) technique described above, with the aim of comparing methanolic extracts obtained by means of SPE from flasks and bioreactor. Residual culture broth was extracted with ethyl acetate (liquid-liquid separation) (Lq_2) to be concentrated and analyzed by means of GC-MS. In these assays, three replicates per treatment were used.

Anti-fungal activity assay with total extracts

Methanolic and ethyl acetate extracted from the culture broth of *L. quieticolor* obtained by means of SPE were concentrated in a rotary evaporator and dried at 40°C, to be subsequently used to assess their anti-fungal activity on *Botrytis cinerea* Pers.: Fr, *Fusarium oxysporum* Sclechtend.:Fr., *F. avenaceum Fr*. Sacc and *Penicillium notatum,* using the agar diffusion method, according to Sottorff et al. (2013). 1 mg of dry extract was diluted in 1 mL ethanol and 5 mm diameter Whatman filter paper impregnated with 10 and 20 µL of the extracts (which corresponded to 10 and 20 µg/mL, respectively) was used. The impregnated disks were placed on Petri dishes with potato-dextrose-agar (PDA) medium inoculated with spores from each fungus. As a negative control, disks impregnated with 10 and 20 µL of the extraction solvent were used. Finally, the zone of inhibition, generated around the extract samples, was measured with calipers (mm) at 72 h. In these assays, three replicates per treatment were used.

RESULTS

Extractions allowed obtained 143.6 mg/L of dry extract through XAD-2 and 48 mg/L through SPE from the *L. quieticolor* culture broth, growing in bioreactor. In contrast, from the cultures growing in flasks, 42 mg/L of dry extract were obtained through SPE and the liquid/liquid extraction with ethyl acetate produced 33.6 mg/L. The abundance percentage of metabolites from the total obtained in the dry extract and standardized to 10 mg/mL concentrations to be evaluated by GC-MS, is shown in Tables 1 and 2.

Fungal extracts obtained from the cultures in the bioreactor and in flasks, were analyzed by means of GC-MS and the main metabolites detected in both, the acetonic and methanolic extracts, are shown in Table 1.

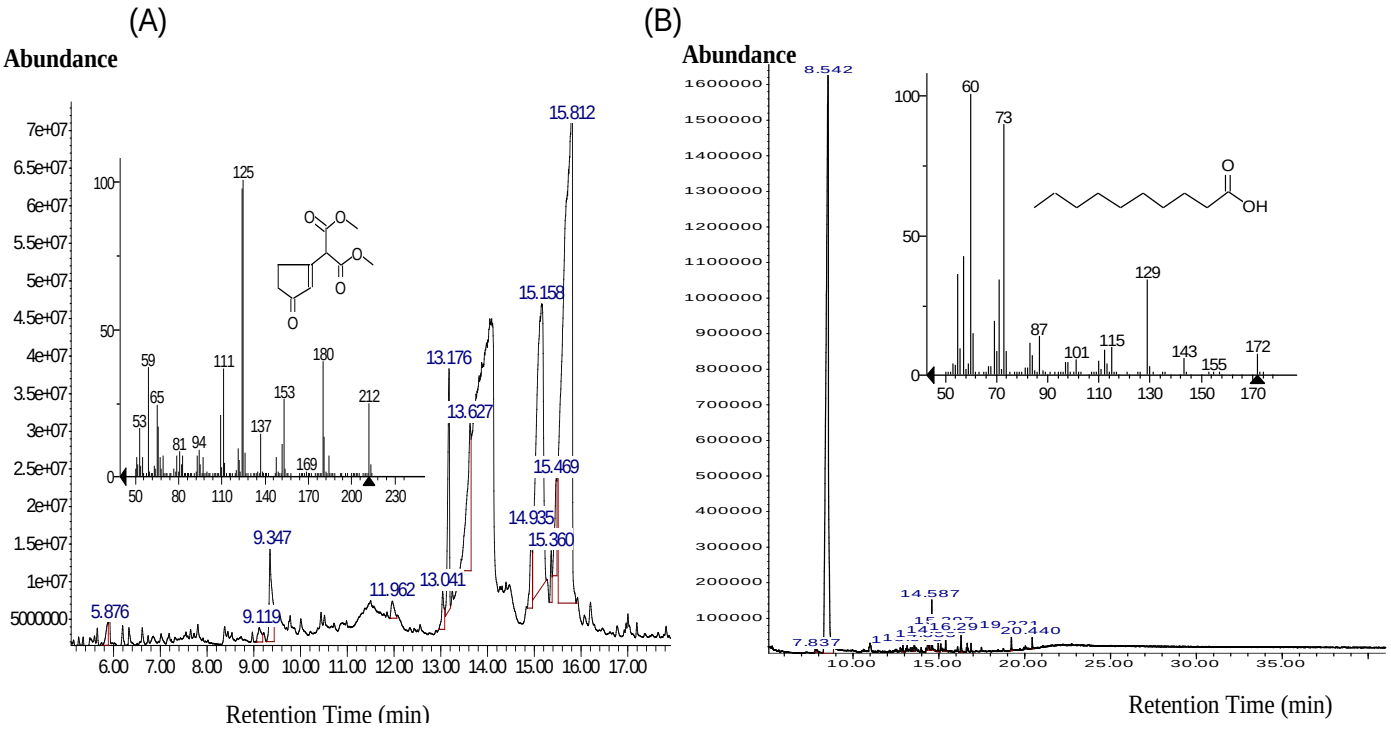

Figure 1. GC-MS chromatogram of the main metabolites obtained from the culture broth of *L. quieticolor* growing in a bioreactor LIFLUS GX (5 L) (A) Propanedioic acid (3-oxo-1-cyclopentane-1-yl) (retention time: 15.8 min), (B) Decanoic acid (retention time: 8.5 min).

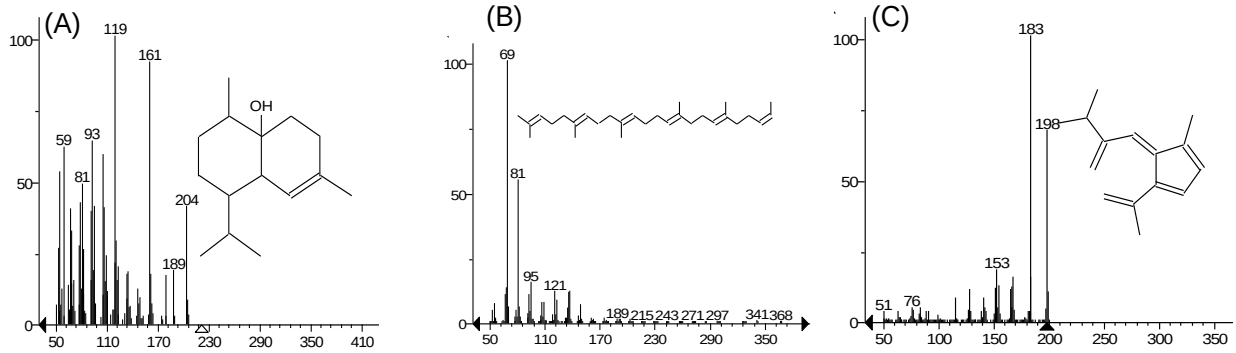

Figure 2. Mass spectrometry of the main metabolites obtained from the culture broth of *L. quieticolor* growing in Erlenmeyer flasks (A) Cubenol, (B) Squalene, (C) 1,4-dimethyl-7-(1-methylethyl)-Azulene, detected by GC-MS.

The metabolites present in the highest percentage in the acetonic extracts were propanedioic acid (3-oxo-1-cyclopentane-1-yl) (Figure 1A and Table 1), hydroquinone derivatives and ergosterol, whereas in the methanolic SPE extracts, it was decanoic acid (Figure 1B and Table 1). Only distinctive compounds in the composition of the culture medium (MMN) were identified in the controls. In addition to the determination of secondary metabolites by GC-MS, the presence of organic acids in the culture broth of *L. quieticolor* was analyzed by HPLC. Under bioreactor culture conditions, only the presence of oxalic and fumaric acids in concentrations of 20 µg/mL and 4 µg/mL respectively were found.

When *L. quieticolor* was cultured in flasks under agitation conditions for eight weeks, the presence of other metabolites, different from those produced in the bioreactor, was detected (Table 2), including cubenol (a sesquiterpene), squalene (a triterpene) and a derivative of azulene (a pigment) (Figure 2A and C).

The antifungal activity of the extracts obtained from the

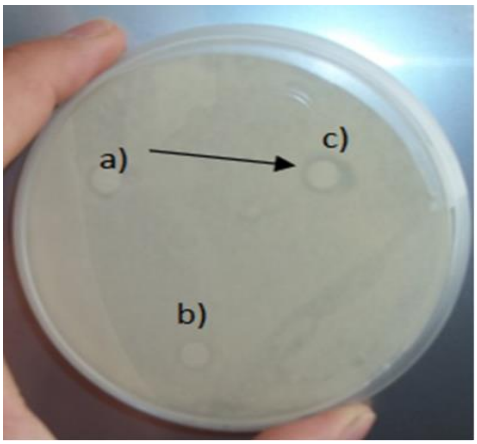

Figure 3. Antifungal activity assays against *P. notatum* with total extracts of *L. quieticolor* Lq_2. a) Control, b) 10 and c) 20 µg/mL.

L. quieticolor cultures in flask and bioreactor was assessed in paper disk assays. Neither extract presented any antifungal activity, independently of the concentration used or the fungal species assayed, except in the case of Lq_2 (culture in flask) extract against *P. notatum*. However, the inhibition percentage of the growth was only around 10% (7 mm diameter) in disks impregnated with 20 µL of *L. quieticolor* extract (Figure 3).

DISCUSSION

Submerged fungal culture in a bioreactor offers several advantages, including but not limited to the control of culture parameters inside the fermentation jar. Chapman et al. (1990) suggested, that ectomycorrhizal fungi had a limited growth in liquid medium. Yet our results show that culturing in a bioreactor is feasible and promotes the growth of *L. quieticolor*, which is consistent with the observations for other mycorrhizal fungi (Kuek, 1996; Carrillo et al., 2004; Oliveira et al., 2006; Rossi, 2006).

In this study, 1.8 g /L of dry fungal biomass was obtained after 20 days of culture in a bioreactor, with an average of 0.09 g/L day^{-1}. Even though this biomass production is lower as compared to the production of other ectomycorrhizal fungi, it is still relevant for *L. quieticolor,* due to the very thin mycelium that this fungus produces. Mycelium production assays for other fungi have obtained a dry mycelial biomass production of 0.25 g/L day^{-1} for *Suillus grevillei* (Baroglio et al., 2000) and yields higher than 1 g/L day^{-1} of mycelial biomass have been obtained with *Rhizopogon nigrescens* (Oliveira et al., 2006; Rossi, 2006).

Along with the good production of biomass by *L. quieticolor*, cultivation in a bioreactor makes it possible to obtain large volumes of broth, containing metabolites that could be of great commercial value, such as decanoic or capric acid (93%), a saturated fatty acid whose main use is focused on the production of perfumes and aromatizing substances, humidifying agents and food additives (Cermak et al., 2007) as well as corrosion protection of galvanized steel and electroplating steel (Lebrini et al., 2009). Currently, it is used as a nanostructure for the micro-extraction of pesticides from natural waters (Moral et al., 2012). Further experiments are needed to separate these metabolites from *L. quieticolor* broth and create protocols to optimize production and assess its possible industrial use. Ergosterol was another major metabolite detected through GC-MS (10%), which is a sterol present in the cell membrane of almost all fungi and an important precursor of vitamin D_2 (Hibbett et al., 2007). The presence of ergosterol in the extracellular medium of the fungus could indicate cell disruption due to the mechanical effect of the agitation inside the fermentation jar. Other studies have demonstrated that the ergosterol content of a species changes depending on the physiological state of the fungus (Müller et al., 1994). In addition, other metabolites such as cubenol, a sesquiterpene that presents an OH- group, have also been detected in the broths of *L. quieticolor* produced in flasks, but not in a bioreactor. In this regard, the presence of an OH- group in cubenol is an efficient decoupling of the bacterial plasma membrane, which is often lethal (Hammond and Lambert, 1981). This suggests that the extracts obtained from cultures in flasks could show antibacterial activity due to the presence of cubenol. The verification of antibacterial activity of the total extracts of *L. quieticolor* is currently being developed.

No antifungal activity was observed in most of the assays with the total extracts of *L. quieticolor*, except against *P. notatum*, although this was very low as compared to the antifungal activity frequently presented by plant extracts (Timothy et al., 2012). However, this could be due to the low concentration of extracts used in the assays (10 and 20 µg/mL). It has been observed that *L. deliciosus* exhibits antifungal activity against *Fusarium* sp. responsible for damping off (Pinto et al., 2006) and antibacterial activity against Gram-positive bacteria (*Bacillus cereus* CECT 148, *Bacillus subtilis* CECT 498 and *Staphylococus aureus* ESA 7 isolated from pus) (Barros et al. 2007).

Besides carotenoids, only a few terpenoids are colored compounds. The injured fruiting bodies and the latex of several species of *Lactarius* (Russulaceae) show color changes due to the presence of sesquiterpene compounds (Yang et al., 2006). Young fruiting bodies of *L. deliciosus* initially present a latex carrot color, but this becomes green as a result of an auto-oxidation process. *Lactarius deterrimus* Gröger has a pale peach color that turns grayish with green tones due to aging (Velíšek and Cejpek, 2011). All these color changes are due to the

presence of sesquiterpenes derived from azulene such as 1,4-dimethyl-7-1-(1- methylethenyl) azulene (lactarzulene blue) and 4-methyl-1-7-(1-methylethenyl) azulene-1-carb-aldehyde (lactaroviolin violet-red) detected in *L. deliciosus* in fruiting bodies (Velíšek and Cejpek, 2011). The first one has been confirmed in the extracellular culture of the Chilean strain of *L. quieticolor* growing in flasks.

When comparing the SPE methanolic extraction from the cultures, both in the bioreactor and the flasks, it was observed that the type and abundance of metabolites changes, depending on the type of cultivation, generating the highest percentages of compounds in the bioreactor, mainly decanoic acid (93%). In the bioreactor, the mycelium of the fungus is constantly subjected to fragmentation, which can generate mechanical stress (Rossi, 2007), thus, stimulates the production of secondary metabolites. On the other hand, a lower variability of metabolites extracted by SPE in the bioreactor was observed, in comparison with the cultures in flask, which could facilitate the purification of the metabolites of interest (e.g. decanoic acid) for further studies.

With these preliminary results, we can conclude that the broths of *L. quieticolor,* discarded as a byproduct during the production of mycelial biomass in the bioreactor for the inoculation of nursery plants, could be used to obtain compounds of industrial interest, such as the decanoic acid. However, it is still necessary to carry out additional studies for the complete identification and verification of the metabolites yield, present in the broth cultures of *L. quieticolor*.

Conflict of interests

The authors did not declare any conflict of interest.

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Optimization of L-glutaminase synthesis by *Aspergillus oryzae* NRRL 32657 in submerged culture

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Various fungal strains have been screened for L- glutaminase synthesis. *Aspergillus oryzae* NRRL 32657 proved to be the highest producer (24.19 U.ml^{-1}) with the largest pink zone diameter (8 mm). Production of L- glutaminase (E.C. 3.5.1.2. L-glutamine amidohydrolase) by *A. oryzae* in batch submerged system was optimized. Several factors: incubation period, source and concentration of carbon, source and concentration of nitrogen, initial pH level, incubation temperature, amino acids source and concentrations and sodium chloride concentration were tested for their effect on enzyme synthesis. The highest L- glutaminase synthesis (46.53 U. ml^{-1}) was achieved when *A. oryzae* was allowed to grow aerobically (30ºC for 3 days) in a buffered medium (pH 7.0) containing 2.5% lactose, 2% yeast extract, 0.5% L- glutamine and 0.5% sodium chloride.

Key words: L-glutaminase, *Aspergillus oryzae*, submerged fermentation, optimization.

INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia (Iyer and Singhal, 2009). The action of glutaminase plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes (Nandakumar et al*.,* 2003). Several species of bacteria: *Pseudomonas* sp, *Vibrio costicola* and *V. cholerae* (Renu and Chandrasekaran, 1992), *Vibrio* sp. M9 (Durai et al., 2014)*, Bacillus subtilis* and *B. licheniformis* (Cook et al*.,* 1981)*, Beauveria* sp. (Sabu et al*.,* 2000)*,* and *Micrcoccus luteus* (Moriguchi et al*.,* 1994); actinomycetes (Teja et al., 2014) and *Streptomyces rimosus* (Sivakumar et al*.,* 2006); molds: *A. oryzae* (Chou et al*.,* 1993), *A. wentii* (Siddalingeshwara et al*.,* 2011), *A. flavus* (Nathiya et al*.,* 2011b) and *Trichoderma koningii* (Pallem et al*.,* 2010) and yeasts: *Debaryomyces* sp. (Durá et al*.,* 2002), *Zygosaccharomyces rouxii* (Kashyap et al*.,* 2002; Iyer and Singhal, 2008 and 2010) were reported to produce L- glutaminase. Glutaminases are classified into two types: intracellular and extracellular and the majority is extracellular (Kashyap et al*.,* 2002; Iyer and Singhal, 2008 and 2010). Recently, microbial L-glutaminases have found several potent applications in various industrial sectors. The enzyme, though originally identified as a potent anti-cancer drug with possible applications in enzyme therapy (Sabu et al*.,* 2000), has been used in food industry to enhance flavor and as a replacement of monosodium glutamate specially in Chinese foods (Wakayama et al*.,* 2005).

Monosodium glutamate (MSG) gives the taste “umami”

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which has been widely recognized as the fifth basic taste besides sweet, acid, salty and bitter. It has been widely used as a flavor enhancer in the food industry. However, there are some questions about its safety, since it may cause some side effects for some people such as wheezing, changes in heart rate and difficulty in breathing (Farombi and Onyema, 2006). Therefore, the need to develop a safer natural flavor enhancer as an alternative to MSG has been increased. In the present study, a stepwise optimization process for fermentation parameters to maximize L-glutaminase production by *A. oryzae* NRRL 32657 has been conducted.

MATERIALS AND METHODS

Fungal strains

The investigated strains: *A. oryzae* NRRL 5590, *A. oryzae* NRRL 32614, *A. oryzae* NRRL 32657, *A. parasiticus* NRRL 1988, *Zygosaccharomyces rouxii* NRRL Y-12622 and *Z. rouxii* NRRL Y-2547 (Northern Regional Research Laboratories, Peoria, IL, USA) and *Saccharomyces cervicea* (Department of Microbiology, Faculty of Agriculture, Cairo University, Giza.) were allowed to grow on malt extract agar (Merck, Darmstadt, Germany) at 30ºC for 3 days. Cultures were then stored in a refrigerator at 4ºC till use. Cultures were activated monthly on malt extract agar.

Spores suspension

For the preparation of *A. oryzae* NRRL 32657 spores, the mold was inoculated on malt extract agar slants and incubated for 5 days at 30°C. After incubation, spores were scrapped and inoculated into 50 ml of saline solution containing of 0.1% tween 80. The collected spores were microscopically counted (1.5×10^7 spores.ml^{-1}), stored at 4°C and utilized as stock inoculum.

Media

Screening medium

The glutamine agar medium (GAM) (Siddalingeshwara et al*.,* 2010) was utilized for the production of the enzyme and it contained (gL^{-1}): glutamine, 10; KH_2PO_4, 0.1; K_2HPO_4, 1.0; NaCl, 0.5; Mg $SO_4.7H_2O$, 1.0; phenol red indicator, 0.125 and agar 15.

L-glutaminase production medium

The medium used for growth and production of glutaminase was referred to Wakayama et al*.* (2005). The medium contained (gL^{-1}): yeast extract, 17.5; glucose, 20; glutamine, 5; KH_2PO_4, 1.5; K_2HPO_4, 3; NaCl, 5 and Mg $SO_4.7H_2O$, 5 at pH 7.0. Fifty milliliters of the medium were placed in 250 ml Erlenmeyer flasks and autoclaved at 121°C for 15 min. The sterilized flasks were inoculated with 1 ml of the previously prepared spores suspension, placed in a rotary shaker (100 rpm) and the growth was aerobically carried out at 30ºC for 3 days. At the end of the incubation period, the mycelia were recovered from each flask by filtration on Whatman no.1 (Whatman Ltd., Maidstone, England) and used for determination of biomass (mg.ml^{-1}). Glutaminase activity was then determined in the culture filtrate.

Methods

Dry weight of the mold mycelia

After filtration, mycelia were washed twice with distilled water and dried at 70°C to constant weight.

Assay of L-glutaminase

L-Glutaminase activity was determined using L-glutamine as substrate and the released ammonia was measured using Nesseler's reagent. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml phosphate buffer (0.1 M, pH 7.0). Then the mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 0.5 ml of 1.5 M trichloro acetic acid. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 430 nm using a spectrophotometer, Model 6300 (Jan way LTD., Essex, U.K). A standard graph was plotted using ammonium chloride (Imada et al*.,* 1973). One international unit of L-glutaminase was defined as the amount of enzyme that liberates one µmol of ammonia under optimum conditions.

Factors regulating glutaminase production

Incubation period

To determine the optimum incubation period for glutaminase production, fermentation flasks were incubated for different time durations (1 to 7 days) and then enzyme activity was daily analyzed.

Carbon source

Glucose, fructose, sucrose, lactose, maltose, mannitol and dextrin (1%, each) were separately added to the fermentation medium.

Carbon source concentration

Different levels of the best carbon source “lactose” (0.5 to 4.0%) were tested.

Nitrogen source

Organic source (peptone, tryptone, yeast extract, beef extract and urea containing 15, 13.2, 9.5, 12.5 and 46.6 % nitrogen, respectively) and inorganic source (ammonium nitrate, sodium nitrate, ammonium sulfate and ammonium chloride) were evaluated for their effect on growth of the tested strain and glutaminase synthesis. The amounts of nitrogen sources were adjusted to give final nitrogen concentration of 0.2%.

Nitrogen source concentration

To determine the optimum concentration of the best nitrogen source, different levels (0.5 to 3.5%) of yeast extract were added to the growth medium.

Effect of temperature

The effect of temperature on glutaminase production was studied

Table 1. Screening of different strains for their glutaminase activity

Strains	Enzyme activity (U.ml^{-1})	Pink zone (mm)
Aspergillus parasiticus NRRL 198	19.36^{b*}±0.03	5.00^{b}±0.06
Aspergillus oryzae NRRL 32614	4.96^{e}±0.03	2.00^{f}±0.03
Aspergillus oryzae NRRL 32657	24.19^{a}±0.10	8.00^{a}±0.01
Aspergillus oryzae NRRL 5590	10.73^{c}±0.00	4.00^{c}±0.00
Zygosaccharomyces rouxii NRRL Y-12622	11.50^{c}±0.02	4.00^{c}±0.05
Zygosaccharomyces rouxii NRRL Y-2547	8.23^{d}±0.00	3.00^{d}±0.02
Saccharomyces cerevisiae	5.19^{e}±0.01	2.50^{e}±0.01

* Means followed by different superscripts within columns are significantly different at the 5% level.

by carrying out the growth at different temperatures (20, 30 and 40°C).

Effect of pH

To study the effect of the initial pH values of the medium on strain growth and glutaminase activity, different pH levels were tested (3, 4, 5, 6, 7, 8, 9 and 10).

Effect of amino acids

Different amino acids (0.5%, w/v) that is glutamine, asparagine, arginine, lysine, glycine, proline, methionine, tryptophan and glutamic acid were separately added to the growth medium with and without yeast extract, tested for their effect on the enzyme synthesis and different levels (0.02 to 0.19% nitrogen) of the best amino acid (glutamine and glutamic acid) without yeast extract were tested.

Effect of sodium chloride concentration

Impact of sodium chloride on enzyme production was determined by adding different concentrations of NaCl (0.25-20 %, w/v) to the fermentation medium.

RESULTS AND DISSCUSSION

Screening of different fungal strains for glutaminase activity

The seven tested strains were screened for their abilities to produce glutaminase. Such screening of strains was based on the semi qualitative method (Katikala et al*.,* 2009; Siddalingeshwara et al*.,* 2010). Cultures were grown on the glutamine media supplemented with a dye indicator (phenol red). The indicator is pH sensitive. Normally it gives yellow color to media in acidic and neutral conditions and gives the pink color to the media when the pH changes from acidic to alkaline condition. The pink zone around colony indicates the pH alteration which originated from ammonia accumulation in the medium. The results from plate assay and the determination of enzyme activity in the culture filtrate are presented in Table 1.

The obtained results show that different strains varied in their ability to produce glutaminase after three days of cultivation. The highest glutaminase activity was recorded for *A. oryzae* NRRL 32657 (24.19 U.ml^{-1}) followed by *A. parasiticus* NRRL 1988 (19.36 U.ml^{-1}), *Z. rouxii* NRRL Y-12622 (11.50 U.ml^{-1}) and *A. oryzae* NRRL 5590 (10.73 U.ml^{-1}), while the lowest was for *A. oryzae* NRRL 32614 (4.96 U.ml^{-1}). Therefore, *A. oryzae* NRRL 32657 was selected for further experiments. With respect to the relation between the qualitative and quantitative methods, the obtained results indicate a high correlation (r = 0.97) between both techniques. Therefore, the pink zone qualitative detection method could give an indication about the approximate glutaminase activity. These results are in agreement with those obtained by Katikala et al. (2009) and Siddalingeshwara et al. (2010) who used both methods to test the ability of different strains for production of glutaminase.

Factors regulating glutaminase production

Effect of incubation period

Enzyme activity was measured every 24 h time intervals (Figure 1). With regard to the different incubation periods, the maximum enzyme production (24.5 U.ml^{-1}) was noted at the 3rd day of fermentation and the activity gradually decreased after that till the end of the incubation period reaching 44.9% of the maximum.

Such reduction in activity was probably due to presence of proteolytic activity which was able to degrade the L- glutaminase present in medium (Kashyap et al*.,* 2002). Also, organism might have reached a stage from which it could no longer balance its steady growth with the availability of nutrient resources (Siddalingeshwara et al*.,* 2011). Similarly, 3 day of fermentation was the time of choice to maximize glutaminase production by *Aspergillus* sp. (Prasanth et al*.,* 2009) and *Aspergillus wentii* (Siddalingeshwara et al*.,* 2011).

Effect of carbon source

Varying the carbon source in culture medium affected

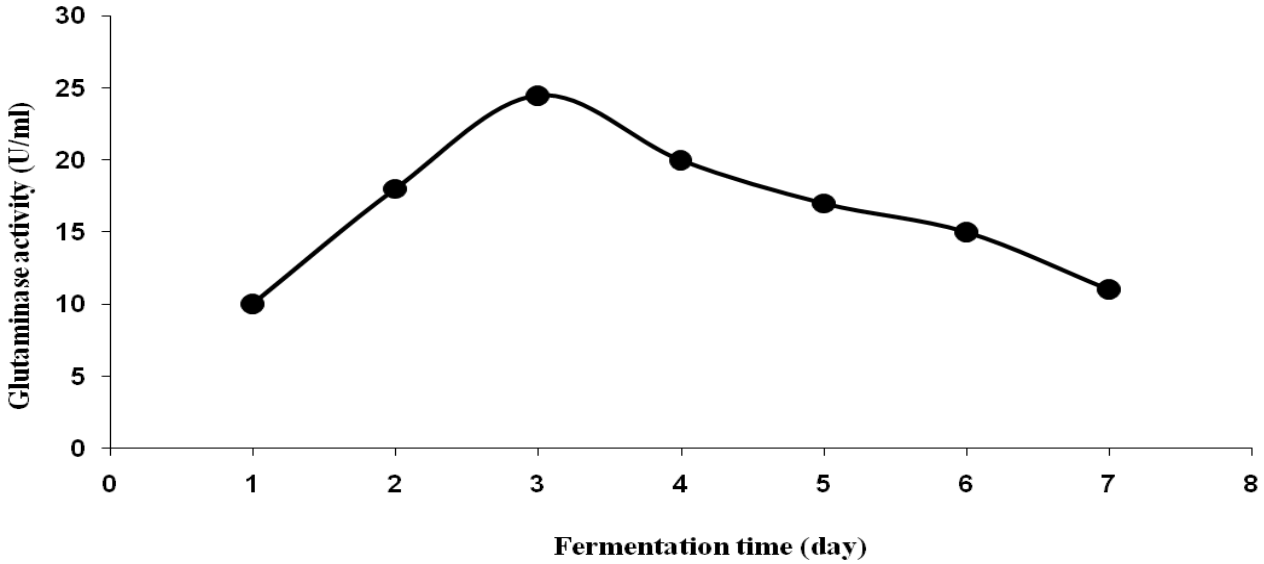

Figure 1. Effect of incubation period on glutaminase production by *Aspergillus oryzae* NRRL 32657.

Table 2. Effect of different carbon sources on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

Carbon source	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1}) * ±SD
Glucose	8.0	9.16	15.24^{d}±0.1
Fructose	8.2	11.62	15.50^{d}±0.3
Sucrose	8.2	11.24	11.97^{f}±0.3
Maltose	8.0	13.78	14.86^{e}±0.4
Lactose	9.0	5.38	38.97^{a}±0.1
Mannitol	8.6	6.70	32.24^{b}±0.5
Dextrin	8.6	7.66	21.40^{c}±0.1

*Means followed by different superscripts within columns are significantly different at the 5% level.

glutaminase production by *A. oryzae*. Results (Table 2) show no relationship between biomass and glutaminase synthesis but the highest pH value (9) was obtained at the highest enzyme activity, while the lowest pH values (8.0 and 8.2) were obtained at the lowest recorded enzyme activity. Maximum glutaminase activity (38.97 U.ml^{-1}) was obtained with lactose followed by that obtained with mannitol (32.24 U.ml^{-1}).

It is of interest to report that both activities were achieved with slowly assimilated sugars (lactose and mannitol). On contrary, the presence of fast metabolized sugars such as glucose, fructose, sucrose and maltose resulted in catabolite repression and therefore, the glutaminase synthesis was reduced to 39.1, 39.8, 30.7 and 38.1% of the maximum obtained activity (38.97 U.ml^{-1}), respectively. The utilization of dextrin as carbon source resulted in an intermediate activity (54.9% of the maximum) and probably due to the enzymatic hydrolysis of dextrin to units of glucose which resulted in catabolite repression. Similarly, Yuasa et al. (2003) reported that the use of relatively slowly assimilated carbon sources such as lactose, mannitol and sorbose eliminated the catabolite repression occurred with the use of fast assimilated carbon sources such as glucose. Also, Prasanth et al. (2009) reported that the replacement of glucose with lactose improved the glutaminase synthesis by *Aspergillus* sp. from 22.97 to 27.64 U.ml^{-1}. On the other hand, carbon sources such as glucose and maltose enhanced glutaminase production by *Trichoderma koningii* in solid-state fermentation using sesamum oil cake (Pallem et al*.,* 2010).

Effect of lactose concentration

The obtained results (Figure 2) indicate that lactose concentrations affected biomass as well as L- glutaminase activity.

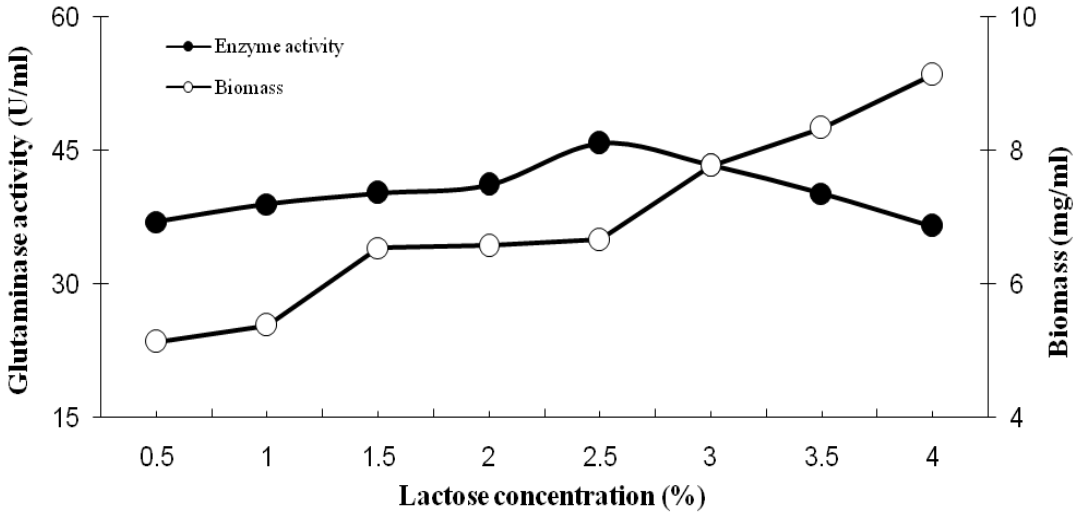

Figure 2. Effect of lactose concentrations on biomass and glutaminase produced by *Aspergillus oryzae* NRRL 32657.

Table 3. Effect of different nitrogen sources on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

Nitrogen source	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1}) * ±SD
Peptone	8.4	5.84	27.32^{c}±0.04
Tryptone	8.8	7.57	35.50^{b}±0.01
Yeast extract	9.0	6.66	45.78^{a}±0.01
Beef extract	9.0	5.21	20.12^{d}±0.03
Urea	8.6	3.85	17.50^{e}±0.02
$(NH_4)_2SO_4$	7.6	3.16	5.35^{h}±0.01
NH_4NO_3	8.6	4.16	10.42^{g}±0.00
$NaNO_3$	9.0	4.23	13.12^{f}±0.00
NH_4Cl	7.0	2.18	3.37^{i}±0.03

*Means followed by different superscripts within columns are significantly different at the 5% level.

Gradual increase in biomass from 5.13 to 9.13 mg.ml^{-1} was noted when the level of lactose was elevated from 0.5 to 4.0%. Also, a gradual increase in glutaminase activity was noted reaching its maximum value (45.78 U.ml^{-1}) at lactose concentration of 2.5% after which a gradual decrease occurred reaching 36.5 U.ml^{-1} at lactose level of 4.0%. Since 2.5% lactose resulted in the highest enzyme synthesis, such concentration was utilized for further experiments.

Effect of nitrogen source

The changes in biomass, medium pH, and enzyme synthesis are shown in Table 3. The results indicated that, for all tested nitrogen sources, the pH values of the fermentation medium increased at the end of incubation period (3 days) comparing to the initial pH (7.0), except the use of NH_4Cl where the pH level remained constant (7.0). The highest pH value (9) was detected when yeast extract, beef extract and sodium nitrate, were used as to nitrogen source.

Data in the same table clearly show that the presence of organic nitrogen (except urea) in the fermentation medium was more favorable for the growth of *A. oryzae* NRRL 32657. The maximum biomass (7.57 mg. ml^{-1}) was obtained in the presence of tryptone as an organic nitrogen source. The obtained biomass in the presence of yeast extract and peptone was 6.66 and 5.84 mg.ml^{-1}, respectively. However, the obtained biomass in case of using urea was the lowest among the tested organic sources and this was probably due to that urea didn't contain growth factors such as vitamins and minerals as present in peptone, tryptone, beef extract and yeast

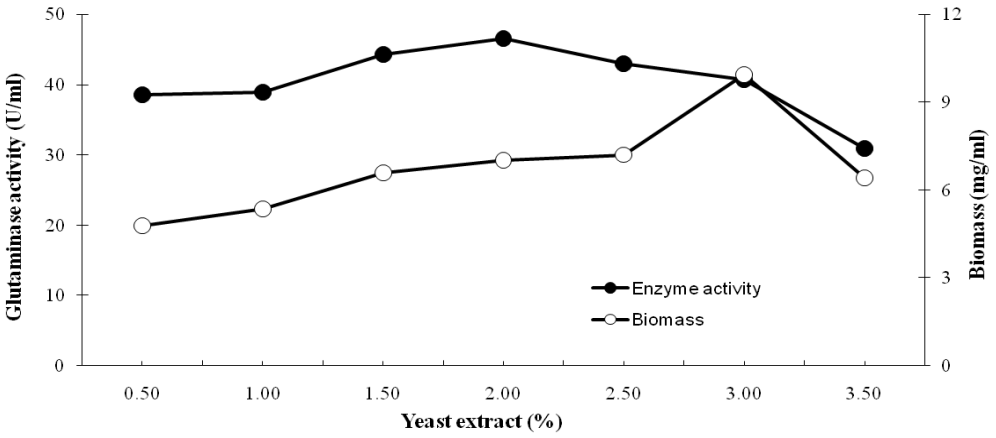

Figure 3. Effect of yeast extract concentrations on biomass and glutaminase produced by *Aspergillus oryzae* NRRL 32657.

extract (Bazaraa and Al-Dagal, 1999). On the other hand, inorganic nitrogen sources did not support good mold growth and the obtained biomass ranged from 2.18 to 4.23 mg. ml^{-1}. Concerning the effect of nitrogen source on glutaminase synthesis, data (Table 3) reveal that, organic nitrogen sources significantly enhanced more enzyme synthesis than the inorganic sources. Among the organic sources, yeast extract was found to be the best nitrogen source for glutaminase production (45.78 U. ml^{-1}) followed in descending order by tryptone (35.50 U. ml^{-1}), peptone (27.32 U. ml^{-1}), beef extract (20.12 U. ml^{-1}) and urea (17.5 U. ml^{-1}) with significant differences between them. These results are in agreement with those reported by Iyer and Singhal (2008), who reported that, the tested organic nitrogen sources were preferable than the inorganic sources and yeast extract was found to be the best nitrogen source for glutaminase synthesis by *Z. rouxii*. . In addition, these results correlate well with those reported by Nathiya et al. (2011a) who indicted that, maximum glutaminase synthesis by *Penicillium brevicompactum* was obtained when yeast extract was used as an organic nitrogen source in fermentation medium. Moreover, Nathiya et al. (2011b) recorded that the maximum L-glutaminase production from *Aspergillus flavus* was achieved when yeast extract was utilized as nitrogen source. Also, Suresh Kumar et al. (2013) reported that the maximum glutaminase yield (25 IU.ml^{-1}) was obtained in the presence of yeast extract. On the other hand, the use of sodium nitrate as the nitrogen source led to the highest glutaminase production by *A.oryzae* (Prasanna and Raju, 2013) and *Streptomyces arvermitilis* (Abdallah et al*.,* 2013). While, Kashyap et al. (2002) and El-Sayed (2009) reported that, the supplementation of agro-wastes with organic sources such as (peptone, yeast extract, malt extract and beef extract) and inorganic sources such as ($(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$) did not improve glutaminase synthesis.

Effect of yeast extract concentration

A positive correlation between biomass and yeast extract concentration was noticed (Figure 3). The obtained biomass increased as yeast extract concentration increased and recorded the highest amount of biomass (9.94 mg. ml^{-1}) in the presence of 3.0% yeast extract and decreased thereafter. Results (Figure 3) also reveal that the synthesis of glutaminase by test organism was influenced by the concentration of yeast extract. The maximum production (46.53 U.ml^{-1}) was achieved at 2.0% yeast extract concentration. Utilizing higher yeast extract concentration above 2% resulted in lower enzyme synthesis, where 42.89, 40.68 and 30.86U.ml^{-1} were obtained at yeast extract concentration of 2.5, 3.0 and 3.5%, respectively. Similar trend for glutaminase production as affected by nitrogen source concentration was obtained by Nathiya et al. (2011a) using *Penicillium brevicompactum*.

Effect of different amino-acids

Amino acids were reported as common growth factors required for the production of enzymes as major nitrogen source (Suresh Kumar et al*.,* 2013). Hence, to study the effect of additional amino acids on glutaminase production, different amino acids were separately added to the medium (Table 4). It was observed that the medium supplementation with different amino acids variously affected the growth, pH and enzyme synthesis. Among the tested amino acids, L-glutamine supplementation followed by glutamic acid resulted in the highest synthesis of L- glutaminase (46.53 and 42.80 U. ml^{-1}, respectively). Results (Table 4) confirm that the addition of the tested amino acids in absence of yeast extract resulted in lower values for final pH, biomass and enzyme activity comparing to in the presence of yeast

Table 4. Effect of different amino acids on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

Amino acids	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1})±SD*	
			With**	without
Glutamine	9.0 (8.6) ***	7.02 (2.07)	46.53^{a}±0.01	24.50 ^{a} ±0.00
Asparagine	8.8(7.8)	5.76 (0.83)	32.04^{c}±0.03	18.40 ^{c} ±0.01
Arginine	8.6(8.0)	6.04 (1.08)	26.20^{d}±0.00	15.20 ^{d} ±0.02
Lysine	8.4(7.5)	6.36 (0.82)	20.80^{e}±0.01	8.90 ^{e} ±0.00
Glycine	9.0(7.5)	6.33(1.64)	13.64^{f}±0.02	6.70 ^{f} ±0.01
Proline	8.6(8.0)	8.14(5.56)	11.59^{g}±0.03	5.80 ^{g} ±0.03
Methonine	8.4(7.5)	5.71(1.20)	20.50^{e}±0.02	8.60 ^{e} ±0.01
Tryptophan	8.4(7.5)	6.36(1.41)	20.31^{e}±0.01	8.30 ^{e} ±0.00
Glutamic acid	8.4(8.0)	7.61(2.08)	42.80^{b}±0.00	20.03 ^{b} ±0.01

* Means followed by different superscripts within columns are significantly different at the 5% level. ** With yeast extract or without yeast extract. *** Data between parentheses were for enzyme production in media without the addition of yeast extract.

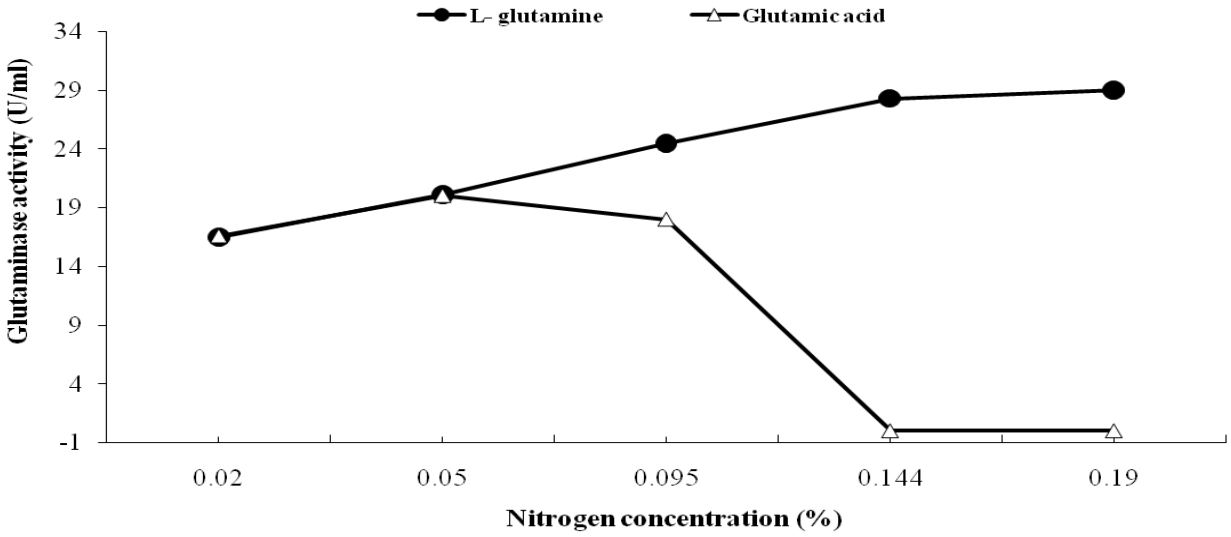

Figure 4. The effect of different glutamine and glutamic acid concentrations (as nitrogen percentage) on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

extract. Data reveal that the biomass ranged from 0.082 to 5.56 mg. ml^{-1} (in absence of yeast extract) and the highest value (5.56 mg. ml^{-1}) was observed in the case of proline. Therefore, the presence of yeast extract in medium was essential for maximum biomass (8.14 mg. ml^{-1}) and glutaminase synthesis (46.53 U.ml^{-1}) since it contained the required growth regulators (Nathiya et al*.,* 2011a). Concerning the effect of amino acids on glutaminase production by *A. oryzae* without the addition of yeast extract, it was found that the highest glutaminase synthesis (24.50 U. ml^{-1}and 20.03 U. ml^{-1}) was obtained with the addition of L- glutamine and glutamic acid, respectively.

To determine the optimal L-glutamine and glutamic acid concentrations for glutaminase activity, various concentrations of nitrogen (ranged from 0.02 to 0.19%) were separately added to the fermentation medium. Results presented in Figure 4 indicate the relation between glutaminase activity and the nitrogen concentration of L-glutamine and glutamic acid. The increase in nitrogen concentration in glutamine from 0.02 to 0.144% led to an increase in glutaminase activity from 16.5 to 28 .3 U. ml^{-1} and persisted after that. On the other hand, in case of the use of glutamic acid the maximum activity (20.03 U. ml^{-1}) was achieved at 0.05% nitrogen, decreased to reach 18.01 U.ml^{-1} at 0.095% nitrogen and a complete loss of activity was noted at the highest tested concentrations (0.144% and 0.190%). Prabhu and Chandrasekaran (1997)

Table 5. Effect of initial pH on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

pH	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1}) ±SD*
3	3.0	0.00	0.00^{f}±0.00
4	4.0	0.00	0.00^{f}±0.00
5	8.6	4.51	18.30^{d}±0.02
6	8.6	7.00	29.50^{b}±0.01
7	9.0	7.02	46.53^{a}±0.01
8	9.2	10.01	19.92^{c}±0.00
9	9.0	0.00	0.00^{f}±0.00
10	10.0	0.00	0.00^{f}±0.00

*Means followed by different superscripts within columns are significantly different at the 5% level.

studied the effect of the additional amino acids on glutaminase production by marine *V. costicola* in solid state fermentation. They reported that all tested amino acids (except glutamine), had a negative impact on glutaminase synthesis. El-Sayed (2009) reported that L-glutamine was the best tested amino acid in enhancing glutaminase synthesis by *Trichoderma koningii*. He explained that this result may be due to the action of glutamine as an inducer.

Effect of incubation temperature

Data indicate that, glutaminase synthesis was apparently variant at different temperatures, and at 30°C the maximum glutaminase synthesis (46.53 U. ml^{-1}) was achieved. A sharp reduction in such activity (63.0 and 41.3% considering 46.53 U. ml^{-1} as 100%) was noted when *A. oryzae* was allowed to grow at 20 and 40°C, respectively. On the other hand, maximum growth (7.61 mg ml^{-1}) was achieved at 20°C. Similarly, Kashyap et al. (2002) indicated that maximum glutaminase production (11.61 U per g dry solids) by *Z. rouxii* was obtained when the fermentation was carried out at 30°C. Obtained results agreed well with those reported for glutaminase synthesis by *T. koningii* (EL-Sayed, 2009), *Vibrio* sp. (Prakash et al*.,* 2010) and *A. oryzae* NCIM 1212 (Prasanna and Raju, 2013). On the other hand, incubation temperatures 33 and 40°C were selected as optimal temperature for the production of glutaminase by *T. koningii* and *P. fluorescens* (Pallem et al*.,* 2010; Chitanand and Shete, 2012).

Effect of pH value

Results presented in Table 5 confirm that *A. oryzae* NRRL 32657 grew well in buffered medium that ranged from 5 to 8. Data reveal that the maximal biomass values (10.01 and 7.02 mg. ml^{-1}) were observed at initial pH values 8 and 7, respectively. Meanwhile, pH values 3, 4, 9 and 10 were not suitable for the growth.

The maximum glutaminase production of 46.53 U. ml^{-1} was obtained at the initial pH value of 7.0. This may be attributed to the balance of ionic strength of plasma membrane (El-Sayed, 2009). These results are in coincidence with that reported by Prasanna and Raju (2013). Also, Balagurunathan et al. (2010) stated that the optimum pH value for glutaminase production by *Streptomyces olivochromogenes* was 7.0. Abdallah et al. (2012) reported that, maximum enzyme productivity (13.47 U.ml^{-1}) was recorded at pH 8.0 and no synthesis was detected at pH 3, 4 and 11. On the other hand, Nathiya et al. (2011b) reported that, the maximum glutaminase production by *Aspergillus flavus* (38.69 U/g) was observed at initial pH 4.0 and a loss of more than 50% of enzyme production was observed at initial medium pH of 7.0.

Effect of NaCl concentration

The relation between salt tolerance of the test organism and glutaminase production was studied and results were recorded in Table 6. Increasing NaCl concentrations from 0.25 to 2.0% dramatically affected mold growth (Table 6). A complete inhibition was noted at 17.5% NaCl, while a good resistance was obvious up to 2.5%. On the other hand, glutaminase activity was highest (46.53 U. ml^{-1}) at 0.5% NaCl and decreased thereafter with a complete loss of activity at 17.5% NaCl. Since growth of mold was very limited or non at pH above 10.0. Therefore, no change in pH level was detected. Suresh Kumar et al. (2013) reported that the production of L-glutaminase from *Serratia marcescens* was maximized at 0.75% NaCl. On the other hand, Krishnakumar et al. (2011) and Abdallah et al. (2013) found that the maximal L-glutaminase activities produced by marine alkalophilic *Streptomyces* sp.-SBU1 and *Streptomyces avermitilis* were observed in a medium supplemented with 2 and 3% NaCl (w/v),

Table 6. Effect of NaCl concentrations on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657

NaCl (%)	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1})±SD*
0.25	8.9	7.13	41.63^{b} ±0.02
0.50	9.0	7.02	46.53^{a}± 0.01
1.00	9.0	7.00	39.42^{c}±0.00
1.50	8.8	6.92	31.00^{d}±0.02
2.00	8.4	6.85	25.40^{e}±0.01
2.50	7.6	6.70	17.36^{f}±0.01
5.00	7.4	4.32	16.23^{g}±0.03
7.50	7.2	2.05	15.50^{h}±0.02
10.0	7.2	1.65	13.38^{i}±0.01
12.5	7.0	0.44	4.70^{j}±0.02
15.0	7.0	0.31	1.21^{k}±0.00
17.5	7.0	0.00	0.00^{l}±0.00
20.0	7.0	0.00	0.00^{l}±0.00

*Means followed by different superscripts within columns are significantly different at the 5% level.

respectively. Therefore, it could be concluded that 0.5 % of NaCl concentration was the optimum for the production of glutaminase from *Aspergillus oryzae* NRRL 32657.

The study indicated the relation between glutaminase activity and the different optimization steps for maximizing glutaminase synthesis by *A. oryzae* as following: fermentation time was tested as the first factor and the activity was 10 U.ml^{-1} after one day of fermentation and increased to reach 24.5 U.ml^{-1} at the 3rd day of fermentation. Second, lactose was chosen as the best carbon source and activity increased from 24.5 to 38.97 U.ml^{-1}. In the third step of optimization, increasing lactose concentration to 2.5% resulted in an additional 27.9% increase in activity. Optimization of nitrogen source, nitrogen source concentration, yeast extract concentration, amino acids, incubation temperature, initial pH value of media and NaCl concentration did not significantly affect the activity under the specified tested conditions. Maximum glutaminase synthesis by the test organism after the stepwise optimization (46.53 U. ml^{-1}) was almost 4.7 folds that of 10 U.ml^{-1} obtained at the beginning of optimization after one day of fermentation. Results were compared with others in literature and among results of synthesis of glutaminase by mold in literature, only *Penicillium brevicompactum* showed higher glutaminase synthesis (66.7 U.ml^{-1}) (El-Shafei et al*.,* 2014). Otherwise, the tested *A. oryzae* NRRL 32657 showed higher or equal results. On the other hand, the bacterial strains *Vibrio costicola* and *Vibrio sp.* significantly showed higher activity as reported by Prabhu and Chandrasearan (1997) and Prakash et al. (2010), respectively.

Conflict of interests

The authors did not declare any conflict of interest.

Conclusion

It could be concluded that *A. oryzae* NRRL 32657 could be considered as a promising mold strain for the production of glutaminase. More research in the area of mutation, protoplast fusion and genetic engineering are needed to enhance the activity of glutaminase from this source.

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Survival analysis for estimating risk factors in incubation period of *Didymella pinodes* on pea (*Pisum sativum* L.)

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Ascochyta blight caused by *Didymella pinodes* (Berk. et Blox.) Vestergr. is one of the most devastating diseases, causing severe damage in pea. A new statistical approach based on factor risk using non-parametric and semi parametric survival analysis was used in this study. Different hypotheses dealing with factors that might influence the incubation period were tested. Survival analysis using Kaplan-Meier estimates and Coxproportional hazards was performed for data analysis. During these investigations, incubation period was regressed against leaf wetness duration (LWD), inoculum concentration, plant age and isolate's aggressiveness. The non-parametric Kaplan-Meier test had shown the importance of leaf wetness duration, inoculum concentration and plant age in the survival curve for the incubation period. Thus, the lowest median incubation period was obtained under the LWD of 72 h. This was 9.0 days (95% CI 8,402-9,598 days). On the other hand, the highest inoculum concentration induced the shortest incubation period with a median value of 9.0 days (95% CI 7,772-9,531 days). Likewise, using the semi parametric Cox proportional hazard regression, only two covariates (leaf wetness, inoculum dose) were associated with survival time with an hazard ratio of 1.144 p=0.03) and 1.015 (p<0.0001). Moreover, neither the plant age inoculation nor the isolate presented a significant hazard ratio for the best fit of the model.

Key words: *Pisum sativum, Ascochyta blight*, Cox regression, *Didymella pinodes.*

INTRODUCTION

The Ascochyta blight caused by *Didymella pinodes* (Berk. et Blox.) Vestergr. is one of the most destructive pathogens of pea (Moussart et al., 1998; Chilvers et al., 2009; Le May et al., 2012). It is wide spread throughout the major pea-growing areas worldwide (Wallen, 1965; Lawyer, 1984; Bouznad, 1988; Bretag et al., 2006).

In recent years, the incidence of Ascochyta blight was observed in different production areas in Algeria, which has led to increased yield loss (Setti et al., 2008). This could be due to an increased pathogenicity of the

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pathogen population or a greater inoculum pressure. Numerous previous studies have shown the key role of the incubation period on epidemics of Ascochyta blight (Tivoli et al., 1999; Roger et al., 1999; Turechek, 2004; Tivoli and Banniza, 2007). On the other hand, Shaner (1981), and Van Ginkel and Scharen (1988) suggested the use of moisture parameters for predicting aerial disease infection. Similarly, Fitt et al. (1998) and Huber et Gillespie (1992) already noted the impact of free water on leaf surface on the incubation and latent period. Furthermore, Tivoli et al. (1999), Roger et al. (1999) and Turechek (2004) considered that a parameter such as the inoculum concentration may have a great impact on the life cycle of *Didymella pinodes* (*D. pinodes*) and hence may determine all the components of disease, including the incubation period. Gibb et al. (1998) suggested that both the incubation and latent period might be influenced by plant age and the isolate's virulence. Many previous studies determined and quantified the latent period in different plant pathogens' interaction (Trapero-Casas and Kaiser, 1992; Pederson and Morrall, 1994; Wallen and Galway, 1977) but little is known about the duration of the incubation period for *D. pinodes* in pea. In our previous study, the incubation period values were calculated only for plants that were symptomatic during the study period, whereas a number of inoculated pea plants had not presented any symptoms at the time when the final disease symptom was recorded. The plants that did not present symptoms during the frame of the study are referred to as "censored data". Survival analysis is a powerful class of nonparametric and semi parametrics tool, especially designed for such data. Survival analysis involves the timing of events (such as infection, germination, pycnidia production, symptoms appareance) while allowing censored observations (Hosmer and Lemeshow, 1999; Klein and Moeschberger, 2003). This type of analysis has a long history in statistical research and practice, particularly in medical studies. Moreover, this statistical method is also used in several others disciplines, and referred to with others names such as event history analysis and failure time in sociology and industry, respectively. Recently, the use of the survival analysis has become a widespread tool for resolving more complicated data in ecological studies such as biodiversity and environmental toxicology (Castro et al., 2004; Vange et al., 2004). In contrast to the medical and ecological fields, survival analysis has rarely been applied in plant pathology (Madden and Nault, 1983; Muenchow, 1986; Westra et al., 1994). Garrett et al. (2004) and Esker et al. (2006) stated that plant pathology research data are often collected in the form of time to event data (until the appearance of the first symptom of disease; appearance ofpycnidia structure and spore germination, etc.). Survival analysis is an interesting method that enables the introduction of censored data in the analysis. In the pea-*D.pinodes* pathosystem, this may permit the inclusion of infected plant that did not present symptoms during the period of study. Hence, this method will help us to obtain a more realistic estimation of incubation period. Such data might have a great importance in the estimation of cultivar resistance, and also in each biotic factor in any plant pathogen's interaction. This information may play a key role for the comprehension of the epidemic structure of the pathogen. In fact, in our previous study, the influence of abiotic parameters was approached, taking into account only the mean and variance analysis (Setti et al., 2008). This is the reason why we tried in this study to introduce a survival analysis approach, which is considered as a robut statistical tooltodeterminate accurately the incubation time length. In fact, the comprehension of this period could have an important consequences on the epidemic development due to the seasonal spore accumulation, given the polycyclicnature of the pathogen (Motisi et al., 2013 ; Leclerc et al., 2014). Moreover, empirical data on the incubation period of numerous plant pathogens are rarely available (*Motisi* et al., 2013). Therefore, the objectives of this study were to investigate the use of survival methods to estimate the incubation period of the *Mycospherella* spp. that infects pea and to assess the effect of four factors on time for the appearance of the first symptom of disease using the Cox semi parametric analysis: (i) isolates' aggressiveness, (ii) leafwetness duration, (iii) inoculum concentration, (iv) and plant age.

MATERIALS AND METHODS

Plant and fungal material

Two *Didymella pinodes* isolates (md0203 and tn0203) that present different degree of aggressivness were used in this study. Isolates were grown on PDA medium for 10 days at 21°C. Conidia from 10 days old culture were collected by adding 10 ml of sterile deionised water to dislodge spores. The concentration of spores was determined using a haemocytometer. The conidial suspension was diluted with sterile deionised water to obtain a final concentration required for each experiment. The cv 'Merveille de Kelvedon', one of the most cultivated cultivars which is considered as moderately resistant to Asochyta blight was used in this study. Seedswere sown in pots containing unsterilized soil/compost mixture. Fifteen seeds were planted per pot and seedlings were thinned to ten. The plants were maintained in glasshouse.

Effect of inoculum concentration, leaf wetness, and plant age on incubation period

Inoculum concentration effect was investigated on 15- and 30- day old plants of cv 'Merveille de Kelvedon'. Plants were inoculated by spraying to runoff with spore suspension. Three inoculum concentrations (IC) were assessed namely $3x10^3$, $5x10^5$, $7x10^7$ spores/ml. Suspensions were applied with a spray atomizer with an adjustable nozzle to form a high density of fine droplets on the aerial parts of the plants. For the investigation of the leaf wetness (LWD) effect, the pea seedlings weresubjected to LWD of 6, 48 and 72 h. Plants were covered with clear polyethylene bags immediately after inoculation and sprayed inside with deionised water to facilitate infection. The plants were thenuncoveredat each LWD, and kept in

uncontrolled glasshouse where temperature ranged from 15 to 25°C.

Risk factor analysis methods

Univariate analysis using Kaplan-Meier estimator

Incubation period is defined as the period from the host inoculation to the onset of the first symptoms referred to as survival data. To estimate the incubation period (IP), plants were observed daily from the time of inoculation up to 10 days, when the experiment was terminated. The Kaplan-Meier method of survival analysis (Kleinbaum, 1996) was used to generate and adjust survival curves using preoperative variables that differed among the treatment groups. The censored observations are plants that did not develop symptoms by the end of the assessment period. In fact, the survivor function S(t) measures the probability that an individual will survive beyond time t: S(t) = P[T > t]. Let T represent survival time. We regard T as a random variable with cumulative distribution function P(t) = Pr(T ≤ t) and probability density function p(t) = dP(t)/dt. The dependent variable is hence considered as a "survival time" (Esker et al., 2006. Scherm and Ojiambo, 2004; Garrett et al., 2004; Padovan and Gibb, 2001). Another representation of the distribution of survival times is the hazard function, which assesses the instantaneous risk at time t:

$$h(t) = \lim_{\Delta t \to 0} \frac{\Pr\left[(t \leq T < t + \Delta t) | T \geq t\right]}{\Delta t}$$

Overall survival rates were calculated by the Kaplan–Meier method and the log-rank test was used for differences between survival curves. A P-value of < 0.05 was accepted as statistically significant. Variables were subjected to univariate analysis. The estimator S(t) that was used to calculate non-parametric estimates of the survivor function is:

$$\hat{S}(t) = \prod_{j:t_j<t} \left(\frac{n_j - d_j}{n_j}\right) = \prod_{j:t_j<t} \left(1 - \frac{d_j}{n_j}\right)$$

Where, dj is the number of individuals that experienced the event in a given interval and nj is the number at risk. Survival curves are monotone non-increasing step functions equal to 1 at time zero, and 0 as time approaches infinity. Statistical differences between survival curves were calculated using the Mantel–Haenszel log-rank test (Rothman and Greenland, 1998).

Multivariate analysis using semi parametric Cox proportional hazards

Cox regression models use the hazard function to estimate the relative risk of failure. The hazard function, h(t) is an estimate of the potential death per unit time at a particular instant, given that the case has survived until that instant (Kelinbaum, 1996).Cox (1972) first introduced his proportional hazards approach as a way to incorporate covariate information into a survival model without having to assume an underlying distributional form for the data. The model is defined in terms of the hazard function as:

$$h(t, \mathbf{X}) = h_0(t) \exp\left(\sum_{i=1}^{p} \beta_i X_i\right)$$

Where, X is the explanatory/predictor variable, and $h_0(t)$ is the unspecified baseline hazard function (that is when X = 0). Here β is a p-vector of parameters. The Cox proportional hazard model examines the influence of potential covariates on the hazard of event for an individual (Collett, 2003; Ojiambo *et al*. 2002; Kleinbaum, 1996; Dungan *et al*., 2003). The hazard at time *t* is the probability that an individual who has survived to time *t* will die in the next small period of time (Ojiambo *et al*., 2002, Scherm and Ojiambo, 2004, Muenchow, 1986). Both the Kaplan-Meier and Cox regression model analyses were performed using the SPSS 17.0.

RESULTS

Kaplan-Meir analysis

Concerning the inoculum concentration, the shortest incubation period (IP) value was seen with an IC of $7x10^7$ with a median value of 8 days (Table 1). The Kaplan–Meier survival curve was statistically significant between the different IC(log-rank = 5,674, P= 0,045). The shortest IP length occurred with the isolate tn0203 at 72 h of LWD. The median value was 8 days (Table 1). Moreover, the IP increased with the decreasing of LWD. The highest IP value was seen at 6 hours of LWD. The IP ranged from 8 to 12.5 days. The Kaplan–Meier survival curve showing a statistically longer differences between the three LWD would be expected to occur by chance (log-rank = 24,88, P < 0,001). Mean and median difference was observed between the isolates for incubation period (Table 2). The median incubation period was estimated using Kaplan-Meier probabilities of developing disease (Figure 1). The median values were respectively12.0 (sd: 0,384) (95% CI 9,939–12,442 days) and 10.39 days (sd: 0,203) (95% (CI 9,531–10,469) for tn0203 and md0202 (Table 1, Figure 1). However, the Kaplan–Meier survival curve showed a statistically non significant survival time between the two isolates (log-rank = 1,163, P = 0,064).

The survival analysis of the incubation time revealed that IP increased with age of the inoculated plants. Hence, the median value of the lowest IP was obtained with the inoculated 15 days old plants. This was 9.780 days (SE:0,420) (95% CI (8,329 – 9,671 days). The estimation of the survival function with the Kaplan Meier estimator has revealed however non significant differences between the IP of the two inoculated plants' ages (Log-rank =24. 88, P= 0,055). However, compared with the Wilcoxon test, the IP has revealed differences between the two plants' ages (P=0. 043) (Table 2).

The Cox's proportional hazards model

The survival analysis estimators such as the log rank test and the Wilcoxon test are used to compare between groups (Figure 1) for one parameter without taking into account the other explanatory variables. This is the reason why it is of great importance for such analysis to apply the semi parametric model known as the Cox's

Table 1. Medians for survival time of incubation period of *Didymella pionodes.*

Parameters		Median		
		Estimate	SD	95CI
Isolate	md0203	12,000	0.384	(9,939; 12,442)
	tn0203	10,397	0.203	(9,531; 10,469)
Inoculum concentration (spores/ml)	$03*10^3$	10,000	0.467	(9,635; 10,915)
	$5*10^5$	10,000	0.239	(9,085; 10,102)
	$7*10^7$	8,000	0.439	(7,772; 9,531)
Plant age (days)	15	9,780	0.420	(8,329; 9,671)
	30	10,000	0.239	(9,531; 10,469)
LWD (h)	6	12,551	0.305	(9,125; 10,002)
	48	10,117	0.322	(9,349; 10,902)
	72	8,000	0.305	(7,402; 9,598)

SE, Standard error; CI, 95% Confidence interval.

Table 2. Kaplan Meier survival estimator of the four parameters.

Parameters	Log rank test		Wilcoxon test	
	Statistics	P value	Statistics	P value
Isolate	1.163	0.064	0.075	0.510
Inoculum concentration	5.674	0.045	6.703	0.035
Plant age	3.831	0.055	4.114	0.043
Leaf wetness duration (LWD)	24.88	0.000	19.01	0.000

proportional hazards that takes into account all the parameters at the same time. Based on the examination of the effect of different covariates on the risk of reducing the IP length, the hazard was estimated for plant age (β= -0,091), IC(β=0,13), LWD(β=0,015), and isolate (β=0,152). This model had indicated that among the covariates tested, two had affected the incubation period with high risk (Table 3).

The overall best fit for the influences of abiotic parameters on the IP time length was provided by a model that included only the inoculum concentration and the leaf wetness duration. Neither the plant age inoculation nor isolates' aggressiveness was significant in this model (Table 3).The IC had an estimated hazard ratio of 1,144 (P=0.034), indicating their influence on the appearance of disease symptoms. On the other hand, the LWD had an estimated hazard ratio of 1,015 (P<0.0001), indicating the importance of this explanatory variable in the Cox regression model.

DISCUSSION

The present investigation examined the incubation period using survival functions for isolates' aggressiveness, plant age inoculation, LWD and inoculum concentration. They were estimated by non-parametric method of Kaplan-Meier and compared by the logrank test and semi parametric techniques, using the Cox regression model. The infection cycle is mainly the period of infection during which the pathogen enters and infects the host, the period of incubation that follows infection, and ends with the appearance of symptoms. In this study, survival analysis of the data has shown that three of the parameters tested were associated with the incubation period lengths when the nonparametric survival analysis was performed. This is in agreement with other studies which suggested that short latent and incubation period length were observed with increase in both inoculum concentration and leaf wetness duration (Scott et al., 1985; Roger et al., 1999).

The Cox semi-parametric model permits evaluation of the effects of all the studied parameters at the same time. The best fit was obtained with only two parameters, LWD and IC when estimated usingthe Log rank test and Wilcoxon test. This confirms the importance of LWD in the infection process due to the estimated hazard ratio which was relatively high (1,015). The LWD in general is an important factor that enables the numerous fungal

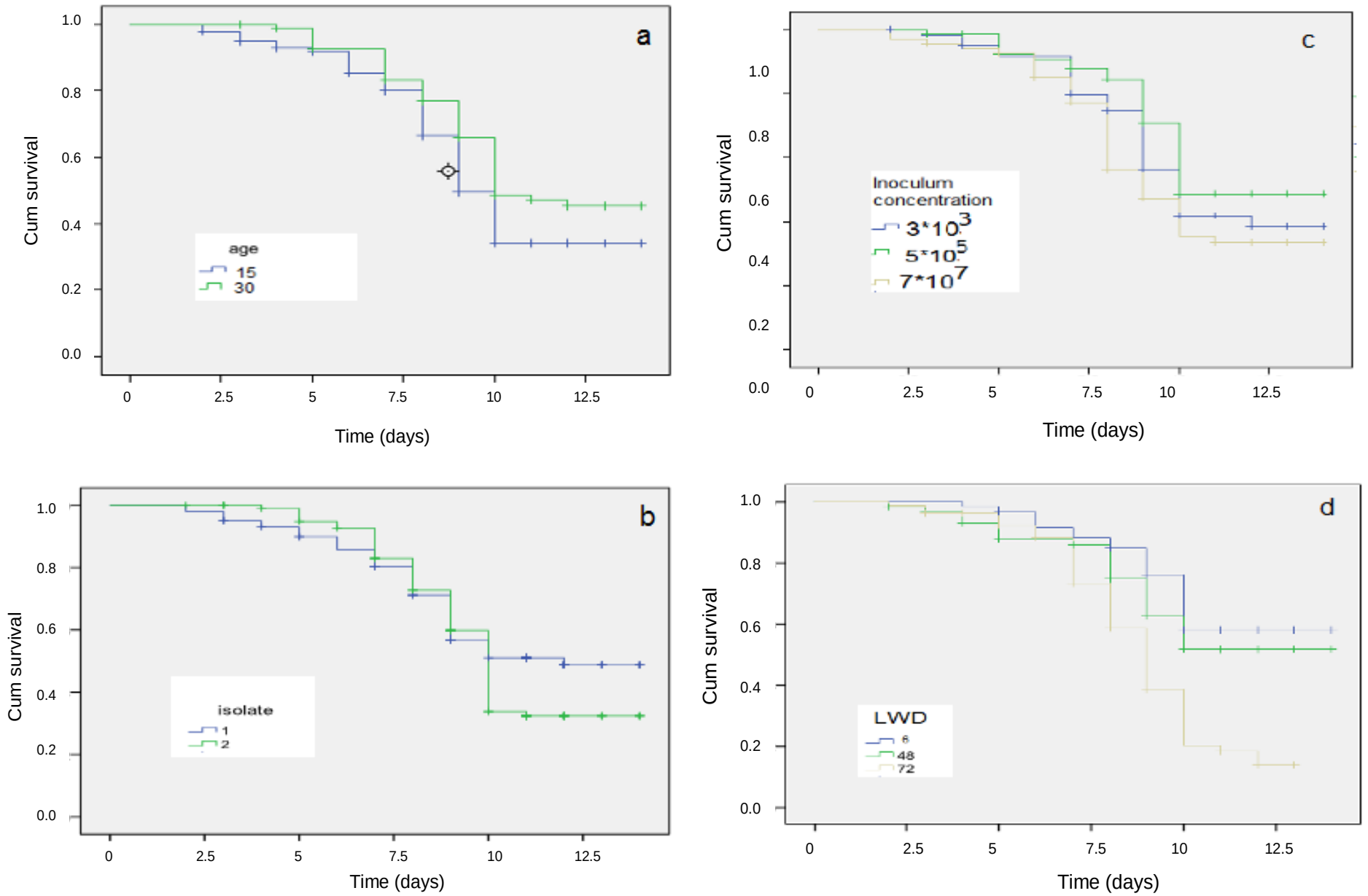

Figure 1. Kaplan-Meier survival for the incubation period of *D. pinodes* on cv 'Merveille de Kelvedon'. Effect of a) plant age, b) isolates, c) inoculum concentration and d) LWD.

Table 3. Results of Cox models for the incubation period length of Ascochyta blight on pea caused by *Didymella pinodes*.

Explanotory variable	β	Exp(β)	SE	Sig.	95.0% CI	
					Lower	Upper
Age	-0.091	0.913	0.041	0.78	0.843	0.989
Inoculum concentration (IC)	0.13	1.144	0.130	0.03	0.887	1.474
Leaf wetness duration(LWD)	0.015	1.015	0.004	0.000	1.008	1.023
isolate	0.152	1.164	0.202	0.65	0.784	1.729

plant pathogens, particularly those infecting F the aerial parts of plants. Weather moisture is frequently used as an indicator of the likelihood of an epidemic (Royle and Butler, 1986). Most foliar fungi can infect the leaves of a plant only while the leaves are wet. The optimal wetness, however, varies depending on the specific pathogen (Trapero-Casas and Kaiser, 1992; Pederson and Morrall, 1994; Gilles et al., 2000). Many previous studies have reported that severe disease was obtained with a LWD of at least 48 h (Shew et al., 1988; Davis and Fitt, 1994; Scott et al., 1985; Roger et al., 1999). Roger et al. (1999) and Setti et al. (2008; 2009) have observed a positive correlation between IC and incubation and latent period and also between the IC and the disease severity for *D. pinodes*. In our experiment, the estimated hazard ration for the IC was 1,144. Such a positive correlation between IC and disease severity was also demonstrated for other Didymella spp. (Scott et al., 1985; Shew et al., 1988; Setti et al., 2010). According to Pederson and Morrall (1994), both the incubation period and the latent period are strongly affected by the IC.

Concerning the isolate effect, non significant differences were observed between the two isolates on the cv 'Merveille de Kelvedon'. The lack of differences could be explained partly by the behavior of this cultivar towards the Ascochyta blight. In fact, the cv 'Merveille de Kelvedon' is considered as moderately resistant against the Ascochyta blight and consequently the effect could be reduced. On the other hand, using the Kaplan Meier estimator namely the Wilcoxon test, our study has also determined the influence of the plant age and the survival curve of the incubation period; however, no evidence of this influence was seen and consequently, the plant age does not appear to best fit the cox regression model. The present study highlights the importance of the incubation period as one of the components of plant disease resistance that can reduce the rate at which disease epidemics develop. Moreover, other important components of resistance must be studied to limit the epidemic propagation of the pathogen such as germination and infection efficiency, and rate and duration of spore production. The integration of the incubation period and latent period associated with other epidemic components is of great importance in disease forecasting systems, especially in systems.

Conflict of interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Occurrence of *Brucella abortus*, *Leptospira interrogans* and bovine herpesvirus type 1 in buffalo (*Bubalus bubalis*) herd under extensive breeding system

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The current study aimed to determine the occurrence of *Brucella abortus*, *Leptospira interrogans* and BoHV-1 in buffalo (*Bubalus bubalis*) herd under extensive breeding system. In order to perform the study, blood samples were collected from 306 female buffaloes belonging to a rural property located in São Mateus County – MA. The samples were tested for the presence of specific antibodies. The number of samples collected in such property was estimated by taking under consideration a minimum prevalence of 6% for each infection, with confidence interval of 95%. Of the 306 analyzed female buffaloes' serum samples, 70.58% were positive for one or more *Leptospira interrogans* serovars, with variable titers between 100 and 800. The most prevalent serovars in the current study were Pomona, Butembo, Icterohaemorrhagiae, Sentoti, Copenhageni, Adamanda, Castelonis, Wolffi, Panama and Grippotyphosa. A BoHV-1 occurrence of 87.25% was identified in the evaluated animals. All animals in the study tested negative for brucellosis. The study results indicate that *L. interrogans* and BoHV-1 were widespread microorganisms within the assessed property and they may contribute to a decrease in the production and reproductive rates of the herd.

Key words: Antibodies, BoHV-1, brucellosis, female buffaloes, leptospirosis.

INTRODUCTION

The reproductive infections caused by *Brucella abortus*, *Leptospira* spp. and by Bovine Herpesvirus type 1 (BoHV-1) are among the main causes of cattle productivity losses in Brazil (Frandoloso et al., 2008).

According to the Food and Agriculture Organization (FAO), World Health Organization (WHO) and to the World Organization for Animal Health (OIE) (Al-Majali et al., 2009; Mekonnen et al., 2010; Abernethy et al., 2011), brucellosis is one of the most important and widespread zoonoses in the world. *Brucella abortus* is highly pathogenic and causes severe illness, especially in bovines (Corbel et al., 2006). This species also has clinical and

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epidemiological importance and it is considered as pathogenic for humans (Maurin, 2005).

The damages caused in bovines by brucellosis are countless: the herd undergoes decrease in milk and meat productivity, sale price devaluation regarding the animals and animal products from endemic regions, increased calving intervals, the occurrence of miscarriage in sick female animals, sterility, indication for sacrificing reactive animals and the consequent expense with purchasing other animals for replacement in the herd (Berhe et al., 2007). Buffalo breeding probably undergoes similar losses (Paulin and Ferreira Neto, 2008).

Brucellosis is considered both a foodborne and an occupational disease, and transmission can occur by contact with infected animal parts, consumption of infected unpasteurised milk products and via the airborne route (Pappas et al., 2008).

Leptospirosis is a zoonotic disease that affects domestic and wild animals, and humans, thus representing an important economic and public health problem. Its transmission is facilitated by the presence of water contaminated by bacteria from genus *Leptospira* (WHO, 2011). The consequences of leptospirosis for animal health reaches the economic sphere, in view of the involvement of producing animals such as cattle, sheep, goats, pigs and horses (Brazil, 1995) with it. In these species, economic losses result mainly from reproductive disorders such as infertility, miscarriage, birth of weak calves and temporary decrease in milk production (Cervantes et al., 2002).

Pathogenic leptospires, the bacteria that causes leptospirosis, enter via the mucous membranes or the damaged skin of the host animals and colonize the proximal renal tubules of the kidneys of the carrier (Athanazio et al., 2008; Gamage et al., 2014).

Bovine herpesviruses (BoHV) are the etiological agents of many impacting diseases in livestock, and they are responsible for major economic losses in cattle breeding (Silva et al., 2000). Bovine herpesvirus type 1 (BoHV-1) is related to respiratory manifestations, such as infectious bovine rhinotracheitis (IBR), genital manifestations (infectious pustular balanoposthitis and vulvovaginitis), reproductive failures, return to estrus and miscariages (Del Médico Zajac et al., 2010). The bovine species is the natural host of bovine herpesvirus type 1 (BoHV-1) and type 5 (BoHV-5). However, serological studies have suggested that buffaloes may be susceptible to BoHV-1 (Cavirani et al., 1997; Galiero et al., 2001) and to other genetically related alpha herpes viruses (Thiry et al., 2007).

BoHV-1 is shed in nasal discharge for 10–14 days during acute respiratory infection and transmission occurs by contact with mucosal droplets from infected cattle. The virus is also shed following reactivation from latency. BHV-1 can also be transmitted to susceptible animals through contaminated materials including semen (Biswas et al., 2013).

Thus, the current study aimed to determine the occurrence of *Brucella abortus*, *Leptospira interrogans* and BoHV-1 in buffalo (*Bubalus bubalis*) herds under extensive breeding system.

MATERIALS AND METHODS

Study area

The study was conducted in São Mateus County, which is located in the Central Mesoregion of Maranhão State, Brazil, and has a land area of 783.335 km² (IBGE, 2011). The studied area was selected according to the existence of buffalo beef cattle ranching. The county has a herd of 26,801 bovids distributed among 304 rural properties (Maranhão, 2012).

Animals

The studied population consisted of female beef cattle buffaloes, over the age of 24 months and vaccinated against brucellosis only. The study excluded postpartum females, females 15 days before or 15 days after delivery, thus following the technical regulations of the National Program for the Control and Eradication of Brucellosis and Tuberculosis – PNCEBT (Brazil, 2001; Brazil, 2004; Brazil, 2006).

Sampling design

The number of blood samples collected from the property was estimated by using Win Episcope 2.0 (Blas et al., 2004) software. The prevalence of at least 6% for each disease, and 95% probability of detecting at least one positive animal was predicted. The number of female buffaloes in this property is of 896 and the total number of analyzed samples was of 306.

Blood samples collection

Blood was collected from the animals' jugular vein by using a system of tubes containing vacuum. After clot retraction, the samples were centrifuged at 3.000 rpm (1,512 g) for 15 min to extract serum and the sera were stored at -20°C until the completion of serological tests (Subharat et al., 2012). An epidemiological questionnaire was applied in order to obtain information pertaining to the management and the health status of the evaluated animals. The current study was approved by the Ethics Committee on Animal Experimentation - CEEA at the Veterinary Medicine School of the State University of Maranhão, Protocol 037/2011.

Diagnostic tests

Brucellosis

The Buffered Acidified Antigen (BAA) was performed as a screening test for detecting anti-*Brucella abortus* antibodies by using an antigen produced by the Laboratory of Technology of Paraná - TECPAR. The reactive samples in BAA were simultaneously subjected to 2-Mercaptoethanol (2-ME) and Slow Serum-agglutination in Tubes (SSA) by using antigen produced by TECPAR, in titers of 1:25, 1:50, 1:100 and 1:200. Results interpretation was performed according to the current legislation (Brazil, 2004).

Leptospira interrogans

The blood sera were submitted to the study of anti-leptospira

Table 1. Ocurrence of antibodies against *Brucella abortus* in 306 buffaloes under extensive breeding system according to the diagnostic test.

Diagnostic tests	Positive animals N (%)	Negative animals N (%)
BAA	27 (8.82)	279 (91.18)
SSA	27 (8.82)	279 (91.18)
2-ME	0 (0)	306 (100)

N = Number of animals; % = Percentage of animals; BAA = Buffered Acidified Antigen; SSA = slow serum-agglutination in tubes; 2-ME = -Mercaptoethanol.

agglutinins against 24 serovars from the *Leptospira* complex kept by the Laboratory of Bacterial Zoonoses, Veterinary Medicine Graduation Course, University of São Paulo (FMVZ-USP). The procedure was performed by using Microscopic Agglutination Test (MAT), according to the standards by the Ministry of Health (Brazil, 1995).

Each serum sample was first diluted at 1:100 in phosphate buffered saline solution, pH 7.2, and tested against 24 serovars: Andamana, Patoc, Shermani, Cynopteri, Butembo, Panama, Hardjobovis, Castelonis, Whitcombi, Tarassovi, Javanica, Australis, Autumnalis, Bataviae, Bratislava, Canicola, Copenhageni, Grippothyphosa, Hebdomadis, Icterohaemorrhagiae, Pomona, Sentot, Wollfi, Pyrogenes.

Samples with agglutination ≤50% were considered as positive for *Leptospira interrogans* when they were compared with the control. The positive samples in the initial titer (screening) were tested again in order to set the antibody titer for each serovar by using increasing dilutions from 1:100 up to 1:800. Samples with titer equal to or less than 100, with 50% agglutination or cells disappearing from the field were considered as positive, according to the dark field microscopy (Brazil, 1995; Santa Rosa et al., 1970). The reactive sera were titrated in geometric series of four dilutions of ratio two, and the titer was the reciprocal of the highest dilution in which there was agglutination.

Bovine Herpesvirus type 1

The qualitative detection of anti-BoHV-1 antibodies was performed by using Enzyme-Linked Immunoabsorbent Assay (ELISA) technique by applying the commercial kit for Indirect ELISA Test (CHEKIT IBR – SERO - Dr. BOMMELI AG/Liebefeld – Bern – Swiss).

Data analysis

The information from the questionnaires, as well as the serology results were stored in a database by using Microsoft Access®.

RESULTS AND DISCUSSION

The property assessed in the current study is a prototype of rural property for beef cattle buffalo breeding currently found in the State of Maranhão, Brazil. The property has Murrah buffaloes and Murrah-crossbred ones reared under extensive production system. The buffaloes feed on pastures and their diet is supplemented with minerals only. The breeders do not apply artificial insemination and, for sanitary control, they vaccinate females for brucellosis only and sporadically test animals for brucellosis and tuberculosis only.

The introduction of new animals in the property is usually made without considering health aspects, and without performing quarantine. Given this scenario, there is a great possibility of introducing animals infected by *B. abortus*, *L. interrogans* and BoHV-1.

The current study investigated the occurrence of *B. abortus*, *L. interrogans* and BoHV-1 in a buffalo herd in São Mateus County, where there was no previously reported epidemiological data on such diseases.

Of the 306 examined serum samples from female buffaloes, 8.82% (n=27/306) tested positive for brucellosis in the screening test (BAA). Of these, 100% were positive in the SSA test and negative in the 2-ME test regarding the four tested dilutions (1:25, 1:50, 1: 100 and 1:200) (Table 1).

No positive result was found for brucellosis in buffaloes from the assessed property. Only vaccination antibodies were detected in the examined animals. The absence of positive animals in the current study may be associated with a set of official health actions performed over the last decades in almost all Brazilian regions covering the control and eradication of such disease.

Of the total serum samples from the analyzed female buffaloes, 70.58% (n = 216/306) were positive for one or more *Leptospira* serovars, with titers ranging from 100 to 800. Of the 24 serovars investigated in the current study, 100% of them were detected, and the most common serovars were: Pomona (29.41%), Butembo (25.49%), Icterohaemorrhagiae (24.50%), Sentoti (22.54%), Copenhageni (20.58%); Adamanda (20.58%), Castelonis (19.60%), Wolffi (18.62%), Panama (18.62%) and Grippotyphosa (17.64%) (Table 2).

In the current study, the occurrence rate of *L. interrogans* found in the buffalo species, regardless the serovar, was higher than the rates reported for buffaloes by some researchers in the country, whose studies showed values ranging between 37.70% (Langoni et al., 2000) and 67.72% (Silva et al., 2009) for measures taken in the States of São Paulo and Pará, respectively.

The differences found among the results obtained in the current study and some results published in the literature can be understood, in part, through the number and types of serovars used in the serologic evaluation, herds' hygienic-sanitary handling, as well as in the degree and type of exposure to other domestic and wild animals and to synanthropic rodents that are known by

Table 2. Serovars ocurrences of MAT-positive and percentages in 216 buffaloes under extensive breeding system.

Serovars	Ocurrences N (%)
1 – Pomona	90 (29.41)
2 – Butembo	78 (25.49)
3 – Icterohaemorrhagiae	75 (24.50)
4 – Sentoti	69 (22.54)
5 – Copenhageni	63 (20.58)
6 – Adamanda	63 (20.58)
7 – Castelonis	60 (19.60)
8 – Wolffi	57 (18.62)
9 – Panama	57 (18.62)
10 – Grippotyphosa	54 (17.64)
11 – Patoc	51 (16.67)
12 – Autuminallis	45 (14.70)
13 – Hebdomadis	39 (12.74)
14 – Batavae	36 (11.76)
15 – Brastilava	36 (11.76)
16 – Australlis	30 (9.80)
17 – Canícola	30 (9.80)
18 – Javanica	30 (9.80)
19 – Hadjo	27 (8.82)
20 – Taransovi	27 (8.82)
21 – Cynope	24 (7.84)
22 – Pyrogenes	18 (5.88)
23 – Shermani	15 (4.90)
24 – Whitcombi	9 (2.94)

MAT = Microscopic agglutination test.

their interference in the epidemiology of this disease, as highlighted by Linhares et al. (2005).

Silva et al. (2009) investigated the occurrence of anti *Leptospira* agglutinins in blood sera from buffaloes in Pará State and they identified Hardjo (hardjioprajitno) Grippotyphosa and Pomona serovars as the most prevalent ones. Langoni et al. (1999) tested 403 sera from buffaloes and found that their samples were positive for Wolffi (44.80%), Icterohaemorrhagiae (33.60%), Hardjo (33.60%) and Castellonis serovars (16.5%). These data partly resemble those obtained in the current study, since Hardjo serovar, despite having reacted in the tested samples, did it less frequently (8.82%) in the current study.

As it is considered that buffaloes' physiology is similar to that of bovines, the current study draws a parallel with the seroepidemiological investigation conducted by Silva (2011) who analyzed 2582 serum samples from cows in reproductive age in the state of Maranhão. The authors reported a seroprevalence of 24.32%, in which Hardjo serovars were identified as the most prevalent ones (24.32%), followed by Wolffi (22.00%), Patoc (12.42%), Shermani (8.85%) Grippotyphosa (8.21%) and hebdomadis serovars (7.35%). These differences may be related to the degree and type of exposure to sources of infection that affect leptospirosis epidemiology.

The large number of *Leptospira* serovars identified in the assessed buffalo herd, such as Pomona, Icterohaemorrhagiae, Sentot, Copenhageni, Andamana, castellonis, Panama and Grippotyphosa, reinforces the suspicion of frequent and intense presence of wild and free living animals in the studied rural property.

Several studies demonstrate the large number of serovars affecting wild and free living animals (Silva, 2011). Silva et al. (2010) reported that opossums (*Didelphis albiventris*) and cervids can be Patoc, Autumnalis, Icterohaemorrhagiae, Andamana and Canicola serovar reservoirs to domestic animals such as cattle, goats, sheep, pigs, horses and dogs.

Incidental serovars such as Castellonis, Sentot and Andamana, which were detected in the current study and which descriptions are related to wild animals (Santa Rosa et al., 1975; Santa Rosa et al., 1980), raise suspicions on the involvement of wild animals as reservoirs of these serovars among the evaluated buffaloes.

These results show the importance of intensifying the studies on leptospirosis in wild and free-living animals that live in the surroundings of rural properties in the State of Maranhão and humans. So, management measures can be implemented in order to reduce the presence of these reservoir species in herds and, thus, prevent and control this disease in a most efficient manner.

Leptospirosis is a globally important zoonotic disease. Humans contract leptospirosis mainly from infected animal sources (human-to-human transmission has yet to be reported). Pathogenic leptospires, the bacteria that causes leptospirosis, enter via the mucous membranes or the damaged skin of the host animals and colonize the proximal renal tubules of the kidneys of the carrier. The known carriers of leptospires are wild and domestic animals, such as rodents, cattle, buffaloes, pigs and dogs (Gamage et al., 2014).

It is worth emphasizing that Icterohaemorrhagiae serovar, which is of great relevance to public health (Brazil, 2005) and is frequently isolated from rodents (Acha and Szyfres, 2001), showed high incidence (24.50%) in the evaluated female buffaloes. Similar results were obtained by Juliano et al. (2000) who reported the occurrence of 20.6% of this serovar among reactive animals in Goiania.

Besides Icterohaemorrhagiae, other serovars foun in the current study, such as Grippotyphosa, may be of importance to public health if the environment is contaminated by urine from infected animals and if the infecting dose is high within an exposed population. In addition, a pre-existing immunodeficiency condition in an individual or a group of individuals exposed to environments by

contaminated other serovars can generate a serious clinical condition in these individuals, even when the infectious dose is low, as outlined by Silva (2008).

Regarding the analyzed period and herd, BoHV-1 antibodies were found in 87.25% (n = 267/306) of the analyzed samples. The occurrence of BoHV-1 was highly diagnosed in the studied property.

This is the only data about BoHV-1 occurrence in female buffaloes in the State of Maranhão, Brazil. The BoHV-1 occurrence observed in the current study was higher than that reported by Medeiros et al. (2011) in southern Rio Grande do Sul, who found a 43.75% prevalence in a total of 80 analyzed samples. Such difference may be related to the sample size used in the research. However, the results from both studies indicate that the infection by this virus occurs in significant percentages of buffalo herds and, despite the claims by many professionals involved in buffalo breeding that this virus is exotic in Brazil, the results from the two studies say otherwise.

Studies by Scicluna et al. (2010) indicate that the buffalo species is susceptible to infection by bovine herpesvirus, thus demonstrating the possible role of buffaloes as hosts or reservoirs of this virus.

There are different reasons for the high occurrence and distribution of *Leptospira sp*. and BoHV-1 in the studied property. However, both diseases have in common the fact that they are caused by microorganisms able to definitively establish themselves in the animals, whether by persistent infection (*Leptospira* spp.) or by latent infection (BoHV-1). Thus, with respect to any of the diseases, the introduction of a single animal contaminated by the microorganism in the property would be enough for the further spread and perpetuation of the infection in the buffaloes. This is a common practice performed in the studied property, as stated by the respondent producer.

In addition to such scenario, the neglect of buffaloes' health aspects in this property and the lack of knowledge on the pathogenicity of the reproductive infections associated with diagnosis difficulties and costs inhibit initiatives to implement control programs prior to the introduction of new animals in the property, or to the investigation of the infectious cause's related to reproductive disorders.

Conclusions

By analyzing the results from the current study, it can be inferred that *B. abortus* was not found in the assessed herd. On the other hand, *L. interrogans* and BoHV-1 were found and showed high occurrence rates. The presence of such microorganisms in the studied population may constitute an important factor to the reduced productivity rates of female buffaloes in São Mateus County, State of Maranhão, Brazil.

Considering that buffalo breeding is important to the State of Maranhão, it is recommended that efforts should be focused on conducting studies that are more comprehensive, with epidemiologically acceptable standards, involving larger numbers of herds and samples. It is worth emphasizing the importance of conducting health education programs.

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Full Length Research Paper

Evaluation of diagnostic polymerase chain reaction (PCR) for the detection of *Escherichia Coli, Staphylococcus aureus and Bacillus cereus* in cheese

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Polymerase chain reaction (PCR) assay was used to detect pathogenic bacteria in white cheese samples using suitable primers which are based on specific genes. A total of 70 samples of white cheese were collected from different parts of Baghdad for detection of the pathogens, *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus.* Cheese was made locally from different sources of milk (buffalo, cows and sheep). Samples were cultured in liquid enrichment medium (Nutrient Broth) and incubated at 37°C for a period of 16-20 h. DNA was extracted by kit extraction and pathogens were diagnosed. Multiplex polymerase chain reaction was used with primers targeting specialized sites of the target gene in one reaction and the results showed the presence of *E. coli* (87%), *S. aureus* (20%) and *B. cerues* (18%). The results of this study revealed that PCR was a rapid and useful tool for detection of pathogenic bacteria in cheese.

Key words: Cheese, polymerase chain reaction (PCR), *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*.

INTRODUCTION

The production of cheese from milk is a very ancient process. Cheese manufacturing started about 8000 years ago in the Fertile Crescent between Tigris and Euphrates rivers (Hayaloğlu et al., 2002). The white cheeses is homemade, fresh, soft, and is still mostly made in traditional ways within small unlicensed health works-hops, or in some rural homes, according to the specifications of old traditional and the high ratio of salt which works to save them for a long time (Bintsis and Papademas, 2002). The fact that the cheese is rich in nutrients, is a compromise suitable for the growth of microorganisms and reproduction, therefore, the cheese is an important source of the spread of many cases of food poisoning and many types of microbial (Rampling, 1996). Milk products constitute a complex ecosystem of bacteria. Contamination of milk products with pathogenic bacteria is mainly due to processing, handling and unhygienic conditions. This soft white cheese is made of pasteurized or raw milk, it is characterized by a high water content of43.0% and low pH, 5.1-5.6 (Freitas et al.,

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Table 1. Primer sequences and anticipated sizes of PCR products for *E. coli, S. aureus, B. cereus* primers used in this study.

Species	Target gene	PCR primers' sequences (5' – 3')	Product size
E. coli	MalB Promoter	Eco-1, GACCTCGGTTTAGTTCACAGA Eco-2, CACACGCTGACGCTGACCA	585
S. aureus	Nuclease gene	SA-1, GCGATTGATGGTGATACGGTT SA-2, CAAGCCTTGACGAACTAAAGC	275
B. cereus	hemolysin gene	BC-1, CTGTAGCGAATCGTACGTATC BC-2, TACTGCTCCAGCCACATTAC	185

1993). Many enteropathogenic microorganisms have been found in milk and dairy products such as cheese, which is usually stored under inadequate temperatures and consumed without any prior thermal treatment. They are frequently associated with outbreaks of food borne diseases. Domestic animals play an important role in causing *Escherichia coli* infections, mainly cattle and sheep that can be asymptomatic vectors of virulent strains (Chapmann et al., 1993).

To better control microbial contaminants of food and consequently to reduce foodborne illnesses, rapid and accurate pathogen detection methods are required for effectively monitoring microbial pathogens in food supplies. Traditional detection methods depend upon selective plating combined with immunological and biochemical identification. The negative aspects of these methods are that they are time consuming, laborious, and take several days to complete. In fact, it is impractical to use traditional microbiological methods for high-throughput screening of large number of food samples for the presence of one or more pathogens (Abubakar et al., 2007). PCR is one of the most promising techniques for rapid detection of microorganisms in food. This process has provided increased sensitivity for detection and therefore enhanced the likelihood of detecting bacterial pathogens (Lampel et al., 2000).

The soft white cheese is widely consumed in Iraq, which is marketed and displays in unhealthy way in shops and on the sidewalks. The aim of this study was to evaluate the use of polymerase chain reaction (PCR) in the detection of *E. coli*, *Staphylococcus aureus* and *Bacillus cereus* in soft white cheese.

MATERIALS AND METHODS

Samples

This study was carried out from the beginning of September 2013 till the end of January 2014. Seventy white cheese sample were collected randomly from different regions of Baghdad city, which were made locally from different sources of milk (buffalos, cows and sheep), samples were collected using sterile bags and transported to the laboratory for detection of pathogenic bacteria (*E. coli*, *S. aureus*, and *B. cereus*). Weight of 10 g from each sample was homogenized with 90 ml of nutrient broth and incubated at 37°C for 16-20 h with continuous shaking for the purpose of homogenization.

DNA extraction

A volume of 1.5 ml of the post-enriched sample was centrifuged at 14,000 g for 1 min, DNA was extracted using Presto Mini g DNA Bacteria Kit according to manufacturer's instructions (Geneaid, Korea). The extracted DNA was stored −20°C until use.

Agarose gel

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA. 10 µl portion of the sample was analyzed by electrophoresis in agarose gel (2%), staining with ethidium bromide (Promega, USA), and visualized in UV light. A DNA molecular weight standard 50 bp was analyzed along with the samples (Wang et al., 1997).

PCR Primers

Oligonucleotide primers for the PCR assay (Table 1) were selected based on the published nucleotide sequence of the genes (Wang et al., 1997).

PCR assay

The PCR amplification was performed in a final volume of 25 µl containing 4 µl of DNA template where 1 µl of each primer was added as given in Table 1, together with 8 µl of nuclease free water, 5 µl master mix, and distributed components at a rate of 16 µl each sample; the samples were transported to a thermal cycle using the amplification program consisting of initial denaturation at 94°C for 3 min, 35 cycles with a denaturation at 94°C for 1 min, annealing at 60°C for 45 s and extension at 72°C for 90 s, followed by the final extension at 72°C for 10 min.

RESULTS

The different size of the amplification products allowed rapid and specific discrimination of *E. coli*, *S. aureus* and *B. cereus.* Table 2 shows the results of pathogenic bacteria PCR detection after 24 h of incubation in enrichment media.

PCR results showed contaminated of 61 samples of *E. coli* (87%), 14 samples *of S. aureus* (20%) and 13 samples of B. *cereus* (18%) (Figure 1). Multiplex PCR

Table 2. Summary of the results of multiplex PCR assay.

No. of sample	Type of milk	*E. coli*	*S. aureus*	*B. cereus*
1		+	+	+
2		+	–	–
3		+	–	–
4		+	–	–
5		+	–	–
6		+	+	–
7		+	–	+
8		+	+	–
9		+	–	–
10		+	–	–
11		+	–	–
12		+	–	–
13		+	–	+
14		+	–	–
15		+	–	–
16		+	–	–
17		+	–	+
18		+	–	–
19	**Buffalo**	+	–	–
20		+	–	–
21		+	+	–
22		+	–	–
23		+	+	–
24		+	–	–
25		+	–	–
26		+	–	+
27		–	+	–
28		+	–	–
29		–	–	–
30		+	–	–
31		+	–	–
32		+	+	–
33		–	–	–
34		+	–	+
35		+	–	–
36		+	–	–
37		+	–	–
38		+	–	–
39		–	–	–
40		+	+	–
41		+	–	+
42		+	–	+
43		+	+	–
44	**Cow**	+	+	–
45		+	+	–
46		+	–	+
47		+	–	–
48		+	–	–
49		+	–	–
50		+	+	–

Table 2. Contd

51		+	–	–
52		–	–	+
53		+	–	–
54		+	–	–
55		–	–	–
56		+	–	–
57		+	–	–
58		+	–	+
59		+	–	–
60		+	–	–
61		+	–	–
62		+	–	+
63		+	–	–
64	**Sheep**	+	+	–
65		+	–	–
66		+	–	–
67		+	–	–
68		–	–	–
69		–	–	+
70		–	+	–

successfully amplified the DNA fragments corresponding in size to each species as follows: *E. coli* 585 bp, *S. aureus* 276 bp, *B. cereus* 185 bp (Figure 2). The mPCR results diverged, some of the cheese samples contain the three types of pathogens under study and some of them did not contain any pathogen and other samples contain only one type of pathogens.

DISCUSSION

The present study attempted to detect *S. aureus* using specific primers for *nuc* gene which is responsible for the ability of *S. aureus* to coagulate plasma and produce a thermostable nuclease (Pinto et al., 2005). Fourteen out of seventy samples were detected to contain *S. aureus* and revealed the presence of amplified product of the size, 276 bp (Figure 1). The amplification of *nuc* gene has potential for the rapid diagnosis of *S. aureus* infection (Brakstad et al., 1992); it is considered the base line in identification and classification of *S. aureus*. Sixty one (87%) from the total cheese samples was positive for *E. coli* by amplification of *lamB* gene which code for maltose transport protein (Figure 1). Other studies have also reported similar finding such as Gonzales et al. (2000) who reported incidence of virulent strains of *E. coli* in unpasteurized milk cheese. Also, *hemolysin BL* gene was specific target for detection of *B. cereus* from cheese sample, thirteen of seventy samples were detected to contain *B. cereus* and revealed the presence of the amplified product of the size, 185 bp (Figure 1). Kumar et al. (2010) reported that the mPCR was able to detect as low as 10^1-10^2 of *B. cereus* organism per ml following overnight enrichment of spiked food samples (vegetable biriyani and milk) in buffered peptone water (BPW), and could also be detected by mPCR in naturally contaminated samples of rice based dishes and milk.

This primer sets (Figure 2) shows specificity for the detection of pathogenic genes when used alone or with each. DNA amplification technique is suitable for assessing complex microbial communities (Huyghe et al., 2008); it can be used for the detection and identification of food-borne pathogenic bacteria and environmental microorganisms, for comparative genomic analyses and strain typing. From the foregoing information, we can say that molecular method can characterize the bacteria that contaminate the white cheese depending on amplification of suitable genes. It is considered as fast, high sensitive and specific method when compared with traditional method which is considered easier, but less sensitive, less specific and slower and time consuming (Techathuvanan et al., 2011; Zhang et al., 2011). The molecular assay also revealed results which were equivalent to those obtained with standard methods used in microbiology, however the time required for analysis was reduced from 7-10 to two working days (Amagliani et al., 2007). This study agrees with the study conducted by Holko et al. (2006) on the detection of *E. coli* in cheeses samples made in the traditional way. Our results show that the above protocol can be used in detection of pathogens in

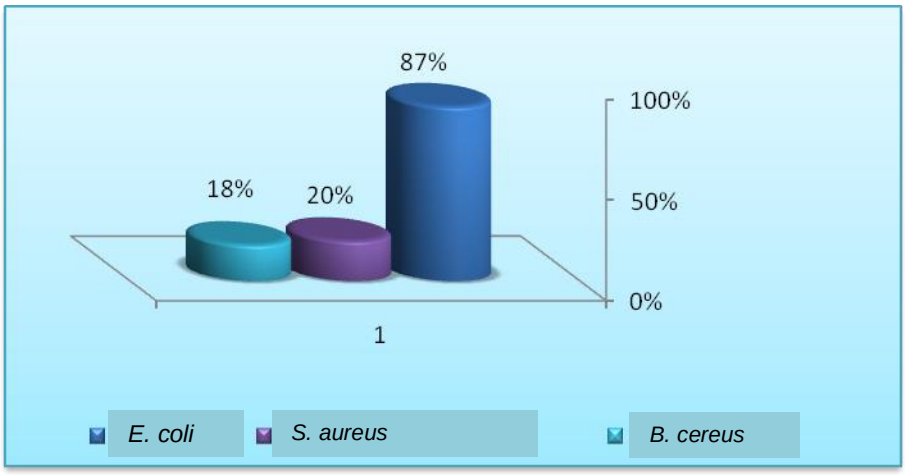

Figure 1. The percentages of microbial species detected in local cheese; the highest percentage of the isolate was *E. coli* (87%).

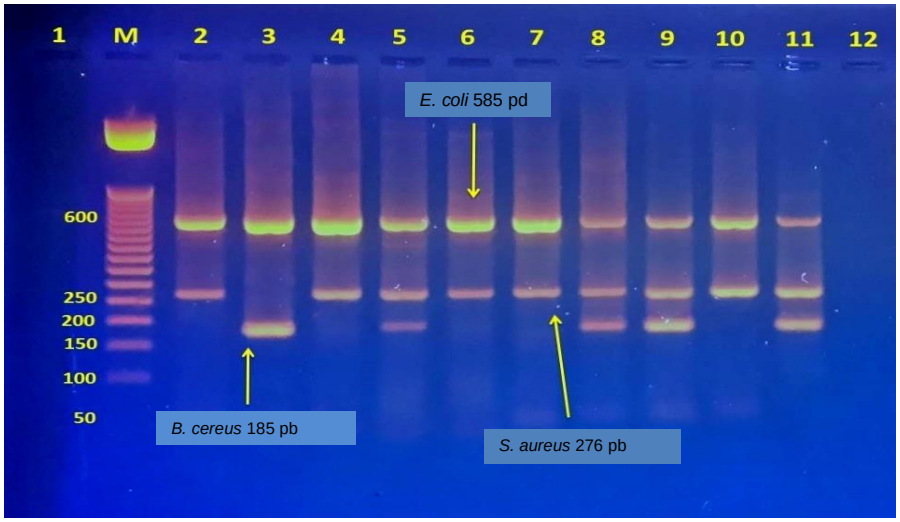

Figure 2. PCR amplification of 585 bp *E. coli*, 276 bp *S. aureus*, 185 bp *B. cereus* analyzed by electrophoresis on a 2% agarose gel, Lane M: 50 bp DNA ladder, Lanes 2, 4, 6, 7, 10: *E. coli* and *S. aureus*. Lanes 1, 12: Negative controls, Lanes 3, 5, 8, 9, 11: *B. cereus*.

white cheese samples because it is easy and fast with high sensitivity and specificity when compared with traditional methods.

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Full Length Research Paper

Comparison of *Brucella* agar, CITA and Farrell media for selective isolation of *Brucella abortus* from semen of bovine bulls

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Brucellosis remains as a public health concern worldwide. In domestic animals, the disease is characterized by reproductive disorders in male and female. Besides extensive use of serological tests and recent development of molecular biology techniques, microbiological culture of *Brucella* species is yet considered a "gold standard" method for diagnosis. Here, semen of 335 bovine bulls was subjected simultaneously to microbiological culture in *Brucella* agar, Farrell media, and CITA media to evaluate comparatively the best selective media for isolation of *Brucella* sp. Among all 335 samples, *B. abortus* B19 strain was isolated from semen of five (1.49%) bulls using the three selective media. However, Farrell media was considered the best selective media for microbiological diagnosis, because of allowed isolation of *B. abortus* B19 strain from bull semen without bacterial commensal or fungal contamination of plates.

Key words: *Brucella* agar, Farrell media, CITA media, bovine bulls, semen.

INTRODUCTION

Brucellosis remains as a public health concern worldwide (Acha and Szyfres, 2003). In addition to zoonotic impact, *Brucella* infections in livestock also represent a great economic significance particularly in developing countries due to reproductive problems, reduced milk production, as well as restrictions to animal movements and trade

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imposed by international regulatory norms (Nielsen and Duncan, 1990; Radostitis et al., 2007).

Brucellae are well-recognized intracellular Gram-negative bacteria that infect humans, wildlife and domestic animals (Quinn et al., 2011). In livestock, the infection is acquired usually by direct contact or ingestion of contaminated foetal tissues and fluids. Alternatively, the infection occurs also by venereal contact, penetration through skin lesions, inhalation or transplacental transmission (Radostits et al., 2007).

Intermittent bacteraemia causes spread and localization of organism in the reproductive organs and associated glands in sexually mature animals. Erythritol is recognized as a polyhydric alcohol which acts as a growth factor for *Brucella* species, present in high concentrations in the placentae of domestic ruminants and pigs. Furthermore, this growth factor is found in epididymo and, mammary gland, which may explain, in part, the targets to the genus *Brucella* for these animal tissues (Quinn et al., 2011).

Bovine and buffaloes brucellosis is caused typically by *Brucella abortus* (*B. abortus*), characterized by signs of reproductive tract in males and females (Hafez, 2004). In cows, the disease is related to abortion, retained placenta and metritis. In bovine bulls, brucellosis is associated with infection of accessory reproductive organs, including seminal vesicles, ampullae, testicles and epididymides (Nielsen and Duncan, 1990). The organism may be eliminated by semen from infected bulls causing decrease libido and impaired fertility (Songer and Post, 2005; Radostits et al., 2007).

In last decades, fluorescent polarization and molecular techniques have been used as alternative methods to conventional brucellosis diagnosis based on microbiological culture, serum agglutination and complement fixation tests. Nevertheless, isolation of *Brucella* sp. is yet considered the “gold standard” method for the diagnosis of animal brucellosis. The Office International des Epizooties (OIE) recommends the simultaneous use of selective media, including Farrell and modified Thayer-Martin media, for the primary isolation of *Brucella* species from animal samples (OIE, 2009).

The Farrell selective media inhibits the growth of most bacterial and fungal contaminants, and it is apparently the most widely selective media used for bacteriological diagnosis worldwide. However, some antimicrobials present in formulation of this media inhibit the growth of some *Brucella* species (Meirelles-Bartoli et al., 2012).

Modified Thayer-Martin medium shows greater sensitivity compared to Farrell media, although it does not inhibit contaminating microorganisms as well. For this reason, CITA media was developed based on modified Thayer-Martin media with addition of different antimicrobials and amphotericin B to inhibit contaminants without impairing the growth of *Brucella* species (De Miguel et al., 2011). Likewise, *Brucella* agar media is another selective media commonly used for microbiological diagnosis of animal brucellosis (Meirelles-Bartoli et al., 2012).

Here, we evaluated comparatively three selective culture media for *Brucella* (*Brucella* agar, Farrell media, and CITA) to investigate the presence of microorganism in semen of bovine bulls.

MATERIALS AND METHODS

Three hundred thirty-five bovine bulls in reproductive age (up 3 years) of different breeds or crossbreeds, without any apparent signs of orchitis or inflammation of the accessory glands were used in the study. The animals came from São Paulo, Mato Grosso, and Paraná States of Brazil where bovine breeding is developed. Semen samples were collected by electroejaculation method (Hafez and Hafez, 2004; Palmer, 2005), and material immediately analyzed for sperm viability. An aliquot of the semen samples were frozen (-20°C) for microbiological diagnosis. All semen samples of 335 bulls were simultaneously subjected to microbiological culture using *Brucella* agar, Farrell media, and CITA.

Brucella agar was prepared using *Brucella* media base (OXOID™) supplemented with 5% fetal bovine serum (Invitrogen™). The same *Brucella* medium base was also supplemented with Farrell antimicrobials (OXOID™), and 5% fetal bovine serum (Farrell medium). CITA media was prepared using defibrinated sheep blood agar base number 2 (OXOID™), supplemented with the following antimicrobials: vancomycin, colistin, nystatin, nitrofurantoin, amphotericin B (Sigma™), and 5% fetal bovine serum. The concentration used of the antimicrobials was described previously (De Miguel et al., 2011).

The plates were maintained under micro-aerobic conditions (10% CO_2), at 37°C, and observed every 24 h, for up to 14 days. Colonies suspected of *B. abortus* were subjected to Gram and Koster’s stains, and conventional biochemical characterization based on requirement for CO_2, catalase, oxidase, urease, citrate, urease, thionin, fuchsin, indol, and nitrate reduction tests (Songer and Post, 2005; Quinn et al, 2011). In addition, phenotypic differentiation of field *B. abortus* and *B. abortus* B19 vaccine strains was carried out by subjecting the isolates to growth in thionin (2 µg/mL), penicillin (5 UI/mL) and rifampicin (50 µg/mL) (Alton et al., 1988).

RESULTS AND DISCUSSION

Brucella agar medium

After four days of incubation, small, smooth, round, translucent colonies in *Brucella* agar medium from semen of five bulls (animal number 331, 332, 333, 334, 335) were observed. Conventional phenotypic tests including Gram and Koster’s stains, production of H_2S, catalase, oxidase, urease, citrate, and nitrate activity, as well as growth containing thionin (20 µg/mL), and fuchsin (20 µg/mL) allowed characterizing the isolate as *B. abortus* B19 strain, used in commercial vaccines. In the same selective culture medium, growth of bacterial contaminants in all plates was observed, predominantly Gram-positive bacteria, identified as *Corynebacterium* spp., *Bacillus* spp., *Micrococcus* spp., and *Rhodococcus equi* (Quinn et al., 2011). Furthermore, three plates of *Brucella* agar

Figure 1. Aspect of *B. abortus* B19 strain from semen of bulls isolated in CITA media.

showed fungal growth, indicating a lack of inhibition of fungal contamination. The isolation of organisms from fungal origin occurred probably due to absence of antimycotic cycloheximide, which is present in the Farrell supplement. Cyclohexamide inhibits the translation of mRNA by ribosomes, preventing fungal protein synthesis (De Miguel et al., 2011). In addition, fungal growth may also inhibit bacterial growth, leading to a reduction in the sensitivity of the bacteriological diagnosis (Marin et al., 1996).

CITA media

B. abortus B19 strain was isolated in CITA media from the same five bulls of which B19 strain was isolated using *Brucella* agar (Figure 1). In contrast to *Bucella* agar, CITA media showed isolation of bacterial contaminants in only two plates (animal 103 and 202). However, no fungal growth was observed in CITA media. The absence of fungal contamination in this media occurred probably due to addition of the amphotericin B, which interacts with a steroid present in the membrane of the fungi, causing fatal loss of selective permeability of the membrane and cytoplasmic components of organisms (De Miguel et al., 2011).

Farrell media

From a similar way, *B. abortus* B19 strain was isolated in Farrell media from the same five bulls of which B19 strain was also identified in *Brucella* agar and CITA. Nevertheless, any contaminants from bacterial or fungal origin were observed using Farrell media, including five positives plates to isolation of *B. abortus* B19 strain. Farrell media prevents the growth of fungal and commensal bacteria that commonly lead to contamination during sample collection. Thus, it is recognized traditionally as one of the most efficient selective media for brucellae isolation (Nielsen and Duncan, 1990).

The semen is considered one of the choice specimens for isolation of *Brucella* species from livestock. Brucellae are organisms that are fastidious and some species and/or biotypes need specific growth requirements for isolation, including blood, amino acids, nicotinamide, thiamine, and magnesium. In the light of this, use of selective culture media is a critical tool to proper microbiological isolation of brucellae from animal specimens (Songer and Post, 2005; Quinn et al., 2011).

More recently, a study compared the same selective culture media (*Brucella* agar, CITA media, and Farrell media) for microbiological diagnosis of *Brucella* species from lymph nodes of pigs and wild boars, and also

observed that Farrell was the best selective media, due to the potential to inhibit the growth of contaminants (Vicente et al., 2014). In fact, Farrell media was the best selective media in the current study, because it allowed isolation of *B. abortus* strain from bull semen without bacterial commensal or fungal contamination of plates. However, some antimicrobials present in Farrell media inhibit the isolation of *B. ovis, B. melitensis, B. suis*, and some strains of B. *abortus*, due to high concentrations of nalidixic acid and bacitracin (Marin et al., 1996; Ferrão-Beck et al., 2006). Thus, combined use of selective media can increase the isolation of *Brucella* species from different samples of animals, despite apparent best effectiveness of Farrell.

Bovine brucellosis is considered endemic in several regions of Brazil (Poester et al., 2002). Vaccination is a critical tool to control or eradicate bovine brucellosis (Nielsen and Ewalt, 2004), because prevents abortion (Megid et al., 2005), and subsequent pasture contamination, and it is recognized as a main route to transmission of *B. abortus* to bovine (Olsen and Tatum, 2010). In the present study, the isolation of *B. abortus* B19 strain from semen of bulls occurred probably secondary to improper vaccination practice of male calves with B19 vaccine strain, since this vaccine is recommended exclusively to female calves between 3-8 months of age (Nardi Júnior et al., 2012) in Brazilian Control Program of Bovine Brucellosis (Brasil, 2009).

Conclusion

Here, we observed that Farrell media was the best selective media compared to *Brucella* agar and CITA for isolation of *B. abortus* from 335 semen samples of bovine bulls, due to its capacity to inhibit commensal bacterial and fungal contamination of samples.

Conflict of interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Random amplified DNA polymorphism of *Klebsiella pneumoniae* isolates from Mansoura University Hospitals, Egypt

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Klebsiella pneumoniae **is one of the major causes of nosocomial infection. Understanding the route and dynamics of dissemination in the outbreaks of infections relies on using accurate typing methods. This is achieved through molecular typing using random amplified polymorphic DNA (RAPD) analysis technique in correlation to the infection source and antibiotic resistance. In this study, a total of 300 clinical isolates were collected from different clinical sources among Mansoura University Hospitals, Dakahlia governorate, Egypt. Ninety six isolates were identified as *K. pneumoniae*. The antimicrobial susceptibility test showed high resistance to the majority of tested antimicrobials, especially to β-lactams. *K. pneumoniae* isolates were categorized into 24 different antimicrobial susceptibility patterns (A1-A24). Furthermore, RAPD analysis was applied as a molecular typing method using two individual primers, AP3 and OPA13. RAPD typing showed 51 distinct patterns (R1-R51) verified into 5 groups (A, B, C, D, and E). The most common patterns were D11, D18 and D19 included in groups B and C. On the other hand, more molecular variable isolates were distributed in groups D and E. Correlation between RAPD analysis and antibiotying established that specific RAPD pattern D11 was associated with multidrug resistant isolates. This confirms that cross acquisition can play an important role in the epidemiology of nosocomial colonization and infection with *K. pneumoniae* at Mansoura University Hospitals. In conclusion, this study emphasized the need for appropriate monitoring of *K. pneumoniae* infections, by using both traditional and molecular methods. RAPD proved to be effective technique in discriminating *K. pneumoniae* isolates.**

Key words: *Klebsiella pneumoniae*, random amplified polymorphic DNA (RAPD), antimicrobial susceptibility, typing, dendrogram.

INTRODUCTION

Klebsiella pneumoniae is an important nosocomial pathogen that causes severe morbidity and mortality worldwide, with immune compromised patients, diabetic, elder and pediatric patients. *K. pneumoniae* predominantly causes respiratory and urinary tract infections as well as surgical wounds infection (Cao et al., 2014).

Extensive use of antibiotics such as aminoglycosides, extended-spectrum cephalosporins and carbapenems was associated with multidrug resistance to *K. pneumoniae* (Souli et al., 2010). *K. pneumoniae* is an important cause of nosocomial infections in many parts of the world, especially in intensive care units (Abdel-Hady et al., 2008). Epidemiological characterization of *K. pneumonia*e is highly significant to monitor the spread of its infection and assists in controlling their resistance and pathogenicity.

Infection control efforts aim to identify the source of the infection and the mode of the transmission (de Sauza et al., 2005).

Traditional techniques for typing *K. pneumoniae* based on phenotypic characteristics including biotyping, antibiogram typing, O-serotyping, bacteriocin and phage typing are insensitive for typing and differentiation between isolates (Bricker, 2011).

Molecular methods such as plasmid-profile analysis, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism, gene sequencing and pulsed-field gel electrophoresis (PFGE) are more perceptive typing techniques (Bricker, 2011). Polymerase chain reaction (PCR)-based typing techniques, such as RAPD analysis is fast and easy to perform (de Sauza et al., 2005).

RAPD typing has been applied successfully for epidemiological investigations of many bacterial and fungal outbreaks (RAPD is based on PCR amplification of a set of fragments by using short arbitrary primers 6-12 bp that target several unspecified genomic sequences (Williams et al.,1990). The resulting amplified fragments function as polymorphisms for DNA fingerprinting. It has been widely used for typing and characterization of bacterial isolates in cases of outbreaks including *K. pneumoniae* (Ben-Hamouda et al., 2003), *Staphylococcus aureus*, *Staphylococcus intermedius*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus rattus* (Truong et al., 2000) and *E. coli* (Lin et al., 2010). Monitoring the spread of specific strains is of epidemiological importance in order to detect the source of infection and control bacterial pathogenicity. *K. pneumoniae* has been associated with nosocomial infection worldwide (Lin et al., 2010). Also, *K. pneumoniae* had been detected in outbreak of infection especially extended spectrum resistant isolates in Egypt (Abdel-Hady et al., 2008). However, no data on the genetic characterization of *K. pneumoniae* isolates by RAPD profile in Egypt was reported to date.

The aim of this study was the epidemiological characterization of molecular variability among *K. pneumoniae* isolates from various clinical sources at Mansoura University hospitals. This was achieved through molecular typing using RAPD analysis technique in correlation to the infection source and antibiotic susceptibility pattern.

MATERIALS AND METHODS

Isolation and identification of *K. pneumoniae* clinical isolates

A total of 96 clinical isolates of *K. pneumoniae* were isolated from 300 different patients distributed among Mansoura University Hospitals, Dakahlia governorate, Egypt in the period between January 2012 to April 2012. The isolated strains were purified and identified according to Crichton (1996). All the purified isolates were maintained in glycerol 30% at -80ºC.The experimental protocol conducted in the study complies with the ethical guidelines and the principals of care, use and handling of human subjects in medical research adopted by "The research Ethics Committee, Faculty of Pharmacy, Mansoura University, Egypt which is in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki involving use and handling of human subjects).

Antimicrobial susceptibility testing

Susceptibility to antibiotics was determined by the disc-diffusion method using Mueller–Hinton agar (Becton, Dickinson and company, USA) according to the Clinical and Laboratory Standards Institute (CLSI) 2011. Ten antimicrobial discs were used including; amikacin (AK, 30 µg) (aminoglycosides), amoxicillin/clavulanic acid (AMC, 30 µg) and ampicillin/sulbactam (SAM, 20 µg) (beta-lactam beta-lactamase inhibitor), cefepime (FEP, 30 µg) (fourth generation cephalosporin), ceftazidime (CAZ, 30 µg) and ceftriaxone (CRO, 30 µg) (third generation cephalosporin), cefuroxime (CXM, 30 µg) (second generation cephalosporin), ciprofloxacin (CIP, 5 µg) and levofloxacin (LEV, 5 µg) (quinolone) and meropenem (MEM, 10 µg) (carbapenem); all discs were supplied from Oxoid products, UK.

Genotypic analysis

Genomic DNA extraction

The chromosomal DNA of all isolates of *K. pneumoniae* was prepared using Thermo Scientific GeneJet Genomic DNA purification Kit #K0721 (Thermo Fisher Scientific, European Union), according to manufacturer instructions. Genomic DNA was eluted by adding 50 µl EB buffer (10 mMTris-HCl, pH 9, 0.5 mM EDTA) and visualized by electrophoresis on horizontal gels containing 1% agarose and stained with ethidium bromide.

RAPD-PCR amplification

DNA typing was carried out by RAPD analysis according to Williams et al. (1990) with some modifications. RAPD analysis was performed using oligonucleotides AP3; 5`…TCACGATGCA…3' (Green et al., 2011) and primer; OPA13; 5'…CAGCACCCAC…3' (Rodrigues et al., 2008). PCR was performed in a 20 µl reaction volume containing 100 ng of genomic DNA, 20 µM of the used primer, 1.5U of FlexiTaq DNA polymerase, 5x GoTaq® Flexi buffer and supplied by manufacturer (Promega, USA). The negative control without DNA was included in each PCR run.

The amplification was performed in thermocycler machine, FPROGO2D, Tchne, LTD, Duxford Cambridge, UK. The amplification protocol consisted of the following steps: initial denaturation 95°C for 5 min., followed by 40 cycles of denaturation at 95°C for 30 sec, annealing temperature at 30ºC for 30 sec, extension at 72°C for 2.5 min. and final extension at 72°C for 10 min. Amplified PCR products were mixed with 6X loading buffer, separated using 1% agarose gels and visualized by UV transillumination. DNA fingerprints were compared by visual inspection.

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Reproducibility of the amplification patterns was confirmed by the repetition of the PCR reactions. Electrophoresed agarose gels were analyzed visually and scored using a binary code. The binary code was analysed using DendroUPGMA: A dendrogram construction utility, Universitat Rovira i Virgili (URV), Tarragona, Spain. The dendrogram was constructed based on Jaccard coefficient and unweighted-pair group method with arithmetic mean clustering method (UPGMA) (de Sauza et al., 2005).

RESULTS

A total of 300 clinical isolates were collected from different clinical sources among Mansoura University Hospitals, Dakahlia governorate, Egypt. Ninety six isolates were identified as *K. pneumonia*e. The sampling information of all isolates including clinical sources, dates and places of isolation are illustrated in Table 1.

Antimicrobial susceptibility and resistance pattern

As determined by disc-diffusion antimicrobial susceptibility testing, a high resistance percentage was observed for most antimicrobials where 76.04% were resistant to cefuroxime (second generation cephalosporins), 73.9% were resistant to ceftazidime and ceftriaxone (third generation cephalosporins), 67.7% were resistant to amoxicillin/clavulanic acid and ampicillin/sulbactam (beta-lactam/beta-lactamase inhibitor combinations) and 56.25% were resistant to ciprofloxacin and levofloxacin (quinolone). On the other hand a lower resistance was noticed for cefepime (fourth generation cephalosporin) (36.45%), amikacin (aminoglycoside) (27.04%) and meropenem (carbapenem) (9.37%). Among isolates, 16 were considered multiple drug resistant (MDR) (resistant to more than two classes of antibiotics) named 8, 9, 10, 13, 15, 17, 21, 22, 23, 24, 37, 57, 71, 72, 88 and 94 (Table 2).

The tested *K. pneumoniae* isolates were distributed into 24 different antimicrobial susceptibility patterns (A1-A24) according to their resistance to different antimicrobial groups (Table 2). Most patterns shared resistance to cephlosporines and beta-lactam/beta-lactamase inhibitor. The most predominant pattern A2 involved 13 isolates showing resistance to all tested antimicrobials except amikacin (aminoglycoside) and meropenem (carbapenem). On the other side, pattern A22 involved 13 isolates with susceptibility to all antimicrobials. The second common pattern was A1 representing 12 isolates showing resistance to all tested antimicrobials except cefepime, amikacin and meropenem (carbapenem). Strain number 94 was the most resistant isolate as it was resistant to all tested antimicrobials.

RAPD analysis

K. pneumoniae isolates were typed by RAPD technique to investigate the patterns of *K. pneumoniae* infection at Mansoura University Hospitals. Two individual primers, AP3 and OPA13 were used. The primer OPA13 could amplify variable amplicons ranging in size from 350 to about 3000 bp. Most isolates (44%) shared a common amplicons size 400, 450, 550, 750, 1350 bp. RAPD analysis using AP3 primer showed amplified bands ranging in size from 200 to about 3000 bp with most common amplicon size 350-850 bp. RAPD profile of all isolates using OPA13 and AP3 primers are represented in Figures 1 and 2 respectively.

Cluster analysis of RAPD profile of both primers AP3 and OPA13 classified 96 *K. pneumoniae* isolates into 5 groups (A, B, C, D, and E) with distinct patterns (D1-D51) representing the 96 isolates (Figure 3). Isolates considered with the same pattern if the level of similarity was ≥70%. The most common patterns were D11, D18 and D19 included in groups B and C. Pattern D18 represented eleven isolates; isolates 7 and 73 were isolated from blood samples from pediatric hospital and isolates 14, 49, 60, 75, 77, 84, 89, 90 and 96 were obtained from urine samples. Samples 14, 49, 60, 84, 89, 90 and 96 were taken from the Kidney center and samples 75 and 77 were isolated from the Digestive center. Also, the pattern D11 included ten isolates (8, 9, 10, 13, 15, 16, 18, 20, 21 and 22), with percentage similarity 100% and all of them were obtained from urine samples except isolate 8 from endotracheal tube (Figure 3). On the same instance, isolates number 8, 9, 10, 13, 15 and 22 had the same antibiotype (A5) (Table 2).These isolates were mainly obtained from kidney center indicating the possibility of cross infection. Also, isolates 33, 85, 91, 93 and 94 chaired the same pattern D21 and they were all obtained from urine samples of kidney center except isolate number 33 from blood. On the other hand, groups D and E included more variable isolates. Thirty five isolates were distributes in groups D and E with 27 variable patterns D24-D51 (Figure 3).

DISCUSSION

The extensive use of antibiotics leads to high incidence of resistance among bacterial populations associated with an ultimate change in the susceptibility profile of microorganisms (Cao et al., 2014). The outbreaks of *K. pneumoniae* infection (Ben-Hamouda et al., 2003) raised the attention in hospitals. Epidemiological investigations are important for successful microbial control to minimize and eliminate the source of infection. Characterization of the organisms in the infection outbreak is very important aseptic in the control of nosocomial infection (Bricker, 2011). In the present study, the antibiotyping of 96 *K. pneumoniae* was investigated. The isolates established different antibiotic sensitivity patterns (A1-A24) indicating the diversity in their susceptibility to the tested anti-microbials (Table 2). Likewise, in the study of Ben-Hamouda et al., 2003 thirteen different antibiotic susceptibility patterns (A1-A13) were detected among 49

Table 1. List of isolates, their clinical sources, isolation centers and dates of isolation.

Isolate number	Clinical source	Isolation center	Date of isolation
1	Sputum	Digestive System Center	1/1/2012
2	Throat	Digestive System Center	1/1/2012
3	Sputum	University Hospital	2/1/2012
4	Nose	University Hospital	2/1/2012
5	Endotracheal aspirate	Emergency Hospital	5/1/2012
6	Sputum	Tumor Hospital	5/1/2012
7	Blood	Pediatric Hospital	5/1/2012
8	Endotracheal aspirate	Emergency Hospital	12/1/2012
9	Urine	Tumor Hospital	12/1/2012
From 10 to 17	Urine	Kidney Center	14/1/2012
From 18 to 26	Urine	Kidney Center	21/1/2012
27	Blood	Pediatric Hospital	26/1/2012
28	Endotracheal aspirate	Emergency Hospital	26/1/2012
29	Blood	Pediatric Hospital	2/2/2012
30	Urine	University Hospital	5/2/2012
31	Blood	Pediatric Hospital	9/2/2012
32	Endotracheal aspirate	University Hospital	12/2/2012
33	Blood	Pediatric Hospital	16/2/2012
34	Sputum	University Hospital	19/2/2012
35	Heart Tube	Pediatric Hospital	23/2/2012
36	Blood	Pediatric Hospital	23/2/2012
37	Urine	Pediatric Hospital	23/2/2012
38	Throat	Digestive System Center	23/2/2012
39	Urine	Kidney Center	25/2/2012
40	Endotracheal aspirate	Emergency Hospital	27/2/2012
41	Urine	Kidney Center	28/2/2012
42	Urine	Pediatric Hospital	1/3/2012
43	Urine	Digestive System Center	1/3/2012
From 44 to 50	Urine	Kidney Center	3/3/2012
51	Endotracheal aspirate	University Hospital	4/3/2012
52	Urine	Digestive System Center	5/3/2012
53 and 54	Urine	Kidney Center	6/3/2012
55	Blood	Pediatric Hospital	8/3/2012
From 56 to 61	Urine	Kidney Center	10/3/2012
From 62 to 70	Urine	Kidney Center	13/3/2012
71	Diabetic foot	University Hospital	18/3/2012
72	Sputum	University Hospital	18/3/2012
73	Blood	Pediatric Hospital	19/3/2012
74	Urine	Digestive System Center	19/3/2012
75	Urine	Digestive System Center	19/3/2012
76	Sputum	University Hospital	21/3/2012
77	Urine	Digestive System Center	22/3/2012
78	Urine	University Hospital	25/3/2012
79	Urine	Digestive System Center	26/3/5012
80	Wound	University Hospital	28/3/2012
From 81 to 86	Urine	Kidney Center	31/3/2012
From 87 to 91	Urine	Kidney Center	7/4/2012
From 92 to 96	Urine	Kidney Center	14/4/2012

Table 2. Source and antibiotic sensitivity patterns of 96 *K. pneumoniae* clinical isolates.

Antibiotype	Isolate	Source of isolate	Resistance phenotype
A1	1 35 11, 12, 14, 18, 26, 39, 62, 67, 78, 91	Sputum Heart tube Urine	-2nd ceph, 3rd ceph, Q, β-β/lactamase
A2	3, 76 4 38 5, 40 69, 74, 83, 84, 86, 95, 96	Sputum Nose swab Throat swab ETA Urine	-2nd ceph, 3rd ceph, 4th Ceph, Q, β-β/lactamase
A3	6 73 54, 75, 93	Sputum Blood Urine	-2nd ceph, 3rd ceph, 4th Ceph, β-β/lactamase
A4	56 7,36	Urine Blood	-2nd ceph, 3rd ceph, 4th Ceph , AG, β-β/lactamase
A5	8 9, 10, 13, 15, 22, 23, 24, 88	ETA Urine	-2nd ceph, 3rd ceph, Q, AG, β-β/lactamase
A6	71 17, 21 37,57 72	Diabetic foot Urine Sputum	-2nd ceph, 3rd ceph,4th Ceph, Q, AG, β-β/lactamase
A7	19,89	Urine	-2nd ceph, β-β/lactamase
A8	34 20, 48, 52, 70, 82, 85, 90	Sputum Urine	-Q
A9	25, 41, 42, 92	Urine	-2nd ceph, 3rd ceph, β-β/lactamase
A10	27	Blood	-2nd ceph
A11	28	ETA	-2nd ceph, 3rd ceph, 4th Ceph , Cap, Q, β-β/lactamase
A12	29 51 47, 63, 64, 65	Blood ETA Urine	-2nd ceph, 3rd ceph
A13	31	Blood	-3rd ceph, 4th Ceph, Cap, AG, β-β/lactamase
A14	32	ETA	-2nd ceph, 3rd ceph, Cap, Q, β-β/lactamase
A15	33	Blood	-2nd ceph, 3rd ceph, 4th Ceph, AG, β-β/lactamase
A16	43 , 79	Urine	-2nd ceph, 3rd ceph, 4th Ceph, Cap, AG, β-β/lactamase
A17	45	Urine	-2nd ceph, 3rd ceph, Cap, β-β/lactamase
A18	46	Urine	-2nd ceph, 3rd ceph, AG, β-β/lactamase

Table 2. Contd.

A19	55 80	Blood Wound	-2nd ceph, 3rd ceph, 4th Ceph, Cap, Q, β-β/lactamase
A20	59	Urine	-2nd ceph, 3rd ceph, Q
A21	61	Urine	-2nd ceph, 3rd ceph, AG
A22	2 16, 30, 44, 49, 50, 53, 58, 66, 68, 77, 81, 87	Throat Swab Urine	-Sensitive to all
A23	94	Urine	-Resistant to All
A24	60	Urine	-AG

AG, aminoglycosides; β-β/lactamase; beta-lactam, beta-lactamase inhibitor; 2nd ceph, second generation cephalosporin; 3rd ceph, third generation cephalosporin; 4th Ceph, fourth generation cephalosporin; Cap, carbapenem; Q, quinolone; ETA, endotracheal aspirate.

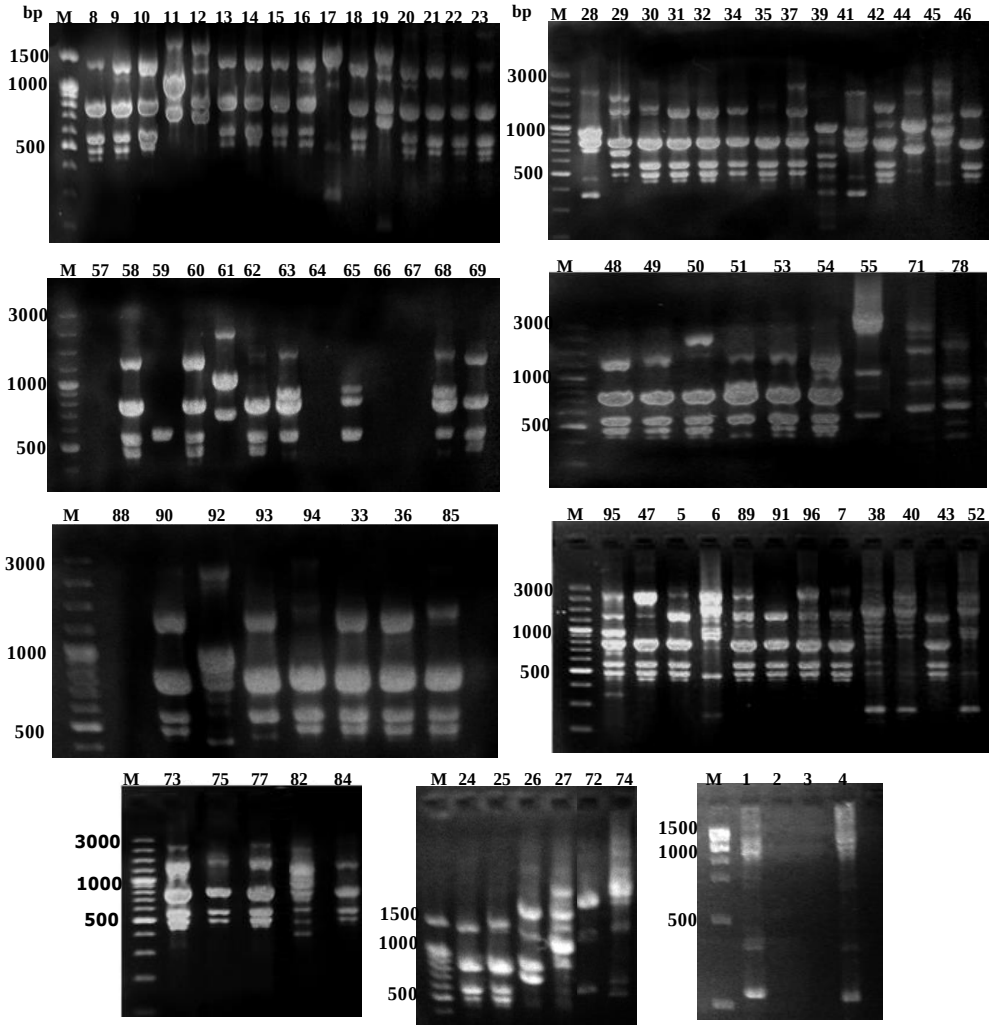

Figure 1. Random amplified polymorphic DNA fingerprinting of clinical isolates of *K. pneumoniae* using (OPA13) primer (Lane M was DNA marker).

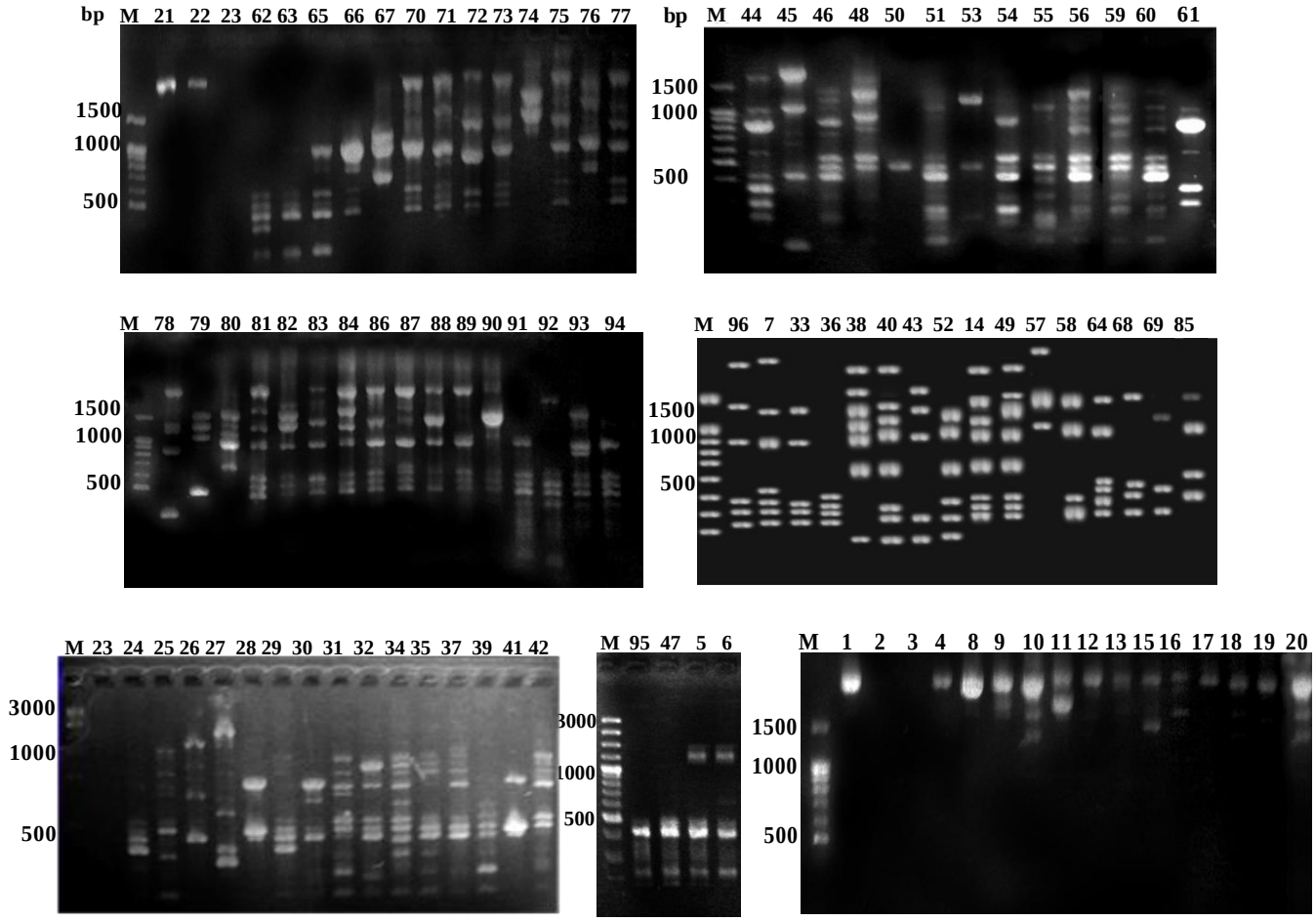

Figure 2. Random amplified polymorphic DNA fingerprinting of clinical isolates of *K. pneumoniae* using (AP3) primer (Lane M was DNA marker).

K. pneumoniae isolates.

In the present study, we also detected that the majority of *K. pneumoniae* isolates were resistant to cephalosporins and beta-lactam/beta-lactamase inhibitor and 16.66% of the isolates were MDR. Also, high incidence of MDR *klebsiella* isolates was established in nosocomial out breaks (Cao et al., 2014). The widespread use of antibiotics especially the expanded-spectrum cephalosporins, was associated with *K. pneumoniae* producing extended-spectrum beta-lactamase in different localities in Egypt (Abdel-Hady et al., 2008) and all over other countries; in Tunisian (Ben-Hamouda et al, 2003), Greece (Souli et al., 2010) and Taiwan (Lin et al., 2010). In contrast, most of the isolates were susceptible to meropenem and amikacin. Similarly, 97% of *K. pneumoniae* isolates were susceptible to carbapenem in the study of lin et al. (2010) and 87.2% of the isolates were susceptible to amikacin in the study of Das et al. (2006).

However, the antibiotic susceptibility patterns did not show enough strain-to-strain variation to discriminate between different isolates (Ben-Hamouda et al, 2003). Differentiation between isolates with slight variations in resistance profiles requires genetic based methods. Therefore, genotypic methods, including plasmid typing, RAPD analysis (de Sauza et al., 2005), PCR fingerprinting and PFGE of chromosomal DNA restriction fragments (Cao et al., 2014) have been used to investigate nosocomial outbreaks of *K. pneumoniae*.

RAPD-PCR is a genotypic identification and characterization system. RAPD profile proves specificity and sensitivity to define bacterial isolates (de Sauza et al., 2005). RAPD analysis has a great ability to type a wide variety of bacterial species and detect the genetic differences between isolates (Lin et al., 2010). It was used in typing various organisms and detection outbreak of nosocomial infections of *K. pneumoniae* at Tunisian neonatal ward (Ben-Hamouda et al., 2003) and Barazil (de Sauza et al., 2005). RAPD analysis is used in identification of phylogenetic diversity and genetic structure of both *K. pneumoniae* and *K. oxytoca* of 120 clinical isolates collected from 22 European hospitals (Brisse and Verhoef,2001).

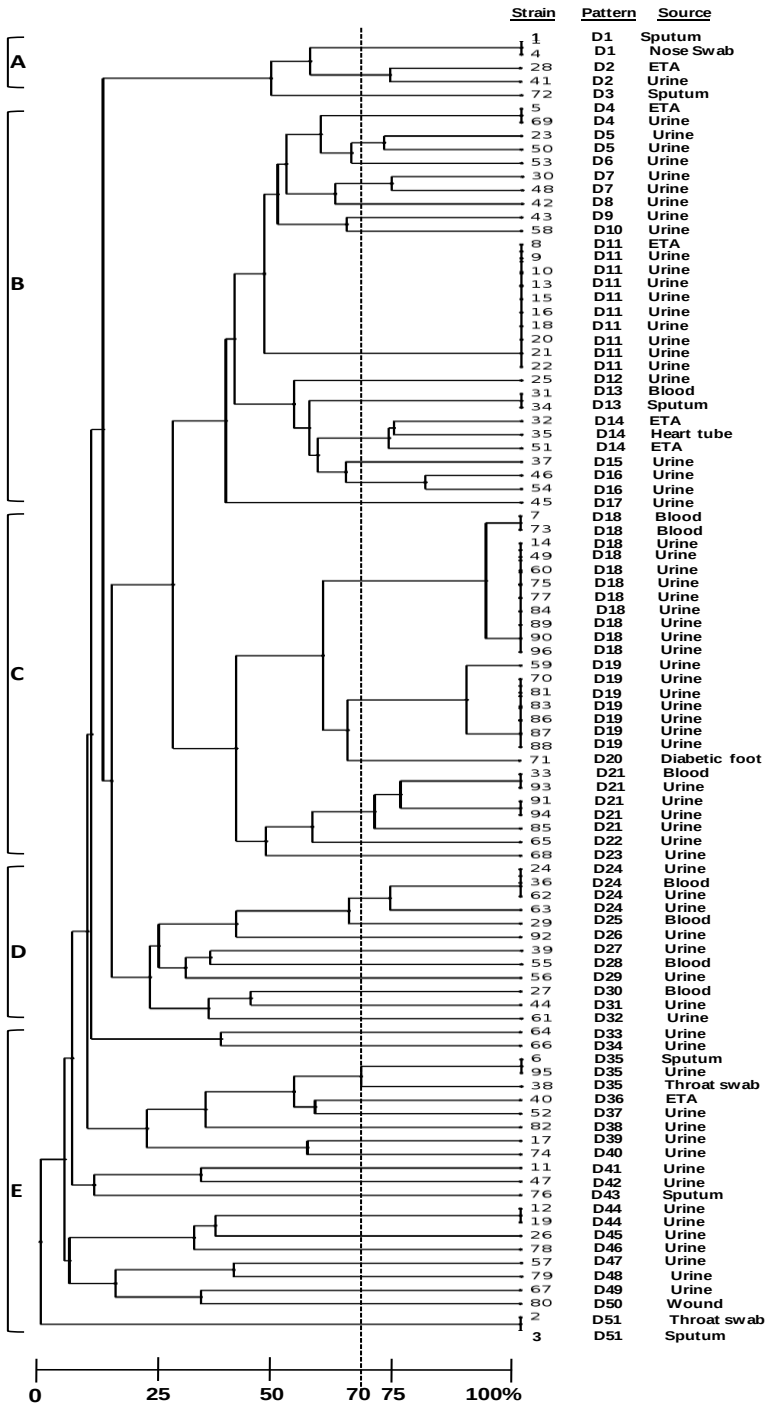

Figure 3. Dendrogram representing random amplified polymorphic DNA analysis of the *K. pneumoniae* isolates with polymorphism percentage (53%) and 52 alleles. The scale indicates similarity percentage. The respective clinical source and the antibiotic sensitivity pattern of each isolate.

However, molecular characterization of *Klebsiella* infection using RAPD profile in Egypt is limited. In the present study the molecular typing of 96 *K. pneumoniae* was performed using RAPD analysis with both primers OPA13 and AP3. RAPD distinguished *K. pneumoniae* into different subtypes based on the numerous fingerprints generated (Figure 3). A specific RAPD patterns D11, D18 and D19 were the most common among *K. pneumoniae* isolates. The main source of these isolates was urine samples obtained from Kidney Center/Mansoura University, ruling

the possibility of the cross infection. In addition, through application of RAPD technique we could detect genetic homogeneity between different isolates obtained from various sources. This can be useful to monitor this pathogen in nosocomial infections. Also, high molecular diversity of the remaining strains (70.83%) could discriminate between isolates on molecular level. Heterogeneous *K. pneumoniae* pathogenic isolates were also observed by de Sauza et al. (2005).

The correlation between antibiotyping and RAPD analysis was also assessed. Two isolates 5 and 69 showed the same antibiotype (A2) and have the same RAPD pattern D4. They were isolated from endotracheal aspirate and urine respectively. In addition six isolates 8, 9, 10, 13, 15 and 22 had the same antibiotype (A5) and the same RAPD pattern D11. Also, the urine isolates 84 and 96 had the same antibiotype (A2) and the same RAPD pattern D18. This confirms that cross acquisition can play an important part in the epidemiology of nosocomial colonization and infection with *K. pneumoniae* at Mansoura University hospitals.

The relationship between RAPD patterns and multiple drug resistant isolates was also analyzed. MDR isolates marked 8, 9, 10, 13, 15 and 22 established the same antibiotype (A5) and the similar RAPD patterns (D11). These isolates represent 37.5% of the total MDR. Horizontal spread has been well-known as the major mechanism for the emergence and maintenance of extended spectrum beta lactamase (ESBL) producing *K. pneumoniae* outbreaks as reported by Paterson et al. (2004). Outbreaks caused by ESBL producing *K. pneumoniae* have been related to the cross transmission. It is predominate in areas with excessive antibiotic use where the potential for patient to patient transmission of organisms is the greatest, such as neonatal units, intensive care units and surgical units (Randrianirina et al., 2009).

In the following study, the outbreak of MDR *K. pneumoniae* among urine samples obtained from Kidney center was monitored by RAPD analysis highlights the need of infection control measures and adoption of new antibiotic policies. Also, improving hospital hygiene and application of stuff training programs should be applied for controlling the spread of microbial infection.

Conclusion

The present work showed a high resistance profile of the studied clinical isolates of *K. pneumoniae.* RAPD analysis showed high molecular diversity among *K. pneumoniae* isolates indicating its discrimination capability in molecular typing. Moreover, this method showed a good potential to identify clonal lineages, as it recognized identical genotypes among *K. pneumoniae* isolates. The present study highlights the need for appropriate epidemiological monitoring of *K. pneumoniae* infections, by using both traditional and molecular methods. As well, monitoring of resistance to broad-spectrum antimicrobials in hospital environment is necessary to control antimicrobial use.

Conflict of interest

There is no conflict of interest.

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Full Length Research Paper

Production of cellulases from *Humicola fuscoatra* MTCC 1409: Role of enzymes in paddy straw digestion

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Cellulases were produced from fungus *Humicola fuscoatra* MTCC 1409 by solid state fermentation under different cultural conditions viz. pH, incubation temperature, inoculum size and days of incubation in order to optimize the conditions for maximum enzyme production. The potential of cellulase pretreatment to increase the digestibility of paddy straw was also ascertained. Maximum enzyme production was achieved at pH 6.0 of Mandel media and at temperature 45°C. Inoculum size of 1×10^7 spores/ml was found to be optimum for maximum enzyme production. Enzyme production increased with the increase in days of incubation from 2 to 6 days and then declined thereafter. Cellulase units at the concentration of 1, 1.5 and 2 µmole/g were exogenously added to paddy straw and change in chemical composition of paddy straw was determined after 18, 24, 30 and 36 h of treatment. With increase in enzyme concentration and incubation period, the content of neutral detergent fibre (NDF), acid detergent fibre (ADF), cellulose and hemicellulose reduced gradually with simultaneous increase in lignin and silica content. The concentrations of NDF, ADF, cellulose and hemicellulose decreased by 9.2, 5.9, 10.8 and 23.4% respectively, however lignin and silica content increased by 9.7 and 6.4% respectively as compared to control (with no cellulase added) at 2 µmole enzyme concentration after 36 h of pretreatment. These results show that the enzyme produced from cellulolytic fungus *H. fuscoatra* is capable of increasing paddy straw digestibility and thus enhancing the utilization of paddy straw for different purposes.

Key words: Cellulase production, *Humicola fuscoatra*, paddy straw, paddy straw digestibility.

INTRODUCTION

Rice being the major cereal crop is produced in large quantities in India. About 136.5-150 million tons of paddy straw is estimated to be produced annually in India (Anonymous, 2010). Punjab had contributed 10.86% of the total rice production in India during 2012, with a production of 104.32 million tons. About 1-1.5 kg of straw is produced from every kilogram of grain harvested (Maiorella, 1985). Paddy straw is a big challenge for agriculture scientists, engineers and environmentalists, as a huge quantity of straw is difficult to handle. In India,

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approximately 75-80% (85-95 million tons) of paddy straw is disposed off by burning. One ton of paddy straw burning releases 3 kg particulate matter, 60 kg CO, 1460 kg CO_2, 199 kg ash and 2 kg SO_2 (Jenkins and Bhatnagar, 2003). Burning of paddy straw in the open fields led to various medical and environmental problems. It is a major source of environmental pollution and climate change. It may also lead to many medical problems such as skin irritation, bronchitis, asthma, eye ailments, respire-tory troubles etc. The straw burning further destroys the soil texture.

Paddy straw is an attractive lignocellulosic material which is one of the most abundant renewable resources. It predominantly contains cellulose (35-40%), hemicellulose (20-24%), lignin (8-12%), ash (14-16%) and extractives (10-12%) (Maiorella, 1985; Saha, 2003). Bioconversion of lignocellulosic biomass contributes significantly to the production of organic chemicals. High cellulose and hemi-cellulose content of paddy straw can also be readily hydrolysed into fermentable sugars. Cellulase is the enzyme that degrades the cellulose into glucose, which in turn can be converted into ethanol, single cell protein and other valuable chemicals. Paddy straw can be mainly used as a source of feed for ruminant livestock, but the high level of lignification and silicification, slow and limited ruminal degradation of the carbohydrates and the low content of nitrogen affect its value as a feed for ruminants (Van Soest, 2006).

There occur several challenges and limitations in the process of bioconversion of rice straw. The barrier to the production and recovery of valuable materials from paddy straw is the structure of lignocellulose. An efficient multi-enzyme system is required for the hydrolysis of agricultural biomass to fermentable sugars. But the lignin and silica complex surrounding the cellulose fibers shields the microbial/enzyme action. Therefore, the paddy straw needs to be pretreated in order to enable cellulose to be more accessible to the microbial or enzymatic attack and to increase its digestibility by removing lignocellulolytic complex. Several pretreatments such as physical (grinding, steaming, γ-irradiation), chemical (alkaline hydrolysis, acid hydrolysis, oxidative delignification and solvent extraction), physico-chemical (ammonia fiber explosion, CO_2, steam explosion) and biological (microorganisms and enzymes) pretreatments have been used to improve rice straw utilization (Sarnklong et al., 2010). However, the physical and chemical pretreatments require high energy and corrosion resistant high pressure reactors, which increase the cost of pretreatment. The physical, chemical and physico-chemical treatments are still restricted in terms of safety concerns, costs and potential negative environmental consequences (Phutela et al., 2011). Biological treatments such as the use of ligninolytic fungus, with their ligninolytic enzymes or specific enzymes degrading cellulose or hemicelluloses is an alternative approach to improve the nutritive value of rice straw. Enzymatic hydrolysis of cellulosic wastes may give a relatively pure product with the consumption of less energy during the process (Fennington et al., 1982).

Complete enzymatic hydrolysis of lignocellulosic waste requires the synergistic action of three types of cellulases; namely exoglucanase or cellobiohydrolase, endoglucanase or carboxymethyl cellulase and β-glucosidases. Endoglu-canase (endo-1,4-glucano-hydrolase) attacks regions of low crystallinity in the cellulose fiber and creates free side chains. Exoglucanase or cellobiohydrolase (1,4-glucan cellobiohydrolase) degrades the molecule further by removing cellobiose units from the free chain ends. β-glucosidase hydrolyses cellobiose to produce glucose. Cellulolytic enzymes are synthesized by a number of microorganisms commonly by bacteria and fungi (Lederberg, 1992). Fungal cellulases have proved to be a better candidate than other microbial cellulases (Lynd et al., 2002), with their secreted free cellulase complexes comprising all the three components of cellulase (endoglucanases, exoglucanases and cellobiases). Filamentous fungi like *Aspergillus*, *Penicillium* and *Trichoderma* had demonstrated a great capability for secreting a wide range of cellulolytic enzymes. Since most industrial processes are carried out at high temperatures, therefore, there is a great demand for thermophilic enzymes (Haki and Rakshit, 2003). Thermophiles are a good source of novel catalysts that are of great industrial interest. The thermophiles have more stable enzymes as compared to mesophiles (Li et al., 2005). Thermophilic enzymes are also active at low temperatures. Thermophiles developed more rapidly to higher peaks as compared to mesophiles and stability of obligate thermophiles increased with process temperature. No doubt, reports are available for biological pretreatment of paddy straw by using mesophilic fungi; however less work has been done on pretreatment using thermophilic fungi. Recently, Phutela and Dar (2014) showed enhancement in paddy straw digestibility by pretreatment with thermophilic fungus *Thermoascus aurantiacus* MTCC 375. However, till now the data on the production of cellulases by thermophilic fungi is very scarce. Therefore, the need of the hour is to produce efficient cellulases which can enhance paddy straw digestibility. So, the present investigation was undertaken with the objective of the optimization of production of cellulases from microbial source i.e. *Humicola fuscoatra* MTCC 1409 and application of cellulases to increase paddy straw digestibility.

MATERIALS AND METHODS

Procurement of materials and maintenance of culture

Paddy straw was procured from the research field of Punjab Agricultural University, Ludhiana after harvesting of the crop. The paddy straw was chopped to 3-4 cm with a chopping machine and ground with a blender and was stored in polythene bags at room

temperature. The culture of *Humicola fuscoatra* MTCC 1409 was procured from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained by sub-culturing on potato dextrose agar (PDA) slants at 45±2°C by monthly transfers.

Preparation of media and production of enzyme

Enzyme cellulase was produced on Mandel media (Mandels et al., 1976). Composition of Mandel medium (per L) was: 0.3 g urea, 0.75 g peptone, 0.25 g yeast extract, 1.4 g $(NH_4)_2$ SO_4, 2.0 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgSO_4.7H_2O$, 0.005 g $FeSO_4.7H_2O$, 0.0016 g $MnSO_4.4H_2O$, 0.0014 g $ZnSO_4.7H_2O$ and 0.020 g $CaCl_2.6H_2O$. Enzyme production was carried out by adding 3 g of paddy straw (substrate) to flasks containing 12 ml of Mandel media. The pH of media was adjusted to 6.0 and media was autoclaved. After cooling to room temperature, the flasks were inoculated with 1 ml of 10^7 spores /ml of spore suspension and were incubated at 45±2°C for 6 days in an incubator. After 6 days of incubation, the enzyme was extracted with acetate buffer (10 times of substrate) and the fermented medium was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used as crude enzyme preparation.

Effect of different cultural conditions on cellulase production

The effect of initial pH on enzyme production was investigated by adjusting the pH of Mandel's medium containing substrate in the range of pH 4.0-8.0 with the interval of pH 1.0 and incubating the flasks for 6 days at 45°C in an incubator. For investigating the effect of incubation temperature on enzyme production, flasks containing Mandel media and substrate having pH 6.0 were incubated at different temperatures ranging from 35 to 60°C with 5°C interval for 6 days in an incubator. The effect of inoculum size was determined by adding the spore suspension of concentrations 10^6, 10^7 and 10^8 spores/ml using 1 ml of spore suspension to each flask containing Mandel media and substrate having pH 6.0 and the flasks were incubated at 45°C for 6 days. To study the effect of days of incubation on enzyme production, spore suspension of 1 ml of 10^7 spores/ml was grown on substrate with Mandel medium at pH 6.0 and incubated at 45°C and the enzyme was extracted after 2, 4, 6 and 8 days interval.

Assay of cellulolytic enzymes

Filter paper assay

To 0.5 ml of enzyme, 1 ml of sodium citrate buffer (pH 4.8) and one whatman # 1 filter paper strip (6×1 cm) was added to each tube. Tubes were incubated in a water bath at 50°C for 1 h. After incubation, 3 ml of DNS reagent was added and tubes were then placed in a boiling water bath for 15 min and 1 ml of sodium potassium tartarate was added. The contents were cooled at room temperature followed by addition of 2 ml distilled water in each test tube. The absorbance was recorded at 575 nm in a UV-VIS spectrophotometer (Miller, 1959). The corresponding enzyme activity was calculated from the standard curve prepared simultaneously using glucose as a standard (10 to 100 µg/ml). One unit of cellulase is defined as the amount of enzyme which will release 1 µmole of reducing sugar in one min per gram paddy straw.

Carboxymethyl cellulase assay

To 0.5 ml of enzyme extract 0.5 ml of substrate (carboxymethyl cellulose) was added and the tubes were incubated at 50°C for 30 min. Reducing sugar produced during this reaction was estimated using DNS as described above.

Cellobiase assay

To 0.5 ml of enzyme extract, 0.5 ml of cellobiose solution was added and the mixture was incubated at 50°C for 10 min. Reducing sugar produced during this reaction was estimated using DNS.

Effect of cellulase on paddy straw digestibility

Chopped paddy straw (10 g) was soaked in water overnight. The excess water was drained off, so as to have approximately 65-70% moisture content. Then, 1, 1.5 and 2 units of enzyme cellulase were added per gram of paddy straw. After proper mixing, paddy straw was incubated at 45±2°C for different time intervals i.e. 18, 24, 30 and 36 h, respectively. After the completion of each incubation period, the paddy straw was oven dried and then each set of paddy straw was analysed for its proximate composition that is total solids, volatile solids, ash, cellulose, hemi-cellulose, lignin and silica content by Standard methods of AOAC(AOAC, 2000). Total sugars were estimated by phenol-sulphuric acid method using glucose as a standard (100 µg/ml) (Dubois et al., 1956).

Statistical analysis

All treatments were completed in triplicate. Critical difference at 5% level was performed for proximate and chemical analysis using Completely Randomized Designs (CRD) in the CPCS software developed by Department of Statistics, PAU, Ludhiana. Standard error was calculated manually for all the experiments.

RESULTS AND DISCUSSION

Optimization of production of cellulose

Effect of different cultural conditions on cellulase production

Effect of initial pH on cellulase production: Production of cellulases increased with increase in pH value, reaching the maximum at pH 6.0, followed by gradual decrease thereafter (Figure 1). Cellobiase activity was higher than CMCase and Fpase activity. In medium at pH 6.0, the activity of enzyme cellobiase was 10.8 U/g, CMCase was 2.99 U/g, and Fpase was 1.42 U/g. Ong et al. (2012) also reported maximum exoglucanase activity of 46.45 FPU/gat pH 6.0 by *Aspergillus niger.* However, Ahmed et al. (2009) observed maximum production of exoglucanase, endoglucanase and cellobiase at pH 5.5 by *Trichoderma harzianum*. While Devi and Kumar (2012) showed maximum cellulase production of 3.9 U/ml by *A. niger* at pH 5.0. Pushalkar et al. (1995) observed that cellobiase activity was very high as compared to CMCase and Fpase activity with the pH in range of 4.0-5.5 when produced from *Aspergillus terreus*.

Effect of temperature on cellulase production:

Maximum activity of cellobiase (9.30 U/g), CMCase (3.83

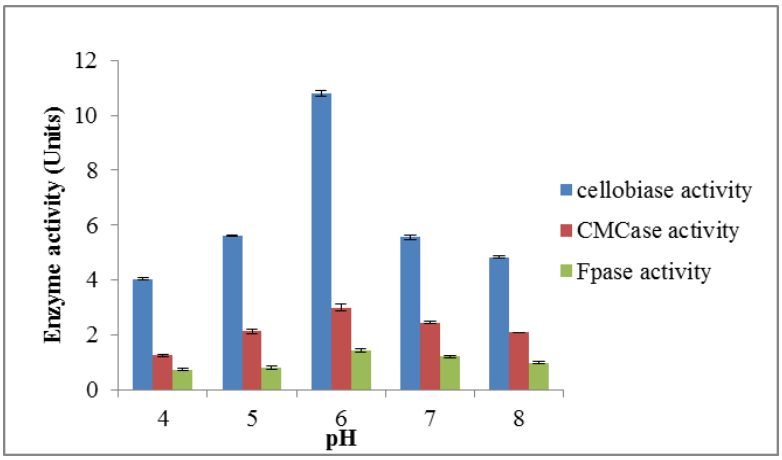

Figure 1. Effect of pH on cellulase production.

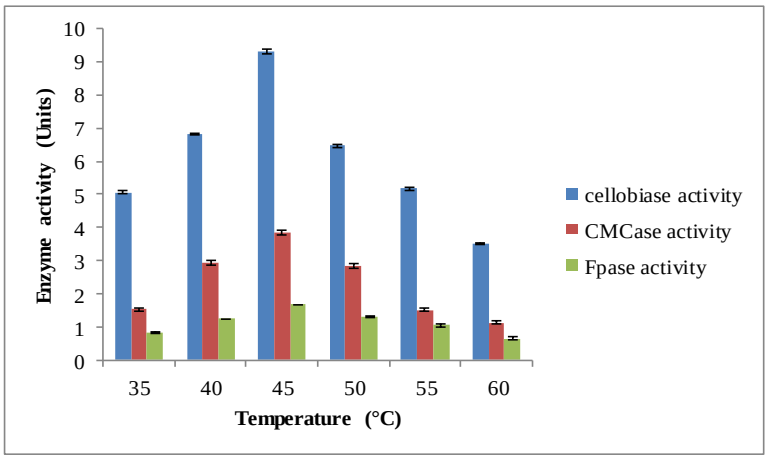

Figure 2. Effect of temperature on cellulase production.

U/g) and Fpase (1.67 U/g) was observed at 45°C (Figure 2). Further increase or decrease in temperature resulted in decrease in enzyme activity indicating that 45°C was the optimum temperature for the maximum production of enzyme. Less production of enzyme at low temperature (35-40°C) and at high temperature (50-60°C) as compared to 45°C might be due to slow growth of fungus at low temperature and inactivation of the enzyme at high temperature. Kaur et al. (2007) also reported maximum cellobiase activity of 132.4 U/g at 45°C from a new strain of thermophilic fungus *Melanocarpus* sp. MTCC 3922. Devi and Kumar (2012) showed that the highest cellulase activity (3.9 U/ml) was obtained at 45°C by *A. niger*. However, Ahmed et al. (2009) reported maximum cellulase production at 28°C by *Trichoderma harzianum*. Ali and El-Dein (2008) showed that optimum temperature for three cellulases was 35°C for *A. niger* and 30°C for *Aspergillus nidulans*.

Effect of inoculum density on enzyme production

Increase in inoculum size from 10^6 spores/ml to 10^7 spores/ml resulted in increase in enzyme production from 5.13 U/g for cellobiase, 1.24 U/g for CMCase and 0.817 U/g for Fpase to 9.72 U/g for Cellobiase, 2.89 U/g for CMCase and 1.60 U/g for Fpase. Further increase in inoculum size that is at 10^8 spores/ml resulted in decrease in all the three enzyme activities which might be due to fast degradation of substrate (Figure 3). At inoculum size of 10^8 spores/ml, 32.71% decline in the production of enzyme was observed with respect to 10^7 spores/ml indicating inoculum size of 1ml of 10^7 spores was found to be optimum for maximum production of cellulases. Grover et al. (2013) showed that with increase in inoculum size from $1\times10^5$to 1×10^7 spores/ml, cellulase production increased from 0.237 to 0.541 IU. However, Pankaj and Satyanarayana (2004) observed maximum

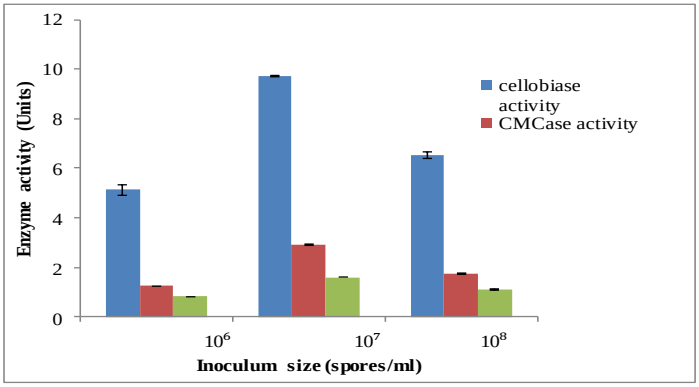

Figure 3. Effect of inoculum size on cellulase production.

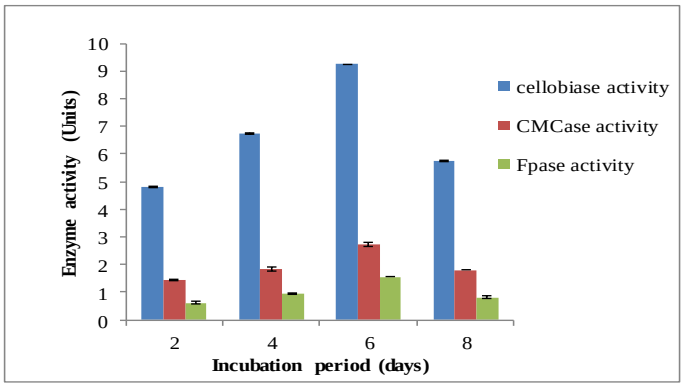

Figure 4. Effect of incubation days on cellulase production.

cellulase production of 7832 U/g of dry moldy bran with an inoculum level of 3×10^6 spores of *Humicola lanuginose*.

Effect of incubation days on cellulase production

After 2 days of incubation the activity was 4.82 U/g for cellobiase, 1.44 U/g for CMCase and 0.62 U/g for Fpase which increased to 6.73 U/g for cellobiase, 1.84 U/g for CMCase and 0.94 U/g for Fpase after 4 days of incubation. After 6 days of incubation the activity further enhanced to 9.24 U/g for cellobiase, 2.74 U/g for CMCase and 1.55U/g for Fpase. Further increase in time did not show any increment in the level of enzyme production as activity of enzyme declined to 5.74 U/g for cellobiase, 1.79 U/g for CMCase and 0.82 U/g for Fpase after 8 days of incubation period. Hence maximum yield of cellulase was observed after 6 days of incubation (Figure 4). The period required for incubation depends on the growth rate of microorganism, on substrate concentration and its enzyme production pattern. Kang et al. (2004) reported the highest cellulase activity after 5-6 days of fermentation by *A. niger* on rice straw. Devi and Kumar (2012) reported maximum cellulase production at 7th day of incubation by *A. niger*. However, *Trichoderma harzianum* showed maximum cellulase activity at 5th day of incubation (Ahmed et al., 2009). Ali and El-Dein (2008) reported that 7 days of incubation was best for maximum cellulase production by *A. niger* and *A. nidulans.*

Evaluation of effect of enzymatic pretreatment on paddy straw digestibility

Effect of enzymatic pretreatment and incubation period on paddy straw digestibility

Pretreatment of paddy straw was done using varied enzyme concentrations (1, 1.5 and 2 units/g paddy straw) and was kept for 18, 24, 30 and 36 h in an incubator at 45°C. Results show that with the increase in enzyme concentration and incubation period; neutral detergent fiber, acid detergent fiber, cellulose and hemicellulose content of paddy straw decreased whereas lignin and silica content increased. After 36 h of incubation, neutral detergent fiber decreased by 5.3% at 1 unit/g, 8.8% at

Table 1. Effect of enzyme concentration and incubation time on lignocellulose composition of paddy straw.

Composition	Enzyme units (µmoles)	Time (h)				CD (5%)
		18	24	30	36	
NDF (%)	1	74.5±0.26 (3.1↓)	73.8±0.37 (4.03↓)	73.4±0.26 (4.5↓)	72.8±0.35 (5.3↓)	T= 0.494 E= 0.427 T×E= 0.855
	1.5	73.5±0.24 (4.4↓)	72.9±0.26 (5.2↓)	72.2±0.26 (6.1↓)	70.1±0.35 (8.8↓)	
	2	72.1±0.21 (6.2↓)	71.0±0.26 (7.7↓)	69.8±0.23 (9.2↓)	69.6±0.38 (9.2↓)	
ADF (%)	1	51.9±0.26 (0.4↓)	51.3±0.29 (1.5↓)	50.9±0.52 (2.3↓)	50.4±0.40 (3.3↓)	T= 0.545 E= 0.472 T×E= NS
	1.5	51.2±0.26 (1.7↓)	50.5±0.32 (3.1↓)	50.3±0.35 (3.4↓)	49.9±0.36 (4.2↓)	
	2	50.8±0.14 (2.5↓)	50.1±0.29 (3.8↓)	49.7±0.18 (4.6↓)	49.0±0.32 (5.9↓)	
Cellulose (%)	1	37.9±0.17 (2.0↓)	37.3±0.26 (3.6↓)	36.5±0.35 (5.7↓)	35.6±0.29 (8.0↓)	T= 0.492 E= 0.426 T×E=NS
	1.5	37.4±0.32 (3.3↓)	36.7±0.32 (4.4↓)	35.7±0.28 (7.7↓)	34.8±0.43 (10.1↓)	
	2	37.0±0.26 (4.4↓)	36.1±0.18 (6.7↓)	35.1±0.21 (9.3↓)	34.5±0.32 (10.8↓)	
Hemicellulose (%)	1	23.1±0.32 (6.8↓)	22.2±0.33 (10.5↓)	21.1±0.32 (14.9↓)	20.0±0.56 (19.3↓)	T= 0.56 E= 0.485 T×E= NS
	1.5	22.4±0.26 (9.7↓)	21.3±0.23 (14.1↓)	20.1±0.30 (18.9↓)	19.2±0.35 (22.6↓)	
	2	21.9±0.31 (11.7↓)	20.9±0.26 (15.7↓)	19.6±0.26 (21.0↓)	19.0±0.34 (23.4↓)	
Lignin (%)	1	7.20±0.06 (2.9↑)	7.35±0.03 (5.0↑)	7.44±0.04 (6.3↑)	7.47±0.01 (6.7↑)	T= 0.058 E=0.051 T×E= NS
	1.5	7.38±0.03 (5.4↑)	7.54±0.03 (7.7↑)	7.57±0.02 (8.1↑)	7.61±0.02 (8.7↑)	
	2	7.55±0.02 (7.8↑)	7.60±0.02 (8.6↑)	7.64±0.03 (9.1↑)	7.68±0.03 (9.7↑)	
Silica (%)	1	6.55±0.03 (2.34↑)	6.63±0.03 (3.6↑)	6.71±0.03 (4.8↑)	6.76±0.02 (5.6↑)	T= 0.039 E= 0.034 T×E= NS
	1.5	6.62±0.02 (3.4↑)	6.66±0.03 (4.1↑)	6.73±0.02 (5.15↑)	6.79±0.02 (6.1↑)	
	2	6.67±0.01 (4.2↑)	6.70±0.02 (4.7↑)	6.77±0.02 (5.9↑)	6.81±0.01 (6.4↑)	

#Data in parenthesis represent percentage increase or decrease as compared to control i.e. untreated paddy straw; T: time, E: enzyme concentration; Untreated paddy straw composition (%): NDF=76.9; ADF=52.1; cellulose=38.7; hemicellulose=24.8; lignin= 7.0; silica=6.4; ± values indicate % standard error for triplicate data; (↓), decrease; (↑), increase.

1.5 units/g and 9.2% at 2 units/g of enzyme concentration whereas acid detergent fiber decreased by 3.3% at 1 unit/g, 4.2% at 1.5 units/g and 5.9% at 2 units/g of enzyme concentration. Cellulose and hemicellulose content also decreased gradually during 36 h of incubation and percentage decrease was 8 to 10.8% for cellulose and 19.3 to 23.4% for hemicellulose (from 1 to 2 units/g of enzyme concentration). Decrease in cellulose and hemicellulose content might be the result of breakdown of cellulose and hemicellulose into fermentable sugars. Lignin content before the start of treatment was 7% which increased to 7.47, 7.61 and 7.68% after treatment with 1, 1.5 and 2 units/g of enzyme concentration respectively. Silica content also increased by 5.6, 6.1 and 6.4 % at 1, 1.5 and 2 units/g of enzyme treatment respectively (Table 1). Similar results were reported by Zafar et al. (1980)

Table 2. Effect of enzymatic pretreatment on total solids, ash, volatile solids and total sugars.

Composition	Enzyme units (µmoles)	Time (h)				CD (5%)
		18	24	30	36	
Total solids	1	24.1±0.32(6.9↓)	23.7±0.17(8.5↓)	23.1±0.23(10.8↓)	22.7±0.26(12.3↓)	T= 0.426
	1.5	23.5±0.23(9.3↓)	23.3±0.23(10.0↓)	22.6±0.29(12.7↓)	22.3±0.32(13.9↓)	E= 0.369
	2	23.0±0.23(11.2↓)	22.5±0.26(13.1↓)	22.2±0.23(14.3↓)	21.9±0.23(15.4↓)	T×E= NS
Ash	1	17.6±0.29(4.8↑)	18.2±0.14(8.3↑)	19.0±0.17(13.1↑)	20.4±0.26(21.4↑)	T= 0.392
	1.5	17.9±0.26(6.5↑)	18.4±0.21(9.5↑)	19.8±0.23(17.8↑)	20.7±0.24(23.2↑)	E= 0.34
	2	18.1±0.20(7.7↑)	18.9±0.20(12.5↑)	20.9±0.20(24.4↑)	21.3±0.31(26.8↑)	T×E= NS
Volatile solids	1	82.4±0.29(0.96↓)	81.8±0.23(0.607↓)	81.1±0.21(1.45↓)	80.0±0.20(2.8↓)	T= 0.413
	1.5	82.0±0.28(0.36↓)	81.3±0.26(1.25↓)	80.2±0.23(2.55↓)	79.6±0.23(3.3↓)	E= 0.357
	2	81.7±0.23(0.73↓)	80.5±0.28(2.2↓)	79.4±0.23(3.5↓)	79.1±0.20(3.9↓)	T×E= NS
Total sugars	1	36.9±0.52(3.4↑)	37.4±0.23(4.8↑)	38.4±0.20(7.6↑)	38.7±0.23(8.4↑)	T= 0.417
	1.5	37.3±0.14(4.5↑)	37.7±0.26(5.6↑)	38.8±0.20(8.7↑)	39.3±0.18(10.1↑)	E= 0.362
	2	37.9±0.18(6.2↑)	38.2±0.18(7.0↑)	39.0±0.26(9.2↑)	39.7±0.14(11.2↑)	T×E= NS

#Data in parenthesis represent percentage increase or decrease as compared to control i.e. untreated paddy straw composition; T, time; E, enzyme concentration; Untreated paddy straw composition (%): Total solids=25.9, volatile solids=83.2, ash= 16.8, total sugars=35.7; ± values indicate % standard error for triplicate data; (↓): decrease; (↑): increase.

who showed decrease in cellulose content after treatment of paddy straw by *Pleurotus sajor caju*. Jafari et al. (2007) also reported decrease in hemicellulose, acid detergent fiber andneutral detergent fiber after pretreatment of rice straw with *Pleurotus spp*. Sahni (2013) also reported reduction in neutral detergent fiber, acid detergent fiber, cellulose and hemicellulose content and increase in lignin and silica content after pretreatment of paddy straw with *Humicola fuscoatra*. Phutela and Dar (2014) investigated the potential of microbial pretreatment under aerobic conditions on paddy straw digestibility by pretreating it with *Thermoascus aurantiacus* MTCC 375 at regular intervals of 1, 2, 3, 4 and 5 days and reported that pretreatment of 5 days significantly reduced the concentrations of cellulose, hemicelluloses, lignin and silica content of paddy straw. A maximum of 30% increase in biogas production was observed from one day pretreated paddy straw as compared to untreated paddy straw.

Effect of enzymatic pretreatment on total solids, ash, volatile solids and total sugars

Treatment of paddy straw with different enzyme concentrations and incubation periods resulted in decrease in total solids and volatile solids and increase in ash content. This increase or decrease in parameters increased with increase in enzyme concentration and incubation period. During 36 h of incubation total solids gradually decreased from 25.9 to 22.7, 22.3 and 21.9% at 1, 1.5 and 2 units/g of enzyme concentration respectively. However, ash content increased by 21.4, 23.2 and 26.8 % respectively at 1, 1.5 and 2 units/g of enzyme concentration. From 18 to 36 h volatile solids decreased from 82.4 to 80% at 1 unit/g, 82 to 79.6% at 1.5 units/g and 81.7 to 79.1% at 2 units/g of enzyme concentration. The percentage increase in total sugars was 8.4 to 11.2% from 1 to 2 units/g of enzyme concentration at 36 h. Total sugars increased gradually with increase in enzyme concentration and incubation period because of hydrolysis of cellulose into fermentable sugars by cellulase action (Table 2) as also indicated by decrease in cellulose and hemicellulose content (Table 1). Phutela et al. (2011) showed that treatment of rice straw with *Trichoderma reesei* MTCC 164 and *Coriolus versicolor* MTCC 138 resulted in decrease in total solids, volatile solids and increase in ash &total sugars. Phutela and Dar (2014) also reported reduction in total solids, volatile solids and enhancement in ash content of paddy straw when pretreated with thermophilic fungus *Thermoascus aurantiacus* MTCC 375.

Conclusion

Cellulases were produced from thermophilic fungus *H. fuscoatra* by solid state fermentation and maximum production was achieved at 6th day of incubation at pH 6.0 and temperature 45°C when spore suspension containing 10^7 spores/ml was used. The pretreatment of paddy straw with this enzyme resulted in decrease in NDF, ADF, cellulose and hemicellulose content of paddy straw. Thus, the cellulases produced from thermophilic fungus *Humicola fuscoatra* are capable of increasing paddy straw digestibility and thus enhancing the utilization of

paddy straw for different purposes such as for biogas production, ethanol production or as a feed for ruminants etc.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Microbial load of operating theatre at Ayder Referral Hospital, Northern Ethiopia

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Microbial contamination of the operating theatre (OT) is a major cause of nosocomial infection (NI). The study assessed the level of microbial contamination and determines the antimicrobial resistance of the bacterial isolates. Settle plate's method was used for air sample collection while swab method was used to collect samples from surfaces and other articles in the major OT. Collected samples were transported and microbiologically processed using standard procedures. One hundred and twenty air, 36 article and 12 surface samples were taken for microbiological evaluation. The highest level of microbial contamination was detected in the OT air before proper cleaning-fumigation as compared to after the intervention. Moreover, microbial growth was found on surfaces and semi-critical articles. On the other hand, articles which were sterilized by autoclave showed no microbial growth. The five types of bacteria isolated were coagulase negative *Staphyllococci* (68; 53.4%), *Staphyllococcus aureus* (42; 33.1%), *Pseudomonas* aeruginosa (13; 10.2%), *E. coli* (2; 1.6%), and *Bacillus* spp. (2; 1.6%). Methicillin resistance *S. aureus* (MRSA) account for 7.7% of the *S. aureus* isolates. The highest resistance was found against penicillin G and ampicillin with a resistance rate of 52.7 and 44.5%, respectively. Multidrug resistance was observed among 23 (36.5%) of the bacterial isolates. In general, the results indicate proper cleaning-fumigation of OT significantly reduced the microbial contamination, and bacterial strains such as coagulase negative *Staphylococci*, and *S. aureus* have a greater propensity to cause contamination in OT. In conclusion, there was high level of microbial contamination in the OT, particularly in air and semi-critical articles. However, it has been dramatically reduced through proper cleaning-fumigation of the OT. Therefore, regular microbiological surveillance of the OT is mandatory in reducing microbial contamination. Furthermore, efforts should be made to ensure strict infection control practices in the OT.

Key words: Microbial contaminants, operating theatre, antimicrobial resistance.

INTRODUCTION

Microbial contamination of the operating theatre (OT) is a major cause of nosocomial infections (NI) (Singh et al., 2013; Okon et al., 2012; Al-Benna, 2012, Ensayef et al., 2009). Its clinical implication is enormous on both the patient and the caring surgical team (Chacko et al., 2003; Okon et al., 2012). Approximately, 10% of all infections can have serious consequences in terms of increased patient mortality, morbidity and length of hospital stay and

Table 1. Antimicrobial resistance of bacterial isolates from major operation theatre (OT) at the Ayder Referral Hospital, Mekelle, Ethiopia.

Antibiotic (disc potency)	CoNS (n=68)	*S. aureus* (n=42)	*P. aeruginosa* (n=13)	*E. coli* (n=2)	Total (N= 125)
	Number (%) of resistance				
penicillin G (10IU)	32(47.1)	26(61.9)	NT	NT	58(46.4)
ampicillin (10 µg)	30(44.1)	19(45.2)	NT	NT	49(39.2)
chloroamphenicol (30 µg)	11(16.2)	10(23.8)	NT	1	22(17.6)
amikacin (30 µg)	2(2.9)	2(4.8)	0	0	4(3.2)
gentamicin (10 µg)	9(13.2)	3(7.1)	0	0	12(9.6)
ciprofloxacin (5 µg)	7(10.3)	0	0	0	7(5.6)
trimethoprim-Sulphamethoxazole (1.25/23.75 µg)	8(11.8)	0	NT	1	9(7.2)
tetracycline (30 µg)	10(11.7)	16(38.1)	NT	1	27(21.6)
vancomycin (30 µg)	0	0	NT	NT	0

NT: not tested, CoNS: Coagulase negative *S. aureus*

overall costs among patient admitted for post-operative surgery (Reddy, 2012); with multi-drug resistant strains like methicillin-resistant *Staphylococcus aureus* (MRSA) (Chacko et al., 2003). Antimicrobial resistance results in increased illness, death cases and health care costs. The emergence of multidrug resistant strains in a hospital environment, particularly in developing countries is an increasing infection control problem and associated with a high frequency of nosocomial infections (NIs) and anti-biotic resistance rate (De Lissovoy et al., 2009).

Multiple reservoirs have been reported as being responsible for the contamination of the OT, including unfiltered air, ventilation systems and antiseptic solutions, drainage of the wounds, transportation of patients and collection bags, surgical team, extent of indoor traffic, theatre gown, foot wares, gloves and hands, use of inadequately sterilized equipment, contaminated environ-ment and grossly contaminated surfaces (Fleischer et al., 2006; Okon et al., 2012). The impact of these sources on the degree of microbial contamination differs, depending on the numbers of pathogens involved. *S. aureus* and coagulase negative *staphylococci* (CoNS), for example, are the major pathogens associated with infection of implantable biomedical devices (Edmiston et al., 2005).

Microbial contamination of the OT can be prevented through adequate application of infection control practices. Reduction of airborne bacteria in the OT by about 13-fold, for example, would reduce the wound contamination by about 50% (Fleischer et al., 2006). This depends primarily on improving cleaning, proper disinfection and regular fumigation of OT (Okon et al., 2012; Chacko et al., 2003). In Ethiopia, data on the microbial contamination of OT is scarce. Therefore, this study was conducted to assess the level of microbial contamination and to determine the antimicrobial resistance of the bacterial isolates from major OT at Ayder Referral Hospital, Mekelle, Ethiopia (Table 1).

MATERIALS AND METHODS

After getting full approval from College of Health Sciences Ethical Review Committee, Mekelle University, the study was conducted to evaluate the microbial contamination of major operating theatres at the Ayder Referral Hospital, Mekelle, Ethiopia. Air and surface samples were collected from five major operating rooms without prior discussion with the cleaning staff. Air sampling (before and after cleaning-fumigation) was performed with passive air sampling (settle plate's methods) according to the 1/1/1 scheme (a Petri dish with a diameter of 9 cm was placed for 1 h, 1 m above the floor, about 1 m away from the walls or any major obstacles) (Ekhaise et al., 20008). Sealed Petri dishes containing 5% sheep blood agar (Oxoid, UK) were transported to major OT in a sealed plastic bag. The plates were labeled with sample number, set within theatre, time and date of sample collection. Then, they were placed at four chosen places in the operating room. Each day, the air samples were collected three times: in the morning between 10 and 11 a.m., lunch between noon and 1 p.m. and in the evening between 5 and 6 p.m. After this exposure, the plates were covered with their lids and then sealed. To check the sterility of articles and surfaces in the major OT, a sterile swab moistened with sterile normal saline was used to collect samples. All the plates were labeled properly and then, the swab was immediately streaked on to 5% sheep blood agar (Oxoid, UK). Upon inoculation, and plates were sealed and transported along with those exposed in the air to the Ayder hospital microbiology laboratory in sealed plastic bags and incubated at 37°C under aerobic conditions for 24 h.

For air samples, the total number of colony forming units (CFU) was enumerated using colony counter and results were expressed in CFU/dm^2/hour (Andersson et al., 2012). The bacterial isolates were identified up to species level based on different criteria

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which includes morpho-cultural and biochemical characteristics (Cheesbrough, 2006). Morpho-cultural characteristics including Gram stain was used to determine the bacterial Gram-reaction and colony appearance such as color (e.g. golden yellow colonies on mannitol salt agar (Oxoid, UK) is indicative of *S. aureus*), pigment production and haemolysis pattern on blood agar. Bacteria were further characterized by the pattern of biochemical reactions they produce; such as motility, indole, urease, oxidase, catalase, and carbohydrate utilization tests such as lactose fermentation on MacConkey agar (Oxoid, UK) for Gram-negative bacteria, and catalase, coagulase test and haemolysis pattern on 5% sheep blood agar (Oxoid, UK) was used for identification of Gram-positive bacteria. Screening of MRSA was done using oxacillin Screen Agar (Mueller Hinton Agar with 6 µg/ml oxacillin and 4% NaCl). The efficacy of the disinfectants used in the OT was also tested by qualitative suspension tests (Siridhar, 2014).

The antimicrobial susceptibility testing of the bacterial species identified before proper cleaning-fumigation was done on Mueller-Hinton agar (Oxoid, UK) against nine antibiotics by Kirby-Bauer disk diffusion method matching the test organism to 0.5 McFarland turbidity standards. Then, the susceptibility result was interpreted according to the Clinical Laboratory Standards Institute (CLSI, 2012) methodology (CLSI M100) (Patwardhan and Kelkar, 2011). *S. aureus* ATCC25923, *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as control bacterial strains to monitor the whole bacteriological procedures. Data obtained was subjected to statistical analysis using SPSS16.0 and Microsoft Office Excel 2007. A significant difference among the mean microbial air counts was tested with Kruskal-Wallis test.

RESULTS AND DISCUSSION

One hundred and twenty air (before and after cleaning-fumigation), 36 article and 12 surface samples were taken from the major OT for microbiological evaluation. The mean microbial count of the major OT air before cleaning-fumigation during morning, lunch, and evening time was 91.8 (SD 54.3), 44.9 (SD 29.3), and 17.2 (SD 17.1) CFU/dm^2/h, respectively, however, after proper cleaning-fumigation, this was significantly reduced to 42.6, 31.3 and 14.7 CFU/dm^2/h, respectively (Figure 1). The difference among the means was statistically significant (p=0.000). According to Fisher's index of microbial air contamination (Ekhaise et al., 20008), air microbial count of OT at rest and in activity should not exceed 9.0 and 91.0 CFU/dm^2/h, respectively. However, the results obtained before proper cleaning-fumigation indicates higher mean air microbial count, that is, at rest (evening) 17.2 CFU/dm^2/h and in activity (morning) 91.8 CFU/dm^2/h. Nevertheless, the microbial load reduced significantly to acceptable levels after proper cleaning-fumigation of the OT. This dictates proper infection control practices in the OT.

The critical articles which were sterilized by autoclave showed no microbial growth, whereas articles like endotracheal tubes and laryngoscope which were heat labile and disinfected by chemicals and surfaces like floors, operating table and OR light showed heavy growth of pathogens. The *in vitro* disinfectants efficacy tests indicate that the disinfectants were effective against the bacterial isolates and control bacterial strains, hence the

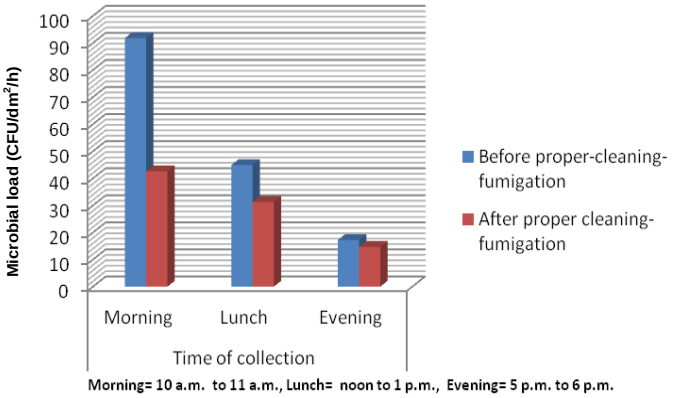

Figure 1. Bar chart showing the microbial load before and after proper cleaning-fumigation of the major operation theatre (OT) at the Ayder Referral Hospital, Mekelle, Ethiopia.

microbial growth on the OR articles may be due to improper preparation and/or application of these chemicals.

Although, the direct involvement of these fomites in disease transmission was not investigated in this study, the isolation of coagulase negative *Staphylococci* (CoNS) (68; 53.5%) *S. aureus* (42; 33.1%), *P. aerugionosa* (13; 10.2%), *Bacillus* spp. (2; 1.6%) and *E. coli* (2; 1.6%) presents a serious concern for possible nosocomial transmission. Among the bacterial pathogens isolated, CoNS (65; 55.1%) and *S. aureus* (38; 32.2%) had the highest percentage of occurrence in air samples. This finding is comparable with studies conducted in Jimma (Genet et al., 2011), India (Chacko et al., 2003), Pakistan (Saadoun et al., 2008) and Iraq (Ensayef et al., 2009). In these cases, the contamination source is usually endogenously from the normal skin flora of patients or exogenously from surgical staff, especially as CoNS was the main isolate in the OT air (Ensayef et al., 2009). However, this result contradicted with related study conducted in Iraq (Alsaimary et al., 2014) where the predominant isolate was *P. aeruginosa* (54%) followed by *Bacillus subtilis* (9.5%). It was observed that operating table, floor and OR light were heavily contaminated with *S. aureus*. This finding is in line with similar studies in India (De Lissovoy et al., 2009). However, this was contradicted with a study conducted in Pakistan (Saadoun et al., 2008), which reported *Bacillus* spp. This might be due to variation in methodology and sample size. Among the *S. aureus* isolated, only 5 (7.7%) were methicillin resistant (MRSA). This finding is significantly lower among similar studies conducted in Jimma (Genet et al., 2011) and Gaza (Al Laham, 2012) with MRSA prevalence rate of 100 and 33.3%, respectively.

The antimicrobial susceptibility pattern of bacterial isolates revealed that the most effective antimicrobials were vancomycin, amikacin, ciprofloxacin and gentamicin with a resistance rate of 0.0, 3.2, 75.6 and 9.6%, respectively. This result is in line with related study

conducted in Gaza ((Al Laham, 2012) and Jimma (Genet et al., 2011). Nevertheless, this finding is contradictory to similar study conducted in Iraq (Alsaimary et al., 2014) in which ciprofloxacin had the highest percentage of resistance (88.88%).

However, the highest resistance was observed against penicillin G and ampicillin with a resistance rate of 46.4 and 39.2%, respectively. On the other hand, *Staphyllococcus* spp. was found to be highly resistant against penicillin G and ampicillin (52.7 and 44.5%, respectively). Multidrug resistance was observed among 23 (36.5%) of the bacterial isolates. This is significantly lower than similar study in Gaza (Al Laham, 2012), in which 81.8% of the bacterial isolates showed resistance to at least two antibiotics.

Conclusion

In conclusion, there was high level of microbial contamination in the OT, particularly in air and semicritical articles. However, it has been dramatically reduced through proper cleaning-fumigation of the OT. Therefore, regular microbiological surveillance of the OT is mandatory in reducing microbial contamination; consequently postoperative infectious episodes can be reduced considerably. Furthermore, efforts should be also be made to ensure strict infection control practices in the OT.

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Effect of nitrogen fertilization associated with diazotrophic bacteria inoculation on nitrogen use efficiency and its biological fixation by corn determined using ^{15}N

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The use of plant growth-promoting diazotrophic bacteria as an alternative to increase nitrogen (N) availability and contribute to its use by corn can be a less costly and ecologically more viable option. Thus, this study aimed to evaluate the effects of the inoculation with *Azospirillum brasilense* and *Herbaspirillum seropedicae* in association with N fertilization on the N use efficiency and its biological fixation by corn, in field conditions, using the ^{15}N-isotope technique. A randomized-block design was used, with nine treatments and six replicates. The following parameters were evaluated: grain productivity, dry matter production, accumulated N in plant, percentage of N from the fertilizer, total N from the fertilizer, use efficiency of N applied as fertilizer and percentage of biological N fixation. Results show that N fertilization associated with *A. brasilense* and *H. seropedicae* inoculations influences positively grain productivity in corn. The inoculation with *A. brasilense* and *H. seropedicae* combined with 30 and 120 kg ha^{-1} of N promotes a reduction of N percentages in corn grains and shoot. The increase in N dose associated with the inoculation of *A. brasilense* and *H. seropedicae* promotes an increase in the N from the fertilizer in corn grains and shoot and reduces the N use efficiency by the crop. Using the ^{15}N natural abundance technique, it was found that the inoculation with *A. brasilense* and *H. seropedicae* contributed, respectively, with in average 19.40% and 9.49% of the N required for the development of corn plants.

Key words: *Zea mays* L., *Azospirillum brasilense, Herbaspirillum seropedicae*, ^{15}N, biological nitrogen fixation.

INTRODUCTION

Corn cultivation has a high demand for nitrogen fertilizers, making necessary the application of this nutrient to obtain high productivities (Bastos et al., 2008; Fidelis et al., 2007). Due to the high economic and environmental costs of the industrial process for nitrogen fixation, combined with the increase in food demand, there arises

the need to incorporate new technologies into agricultural activities, aiming to rationalize the use of nitrogen fertilizers. One option is using the benefits provided by the association between diazotrophic bacteria and crops of great economic interest, since these microorganisms are capable of promoting plant growth and increasing plant development and productivity (Baldani et al., 1997).

According to Hungria (2011), diazotrophic bacteria associated with grasses can stimulate plant growth in different ways. In addition to their capacity for biological nitrogen fixation (BNF) (Han et al., 2005; Huergo et al., 2008), they can act in the increase of nitrate reductase activity, when they grow endophytically (Cássan et al., 2008); in the production of plant hormones, such as auxins, gibberellins, ethylene etc. (Khaliq et al., 2004; Donate-Correa et al., 2004; Radwan et al., 2004; Creus et al., 2004; Dobbelaere et al., 2003); in the solubilization of zinc phosphates and oxides (Rodriguez et al., 2004; Baldotto et al., 2010); and in the biological control of pathogens (Mariano et al., 2004).

The genera *Azospirillum* and *Herbaspirillum* include a group of plant growth-promoting bacteria with a good capacity to associate with corn plants. These bacteria have nitrogenase enzymatic complex and are able to break the triple bond connecting the two nitrogen atoms and reduce N_2 to ammonia. However, N_2 fixation efficiency has not proved to be enough to meet corn demand (Hungria, 2011), thus complementary nitrogen fertilization is required in addition to the inoculation.

In general, nearly 70% of field experiments with diazotrophic bacteria inoculation, using various crops and under different soil and climate conditions, showed increases in productivity of up to 30% (Okon and Labandera-González, 1994), and this contribution is higher when plants receive variable doses of nitrogen fertilizers (Dobbelaere et al., 2003).

Currently, the ^{15}N isotope technique is the most used method to determine N use efficiency (NUE), when N is applied as a fertilizer in corn cultivation. Among the various results obtained, it is verified great variation of NUE in corn, with values ranging from 10 to 65% of the applied N (Lara Cabezas et al., 2000; Cantarella et al., 2003; Silva et al., 2003; Gava et al., 2006; Gava et al., 2010). BNF contribution to corn can also be quantified through the ^{15}N isotope technique, based on its natural abundance.

The use of plant growth-promoting bacteria (PGPB) as an alternative to increase N availability and contribute to its use by corn can be a less costly and ecologically more viable option. Given the aforementioned, this study aimed to evaluate the effects of *Azospirillum brasilense* and *Herbaspirillum seropedicae* inoculations associated with N fertilization on the N_2 use efficiency and its biological fixation by corn, in field conditions, using the ^{15}N-isotope technique.

MATERIALS AND METHODS

The experiment was carried out at the experimental field of Embrapa Western Region Agriculture, in Dourados-MS, from March to July 2012. Geographical coordinates of the area are 22° 14' S and 54° 9' W, with average altitude of 450 m. The climate of the region is classified as Cwa, according to Köppen's classification system. The soil was classified as Distroferric Red Latosol with very clayey texture (Embrapa, 2013). Average data of temperature and rainfall during the experiment were obtained from the Weather Station at Embrapa Western Region Agriculture, in Dourados-MS (Figure 1).

Results of the soil chemical analysis in the layer of 0-20 cm, before the experiment installation, were, pH ($CaCl_2$), 4.5; Organic matter, 31.18 g dm^{-3}; C, 18.13 g dm^{-3}; P (Mehlich), 22.07 mg dm^{-3}; K, 6.0 $mmol_c$ dm^{-3}; Ca, 35.4 $mmol_c$ dm^{-3}; Mg, 8.7 $mmol_c$ dm^{-3}; Al, 4.8 $mmol_c$ dm^{-3}; H+Al, 62.1 $mmol_c$ dm^{-3}; SB, 50.1 $mmol_c$ dm^{-3}; CEC, 112.2 $mmol_c$ dm^{-3}; Base saturation, 44.65%; Zn, 1.65 mg dm^{-3}; Cu, 9.27 mg dm^{-3}; Fe, 29.14 mg dm^{-3}; and Mn, 24.06 mg dm^{-3}. Granulometric analysis showed 215 g kg^{-1} of sand, 115 g kg^{-1} of silt and 670 g kg^{-1} of clay. Soil pH correction was performed a month before sowing, with 1720 kg ha^{-1} of dolomitic limestone (RNV 100%), considering soil analysis results, in order to increase base saturation to 60%. The area was irrigated after crop implementation and in periods with higher water deficit.

A randomized block design was used, with nine treatments and six replicates, as follows: 1) Control without N and without inoculation; 2) Inoculation with *A. brasilense*, without N; 3) Inoculation with *H. seropedicae*, without N; 4) 30 kg ha^{-1} of N at sowing; 5) *A. brasilense* + 30 kg ha^{-1} of N at sowing; 6) *H. seropedicae* + 30 kg ha^{-1} of N at sowing; 7) 30 kg ha^{-1} of N at sowing + 90 kg ha^{-1} of N in covering; 8) *Azospirillum brasilense* + 30 kg ha^{-1} of N at sowing + 90 kg ha^{-1} of N in covering; and 9) *H. seropedicae* + 30 kg ha^{-1} of N at sowing + 90 kg ha^{-1} of N in covering.

The seeds of the simple hybrid P3646H used in the study had been previously inoculated with a combination of two strains of *A. brasilense* (Ab-V5 and Ab-V6) (inoculant cell concentration of about 10^8), using a liquid inoculant, and the Z-94 strain of *H. seropedicae* (inoculant cell concentration of about 10^9), using a peat-based inoculant produced by Embrapa Agrobiology, Seropédica-RJ. The applied doses were 150 mL of the liquid inoculant for each 50 kg of corn seeds, and 250 g of the peat-based inoculant for each 10 kg of corn seeds. For the inoculation with the Z-94 strain of *H. seropedicae*, 60 mL of a 10% sugar solution (m/v) were added in order to increase the adhesion of the inoculant to the seeds.

Base fertilization was applied by broadcasting at sowing, with later incorporation, using 300 kg ha^{-1} of a 0-20-20 formulation to supply 60 kg ha^{-1} of P_2O_5 and K_2O, respectively. Planting was performed manually, with the aid of a hand-held corn planter (known as "matraca"), placing two seeds per hole and leaving six plants per linear meter after thinning.

Each experimental unit was composed of five 5-m rows, spaced 0.90 m apart. The three central rows were considered as the useful plot area, excluding the last 0.5 m of each row. A microplot was

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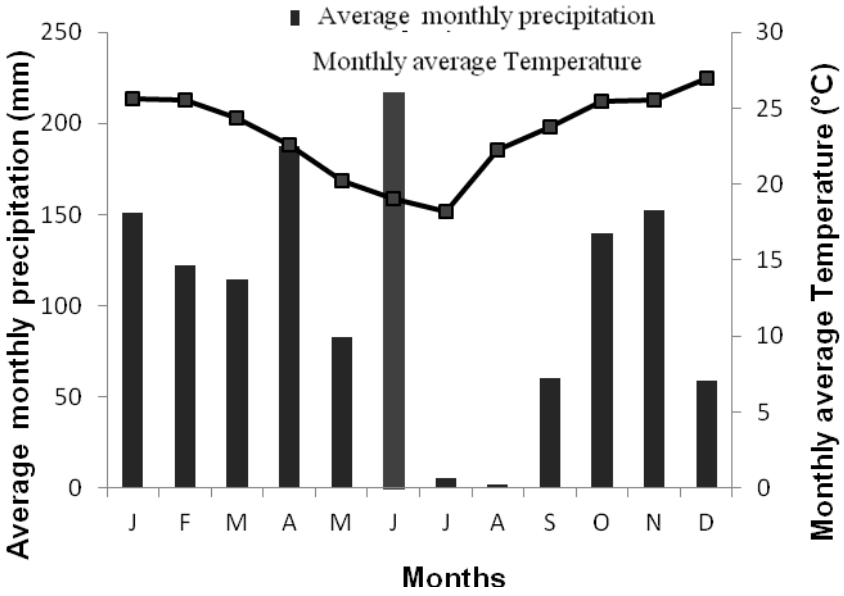

Figure 1. Monthly average rainfall (mm) and temperature (°C), recorded at the Weather Station of Embrapa Western Region Agriculture, in Dourados-MS, Brazil in 2012.

installed in each plot and urea marked with ^{15}N was applied. Each microplot had one 2-m row, and the sampling area was restricted to 1 m in the center of the line.

As for N fertilization, 30 kg ha^{-1} of N were applied in the planting furrow and 90 kg ha^{-1} of N were divided into two applications of 45 kg ha^{-1} and applied in covering as urea (45%) in the whole plot, except for the microplots.

In the treatments 4, 5 and 6, N fertilization was applied as urea enriched with 2.6% of ^{15}N atoms in excess. For these treatments, 12 g of marked urea were applied in each 2-m microplot.

In the treatments 7, 8 and 9,fi N fertilization was applied as urea enriched with 0.7% of ^{15}N atoms in excess, totaling 120 kg ha^{-1} of N, with 30 kg ha^{-1} of N applied at sowing and 90 kg ha^{-1} of N applied in covering, which were divided into two times of 45 kg ha^{-1}. In these treatments, 12 g of urea enriched with 2.6% of ^{15}N atoms in excess were applied at sowing and 36 g of urea enriched with 0.7% of ^{15}N atoms in excess, divided into two times of 18 g, were applied in covering in order to meet the 90 kg ha^{-1} of N. The first N application in covering was performed during the V4 development stage, which corresponds to 4 leaves fully expanded, and the second application during the V7 development stage, corresponding to 7 leaves fully expanded.

Corn harvest was performed manually, collecting all plant ears from the plot useful area (9.0 m²). In order to determine grain productivity, ears were threshed with the aid of a manual device and then weighed. The obtained results were converted to kg ha^{-1}, correcting the humidity to 13% on a wet basis. Plant dry matter (straw) was estimated through the sampling of three plants inside each microplot. Dry matter was determined by drying the samples in a forced-air oven at constant temperature of 65ºC for 72 h. Dry matter data were expressed in kg ha^{-1}.

At the harvest, plants were divided into shoot (straw) and grains. All collected plant material was washed in a detergent solution (3 mL L^{-1}), running water, 0.1 mol L^{-1} solution of HCl and deionized water, respectively. Then, samples were accommodated in paper bags and dried in a forced-air oven at 65ºC for 72 h. After drying, all the plant material was weighed and ground in a Wiley-type mill for the analyses of Total N and N isotopic composition.

Total N in different plant parts (straw and grains) was determined through the Kjeldahl method, according to the methodology described in Embrapa (2009). As for the ^{15}N isotopic composition analyses, samples were processed according to the methodology in Rittenberg (1946); using the final distillate obtained in the Total N% analysis, the extracts were again acidified with 0.5 mol L^{-1} H_2SO_4 and concentrated through evaporation, and the $N\text{-}NH_4^+$ was converted to N_2 through oxidation with lithium hypobromite (LiOBr) (Porter and O'deen, 1977). ^{15}N isotopic composition analyses were performed in a Delta Plus mass spectrophotometer, from the John M. Day Stable Isotope Laboratory at Embrapa Agrobiology. With the results of nitrogen isotopic composition (% of ^{15}N atoms) in the samples, the following parameters were calculated:

Total N content accumulated in the plant (TN, mg/plant):

$$TN = \frac{DMY \times N}{100}$$

Where, DMY is the dry matter yield and N is the N content in the plant (g kg^{-1}).

b) Percentage of N in the plant coming from the fertilizer (%PNF):

$$\%PNF = \left(\frac{\%\text{ in 15N atoms excess plant test}}{\%\text{ in 15N excess fertilizer}}\right) \times 100$$

c) N in the plant coming from the fertilizer (PNF):

$$PNF = \frac{\%PNF \times DMY}{100}$$

d) Use efficiency of N applied as a fertilizer as a function of the

Table 1. Productivity (PRO), total dry matter (MST), grain N percentage (%NG), shoot N percentage (%NPA), total grain N (NTG) and total shoot nitrogen (NTPA) of corn plants in response to nitrogen fertilization and inoculation with *Azospirillumbrasilense* and *Herbaspirillum seropedicae*. Dourados-MS, 2012.

Treatment	PRO	MST	NG	NPA	NTG	NTPA
	kg ha^{-1}		%		kg ha^{-1}	
1. Control	9231.71b	9977.91	1.66ab	0.86ab	154.46ab	83.61
2. *A.brasilense*	9078.41b	9802.84	1.82ab	0.90a	165.67ab	89.85
3. *H. seropedicae*	9023.88b	10098.58	1.99a	0.79abc	180.83a	80.02
4. 30 kg ha^{-1} N	9302.05b	10633.24	1.29cd	0.62bc	121.33c	67.64
5. *A.brasilense* + 30 kg ha^{-1} N	9531.75ab	11587.76	1.23d	0.58c	117.58c	68.76
6. *H. seropedicae* + 30 kg ha^{-1} N	9133.32b	9531.11	1.25d	0.66bc	114.92c	63.74
7. 30 kg ha^{-1} N + 90 kg ha^{-1} N	10146.52a	10198.58	1.26cd	0.85ab	128.31bc	87.46
8. *A. brasilense* + 120 kg ha^{-1} N	9861.37ab	11197.74	1.35cd	0.75abc	134.02bc	84.37
9. *H. seropedicae* + 120 kg ha^{-1} N	9858.23ab	11867.71	1.47bcd	0.76abc	144.98bc	92.88
Média	9463.03	10543.89	1.48	0.75	140.23	79.81
Teste F	4.76*	1.55^{ns}	10.02*	4.52*	6.39*	1.81^{ns}
CV (%)	4.81	15.42	14.50	16.95	15.85	24.05

*and ^{ns}significant 5% probability and non-significant, respectively. Medium followed by the same letter in the columns, do not differ statistically between themselves by Tukey test, the 5% probability.

applied quantity (NAQ):

$$\text{NUE} = \frac{\text{PNF}}{\text{NAQ}} \text{x } 100$$

BNF contribution to corn was quantified through the ^{15}N-isotope dilution technique, based on the natural abundance of ^{15}N (Shearer and Kohl, 1986). In addition to the corn plant samples obtained at the R6 stage, non-leguminous spontaneous species were collected to serve as a reference for the natural abundance of ^{15}N available in soil. Thus, three plants of each control treatment replicate were collected as a reference, which were used to estimate BNF: *Commelia benghalenis* L., *Digitaria insularis* and *Cenchrus echinatus*. The collected material was dried in an oven at 65ºC, ground and analyzed for ^{15}N natural abundance (Okito et al., 2004).

BNF estimate through the ^{15}N natural abundance was calculated using the equation:

$$\%\text{FBN} = (1 - \frac{\%\text{ atoms of 15N in excess in plant test}}{\%\text{ atomos of 15N in excess control}}) \text{x}100$$

Results were subjected to variance analysis and means were compared using Tukey test at 5% of probability, using the statistical software SISVAR (Ferreira, 2000).

RESULTS

The highest corn grain productivities were obtained in the treatment fertilized with 120 kg ha^{-1} of N, which did not differ statistically from that inoculated with *H. seropedicae* and supplied with 120 kg ha^{-1} of N and that inoculated with *A. brasilense* and supplied with 30 and 120 kg ha^{-1} of N (Table 1). It should be pointed out that the treatment fertilized with 120 kg ha^{-1} of N promoted an increase of about 10% in grain productivity compared to the control, without either N fertilization or inoculation. The treatment inoculated with *H. seropedicae* and supplied with 120 kg ha^{-1} of N and that inoculated with *A. brasilense* and supplied with 30 and 120 kg ha^{-1} of N promoted increases in grain productivity of about 6.78, 6.82 and 3.25%, respectively, although they had not shown any significant difference compared to the control. In average, these treatments promoted an increase of 518.74 kg ha^{-1} in corn grains compared to the control, which represents a gain of 8.64 bags per hectare, suggesting the applicability of inoculation associated with nitrogen fertilization for corn cultivation.

The percentage of N in grains (%NG) and the total N in grains (NTG) of corn plants showed significant differences between treatments inoculated with diazotrophic bacteria and supplied with 30 and 120 kg ha^{-1} of N compared to the control (without either N fertilization or inoculation) (Table 1). The treatments inoculated with *A. brasilense* and *H. seropedicae* supplied with 30 and 120 kg ha^{-1} of N showed reduction in %NG and, as a consequence, in NTG. As for the N shoot percentage (%NPA), the inoculation with *A. brasilense* promoted the highest increase, not differing from the control and the treatments inoculated

Table 2. Percentage of N in grains from the fertilizer (%NGPF), percentage of N in the shoot from the fertilizer (%NPAPF), total grain N from the fertilizer (QNGPF), total shoot N from the fertilizer (QNPADF), N use efficiency in grains (EUNG), N use efficiency in the shoot (EUNPA) and N use efficiency by corn plants (EUNPL) in response to nitrogen fertilization and inoculation with *Azospirillum brasilense* and *Herbaspirillum seropedicae*. Dourados-MS, 2012.

Treatment	NGPF	NPAPF	QNGPF	QNPAPF	EUNG	EUNPA	EUNPL
	%		kg ha^{-1}			%	
4. 30 kg ha^{-1} N	11.70b	17.66b	14.33b	11.77b	47.76	39.26	87.03a
5. A.brasilense + 30 kg ha^{-1} N	11.58b	17.73b	13.70b	12.22b	45.68	40.73	86.44a
6. H. seropedicae + 30 kg ha^{-1} N	11.63b	17.30b	13.26b	10.90b	44.22	36.34	80.57a
7. 30 kg ha^{-1} N + 90 kg ha^{-1} N	38.74a	43.83a	49.33a	38.36a	36.54	28.42	64.96b
8. A. brasilense + 120 kg ha^{-1} N	36.86a	39.69a	49.15a	33.61a	36.40	24.89	61.30b
9. H. seropedicae + 120 kg ha^{-1} N	37.27a	40.19a	52.84a	38.48a	39.14	28.50	67.64b
Average	24.63	29.40	32.10	24.22	41.63	33.79	74.66
Test F	45.28*	37.63*	76.41*	14.22*	2.08^{ns}	2.09^{ns}	3.31*
CV (%)	21.05	17.72	17.54	37.29	19.99	21.37	21.31

* and ^{ns}significant 5% probability and non-significant, respectively. Medium followed by the same letter in the columns, do not differ statistically between themselves by Tukey test, the 5% probability.

and fertilized with 120 kg ha^{-1} of N (Table 1).

In average, the percentage of N in grains from the fertilizer (%NGPF) was equal to 11.63% in the treatment fertilized with 30 kg ha^{-1} of N and in the treatments with *A. brasilense* and *H. seropedicae* supplied with 30 kg ha^{-1} of N, not differing statistically (Table 2). However, when supplied with 120 kg ha^{-1} of N in the absence and presence of inoculation with *A. brasilense* and *H. seropedicae*, %NGPF was in average 37.62%, statistically differing ($p \leq 0.05$) from the treatments inoculated and supplied with 30 kg ha^{-1} of N (Table 2). The percentage of shoot N from the fertilizer (%NPAPF) was equal to 17.56% in the treatments fertilized with 30 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae*, while %NPAPF was equal to 41.23% when there was the addition of 120 kg ha^{-1} of N. However, there was an average increase higher than 100% with the addition of 120 kg ha^{-1} of N compared to the addition of 30 kg ha^{-1} of N (Table 2).

The total N in grains from the fertilizer (QNGPF) and the shoot N from the fertilizer (QNPAPF) followed the same trend observed for %NGPF and %NPAPF. There were average increases of 266 and 216 kg ha^{-1} of N in corn grains and shoot, respectively, when fertilized with 120 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae* compared to the treatments fertilized with 30 kg ha^{-1} of N and inoculated (Table 2).

However, N use efficiency by the plant (EUNPL) was contrary to the previous parameters. Corn plants fertilized with 30 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae* showed the highest percentages of N use efficiency. In average, EUNPL in these treatments was equal to 84.65% compared to 64.63% of the ones fertilized with 120 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae* (Table 2). It is verified that N use efficiency was not calculated for treatments without N application, since it takes into account the N applied to soil, which did not happen in these cases.

Results of N use efficiency for the grains (EUNG) and for shoot (EUNPA) were not significantly different ($p \geq 0.05$). However, EUNG and EUNPA had average values of 45.88 and 38.77%, respectively, in the treatments fertilized with 30 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae,* corroborating the results found for EUNPL (Table 2).

The evaluation of BNF quantification (Tables 3 and 4) shows the capacity of the P3646H corn genotype to obtain N from BNF for its development. For these evaluations, *Commelia benghalenis* L., *Digitaria insularis* and *Cenchrus echinatus* were used as reference plants (RP) to calculate BNF, along with plots without the addition of mineral N.

Table 3 shows the results of RP used as a control in the determination of BNF contributions. It was observed that BNF values for the P3646H corn hybrid inoculated with both *A. brasilense* and *H. seropedicae* were not significant. RP values for ^{15}N natural abundance were lower than the ones for corn plants, which did not allow the application of the ^{15}N technique described in Shearer and Kohl (1986) to estimate BNF contribution for corn.

Table 4 shows the results for non-inoculated plants that were used as a control in the determination of BNF contributions. For that, it was assumed that the BNF contributions to these plants through the association with native diazotrophic microbes (not inoculated) occurred uniformly for all tested treatments. Therefore, the values refer to the BNF gains obtained with the inoculation of the selected strains of *A. brasilense* and *H. seropedicae*. It was verified that the BNF rate for the P3646H corn hybrid inoculated with *A.brasilense* in these evaluations was significantly higher ($p \leq 0.05$) than that inoculated with

Table 3. Mean values of δ ^{15}N of inoculated and non-inoculated treatments, estimated by the analysis of ^{15}N natural abundance for corn plants in the second cropping season.

Treatment	δ ^{15}N	δ ^{15}N PR1	δ ^{15}N PR2	δ ^{15}N PR3	δ ^{15}N media
1. Control	8.0 ± 0.56				
2. *A.brasilense*	6.7 ± 0.32	6.4 ± 0.04	6.26 ± 0.06	6.60 ± 0.35	6.45 ± 0.12
3. *H. seropedicae*	7.3 ± 0.74				

Averages of four repetitions, using plants as reference control for BNF. PR1, *Commelia benghalenis L.*; PR2, *Digitaria insularis*; PR3, *Cenchrus echinatus*.

Table 4. Mean values of δ ^{15}N and BNF of inoculated treatments, estimated by the analysis of ^{15}N natural abundance for corn plants in the second cropping season.

Treatment	δ ^{15}N inoculado	δ ^{15}N não inoculado	FBN (%)
1. *A.brasilense*	6.7 ± 0.32	8.0 ± 0.56	19.40
2. *H. seropedicae*	7.3 ± 0.74		9.49b

Averages of six repetitions, using plants inoculated as control to BNF.

H. seropedicae. The inoculation with *A. brasilense* contributed with 19.40% of the N from BNF, while the inoculation with *H. seropedicae* contributed with 9.49% of the N from BNF (Table 4). These results confirm corn potential for BNF, corroborating results from other studies, claiming that BNF is responsible for meeting part of the crop N demand (Riggs et al., 2001; Dobbelaere et al., 2002).

DISCUSSION

In a study conducted by Neto (2008), the inoculation of a product based on *A. brasilense* provided significant increase in grain productivity, from 9021 to 9814 kg ha^{-1}, that is an average increase of 9%. Alves et al. (2014) observed increases of 34 and 24% in corn productivity with the use of *H. seropedicae* in the first and second cropping seasons, and found that the inoculation can supply up to 40 kg ha^{-1} of N. Dalla Santa et al. (2004), studying inoculations of the RAM-7 and RAM-5 strains of *Azospirillum* sp., observed a reduction of 40% in the amount of nitrogen fertilizer required for corn. Hungria et al. (2010) and Lana et al. (2012) verified that the inoculation with *A. brasilense* promoted increases of 26 and 15.4%, respectively, in corn grain productivity. In 2013, Canellas and co-workers observed increase of 65% in corn grain productivity, compared to the control, when inoculated with *H. seropedicae* in combination with humic substances.

According to Huergo et al. (2008) and Hungria et al. (2011), increases in corn dry matter production and grain productivity in response to inoculation can be attributed to the stimulus that diazotrophic bacteria provide to root system development, increasing root hair density, length, volume and number of lateral roots, resulting in higher capacity to absorb and use water and nutrients.

It should be pointed out that the treatments with inoculation of diazotrophic bacteria and addition of 30 kg ha^{-1} of N show similar results to the treatment with the highest N dose (120 kg ha^{-1} of N) for grain productivity (Table 1), which suggests that the application of 30 kg ha^{-1} of N is less costly compared to the dose of 120 kg ha^{-1} of N. Therefore, the tendency to adopt this technology in corn cultivations can promote a reduction in the use of synthetic nitrogen fertilizers and, consequently, a reduction in production costs.

Rodrigues et al. (2006) and Pedraza et al. (2009), verified significant increase in % N in wheat and rice grains with the inoculation of A*zospirillum* spp., without N addition. Guimarães (2006) observed increases of 64% in the N accumulation in the grains of rice plants (IR 42 variety) inoculated with the ZAE-94 strain and fertilized with 50 kg ha^{-1} of N, compared to the control, without either N fertilization or inoculation.

The results reveal that both the percentage and the total N from the fertilizer were proportional to the applied N doses and the inoculations with *A.brasilense* and *H. seropedicae.*

Studies on N use efficiency in production systems are essential, because as the applied quantity exceeds plant's capacity to absorb it for production, nitrogen can be leached or accumulated in plant tissues, reducing its use efficiency. In the present study, the highest N use efficiency values were obtained for plants under 30 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae*, which did not differ statistically. These results reveal that corn plants did not have the potential to use N more

efficiently in doses higher than 30 kg ha^{-1} of N in field conditions and inoculated with diazotrophic bacteria. Reduction of N use by corn plants as N doses increase has also been reported by other authors (Fernandes et al., 2005; SILVA et al., 2009). Alves et al. (2006), evaluating N fertilization in corn in a Distroferric Red Latosol, found EUNPL values of 18% for the dose of 25 kg ha^{-1} of N applied at sowing; while even higher EUNPL values, 62% for corn, were found for the dose of 45 kg ha^{-1} of N when applied 26 days after emergence.

Many studies on corn, using ^{15}N-isotope methods in a Red Latosol, have shown differences in the efficiency to recover N from the fertilizer: 26 to 49% (Figueiredo et al., 2005); 40 to 50% (Silva et al., 2006); 45% (Gava et al., 2006) and 39% (Duete et al., 2008). The variation in the use of N from mineral fertilizers by corn is due to many factors, especially to soil and climate conditions, type of fertilizer, fertilization management (dose, time and way of application) and cultivation system (direct seeding or conventional tillage) (Lara-Cabezas et al., 2004; Duete et al., 2008). N losses by leaching, volatilization, denitrification, erosion and the microbial immobilization of N also influence the use of N from mineral sources (Lara-Cabezas et al., 2004; Figueiredo et al., 2005).

Many diazotrophs have been identified in corn (Baldani et al., 1980, 1986; Chelius and Triplett, 2000, 2001; Perin et al., 2006) and described as plant growth-promoting bacteria, but the BNF contribution to corn has not been clearly documented (Chelius and Triplett, 2000; Gutiérrez-Zamora and Martínez-Romero, 2001). García de Salamone et al. (1996) verified through the ^{15}N-isotope technique that *Azospirillum* spp. inoculation contributed significant BNF levels depending on the corn genotype. Alves et al. (2006), using the ^{15}N natural abundance technique, found that the SHS 5050 corn hybrid had N contributions of 45 and 36% from BNF in experiments in the first and second cropping seasons, respectively, when inoculated with the BR 11417 strain of *H. seropedicae*. Montanez et al. (2009) observed a BNF variation of 12 to 33% in a series of commercial corn cultivars in Uruguay using the ^{15}N-isotope dilution method.

Conclusions

Nitrogen fertilization associated with the inoculation of *A. brasilense* and *H. seropedicae* influences positively corn grain productivity.

Inoculation with *A. brasilense* and *H. seropedicae* combined with 30 and 120 kg ha^{-1} of N promotes a reduction in the percentage of N in corn grains and shoot.

The increase of the N dose associated with the inoculation of *A. brasilense* and *H. seropedicae* causes increase in the total N from the fertilizer in corn grains and shoot, and reduces N use efficiency by the crop.

Using the ^{15}N natural abundance technique, the inoculation of *A. brasilense* and *H. seropedicae* contributed, respectively, with an average of 19.40 and 9.49% of the N needed for corn crop development.

Conflict of interests

The authors did not declared any conflict of interests.

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Full Length Research Paper

Molecular typing of *Salmonella enterica* serovars Typhimurium and Enteritidis isolated from Taif area of Saudi Arabia by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)

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Twenty three isolates of *Salmonella enterica* representing two serovars, *Salmonella* Typhimurium and *Salmonella* Enteritidis isolated from three hospitals in Taif province, Kingdom of Saudi Arabia, were allocated to 23 genomic types using random amplified polymorphic DNA RAPD-PCR employing four 10-mer arbitrary primers. Jaccard's similarity coefficients were used to reveal the genetic diversity among isolates. Random amplified polymorphic DNA-polymerase chain reactioin (RAPD-PCR) amplified a total of 55 distinct bands (54 polymorphic bands or 98%). Cluster analysis based on the combined data from the four primers indicated that each of the *S.* Enteritidis or *S.* Typhimurium isolates had a distinct RAPD type. Principal component analysis (PCA) showed that the first three components explained about 65 and 33% of the total variability among RAPD types belonging to serovars Enteritidis and Typhimurium, respectively. The isolates belonging to serovar Enteritidis exhibited considerable genetic variation between locations, while those belonging to serovar Typhimurium exhibited considerable genetic variation both within and between locations. RAPD-PCR yielded highly reproducible results that demonstrated the high genetic heterogeneity of *Salmonella* isolates. Several genovars were present among *Salmonella* serovars and no specific RAPD type (or genovar) was found to be predominantly circulating in Taif province. The results presented here indicated that RAPD analysis has a great discriminatory power for the differentiation of *Salmonella* isolates and, therefore, can be a useful tool for the analysis of *Salmonella* genovars.

Key words: *Salmonella* genovars, Taif province, random amplified polymorphic DNA (RAPD) profiles, RAPD types, discrimination index, cluster analysis, principal component analysis.

INTRODUCTION

Salmonella food poisoning is one of the most common and widely distributed diseases in the world (Rodrigues et al., 1990; WHO, 2005), estimated to cause 1.3 billion cases of gastroenteritis and 3 million deaths worldwide (Bhunia,

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2008). *Salmonella* is a Gram negative facultative rod-shaped bacterium in the same proteobacterial family as *Escherichia coli*, the family Enterobacteriaceae, trivially known as "enteric" bacteria (Andrews and Baumler, 2005; Parry, 2006; Bhunia, 2008). *Salmonella* is associated mainly with raw meats, poultry and dairy products (Yang et al., 2010). However, many other foods have been implicated in outbreaks caused by *Salmonella*, and these include mayonnaise, salads, milk, unpasteurized orange juice, seafood, sprouted seeds and eggs (Pui et al., 2011). It gets into other foods by cross contamination from contact with raw foods, utensils, equipment and hands. Infection by non-typhoid salmonella (NTS) is much more common and usually causes gastroenteritis, ranges from mild self-limiting diarrhea, abdominal pain, nausea and vomiting lasting from 1-7 days to severe diarrhea that requires hospitalization (Panhotra et al., 2004; Parry, 2006). Healthy adults rarely suffer other symptoms and the mortality rate is <1%. Children, the elderly and the immuno-compromised patients may develop much more severe infections, such as septicaemia (Grassl and Finlay, 2008). Salmonella infection has also been associated with wound infections, meningitis and urinary tract infections (CDC, 2011).

To decrease morbidity and mortality due to systemic salmonellosis, antimicrobial therapy must be started immediately using one of the three commonly used antibiotics in tropical countries for the treatment of systemic NTS infections, that is, ampicillin, co-trimoxazole and chloramphenicol (Crump et al., 2011).

There are over 2,500 different serotypes of *Salmonella*, but most commonly reported, *Salmonella* Typhimurium and *Salmonella* Enteritidis, together account for at last 70% of reported human infection in Europe (Pires et al., 2010; CDC, 2011). In Saudi Arabia, serovar Typhimurium and Enteritidis were the most frequently isolated serovars from humans and animals (Halawani and Shohyeb, 2006; Boyen et al., 2008; Abdullahi, 2010). The biochemical, serological and molecular techniques were employed to detect and characterize Salmonella isolates in human fecal samples obtained from the western region of Saudi Arabia by amplifying the *inv*A and *hil*A genes to understand the genetic links between those isolates which spread in Taif Province (Ohud et al., 2010). The commonest serovars associated with human disease are *S.* Typhimurium and *S.* Enteritidis, but many others have been shown to cause disease, such as *Salmonella* notably *Salmonella* Infantis, *Salmonella* Virchow and *Salmonella* Newport (Mishu et al., 1994; Andrews and Baumler, 2005).

One hundred and forty-two cases of Salmonella infection were seen in Asir region, Southern Saudi Arabia, during the period of 1989-1991(Malik et al., 1994). Thirty-six cases of Salmonella infection were reported in Taif region, Saudi Arabia during 2002-2003. The two main serotypes were *S.* Typhimurium (38.9%) and *S.* Enteritidis (36.1%) (Halawani and Shohayeb, 2006). Improperly prepared fruits, vegetables, dairy products and shellfishs have also been implicated as sources of *Salmonella*. In a wedding ceremony in 2010 in Riyadh, Kingdom of Saudi Arabia, 283 individual developed infection with *Salmonella enterica*, 88% developed gastroenteritis, most commonly manifested by diarrhea (100%), abdominal pain (94.31) and fever (86.4%).

Individual serovars can be further characterized (typed) by a number of methods, including an antibiotic resistance profiles (Hanes, 2003; Nayak et al., 2004; Bhunia, 2008, Halawani and Shohyeb, 2008).

Classical typing methods for identification of isolates involve serotyping based on the Kauffmann-White serological scheme that targets the cell surface O and H antigens. This system currently identifies more than 2500 serotypes worldwide (Grimont and Weill, 2007). Phage typing systems have been commonly used method of *Salmonella* typing since 1950, and developed for further discrimination of serovars commonly associated with disease such as *S. enterica* serovar Typhimurium and Enteritidis (Anderson et al., 1977; Ward et al., 1987). More recently, molecular typing methods have been assessed and adopted for further discrimination of *Salmonella* isolates (Weigel et al., 20004; Halwani and Shohayb, 2008; Bugarel et al., 2011).

In recent years, *S.* Typhimurium and *S.* Enteritidis have emerged as a major serovars in the world (Rodrigues et al., 1990; WHO, 2005). Because of the increasing role of *S. enterica* serovar Typhimurium and Enteritidis in salmonella infections in Saudi Arabia (Nabbut et al., 1982; Al-Nakhli et al., 1999; Panhotra et al., 2004; Halawani and Shohayeb, 2008; Moussa et al., 2010), establishment of molecular typing data for this *Salmonella* serovars is important.

A PCR-based typing method, random amplified polymorphic DNA (RAPD)-PCR, has been described as a simple and rapid method able to offer detailed fingerprinting of the genomic composition of the organism (Williams et al., 1990; Weigel et al., 2004; Ammari et al., 2009; Stella et al., 2011; Rezk et al., 2012). The success of this method is due to the fact that no prior sequence information about the target is needed and a single short 10- mer oligonucleotide primer can be used in the reaction. The amplification happens at low stringency, allowing the primers to anneal to several locations on the two strands of the DNA. These primers detect polymorphisms in the absence of specific sequence information and the DNA sequence variations may work as genetic markers that can be used in epidemiologic studies (Quintaes et al., 2002; Lim et al., 2005).

The molecular typing data may be useful in recognizing and identifying the infectious strains and the clonality of isolates which cause the food borne outbreaks and this would determine the epidemiologic and prevalence of strains of salmonella food poisoning infections (Terajima et al., 1998; Tsen and Lin, 2001; Halawani and Shohayeb, 2008; Moussa et al., 2010; Moussa et al., 2011). In the

present study, molecular typing method (RAPD-PCR) was performed in order to establish the genetic relationships among salmonella clinical isolates obtained from the Western region of Saudi Arabia and to understand the epidemiological links between these isolates which spread in this area.

MATERIALS AND METHODS

Bacterial samples

Twenty three *Salmonella* strains were isolated from human fecal samples obtained during 2010 from local general hospitals and private clinical laboratories in Taif province, KSA. *Salmonella* isolates were characterized biochemically using API 20E strip (bioMe´rieux®, Inc., France), serologically by using Salmonella O and H antisera (Remel Europe Ltd., UK), and molecularly by strain specific PCR (Ohud et al., 2010) and RAPD-PCR (This study). Twenty serovars were confirmed positive by using Salmonella O and H antisera while three isolates could not be classified as *Salmonella* spp (Ohud et al., 2010). The isolates were as follows: S1, S5, S15, S16, S17, S18, S18, S20, S21, S22 and S23: Pediatric hospital; S2, S3 and S4: King Faisal Hospital; S6, S7, S8, S9, S10, S11, S12, S13 and S14: Al Edwani Hospital, Taif Province.

Random primers

RAPD fingerprinting of *S.* Typhimurium and *S.* Enteritidis was carried out using four decamer random primers designated OPA-13(5'-CAGCACCCAC-3'), OPB-10 (5'-CGTCTGGGAC-3'), OPB-18 (5'-CCACAGCAGT-3') and OPJ-10 (5'-AAGCCCGAGG-3' (Un-Ho Jin et al., 2000; Ohud et al., 2010). The oligonucleotide primers were commercially synthesized by Operon, A Qiagen Company, Germany. All primers used were resuspended in dd H_2O and stored at -20°C in aliquots to be used in PCR.

Preparation of genomic DNA

Genomic DNA was extracted from cell suspensions of bacteria grown overnight on xylose-lysine-deoxycholate (XLD) broth at 37°C using QIAamp® DNA Mini kit from Qiagen according to Lee et al. (2009). To elute bacterial DNA, 100 µl elution buffer (AE) was added to the center of the column, and the column was incubated at 37°C for 5 min and centrifuged at 20,000 xg for 1 min. DNA purity and quantity was determined using a GeneSys 10 UV spectrophotometer (Thermo Scientific, USA). The quality of *Salmonella* DNA prepared in this study was verified by PCR amplification of the *inv*A and *hil*A genes from the bacterial genome.

RAPD-PCR

The reaction mixture (25 µl) contained 10 mM Tris–HCl, pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 0.1 mM dNTPs, 15 pmol of the RAPD primer, 20 ng genomic DNA and 0.8 U of Taq DNA polymerase. Amplification was carried out in a heated-lid PXE 0.5 Thermal cycler (Thermo Electron Corporation, USA) for 40 cycles, each consisting of a denaturing step of 1 min at 94°C, followed by annealing step of 1 min at 36°C and an extension step of 2 min at 72°C. The last cycle was followed by 7 min of long extension at 72°C. The amplification products were separated by gel electrophoresis in 2% agarose (Ambion , USA) in 45 mM Tris-borate, 1 mM EDTA buffer (pH = 8.0), containing ethidium bromide at 0.5 g/ml at a constant voltage of 5 V/cm. The gels were photographed under UV transillumination (Biometra, Germany) using a digital camera.

Analysis of RAPD-PCR data

Gel images were analyzed for genetic similarity among isolates using the AlphaEase Software (Alpha Innotech, CA, USA). Dendrograms were analyzed using MultiVariate Statistical Package software (MVSP 3.2., UK). RAPD bands were scored as discrete variables, using ‘‘1’’ to indicate presence and ‘‘0’’ to indicate the absence of a band in the profile. The PCR profiles are defined by the pattern of presence or absence of bands on the gel. The discrimination index (D) was calculated for each primer by using Simpson’s index of diversity as described by Hunter and Gaston (1988) as follows:

$$D = 1 - (\Sigma n_j(n_j - 1) / N(N - 1)$$

Where D is the diversity, N is the total number of strains, and n is the number of strains in each RAPD profile/type.

The similarities between DNA fingerprints were calculated with the band-matching Jaccard’s coefficient that ranges from 0 to 1.0, where 1.0 represents 100% identity (presence and position) for all bands in the two PCR fingerprints being compared. A pairwise similarity (or distance) matrix was developed and cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic averages (UPGMA) method. Principal component analysis (PCA), a mathematical procedure that uses orthogonal linear transformation, was used to recognize patterns in the RAPD-generated markers and to highlight the relationships between the genotypes examined.

RESULTS

In the present study, RAPD-PCR was used for characterizing *S. enterica* isolates in Taif province according to Ohud (2012). RAPD PCR using four 10-mer arbitrary oligonucleotide primers (OPA-13, OPB-10, OPB-18 and OPJ-10) was used in this study to evaluate the genetic diversity among *S. enterica* isolates (Figure 1). A total of 55 distinct and reproducible RAPD bands (54 polymorphic bands or 98 %) were amplified from the twenty three *Salmonella* isolates.

The total numbers of RAPD bands amplified were 10 (all polymorphic), 14 (all polymorphic), 14 (all polymorphic) and 17 (16 polymorphic), for RAPD primers OPA-13, OPB-10, OPB-18 and OPJ-10, respectively. Ten distinct polymorphic amplicons ranging from 400 to 1100 bp were generated by primer OPA-13. Cluster analysis based on RAPD primer OPA-13 and Jaccard's similarity coefficients distinguished 7 different RAPD profiles among *Salmonella* Enteritidis and 14 different RAPD profiles among *S.* Typhimurium. The index of discrimination (D) for RAPD primer OPA-13 was 0.94 for *S.* Enteritidis and 1 for *S*. Typhimurium according to Simpson's index of diversity. RAPD profiling using OPA-13 primer showed that the *S*. Enteritidis isolates S19 and S20 isolated from pediatric hospital were identical and had a RAPD profile #2. Isolates S12 and S14 isolated from Al-Edwani hospital were also identical and had a RAPD profile #6

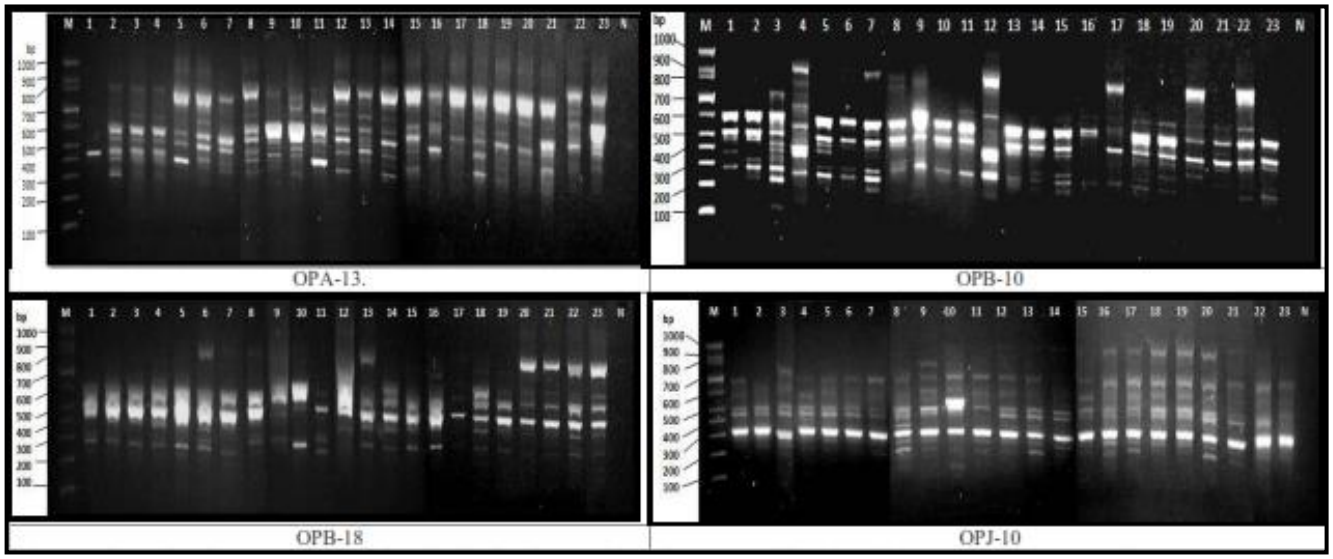

Figure 1. The results of agarose gel electrophoresis showing the separation of RAPD amplification products amplified by RAPD OPA-13, OPB-10, OPB-18, OPJ-10. The amplicons were electrophoresed onto 2% agarose in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA) containing ethidium bromide (0.5 µg/ml) at constant voltage of 5 V/cm. Lanes (1-23): PCR produced from *Salmonella* isolates. M: Molecular size markers (100bp DNA ladder); N: Negative control (DNA-less, that is, ddH_2O instead of DNA).

while each of the remaining isolates produced a distinct RAPD profile. The RAPD primer OPA-13 produced an immense genetic variability to the maximum extent among the fourteen *S*. Typhimurium isolates and this is consistent with the discriminating ability of the primer (D = 1) according to Simpson's index of diversity. Cluster analysis based on RAPD primer OPB-10 and Jaccard's similarity coefficients distinguished 8 different RAPD profiles among *S.* Enteritidis and 13 different RAPD profiles among *S.* Typhimurium isolates. The index of discrimination (D) for RAPD primer OPB-10 was 0.97 for *S*. Enteritidis and 0.99 for *S*. Typhimurium according to Simpson's index of diversity. Cluster analysis based on RAPD primer OPB-18 and Jaccard's similarity coefficients distinguished 7 different RAPD profiles among *S.* Enteritidis and 9 different RAPD profiles among *S.* Typhimurium isolates. The index of discrimination (D) for RAPD primer OPB-18 was 0.94 for *S*. Enteritidis and 0.96 for *S*. Typhimurium according to Simpson's index of diversity.

Cluster analysis based on RAPD primer OPJ-10 and Jaccard's similarity coefficients distinguished 4 different RAPD profiles among *S.* Enteritidis and 7 different RAPD profiles among *S.* Typhimurium isolates. The index of discrimination (D) for RAPD primer OPJ-10 was 0.94 for *S*. Enteritidis and 0.95 for *S*. Typhimurium according to Simpson's index of diversity.

RAPD profiling using OPJ-10 primer showed that seven *S.* Typhimurium isolates S1 (pediatric hospital), S2 and S4 (King Faisal hospital), S5 (pediatric hospital), S6, S7 and S8 (Al-Edwani hospital) were identical and belonged to RAPD profile #11. These RAPD types were designated E1 to E9 (Figure 2A). Likewise, the fourteen *S.* Typhimurium isolates were clustered based on the combined data of the four primers and each isolate had a distinct RAPD type and fourteen RAPD types were designated (T1 to T14) (Figure 2B). Two RAPD types (T11 and T12) were found in King Faisal hospital, five RAPD types (T1, T2, T3, T9 and T14) were found in pediatric hospital, while seven RAPD types (T4, T5, T6, T7, T8, T10 and T13) were found in Al-Edwani hospital (Table 1).

Principal component analysis (PCA) showed that the first three components explained about 65 and 33% of the total variability among RAPD types belonging to serovars Enteritidis and Typhimurium, respectively (Tables 2 and 3) .

DISCUSSION

Four arbitrary RAPD primers were used with all twenty three *Salmonella* isolates genomes according to Ohud (2012). The four primers were found suitable for typing of *Salmonella* isolates on the basis of polymorphic RAPD markers after agarose gel electrophoresis. Interestingly, each of the studied *Salmonella* isolates had a distinct amplification pattern by RAPD-PCR with few exceptions. Cluster analysis calculated from the pairwise comparisons based on RAPD primer OPA-13 and Jaccard's similarity coefficients distinguished 7 different RAPD profiles among *S.* Enteritidis and 14 different RAPD profiles among *S.* Typhimurium. The results obtained by RAPD analysis revealed two identical RAPD profiles, profile #2 contained S19 and S20 isolated from pediatric hospital

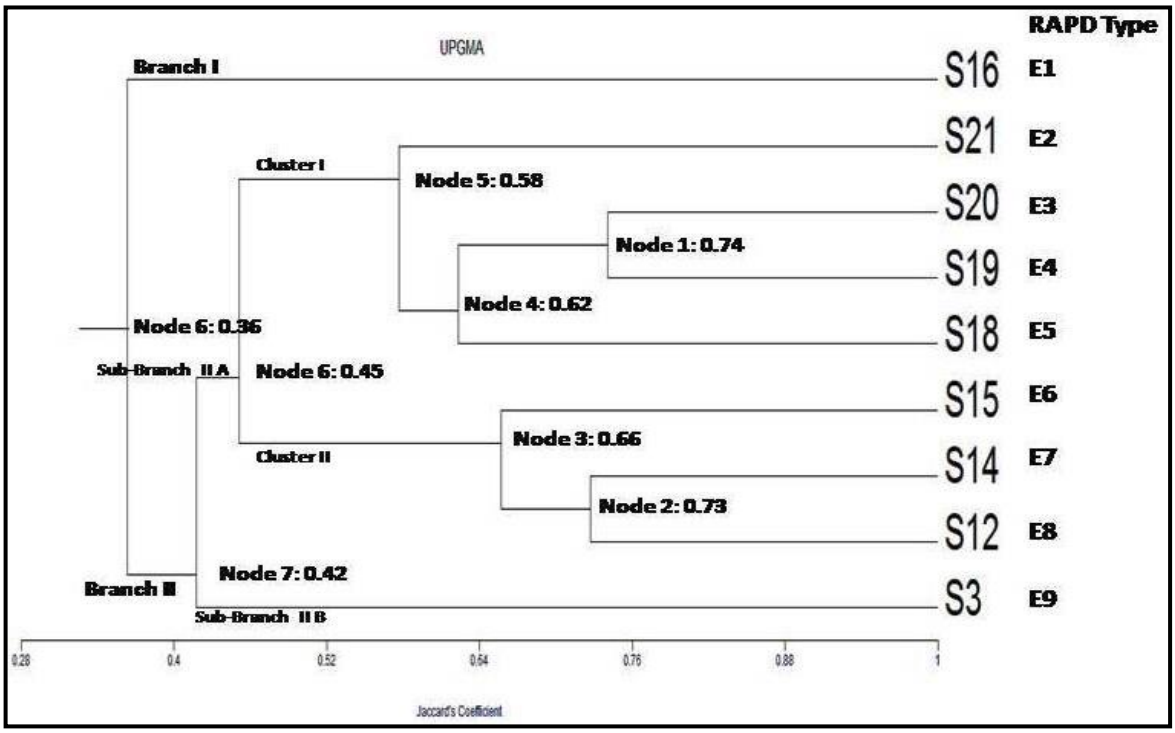

Figure 2A. A combined dendrogram constructed from the pairwise comparisons of Jaccard's similarity coefficients with clustering by UPGMA, based on the presence or absence of amplified product calculated identified nine RAPD type of *S.* Enteritidis. Similarity is given at each node.

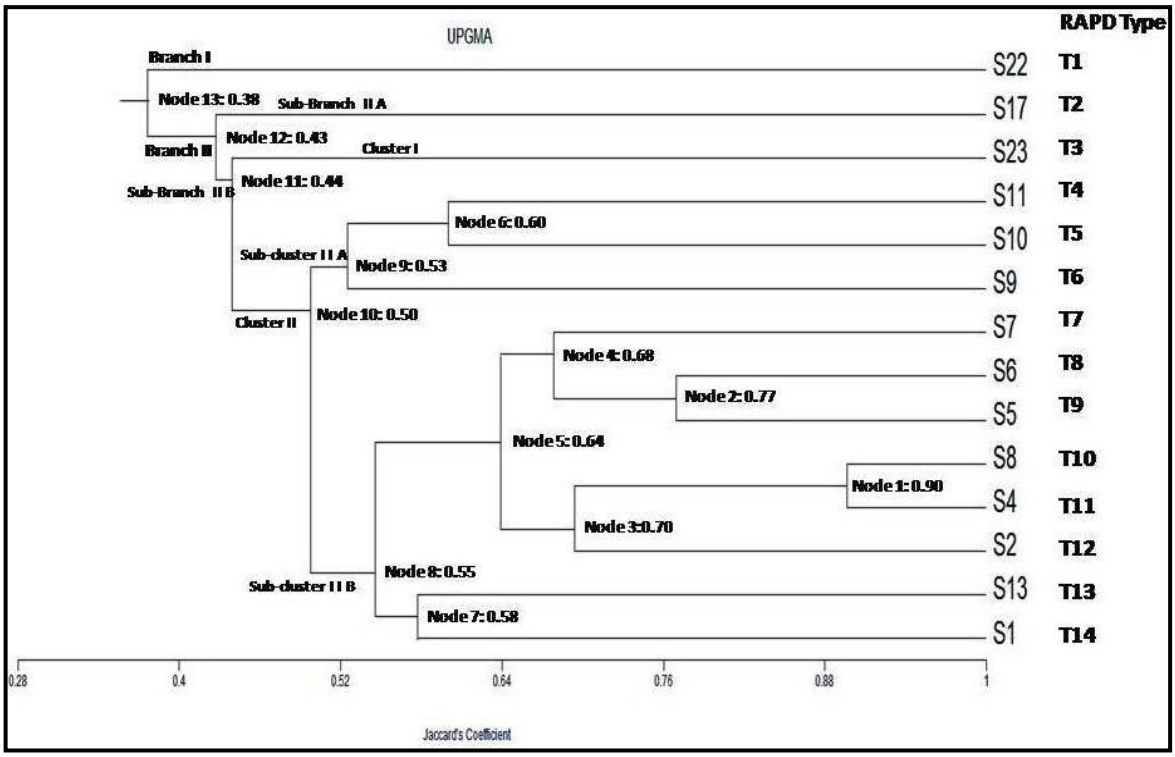

Figure 2B. A combined dendrogram constructed from the pairwise comparisons of Jaccard's similarity coefficients with clustering by UPGMA, based on the presence or absence of amplified product calculated identified 14 RAPD type of *S.* Typhimurium. Similarity is given at each node.

Table 1. RAPD PCR profiles and types generated from *Salmonella* enterica isolates using four RAPD primers.

RAPD type	RAPD profiles amplified by each primer				Isolate/Hospital	
	OPJ-10	OPB-18	OPB-10	OPA-13		
E9	4	7	5	7	S3- King Faisal	
E8	3	2	6	6	S12- Al-Edwani	
E7	3	5	2	6	S14 -Al-Edwani	
E6	3	1	2	5	S15- Pediatric	
E1	2	6	1	3	S16 -Pediatric	*Salmonella* Enteritidis
E5	2	5	3	4	S18 -Pediatric	
E4	2	3	4	2	S19- Pediatric	
E3	2	4	7	2	S20- Pediatric	
E2	1	4	8	1	S21- Pediatric	
T14	11	16	21	21	S1P-ediatric	
T12	11	14	20	20	S2 -King Faisal	
T11	11	11	16	18	S4- King Faisal	
T9	11	11	18	10	S5- Pediatric	
T8	11	10	11	9	S6- Al-Edwani	
T7	11	11	10	17	S7- Al-Edwani	
T10	11	12	15	19	S8- Al-Edwani	*Salmonella* Typhimurium
T6	8	13	13	11	S9- Al-Edwani	
T5	7	12	17	16	S10- Al-Edwani	
T4	10	13	19	8	S11- Al-Edwani	
T13	10	15	12	14	S13- Al-Edwani	
T2	9	13	9	12	S17- Pediatric	
T1	6	8	14	13	S22- Pediatric	
T3	5	9	12	15	S23- Pediatric	

Table 2. PCA analysis of nine RAPD profiles and 55 RAPD amplicons for *S.* Enteritidis isolates.

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7	Axis 8
Eigenvalues	2.845	1.672	1.412	0.960	0.826	0.789	0.376	0.259
Percentage	31.126	18.292	15.446	10.504	9.043	8.631	4.119	2.839
Cum. percentage	31.126	49.418	64.864	75.368	84.411	93.042	97.161	100.00

Table 3. PCA analysis of fourteen RAPD profiles and 55 RAPD amplicons for *S.* Typhimurium isolates.

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7	Axis 8	Axis 9	Axis 10	Axis 11
Eigenvalues	1.557	1.056	0.982	0.930	0.715	0.693	0.527	0.402	0.363	0.295	0.212
Percentage	19.759	13.399	12.459	11.799	9.071	8.793	6.691	5.105	4.604	3.739	2.688
Cum. percentage	19.759	33.158	45.618	57.416	66.487	75.281	81.972	87.077	91.680	95.419	98.107

with 100% similarity and profile #6 contained S12 and S14 isolated from Al-Edwani hospital with 100% similarity. This two RAPD profiles may represent the most common RAPD profiles distributed among other different minor genotypes in Taif province while each of the remaining isolates produced a distinct RAPD profile. The discrimination index (D) for RAPD primer OPA-13 among *S.* Enteritidis was 0.94 according to Simpson's index of diversity. Cluster analysis based on this primer grouped *S.* Typhimurium into 14 different RAPD profiles. Among the 14 RAPD profiles, RAPD profile #20 isolate S2 found in King Faisal hospital and RAPD profile #19 isolate S8 found in Al-Edwani hospital had the highest similarity (86%) among the *S.* Typhimurium profiles. The index of

discrimination (D) for RAPD primer OPA-13 was 1 according to Simpson's index of diversity. Among all the 4 primers used, OPB-10 generated fourteen distinct fragments ranging from 145 to 1159 bp producing high genetic variability to the maximum extent and this is reflected in the high discriminating index of the primer (D=0.97). A report by Un-Ho et al. (2000) showed maximum genetic variability among *S*. Typhimurium isolates using the OPB-10 primer as compared to OPA-13, OPB-18 and OPJ-10. Cluster analysis based on OPB-10 grouped *S.* Enteritidis isolates into 8 different RAPD profiles and *S.* Typhimurium isolates with 13 RAPD profiles. Among the eight RAPD profiles found for *S*. Enteritidis isolates, RAPD profile #2 (S14) was found in Al-Edwani hospital and (S15) was found in Pediatric hospital with 100% similarity. The index of discrimination (D) for RAPD primer OPB-10 among *S.* Enteritidis was 0.97 according to Simpson's index of diversity. Among *S.* Typhimurium, 14 different RAPD profiles were elucidated by using OPB-10 RAPD primer. The two isolates S13 isolated from Al-Edwani hospital and S23 isolated from pediatric hospital were identical and belonged to RAPD profile #12, indicating that this profile might be more common among the remaining isolates in Taif province. This RAPD profile, therefore, was more frequent as compared to the other *S.* Typhimurium profiles. The index of discrimination (D) for RAPD primer OPB-10 among *S.* Typhimurium was 0.99 according to Simpson's index of diversity. Fourteen distinct polymorphic amplicons ranging from 200 to 850 bp were generated by OPB-18 primer. Cluster analysis distinguished 7 different RAPD profiles among *S.* Enteritidis, with isolates S20 and S21 (RAPD profile #4) and S14 and S18 (RAPD profile #5) being identical and each of the remaining isolates having a distinct RAPD profile. Isolate S19 (RAPD profile #3) was more close to isolate S20 and S21 (RAPD profile #2) as they had a similarity of 79%. The index of discrimination (D) for RAPD primer OPB-18 among *S.* Enteritidis isolates was 0.94 according to Simpson's index of diversity. Nine different RAPD profiles were resolved among *S.* Typhimurium isolates by using this primer. Four isolates, S4 (King Faisal hospital), S5 (pediatric hospital), S7 and S8 (isolated from Al-Edwani hospital), had the same RAPD profile (profile #11). Three isolates S9, S11 (Al-Edwani hospital) and S17 Pediatric hospital had the same RAPD profile (profile #13). Both profiles 11 and 13 were probably the most common profiles as compared to the other *S.* Typhimurium profiles. The index of discrimination (D) for RAPD primer OPB-18 among *S*. Typhimurium was 0.96 according to Simpson's index of diversity. Seventeen RAPD amplicons ranging in size from 160 to 1100 bp were generated by OPJ-10 RAPD primer, with only one monomorphic amplicon. Four different RAPD profiles were resolved among *S.* Enteritidis isolates by using OPJ-10 RAPD primer. Four isolates, that is, S16, S18, S19 and S20 (isolated from pediatric hospital) were identical and belonged to RAPD profile #2 and three isolates, that is, S12, S14 (Al-Edwani hospital) and S15 (pediatric hospital) were also identical and belonged to RAPD profile #3, indicating that the two RAPD profiles 2 and 3 were the most common profiles among *S.* Enteritidis isolates while each of the remaining isolates produced a distinct RAPD profile. The index of discrimination (D) for RAPD primer OPJ-10 among *S*. Enteritidis was 0.94 according to Simpson's index of diversity.

Cluster analysis distinguished seven different RAPD profiles among *S.* Typhimurium, with two common RAPD profiles #10 and #11. RAPD profile #10 contained two identical isolates, that is, S11 and S13 isolated from Al-Edwani hospital while RAPD profile #11 contained seven identical isolates, that is, S1 (pediatric hospital), S2 and S4 (King Faisal hospital), S5 (pediatric hospital), S6, S7 and S8 (Al-Edwani hospital). This might suggest the predominance of this two RAPD profiles in Taif province. The index of discrimination (D) for RAPD primer OPJ-10 among *S.* Enteritidis isolates was 0.95 according to Simpson's index of diversity.

All the four primers, that is, OPA-13, OPB-10, OPB-18 and OPJ-10 produced reproducible results and useful as efficient RAPD primers regardless of their short oligonucleotide sequence. Similar results have been found by Hilton et al. (1996), Chansiripornchai et al. (2000) and Mare et al. (2001). These results revealed that RAPD-PCR can be used as an epidemiological tool in combination with other molecular and phenotypic typing techniques. Similar interpretations have been reported by several investigators on the same serotypes as well as other serotypes (Meenu, 2002; Jin et al., 2006; Yaqoob et al., 2007; Maripi et al., 2007; Dos Santos et al., 2008; Morshed and Peighambari, 2010).

The RAPD profiles generated by the four primers were combined and the combined dendrograms were highly branched, suggesting a genetically diverse population. The majority of *Salmonella* isolates within a cluster belonged to different RAPD types. The differences in the percent similarity could be the result of strain variation, as described by Hilton et al. (1996) and Franklin et al. (2011). Similar results have been described by Millemann et al. (1996), Chansiripornchai et al. (2000) and Mare et al. (2001). Dendrogram branch lengths were proportional to genetic distance between isolates. Phylogenetic comparisons of *S. enterica* isolates from different geographical regions have been useful in taxonomical and epidemiological investigations.

The nine *S.* Enteritidis isolates were clustered based on the results of the four primers. Each of the nine *S.* Enteritidis isolates had a distinct RAPD type. The nine RAPD types (E1 to E9) were designated No E1 to E9 (Figure 1A). One RAPD (E9) was found in King Faisal hospital, two RAPD types (E7 and E8) were found in Al-Edwani hospital, while six RAPD types (E1, E2, E3, E4, E5 and E6) were found in pediatric hospital. These results suggested that several genovars were present

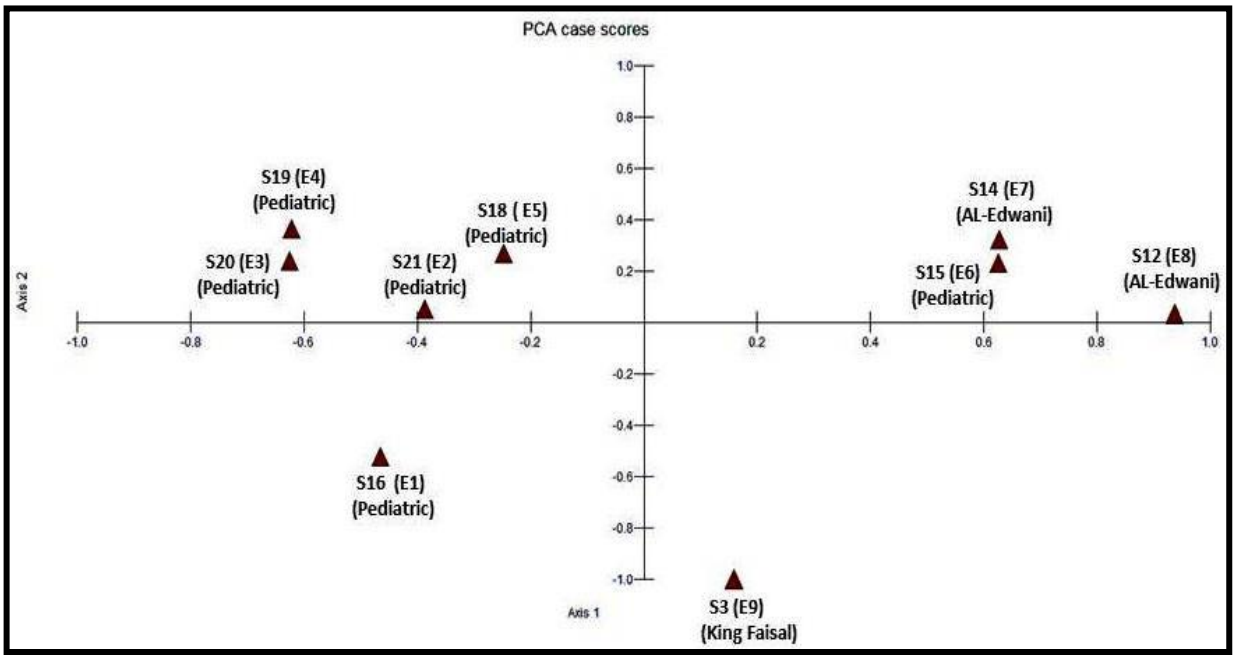

Figure 3A. Scatter plots showing two dimensional distribution of *S.* Enteritidis isolates derived from principal component analysis (PCA) of RAPD-PCR data. *Salmonella* codes are shown on the arrows.

among *Salmonella* serovars. This result indicates that there is no specific *S.* Enteritidis strain that circulates in Taif province. Likewise, the fourteen *S.* Typhimurium isolates were clustered based on the four primers and each isolate had a distinct RAPD type. The fourteen *S.* Typhimurium RAPD types were designated T1 to T14 (Figure 1B). Two RAPD types (T11 and T12) were found in King Faisal hospital, five RAPD types (T1, T2, T3, T9 and T14) were found in pediatric hospital, while seven RAPD types (T4, T5, T6, T7, T8, T10 and T13) were found in Al-Edwani hospital.

The results presented in this study indicated that RAPD analysis is a useful tool for the analysis of *Salmonella* genovars, provided that strict experimental protocols are maintained and might be used as a cost-effective tool for molecular epidemiology research (Garaizar et al., 2000).

The principal component analysis (PCA) based on RAPD data of the nine RAPD Types of *S.* Enteritidis showed four components with Eigenvalues more than one. The first two components explained about 49.4% of the RAPD variation, while the first three components explained about 65% of the total variance (Table 2). The remaining components accounted for about 35% of the total variance. The scatter plot defined by the first two principal components (Axis 1 and 2) distinguished the RAPD Type E9 (found in King Faisal hospital) from the other RAPD Types (Figure 2A). The two RAPD Types E7 and E8 (found in Al-Edwani hospital), and the six RAPD Types E1 to E6 (found in Pediatric hospital) were also distinguishable in the scatter plot (Figure 2B). This result indicated that *S. enterica* subspecies *enterica* serovar Enteritidis may exhibit considerable genetic variation between locations (Figure 3A).

The PCA based on RAPD data of the fourteen RAPD types of *S.* Typhimurium showed that there were two components with Eigenvalues more than one. The first component explained about 20% of the RAPD variation, while the first two components explained about 33% of the total variance (Table 3). The remaining components accounted for about 67% of the total variance. No RAPD types could be appropriately grouped in the scatter plot defined by the first two principal components (Axis 1 and 2). This result confirmed the results of cluster analysis and showed that *S. enterica* subspecies *enterica* serovar Typhimurium exhibit considerable genetic variation within and between locations (Figure 3B).

The results of PCA for the nine RAPD types of *S.* Enteritidis indicated that *S. enterica* subspecies *enterica* serovar Enteritidis may exhibit considerable genetic variation between locations. The results of principal component analysis (PCA) for fourteen RAPD types of *S.* Typhimurium confirmed the results of cluster analysis and showed that *S. enterica* subspecies *enterica* serovar Typhimurium exhibit considerable genetic variation within and between locations (Figure 3B). The results of PCA (Tables 2 and 3) indicated that the isolates belonging to serovar Enteritidis exhibited considerable genetic variation between locations, while the isolates belonging to serovar Typhimurium exhibited considerable genetic variation both within and between locations. In conclusion, RAPD-PCR yielded highly reproducible results that demonstrated the high genetic heterogeneity of

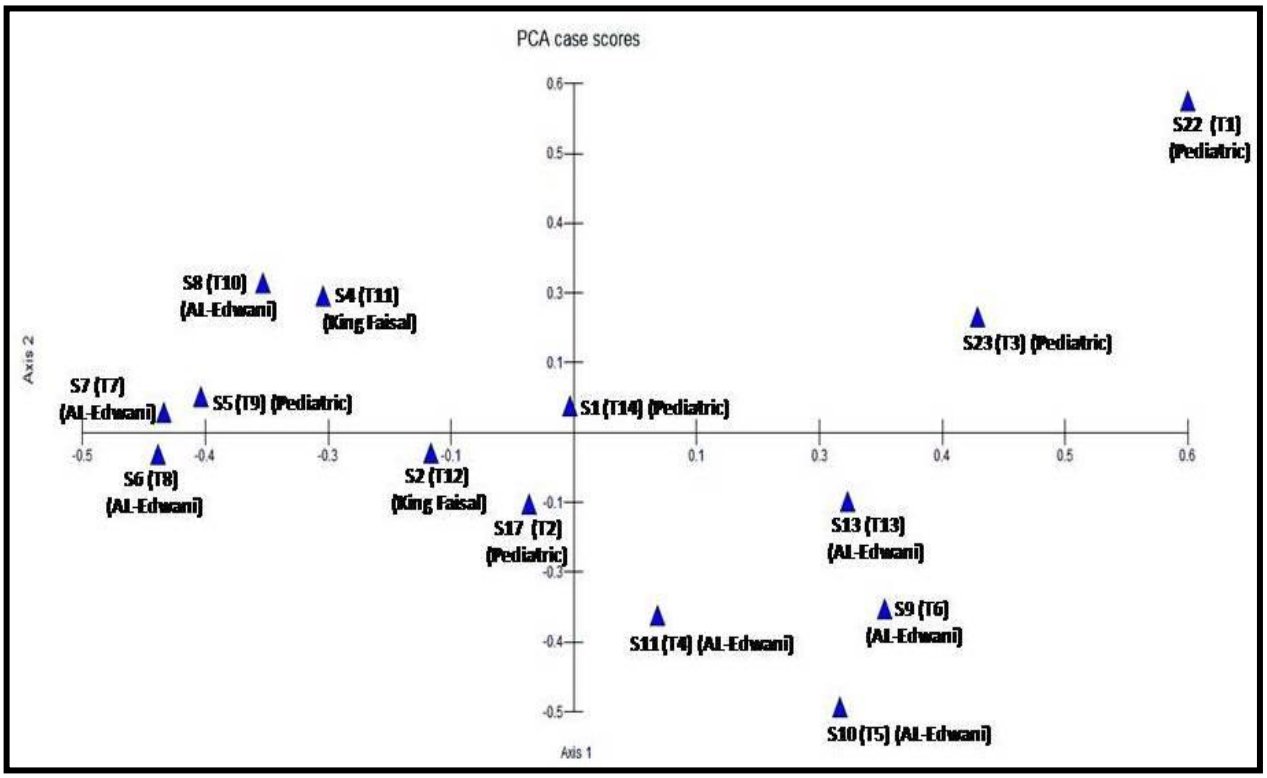

Figure 3B. Scatter plots showing two dimensional distribution of *S.* Typhimurium isolates derived from principal component analysis (PCA) of RAPD-PCR data. *Salmonella* codes are shown on the arrows.

Salmonella isolates. Several genovars were present among *Salmonella* serovars and no specific RAPD type (or genovar) was found to be circulating predominantly in Taif province.

Our results were in agreement with the work of Un-Ho Jin et al. (2000) and Jegadeeshkumar et al. (2010) in identifying and differentiating *S.* Typhimurium from other Gram negative bacteria by using DNA (RAPD) fingerprinting of *S.* Typhimurium. Un-Ho Jin et al. (2000) used the same andom primers designated OPA-13, OPB-10, OPB-18 and OPJ-10, and its patterns compared with 6 representive intestinal, Gram negative bacterial strains. The results showed that *S.* Typhimurium had unique and distinct fingerprinting patterns and the RAPD fingerprinting is thus concluded to be a rapid and sensitive method for the identification of *S.* Typhimurium as compared to conventional culturing procedures or immunoassays.

The results shown in the present study was not reported elsewhere about the genetic diversity among *Salmonella* isolates using RAPD-PCR in Saudi Arabia. The results presented in this study indicated that RAPD analysis has a great discriminatory power for the differentiation of *Salmonella* isolates. RAPD analysis, therefore, can be a useful tool for the analysis of *Salmonella* genovars. Of course, this should have important implications in the fields of food industry, human health and epidemiology.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Antibacterial resistance pattern among *Escherichia coli* strains isolated from Mansoura hospitals in Egypt with a special reference to quinolones

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Extensive use of fluoroquinolone antibacterial in clinical practice has been associated with increasing frequency of quinolone-resistant *Escherichia coli* strains. In the current study, a total of 80 *E. coli* clinical isolates from Mansoura hospitals patients in Egypt were studied for antibacterial susceptibility pattern against 15 different antibacterials. These strains were tested for quinolones resistance by minimum inhibitory concentration (MIC) determination using broth micro-dilution method. The resistance rate of ciprofloxacin and levofloxacin for *E. coli* isolates was found to be 60%. PCR was performed for detection of plasmid-mediated quinolone resistance genes including *qnrA, qnrB* and *qnrS*. 30 and 61.3% of *E. coli* isolates were positive for *qnrA* and *qnrB*, respectively, whereas *qnrS* was identified in only 15% of isolates. Quinolone resistance-determining region (QRDR) of *gyrA* and *ParC* genes was characterized for 17 ciprofloxacin and levofloxacin resistant *E. coli* isolates (MIC 12.5-200 µg mL^{-1}). Two mutation sites in *gyrA* were detected in 17 tested *E. coli* isolates. However, two mutation sites in *parC* were detected in four *E. coli* isolates. The amino acid change at Ser-83 and aspartic-87 in GyrA were the most common mutation sites identified in the isolates. These results indicated that multiple mechanisms of quinolone-resistance are commonly found in *E. coli* isolated from Mansoura hospitals.

Key words: Quinolone resistance, *gyrA*, *parC, qnr* gene.

INTRODUCTION

Quinolones are powerful broad-spectrum antibacterial agents commonly used in both human and veterinary medicine for the treatment of a wide variety of infections. In the last decade, fluoroquinolones have become first and second-line antibacterials of choice for acute respiratory, enteric and urinary tract infections as well as serious systemic infections such as bacteremia (Jamison, 2006). Their extensive use has been associated with raising level of quinolone resistance in different microorganisms (Robicsek et al., 2006). Fluroquinolones act by increasing levels of enzyme-mediated DNA cleavage affecting DNA gyrase enzyme which catalyzes the negative supercoiling of DNA and topoisomerase IV enzyme which decatenates or removes the interlinking of daughter chromosomes at the completion of a round of DNA replication allowing their segregation into daughter cell (Ambrozic et al., 2007; Wang et al., 2009). Quinolone resistance has traditionally been attributed mainly to chromosomal mutations

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in the *gyrA* and *gyrB* genes of DNA gyrase and in *parC* and *parE* genes of topoisomerase IV or due to decreased intracellular concentration as a result of decreased permea-bility of the membrane or over expression of efflux pump systems (Poirel et al., 2006; Oktem et al., 2008; Allou et al., 2009).

Alteration in quinolone resistance determining region (QRDR) is considered as the most important mechanism of quinolone resistance. Mutations in QRDRs of *gyrA* and *parC* are most commonly documented, however resistance is also conferred by mutations in *parE* (Hopkins et al., 2005). These mutations block the action of quinolones resulting in increased level of resistance to fluoro-quinolones (Ruiz, 2003). In *Escherichia coli* and related Gram negative bacteria, DNA gyrase is the first target for fluoroquinolones. Alterations in *gyrA* are reported much more often than alterations in *gyrB* (Frank et al., 2011).

Plasmid-mediated quinolone resistance has been also previously described. The study of Martinez-Martinez et al. (1998) was the initial report of this transferable mode of resistance associated with *qnr* gene (now named *qnrA*). This gene was identified for the first time in *Klebsiella pneumoniae* in the United States. Several studies reported a world wide distribution of *qnr* deter-minants among bacterial isolates (Cheung et al., 2005; Cerquetti et al., 2009). The *qnr* gene encodes a 218-amino-acid protein which protects DNA gyrase and topoisomerase IV activity from the action of quinolones (Tran and Jacoby, 2002; Strahilevitz et al., 2009). The plasmid-mediated quinolone resistance determinants are widely distributed in clinical Enterobacteriaceae isolates around the world. These genes are usually located on mobile elements, including integrons, insertion sequences and transposons (Martinez-Martinez et al., 1998; Robiesek et al., 2006; Yamane et al., 2008). Furthermore, the *qnr* gene carrying plasmids, which are classified as Class I integron-carrying plasmids, usually carry in addition to *qnr* gene multiple resistance determinants providing multidrug resistance to different antimicrobials including: aminoglycosides, B-lactams and sulfonamides (Martinez-Martinez et al., 1998; Tran et al., 2005).

The prevalence and distribution of *qnr* genes were different in various geographical areas. *qnrA* genes have been identified worldwide in a variety of Enterobacterial species. Six variants have been identified (*qnrA1* to *qnrA6*). These genes can increase the MIC of fluoro-quinolones up to 32-fold in *E. coli* isolates (Poirel et al., 2006; Allou et al., 2009). In addition, *qnrA* gene enhances the selection of chromosomal encoded quinolone resistance determinants which confer additional resistance to fluoroquinolones. Other plasmid-mediated quinolone resistance determinants *qnrB* (*qnrB1* to *qnrB6*) and *qnrS* (*qnrS1* and *qnrS2*) have been also identified in entero-bacterial species, sharing 41 and 60% amino acid identity with *qnrA*, respectively (Nordmann and Poirel, 2005; Shin et al., 2009). Another mechanism of quinolone resistance relies on upregulation of efflux pump which exports quinolones and other antimicrobials out of the bactereial cell. Although multiple mechanisms of quinolone resistance have been reported from many continents, there are few data from Africa on the molecular basis for quinolone resistance. In the current study, we focused on the prevalence of *E. coli* resistance to quinolones and the frequency of *qnrA, qnrB* and *qnrS* among clinical isolates of *E. coli* in Mansoura Hospitals. Also, this study was undertaken to determine the mechanism of quinolone resistance among *E. coli* isolates from Mansoura Hospitals.

MATERIALS AND METHODS

Bacterial strains

Eighty clinical *E. coli* isolates were collected from Mansoura university hospitals in Dakahlia governorate, Egypt during March 2011 to February 2013. The isolates were collected from urine, wound and sputum samples. These isolates were identified using standard biotyping methods (Crichton, 1996).

Antibacterial susceptibility testing

Each strain was screened for susceptibility to fifteen antimicrobials using the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) disc diffusion method (NCCLS, 2003). Discs used contained ampicillin (10 µg), imipenem (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefoperazone (75 µg), ceftizoxime (30 µg), cefoxitin (30 µg), cefotaxime (30 µg), norfloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), tobramycin (10 µg), gentamicin (10 µg), azithromycin (15 µg) and amikacin (30 µg) (Oxoid). Inhibition zone diameters were interpreted in accordance with CLSI guideline using WHONET software version 5.3 (O'Brien and Stelling, 1995)

Determination of MIC (Minimal inhibitory concentration) for the isolated strains

The minimal inhibitory concentrations of the isolates for ciprofloxacin and levofloxacin were determined using broth micro-dilution method following the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (CLSI, 2007). In this method, 96-well microtitre plates were filled with small volumes (200 µl) of serial two-fold dilutions of each tested antibacterial. The final concentrations of each antibacterial in the wells ranged from 1.56 to 200 µg mL^{-1}. The turbidity of overnight culture is adjusted to obtain visually comparable turbidity to that of the 0.5 McFarland turbidity standards, then an aliquot of 20 µl was added to each wells of the microtitre plate. The plates were incubated for 24h at 30°C before determining the results. The MICs were read visually and were defined as the lowest concentration where no viability was observed in the wells of the microplates after incubation. The MICs values indicate resistance > 1 and > 2 μgmL^{-1} for ciprofloxacin and levofloxacin, respectively

Screening for the *qnr* genes in clinical strains

Screening was carried out by polymerase chain reaction (PCR) amplification of *qnrA, qnrB* and *qnrS* using the primed sets listed in Table 1. DNA templates were prepared by transferreing bacteria isolates to distilled water in Eppendorf tubes and then boiling for 10 min then prepared DNA templates were directly used in the PCR

Table 1. Primers used in this study.

Target gene	Name	5'-3' sequence	References
	gyrA12004	TGC CAG ATG TCC GAG AT	Wang et al., 2001
*gyr*A	gyrA11753	GTA TAA CGC ATT GCC GC	Wang et al., 2001
	EC-PAR-A	CTG AAT GCC AGC GCC AAA TT	Deguchi et al., 1997
*par*C	EC-PAR-B	GCG AAC GAT TTC GGA TCG TC	Deguchi et al., 1997
	qnrA-1A	TTC AGC AAG ATT TCT CA	Wu et al., 2007
*qnr*A	qnrA-1B	GGC AGC ACT ATT ACT CCC AA	Wu et al., 2007
	qnrB-CS-1A	CCT GAG CGG CAC TGA ATT TAT	Wu et al., 2007
*qnr*B	qnrB-CS-1B	GTT TGC TGC TCG CCA GTC GA	Wu et al., 2007
	qnrS-1A	CAA TCA TAC ATA TCG GCA CC	Wu et al., 2007
*qnr*S	qnrS-1B	TCA GGA TAA ACA ACA ATA CCC	Wu et al., 2007

assay. A reaction mixture containing 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq polymerase (Thermoscientific Dream Taq Green DNA polymerase), 5 µl of template DNA and nuclease free water was added for a total volume of 25 µl per reaction. PCR reactions were carried out by using Techne progene thermocycler under the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 46°C for *qnrA* or 54°C for *qnrB* or 48°C for *qnrS* for 30 s, and extension at 72°C for 1 min; final extension at 72°C for 5 min. PCR products aliquots were analyzed by agarose gel electrophoresis on 1% agarose gel and visualized by ethidium bromide staining

Mutational analysis of the quinolone-resistance determining regions of *gyrA* and *parC*

The QRDR of the *gyrA* and *parC* genes were amplified in seventeen quinolone resistant *E. coli* strains by PCR using the primer pairs listed in Table 1. PCR reactions began with 10 min primary denaturation at 94°C followed by 40 cycles of 94°C for 30s, annealing temperature for 30 s and 72°C for 30 s. *gyrA* and *parC* amplification primers were annealed at 55 and 62°C, respectively.

PCR amplified gene fragments were purified using the PCR Purification Kit (MEGA quick-spin fragment DNA purification INtRON biotechnology, Korea) for subsequent sequencing. Purified PCR products were used as a template in sequencing reactions carried out with the ABI PRISM ® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA). The reaction mixtures were analysed on an ABI 3730 DNA analyser (Applied Biosystems, Foster City, USA).

Amplicons were sequenced on both strands and predicted peptide sequences were analysed by the online BLAST of the NCBI website software (http://www.ncbi.nlm.nih.gov/BLAST/). Then point mutations were identified by comparing the identified sequences to the corresponding genes by pair-wise FASTA alignments.

Nucleotide sequences of the antibacterial resistance genes from the fragments of *gyrA* and *parC* were deposited in GenBank under the following accession numbers: KF994612, KF994613, KF994614, KF994615, KF994616, KF994617, KF994618, KF994619, KF994620, KF994621, KF994622, KF994623, KF994624, KF994625, KF994626, KF994627, KF994628, KF994629, KF994630, KF994631, KF994632, KF994633, KF994634, KF994635, KF994636, KF994637, KF994638, KF994639, KF994640, KF994641, KF994642, KF994643, KF994644, KF994645, KF994646.

RESULTS

Antibacterial susceptibility testing

All *E. coli* isolates were resistant to ampicillin. On the other hand, they were all sensitive to imipenem. In addition, the highest resistance rate was recorded against ceftazidime (72.5%), ceftriaxone and cefoperazone (71.3%), ceftizoxime and cefoxitin (70%), norfloxacin, ciprofloxacin and azithromycin (67.5%), cefotaxime (65%), tobramycin and levofloxacin (62.5%) and gentamicin (51.3%). In contrast, resistance to amikacin was less common but was seen in only 23.75% of the isolates. Quinolone resistance was always seen in multiple-resistant *E. coli*, as all quinolone resistant *E. coli* were resistant to at least one other antimicrobial (Figure 1).

Determination of minimum inhibitory concentrations (MICs) of ciprofloxacin and levofloxacin against *E. coli* isolates

Ciprofloxacin MICs values for 80 *E. coli* isolates are shown in Figure 2. The susceptibility of isolates to ciprofloxacin showed that 48 (60%) of 80 *E. coli* were resistant while 22 (27.5%) of *E. coli* isolates were sensitive to ciprofloxacin in addition to 10 isolates (12.5%) exhibiting intermediate susceptibility to ciprofloxacin. The distribution of levofloxacin MICs values are shown in Figure 3. 48 isolates exhibited high-level levofloxacin resistance producing MICs of ≥ 12.5 µg mL^{-1}. However, 25 isolates exhibited susceptibility to levoflo-xacin. Only 7 isolates showed intermediate susceptibility.

Characterization of quinolone resistance mechanisms in *E. coli*

Multiple horizontally-transmitted quinolone resistance genes were detected among *E. coli* strains isolated from

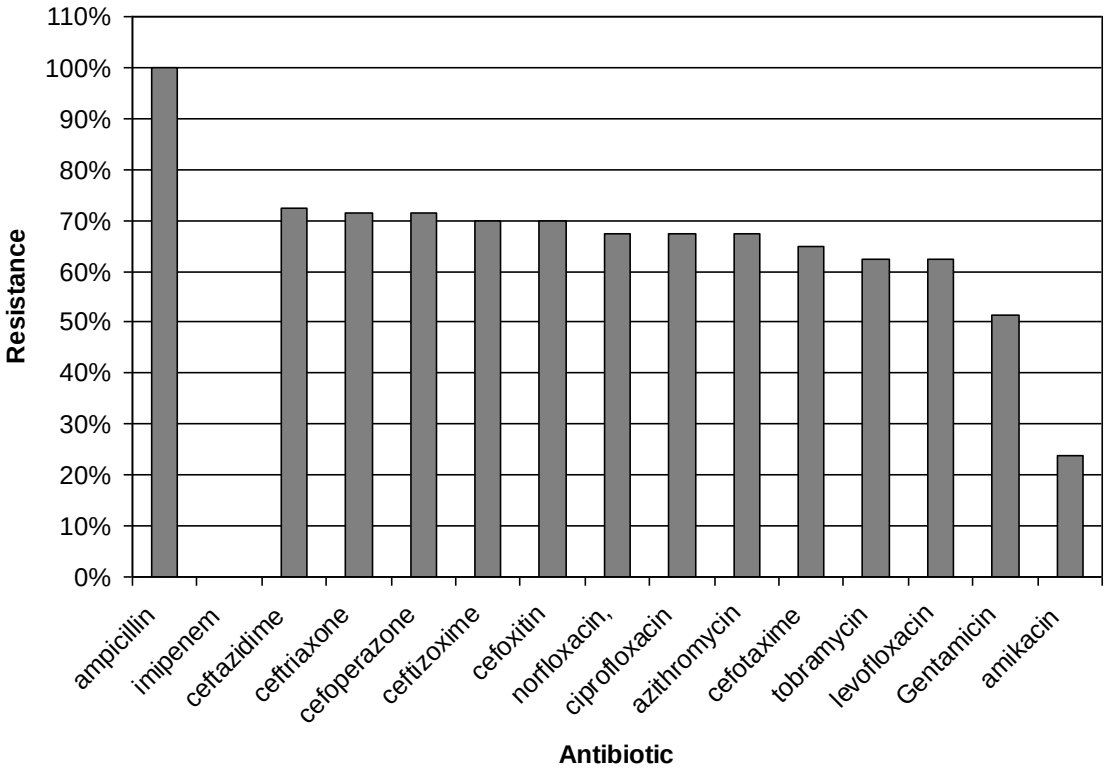

Figure 1. Proportion of *E. coli* isolates resistant to each of the fifteen antimicrobial agents.

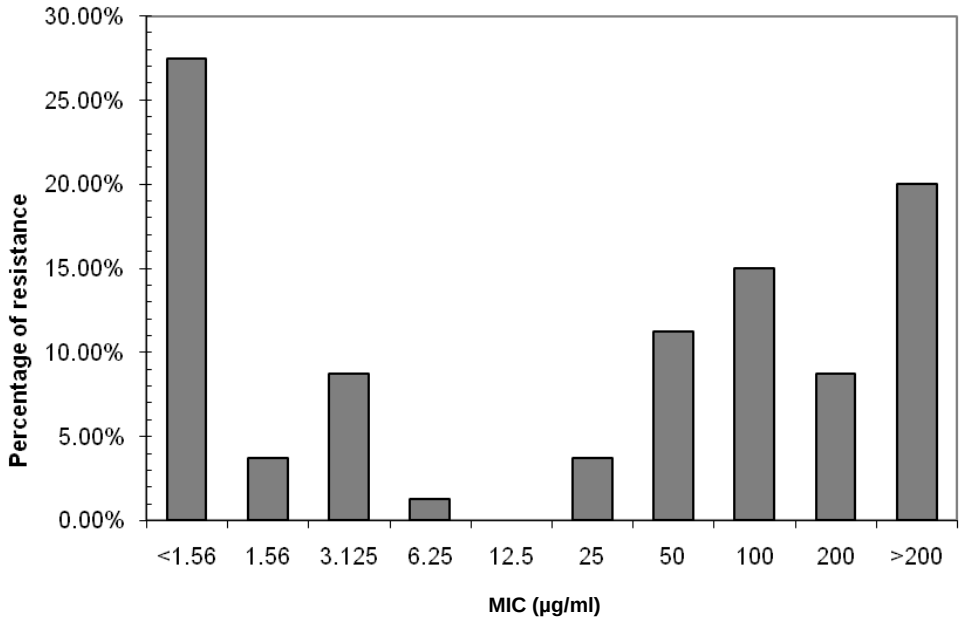

Figure 2. Ciprofloxacin MIC distributions of 80 *E. coli* isolates.

Mansoura Hospitals. By applying PCR technique, *E. coli* isolates were screened for the presence of *qnrA, qnrB* and *qnrS* genes as shown in Figure 4. In 80 *E. coli* isolates, 59 carried one or more of horizontally acquired quinolone resistance gene. These genes were *qnrS* (12 isolates), *qnrA* (24 isolates) and *qnrB* (49 isolates). The tested horizontally acquired quinolone resistance genes were detected in all resistant and intermediate resistant

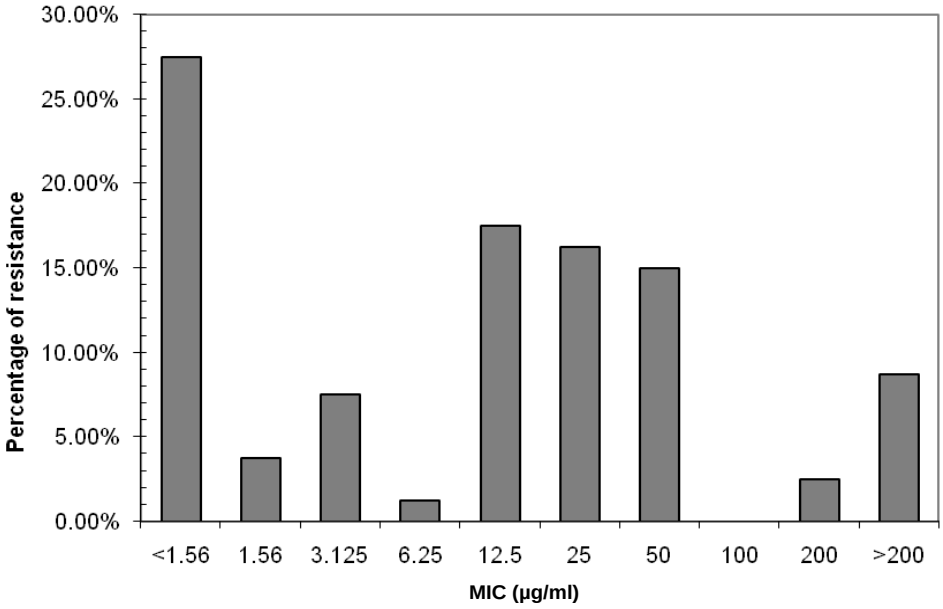

Figure 3. Levofloxacin MIC distribution of 80 *E. coli* isolates.

of the tested isolates. However, 9 of 59 *qnr* positive strains showed low level of resistance to ciprofloxacin and levofloxacin (MIC of 3.125-1.56 µg mL^{-1}). In contrast, only 4 isolates of 16 *qnr* negative isolates had higher level of resistance to ciprofloxacin and levofloxacin (200-12.5 µg mL^{-1}).

Seventeen highly resistant *E. coli* isolates were selected in this study to perform *gyrA* and *parC* sequence. Mutations in *gyrA* and *parC* subunits are summarised in Table 2. Quinolone-resistant isolates had at least two non-synonymous substitutions in the QRDR of *gyrA* and some of these isolates also had one additional mutation in *parC*. Sixteen from the tested isolates having two mutations in GyrA had a serine to leucine substitution at position 83 and aspartic acid to asparagine substitution at position 87, one of the most commonly documented resistance conferring mutations. All of these isolates had MIC values of at least 12.5 and 25µg mL^{-1} levofloxacin and ciprofloxacin, respectively. Only one isolate had S83L and additional GyrA substitution, Aspartic acid with Tyrosine (D87Y) had MIC values of 50 and 200 µg mL^{-1} levofloxacin and ciprofloxacin, respectively. Four of *E. coli* isolates also harboured the frequently documented non-synonymous mutations in the QRDR of *parC.* These ParC substitutions were identified as glutamic acid substituted with glycine E84G, alanine with valine A108V and glutamic acid with valine E84V.

DISCUSSION

The emergence of fluoroquinolone-resistant *E. coli* is increasing in many parts of the world (Levermore, 2009). In African countries with a high infectious disease burden, formal and informal health systems depend heavily on broad spectrum orally-administrable antibacterials. In this study, most of *E. coli* isolates from Mansoura hospitals in Egypt were resistant to ceftazidime, ceftriaxone, cefoperazone, ceftizoxime, cefoxitin, cefotaxime, azithromycin, tobramycin and gentamicin. Fluoroquinolone antibacterials have been recently introduced as an effective alternative to antibacterials that have been compromised by resistance. Our results indicated high level of quinolone resistance with elevated MIC levels identified among the isolated *E. coli* in this study. However, lower resistance rates were markedly identified previously for this class of drugs (Sreela et al., 2011).

In our study, 60% of *E. coli* isolates exhibited high level of levofloxacin and ciprofloxacin resistance. The rate of *E. coli* resistance to fluoroquinolones is increasing worldwide. In USA, the rate of resistance in *E. coli* isolates was 3.5 and 1.9 to 2.5% for nalidixic acid and fluoroquinolones, respectively (Karlowsky et al., 2003). Quinolones target the bacterial enzymes DNA gyrase and topoisomerase IV, which are essential for cell growth and proliferation. DNA gyrase and topoisomerase IV are both tetrameric enzymes comprising two subunits *gyrA* and *gyrB* in DNA gyrase and two subunits *parC* and *parE* in topoisomerase IV.

The association between mutations of DNA gyrase and topoisomerase IV with fluoroquinolone resistance has been previously established for both Gram-negative and Gram-positive organisms (Frank et al., 2011). Accumulation of alterations in *gyrA* and the simultaneous presence alterations in *parC* play fundamental role in developing high level of resistance to ciprofloxacin in clinical isolates. In Gram negative bacteria, the primary target of quinolones is the *gyrA* subunit of DNA gyrase, and point mutations

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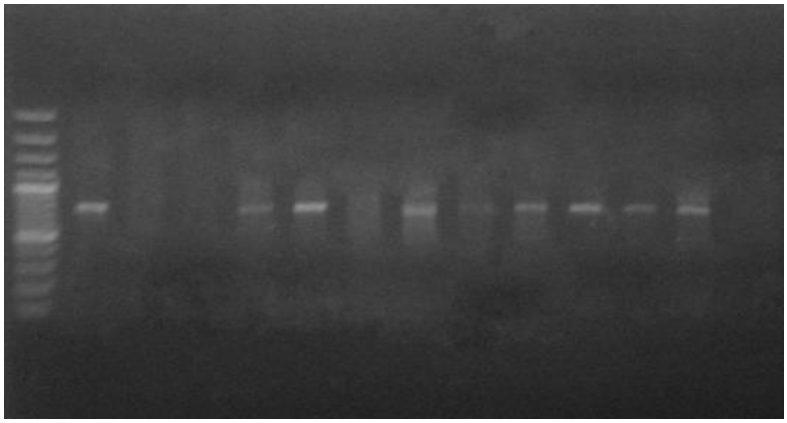
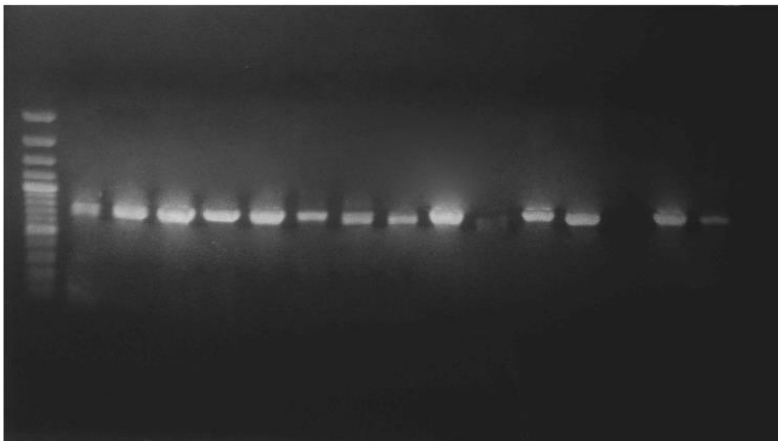


B. M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



C. M 1 2 3 4 5 6 7 8 9 10 11 12 13

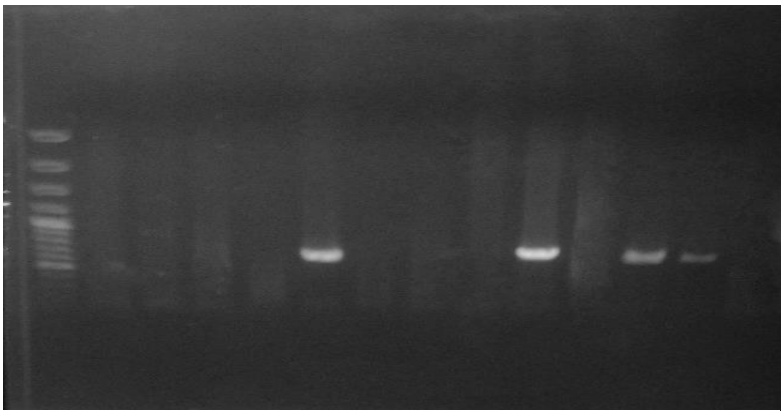


Figure 4. Agarose gel electrophoresis of genes. Lane M is 100 bp ladder molecular weight marker. A. Lanes 1 to 12 were amplicons of *qnrA* gene from *E. coli* isolates No.1, 4, 18, 34, 35, 50, 53, 59, 64, 65, 59, 70; B. Lanes 1 to 15 were amplicones of *qnr B* gene from *E. coli* isolates No. 2, 8, 9, 10, 11, 12, 15, 16, 27, 31, 33, 38, 80, 83, 90; C. Lanes 1 to 13 were amplicons of *qnrS* gene from *E. coli* isolates No. 6, 10, 16, 13, 19, 37, 50, 53, 60, 71, 69, 80, 84.

Table 2. Correlation between susceptibilities of levofloxacin and ciprofloxacin and alterations in GyrA and ParC in17 *E. coli* isolates.

Number of isolate	Ciprofloxacin MIC (µg mL^{-1})	Levofloxacin MIC (µg mL^{-1})	Horizontally acquired	Mutations in QRDR	
				GyrA	ParC
2	> 200	50	*qnrB*	S83L,D87N	-
3	> 200	50	*qnrA, qnrB*	S83L,D87N	E84G
4	> 200	> 200	*qnrA*	S83L,D87N	-
29	> 200	200	*qnr B*	S83L,D87N	-
54	> 200	50	*qnrA, qnrB*	S83L,D87N	-
68	> 200	50	*qnrA, qnrB*	S83L,D87N	-
77	> 200	25	*qnr B*	S83L,D87N	-
18	200	50	*qnrA, qnrB*	S83L,D87N	A108V
38	200	50	*qnrA, qnrB*	S83L,D87Y	E84G
60	200	25	*qnrS, qnrB*	S83L,D87N	-
69	200	25	*qnrA, qnrS*	S83L,D87N	-
33	100	25	*qnr B*	S83L,D87N	-
36	100	25	*qnr B*	S83L,D87N	-
41	100	12.5	*qnr B*	S83L,D87N	E84V
63	100	50	*qnrA, qnrB*	S83L,D87N	-
67	50	50	*qnrA*	S83L,D87N	-
53	25	12.5	*qnrA*	S83L,D87N	-

are mostly observed within a highly conserved domain of the *gyrA* N-terminus, known as the quinolone resistance determining region (QRDR) which is in close proximity with the DNA-binding region near the putative active site tyrosine-122 (Piddock, 1999).

Two positions for mutations in GyrA were identified in 17 highly resistant isolates, representing serine-83 and aspartic-87. These two sites were previously identified as being most often observed and their presence has been validated experimentally in resistant strains (Piddock, 1999). High-level fluoroquinolone resistance was detected in *E. coli* isolates in our study. These isolates harbored two GyrA substitutions and in some isolates another additional substitution in ParC. However, no isolate had a mutation in the *parC* gene without the simultaneous presence of quinolone resistance-associated mutations in the *gyrA* gene.

Our *E. coli* isolates exhibited MIC ranging from 12.5-200 and 50-200 µg/ml of levofloxacin and ciprofloxacin, respectively. Four of these isolates also harbored point mutations in the topoisomerase IV subunit genes *parC* which is previously identified in Gram negative bacteria but at a significantly lower frequency than *gyrA* mutations (Ling et al., 2003). These ParC substitutions were detected in Glutamic acid-84 and Alanine-108 amino acid positions in the protein sequence of ParC. The QRDR polymorphisms most commonly detected in this study are those most frequently reported in the literature in *E. coli* (Namboodiri et al., 2011). It is generally believed that *parC* gene mutations arise after *gyrA* gene mutations, as DNA gyrase (rather than topoisomerase IV) is the preferred target of quinolones in Gram negative bacteria (Ling et al., 2003).

This study suggests that in clinical isolates of *E. coli*, DNA gyrase is a primary target of quinolones. In addition, two amino acid changes at Ser-83 and Asp-87 in GyrA were identified in all of high-level quinolone resistant *E. coli* with decreased susceptibility to both ciprofloxacin and levofloxacin. Moreover, the simultaneous presence of the ParC alterations play additional role in developing high-level resistance to quinolones which is supported by lower frequency ParC substitutions in highly quinolone resistant strains.

qnr gene, a naturally occurring gene encoding a pentapeptide repeat protein that confers reduced susceptibility to nalidixic acid or fluoroquinolone can be easily transferred between bacterial isolates due to its presence on mobile genetic elements (Jacoby et al., 2008; Strahilevitz et al., 2009). The first *qnr* gene, now known as *qnrA*, was found to protect *E. coli* DNA gyrase from inactivation by ciprofloxacin (Tran et al., 2005). Other *qnr* genes have been isolated, including *qnrS1* from *Shigella flexneri* (Tran et al., 2005) and *qnrB* (Jacoby et al., 2006).

In this study, the presence of *qnr* genes was investigated in some isolates. *qnr* positive isolates identified at higher level were: *qnrB* (49 isolates) as compared to *qnrS* and *qnrA* (14 isolates and 24 isolates,

respectively). It is important to note that plasmid-encoded *qnr* genes do not confer quinolone resistance by themselves, but facilitate the selection of bacteria bearing higher-level resistance, thereby augmenting the effect of other resistance mutations. The presence of horizontally-acquired genes accounted in part for elevated nalidixic acid MICs in strains that harboured these genes, but not completely. It is therefore possible that other resistance mechanisms, such as *ParE* polymorphisms, other horizontally acquired resistance genes, over-active efflux, or even novel mechanisms are present in some of the isolates (Martínez et al., 1998).

Conclusion

Fluoroquinolones, largely ciprofloxacin and levofloxacin, are considered as antimicrobials of high use in Mansoura hospitals in Egypt. As expected, this study demonstrates that resistance to these drugs is common, present at high numbers in *E. coli* isolates and occurs through multiple mechanisms. The main mechanism is associated with *gyrA* alterations in QRDR. Additionally, horizontally-acquired resistance to the quinolones was also identified at high frequency in this study this is supported by the hypothesis that these genes are present on mobile elements that could be transmitted to different pathogens. Finally, resistance to other antimicrobials except imipenem was also documented in this study which may limit the use of other antimicrobials as alternatives.

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Conflict of interest

The authors declare that they have no conflict of interest.

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