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Table of Content

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Review

An overview of pathogenesis and epidemiology of *Helicobacter pylori* infection

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Helicobacter pylori **induces chronic gastritis, the strongest known risk factor for peptic ulcer disease, distal gastric cancer and a number of extra gastric related morbidity. More than 50% of the world's population is infected with this organism lifelong without effective bacterial eradication. Clinical sequelae are dependent upon bacterial virulence factors and host genetic diversity, particularly within immune response genes. The organism is able to evade the harsh acidic environment in the gastric mucosa and host immune response by elaborating a number of factors that aid in the achievement of its persistent colonization.** ***H. pylori*** **possess numerous virulence proteins (*cagA, vacA* and *iceA*) and enzymes (urease, catalase, lipase, phospholipase and proteases) with substantial genotypic diversity, which engenders differential host inflammatory responses that influence the pathologic outcome. The hallmark of** ***H. pylori*** **infection is a marked inflammatory response with the infiltration of various immune cells into the infected gastric mucosa; with a polarized Th1 immune response which further attracts inflammatory cells to the gastric mucosa leading to damage. Knowledge on** ***H. pylori*** **reservoirs and transmission remains elusive. However, studies have described the gastro-oral, oral-oral and faecal-oral as possible routes of acquisition and transmission. This paper provides an understanding of** ***H. pylori*** **persistence and pathogenesis as well as its route of transmission.**

Key words: *Helicobacter pylori*, pathogencity, virulence factors, epidemiology, risk factors, immune evasion.

INTRODUCTION

The human gastrointestinal tract is colonized by an abundance of bacteria, which are in constant interaction with the epithelial lining usually leading to an intricate balance between tolerance and immunological response (Mbulaiteye et al., 2006; Rasmus et al., 2007). There is ample evidence that the abundant presence of bacteria thus play a role in the maintenance of human health, as well as in the induction of chronic inflammatory diseases of the gastrointestinal tract (Kuipers and Michetti, 2005).

Helicobacter pylori, the principal species of the genus *Helicobacter,* is a common human pathogen that is responsible for a variety of gastro–duodenal pathologies in the developed and developing world (Perez-Perez et al., 1991; Abdulrasheed, 2005). *H. pylori* is a small, curved, highly motile, gram-negative bacillus which is recognized as a chronic colonizer of the human stomach; and known to be one of the most genetically diverse of bacterial species (Ndip et al., 2003; McNulty et al., 2004; Hovey et al., 2007; Talekhan et al., 2008). It has been implicated as the major cause of various diseases since its Nobel-prize-winning discovery by Warren and Marshall in 1982 (Warren and Marshall, 1983; Dixon, 1991; Parsonnet et al., 1991; Ndip et al., 2003). It is closely associated with adenocarcinoma of the distal stomach, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma as well as a number of extra gastric diseases (Permin and Andersen, 2005) A number of factors have been implicated in the pathogenesis of *H. pylori* infection. The risk of disease involves specific interactions between the pathogen and host, which, in turn, are dependent upon strain-specific bacterial factors and/or inflammatory responses governed by host genetic diversity (Peek, 2005; Rasmus et al., 2007). A comprehensive understanding of how *H. pylori* infection causes gastritis, peptic ulcer or gastric cancer is very important in the prognosis

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and management of infection.

The prevalence of *H. pylori* infection varies widely according to geographical area, patient age and socio-economic status (Graham et al., 1991; Segal et al., 2001). Rates of isolation range between 70 - 90% in developing countries and 25 - 50% in developed countries (Logan and Walker, 2002; Tay et al., 2009). In various regions of sub-Saharan Africa, for example, 61 - 100% of the population may harbour the pathogen (Holcombe, 1992; Asrat et al., 2004). Although there is geographical and socio-demographic variation in the prevalence of human infection with the organism (Mukhopadhyay et al., 2000; Ndip et al., 2004; Asrat et al., 2004), prevalence has been reported to be discordant with the incidence of morbidity caused by the infection. In Africa, for example, the prevalence of infection is very high but the incidence of gastric carcinoma and other *H. pylori*-associated morbidities is relatively low (Ahmed et al., 2007). This apparent anomaly has been termed the 'African enigma' (Holcombe, 1992; Tanih et al., 2009).

The principal reservoir of *H. pylori* is man, but there have been descriptions of infection spread by means of water or uncooked vegetables contaminated with sewage and a host of other factors (Feldman et al., 1998; Dube et al., 2009a). The role of domestic animals in the spread of infection still remains unclear (Fox et al., 1995). The putative routes of transmission of the organism have been reported to be faecal-oral, oral-oral, and gastric-oral (Brown, 2000).

This review appraises the factors and or mechanisms that govern *H. pylori* pathogenesis and epidemiology.

PATHOGENESIS OF *H. PYLORI* INFECTION

Virulence proteins

The role of *H. pylori* in gastroduodenal diseases has been firmly established. Infection by the organism is presumed to be from the gastric antrum and then extending down to the corpus after extensive mucosal damage (Akada et al., 2003). Intense research into *H. pylori* has lead to the discovery of virulence factors such as *vacA, cagA* and other proteins like *iceA*. These proteins have revealed many aspects of the relationships between this bacterium, the gastric mucosal surface, and the induction of disease (van Doorn et al., 1998; Smith et al., 2002). Disease outcome is the result of the intricate, ongoing interplay between environmental, bacterial, and host factors. Strain-to-strain genetic variability in bacterial virulence factors does not only affect the ability of the organism to colonize and cause disease but also affects inflammation and gastric acid output (Figueiredo et al., 2002; Kuster et al., 2006).

The vacuolating cytotoxin, *vacA*, is a protein complex which is present in about 50% of all *H. pylori* strains (Haas, 2002). *vacA* leads to the formation of acidic vacuoles in epithelial cells and consequently to their death following infection and colonization of an *H. pylori* strains carrying the gene. *IceA* is a novel gene signaling: induced by contact with epithelium. There are two main allelic variants of the gene: *iceA1* and *iceA2* (van Doorn et al., 1999; Wong et al., 2001; Smith et al., 2002). The function of *iceA* is not yet clear but there is significant homology to a type II restriction endonuclease. The expression of *iceA1* is up-regulated following contact between *H. pylori* and human epithelial cells and may be associated with peptic ulcer and other gastric related diseases (van Doorn et al., 1999; Arents et al., 2001).

The well-characterized *H. pylori* virulence determinant is the *cagA* gene (Ally et al., 1999; Andreson, 2002). Although all *H. pylori* strains induce gastritis, *cagA+* strains significantly augment the risk for severe gastritis, peptic ulcer disease, and distal gastric cancer compared to that incurred by *cagA*– strains. A combination of these genotypes is responsible for prolong and severe risk of disease associated with *H. pylori* infection (Atherton et al., 1995; Asrat et al., 2004).

Initial infection by highly pathogenic strains possessing a cluster of genes known as the *cag* pathogenicity island result in altered expression of several genes associated with glycan biosynthesis, especially β3GlcNAc T5, a GlcNAc transferase required for the biosynthesis of Lewis antigens (Marcos et al., 2008). Resultant over-expression of β3GlcNAc T5 in human gastric carcinoma cell lines lead to increased sialyl-Lewis x expression, a specific kind of sugar molecule that these cells display on their surface as a flag to attract immune cells to the infection site (Nagorni, 2000; Bor-Shyang et al., 2006; Marcos et al., 2008).

While the *rocF* gene is not essential for the initiation of an infection, it encodes arginase, an enzyme responsible for the hydrolysis of L-arginine to L-ornithine and urea. Unavoidable, arginase allows the bacterium to evade host immune response by competing with macrophage iNOS for L-arginine. Due to bacterial cell deficiency in arginine synthesizing enzymes, this organism exploits the host's arginine to maintain the nitrogen balance (Hovey et al., 2007).

Gastric colonization

H. pylori have been shown to employ multiple mechanisms to antagonize, impair, or subvert host responses (Ernst et al., 2006). The stomach is protected by a mucosal barrier that prevents gastric secretions and other destructive agents from injuring the epithelial and deeper layers of the stomach wall (Radosz-Komoniewska et al., 2005). The integrity of the mucosal layer is maintained by tight cellular junctions and the presence of a protective mucus layer. Prostaglandin is derived from the cell membrane lipids and serves as a chemical messenger that protects the stomach lining by improving

blood flow, increasing bicarbonate secretion, and enhancing mucus production (Porth, 2002).

Controversy still persists on the duration of the relationship between *H. pylori* and humans considering how this pathogen has adapted to having a complete life in the human stomach (Scott et al., 2007). However, co-evolution of *H. pylori* with humans over thousands of years has effectively refined the interactions that occur between bacterial and host factors, transmission between hosts, survival during acidic stress within hosts, and avoidance of immune response (Blaser, 1997; Scott et al., 2007). Gastric acidity and peristaltic muscle movement of the alimentary canal have the potential to preclude bacterial colonization of the human stomach.

H. pylori have evolved several mechanisms to evade primary host defences such as acidity and peristalsis in order to establish persistent infection within the stomach. The organism elaborates a number of enzymes of which urease is one of the most important (Malcolm et al., 2004; Peek, 2005). Urease is conserved among all known *Helicobacter species* and is a necessary factor for the establishment of chronic infection *with* the organism. Two major subunits of this enzyme have been identified (*ureA* and *ureB*). This accessory protein, catalyses the cleaving of urea into ammonia and hydrogen carbonate, achieving a local neutralization of the acid pH in the cytoplasm and on the periplasm (Tanahashi et al., 2000; Peek, 2005; Suarez et al., 2006). Thus, the pathogen can successfully survive in the gastric lumen (pH 1 - 2) for a short time before it penetrates into the bicarbonate-buffered mucus layer of the gastric mucosa, its real habitat (Benanti and Chivers, 2009). The mucus layer has a pH gradient reaching from the epithelial cell surface (pH 7) to the lumen (pH 2), and the pathogen reacts chemotactically to this gradient (Haas, 2002).

Isolates that lack the ability to produce urease correspondingly fail to colonize rodent models indicating the importance of this conserved enzyme (Peek, 2005). In addition to urea that can be bacterial derived or obtained from the host (Hovey et al., 2007), other enzymes such as catalase and oxidase are produced (Kusters et al., 2006).

Motility within the gastric mucosal is aided by five or six polar flagella that are comprised of two major structural subunits: *flaA* and *flaB*. The genes encoding these two flagellar components are located at distant sites on the *H. pylori* chromosome and are transcriptionally regulated by different promoters (O'Toole et al*.,* 2000). Similar to urease production, motility is required for persistent infection, and recent data have shown that a component of the flagellar secretion apparatus, which regulates flagellar biosynthesis, also regulates urease activity (Peek, 2005). They are coupled by the *FlbA* gene.

Other very important virulence factors are adhesins, which allows binding of the bacterium to the gastric cells. Many different molecules such as *SabA, OipA, AlpA*, and *AlpB*, show adhesion activity, including the BabA2 outer membrane protein, which is encoded by the *bab* (blood group antigen binding) genes (Maeda and Mentis, 2007). The BabA2 protein can bind fucosylated polysaccharides, which are blood antigens known as Lewis blood antigens (Ilver et al., 1998; Sheu et al., 2003). These antigens have been found both on the surface of the mucous membrane and in *H. pylori* lipopolysaccharide.

Inter-species and intra-species entero-coexistence has been highlighted in several studies, with competitive exclusion failing to take its toll (Gibson et al., 1998; Nagorni, 2000; Akada et al., 2003; Fritz et al., 2006; Samie et al., 2007). Coexistence is enhanced by failure of competitive exclusion by *H. pylori* strains suggesting that different strains occupy different gastro-mucosal micro-niches (Akada et al., 2003). The organism has the capacity for horizontal gene exchange hence enabling genetic variability within the population. In addition, it shows competency in the uptake of DNA from other *H. pylori* cells (Blaser and Artherton, 2004; Schwarz et al., 2008). The profound diversity exhibited by this organism can play an influential role in the survival of the population in its niche. Flexibility or adaptability in this population, allows for maximised use of resources in a variety of niches and the size or availability of these gastro-mucosal micro-niches is affected by host genotype and age or physiology (Blaser and Artherton, 2004).

Avoidance of the immune response

If a bacterial species is to persistently colonize its host, its most formidable challenge is to evade immune clearance. *H. pylori* evade immune clearance including the harsh environment in the gastric mucosa, and elicit systemic and mucosal immune responses which, however, are unable to clear the infection (Suarez et al., 2006). Multiple lines of evidence suggest that the immune response contributes to the pathogenesis associated with the infection (Suarez et al., 2006).

Instead of killing the colonizing bacteria, the immune response may lead to destruction of epithelial cells and thinning of the mucosal lining leading to increased mucosal contact with luminal acid (Fan et al., 1998; Beswick et al., 2005). This process is first associated with up-regulation of various genes that are associated with the innate immune system including various Toll-like receptors; complement factor C3, lactoferrin, and bactericidal/permeability-increasing protein. The Toll-like receptor induction in particular occurs through the bacterial LPS. Signaling pathways utilized by these receptors all appear to eventuate in NF-κB activation and proinflammatory gene expression (Peek, 2005). Another mechanism through which *H. pylori* may persist is by limiting the bactericidal effects of proinflammatory molecules, such as nitric oxide. The organism has evolved strategies to avoid global activation of this system (Permin and Andersen, 2005; Peek, 2005; Ernes et al., 2006).

Both natural and acquired specific immune responses

to the organism are elicited at gastric mucosal level. Response to gastro duodenal infection by the organism is characterized by mucosal infiltration of lymphocytes, plasma cells, neutrophils and monocytes. Those infected with the organism have been reported to have elevated titres of IgG and IgA antibodies directed at membrane proteins (MP), flagelin, urease, lipopolysaccharide (LPS), adhesin A (HpaA) as well as IgM- and IgA-producing cells in biopsies from the antral region of the patients' stomaches. These suggest that the infection induces a large recruitment of immune cells into the gastric mucosa, particularly IgA-producing cells (Mattson et al., 1998).

The inflammatory process is further characterized by the production of various cytokines such as IL-2, IL-3, IL-12, as well as IFN-γ (Harris et al., 1996; Kuipers and Michetti, 2005). Colonisation unavoidably stimulates nuclear factor-kappa B (NF-kappa B) activation and interleukin-8 (IL-8) expression in gastric epithelial cells (Kim et al., 2003; Lundgren et al., 2003). Toll-like receptor 2 (TLR2) and 5 (TLR5) recognize *H. pylori* and initiate signalling pathways that result in enhanced activation of NFKB; IL-8 is secreted by the host cells to attract components of the innate and adaptive immune systems to the site of infection. This polarises the immune response towards a Th1 response, further attracting inflammatory cells and T-lymphocytes (Harris et al., 1996; Kim et al., 2003; Lundgren et al., 2003; Yamasaki et al., 2004; Suarez et al., 2006). An effective CD4+ T-cell response is essential to clear *H. pylori*, however this organism has been shown to inhibit CD4+T-cell proliferation and arresting IL-2 cell-cycle progression resulting in avoidance of clearance thereby staging an infection (Gebert et al., 2003; Sundrud et al., 2004; Rasmus et al., 2007).

However, some infection with *H. pylori* elicits a Th2 instead of Th1-dominant immune response to thwart their elimination and could plausibly modulate *H. pylori*-induced immune response towards one less damaging to the gastric mucosa (protective). This response results in the elaboration of pro-inflammatory cytokines such as IL-4, IL-5 and IL-10 (Rasmus et al., 2007). It has been suggested that persons living in high *H. pylori*-prevalence areas with low gastric-cancer incidence like in Africa might have Th2-type dominant *H. pylori*-specific responses (Mbulaiteye et al., 2006).

Besides presenting *H. pylori* antigens to the specific T cells recruited into the gastric antrum, antigen presenting cells release several cytokines, such as IL-1, IL-6, TNF-α and IL-12, whose local concentration strongly influence the developing specific T-cell response (Harris et al., 1996; Yamasaki et al., 2004). Other than inhabiting superficial glycoprotein-rich mucosal niche meant to protect stomach cells from the secreted acids in the stomach cavity, a micro-distance from inflamed glandular cells (Mahdavi et al., 2002; Delport et al., 2006), the organism also avoids recognition by producing specific bacterial factors that stimulate selective expression of host genes and also by inducing an ineffective T-cell response. Genetic diversity of this organism also plays a paramount role in its persistence (Mahdavi et al., 2002).

In uncomplicated chronic gastritis and gastric MALTomas, most of gastric *H. pylori*-induced specific T cells have been reported to elaborate the secretion of both Th1- and Th2-type cytokines (Yamasaki et al., 2004; Rasmus et al., 2007). Existing data suggest that host gastric immune response to *H. pylori* can influence the clinical picture and that gastroduodenal disease may be an immunopathological consequence of a Th1-polarized response to some *H. pylori* antigens, whereas exhaustive and deregulated *H. pylori* -induced T cell-dependent B-cell activation may support the onset of low-grade gastric B-cell lymphoma (De Jong and Enbald, 2008).

PATHOPHYSIOLOGY AND CLINICAL MANIFESTATIONS

Gastritis

Gastritis refers to inflammation of the gastric mucosa. There are many causes of gastritis; most of which can be grouped as acute or chronic gastritis. Chronic infection with *H. pylori* can lead to gastric atrophy and intestinal metaplasia (Kuipers et al., 1995). Acute gastritis refers to the transient inflammation of the gastric mucosa (Kuipers et al., 1995). It is most commonly associated with local irritants such as bacterial endotoxins, caffeine, alcohol, and aspirin (http//www.tjclarko.com/d_ulcers.htm, Furuta and Delchier, 2009). Depending on the severity of the disorder, the mucosal response may vary from moderate oedema and hyperaemia to haemorrhagic erosion of the gastric mucosa (Porth, 2002). Clinical manifestations of acute gastritis include heartburn or sour stomach, transient gastric distress, which may lead to vomiting and, in more severe situations, to bleeding and hematemesis. Acute gastritis is usually a self-limiting disorder; complete regeneration and healing usually occur within several days (Porth, 2002).

Chronic gastritis is characterized by the absence of grossly visible erosions and the presence of chronic inflammatory changes leading eventually to atrophy of the glandular epithelium of the stomach (Kuipers et al., 1995). The changes may become dysplastic and possibly transform into carcinoma. *H. pylori* and a number of factors such as chronic alcohol abuse, cigarette smoking, and chronic use of non steroid anti-inflammatory drugs (NSAIDs) may contribute to the development of the disease (Palmer et al., 2002; Furuta and Delchier, 2009). There are four major types of chronic gastritis: *H. pylori* gastritis, autoimmune gastritis, multifocal atrophic gastritis and chemical gastritis (Porth, 2002; Ernst et al., 2006; Furuta and Delchier, 2009). *H. pylori* gastritis is a chronic inflammatory disease of the antrum and body of

the stomach. It is the most common type of chronic non-erosive gastritis in the United States.

Peptic ulcer disease

Since the early 1980s, there has been a radical shift in thinking regarding the cause of peptic ulcer. No longer is peptic ulcer thought to result from a genetic predisposition, stress, or dietary indiscretions (http//www.tjclarko.com/d_ulcers.htm). Much of the familial aggregation of peptic ulcer whose development was formerly linked to genetic factors is now thought to be due to intra-familial infection with *H. pylori* rather than genetic susceptibilities (van Doorn et al., 1999; Smith et al., 2002; Porth, 2002; Ndip et al., 2008). The most common forms of peptic ulcer are duodenal and gastric ulcers (van Doorn et al., 1999; Figueiredo et al., 2002). It has been documented that virtually all persons with duodenal ulcer and 70% of those with gastric ulcer have *H. pylori* infection. However, two other forms of gastric ulcers, Zollinger-Ellison syndrome and stress ulcers have different causes (Porth, 2002).

Peptic ulcer disease, with its remissions and exacerbations, represent a public health problem (Smith et al., 2002). It has been documented that approximately 10% of the population have or will develop peptic ulcer (Porth, 2002). Duodenal ulcers occur five times more commonly than gastric ulcers; it occurs at any age and frequently is seen in early adulthood. Gastric ulcers turn to affect the older age group, with a peak incidence between 55 and 70 years of age. Both types of ulcers affect men three to four times more frequently than women (Porth, 2002).

Peptic ulcer can affect one or all layers of the stomach or duodenum. It may penetrate only the mucosal surface, or it may extend into the smooth muscle layer. Occasionally, an ulcer may penetrate the outer wall of the stomach or duodenum; with spontaneous remissions and exacerbations being common. The second most common cause of peptic ulcer is NSAID and aspirin use (Porth, 2002; Furuta and Delchier, 2009). There is a 10 - 20% prevalence of gastric ulcers and 2 - 5% prevalence of duodenal ulcers among chronic NSAID users. Aspirin appears to be the most ulcerogenic of NSAIDs. Ulcer development in NSAID user is dose dependent, but some risk occurs even with aspirin doses of 325 mg/day (http//www.tjclarko.com/d_ulcers.htm, McQuaid, 2001; Furuta and Delchier, 2009). The pathogenesis of NSAID-induced ulcers is thought to involve mucosal injury and inhibition of prostaglandin synthesis. In contrast to peptic ulcer from other causes, NSAID-induced gastric injury is often without symptoms, and life-threatening complications can occur.

Clinical manifestations of peptic ulcer include discomfort and pain. The pain, which is burning, gnawing, or cramp-like, is usually rhythmic and frequently occurs when the stomach is empty - between meals and at 1 or 2 O'clock in the morning. The pain is usually located over a small area near the midline of the epigastrium near the xiphoid, and may radiate below the costal margins, into the back, or, rarely, to the right shoulder (Furuta and Delchier, 2009). Superficial and deep epigastric tenderness and voluntary muscle guarding may occur with more extensive lesions. An additional characteristic of ulcer pain is periodicity. The pains turn to recur at intervals of weeks or months. During an exacerbation, it occurs daily for a period of several weeks and then remits until the next recurrence. Characteristically, the pain is relieved by food or antacids (Porth, 2002).

Complications of peptic ulcers include haemorrhage, obstruction, and perforation. Haemorrhage is caused by bleeding from granulation tissue or from erosion of an ulcer into an artery or vein. It occurs in up to 10 - 20% of persons with the condition (Graham et al., 1993; McQuaid, 2001). Evidence of bleeding may consist of hematemesis or melena. Bleeding may be sudden, severe, and without warning, or it may be insidious, producing only occult blood in stool. Up to 20% of persons with bleeding ulcers have no antecedent symptoms of pain; this is particularly true with person's receiving NSAIDs. Acute haemorrhage leads to a sudden onset of weakness, dizziness, thirst, cold and moist skin, the desire to defecate, and the passage of loose, tarry, or even red stools and coffee-ground emesis. Signs of circulatory shock develop depending on the amount of blood lost (Graham et al., 1993).

Obstruction of blood flow is caused by oedema, spasm, or contraction of scar tissue and interference with the free passage of gastric contents through the pylorus or adjacent areas. There is feeling of epigastric fullness and heaviness after meals. With severe obstruction, there is vomiting of undigested food. Perforation occurs when an ulcer erodes through all the layers of the stomach or duodenum wall. Perforation develops in approximately 5% of persons with peptic ulcers usually from the anterior wall of the stomach or duodenum (McQuaid, 2001). With perforation, gastrointestinal contents enter the peritoneum and cause peritonitis, or penetrate adjacent structures such as the pancreas. Radiation of pain into the back, severe night distress, and inadequate pain relief from eating foods or taking antacids in persons with a long history of peptic ulcer may signify perforation (Graham et al., 1993).

Gastric carcinoma

Infection with *H. pylori* appears to serve as a cofactor in some types of gastric carcinomas (Wotherspoon, 1998). Although its incidence has decreased during the past 50 years, stomach cancer is the seventh most frequent cause of cancer mortality in the United States. In 2001, it was estimated that approximately 21,700 Americans

were diagnosed with stomach cancer and 12,800 died of the disease (Porth, 2002). The disease is much more common in other countries and regions, principally Japan, Central Europe, the Scandinavian countries, South and Central America, Soviet Union, China, and Korea; and is the major cause of cancer death worldwide (Porth, 2002). Among factors that increase the risk of gastric cancer is genetic predisposition, carcinogenic factors in diet (e.g., *N*-nitroso compounds and benzopyrene found in smoked and preserved foods), autoimmune gastritis, and gastric adenomas or polyps (Ernst et al., 2006; Furuta and Delchier, 2009).

Virtually all tumours are adenocarcinomas arising from mucus-secreting cells in the base of the gastric crypts. Most develop upon a background of chronic atrophic gastritis with intestinal metaplasia and dysplasia. Stomach cancers are either 'intestinal', arising from areas of intestinal metaplasia with histological features reminiscent of intestinal epithelium, or 'diffuse', arising from normal gastric mucosa (Palmer et al., 2002). Intestinal carcinomas are more common, and arise against a background of chronic mucosal injury. Diffuse cancers tend to be poorly differentiated and occur in young patients. Between 50 and 60% of gastric cancers occur in the pyloric region or adjacent to the antrum (Furuta and Delchier, 2008). Compared with a benign ulcer, which has smooth margins and concentrically shaped, gastric cancers tend to be larger, irregularly shaped, and have irregular margins. Unfortunately, stomach cancers often are asymptomatic until late in their course. Symptoms, when they do occur, are usually vague and include indigestion, anorexia, weight loss, vague epigastric pain, vomiting and an abdominal mass (Porth, 2002).

Gastric lymphoma

H. pylori are closely associated with the development of a low-grade lymphoma ('MALToma') (Wotherspoon, 1998). Lymphoid tissue is not found in the normal stomach but lymphoid aggregates develop in the presence of *H. pylori* infection (Seymour et al., 1997). Superficial MALTomas may be cured by *H. pylori* eradication (Palmer et al., 2002). Primary gastric lymphoma comprises about 3-6% of all the gastric malignancies (Wotherspoon, 1998). The stomach is however, the most common site for extranodal non-Hodgkin's lymphoma and 60% of all primary gastrointestinal lymphomas occur at this site. The clinical presentation is similar to that of gastric cancer and endoscopically the tumour appears as a polypoid or ulcerating mass.

EPIDEMIOLOGY AND ANTICIPATED MODES OF TRANSMISSION

Trends in Prevalence

H. pylori have a computed high prevalence worldwide. Several studies have highlighted inconsistencies between the prevalence rates for *Helicobacter* and disease. In industrialized countries there is generally a low prevalence and yet a relatively high prevalence of gastric cancer. On the other hand, some countries with high *Helicobacter* prevalence rates have low gastric cancer prevalence. The prevalence of *H. pylori* infection though declining in the developed world (Graham et al., 1991; Segal et al., 2001) still varies widely by geographic area, age, race, and socioeconomic status (Malcolm et al., 2004).

It is not possible to ascertain when infection occurs clinically hence most of the information on the rates of *H. pylori* in geographically and demographically diverse populations are drawn from seroprevalence studies. Retrospective seroepidemiological studies have shown a cohort effect consistent with the hypothesis that infection is mainly acquired in early childhood (Logan and Walker, 2002; Thomas et al., 2004). In a rural village of Linqu Country, Shandong Province, China, a study of 98 children found that nearly 70% of those aged 5-6 years were infected with the organism, a rate similar to that reported for adults in that area, suggesting that most infection takes place early in childhood (Dale et al., 1998).

Infection with this organism is relatively common in Africa (Asrat et al., 2004; Ndip et al., 2004; 2008; Dube et al., 2009b) and the organism is the main cause of at least 90% of duodenal ulcers and 70% of gastric ulcers (Ndip et al., 2008; Tanih et al., 2010). Studies conducted in various parts of Africa have revealed high sero-prevalence of infection (61-100%) which differs from country to country and between different racial groups within each country (Holcombe, 1992). Childhood acquisition is the rule with more than 50% of all children in Africa being infected by the age of 10 years, with prevalence rising to 80% in adults (Segal et al., 2001).

More recent reports also show a wide variation of infection rates, with anti-*H. pylori* IgG antibodies reported in 85.6% in 215 dyspeptic individuals in Ethiopia. Kidd et al. (1999) documented a prevalence of 25 and 97% in Uganda and Ghana respectively. Nabwera et al. (2000) in their study observed a high prevalence among Kenyan children aged between 3 - 5 years, indicating that most children in the study area were infected before they reached their third birthday. In Mozambique, Carrilho et al. (2009) reported a high prevalence of 94.5% while in Cameroon Ndip et al. (2008) equally documented a high prevalence of 92.2% in their study population. In Ethiopia, a prevalence of 93% was found in a study by Henriksen et al. (1999) on patients with peptic ulcer disease. In the Democratic Republic of Congo, a seroprevalence of 62.4% was delineated among the study participants (Longo-Mbenza et al., 2007).

H. pylori infection also appears to be common in South Africa; Pelsar et al. (1997) documented a high prevalence (67 - 84%) of *H. pylori* antibodies in children in Bloemfontein, while Mosane et al. (2004) also reported *H. pylori* IgG antibodies in South African mothers and their

children. Recently, Samie et al. (2007) described a prevalence of 50.6% in their study in Venda, North of South Africa. In a recent study of asymptomatic individuals in the Eastern Cape Province, *H. pylori* antigenemia was observed in 86.8% in the stools of our study subjects (Dube et al., 2009b).

In the Western world a number of studies have also reported a high prevalence of the organism in children (Thomas et al., 2004). The difference in the rate of childhood acquisition of infection is probably responsible for the differences seen, in the prevalence of infection, between developed and developing countries (Segal et al., 2001). The prevalence of infection and the incidence of gastric cancer are higher in Asia, South America, and the Caribbean than in Europe and the United States (Segal et al., 2001).

A study carried out in Guatemala revealed the presence of this organism in about 58% of the participants' enrolled (Dowsett et al., 1999). Ghose et al. (2005) in Venezuela also found a high prevalence of the organism [121/127(95.3%)] in their study population. Prevalence of infection has been reported to be higher among blacks than Caucasians in the United States (Hisada et al., 2001). A study which enrolled a Hispanic population reported a prevalence of about 79% (Dehesa et al., 1991). The epidemiology of *H. pylori* infection in the Caribbean islands remains an important concern for public health investigation because of the high prevalence of this infection and its association with gastric cancer. In Jamaica a high prevalence of 70% has been described (Hisada et al., 2001).

Plausible factors exacerbating spread

The prevalence of *H. pylori* has been reported to be high in African Americans, Hispanic, Asian and Native American populations with similar infection rates in males and females (Dehesa et al., 1991). Until recently, it has been difficult to assess accurately the incidence (or route) of infection because of the inaccuracy and cost of detecting (non-invasively) *H. pylori* in young children. Primary acquisition in adults, or re-infection after successful eradication, does occur but is less common, with an annual incidence of 0.3-0.7% in developed countries and 6-14% in developing countries (Logan and Walker, 2002).

The generally high prevalences of human infection seen in Africa and the world at large are an indication that effective public-health interventions need to be developed; while the variations seen in the prevalence of infection between and among populations may point to the fact that parameters such as age, cultural background, genetic predisposition, socio - economic status and environmental factors all play a role in the acquisition and transmission of *H. pylori* (Graham et al., 1991; Segal et al., 2001; Chong et al., 2008; Dube et al., 2009b).

A number of authors have emphasized the role of other factors such as smoking, alcohol consumption, occupational exposure, waterborne exposures, hygienic practices, density/crowding, social factors and family history of gastric disease (Ogihara et al., 2000; Brown et al., 2000; Iso et al., 2005). Within countries, there may be a similarly wide variation in prevalence between the more affluent urban populations and the resource-poor rural populations. A lack of proper sensitization, good drinking water and poor diet seem to play a role in the high prevalence of infection (Dale et al., 1998; Ndip et al., 2004; Dube et al., 2009b).

There appears to be a substantial reservoir of *H. pylori* aside from the human stomach. Animals, e.g. cats, monkeys etc harbour organisms that resemble *H. pylori* (Dubois et al., 1994) but under particular circumstances (Fox et al., 1995). These animals could be reservoirs for human infection. Although possibly important in some circumstances, neither a zoonotic reservoir nor food appears to be significantly involved in acquisition of *H. pylori*. Thus the major question of transmission is how *H. pylori* move from the stomach of one person to that of another.

MODES OF TRANSMISSION

Although epidemiologic studies have addressed a variety of factors such as bacterial, host genetic and environmental factors to delineate the causative links of *H. pylori* infection; knowledge of reservoirs and transmission modes remain poor (Thomas et al., 2004; Asrat et al., 2004; Ndip et al., 2004). However, some routes have been described (Tanih et al., 2008).

Gastro-oral routes

With human being the only known reservoir of infection, it is likely that in developed countries *H. pylori* is picked up from siblings, other children, or parents predominantly via the gastro-oral route (Brown et al., 2000). The organism has been recovered from vomitus after specific culture based approaches on selective media (Leung et al., 1999; Ndip et al., 2003)

Iatrogenic transmission

Iatrogenic transmission is the mode, in which tubes, endoscopes or specimens in contact with the gastric mucosa from one person are introduced to another person (Akamatsu et al., 1996). Adequate sterilization and disinfection of endoscopes has reduced the incidence of transmission (Tytgat, 1995). Endoscopists, especially those who do not wear protective clothing during procedures, are occupationally exposed

(Sobala et al., 1991; Kikuchi and Dore, 2005). This is however the least common form of transmission.

Faecal-oral transmission

Faecal-oral transmission appears to be the most important route of transmission (Tanih et al., 2008). Albeit being isolated from the faeces of young children infected with the organism (Thomas et al., 2004), faecal isolation is not common; this could indicate that shedding is intermittent (Mackay et al., 2003). Faecally contaminated water may be a source of infection (Klein et al., 1991; Dube et al., 2009a) but the organism proves difficult to be isolated from water. Food-borne transmission and unclean hands have also been substantiated (Kersulyte et al., 1999). *Helicobacter* seropositivity increased with consumption of uncooked vegetables in Chile which was perhaps related to contaminated water used on the vegetables (Hopkins et al., 1993).

Oral-oral transmission

Oral-oral transmission has been identified in African women who feed their children with premasticated foods (Me´graud, 1995). Premastication of food was common in Burkino Faso families with both mother and child sero-positive for *H. pylori* when compared to frequencies in families with a sero-positive mother and a sero-negative child (Aditya et al., 2009). There is evidence of intrafamilial transmission; in which poor living conditions has been shown to increase the risk of infection (Aguemon et al., 2005). Although dental plaque has been proposed to be a possible route of transmission (Majmudar et al., 1990; Desai et al., 1991) this has failed in other studies (Bernander et al., 2003).

Sexual transmission

There is no identified association of infection with sexual transmission (Perez- Perez et al., 1991) and such transmission, if it occurs, must be uncommon.

CONCLUSION

The data presented in this review demonstrates the importance of the interactions between virulence factors of *H. pylori* and host cells, and the consequences that follow interplay between the bacterium and cells of the immune system. *Helicobacter* infections induce inflammation and stimulate an ineffective immune response. Alteration of epithelial cell growth and en-hanced apoptosis play a role in disease manifesttations; failure of mucosal adaptation, ulceration, and abnormal repair may predispose to malignancy. However, many questions still remain unanswered on *H. pylori* epidemiology; further studies are therefore required to gain a better understanding of the transmission pathway of this notorious pathogen.

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Review Paper

Advances in research of pathogenic mechanism of pine wilt disease

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Pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus*, is the most serious disease of pine tree with great economic losses. So far it is not clear why the pine trees turn wilting, though several hypotheses about the pathogenic mechanism of pine wilt disease have been presented, such as phytotoxins causing death of pine trees; cellulases hydrolyzing celluloses of pine tree; terpenoids causing cavitation and water column breakage of pine tree, etc. Recently, it was found that certain bacteria, symbiotically associated with the pinewood nematode, may play some roles in the pathogenicity of the disease. Since the pine wilt disease is a complex interrelationships among beetle, pine tree, fungi, bacterium and nematode, all the pathogenic factors are not mutually exclusive, which means a variety of factors make pine tree for death, rather than a single factor. Pinewood nematode and bacteria produce phytotoxins and cellulases, which cause the defense of pine tree and stimulate the production of terpenoids to form cavitation, break water columns and finally make pine trees wilting. Pinewood nematode is involved in the production of phytotoxins, cellulases and terpenoids; therefore it is a vital and indispensable factor for pine wilting disease.

Key words: Pinewood nematode, *Bursaphelenchus xylophilus*, pathogenic mechanism, terpenoid, cavitation, cellulase, phytotoxin, bacteria.

INTRODUCTION

Pine wilt is a disease of pine (*pinus* spp.) caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. PWN is native to North America and is not considered as a primary pathogen of native pines, but is the cause agent of pine wilt for some non-native pines. Although the first occurrence of pine wilt disease (PWD) was reported in 1905 in Nagasaki City, Japan (Yano, 1935), PWN was not identified as the causal agent of the disease until 1971 (Mamiya and Kiyohara, 1972). So far PWD has been reported from North America (Canada, the United States and Mexico), East Asia (Japan, Korea and China) and Europe (Portugal) (Yano, 1913; Cheng, 1983; Tzean and Jan, 1985; Guiran and Bruguir, 1989; Yi et al., 1989; Dwinell, 1993; Mota et al., 1999). It has become a world-wide threat to pine forests and forest ecosystem with great economic losses. In 2003, this disease had killed about 1,000,000 m^3 of pine trees in Japan (Forestry agency, 2004).

However, until now the pathogenic mechanism of PWD has not been clearly illustrated. For a long time, it was thought that the PWN was the only pathogenic agent causing the disease (Mamiya, 1975; Nickle et al., 1981; Nobuchi et al., 1984; Fukuda et al., 1992), to be exactly, phytotoxins, cellulases, which are produced by PWN, ethylene and terpenoids produced by pine trees which are stimulated by invasion of PWN. Recently, it was found that some bacteria are symbiotically associated with the PWNs and may play some roles in the pathogenicity of the disease (Oku et al., 1979; Kawazu, 1998; Han et al., 2003; Zhao et al., 2003; Zhao et al., 2005). In order to be sure of the real cause of pine wilt disease, some hypo-

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theses were introduced and discussed in this paper.

PHYTOTOXIN HYPOTHESIS

During the 70s and 80s of the 20th century, some scientists considered that phytotoxins which was isolated from infected pine trees or from *B. xylophilus* can directly cause wilt symptoms. Oku et al. (1979) found that the filtrate of pine leaf juice in which the pathogenic nematodes were infected could cause wilting of the seedlings, boiled extract of pine wood also contained toxin product. Subsequently, some chemical compound such as benzoic acid, catechol, dihydroconifery alcohol, 8-hydroxycarvotanacetone (carbone hydrate) and 10-dydroxyverbenone were isolated from infected PWN pine trees and could cause wilt symptoms of susceptible pine trees (Oku, 1979). Among them, 8-hydroxycarvotanacetone, dihydroconifery alcohol and 10-dydroxyverbenone could inhibit the reproduction of *B. xylophilus*. Shaheen (1984) also got a similar conclusion that the phytotoxins, which were lipid materials with low molecular weight and isolated from *B. xylophilus* infected scots pine, caused wilting of 45-day-old and 2-year-old pine seedlings in a certain dose.

However, Kozlowski (1962, 1968) held a contrary opinion that PWD kills tree by interfering with water translocation rather than immediate and direct toxic effects. Cao and Shen (1996) studied the toxicity of extraction of PWN, which was cultured on an artificial medium and found that nematode extraction was not toxic to 30-day-old seedlings of *Pinus thunbergii* and *Pinus massoniana*. They concluded that wilt toxins were not produced by PWN under artificial culture conditions. In addition, some scientists hold the alternate opinion that bacteria associated with the PWN produce toxin (Kawazu, 1998; Han et al., 2003; Zhao et al., 2003).

CELLULASE HYPOTHESIS

Cellulases were detected by the analysis of the homogenates and extracts of the nematode species in more than ten genera, including the genus *Bursaphelenchus* (Tracey, 1958; Krusberg, 1960; Dropkin et al., 1962; Morgan and Mcallan, 1962; Dropkin, 1963; Odanil et al., 1985). Cellulases were exuded outside of PWN and left in PWN migrating track which were detected by Yamanoto (1986). After that he collected 9.8×10^6 *B. xylophilus* in 10 ml of distilled water. And then 0.5 ml of the supernatant of this suspension was filtered and injected to pine seedlings. No complete necrosis of the needles was observed in the seedlings, but sporadic distribution of the necrotic needles was observed. Close observations of pine tissues infected with *B. xylophilus* indicated that the destruction of pine cells might be a result of cell wall degrading enzymes such as cellulase (Ishida et al., 1997; Ichihara et al., 2000).

Feeding of 0.5 ml 1% solution of cellulase Onozuka R-10 (Kinki Yakult) to 3-year-old *P. densiflora* seedlings resulted in a complete necrosis of living shoots less than 72 h. Other symptoms such as oleoresin leaks and partial blocking of xylem water conduction were also reproduced by feeding seedlings the crude or high molecular weight fractions of the extracts of the PWN. Therefore Odani et al. (1985) thought that cellulase produced by the PWN is responsible for the development of the early symptom and is a strong candidate of the pathogen. The glycosyl hydrolase family have been isolated from the *B. xylophilus* and characterized (Kikuchi, 2004). In 45 kinds of cellulases, Bx-ENG-1, 2 and 3 could be secreted through the nematode stylet into plant tissues and participate in the weakening of the cell walls, allowing nematodes to feed and migrate more easily in pine tissues. From total homogenates of *B. xylophilus*, Zhang (2006) found a high molecular weight cellulase antigen, which was able to hydrolyze carboxymethyl cellulose efficiently (155.65 U/mg) and had an approximate molecular mass of 58.9 kDa. The style of secreting cellulase from the stylet and using hydrolyze cellulase to facilitate the PWN entering host cells is consistent with other plant parasitical nematodes. Moreover, strong fluorescence signals from cellulase staining were observed in tracheid cells which were naturally infected by PWN, in addition to ray cells and the resin canal zone (Zhang, 2006).

These results support that the nematode–originated cellulase is one of the strong candidate of the pathogenic substances responsible for the development of the pine wilt disease. It is also reported that endoglucanases used by the nematode to degrade the cell walls of fungi on which it feeds as cellulose, as well as chitin and other polysaccharides, have been shown to be present in the walls of some of the fungi on which *B. xylophilus* feed (Cherif et al., 1993).

TERPENOID HYPOTHESIS

Kuroda et al. (1988) proposed that the ultimate death of pine tree is due to water deficit induced by extensive cavitation of sapwood. The pathway of water movement in a tree is via a bundle of capillary water columns. Once any breaks in the water column disrupt water flow, water conduction of xylem tracheids could be impeded due to embolism. Embolized tracheids are filled with air, resulting from cavitation produced by the breakage of water columns in xylem conduits (Ikeda, 1992). By using the acoustic emission technique, Ikeda approved that the occurrence of cavitation events in Japanese black pine growing under field conditions is comparatively rare, even in summer. Based on the results, it seemed that xylem cavitation is caused by pathogenic factor, not by cultivation condition. Cavitation of tracheids is a remarkable initial symptom of PWD caused by the PWN (Kiyohara,

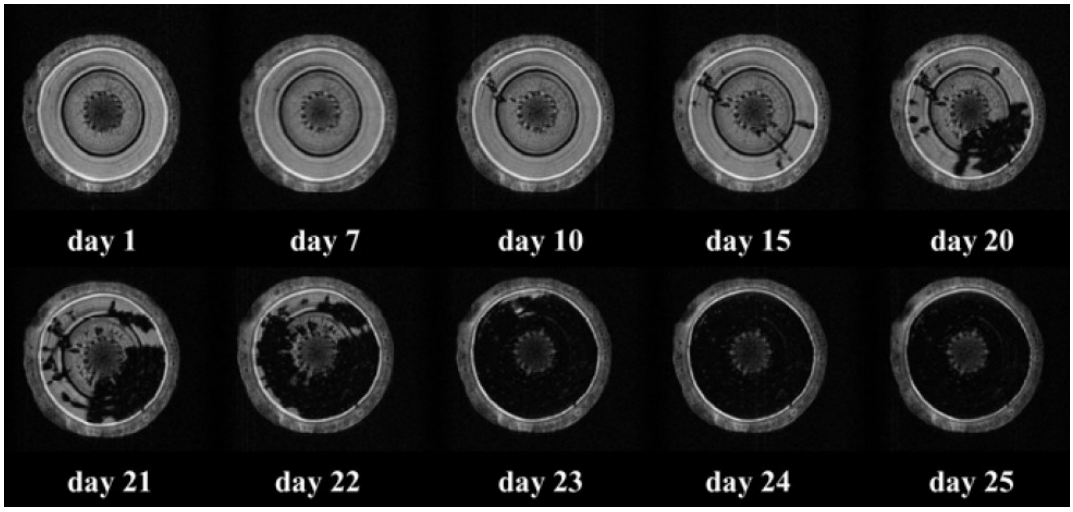

Figure 1. Spin–echo T1–weighted magnetic resonance images (transverse–slice) of inoculated seedling from 1 to 25 days after inoculation. Cavitated xylem appears as dark patches. Repetition time (TR) =500 ms, echo time (TE) = 22 ms, 256 × 256 pixels. Stem diameter 9 mm. Reproduced from Utsuzawa SK (2005).

1990). By observation Kuroda (1992) found that xylem water–blockage caused by cavitation started one week after nematode inoculation.

Kuroda (1989) found that parenchyma cells, which were injured by moving and feeding of nematode, synthesized terpenoids. Seven monoterpenes from infected pine trees: α–pinene, , camphene, β–pinene, myrcene, limonene, β–phellandrene, and p–cymene, were detected. Volatile terpenoids evaporated in tracheids under negative pressure and made bubble. Refilling of cavitated tracheids with water was prevented by hydrophobic effects of terpenoids, therefore, permanent cavitation enlarged gradually. Consequently, cavitated areas reached to cambium, water translation was broke, finally pine trees died due to the water deficit.

Ethylene acts as a signal transduction material to cause terpenoid produce in pine trees, experiments associated with ethylen have been done. Ethylene product was inoculated into seedlings which increased several times greater in the disease development which began a few days earlier than the water potential decrease and chlorosis in needles. Ethylene increase occurred synchronously with cambial death, and they were followed by water deficiency in leaves. After being injected with 0.1–1% ethrel (2–chlorophenylphosphonic acid) solution to seedlings, embolism in tracheids widely occurred and needles turned yellow quickly. A large embolized area was produced in xylem both above and below the injection site. Some days later it developed to almost the whole transverse area of the xylem. Furthermore, denaturation of xylem and cortex parenchyma cells were produced. Ethylene produced in xylem seems to trigger the cytological changes in xylem parenchymatous cell, embolism in tracheids and chlorosis of old needles (Fukuda, 1997).

Utsuzawa (2005) has observed the xylem cavitation caused by PWD by using the magnetic resonance for living trees, rather than by staining part of tree xylem which have to be sectioned. Through the nondestructive observation, he found that cavitaiton was limited to the inner xylem for about 10 days after infection, and the number of cavitated patches and the area of cavitation slowly increased. After 15 days, the cavitation area enlarged rapidly and reached the cambium, and at 21 days the relative area of cavitaion reached near 100%. Water conduction was completely dysfunction and the tree became wilting and dead (Figure 1). According to the symptoms, the development of pine wilt disease was divided into two stages: early and advanced stage (Fukuda, 1997). In the early stage, nematodes migrate through cortical and xylem resin canals in pine stems. They induce cavitation (breaking off the water column in tracheids), embolism (filling of the tracheids with gas) and occlusion of the tracheids with resin; in transverse sections of the stem, the affected tissues appear as dry patches. In the advanced stage, the nematodes multiply and destroy the cambium, which induces dysfunction of water conduction in the entire xylem and causes water potential, transpiration, and photosynthesis to rapidly decrease. Consequently, the needles wilt and the tree dies suddenly. The first stage is within 7 to 14 days and the advanced stage is during 15 to 30 days (Figure 2).

PWN AND BACTERIA HYPOTHESES

The bacteria carried by the PWN play an important role in pathogenicity of PWD. Oku et al. (1980) and Higgins et al. (1999) reported that bacteria were associated with

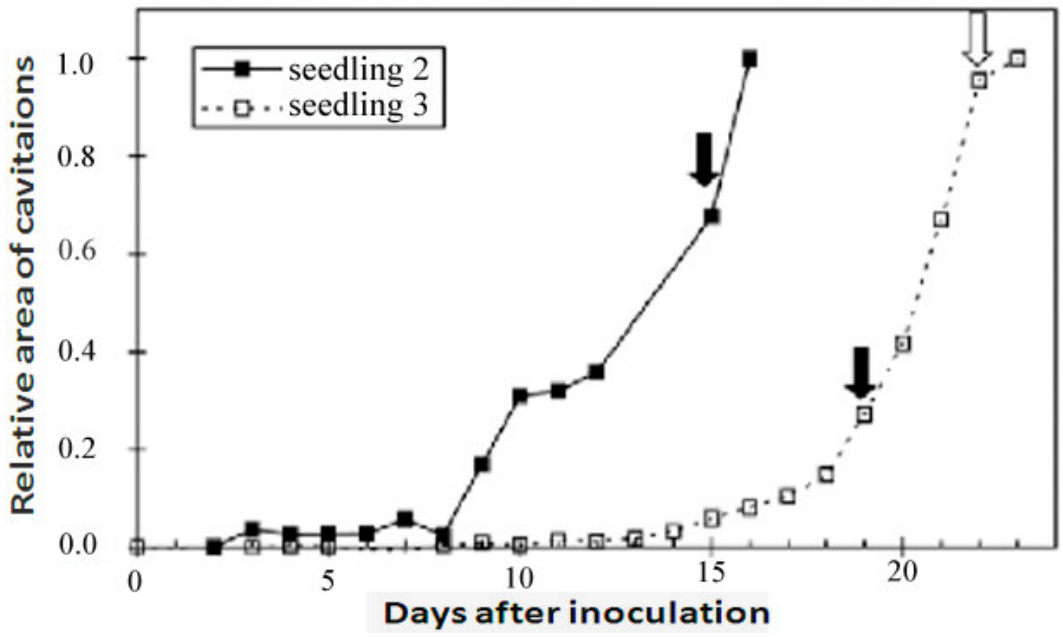

Figure 2. Cavitation development in seedlings 2 and 3 as revealed by magnetic resonance images (transverse–slice). The *y* axis refers to the proportion of the xylem that was cavitated. Image data are missing from seedling 2 on days 0, 1, 13, and 14. Solid and open arrows indicate the days when yellowing of old needles and wilting of current needles, respectively, were first observed. Reproduced from Utsuzawa SK (2005).

PWN. Bacteria adhered onto the body wall of PWN was observed by using electron microscopy and the average number of bacteria carried by one nematode isolated from infected pine tree was 2.9×10^2 (Zhao et al., 2000; Guo et al., 2002).

Some experiments indicated that aseptic PWN does not cause PWD of aseptic pine trees, while PWN associated with infecting bacterium causes wilting symptom. Oku et al. (1980) inoculated 3-year-old seedlings with a suspension of bacterium of the genus *Pseudomonas* isolated from pathogenic PWN. Three out of five of the treated seedlings subsequently wilted. Kawazu and Kaneko (1997) and Chi et al. (2006) reported aseptic *Pseudomonas densiflora* seedlings and 10-year-old *Pseudomonas thunbegii* trees did not wilt after being inoculated with aseptic PWN. Tan et al. (2004) reported that 1– or 2–year–old branches of *Pseudomonas massoniana* were inoculated with aseptic *B. xylophilus* and bacterium *Bacillus firmus* turned diseased. Therefore PWN associated with bacteria plays a significant role in the rapid wilting of pine trees.

Inoculating callus and aseptic black pine seedlings with aseptic PWN and the bacteria which isolated from PWN in the genus *Pseudomonas* showed severe symptoms, but only inoculating with aseptic PWN did not lead to browning (Han et al., 2003). In addition, the filtered liquid which bacteria were cultured in was directly applied to the callus of Japanese black pine induce browning. Han et al. (2003) and Zhao et al. (2003) concluded that wilting was due to toxins in the bacterial culture filtrate. Jiang et al. (2005) and Guo et al. (2007) isolated two chemical compounds, which showed obvious toxicity to both suspension cells and seedlings of *P. thunbergii*, from the culture of a strain of *Pseudomonas fluorescens* (*P. fluorescens GcM5–1A*) carried by PWN. The bacteria carried by PWN from isolated regions may be different. Such differences could explain why *Cedrus deodara* is sensitive to PWD in USA and Japan (Dropkin, 1981), while it is resistant in China (Zhao, 2003). It suggested that the disease was caused by co-infection of both PWN and bacteria and possible toxic effect of bacteria (Han, 2003).

Guo et al. (2006) reported that both the homogenates from live PWN and dead nematodes promoted the reproduction and pathogenicity of the bacterium by providing essential metabolites or nutrients, and that the promotion effect of living nematodal homogenates was stronger than that of dead ones. Furthermore, Zhao (2005) found that there is a mutualistic symbiotic relationship between PWN and 10 bacterial species in the genus *Pseudomonas*. The bacterial mutualistic symbionts are organized whole, which may have co-evolved with PWN rather than being accidentally associated. The finding provides that PWD is a complex process, induced by both PWN and associated phytotoxin-producing bacteria.

However, migration speed of PWN and bacteria is different, so how do the bacteria produce enough toxins to make pine tree wilting before its mass rearing need to

be researched.

DISCUSSION

Phytotoxins and cellulases produced by PWN, such as benzoic acid, catechol, dihydroconifery alcohol, 8-hydroxycarvotanacetone (carbone hydrate), 10–dydroxyverbenone and glycosyl hydrolase family, could cause dysfunction of pine trees in some degree and wilt symptoms of susceptible pine trees, even death. It needs a large number of PWN to produce enough quantity of phytotoxins and cellulases to cause wilt symptoms of pine trees. Consequently, the bigger pine trees should suffer more PWN and longer time to cause disease than smaller ones and the bigger pine trees survive longer time than smaller ones. However, infact the bigger pine trees show wilt symptoms earlier than small pine trees. Phytotoxin hypothesis and cellulase hypothesis could not explain the phenomenon adequately.

Terpenoid hypothesis explained how PWN causes dysfunction of water translocation of pine trees. Migrating of Nematodes through cortical and xylem resin canals in pine stems induces cavitation, embolism, and occlusion of the tracheids with resin. In the advanced stage, nematodes multiply and destroy the cambium, inducing dysfunction of water conduction and causing water potential, transpiration, and photosynthesis to rapidly decrease. Consequently, the needles wilt and the tree dies suddenly. Utsuzawa (2005) has observed the process of xylem cavitation forming caused by PWD by using the magnetic resonance for living trees, which was strong evidence to the terpenoid hypothesis.

PWN and bacteria hypothesis is an important complimentarily to pathogenic mechanism of PWD. The bacteria carried by PWN play a vital action to PWD; however, some scientists take a controversial standpoint about it.

CONCLUSION

In summary each hypothesis approves pathogenic factors exist and take effect to PWD. However, pathogenic factors are not mutually exclusive, which means a variety of factors make pine tree for death, rather than a single factor. PWN associated with bacteria diffuses from cortex resin canals to xylem resin canal, producing phytotoxins and cellulases, induce cytological changes in xylem ray and axial parenchyma cells as a defense reaction which produces ethylene. Subsequently, ethylene acts as a signal transduction material to cause the mass production of terpenoid in pine trees. Ethylene and terpenoid result in cavitation and embolism of tracheids, subsequent decrease in leaf water potential and photosynthesis. During the wilting process of pine tree, PWN is involved in the production of phytotoxins, cellulases, terpenoids; it therefore is a vital and indispensable factor for PWD.

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Full Length Research Paper

Separation and identification of lipase - producing strains and immobilized transesterification efficiency

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12 strains of lipase - producing were isolated from 36 samples collected from oil - contaminated soil of machinery - repair plant, dining room, and vegetable market, respectively, of which strain (3 - 2) had the highest hydrolytic activity (17.1 U/ml). Immobilized cell of strain 3 - 2 onto sodium alginate and immobilized crude enzyme solution of strain (3 - 2) onto Sodium alginate were respectively used in two transesterification programs to transfer the Fatty acid methyl ester (FAME). Results showed that both immobilized crude enzyme solution and cell were of high transesterification efficiency for Transesterification Program I, respectively 32.72 and 26.84%. Cycle test done in this Program found that immobilized crude enzyme solution and cell still keep some transesterification efficiency after being recycled thrice at least. Meanwhile, hydrolytic activity of crude enzyme solution had some correlation with transesterification efficiency. However, hydrolytic activity of immobilized cell did not have a clear correlation with the latter. Strain (3 - 2) belonged to *Sphingobacterium multivorum.* Up to now, the transesterification research has not been reported.

Key words: Lipase, crude enzyme solution, bacterial cell, immobilized, transesterification.

INTRODUCTION

Biodiesel produced by lipase defines fatty acid monoester obtained by transesterification or esterification of triglyceride (or fatty acid) with short - chain alcohol (mostly methanol) under lipase catalysis. The main composition of biodiesel is Fatty Acid Methyl Esters (FAME). Relative to chemical method, Enzymatic method has simpler recycle process for byproduct (that is glycerin), without saponification and moderate reaction condition (Yang et al., 2003; Zhou et al., 1999; Song and Yinbo, 1999; Zhang et al., 2003; Ren and Yingkuo, 1996)

Biodiesel production by enzymatic method features extremely extensive potential and has been massively concerned with nowadays (Wang et al., 2005).

Lipase comes from microorganism, insoluble in solvent and easy agglomeration while reaction, so these greatly reduce lipase utilization. Immobilized lipase may improve its dispersion and thermodynamic stability in solvent and fit for recycle and continuous production (Kirsty et al., 2000; Qiao et al., 1998). Immobilization methods for lipase includes covalent method, cross-link method, entrapping method and adsorption method. Adsorption carrier relies on adhesion link with protein. This simple immobilized process is one of the most economic methods (Chun et al., 2008). Carriers frequently used in adsorption include diatomite and quartz sand etc. (Shi Qiaoqin, 1981; Chen et al., 2006).

We immobilized cell and crude enzyme solution of lipase - producing stain (3 - 2) (isolated from oil-contaminated soil), study on their transesterification and hydrolytic activity in two different transesterification system. This assay evaluates their transesterification efficiency and its application value in biodiesel production industry.

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Table 1. Immobilized transesterification program.

Key compositions for transesterification program	Program I (Chen et al., 2006)	Program II (Chun et al., 2008)
Olive oil (ml)	5	4.8
Methanol (ml)	0.8	0.09
Immobilized bacterial cell (crude enzyme) (g)	1.2	1.2
n-hexane (ml)	44.2	1
Water (ml)	0	0.18
Volume (ml)	50	7.3
Temperature (ºC)	32	32
Rotational speed (r/min)	200	200
Cultivation time (h)	24	24

MATERIALS AND METHODS

Materials

Lipase producing strain 3 - 2 isolated from Chengdu China, olive, diatomite, acetone, sodium alginate, n-hexane, and ethanol (95%). Enrichment medium, screening medium, fermentation medium and others, were all AR and supplied by Chengdu Kelong Reagent Co. Ltd, peptone were supplied by OXOID Reagent Co., Ltd (America) (AR).

Methods

Separation and identification of lipase - producing strain

36 samples were collected from oil-contaminated soil of machinery-repair plant, dining room and vegetable market in Chengdu and peripheral area. After enrichment cultivation, Strains with obvious transparent circle on initially screening medium (contain bromcresol purple) were screened out and then the strain with the highest hydrolytic activity (olive oil emulsion method Gao et al., 2002) were further screened out. BIOLOG MicrostationTM System MicroLog3 4.20 (BIOLOG Inc., Hayward, CA) was used to identify the classify-cation of that strain by metabolic modes of 95 kinds of substrate utilization on GN2 identification plate. At the same time, 16S rRNA gene sequence of this strain was analyzed (EL Herry et al., 2008). The strain was used in the following study.

Fermentation of lipase - producting strain

The activated strain was inoculated to 100 ml fermentation medium by 1 % (scale) and was shaking cultured for 48 h with speed of 160 r/min at 32ºC.

Immobilization of crude enzyme solution with diatomite

We added 8 g diatomite (dried under 300ºC before use) to 100 ml crude enzyme solution (supernatant of fermentation medium culture by centrifuge, 6000 r/min), and mixed them for 3 h at 30 - 35ºC, centrifuged to obtain precipitation, washed it 3 - 5 times by acetone to make particle dispersed, and storage it at 4ºC after freeze drying.

Immobilization of bacterial cell by sodium alginate

The fermented culture was centrifuged 10 min by 6000 r/min to collect bacterial cell. And then mixed 18.0 g of it with saline (the ration was 1 g: 1 ml) and with 4% sodium alginate (90 ml), then dropped into $CaCl_2$ solution (0.15 M) and mixed them all. The mixture was curinged at 4℃ for 3 h to be formed into sodium alginate immobilized ball (2 - 2.5 mm), at last washed them with saline, filtered and dried out, then stored at 4ºC.

Transesterification conditions with immobilized crude enzyme solution and bacterial cell

Immobilized crude enzyme solution and immobilized bacterial cell was undergoing transesterification test according to the following two programs (Table 1).

Cycle transesterification measurement for immobilized crude enzyme solution and bacterial cell

After mentioned test above, we found a better transesterification program. Immobilized crude enzyme solution and Immobilized bacterial cell were tranesterified circularly in this system. The change of hydrolytic activity was measured after each cycle with olive oil emulsion method (Chun et al., 2008) GC-MS was used to analyze the content of methyl ester.

GC-MS analysis for transesterification product

The transesterification product was transported through chromatographic column Rtx-5si1MS (30 m × 0.25 mm × 0.25 μm), with following conditions: helium acted as carrier gas, ion source under 200ºC and 1000 cm/s sample injection rate and no shunt. Sample injection volume was 1 μl, and 50 min analyzing time. With area normalization method, the content of FAME in the product was analyzed.

RESULTS

Separation and identification for lipase - producing strain

12 strains of lipase - producing bacteria were obtained from 36 soil samples, and their hydrolytic activity were tested (Gao et al., 2002), shown in Table 2, including highest hydrolytic activity for strain (3 - 2) with 17.1U/ml. Strain (3 - 2) was used to study on transesterification efficiency in following.

Table 2. Comparison with 12 strians of lipase-producing bacteria.

Strain no.	Hydrolytic activity (U/ml)
4 - 1	0.5
4 - 3	0.6
4 - 4	10.2
4 - 5	0.7
4 - 6	0.3
5 - 2	3.3
5 - 3	1.1
5 - 4	1.2
3 - 1	3.7
(3 - 2)	17.1
N10-H	4.8
N1-H	0.5

16S rRNA sequence of strain (3 - 2) was obtained by 16S rDNA PCR. Through BLAST alignment, the similarity of strain (3 - 2), *Sphingobacterium* sp. GF2B, *Sphingobacterium* sp. QMT3-2 and *Sphingobacterium multivorum* was 97%. Construction of Evolutionary Tree of strain (3 - 2) with UPGMA (MEGA4.0 software) showed that strain (3 - 2) and *S. multivorum* were on the same evolutionary branch (Figure 1) at the same time; strain (3 - 2) was identified as *S. multivorum* by BIOLOG. On the basis of the characteristics of morphology, physiology and biochemistry, and analysis of 16S rRNA gene sequence, strain (3 - 2) was identified as *S. multivorum*.

Transesterification efficiency for immobilized crude enzyme solution and bacterial cell

Sodium alginate immobilized bacterial cell were white small spherical, easy for separation from the reactant. Diatomite - immobilized crude enzyme had the main characters, as follows: white powder, insoluble in water and organic solvents. The same weight of immobilized enzyme and bacterial cell (each with 1.2 g) was added individually to the transesterification test (Chen et al., 2006; Chun et al., 2008). GC-MS was used to analyze transesterification efficiency through analyzing the content of FAMEs in products, and results showed that both immobilized crude enzyme solution and immobilized bacterial cell showed higher transesterification efficiency in transesterification program I (Table 3) compared to program II, respectively as 26.84 and 32.72%.

Analysis on transesterification product (GC-MC) in immobilized crude enzyme solution under program I, showed that (Figure 2), it has highest content of FAMEs, including Methyl Heptadecanoate, Methyl Linoleate, Methyl Oleate, Methyl Separate (arrow showed in Figure 2), totally 32.72% among products. Secondly, fatty acid (retention time: 42.928, 21.99% content), in addition, there also had fatty acid ethyl ester (retention time: 43.661, 7.52% content) and alkane (18.7% content).

Study on transesterification character of immobilized crude enzyme solution and bacterial cell under different cycle times

Change of hydrolytic activity for immobilized crude enzyme solution and bacterial cell under different cycle times

Optimal transesterification program I was used for transesterification test for cycle times with immobilized crude enzyme solution and bacterial cell as catalyst (shown in Table 4), and its results showed that, along with increased cycle times, immobilized crude enzyme solution may recycle, its hydrolytic activity gradually lowers with increasing cycle times. Immobilized bacterial cell has relatively less hydrolytic activity and much lower along increase in cycle times.

Change of transesterification efficiency under different cycle times

From Table 5, Program I with the immobilized crude enzyme solution and bacterial cell as catalyst separately show reduced transesterification efficiency with the increased cycle times. After three cycles, they also still had some transesterification efficiency.

DISCUSSION

Effects of immobilized material on transesterification

The gap between diatomite used to fix crude enzyme solution is small, it can prevent tiny particles to come in, free from chemical reaction with enzyme, free from affecting physical or chemical properties for enzyme, moreover, immobilized crude enzyme solution with diatomite recovered easy, and increase their interaction with cleaning up by acetone. Reuse of immobilized crude enzyme solution may reduce the cost of biodiesel production (Margolin et al., 1987). Long Zhangde et al. (2007) found that, *Serratia marcescens* lipase immobilized by diatomite and Eupergit C may increase its thermal stability and storage time. Lipase can dissolve in organic solvent, and need not covalent combination between immobilized lipase and, carrier, so diatomite adsorption method have been widely used (Sànchezetal, 2002; Kagaetal, 1994; Kharean and Nakajima, 2000) (Shuo-FenChang et al., 2007). This Article found that although the transesterification efficiency of immobilized bacterial cell was less than that of crude enzyme solution, its transesterification reducing rate is much lower than that of immobilized crude enzyme solution along with the increasing cycle. This may result from that immobilized

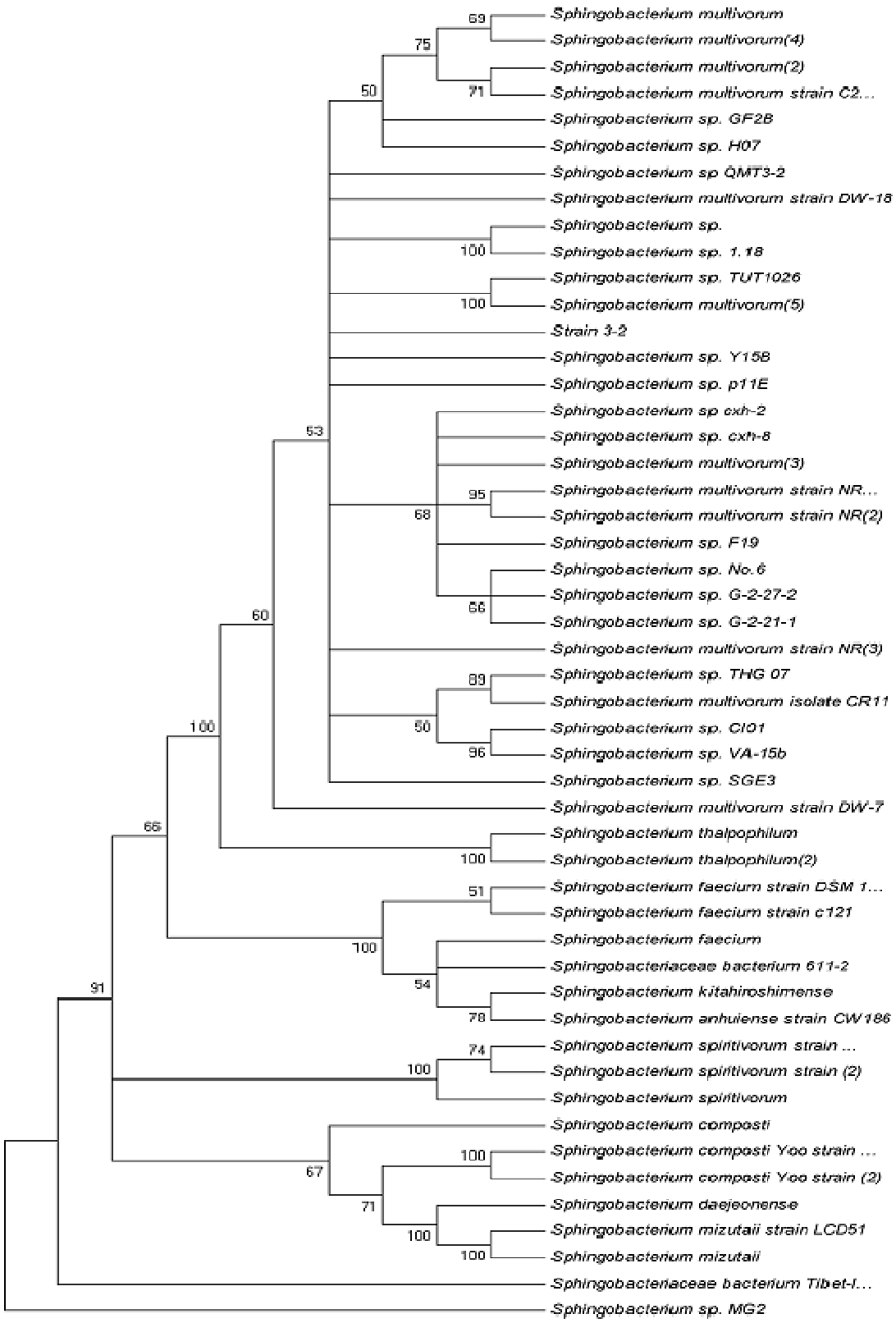

Figure 1. Construction of evolutionary tree of strain (3 - 2) with UPGMA (MEGA4.0 software).

bacterial cell continuously produce lipase through trans- esterification process, this is one of the benefits of immo-

Table 3. Transesterification efficiency in two programs for immobilized crude enzyme solution and Immobilized bacterial cell (unit: %).

Catalyst	Program I	Program II
Immobilized crude enzyme solution	32.72	10.85
Immobilized bacterial cell	26.84	10.91

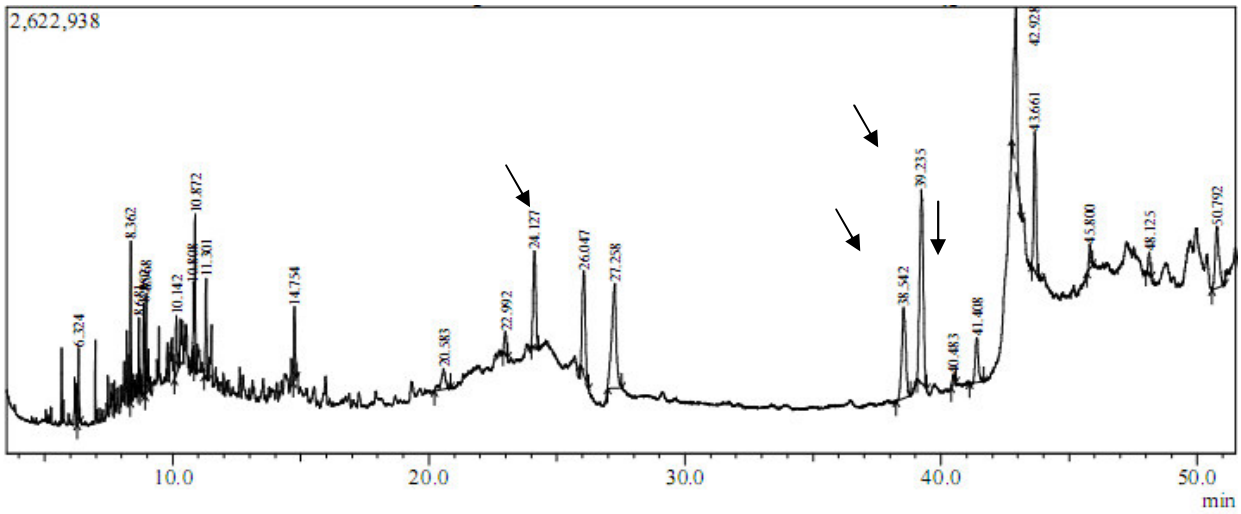

Figure 2. Product (GC-MS) analysis of immobilized crude enzyme solution in transesterification Program I.
Note: a, Methyl Heptadecanoate, b. Methyl Linoleate, c, Methyl Oleate, d. Methyl Separate;

Table 4. Hydrolytic activity change for different cycle times for immobilized crude enzyme solution and bacterial cell (Unit: U/ml).

Cycle times	Immobilized crude enzyme solution	Immobilized bacterial cell
1	16.8	9.3
2	10.2	5.7
3	2.5	0

Table 5. GC analysis result for transesterification product from immobilized crude enzyme solution and bacterial cell under different cycle times (Unit: %).

Cycle times	Immobilized crude enzyme solution	Immobilized bacterial cell
1	32.72	26.84
2	18.38	16.13
3	12.24	10.51

bilized bacterial cell in actual production.

Relationship between transesterification efficiency and hydrolytic activity

Goujard et al. (2009) reported there may no correlation between hydrolytic activity and transesterification efficiency of lipase. Xiao Yan Wu et al. (1996) utilized nine kinds of commercial lipases to research on their hydrolytic activity, esterification and transesterification activity, and found that there was some correlation between esterification and transesterification activity only for *Black Rhizomuco* lipase, but there was no correlation with hydrolytic activity. Hydrolytic and esterification activity for lipase from other source (including *purple bacteria*) has no correlation at all; even lipase with high hydrolytic activity has no transesterification activity. Toshiyuki et al.

(1995) found that, in 50% dioxide solution, transesterification activity for lipase might be estimated by hydrolytic activity by Colormetric Testing only when they use p-Nitrophenyl esters as substrate. Our results showed that, hydrolytic and transesterification activity for strain (3 - 2) crude enzyme and bacteria cell were in turn lowers with increasing cycle times. It showed that there was certain correlation between the two activities. While the immobilized bacterial cell had lower hydrolytic activity, this might result from exoenzyme secrete time, the test only had 10 min reaction time while measuring. If there was not full time to excrete enzyme, this may affect test result. So immobilized bacterial cell hydrolytic activity and transesterification efficiency has no obvious correlation; immobilized bacterial cell still has 10.51% transesterification efficiency even if hydrolytic activity measurement value is zero.

Transesterification reaction analysis for strain (3 - 2)

Two transesterification programs was used in the study, both refer to commercial lipase transesterification method, immobilized crude enzyme solution and immobilized bacterial cell both had higher efficient for transesterification under program I than program II, which may be resulted from that alcohol/oil molar ratio of program I is higher than that of program II, namely 0.17:1, and program I reaction volume reaches 50 ml in total, which made immobilized crude enzyme solution or immobilized bacterial cell fully contact with reactant; but program II has less volume, worse reaction contact. Even if alcohol/oil molar ratio of the two programs are not high, this may find that strain (3 - 2) also has some potential for study of production of FAME in biodiesel industry, fatty acid ethyl ester and alkane of esterified products also valuable in diesel compound. After identification, strain (3 - 2) are *s. multivorum*, such a microbial stain is ε-poly-L-lysine tolerant strain, which has excellent solubility and biological degradation, edibility and nontoxicity. It has extensive prosperity in food preservation, biodegradable fiber, drug carrier (Ing-LungShih et al., 2006). Most important, such microbial strain has not been reported yet with respect to study of lipase and applied to transesterification.

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Full Length Research Paper

Study on β-galactosidase enzyme produced by isolated lactobacilli from milk and cheese

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***β*-galactosidases enzyme have been used in the dairy industry for the improvement of lactose intolerance. The aim of this study was to detect β-galactosidase enzyme produced by isolated lactobacilli from milk and cheese. Isolated lactobacilli were cultured on MRS agar. Lactobacilli were identified by Gram stain and standard bacteriological and biochemical methods. Their ability to hydrolyze 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and O-nitrophenyl-β-D-galactopyranoside (ONPG) was determined. A protein band of indicated β-galactosidase enzyme was also detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method. The colonies that produced green color on X-Gal plates were lactobacilli with β-galactosidase enzyme which had ONPG positive results. The highest enzymatic value (1,966 U/ml) was observed in one strain of *Lactobacillus delbrueckii*. A 116 kDa protein band was detected in some strains (37%) with highest enzyme value and in others (63%), protein band was weak by SDS-PAGE method. By adding *Lactobacilli* producing β-galactosidase enzyme as probiotic to dairy products, could help lactose intolerant people.**

Key words: *Lactobacillus*, β-galactosidase, X-Gal, ONPG, SDS-PAGE.

INTRODUCTION

Lactic acid bacteria (LAB) that used as starters for production of dairy products are the main factors of fermentation and protection of fermentative foods and also have a significant role in texture and flavour of food products (Chammas et al., 2006). One of the glycosidases, is β-galactosidase enzyme that widely used in dairy industry and is produced by most lactobacilli (Karasova et al., 2002; Corral et al., 2006; Nguyen et al., 2007). This enzyme hydrolyzes lactose, the main carbohydrate in milk, into glucose and galactose, which can be absorbed across the intestinal epithelium (Troelsen, 2005; Vasiljevic and Jelen, 2001; Heyman, 2006). β-galactosidase has two enzymatic activities: one is responsible for the hydrolysis of lactose and also cleaves cellobiose, cellotriose, cellotetrose and to a certain extent cellulose and the other, splits β-glycosides (Troelsen, 2005; Heyman, 2006). Low activity of β-galactosidase causes digestive insufficiency, called lactose intolerance in most cases (Karasova et al., 2002; Vasiljevic and Jelen, 2001). The symptoms of lactose intolerance such as abdominal pain and diarrhea, nausea, flatulence, and or bloating after the ingestion of lactose or lactose containing food substances which can lead to decrease quality of life, and daily activities. Treatment is relatively simple by eliminating lactose from the diet or by using of supplemental β-galactosidase enzyme replacement (Vasiljevic and Jelen, 2001). The bacterial species currently used by the dairy industry which produced β-galactosidase enzyme belong to genera of *Lactobacillus* and *Bifidobacterium* and comprise a limited collection of strains (Fernandez et al., 1999; Xanthopoulos et al., 1999; He et al., 2008). These bacteria have become a focus of scientific studies for three particular reasons (Somkuti et al., 1998): a) lactose maldigesters may consume some fermented dairy products with little or no adverse effects, b) These bacteria are generally regarded as safe (GRAS) so the β-galactosidase enzyme derived

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from them might be used without extensive purification (Vasiljevic and Jelen, 2001). c) Some strains have probiotic activity such as improved digestion of lactose and a suitable strain selection must be carried out to manufacture probiotic dairy products (Vinderola and Reinheimer, 2003). The aim of this study was to compare the isolated lactobacilli with β-galactosidase production in milk and cheese for selection of lactobacilli producing high β-galactosidase enzyme as probiotic, by biochemical (X-gal and ONPG substrate) and molecular (SDS-PAGE) methods (Ausubel, 1994). Because, the existence or addition of lactobacilli producing high β-galactosidase enzyme as probiotic can be use for more improvement of lactose digestion in dairy products especially in milk.

MATERIALS AND METHODS

Sampling

This cross-sectional study was conducted from 2006 to 2008 in Iran University of Medical Science. In this study, 50 samples of pasteurized and unpasteurized milk and cheese that bought from different shops throughout Tehran were studied.

Culture media and incubation conditions: For isolation of bacteria, 2 g of cheese and 2 ml of milk was added to 5 ml of MRS broth (Merck, Germany) and incubated anaerobically (24 h at 37°C). Then, 50 µl of them were spread onto MRS agar (Merck, Darmstadt, Germany). Plates were incubated in anaerobic jars at 37°C for 48 h. To determine β-galactosidase activity, MRS broth without glucose (MRS-lac) but contained 1% of lactose was used (Vinderola and Reinheimer, 2003).

Identification of isolated bacteria: Bacteria were examined by Gram stain, and identified by standard bacteriological and biochemical methods (Sneath et al., 1984). Acid production from carbohydrates (glucose, galactose, maltose, mannitol, ribose, sucrose, arabinose, lactose, mannose, raffinose, rhamnose, xylose, sorbitol, salicin) in MRS broth base (without glucose and beef extract) was evaluated. Their ability to grow at various temperatures (4, 15, 25, 30, 37 and 40°C) was examined after 3 - 10 days. Motility, production of indol and H_2S tests were carried out by using SIM medium (Merck, Germany).

Study of β-galactosidase production

X-gal substrate: One colony of isolated bacteria were grown on MRS agar plates containing 60 µl X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, #R0401, fermentas, 20 mg/ml DMF) and 10 µl of IPTG (iso-propyl-thio-β-D-galactopyranoside, dioxane free #R0391, fermentas) solution as an inducer. Plates were incubated at 37°C for 24 h to 3 days. Colonies producing β-galactosidase were green (Vinderola and Reinheimer, 2003).

ONPG substrate: All bacteria were inoculated into tubes containing ONPG (O-nitrophenyl-β-D-galactopyranoside) (0.5 ml) and 0.01 M sodium phosphate buffer (pH 7.0) (5 ml) and peptone water. Production of yellow color was indicated positive ONPG results (Miller, 1998).

Value of β-galactosidase

This test was performed according to the method of Miller (1998), Vinderola Reinheimer (2003). Briefly, all bacteria were harvested in MRS broth. After centrifugation at 12000 × g for 5 min. at 5°C, washed twice in 60 mM $Na_2HPO_4 \times 7H_2O$, 40 mM NaH_2PO_4 buffer (pH 7.0) and inoculated in MRS-lac broth. Then, absorbance 560 nm was adjusted with the same buffer and recorded. One ml of the cell suspension was premeabilized with toluene/acetone (1:9 v/v) solution (50 µl), vortexed for 7 min. and immediately assayed for β-galactosidase value. An aliquot (100 µl) of the permeabilized cell suspension was placed in a microtube and phosphate buffer (900 µl) and O-nitrophenyl-β-D-galactopyranoside (200 µl of ONPG 4 mg/ml, Merck) were added. Microtubes were placed into a water bath (37°C) for 15 min. Then, 1M Na_2CO_3 (0.5 ml) was added to each tube to stop the reaction. The contents of each microtube were centrifuged at 12000 × g for 5 min. to remove the cells. Absorbance values at both 420 and 560 nm were recorded for each microtubes and β-galactosidase value was calculated in Miller units (Vinderola Reinheimer, 2003).

Protein extraction and SDS-PAGE

Lactobacillus strains were cultured in MRS broth (100 ml) without peptone and beef extract. Then, were centrifuged at 3500 × g for 20 min. After centrifugation, once, supernatant was added to Tris HCl and again to saccharose, Tris HCl and lysosyme. Then, EDTA was added. After centrifugation, solution was dissolved in SDS, Tris HCl, glycerol, mercaptoethanol, bromophenol blue and distilled water. After preparation, protein solution was precipitated with 40% of ammonium persulfate and then, centrifuged at 3500 × g at 5°C for 20 min. Precipitate was dialyzed by dialysis tubes (0.2 µ) in distilled water for 72 h. Then, samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli method on vertical slab gels (Laemmli, 1970). Resolving and stacking gel conditions were 12 and 4% acrylamide, respectively. Unstained protein molecular weight marker (#SM0431) was purchased from Fermentas. Gels were run for 4 h at 110 V. Protein bands were visualized by staining with Coomassie Brillint Blue (Merck) R-250 (Dunn et al., 1993).

RESULTS

Identification of isolated *lactobacillus* strains

Forty one *Lactobacillus* strains were isolated from 50 samples (Table 1). All bacteria did not grow at 4°C but all of them grew at the maximum rate at 25°C and weakly growth was detected at 40°C.

β-galactosidase screening with X-gal, ONPG

All bacteria produced green color colonies on X-Gal plates that indicating the presence of β-galactosidase enzyme. All lactobacilli were produced a dark green colonies with or without halos (Figure 1). Some of them (37%) produced dark green colonies after 24 h incubation (rapid enzymatic activity) and others (63%) had delay (slow) enzymatic activity after 2 - 4 days of incubation.

All bacteria had positive ONPG results (production of yellow color). In ONPG method, values of β-galactosidase enzyme were the range of 22.7 to 1,325 (U/ml) in milk isolates and of 103.1 to 1,966 (U/ml) in cheese isolates. High value of β-galactosidase enzyme was detected in *L. delbrueckii* subsp. *bulgaricus* and *L. casei* subsp. *casei* (ranging from 867 to 1,966 U/ml) isolated from cheese and in *L. casei* subsp. *casei*, *L. casei* subsp.

Table 1. Isolated *Lactobacillus* from milk and cheese.

Isolate no.	Strains	Origin		Total	Isolated %
		Milk	Cheese		
1	*L. acidophilus*	1	2	3	7
2	*L. bifermentans*	1	1	2	5
3	*L. brevis*	---	1	1	2
4	*L. casei* subsp. *casei*	2	4	6	15
5	*L. casei* subsp *rhamnosus*	3	4	7	18
6	*L. casei* subsp. *tolerans*	2	2	4	10
7	*L. curvatus*	1	---	1	2
8	*L. delbrueckii* subsp. *bulgaricus*	---	2	2	5
9	*L. delbrueckii* subsp. *delbrueckii*	---	1	1	2
10	*L. delbrueckii* subsp. *lactis*	---	3	3	7
11	*L. fructosus*	---	1	1	2
12	*L. fermentum*	1	---	1	2
13	*L. helveticus*	---	2	2	5
14	*L. plantarum*	3	4	7	18
Total		14	27	41	100

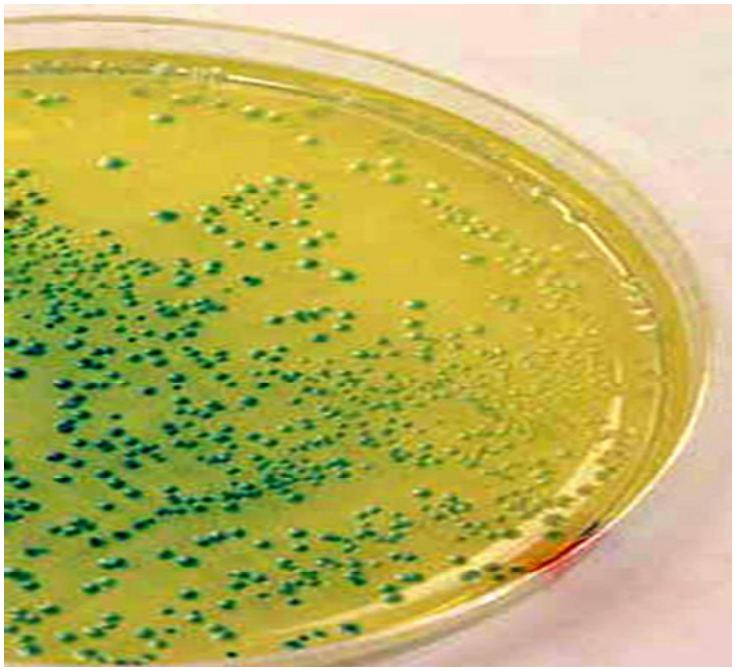

Figure 1. Green colonies of *Lactobacillus* strains on X-gal plates.

tolerans L. plantarum (ranging from 606 to 1,326 U/ml) isolated from milk. Low value of β-galactosidase enzyme was detected in two strains of *L. casei* subsp. *rhamnosus* and in one strain of *L. plantarum* was 22.7, 44.4 and 64.4 (U/ml) in milk lactobacilli, respectively.

Our results showed that the addition of 1% lactose could increase β-galactosidase values. In the presence of 1% glucose as sole of carbon source, a β-galactosidase value was decreased. In this study X-gal and ONPG results were the same (positive results).

SDS-PAGE results

A protein band with a molecular weight of ~116 kDa was observed in some strains (37%) with the highest β-galactosidase enzyme value and in many of them (63%), protein band was weak and could not detected very clearly (Figure 2).

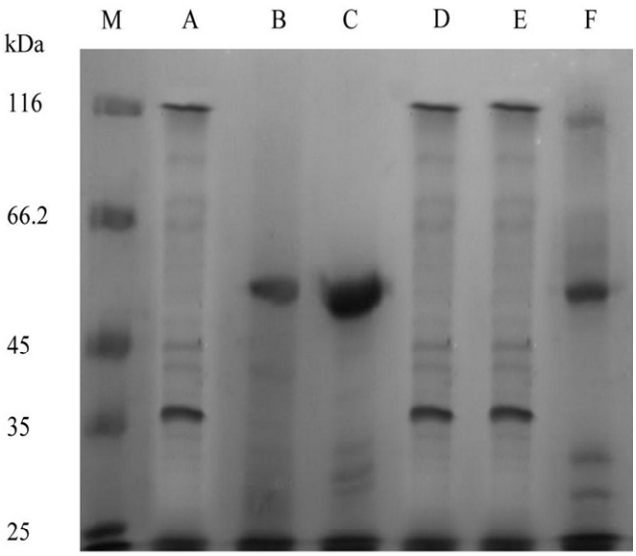

Figure 2. Protein bands of *Lactobacillus* strains in SDS-PAGE. A protein band with a molecular weight of ~116 kDa was observed in A: *L. delbruekii* subsp. *bulgaricus,* D: *L. delbruekii* subsp. *lactis* and E: *L. casei* subsp. *casei* which had the highest β-galactosidase enzyme value. M: Molecular weight marker (left hand side of the pattern): β-galactosidase (116 kDa).

DISCUSSION

Lactobacilli are the most abundant bacteria in gastrointestinal tract. They are the most important food fermentators and used as starters. Also these bacteria have the role in texture and flavour of dairy products, sausage and some other of fermentative foods. β-galactosidase is an enzyme that produced by some of bacteria, especially lactobacilli in dairy products that is yoghurt, cheese and milk.[4] Lactose intolerance has been recognized for many years as a common problem in many children and most adults throughout the world (Heyman, 2006). Therefore, by addition of *Lactobacilli* producing β-galactosidase as probiotic to milk and cheese and other dairy products could help lactose intolerance symptoms.

The fastidious nature of *Lactobacilli*, requiring rich medium for the growth and β-galactosidase activity. It was reported that by using skim milk (it is a source of lactose), the production of β-galactosidase enzyme value would increase (Vasiljevic and Jelen, 2001) and β-galactosidase production in *Bacillus megaterium* is subject to catabolite repression by glucose and is lactose inducible (Show et al., 1998). The results of this study indicated that, enzymatic values were increased by adding of 1% lactose (because of lac operon stimulation), instead of 1% glucose into MRS broth. It was suggested that lactose is an inductive factor to increase β-galactosidase enzyme. On the other hand, in present study, by adding of 1% glucose as sole of carbon source to MRS broth, β-galactosidase values was decreased. So, our results confirmed the Vasiljevic and Jelen results and were similar to the results that they have reported about increase of β-galactosidase enzyme by adding of lactose. Then it is better somehow glucose is eliminated and lactose is replaced it.

Thermophilc bacteria have become an object of interest for the commercial production of β-galactosidase enzyme (Petzelbauer et al., 1999; Tabasco et al., 2007). The β-galactosidase of yogurt mixed culture, consisting of *L. delbrueckii* sub sp. *Bulgaricus* has been characterized (Greenberg and Mahoney, 1982). Showing high activity and stability at temperatures above 50°C. Such conditions can enhance the rate of lactose hydrolysis. In this study, no *Lactobacillus* species with the exception of one species (*L. casei*) was isolated from pasteurized milk, whereas different *Lactobacilli* was isolated from unpasteurized milk. From these observations, it can be concluded that *L. casei* was more stable at pasteurization temperatures and also probably lactobacilli in pasteurized milk were sensitive to high temperatures. These results in agreement with the various strategies, which have showed and proposed to improve the growth rate of bacterial probiotics in milk, mostly by adding of thermostable *Lactobacillus* strains to milk (Gaudreau et al., 2005).

Vinderola and Reinheimer reported that high value of β-galactosidase enzyme in commercial strains of *L. delbrueckii* subsp. *Bulgaricus* isolated from cheese (Vinderola and Reinheimer, 2003). In our study, two strains of *L. delbrueckii* with high and rapid enzyme values from cheese were detected, which were in the range of values previously reported (Vinderola and Reinheimer, 2003). So, it seems that the conditions in cheese for growth of *Lactobacilli* with production of β-galactosidase enzyme is suitable. These results observed among *L. delbrueckii strains* further emphasize the importance of selecting appropriate strains for use as dietary adjuvants.

On the other hand, considering that milk avoidance has a negative effect on calcium and vitamin D intake in infants, children and adolescents which must provided by other dairy products (Heyman, 2006), therefore, consumption of cheese or modified milk (containing lactobacilli with high enzyme value) is recommended.

Favier et al. reported a method to detect bacteria with β-galactosidase activity by X-gal. Colonies growing on X-gal medium with green color were regarded as bacteria containing β-galactosidase enzyme (Favier et al., 1996). In this study, when the method of Favier et al. was applied to 41 *Lactobacilli*, all isolated lactobacilli containing β-galactosidase enzyme had green colonies (some producing strong and rapid and many low and slow enzyme). Our results detected by different biochemical methods (ONPG, X-gal methods) confirmed each other which were similar to previous works (Favier et al., 1996).

It was reported that β-galactosidase enzyme is tetrameric enzyme which consists of identical subunits with a molecular weight of 116 kDa.[21] In this study, by using of SDS-PAGE method, an intensive 116 kDa protein band

was observed in *Lactobacilli* with high values of β-galactosidase enzyme (such as *L. delbruekii* subsp. *bulgaricus*, *L. delbruekii* subsp. *lactis*, *L. casei* subsp. *casei*). Our results were in accordance with results observed in previous studies (Nichtl et al., 1998). Therefore, the SDS-PAGE method is not suitable for all cases to detect β-galactosidase activity. Since all of the isolated lactobacilli that produce high and low or weak β-galactosidase, can be detected by X-gal and ONPG tests, so, biochemical tests, which are rapid, cheap and simple in all laboratory and do not need any expensive molecular materials and apparatus, is recommended.

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Full Length Research Paper

An efficient protoplast isolation and regeneration system in *Coprinus comatus*

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An efficient protocol for protoplast isolation and regeneration was first established in *Coprinus Comatus*. The highest yield of protoplasts was up to 8.9 × 10^6 cells/ml in digestion solution containing 2.0% lywallzyme and 0.6 M KCl after incubation of four-day-old mycelia at 30°C for 3 h; among which, about 1.4% protoplasts could be regenerated into mycelia after 4 - 6 days of incubation at 25°C in CYM medium with 0.6 M mannitol as osmotic stabilizer. The results are beneficial for breeding new cultivars by the methods such as protoplast fusion, mutagenesis as well as transformation. Moreover, the stepwise procedure for protoplast liberation and regeneration could be referred in other species.

Key words: *Coprinus comatus*, mycelia, lywallzyme, protoplast, liberation and regeneration.

INTRODUCTION

Wild *Coprinus comatus* (*C. comatus*) belonging to edible fungi is widely distributed in the world, and in P. R. China, it has been cultured in force in many provinces and regions (Huang, 1997), and the biological efficiency is over 100%. *C. comatus* has very important medical and edible values, for example, it can bring down blood sugar and blood fat, improve immunity and inhibit tumor growth (Liu and Zhang, 2003), so it was referred by FAO and WHO, to one of the 16 species rare mushrooms because it is characterized by nature, nutrition and health. We can search many literatures about the screening of culture media and comparing among strains (Tang et al., 2006; Mi, 2007; Li et al., 2008; Mi and Wang, 2008), but at present, its quality is descreasing and yield is falling, owing to the disease called “Black cap”. So, it is very nessary and significant to breed resistant strains against the pathogen resulting in the disease. Whereas conventional breeding methods including spore cross and mutagenesis are difficult to be used for breeding resistant strains in *C. comatu*, because the pure spores are hardly harvested by spore emission owing to the fruit body autolysis when it grows up into maturation. A feasible solution for screening resistant strains is to regenerate protoplasts from mycelia of *C. comatus*, and the basal work is to establish an efficient system suitable for liberation and regeneration of protoplasts. At present, the protoplasts from many species including some bacteria, yeasts, fungi and plants have successfully been regenerated (Morinaga et al., 1985; Yanagi et al., 1985; Sonnenberg et al., 1988), and many excellent strains have also been selected out in edible mushroom breeding (Gold et al., 1983; Kiguchi and Yanagi, 1985; Mukherjee and Sengupta, 1986; Kim et al., 2000; Sun et al., 2001; Dhitaphichit and Pornsuriya, 2005; Wang et al., 2005; Zhu et al., 2008; Chen et al., 2009). However, there are no reports on isolation and regeneration of protoplasts in *C. comatus* to date.

In the present study, an efficient protoplast isolation and regeneration system has been established in *C. comatus*, and the system is beneficial for screening the elite resistant strains against the pathogen resulting in “Black cap”.

MATERIALS AND METHODS

Strain and mycelial culture

The strain *C. comatus* was conserved in PDA medium (Table 1) in our labarary. A block of spawn was inoculated into 30 ml CYM broth (Table 1) in 500 ml Erlenmeyer flasks and incubated at 25°C to

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Table 1. Media and their compositions.

Media	Compositions (in 1000 ml water)
PDA	200 g potato, 20 g sucrose and 15 g agar
CYM	20 g glucose, 2 g peptone, 1 g yeast extract, 0.5 g $MgSO_4$, 0.46 g KH_2PO_4 1 g K_2HPO_4, 20 g agar(only used in solid medium)
MYG	5 g maltose, 5 g yeast extract, 10 g glucose, 30 mg VB_1, 20 g agar

Table 2. The designed factors affecting protoplast release in *C. Comatus.*

Factors	Ladders or componentsbnb
Lywallzyme concentration (%)	1.5, 2.0, 2.5, 3.0
Osmotic stabilizer (0.6M)	$MgSO_4.7H_2O$, KCl, sucrose, mannitol
[a]pH of enzymatic solution	5.0, 5.5, 6.0, 6.5
Enzymatic digestion time (h)	2, 3, 4, 5
Mycelia age (d)	3, 4, 5, 6, 7
Enzymatic digestion temperature (℃)	25, 30, 35

[a]pH buffer was prepared by citrate-disodium hydrogen phosphate.

Table 3. The designed factors affecting protoplast regeneration in *C. Comatus.*

Factors	Ladder or components
Osmotic stabilizer	KCl, mannitol, sucrose, $MgSO_4.7H_2O$
Media	CYM, MYG, PDA
[a]Protoplast (h)	2.0, 2.5, 3.0, 3.5, 4.0
[b]Protoplast (d)	2 , 3 , 4 , 5 , 6

[a]Protoplasts from different incubation time; [b]Protoplasts from different mycelia age.

proliferate mycelia.

Protoplast liberation and purification

The basic procedure of protoplast liberation of *C. comatus* was conducted as follows:

(1) Mycelia collection and pretreatment. The proliferated mycelia were picked out with inoculation loop, and then rinsed thrice by osmotic stabilizer.
(2) Enzyme digestion of mycelia. 0.1 g mycelia were suspended in 1ml enzymatic mixture for digestion. The mixture containing lywallzyme (Guangdong Institute of Microbiology, P. R. China) and osmotic stabilizer was first prepared in pH buffer, and then sterilized by 0.22 μm of pore membrane.
(3) Counting protoplast and stopping the reaction. The number of protoplast liberated was counted once every hour with globulimeter. When the highest concentration took place, the reaction was stopped.
(4) Removal of undigested mycelia fragment. The residual mycelia fragment in digesting solution was removed with a column prepared by filling a 2 - 3 mm thickness of cotton wool into 5 ml syringe and compressing gently.
(5) Protoplast purification. The protoplasts were purified by washing twice in the osmotic stabilizer the same with that used in enzymatic digestion, then the pellets were collected *via* centrifugation at 4℃ and 4000 rmp for 10 min.

To optimize the conditions involved in protoplast release, in this experiment, six factors were considered (Table 2). All the digestion reaction was conducted under a gentle vibration of 50 rpm.

Protoplast regeneration and phenotypic observation

The basic procedure of protoplast regeneration of *C. comatus* was conducted as follows:

(1) Dilution of protoplast. The obtained protoplasts were first centrifugalized to remove the osmotic stabilizer, and then, the pellets were diluted with 0.6 M mannitol or sterile water to about 10^5 cells/ml. Here, the sterile water was used to eliminate the regeneration errors derived from the mycelia fragments in pellets.
(2) Plating protoplasts. 0.1ml diluted protoplasts were plated into Petri dishes (9 cm in diameter) containing 25 ml regeneration medium and incubated at 25℃ for 4 - 6 days. The regeneration rate was calculated according to the following formula:

Regeneration rate (%) = (A-B) / plated protoplast number × 100%
A: colony number regenerated from protoplasts diluted with 0.6M mannitol.
B: colony number regenerated from protoplasts diluted with sterile water.
To screen the optimum regeneration condition, the following factors were designed to compare their effects on regeneration rate (Table 3). Moreover, The process from protoplasts to visible colonies were observed by microscope or naked eye, and partial visions were taken photographs.

RESULTS

Protoplast isolation

The protoplast concentration obtained under different factors was different, with a range from 2.6×10^6 - 8.9×10^6 protoplasts/ml. All the considered factors could affect protoplast release (Table 4), among of them, osmotic stabilizer seemed to have more significant effect than

Table 4. Effects of different factors on protoplast liberation.

Factors		Yield of liberated protoplast (×10^6 protoplasts/ml)	Factors		Yield of liberated protoplast (×10^6 protoplasts/ml)
[a]Lywallzyme concentration (%)	1.5	5.8	[d]Digestion time (h)	2	5.8
	2.0	8.1		3	8.1
	2.5	5.6		4	6.4
	3.0	3.6		5	6.2
[b]Osmotic stabilizers (0.6M)	$MgSO_4.7H_2O$	6.4	[e]Mycelia age (d)	3	5.0
	KCl	8.9		4	8.0
	Sucrose	2.6		5	6.3
	Mannitol	7.8		6	6.2
[c]Buffer pH	5.0	6.2	[f]Digestion temperature (℃)	7	4.8
	5.5	8.8		25	6.0
	6.0	7.7		30	8.0
	6.5	6.3		35	4.8

[a]The same condition was to incubate 6-d-old mycelia at 30℃ for 3 h in the mixture of pH5.5 containing 0.6M KCl as osmotic stabilizer; [b]the same condition was to incubate 6-d-old mycelia at 30℃ for 3 h in the mixture of pH5.5 containing 2% lywallzyme; [c]the same condition was to incubate 6-d-old mycelia at 30℃ for 3 h in the mixture of pH 5.5 containing 2% lywallzyme and 0.6 M KCl as osmotic stabilizer; [d]the same condition was to incubate 6-d-old mycelia at 30℃ in the mixture of pH5.5 containing 2% lywallzyme and 0.6M KCl as osmotic stabilizer; [e]the same conditions was to incubate 3 - 7 days of mycelia at 30℃ for 3 h in the mixture of pH 5.5 containing 2% lywallzyme and 0.6 M KCl as osmotic stabilizer; [f]the same conditions was to incubate 6-d-old mycelia for 3 h in the mixture of pH 5.5 containing 2% lywallzyme and 0.6 M KCl as osmotic stabilizer.

others, the best one was 0.6M KCl, with a highest yileds of 8.9 × 10^6 protoplasts/ml; the worst was 0.6 M sucrose, with a lowest yileds of 2.6 × 10^6 protoplasts/ml. As for the others factors, there was no significant difference in protoplast release. From the data in Table 4, it was concluded that the optimal condition for protoplast liberations was to incubate 4 day old mycelia at 30℃ for 3 h in the mixture of pH 5.5 containing 2.0% lywallzyme and 0.6 M KCl as osmotic stabilizer.

Protoplast regeneration

Six factors were designed for optimizing regeneration conditions, and the obtained data were shown in Table 5. Four osmotic stabilizers were used in medium to regenerate protoplasts, and 0.6M mannitol presented the highest regeneration rate (1.40%), followed by 0.6 M $MgSO_4.7H_2O$ (1.05%), while, both 0.6 M sucrose and 0.6 M KCl provided zero of regeneration rate, which suggested that sucrose and KCl were not adapt to act as osmotic stabilizer in regenerating protoplasts of *C. comatus*. Three media were employed in protoplast regeneration; as a result, CYM medium showed the highest regeneration rate 1.42%, but the difference between CYM and MYG was not significant. PDA medium displayed 0.51% of regeneration rate, much less than the others. The results revealed that the two media CYM and MYG were preferable to PDA in regenerating protoplasts of *C. comatus*. The protoplasts from different digestion time and mycelia age were also applied for evaluating the regeneration rate, the results showed no significant differences among the protoplasts from different digestion time, but, the regeneration rate from the protoplasts of different mycelia age seemed to have significant differences, among of them, 5-day-old mycelia showed the best regeneration rate 1.41%, whereas the 2-day-old mycelia showed the lowest regeneration rate 0.92%.

From the data in Table 5, it was concluded that the optimum regeneration conditions was to regenerate protoplasts isolated from 3 h of incubation of 5-day-old mycelia into mycelia in CYM medium supplemented 0.6 M mannitol as osmotic stabilizer.

Observation of protoplast regeneration process

Figure 1 showed the regeneration process from protoplasts to visible colonies in regeneration medium CYM. The isolated protoplasts (Figure 1a) were transferred onto regeneration media; they were first enlarged and then developed into a small yeast-like bud (Figure 1b). When more than one yeast-like buds occurred continuously from one mother protoplast, a lot of budding cells with different size was observed (Figure 1c), otherwise, when the new yeast-like buds appeared from one mother protoplast in a synchronous manner, the similar sizes of budding cells could be observed (Figure 1d). Every budding cells also began to produce new yeast-like buds

Table 5. Effects of different factors on protoplast regeneration.

Factors		Regeneration rate
[a]Osmotic stabilizer (0.6M)	KCl	0
	Mannitol	1.40
	Sucrose	0
	$MgSO_4.7H_2O$	1.05
[b]Media	CYM	1.42
	MYG	1.36
	PDA	0.51
[c]Protoplast quality (related to the incubation time) (h)	2.0	1.24
	2.5	1.33
	3.0	1.38
	3.5	1.30
	4.0	1.21
[d]Protoplast quality (related to the mycelia age) (d)	2	0.92
	3	1.21
	4	1.31
	5	1.41
	6	1.30

[a]The same condition was that protoplasts isolated from 3 hours of incubation of 4-day-old mycelia regenerated in CYM medium; [b]The same condition was that protoplasts isolated from 3 h of incubation of 4-day-old mycelia regenerated in medium supplemented 0.6M mannitol as osmotic stabilizer; [c]The same condition was that protoplasts isolated from 4-day-old mycelia regenerated in CYM medium supplemented 0.6M mannitol as osmotic stabilizer; [d]The same condition was that protoplasts isolated from 3 h of incubation regenerated in CYM medium supplemented 0.6M mannitol as osmotic stabilizer.

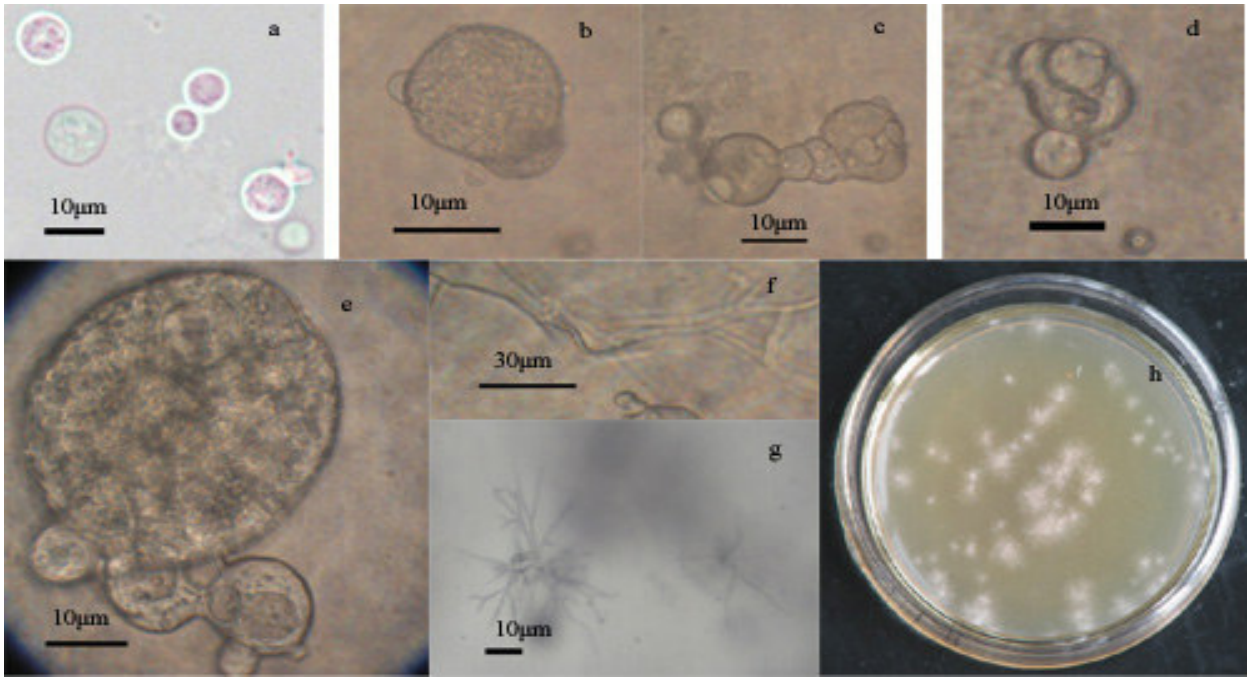

Figure 1. Regeneration process of protoplast of *Coprinus comatus.*
a: the isolated protoplasts; b: the enlarged protoplast; c: asynchronous protoplast budding; d: synchronous protoplast budding; e: new yeast-like buds continuously came out; f and g: the regenerated mycelia; h: the visible colonies.

as they grew up to a certain size (Figure 1e), meanwhile, the mother protoplasts could continue to budding, thus resulted in a continuous sizes of budding cells derived from one mother protoplast. When thesebudding cells regenerated to form cell walls, the mycelia could be observed (Figures 1f and g), and eventually, the propagated mycelia in regeneration medium formed visible colonies with different sizes (Figure 1h). The result was similar to the previous reports in other protoplast regeneration (Lau et al., 1985; Morinaga et al., 1985; Yanagi et al., 1985; Mukherjee and Sengupta, 1986).

DISCUSSION

C. comatus belonging to edible fungi has very important medical and edible values (Liu and Zhang, 2003). But at present, its quality and yield are falling, duo to the disease called “Black cap”. So, it is very nessary and urgent to breed resistant strains against the pathogen resulting in the disease. However, conventional breeding methods including spore cross and mutagenesis can not efficiently be used to breeding resistant strains because of the autolysis of fruit body of *C. comatus* during maturation. An alterative method is to regenerate *C. comatus* from protoplasts, and the primary work is to establish an efficient system suitable for isolation and regeneration of protoplasts. At present, many species have successfully been regenerated from protoplasts (Morinaga et al., 1985; Yanagi et al., 1985; Sonnenberg et al., 1988), however, there are no reports in *C. comatus* to this day. To obtained an efficient protocol suitable for protoplast isolation and regeneration in *C. comatus*, in our studies, several factors affecting experimatal results were designed and evaluated, of which the four main factors including lytic enzyme concentrations, mycelia age, osmotic stabilizers and regeneration media were intensively discussed.

Lytic enzyme concentration is one of the most important factors affecting protoplast release. If too low, mycelia can not be digested sufficiently; too high, protoplast membranes will be injured in spite of high yields, which will lead to a low regeneration frequency (Kitamoto et al., 1988; Zhang and Li, 1993; Rao and Prakash, 1995; Naseema et al., 2008). Lywallzyme, a lytic enzyme produced by GuangDong Institute of Microbiology, P. R. China, has successfully been used to liberate the protoplasts of more than ten *basidiomycetes*, the previous used concentrations ranged from 1.5 - 2.5% (Sun et al., 2001; Wang et al., 2004; Li et al., 2006). The same lytic enzyme was also applied in our study, and four concentration values including 1.5, 2.0, 2.5 and 3.0% were designed to obtain the optimum concentration in *C. comatus*, the results showed that 2.0% is the best, with high up to 8.1×10^6 protoplasts in 1 ml digestion solution (Table 4).

Mycelia age can affect protoplast isolation and its regeneration, although both juvenile and older mycelia of *basidiomycete* could be used for protoplast isolation and regeneration, the protoplasts from elder mycelia can be regenerated into mycelia more rapidly and easily, this cause is probably that elder mycelia have more intact nucleus and organelles (Santiago et al., 1982a, 1982b; Peng et al., 1993; Wang et al., 2005). Nevertheless, too old mycelia are not preferred, because their protoplasts were not easy to be isolated by lytic anzyme, in addition, the obtained protoplasts are hardly regenerated into mycelia duo to bad quality. Thus, in our experiments, to make sure the optimum mycelia age, five mycelia possessing different ages (3, 4, 5, 6 and 7d) were evaluated on the protoplast liberation, the results showed that 4-day-old mycelia have the highest protoplast concentration (8.0×10^6 protoplasts in 1 ml digestion solution) (Table 4). Another five mycelia (2, 3, 4, 5 and 6d) were investigated on protoplast regeneration, and 5-day-old mycelia presented the highest regeneration frequency (1.41%) (Table 5).

The osmotic stabilizers, used for rinsing mycelia, diluting lytic enzyme and preparing regeneration media, is also an important factor influencing protoplast liberation and regeneration. As for protoplast liberation, an osmotic stabilizer is only suitable for a few species owing to the differences of cell wall compositions in different species (Cheng and Bélanger, 2000). For example, mannitol was the best for *Agrocybe aegerita* (Zhang et al., 2004), while, MgSO4 is the best one for *Sclerotium rolfsii* (Morinaga et al., 1985; Farińa et al., 2004). To ascertain the optimum osmotic stabilizer used to liberate protoplasts of *C. comatus*, in our experiments, four including sucrose, mannitol, $MgSO4.7H_2O$ and KC1 were selected from many available osmotic stabilizers and analyzed on protoplast liberation, as a result, 0.6 M KCl provided the highest yield (8.9×10^6 protoplasts per 1 ml digestion solution) (Table 4).

Regarding protoplast regeneration, the optimum osmotic stabilizers supplemented into regeneration media were also different in light of different species. For instance, sucrose was the best one in *A. bisporus* and *A. bitorquis* (Yanagi et al., 1985; Sonnenberg et al., 1988), while in *P. brevicompactum*, 0.8M KCl was much better then others (Varavallo et al., 2004).

In this present experiments, four osmotic stabilizers were respectively added into CYM medium (Table 5), as a reult, the CYM medium containing 0.6 M manitol was proved the best, with 1.4% of regeneration percentage, followed by the CYM appended with 0.6 M $mgSO_4.7H_2O$; whearas, the two CYM supplemented with 0.6 M KCl or 0.6 M sucrose could not regenerate protoplasts into mceylia at all. Mannitol, as osmotic stabilizer, has also been used for protoplast regeneration in plant and other fungi, according to speculation, it was the direct precursor of cell wall synthesis or the indirect one by metabolism and transformation, and thus, could speed up the cell wall synthesis and make protoplast regenerate more easily

(Lau et al., 1985; Peng et al., 1993; Kim et al., 2000; Chitnis and Deshpande, 2002; Balasubramanian et al., 2003).

Besides osmotic stabilizers, media can also influence significantly protoplast regeneration, for example, in the studies by Fariña et al (2004) on protoplast regeneration of *Sclerotium rolfsii* ATCC 201126, the MYG medium supplemented 0.6M sucrose (26%) or 0.7M NaCl (17%) displayed the highest regeneration rate, when CYM media with 0.6M sucrose was used as regeneration culture media, the regeneration rate decreased almost twofold, moreover, the protoplasts on MR medium could not be differentiated into mycelia. Thus, in our study, three media supplemented with the same osmotic stabilizer (0.6M mannitol) were used for protoplast regeneration; as a result, medium CYM was proved the best one, with 1.4% of regeneration percentage (Table 5).

Although the regeneration percentage reported here was not high in *C. comatus*, even much lower than those reported in other species within same genus (Morinaga et al., 1985; Yanagi et al., 1985), the value could sufficiently meet our demands in most studies, such as protoplast fusion, mutagenesis and even transformation. Certainly, we could also increase the regeneration rate by improving regeneration media and protoplast quality.

In summary, an efficient protocol for protoplast isolation and regeneration was first established in *C. comatus*, the highest protoplast yield reached 8.9×10^6 protoplasts / ml in digestion solution, among which, about 1.4% could be regenerated into mycelia. The results are beneficial for breeding new cultivars by these methods such as protoplast fusion, mutagenesis as well as transformation, moreover, the stepwise procedure for protoplast liberation and regeneration contributes to the related studies in other species. Of course, the resistant strains against the pathogen resulting in “Black cap” could be obtained by protoplast techneques.

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Full Length Research Paper

The antifungal susceptibilities of oral *Candida* spp isolates from HIV-infected patients

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Oropharyngeal candidiasis (OPC) is the most common HIV related oral lesion. Most patients are infected with a strain originally present as a commensal of the oral cavity. The resistance of *Candida* isolates to antifungal drugs is important due to morbidity and mortality. The aim of our study was to investigate the antifungal susceptibility profiles of oral *Candida* spp which were isolated from HIV-infected patients. *In vitro* susceptibility tests were performed using the broth microdilution method recommended by the Clinical and Laboratory Standard Institute (CLSI). A total of 67 oral *Candida* isolates from colonized HIV-infected patients, which were previously isolated and identified were included in this study. MIC ranges were 0.12 - 4.0, 0.12 - 16, 0.03 - 1.0, 0.03 - 1.0, and 0.03 - 0.25 µg/ml for amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole, respectively. All isolates were fully susceptible to voriconazole. Sixty five (97%) of all isolates were determined fully susceptible to amphotericin B, 66 (98.5%) to fluconazole, 64 (95.5%) to ketoconazole and 50 (88%) to itraconazole. No resistance was detected to fluconazole and voriconazole in oral *Candida* strains isolated from colonized Turkish HIV positive patients. Antifungal resistance was detected in 8.96% (6 strains) of all isolates tested.

Key words: HIV, *Candida*, oropharyngeal carriage, antifungal agents, antifungal susceptibility.

INTRODUCTION

Although the introduction of highly active antiretroviral therapy (HAART) has reduced the prevalence of most opportunistic infections (Ceballos-Salobreña et al., 2000; Diz et al., 2001). Oropharyngeal candidiasis (OPC) is the most common HIV related oral lesion. About 90% of patients were found to suffer from oropharyngeal or esophageal candidiasis in various stages of AIDS (Kamiru and Naidoo, 2002). The presence of *Candida* in the oral cavities of HIV/AIDS patients predicts the subsequent development of oral candidiasis (Gugnani et al., 2003; Sánchez-Vargas et al., 2005).

The high incidence of mucosal and deep seated forms of candidiasis has resulted in the use of systemic antifungal agents, especially fluconazole and itraconazole (Ellepola and Samaranayake, 2000; Nenoff et al., 1999). The widespread use of these antifungals causes resistance both in *C. albicans* and non-albicans strains. OPC due to drug resistant fungi is a major problem for HIV infected patients (Vanden et al., 1998). The resistance of *Candida* isolates to antifungal drugs is important due to morbidity and mortality. Consequently, *in vitro* susceptibility tests should be performed to detect resistant strains (Colombo et al., 2002; Sojakova et al., 2004).

The aim of our study was to investigate the antifungal susceptibility profiles of oral *Candida* spp which were isolated from HIV-infected patients. This is the first study which investigated the antifungal susceptibility of various *Candida* spp isolated from the oral cavity of Turkish HIV positive patients. Until now, only one study from Turkey (Tekeli et al., 2005) which reported only the susceptibility of oral *C. dubliniensis* strains isolated from HIV patients was published.

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Table 1. Distribution of oral *Candida* spp isolated from HIV-positive patients and presence of previous treatment.

Species	Number of isolates (%)	Without treatment (%)	With previous treatment (%)
C. albicans	49 (73.1)	32 (65.3)	17 (34.7)
C. glabrata	9 (13.4)	5 (55.5)	4 (44.5)
C. dubliniensis	4 (6)	4 (100)	-
C. krusei	2 (3)	-	2 (100)
C. kefyr	1 (1.5)	1 (100)	-
C. lusitaniae	1 (1.5)	-	1 (100)
C. tropicalis	1 (1.5)	-	1 (100)
Total of all isolates	67	42 (62.7)	25 (37.3)

MATERIALS AND METHODS

Isolates

A total of 67 oral *Candida* isolates from colonized HIV-infected patients, which were previously isolated and identified (Erkose and Erturan, 2007) in the Department of Microbiology and Clinical Microbiology, Istanbul Faculty of Medicine, Istanbul University, were included in this study. Informed consent was obtained from participants and procedures were performed according to institutional board of ethical committee.

Antifungal agents

The following agents were supplied as standard powders: Amphotericin B (Sigma-Aldrich, MO, USA), fluconazole (Sigma-Aldrich, MO, USA), itraconazole (Sigma-Aldrich, MO, USA), voriconazole (Pfizer, NY, USA), ketoconazole (Sigma-Aldrich, MO, USA). Fluconazole was dissolved in sterile distilled water. Amphotericin B, itraconazole, voriconazole and ketoconazole were dissolved in dimethyl sulfoxide (DMSO) (Sigma chemical Co, St. Louis, MO, USA) to make stock solutions.

Antifungal susceptibility testing

In vitro susceptibility tests were performed using the broth microdilution method recommended by the Clinical and Laboratory Standard Institute (CLSI) (CLSI M27-A3, 2008; CLSI M27-S3, 2008; NCCLS M27-A2, 2002). The range of concentrations tested was 0.125 - 64 µg/ml for fluconazole and 0.0313 - 16 µg/ml for amphotericin B, ketoconazole, itraconazole and voriconazole. The broth microdilution test was performed by using sterile, disposable multiwell microdilution plates (96 U-shaped wells) (LP Italiano SPA, Milano, Italy). Aliquots of 100 µl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the wells of the plates. The suspension of yeasts after 48 h of incubation onto Sabouraud dextrose agar (BBL, Sparks, MD, USA) was prepared in sterile saline (0.85%), adjusted spectrophotometrically at 530 nm to match the turbidity of a 0.5 Mc Farland standard and was diluted in RPMI 1640 in order to obtain a final concentration of 0.5×10^3 to 2.5×10^3 CFU/ml (CLSI M27-A3, 2008; CLSI M27-S3, 2008; NCCLS M27-A2, 2002). A constant volume (100 µl) of the inoculum was added to each microdilution well containing 100 µl of the serial dilution of antifungal agents to reach final concentrations. The microplates were incubated at 35°C for 48 h. For amphotericin B and the four azoles, minimal inhibitory concentrations (MICs) were defined as the lowest concentration of the drug which resulted in a complete inhibition of visible growth and the lowest concentration of drug that produced a 50% reduction in fungal growth compared to that one of drug-free growth control, respectively (NCCLS M27-A2, 2002). In the case of the spectrophotometer readings, the azole cut-off value was 50% of the reading of growth control wells while for polyene antifungals, a cut-off value of 100% was used. *C. parapsilosis* ATCC 22019 was used for quality control.

The MIC values for fluconazole, itraconazole, voriconazole, ketoconazole and amphotericin B were compared to the CLSI interpretative guideline on antifungal susceptibility testing or based on previous investigations (Cheng et al., 2006; CLSI M27-A3, 2008; CLSI M27-S3, 2008; NCCLS M27-A2, 2002). For fluconazole: ≥64 µg/ml was used for resistant, 16 - 32 µg/ml was used for susceptible dose dependent and ≤8 µg/ml were used for susceptible. For itraconazole: ≥1 µg/ml was used for resistant, 0.25 - 0.5 µg/ml was used for susceptible dose dependent and ≤0.125 µg/ml were used for susceptible. For voriconazole: ≥4 µg/ml was used for resistant, 2 µg/ml were used for susceptible dose dependent and ≤1 µg/ml was used for susceptible. For ketoconazole: >0.125 µg/ml was used for resistant. For amphotericin B: ≥2 µg/ml was used for resistant.

RESULTS

A total of 67 oral *Candida* isolates from colonized HIV-infected patients, which were previously isolated and identified (Erkose and Erturan, 2007) were included in this study. The species distribution as presented in Table 1, *C. albicans* was the most frequently isolated species (49, 73.1%) followed by *C. glabrata* (9, 13.1%) and *C. dubliniensis* (4, 6%). Of the *Candida* isolates, 42 (62.7%) were from HIV-positive patients without any previous antimycotic treatment, while 25 (37.3%) were from HIV-positive patients with previous antimycotic treatment. *In vitro* susceptibility results of the *Candida* isolates are summarized in Table 2. The MICs for the quality control strains were within the accepted limits.

The determined MIC ranges were 0.12 - 4.0, 0.12 - 16, 0.03 - 1.0, 0.03 - 1.0 and 0.03 - 0.25 µg/ml for amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole respectively. The isolates demonstrated very low voriconazole MICs, in which 92.5% (62/67) presented values of 0.03 µg/ml, followed by ketoconazole, in which 89.6% (60/67) presented values of 0.03 µg/ml. All isolates were susceptible to voriconazole.

Table 2. *In vitro* antifungal susceptibilities of oral *Candida* isolates.

Species (number/%)	Antifungal agent	MIC (µg/ml)			SDD (%)	R (%)
		Range	MIC 50	MIC 90		
C. albicans (49/73.1)	Amphotericin B	0.25 - 1.0	1.0	1.0		-
	Fluconazole	0.12 - 4.0	0.12	0.25	-	-
	Itraconazole	0.03 - 0.25	0.06	0.12	3 (6.1)	-
	Ketoconazole	0.03 - 1.0	0.03	0.03		1(2)
	Voriconazole	0.03 - 0.25	0.03	0.03	-	-
C. glabrata (9/13.4)	Amphotericin B	0.25 - 4.0	1.0			1 (11.1)
	Fluconazole	0.12 - 4.0	0.5		-	-
	Itraconazole	0.03 - 1.0	0.12		1 (11.1)	2 (22.2)
	Ketoconazole	0.03 - 0.06	0.03			-
	Voriconazole	0.03	0.03		-	-
C. dubliniensis (4/6)	Amphotericin B	0.5 - 1.0	1.0			-
	Fluconazole	0.12	0.12		-	-
	Itraconazole	0.03 - 0.12	0.06		-	-
	Ketoconazole	0.03	0.03			-
	Voriconazole	0.03	0.03		-	-
C. krusei (2/3)	Amphotericin B	1.0 - 2.0	1.0			1 (50)
	Fluconazole	8.0 - 16	8.0		1 (50)	-
	Itraconazole	0.5	0.5		2 (100)	-
	Ketoconazole	0.25	0.25			2(100)
	Voriconazole	0.06	0.06		-	-
C. kefyr (1/1.5)	Amphotericin B	1				-
	Fluconazole	0.25			-	-
	Itraconazole	0.12			-	-
	Ketoconazole	0.03				-
	Voriconazole	0.03			-	-
C. lusitaniae (1/1.5)	Amphotericin B	0.12				-
	Fluconazole	0.12			-	-
	Itraconazole	0.03			-	-
	Ketoconazole	0.03				-
	Voriconazole	0.03			-	-
C. tropicalis (1/1.5)	Amphotericin B	1.0				-
	Fluconazole	0.12			-	-
	Itraconazole	0.03			-	-
	Ketoconazole	0.06				-
	Voriconazole	0.06			-	-
All *candida* (67/100)	Amphotericin B	0.12 - 4.0	1.0	1.0		2 (2.9)
	Fluconazole	0.12 - 16	0.12	2.0	1 (1.5)	-
	Itraconazole	0.03 - 1.0	0.06	0.25	6 (8.9)	2 (2.9)
	Ketoconazole	0.03 - 1.0	0.03	0.03		3(4.5)
	Voriconazole	0.03 - 0.25		0.03	-	-

Table 2. Continued.

	Amphotericin B	1.0 (0.5 - 4.0)
	Fluconazole	1.0 (1.0 - 4.0)
C. parapsilosis (ATCC22019)	Itraconazole	0.12 (0.12 - 0.5)
	Ketoconazole	0.06 (0.06 - 0.5)
	Voriconazole	0.03 (0.03 - 0.25)

C. parapsilosis (ATCC 22019) was used as reference strain; MIC ranges for 48 h were shown in parenthesis (22, 23, 24); S: Susceptible; SDD: Susceptible dose dependent (for fluconazole, itraconazole and voriconazole); R: Resistant.

65 (97%) of all isolates were determined susceptible to amphotericin B, 66 (98.5%) to fluconazole, 64 (95.5%) to ketoconazole and 50 (88%) to itraconazole.

Two (2.9%) of all isolates were found resistant to amphotericin B, 2 (2.9%) to itraconazole and 3 (4.5%) to ketoconazole. One of the two amphotericin B resistant strains was *C. glabrata* and the other was *C. krusei*. Both of the itraconazole resistant strains were *C. glabrata*, while one of three ketoconazole resistant strains was *C. albicans* and the remaining were *C. krusei*. Six (8.9%) of all isolates were found susceptible dose dependent (SDD) to itraconazole and 1 (15%) isolate to fluconazole. The strains SDD to itraconazole and fluconazole were *C. albicans* (3, 6.1%), *C. krusei* (2, 100%), *C. glabrata* (1, 11.1%) and C*. krusei* (1, 50%), respectively.

DISCUSSION

A variety of antifungal agents are now available for the treatment of OPC infections. Amphotericin B is both used topically in the treatment of superficial and systemic infections of hospitalized individuals (Ellepola and Samaranayake, 2000). Our findings showed that oral *Candida* spp isolated from HIV infected patients were highly sensitive to amphotericin B and only two (2.9%) isolates (one *C. glabrata* and one *C. krusei*) were resistant. The MIC distribution was concentrated in a very narrow range. HIV infected patients may have higher frequencies of amphotericin B resistant non-albicans *Candida* isolates (Blignaut et al., 2002; Kuriyama et al., 2005). Some studies have also shown low (Magaldi et al., 2000; Pfaller et al., 2001) resistance or no resistance (Costa et al., 2006; Gugnani et al., 2003; Hamza et al., 2009; Sánchez-Vargas et al., 2005) for amphotericin B, which is in accordance with our findings. Authors noted out that all *Candida* isolates that presented *in vitro* resistance to amphotericin B were recovered from patients who had previously received this antifungal therapy, this was also the case in our study (Kuriyama et al., 2005; Luque et al., 2009; Magaldi et al., 2000).

Fluconazole is a triazole agent with a broad therapeutic range and little toxicity that is established as a first-line antifungal for the treatment of oral candidiasis (Sheehan et al., 1999). Several recent studies have reported fluconazole resistance in *Candida* strains isolated from HIV-infected patients with OPC (Barchiese et al., 1994; Luque et al., 2009; Vanden Bossche et al., 1994). In the present study, almost all *Candida* isolates were found to be susceptible to this drug. Only one strain (*C. krusei*) (1.5%) was SDD to fluconazole. This result is similar to that reported by other previous studies (Barchiese et al., 2002; Blignaut et al., 2002; Kamiru and Naidoo, 2002; Kuriyama et al., 2005; Sánchez-Vargas et al., 2005).

Itraconazole is used as an alternative to fluconazole for treating oral candidiasis. In our study two (2.9%) (*C. glabrata*) isolates were determined as resistant to itraconazole, while six (8.9%) strains were SDD (three *C. albicans*, one *C. glabrata*, and two *C. krusei*), which is a result similar to some other investigator's findings (Costa et al., 2006; Hamza et al., 2009; Kuriyama et al., 2005; Luque et al., 2009; Magaldi et al., 2000; Sánchez-Vargas et al., 2005).

Ketoconazole is usually preferred as a topical anti-fungal agent because of high hepatotoxicity in systemic use. In the present study sixty four (95.5%) of all isolates were determined susceptible to ketoconazole, a finding which supported some other studies results (Blignaut et al., 2002; Kuriyama et al., 2005; Magaldi et al., 2000).

Voriconazole belongs to a new generation of triazoles and possess potent broad-spectrum activity and a favorable pharmacokinetic profile (Odds, 2006; Sabatelli et al., 2006). In this study all isolates were found to be susceptible to voriconazole. Previous studies have shown low MICs of voriconazole against tested strains (Costa et al., 2006; Gugnani et al., 2003; Kuriyama et al., 2005) which are in accordance with our findings.

Our results show that 2 *Candida* isolates (8%) from patients with previous treatment were resistant to amphotericin B, 2 (8%) to itraconazole, and three (4.5%) to ketoconazole with a further 1 (4%) isolate SDD to fluconazole and 6 (24%) to itraconazole.

In our study, the *in vitro* susceptibility of *C. glabrata* and *C. krusei* to antifungal agents was tested for a low number isolates, but these showed a resistance to imidazolic compounds as previously described by other authors (Luque et al., 2009).

In conclusion, all *Candida* strains isolated from the oral cavity of Turkish HIV-infected patients were susceptible to fluconazole and voriconazole. In our study, only one *C.*

albicans, three *C. glabrata* and two *C. krusei* strains were found to be resistant to antifungals and all of antifungal resistant strains have been associated with prior use of antimycotics. Primary colonizing in the oral cavity strain may cause an infection once the immune function of the HIV positive patient has been destroyed (Li et al., 2006). Oral colonization by isolates resistant to the most commonly used antifungal agents could represent a serious therapeutic problem among immunocompromised individuals.

AUTHORS' CONTRIBUTIONS

All the authors participated in the design, implementation, analysis and interpretation of study and commented on the draft of the report.

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Full Length Research Paper

Investigation of accessory gene regulator (*agr*) in *Staphylococcus aureus* isolated from clinical and subclinical bovine mastitis in Iran

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Mastitis is one of the common diseases of dairy cattle and an inflammatory response of the mammary glands tissue. Mastitis causes considerable loss to the dairy industry. Among several bacterial pathogens that can cause mastitis, *S. aureus* is probably the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured. The present study was to detect *agr* group genes in the *S. aureus* isolated from 360 mastitis milk samples in Chaharmahel va Bakhtiari and Isfahan provinces of Iran via PCR by using specific primers. Among 360 milk samples, 86 samples contained 1250 bp fragment of the 23srRNA gene, 10 samples contained *agr*I gene, 42 samples contained *agr*II gene, 19 samples contained *agr*III gene and 15 samples contained *agrIV* gene.

Key words: Bovine mastitis, *S. aureus*, *agr* group genes, PCR, Iran.

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that has remained a persistent pathogen, causing such infections as endocarditis, meningitis, and toxic shock syndrome in humans. *S. aureus* also is the leading cause of intramammary infections (mastitis), especially in dairy animals, from whose milk it is frequently isolated (Yancey, 1999). Neutrophils are the principle line of defense during the initial stages of mastitis, and the ability of these cells to phagocytize and kill invading bacteria is critically related to the establishment of new intramammary infections (Paape et al., 1979). Therefore, any bacterially derived component that may compromise neutrophil function would constitute an important virulence factor in the pathogenesis of *S. aureus* mastitis. Although a number of different virulence factors involved in the pathogenesis of *S. aureus* mastitis have been identified (Yancey, 1999), the differential expression of these factors as it relates to field strain prevalence of *S. aureus* genotypes has not been investigated. A better understanding of the epidemiology of *S. aureus* mastitis as it pertains to virulence will provide insight concerning important host-pathogen interactions during the pathogenesis of disease. Subtyping is an important tool for epidemiologic investigation of bacterial infections. In the past decade, numerous molecular techniques such as multilocus enzyme electrophoresis, phage typing, plasmid DNA restriction patterns, random amplified polymorphic DNA ribotyping, and coagulase genotyping have proved useful in identification and comparison of *S. aureus* isolates in epidemiological studies (Baumgartner et al., 1984; Matthews, 1993; Saulnier et al., 1993; Thomson-Carter et al., 1989; Wang et al., 1993).

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Abbreviations: *S. aureus*, *Staphylococcus aureus*; ***agr*,** accessory gene regulator.

However, very few studies have identified *S. aureus* isolates by the gene polymorphisms among important virulence-related genes. Among the virulence-related genes in *S. aureus*, we were particularly interested in the accessory gene regulator (*agr*), which has been shown to regulate the synthesis of many virulence factors during bacterial growth (Balaban and Novick, 1995b; Novick et al., 1993). The *agr* system coordinately down-regulates the production of cell wall-associated proteins and up-regulates secreted proteins at late to stationary growth phase in vitro (Ji et al., 1995; Novick et al., 1993; Novick et al., 1995; Recsei et al., 1986). The *agr* locus encodes a two-component signal-transducing system consisting of two divergent transcription units driven by promoters P2 and P3 (Ji et al., 1997). The P3 operon encodes the transcript for RNAIII, the effector of the *agr* response, while the P2 operon contains transcripts for four open reading frames designated *agrA*, -*B*,-*C*, and -*D* (Balaban and Novick, 1995a). *agrB* and -*D* generate an auto-inducing peptide that acts as an activating ligand for *agrC*. The present study was designed to investigate *S. aureus* isolates from cattle with bovine clinical and subclinical mastitis from different region of Iran, genotypically and by the identification of *agr* gene.

MATERIALS AND METHODS

Sample collection and identification

A total of 86 *S. aureus* isolates were collected from milk samples from 360 cows with mastitis from 10 different farms in tow region of Iran (140 samples from Chaharmahal va Bakhtiari and 220 specimens from Isfahan provinces). All of the isolates were identified by culture properties, by the detection of hemolysis (Skalka et al., 1979), and by the tube coagulase reaction.

Nucleic acid purification

For DNA preparation, 5 - 10 colonies of the bacteria were incubated in 100 µl of TE buffer (10 mmol of Tris-HCl/liter, 1 mmol of EDTA/liter, pH 8.0) containing 5 µl of lysostaphin (1.8 U/ µl; Sigma, Deisenhofen, Germany), for 1 h at 37°C and subsequently treated with proteinase K (14.0 mg/ml; Fermentas) for 120 min at 56°C. To inactivate the proteinase K, the suspension was heated for 10 min at 100°C and centrifuged at 10,000× *g* for 20 s. Ninety microliters of the supernatant was treated with 10 µl of 5 mol/liter $NaClO_4$ and 50 µl of isopropanol (99.7%; Merck, Germany), mixed, placed on an ice block for 10 min, and centrifuged for 30 min at 13,000 rpm. The supernatant was discarded, 250 µl of ethanol (70%) was added, and the tube was again centrifuged for 5 min at 13,000 rpm. The supernatant was again discarded, and the pellet was dried in a desiccator for 5 min. After the addition of 50 µl of sterilized aqua dest, the tubes were cooled until they were used.

PCR amplification of the variable region of the *agr* operon

PCR amplification of the 1070 bp variable region of the *agr* operon was performed with primers B1(5-TAT GCT CCT GCA GCA ACT AA-3) and C2 (5-CTT GCG CAT TTC GTT GTT GA-3) described by van Leeuwen et al. (van Leeuwen et al., 2000). The variable *agr* region was amplified from 2 µl of the purified nucleic acid solution in a 100 µl reaction mixture containing 2.5 U of *Taq* DNA polymerase (Fermentas), 200 µM deoxynucleotide triphosphates (dNTPs) (Fermentas), 0.5 µM primer B1, 0.5 µM primer C2, 2 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris HCl (pH 9.0). Amplifications were carried out in a thermocycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) through the following temperature program: 1 cycle of 4 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 74°C; and finally 1 cycle at 74°C for 3 min (Gilot et al., 2002).

Agr group-specific multiplex PCR

The *agr* sequences were amplified from 2 µl of the purified nucleic acid solutions in a 25 µl reaction mixture containing 1.25 U of *Taq* DNA polymerase (Fermentas), 200 µM dNTPs (Fermentas), 5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris HCl (pH 9.0), and a 0.3 µM concentration of each of the following primers: Pan (5'-ATG CAC ATG GTG CAC ATG C-3'), agr1 (5'-GTC ACA AGT ACT ATA AGC TGC GAT-3'), agr2 (5'-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3'), agr3 (5'-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3'), and agr4 (5'-CGA TAA TGC CGT AAT ACC CG-3'). These primers allow the amplification of a 441-bp DNA fragment of the *agr* group 1 strains, of a 575-bp DNA fragment of the *agr* group 2 strains, of a 323-bp DNA fragment of the *agr* group 3 strains, and of a 659-bp DNA fragment of the *agr* group 4 strains. Amplifications were carried out in a thermocycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) through the following temperature program: 1 cycle of 5 min at 94°C; 26 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; and finally 1 cycle of 72°C for 10 min (Gilot et al. 2002). Amplification products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized by transillumination under UV.

Statistical analyses

The analysis of all data was calculated by using SPSS software, version 16 and X^2 statistical test.

RESULTS

A total of 360 raw milk samples from several major herds in the Chaharmahel va Bakhtiari (n = 140) and Isfahan (n = 220) provinces of Iran were used in this study. 86 specimens (23.88%) of 360 milk samples in microbiological studies were identified to infected with *S. aureus*.

The PCR assay was able to detect, *S. aureus* DNA from 86 samples of milk by using primers mentioned in materials and methods. The existence of 1250 bp fragment in samples that showed positive PCR assay. All of the samples contained 1250 bp DNA fragment bands.

Of the 86 samples 10 specimens contained the *agr*I gene, 42 specimens contained the *agr*II gene, 19 specimens contained the *agr*III gene and 15 samples contained the *agr IV* gene. The results are shown in Table 1.

S. aureus strains isolated in this study was relevant to the clinical and sub clinical mastitis cases which in the CMT test had show a positive reaction. From the total number of 86 strains, 20 strains were relevant to mastitis +1 (in the CMT test), 45 strains were relevant to mastitis

Table 1. Frequency of presence of the *agr* genes in the *S. aureus* strains isolated from bovine mastitis milk in Iran.

Specimens	*agr*I	*agr*II	*agr*III	*agr*IV
86	10	42	19	15

Table 2. Number of virulence genes in bovine mastitis milk samples CMT 1+ to 3+ in Iran.

*agr*IV	*agr*III	*agr*II	*agr*I	Samples	Degree of CMT test
4	3	2	1	20	+
4	6	11	3	45	++
7	10	29	6	21	+++

+2, and 21 strains were relevant to mastitis +3. Type and the number of understudied virulence genes obtained from these mastitis cases are shown in Table 2.

DISCUSSION

S. aureus is one of the commonest causes of bovine mastitis which is of economic importance to the industry. *S. aureus* produces a large number of potential virulence factors. The staphylococcal accessory gene regulator (*agr*) is the most important locus responsible for the regulation of virulence factors (Robinson et al., 2005).

The *agr* locus of *S. aureus* recognized a quorum-sensing gene cluster, encodes a two-component signal transduction system that leads to down-regulation of surface proteins and up-regulation of secreted proteins during *in vitro* growth. A role for *agr* in virulence has been demonstrated by the attenuated virulence of *agr* mutants in different animal infection models (Mullarky et al., 2001; Robinson et al., 2005). The *agr* locus consists of the divergently transcribed P2 and P3 operons. The P2 operon consists of the genes *agrB*, *agrD*, *agrC*, and *agrA*. In essence, *agrB* activity leads to secretion of the auto inducing pheromone, *agrD,* which binds to and activates the histidine kinase receptor, *agrC*, which subsequently activates the response regulator, *agrA*. The P3 operon consists of the regulatory effector molecule of the *agr* system, RNAIII, and the gene encoding delta-hemolysin, *hld*. interestingly, amino acid changes within the *agrD* pheromone can cause inhibition of *agr* activity. Four allelic groups of *agr* have been characterized in *S. aureus* (numbered I to IV) that generally induce *agr* activity within a group and inhibit *agr* activity between groups. The inhibitory activity of these *agr* groups represents a form of bacterial interference that affects virulence gene expression (Robinson et al., 2005).

The study was conducted to determine the prevalence of the *agr* gene in *S. aureus* strains isolated from bovine mastitis milk in Chaharmahal va Bakhtiari and Isfahan provinces of Iran. Our results indicated that among the four types of *agr* gene, *agr*II with frequency 48.83% was the most frequently present type among the isolated *Staphylococcus* strains (Table 1).

Expression of *agr* gene in the pathogenic staphylococci is very crucial in bacterial colonization. Regarding the *agr* gene being polymorphic, it is probable that expression of this gene could be effect in ability of *S. aureus* for transfer from one host to another. Involvement of the *agr* genetic locus to regulation expression of virulence gene, incited researchers to use the *agr* system for identifying different *Staphylococcus* spp. (Robinson et al., 2005).

Many researches has been done in different countries to determine the genotype of the *agr* gene , for example , In the research conducted by Shopsin et al. from 196 *S. aureus* strains isolated from children and 64 *S. aureus* isolated from adults, polymorphism of the *agr* gene was studied. In this study it was found out that the *Staphylococcus spp.* caring the *agr* gene are capable to colonization (Shopsin et al., 2003).

In another study performed in 2008 by Reinoso et al. from 45 *Staphylococcus* strains isolated from various sources such as human infections and mastitis were studied from of different virulence factors. In human samples 8 specimens (36%) belonged to the *agr*III group and from 14 human samples belonged to the groups I (14%) and II (27%). Among the samples isolated from the bovine mastitis, 7 specimens (47%) belonged to the *agr*I group 8 specimens belonged to the *agr*I (27%) and *agr*I (13%) groups. 10 specimens were reported to be negative for *agr*I to *agr*III groups (Reinoso et al., 2008).

In the present study most of the *S. aureus* strains contained the *agr*II gene which corresponds, with results obtained by Reinoso et al. (Reinoso et al., 2008).

From the total of 42 indicated that samples containing the *agr*II gene, 29 strains were 3+ CMT test. In the statistical analysis by Chi-square test was statistically significant relationship between the presence of *agr* gene and degree of bovine mastitis in CMT test ($P \leq 0.05$). This result indicates that, the expression of the *agr* gene is effect on pathogencity of *S. aureus* in developing mastitis. As a whole, different results reported from various studies indicate that many factors such as geographical situation and main source of bacteria involved in developing mastitis can play a role in type and percentage of presence of virulence genes in the *S. aureus* strains.

Results of this study can be an introduction for more complete studies on distribution of *agr* genes in strains of *Staphylococcus* isolated from mastitis cases and the rote of involvement of these genes in pathogencity of the bovine mastitis.

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Full Length Research Paper

Irrigation influence by treated domestic wastewater instead of agronomical water on essential oil yield of basil (*Ocimum basilicum* L.)

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In this experiment, we have used the soil profile as a biological filter that can to absorb pollution and microbes in the domestic wastewater at Iran during 2009. In this experiment, we had 15 lysimeters, that were planted canola (*Brassica napus* L.) in 1 to 5 lysimeters and were irrigated by domestic wastewater with BOD_5 about 150 mg/lit and primary drainage water were accumulated. In the 6 to 9 lysimeters was planted alfalfa (*Medicago sativa* L.) and were irrigated by primary drainage water and then, were accumulated secondary drainage water. We have irrigation 10, 11 and 12 lysimeters by secondary drainage water that was planted inside the basil (*Ocimum basilicum* L.). In order to compare plants characteristics, in 13, 14 and 15 lysimeters were planted canola, alfalfa and basil respectively and were irrigated by agronomical water. The results showed that the soil could reduce BOD_5 and COD from 150 and 232 mg/lit to 11 and 18 mg/lit respectively in secondary drainage water. The essential oil yield of basil 17% increased under irrigation by secondary drainage water into irrigation by agronomical water. Therefore, the use of secondary drainage water can be increase the quantity and quality yields in plants and the other hand, protects sweet water resources.

Key words: Domestic wastewater, agronomical water, essential oil yield, basil.

INTRODUCTION

Basil (*Ocimum basilicum* L.) is an annual plant that prefers warm growing weather but nevertheless is tender and killed by sunstroke. Depending on variety and culture, the plants grow 20 - 35 in height and the leaves are violet and typically about 12 (Khalid, 2006). Domestic wastewater treatment is focused generally on treating blackwater. Blackwater is the perfect medium for the growth of pathogenic bacteria. Therefore, it is extremely necessary to treat it before reuse or to be discharged into rivers and lakes. Discharge of untreated or partially treated wastewaters containing carbon (C), nitrogen (N), and phosphorus (P) into receiving waters can lead to eutrophication. As a result, it is necessary to develop treatment systems that efficiently and economically remove nutrients from these wastewaters. Biological nutrient removal methods have advantages over physical and chemical methods, including low waste sludge production and low capital and operational costs. Biofilm wastewater treatment systems, which are characterized by their compactness, simple operation, and easy maintenance, can be more stable in treating wastewaters with high flow and substrate variations than suspended-growth activated sludge systems. In addition, biofilm systems can be more suitable for small-scale wastewater or industrial wastewater treatment than activated sludge systems (Rodgers et al., 2008). Federal and state laws require that domestic wastewater be treated through a two- or three-step process with the end products being sewage effluent and biosolids. Domestic wastewater effluent is essentially clear water that contains low concentrations of plant nutrients and traces of organic matter. It is chlorinated to destroy any pathogens (Kidder, 2001). Perennial aromatic plants are cultivated as cash-crops for fresh or dry herb production, or as a source of essential oils and natural antioxidants. These summer crops require substantial amounts of water, up to 7000 to

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Table 1. The analysis of lysimeters soil before irrigation by domestic wastewater.

Parameters	Density	Parameters	Density
Cd (meq/lit)	0.01	HCO_3^- (meq/lit)	4
Cu (meq/lit)	3.51	Cl (meq/lit)	6
Mn (meq/lit)	8.08	P (meq/lit)	38
Zn (meq/lit)	1.83	K (meq/lit)	21
Ni (meq/lit)	0.005	Ca (meq/lit)	29.8
Mg (meq/lit)	27.4	SO_4^{-2} (meq/lit)	2.08
Fe (meq/lit)	11.03	$Ca(OH)_2$ (%)	11
Pb (meq/lit)	1.73	Humus (%)	0.88
CO_3^{-2} (meq/lit)	0	pH	7.9

9000 m^3 ha^{-1} throughout the growing season, to satisfy their potential for intensive biomass production (Dudai, 2005). Hundreds of hectares of these crops are required to facilitate an economically viable industrial production system. Therefore, shortage of fresh water for irrigation in arid and semiarid regions restricts utilization of aromatic plants as industrial crops. Replacement of fresh water with treated effluent for irrigation of these plants could promote development of large-scale production systems for biomass, essential oil, and natural antioxidants in arid and semiarid zones. No information is currently available concerning the effect of irrigation with treated municipal effluent on growth and development of crops, essential oil yield or antioxidant production. Salinity and heavy metals contained in treated effluents may increase antioxidant activity and reactive oxygen production in plants. Increased antioxidant content and antioxidant activity were demonstrated in many plants in response to environmental stresses (Mittler, 2002). In some medicinal plants, water resources induced changes in antioxidants which were suggested to be involved in prevention of plant tissues damage (Aliabadi et al., 2009). Shortage of water in arid and semiarid regions throughout the world dictates utilization of marginal water, of low quality, for irrigation. Treated urban effluents, which may affect yield quantity and quality, are the most common alternative for agricultural irrigation (Dudai, 2005). Despite the cost of waste water treatment and distribution, annual crop costs are lower when irrigating with effluents because the price of effluent water in some areas is lower compared to potable water (Fine et al., 2006). Therefore, the objective of this study was to evaluate the effect of irrigation by treated domestic wastewater instead of agronomical water on essential oil yield of basil (*Ocimum basilicum* L.).

MATERIALS AND METHODS

This study was conducted on experimental lysimeters of Islamic Azad University, Science and Research Branch of Tehran at Iran (35°48' N, 51°01' W; 1320 m above sea level) in 2009, relative humidity (68%), mean annual temperature (16°C) and rainfall in the study area is distributed with an annual mean of 235 mm. The volume of each lysimeter was 150 lit (Height = 100 cm and Radius = 30 cm) filled by clay loam soil consisted of 19.9% clay, 22.09% silt and 58.72% sand and in order to prevent water influx from field to lysimeters, those placed on metal legs (height = 40 cm). After filling lysimeters by clay loam soil, plants seeds were planted and were irrigated with agronomical water and apparent specific weight of soil was 1.52 g/cm^3 (Table 1).

Canola (*Brassica napus* L. cul. Opera), alfalfa (*Medicago sativa* L. cul. Hamedani) and basil (*Ocimum basilicum* L. var. Mianeh) were used in this experiment. In this experiment, we had 15 lysimeters, that were planted canola in 1 - 5 lysimeters and were irrigated by domestic wastewater with BOD_5 about 150 mg/lit and primary drainage water were accumulated. In the 6 - 9 lysimeters was planted alfalfa and were irrigated by primary drainage water and then, were accumulated secondary drainage water. We have irrigation 10, 11 and 12 lysimeters by secondary drainage water that was planted inside the basil. In order to compare plants characteristics, in 13, 14 and 15 lysimeters were planted canola, alfalfa and basil respectively and were irrigated by agronomical water (Figure 1).

At the maturity, we collected plants from each lysimeters for determination of flowering shoot yield and total dry matter. Then, were selected 100g flowering shoot dry matter for determination of essential oil percentage by Clevenger. Finally, essential oil yield was determined by the following formula (Aliabadi Farahani et al., 2008).

Essential oil yield = Essential oil percentage × Flowering shoot yield

Also, the chemical quality of domestic wastewater, primary drainage water and secondary drainage water were determined for compare with standards of Iran and FAO. Finally, data were subjected to repeated measure analysis.

RESULTS

The chemical quality of domestic wastewater, primary drainage water and secondary drainage water is shown in Table 2.

Also, in the Table 3 is shown amount removed of biological, chemical and microbial pollutants in domestic wastewater by crossing the soil profile in 2 stages.

The final results showed that use the secondary drainage water for basil irrigation increased plat characteristics to compare with agronomical water. In the agronomical water irrigation condition the amount of essential oil yield, biological yield, flowering shoot yield and essential oil percentage were 10.2 kg/ha, 4900 kg/ha, 410 kg/ha and

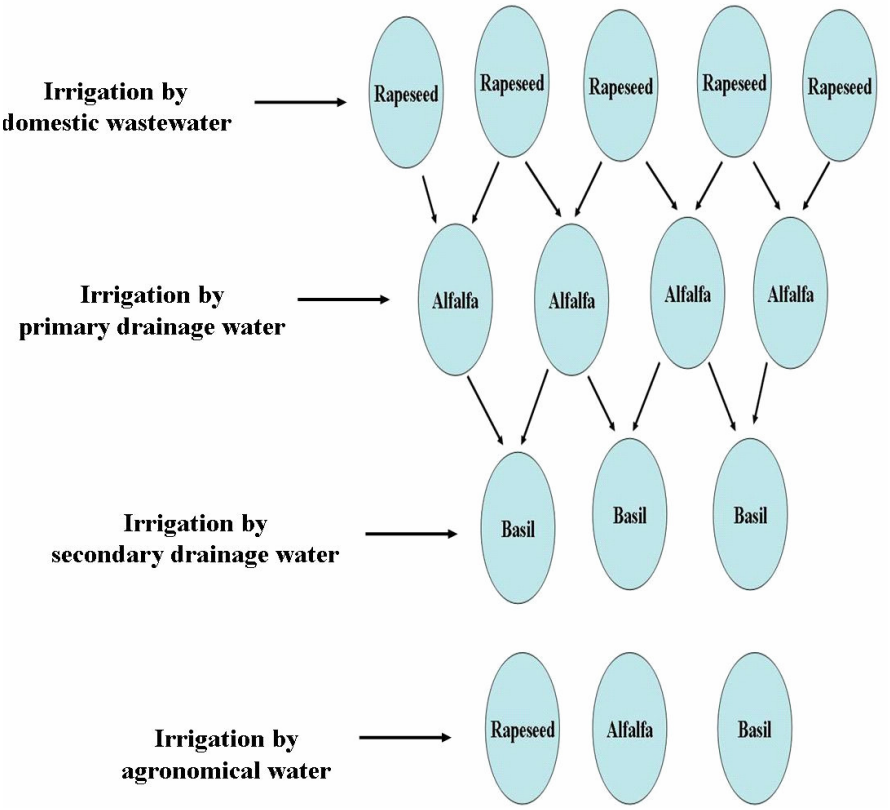

Figure 1. Experimental lysimeters

Table 2. The chemical quality of domestic wastewater, primary drainage water and secondary drainage water and compare them with standards of Iran and FAO.

Parameters	Domestic wastewater	Primary drainage water	Secondary drainage water	Standard of Iran	Standard of FAO
Cd (meq/lit)	0.04	0.01	0	0.1	0.01
Ni (meq/lit)	1.83	0.98	0.01	2	0.06
Mg (meq/lit)	2.71	1.93	1.8	4.1	---
HCO_3^- (meq/lit)	11.18	9.4	8.8	---	0.14
Cl (meq/lit)	8.2	7.92	6.21	16.9	4-10
P (mg/lit)	3.41	2.12	1.8	---	---
K (mg/lit)	2.71	1.93	1.8	4.1	---
Ca (meq/lit)	3.51	3.02	2.64	---	---
SO_4^{-2} (meq/lit)	3.42	3.12	3	4.16	---
Na (meq/lit)	13.82	12.01	11.85	---	3-9
C (mg/lit)	150.47	58.02	30.02	---	---
N (mg/lit)	32.11	24.14	20.11	10	5-30
Salinity (ds/m)	1.83	1.62	1.52	---	< 3
pH	7.9	7.75	7.34	6.5-8.5	6.3-8.4

0.48% respectively. But highest essential oil yield (12 kg/ha), biological yield (5400 kg/ha), flowering shoot yield (500 kg/ha) and essential oil percentage (0.66%) were obtained under irrigation by secondary drainage water (Table 4).

Evaluation of accumulation of different elements in basil shoot showed that accumulation of elements such as nitrogen, phosphorus, potassium, calcium and protein were increased under irrigation by secondary drainage water but the cadmium element was reduced under this

Table 3. The amount of biological, chemical and microbial pollutants in domestic wastewater, primary drainage water and secondary drainage water.

	Domestic wastewater	Primary drainage water	Secondary drainage water
Coliform (n/100ml)	1.2×10^{18}	3×10^{5}	1.5×10^{3}
Fecal Coliform (n/100ml)	1.2×10^{18}	3×10^{5}	1.5×10^{3}
Parasite eggs	1.42	0	0
COD (mg/lit)	232	30	18
BOD_5 (mg/lit)	150	15	11

BOD_5 = Biological oxygen demand
COD = Chemical oxygen demand.

Table 4. Effect of irrigation by secondary drainage water on basil characteristics to compare with agronomical water.

	Secondary drainage water	Agronomical water	Percent of increasing (%)
Biological yield	5400 kg/ha	4900 kg/ha	+ 10
Flowering shoot yield	500 kg/ha	410 kg/ha	+ 21
Essential oil yield	12 kg/ha	10.2 kg/ha	+ 17
Essential oil percentage	0.66 %	0.48 %	+ 37

Table 5. Accumulation of different elements in basil shoots.

	N (%)	P (%)	K (%)	Protein (%)	Ca (mg/kg)	Cd (mg/kg)
Secondary drainage water	7.14	0.18	3.81	18.12	236	0
Agronomical water	6.84	0.14	3.42	14.07	228	0.01

condition (Table 5).

DISCUSSION

The understanding of how the plants respond to the agronomic growing conditions is a prerequisite for the prediction of essential oil and for controlling oil quality. This is especially important since changes in the chemical composition affect the commercial value of the oil, with consequences to the grower's income. As it was shown in our results, the use of secondary drainage water had a positive effect on most of the emphasized growth compounds. In contrary, secondary drainage water supply in soil achieved a situation for plant to absorb by root growth the nutrients. However, secondary drainage water element isn't in essential oil components, but the our final results indicated that applications secondary drainage water increased essential oil content of basil, because the secondary drainage water element (N, P, K, …) develops leaf area, lateral stem, number of flower and because of increase of the essential oil yield, because elements are the major nutrients that influence plants yield and protein concentration. When the amount of available soil nutrients limits yield potential, additions of secondary drainage water can substantially increase plants yield. The interaction between the amount of the essential oil percentage and flowering shoot yield is considered important as two components of the essential oil yield. The essential oil percentage increased under the use of secondary drainage water and also, essential oil yield increased under this condition. Therefore, each increaser factor of essential oil percentage and flowering shoot yield, can increases essential oil yield. Our results were similar to the findings of Bernstein et al. (2009). They to evaluate the effect of irrigation with secondary-treated effluent on plant development, essential oil yield, antioxidant activity and selected antioxidant phenolic compounds in two commercial cultivars of the aromatic species, oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis* L.). The applied treated effluent contained higher levels of Na, Cl, $HCO_{3,}^{-1}$ P, K, NH_4^{+1}, NO_3^{-1}, Ca+Mg, B, Mn, and Fe than the local potable water used as control, and were characterized by higher values of electrical conductivity (EC), pH, and sodium absorption ratio (SAR). The results demonstrate that both oregano and rosemary are suitable as industrial crops for essential oil and antioxidant production under irrigation with secondary-treated municipal effluent because their yield quantity and quality were not affected. In addition to affects on the irrigated crops, much effort is currently made to study potential effects of irrigation by wastewater

on chemical and physical properties of soils. In the present study, the secondary drainage water used were of homely origin, contained only moderate levels of salts, and did not contain elevated levels of heavy metals. Heavy metal accumulation therefore did not appear in the soil or the plant tissues and salinity effects on the plants were moderate.

Conclusion

Our results demonstrate that secondary drainage water is suitable for growth and quality production of basil essential oil production in areas where water supply is limited. In addition, for large-scale production not otherwise possible due to lack of water, cultivation with effluents has an additive economical benefit to the farmers. Therefore, irrigation of basil with the secondary drainage water can reduce stress on the plants, increase essential oil production, and may lead to an economic advantage over regular water irrigation. Practically, our findings may suggest farmers and agricultural researchers to consider carefully on limiting or control the huge water resources by the use of secondary drainage water.

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Full Length Research Paper

Adherence and damage to epithelial cells of human lung by *Ureaplasma urealyticum* strains biotype 1 and 2

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***Ureaplasma* sp. is associated with chronic lung disease and it has been implicated in the morbidity and mortality of new born babies (human). Adherence is an important process for lung infection, and scarce information is available in this respect. This work shows its adhesion to epithelial cells from the respiratory tract in culture, and evaluates the *Ureaplasma* sp. references and clinical strains isolated from premature newborns. *Ureaplasma parvum and Ureaplasma urealyticum* serotype 8 and five clinical samples were evaluated, using the Bertholet assay. They were observed by scanning and transmission electron microscopy to A549 cell line. Adherence was abolished by pre-treatment of ureaplasmas with A549 cell extracts and inhibited by pretreatment of ureaplasmas with N-acetylneuraminic acid, trypsine, glucose and monoclonal antibodies (mAb 106.C anti-MB against *U. parvum* serotype 3). *Ureaplasma* sp. (biotypes 1 y 2) adherence had no significant difference (P = 0.127). The clinical strains by scanning electron microscopy showed a similar damage. Both reference and clinical strains produced evident changes in cell morphology. Micro-colonies were observed on the cells. By transmission electron microscopy, ureaplasmas attached on the cell surface and into the cytoplasm were observed. The A549 cellular line is a good model for the study of adherence of *Ureaplasma* sp. The urease test by Bertholet assay is a good indicator of colonization. These data suggested that ureaplasma adhesins are proteinaceous antigenic substance. Inhibition assays with neuraminidase and glucose showed binding to sialic acid residues and suggested as possible adhesin to MB (multi banded antigen).**

Key words: *Ureaplasma urealyticum*, adherence, epithelial cells, damage.

INTRODUCTION

Ureaplasma sp. causes pneumonia in premature new born babies (human) and is associated to bronchopulmonar dysplasia (BPD); furthermore it is associated with a high morbidity and mortality in the perinatal period (Alpha et al., 1995; Brus and Van Warde, 1991; Cassell et al., 1991; Cassell, 1993; Waites et al., 2005; Faye-Petersen, 2008). *Ureaplasma urealyticum* is subtyped into two biovars and 14 serovars (Cassell, 1993) based on significant genotypic differences between the two biovars, it has been proposed to separate the species previously known as *U. urealyticum* (or *Ureaplasma* sp. human) into two new species, *Ureaplasma parvum* (biovar 1 containing serovars 1, 3, 6 and 14) and *U. urealyticum* (biovar 24 containing serovars 2, 4, 5 and 7 to 13) (Cassell, 1993; Harasawa et al., 1991; Kang et al., 2000; Lee et al., 1991). Biovar 1 (*U. parvum*) is the more common of the two biovars isolated from clinical samples, but both species may occur simultaneously in some people. Some serovars have been implicated with disease more commonly than others (Abele-Horn et al., 1997; Naessens

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et al., 1998; Zheng et al., 1992) but any differences in pathogenicity among serovars are unproven. All *Ureaplasma* sp. serovars contains an external surface protein named multi banded antigen (MB), which is considered a virulence factor. Actually, its biologic activity is unknown (Zheng et al., 1995). Previous reports have examined the relationship between ureaplasma colonization of the neonatal respiratory tract release of inflammatory mediators that may be involved in damage and pathogenesis of BPD.

Ureaplasma sp. in the lower respiratory tracts with progression to BPD has been published since 1998 (Cassell, 1993; Sanchez and Regan, 1988; Wang et al., 1988); and certainly BPD occurs in premature infant who received mechanical ventilation. Evaluation of fibroblast of mouse infected with ureaplasmas without oxygen showed that proinflammatory cytokines play an important role in mediating pathology in a variety of lung disease, including BPD (Stancombe et al., 1993). Apoptosis of pulmonary cells may also be related with development of BPD, the proliferation of neutrophils at the site of lung infection will lead to prolonged inflammation by cytokine production and release of proteases and oxygen free radicals. Li et al. (2002) using human macrophage and lung epithelial cell lines demonstrated that when these cells are stimulated with *Ureaplasma* antigen, apoptosis will occur "*in vitro*" as evidenced by morphological evaluation and analysis of DNA fragmentation. Damage to lung epithelial cell is not necessarily due to ureaplasmas itself. Ronald et al. (1997) infected A549 cells with *Mycoplasma hominis* and *U. urealyticum* serotype 8 and evaluated the interleukins effect; IL-8 levels were higher in A549 cells infected with *M. hominis* than A549 cells infected with *U. urealyticum* serotype 8. These results suggested that *M. hominis* and *U. urealyticum* serotype 8 participated for inflammatory response that Chronic Lung Disease (CLD) preceded directly for cytokines productions by cells type II stimulation. Although, ureaplasmas causes respiratory tract infection of preterm, neonates suggest that lung disease could be associated with these organisms and stimulation of proinflammatory cytokines (TNF-α, IL-10 and IL-8) or blockage of counter-regulatory cytokines (IL-6 and 1L-10) (Waites et al., 2005; Shimizu et al., 2008). A juvenile mouse model of *Ureaplasma* pneumonia showed a persistent focal loss of airway ciliated epithelium and a mild increase in interstitial cellularity. *Ureaplasma* infected mice increased TNF-α at 14 days and increased granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-10 at 28 days. These data suggest that *Ureaplasma* alone may cause limited inflammation and minimal tissue injury in the early phase of infection, but may promote a mild chronic inflammatory response in the later phase of infection (days 14 to 28), similar to the process that occurs in human newborns (Viscardi et al., 2002; Novy et al., 2009).

Ureaplasma sp. is a very tiny microorganism, which cannot be observed by using conventional stains or light microscope; therefore, it is more difficult to study how the binding between the microorganism and the host cell is realized. The studies about adherence of this microorganism are few, using ureaplasmas of animal species and different cell lines through immunofluorescence; the adherence was not definitive (McGarrity et al., 1986). Adherence of mycoplasmas on the culture cells has been controversial to demonstrate (Mernaugh et al., 1993), however, other mycoplasmas showed adherence by immunocytochemistry and scanning electron microscopy techniques (Basolo et al., 1984; Diaz-Garcia et al., 2006; Jennsen et al., 1994; Robinson et al., 1991; Winner et al., 2000), inclusive; these powerful techniques showed that the invasion of host cells occurs and the organisms reside intracellularly (Diaz-Garcia et al., 2006; Jennsen et al., 1994; Winner et al., 2000). Recently, *Ureaplasma* sp., adherence showed that adherence is realized but, this is controversial (Robertson, 1991; Saada et al., 1991; Smith et al., 1994).

Adherence is a virulence factor for lung infection. The aim of this study was to evaluate a type II respiratory epithelial cell line (A549 cells) as a model to study the *Ureaplasma* sp. of human adherence by transmission and scanning microscopy which had not been performed before, and to evaluate the variability of adhesion of some clinical samples of *U. parvum and U. urealyticum* serotype 5 isolated from premature newborns and how MB antigen could participate as a possible adhesin.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Reference strains, *U. parvum* serovar 3 and *U. urealyticum* serovar 8 were obtained directly from the American Type Culture Collection (strains referred after as *U. parvum* ATCC700970 and *U. urealyticum* ATCC27618, respectively) (Kang et al., 2000) and also 5 clinical strains (3 strains *U. parvum* serotype 3 and 2 strains *U. urealyticum* serotype 5), isolated from premature newborn with low birth weight (≤ 2,500 g) and/ or < 36 weeks gestational, were obtained from endotracheal specimens from 1 to 7 years of age. Reference strains and clinical isolates were grown in 5 ml of 10B broth (Shepard, 1983) and incubated at 37°C/ 18 h (1×10^5 UCC/ml, Log phase). All ureaplasmas strains were transferred to 200 µl of culture and frozen to - 70°C before adherence assays. Two different strains were used like adherence control; *Escherichia coli* 2348/69, negative urease microorganism (localized adherence on Hep-2 cells), and *Proteus* sp. positive urease microorganism (clinical strain). Both strains were grown in BHI broth at 37°C/ 18 h. For the adherence assays cultures were centrifuged at 2,500 rpm for 5 min and pellets were washed three times with sterile phosphate-buffered saline (PBS). The pellets were mixed with PBS and diluted at 1.5×10^8 UFC/ml (0.5 MacFarland tube) concentration.

A549 human lung cells culture

A549 cells were obtained from the American Type Culture Collection, Rockville, Md. (CCL-185); this cell line was grown in Dulbecco Modified Eagle-Earle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone Inc., Logan, Utah) with and without antibiotics. A549 cells were incubated at 37°C in 5% CO_2.

Polyclonal and monoclonal antibodies

U. parvum serotype 3 and *U. urealyticum* serotype 8 were grown in 1000 ml of 10B broth (Shepard, 1983) and incubated at 37°C/ 48 h. When we observed a dark red color, this culture was centrifuged at 16,000 rpm for 2 h, the supernatant was removed careful and transferred to an Eppedorff tube, and this pellet was washed three times with sterile PBS (centrifuged at 16,000 rpm for 30 min). The ureaplasma's whole protein was suspended with PBS and diluted at 40 µg/500 ul (to change by 80 µg/ml) protein concentration. Rabbits were injected subcutaneously with Complete Freund's Adjuvant (CFA, Sigma F-5506) and ureaplasma's whole protein (Inoculum's first day), and after 15 days same conditions previously mentioned, the rabbits were immunized with Incomplete Freund's Adjuvant (IFA) and ureaplasma's whole protein. 30 to 32 days, the rabbits were injected subcutaneously with ureaplasma's whole protein and saline solution. At 39 day, 100 ml of blood was drawn from each rabbit. At least 40 ml of serum was then obtained, which was then utilized at a dilution from 1:1,000 to 1:10,000. MAbs 10C6.6 directed to *U. parvum* serotype 3 were obtained of the Multipurpose Arthritis Center at the University of Alabama at Birmingham, previously described (Watson et al., 1988).

Adherence assay

We used assay condition as a previous report (Smith et al., 1994) with the following changes: A549 cells were washed with PBS pre-incubated at 37°C and incubated with PBS/BSA 3% at 37°C in 5% CO_2 for 30 min, and A549 cells were washed with PBS pre-incubated at 37°C before adherence assays. Ureaplasmas inoculum of 4 ml of 10B culture with 1×10^5 UCC/ml was seeded onto six-well plastic plates (with different confluence 100%, 70% and 2.5 $\times 10^5$ cells per well) and incubated for 1, 2 and 3 h with 10B medium, DMEM-10% FBS, A549 cells. The culture of cells A549 plus *E. coli* 2349/68 and A549 plus *Proteus* sp. were used as controls. Adherence of *Ureaplasma* sp. (human) was evaluated by a colorimetric method and electron microscopy. All studies were performed in triplicate.

Adherence colorimetric assay

Adherence of *Ureaplasma* sp. reference and clinical strains of eukaryotic cell monolayers were quantified, using the Bertholet assay to monitor ammonia produced from urea by ureaplasma urease, just like previous reports (Smith et al., 1994; Thirkell et al., 1989).

Adherence abolitions

Adherence was abolished by pre-treatment of ureaplasmas with A549 cell extracts (1 mg/ml) and inhibited to different concentration by pretreatment of the ureaplasmas with N-acetylneuraminic acid (0.15 U/ml), glucose (100 mM), trypsine (250 µg/ml y 500 µg/ml), specific anti-sera (1:200, 1:500, 1:3000 and 1:5000 dilution) and monoclonal antibodies 10C6.6 (1:1000, 1:5000 and 1:10,000 dilution). They were incubated for 30 min at 20°C before abolitions assay. All studies were performed in triplicate.

Transmission electron microscopy

Cultures of cells A549 controls or from *Ureaplasma* sp. (human) adherence assays were fixed with 2.5% glutaraldehyde in 0.1 M, pH 7.2 cacodilate buffer at 37°C, and post-fixed with 1% osmium tetroxide in the same buffer. They were dehydrated by crescent concentrations of ethanol, and embedded in plastic resins; after polymerization at 60°C, longitudinal thin sections were obtained. Once contrasted with lead citrate and uranyl acetate, the thin sections were observed in a JEOL 100-SX transmission electron microscope (Chavez-Munguia et al., 1997; Dykstra, 1993).

Scanning electron microscopy

Plastic circles with the fixed samples were obtained from the culture dishes, dehydrated in ethanol, critical-point dried from CO_2 in a Samdri Tousimis apparatus and gold-coated in an ion sputtering device (JEOL JFC-100) as described previously (Chavez-Munguia et al., 1997; Dykstra, 1993). All samples were analyzed in a JEOL JSM-35 C scanning electron microscope.

Statistical analyses

Mann-Whitney U test was used to evaluate significance in the adherence inhibition and adherence assays, and differences were considered significant at the $P < 0.05$ (INSTAT version 2.0 Software).

RESULTS

Adherence assay conditions

First experiments were performed under the same conditions reported by Smith et al. (1994); however, adherence was not obtained. New assays were designed for A549 cell line, considering standardized conditions: cellular confluence, bacterial incubation time and Bertholet method (Smith et al., 1994).

A549 cell confluence of 2.5×10^5 cell/well infected with ureaplasmas showed the highest adherence (OD600 = 1.1) compared with 100% of A549 cell confluence, which showed an absorbance of ≤ 0.4 very similar to negative controls and adherence controls (*E. coli* and *Proteus*) (Figure 1). These assays were evaluated; with different confluences 100, 70% and 2.5×10^5 cell/well (40% approximately). Non-specific binding of ureaplasmas was observed in plastic ($P = 0.021$).

Adherence effect on A549 cell line of ureaplasmas at different times (1, 2 and 3 h) with 2.5×10^5 cell/well was done three times The optical density was decreased when the incubation time increased at 2 and 3 h (Figure 2).

Adherence abolition

A549 cell confluences of 2.5×10^5 cell/well and incubation time of 1 h were used for all abolition assays and they were evaluated by Bertholet method.

Pre-incubation of ureaplasmas with homologous polyclonal antibodies reduced the adherence to 44, 60, 88 and 91% (dilutions; 1:5000, 1:3000, 1:500 and 1:200 respectively) of the positive adherence control level. With 1:200 dilutions, the adherence was significantless ($P < 0.05$) than other dilution ($P > 0.05$) (Table 1).

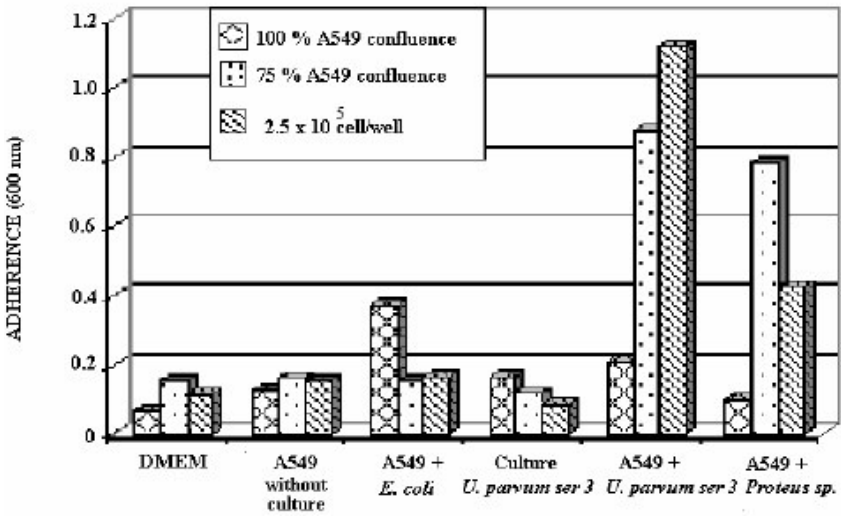


Figure 1. Adherence of *Ureaplasma parvum* serotype 3 to A549 cells with different confluences. The confluences used in adherence assays were 100%, 70% and 2.5 × 10^5 cell/well. *E. coli* 2348/69 was used like control positive of adherence but negative urease and *Proteus* sp. positive control of adherence but positive urease. All assays were performed in triplicate.

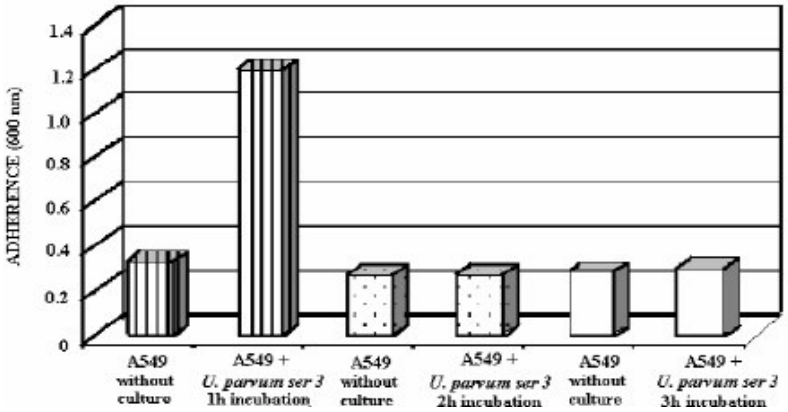


Figure 2. Adherence kinetic of *Ureaplasma parvum* serotype 3 to A549 cell. The adherence was done at different times 1, 2 and 3 h. All assays were performed in triplicate.

The specific binding of ureaplasmas to A549 cells was confirmed by pre-treatment of ureaplasmas with A549 cells extracts ($P < 0.05$), whose adherence was reduced to 66% compared with positive control (Table 1). Both assays demonstrate an antigen-specific recognition from *U. parvum and U. urealyticum* at the A549 cell.

Pre-treatment of A549 cells with glucose and neuraminidase abolished adherence to 61 and 70% respectively, suggesting that a high proportion of the cell receptors terminate in sialic acid in these cells. Both of them were statistical significant ($P < 0.05$) (Table 1).

Pre-incubation of ureaplasmas with trypsin at 250 and 500 µg/ml reduced the adherence to 26 and 55% respectively (Table 1). This abolition assay indicates that some of the *Ureaplasma*'s adhesions appear to be protease-sensitive.

Adherence abolition with monoclonal antibody 10C6.6 against multi-banded antigen (MB 10C6.6) of *U. parvum* reduced attachment to 25, 48 and 63% (1:10,000, 1:5000 and 1:1000 respectively) in a dose-dependent manner, suggesting ($P < 0.05$) that MB antigen could be an adhesin (Table1).

Scanning electron microscopy

Adherence changes correlated with damage observed on electron microscopy. Figure 3a showed an A549 cell

Table 1. Statistic analysis of *Ureaplasma parvum* adherence abolition to A549 cell.

Adherence Inhibitors	% Abolition adherence	P
A549+*U. parvum*	0	
Polyclonal Ab anti *U. parvum* 1:5000	44	0.513
Polyclonal Ab anti *U. parvum* 1:3000	60	0.513
Polyclonal Ab anti *U. parvum* 1:500	88	0.513
Polyclonal Ab anti *U. parvum* 1:200	91	< 0.05 *
A549 extract (1mg/ml)	66	< 0.05 *
Neuromanidase	70	< 0.05 *
Glucose 100 mM	61	< 0.05 *
Trypsin 250 µg/ml	26	0.275
Trypsin 500 µg/ml	55	< 0.05 *
Monoclonal Ab 10C6.6 dil. 1:10,000	25	0.275
Monoclonal Ab 10C6.6 dil. 1:5,000	48	< 0.05 *
Monoclonal Ab 10C6.6 dil. 1:1000	63	< 0.05 *

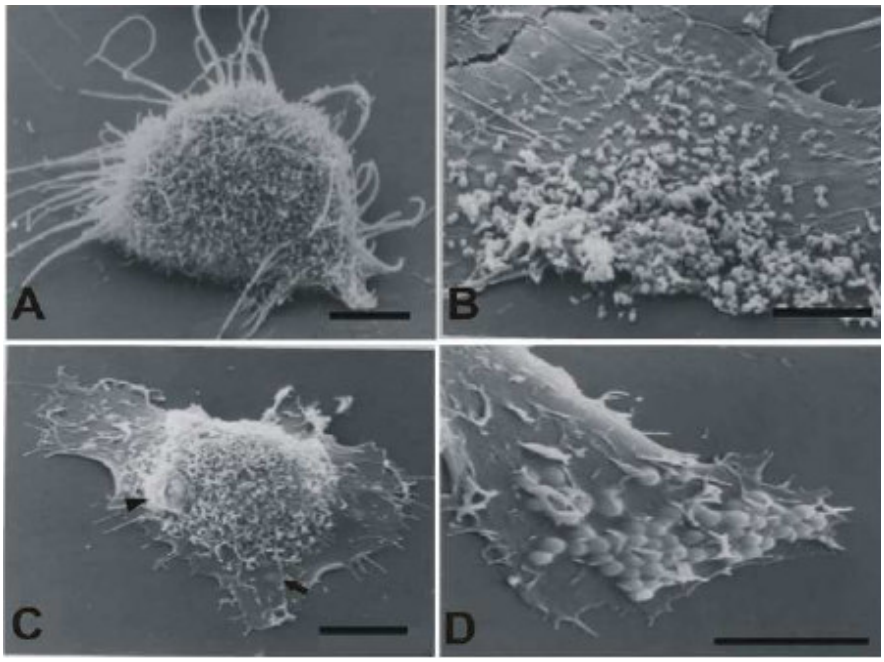


Figure 3. Scanning electron micrograph. (A) Uninfected A549 cell. (B) A549 cell showed several ureaplasmas adhered, the cell looks a plane form or distending and without microvillus. (C) A micro-colony of ureaplasmas was signaling with a head arrow and one ureaplasma covered by membrane cellular was signaling with an arrow. (D) Close up of A549 cell showed several ureaplasmas adhered, someone look covered by cellular- like membrane figure C. Size of the bar corresponding to 1 µm.

uninfected as negative control; it looks whole with its microvillus and oval shape. Loss of microvillus was observed in an A549 cell infected with ureaplasmas (Figure 3B). Ureaplasmas micro-colonies or clumps were observed on the A549 cell (Figure 3c). An A549 cell infected with ureaplasmas showed someone covered apparently by cellular membrane (Figure 3D). An amplification of an A549 cell showed ureaplasma with structures similar as a pedestal (Figure 4a). Figure 4b showed an A549 cell infected with *E. coli*, it looks its microvillus and a little damage; it was used like a control adherence and different size compared with *U. parvum* serotype 3 and other bacteria.

An adherence kinetic was made at 15, 30, 45 min, 1 and 2 h. Scanning electron microscopy of *U. parvum* serotype 3 was observed bound to A549 cell at 15, 30 and 45 min of incubation period time with a little damage (Figures 5a, b and c). Severe damage was observed

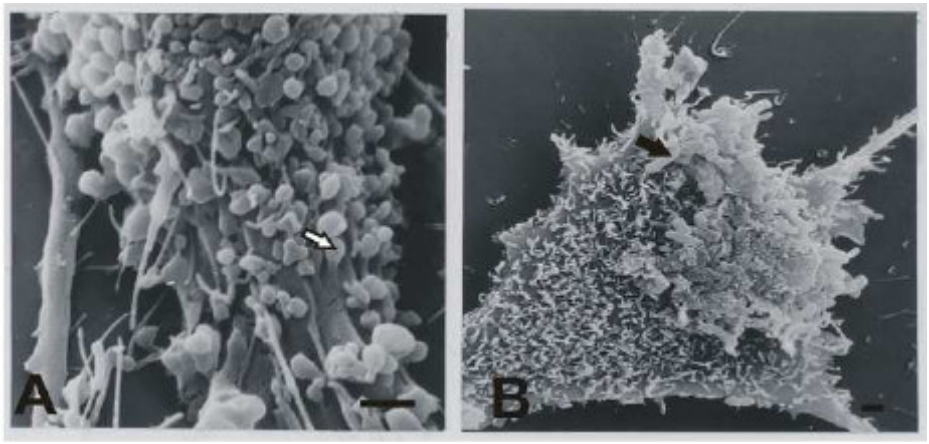


Figure 4. Amplification of A549 cells infected with *U. parvum* serotype 3 (A) and *E .coli* (B). One ureaplasma is on a structure like pedestal, it is signaling by a white arrow (A). The black arrow signaling an *E. coli* bacillus; we can differentiate the size between microorganisms. Size of the bar corresponding to 1 µm.

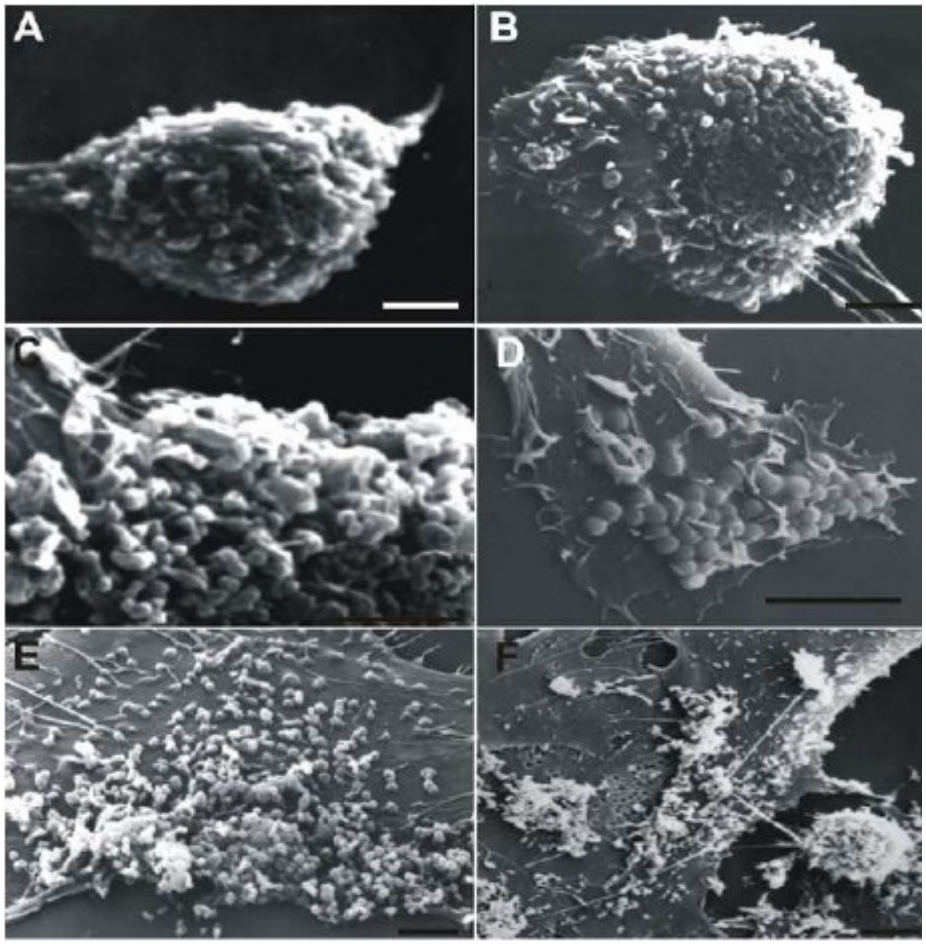


Figure 5. Kinetic of adherence of *Ureaplasma parvum* serotype 3 to A549 cell. (A) Incubation at 15 min showed a little damage. (B) Incubation at 30 min, and (C) Incubation at 45 min showed several ureaplasmas adhered and a little damage. (D) A549 showed several ureaplasmas and it presented severe damage at 1 h of incubation. (E) Incubation at 2 h and (F) at 3 h showed a little ureaplasmas adhered but with a severe damage. Size of the bar corresponding to 1 µm.

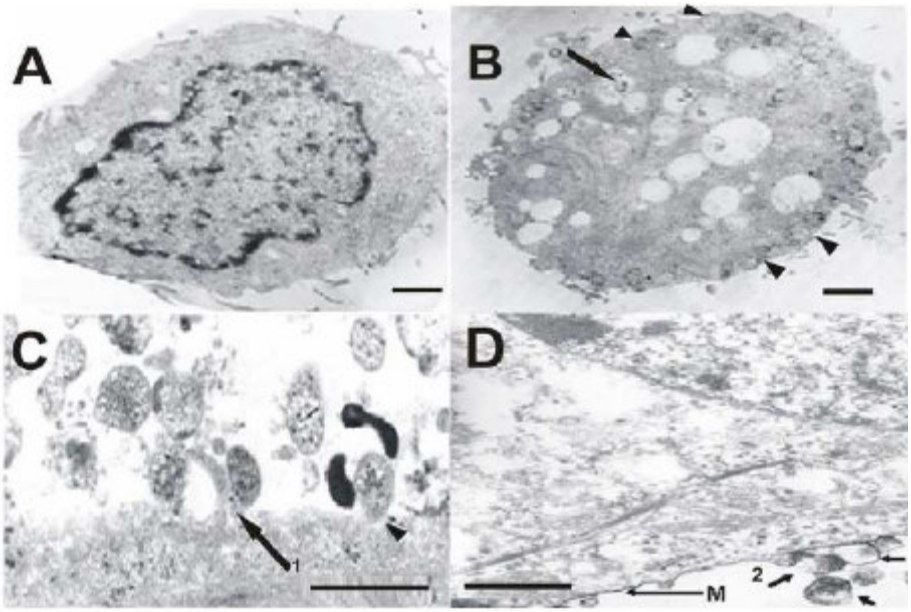


Figure 6. Transmission electron micrograph. (A) Longitudinal cut of uninfected A549 cell. (B) Longitudinal cut of A549 cell infected with ureaplasmas; arrows signaling ureaplasmas into the cell and into some vacuoles. (C) Close up of border A549 cell plus ureaplasmas, the arrow number 1 showed one ureaplasma entered to the cell. (D) Close up of border A549 cell plus ureaplasmas dyeing with red ruthenium and it highlight membranes of the A549 cell and *U. parvum* serotype 3, the arrow number 2 showed one ureaplasma with an structure like a "tip".

after 1 h of incubation (Figure 5d). A few ureaplasmas were observed after 2 or 3 h of incubation (Figures 5e and f).

Transmission electron microscopy

Figure 6A showed a longitudinal cut of an A549 cell uninfected as a negative control. We observed several vacuoles forming into the A549 cell infected with *U. parvum* serotype 3 and someone too near to the border of the cell (Figure 6b). An amplification of the A549 border cell showed some ureaplasmas adhered and one showed a structure like a "Tip". It could be in an enter process (Figure 6c). Similar results were observed when we used red ruthenium, which highlighted membrane of the ureaplasmas and A549 cell; adherence of *U. parvum* serotype 3 to A549 cell and a structure forming like a "Tip" (Figure 6d).

Adherence of *Ureaplasma* sp. clinical strains

Five clinical isolates were evaluated by Bertholet assay and scanning electron microscopy. *U. parvum* serotype 3 and *U. urealyticum* serotype 8 were used like controls (Figures 7a and b).

Bertholet assay did not show statistical significance between *U. parvum* and *U. urealyticum* (Figure 7b). Clinical strains were less adherent than reference strains and they were statistical significant ($P \leq 0.05$) (Figure 7b). Scanning electron microscopy showed similar images among all strains; A549 cells infected with clinical ureaplasma presented severe damage like a distending form or stretching and without microvillus (data not shown).

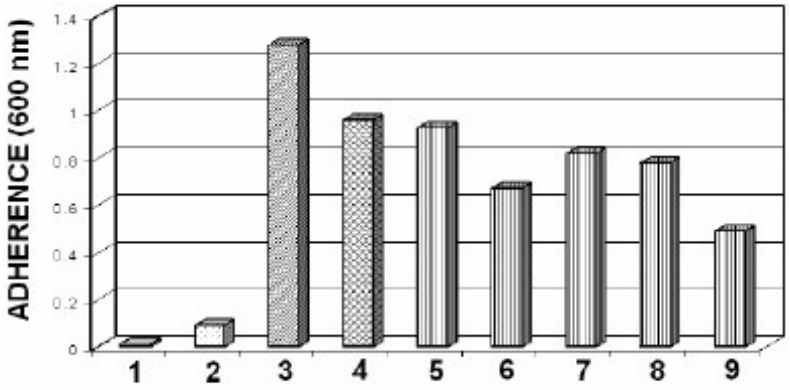
DISCUSSION

Ureaplasma sp. as human pathogen of adults and neonatal infections is supported by several reports (Cassell, 1993; Sanchez and Regan, 1988; Deguchi et al., 2004; Sanchez and Regan, 1990; Yoder and Albertine, 2008). However, there are many questions about its pathogenicity because there is a high prevalence in healthy persons (Cassell, 1993; Waites et al., 2005; Duncan et al., 1992).

Localization and attachment on host cell surface is important as virulence factor to *Ureaplasma* sp. to colonize and subsequently produce pathological lesions (Waites et al., 2005; Tarrant et al., 2009; Oue et al., 2009).

Previous reports have mentioned ureaplasma adherence to a variety of human cells including erythrocytes (Saada et al., 1991), spermatozoa (Basolo et al., 1984; Diaz-Garcia et al., 2006) and urethral epithelial cells (Smith et al., 1994). Principal goal of this work was to prove ureaplasma's adherence to human respiratory cells

A



B

Ureaplasma sp.	*U. parvum* ser 3 (biovar 1) vs	*U. urealyticum* ser 8 (biovar 2) vs
U. urealyticum ser 8	P=0.275	
Sample 11	P=0.127	P=0.827
U. urealyticum ser 5		
Sample 61	P=0.05*	P=0.275
U. parvum ser 3		
Sample 74C	P=0.05*	P=0.513
U. urealyticum ser 5		
Sample 85	P=0.127	P=0.275
U. parvum ser 3		
Sample 121A	P=0.05*	P=0.05*
U. parvum ser 3		

Figure 7. Adherence of reference and clinical ureaplasmas strain to A549 cells. (A) Columns: 1, *Ureaplasma parvum* serotype 3 alone; 2, A549 cell alone; 3, A549 cells plus *Ureaplasma parvum* serotype 3; 4, A549 cells plus *Ureaplasma urealyticum* serotype 8. Columns 5 to 9, A549 cells plus clinical strains (61, 85 and 121A clinical strains corresponding to *Ureaplasma parvum* serotype 3; and 11 and 74C clinical strains belong to *Ureaplasma urealyticum* serotype 5). (B) Table of statistic analysis of adherence of reference and clinical ureaplasmas strain to A549 cells.

using clinical strains isolated from preterm with someone respiratory disease.

A549 cell line was a good adherence and invasive model for *Streptoccocus pneumoniae* (Bérube et al., 1999; Talbot et al., 1996). We evaluated the adherence of *Ureaplasma* sp. (human) to A549 cell line by Bertholet assay and electron microscopy.

A previous report on adherence of *U. urealyticum* serovar 8 to eukaryotic cell monolayers was quantified, using Bertholet assay to monitor ammonia produced from urea by ureaplasma urease; they used HeLa cells with 100% of confluence and 2×10^5 UCC/ml at 1 h of incubation and observed 20% approximately ureaplasmas adhered to monolayers. Using the same conditions from a previous report (Smith et al., 1994), we could not observe adherence. But A549 cells to 2.5×10^5 cell/well and 4×10^5 UCC/ml with 1 h of incubation, we had better adherence effects.

Pathogenic bacteria have selected receptors on the host cells and specific ones were recognized. Adherence patterns as *E. coli* (Donnerberg et al., 1997), *Proteus* sp. on the A549 cells showed minimal adherence than *U. parvum* serotype 3 by Bertholet assay. *U. parvum* serotype 3 is a respiratory tract pathogen and the adherence results compared with *Proteus* sp. suggested that A549 line cell is a selective good model adherence of *Ureaplasma* sp. human strains isolated of the tract respiratory from newborn.

Saada et al. (1991) evaluated the adherence of *U. urealyticum* serotype 8 to human erythrocytes using [35S] methionine labeling; they observed decreased adherence when the incubation time was increased. These results agreed when the time incubation was 2 and 3 h with Bertholet method; the optical density decreased too.

Abolition adherence assay monitored by Bertholet assay showed that specific receptors could be involved on ureaplasmas adherence to A549 cells when there is pre-incubation of ureaplasmas with A549 cells extracts; this abolition assay supports the specific adherence to A549 cells and it was similar to previous report with HeLa cells extracts (Smith et al., 1994).

Adherence of *U. parvum* serotype 3 to A549 cells was reduced being dose-dependent, with polyclonal antiserum; this suggested that a specific-antigen was recognized from *U. parvum* serotype 3 to A549 cell.

Bacteria-host relationships involve many interactions and they are biologically important. Attachment to carbohydrates is an important interaction with host cell to bacteria protein, as lectins and adherence abolition assay with carbohydrates and neuraminidase are used to block receptors with mono or oligosaccharides (Kahane and Jacobs, 1995). Pretreatment of A549 cells with neuraminidase or ureaplasmas suspensions with glucose resulted in statistically significant reduction in adherence to 78%; similar value was reported by Saada et al. (1991). Both results were higher than 13% reported by Smith et al. (1994). These results confirmed the importance of sialic acid residues in the recognition cellular process and agreed with previous ureaplasma reports. They are also similar to others observed with *Mycoplasma* sp. and *M. pneumoniae* (Waites et al., 2005; Razin et al., 1998; Thirkell et al., 1989).

A dose-dependent manner adherence reduced more to 50% with pre-treatment of ureaplasmas suspension with trypsin, suggesting that they had adhesins proteinaceous moiety; this is similar to previous reports (Saada et al., 1991; Smith et al., 1994).

The lack of a cell wall or other appendix in mycoplasmas may facilitate the direct contact of the mycoplasma's membrane to its eukaryotic host. Like other mycoplasmas they possess variable surface proteins (Kahane and Jacobs, 1995; Baseman et al., 1997; Waites and Talkington, 2004; Watson et al., 1988; Shimizu et al., 2008). The multiple banded (MB) protein shows a striking variability of its molecular weight. This is caused by changes of the number of C-terminal repeating units (Zheng et al., 1995; Chavez-Munguia et al., 1997). *Mycoplasma bovis* has a membrane protein (pMB67) with spontaneous changes and variable surface lipoproteins (Vsps) that play an important role like adherence mediators (Beehrens et al., 1996; Sachse et al., 2000). The MB antigen of *Ureaplasma* sp. was identified as highly immunogenic outer membrane protein of serum human in clinical sample (Zheng et al., 1995; Monecke et al., 2003). Adherence abolition with mAbs 10C6 against multi-banded antigen (MB) reduced attachment of dose-dependent manner, suggesting that the MB antigen could be an adhesin. The size of *Ureaplasma* sp. is 330 nm; that is the principal reason why ureaplasmas are difficult to see with light microscopy (Duncan et al., 1992). A methodology for the ureaplasmas adherence studies needs an easy technique to evaluate attachment. Bertholet assay facilitated our work as a previous report (Smith et al., 1994). However; we used electron microscopy techniques as a support of adherence mediated by colorimetric method.

The scanning electron microscopy showed that the A549 cells lost all microvillus, and they looked like a plane form or stretched when *Ureaplasma* sp. was adhered to the cell. This damage is similar to attachment and effacing (A/E) produced by *E. coli* EPEC (Donnenberg et al., 1997).

Several pathogenic bacteria produced severe changes altering the host cytoskeleton architecture as manifested by dramatic rearrangement of microtubule and microfilament proteins (Rosenshine et al., 1992; Scharamm and Wyrick, 1995; Young et al., 1992). This damage has been observed with *Mycoplasma penetrans* (Giron et al., 1996). An amplification of A549 cell showed one ureaplasma on structure like a pedestal.

Micro-colonies of ureaplasmas were observed on A549 cell. It is known that *Ureaplasma* sp. cultured in 10B broth or agar (A7 or A8) would produce "tiny" colonies and because it reproduces by binary fusion (Shepard, 1983). Micro-colonies have been observed with other mycoplasmas too. By Giemsa staining micro-colonies of *M. penetrans* on Hep-2 cells were observed and confirmed by immunofluorescence microscopy (Giron et al., 1996).

Kinetic adherence of *U. parvum* serotype 3 to A549 cell by scanning electron microscopy showed ureaplasmas binding to A549 cell at 15 min of incubation. After 2 h a few ureaplasmas were adhered on the cells and a severe damage was observed.

Ureaplasma sp. has been isolated from cord blood and there have been numerous reports on their isolation from bloodstream of neonates and young infants, sometimes in associations with meningitis. It can be isolated from amnion liquid during early 20 weeks of gestation and without labor and intact membrane, indicating that *Ureaplasma* sp. could be an invasive microorganism (Brus and Van Warde, 1991; Cassell et al., 1991; Waites et al., 2005). Our scanning electron microscopy results showed ureaplasmas covered by cellular membrane (Figure 3D), suggesting that *U. parvum* serotype 3 could enter into the A549 cell.

A previous report showed *Mycoplasma genitalium* intracellular and Vero cells, with several vacuoles at 72 h after infection (Mernaugh et al., 1993; Jennsen et al., 1994; Readdy et al., 1996). *Ureaplasma* sp. that gets into A549 cells adherence was observed at less than 1 h, as *Mycoplasma gallisepticum* which has the capacity to invade host cell at 20 min after infection and to survive around 48 h into the cell (Jennsen et al., 1994; Winner et al., 2000); but by transmission electron microscopy, we observed several vacuoles in A549 cells infected with ureaplasmas. Immunoperoxidase staining assay showed that *U. urealyticum* T960 (serotype 8) attached to 3T6

cells and produced vacuolization of cytoplasm (Kotani and McGarrity, 1986).

We observed a structure like a “Tip” in some ureaplasmas by transmission electron microscopy. This structure has been reported in *M. pneumoniae* and other mycoplasmas (Razin et al., 1998; Razin, 1999; Krause et al., 1983; Krause et al., 1982; Razin and Jacobs, 1992).

Membranes of ureaplasmas dyed with ruthenium red highlight a structure like a “Tip” organelle was observed in *U. parvum* serotype 3. Adhesins have been found by immunoelectron microscopy to cluster at the surface of the “Tip” organelle. The high concentration of the adhesins at the “Tip” is apparently responsible for the remarkable strength of attachment of the mycoplasmas to erythrocytes through the attachment “Tip” (Kahane and Jacobs, 1995; Baseman et al., 1997; Baseman and Tully, 1997). “Tip” organelle plays an important role when entering the host cell (Razin et al., 1998; Razin, 1999; Razin and Jacobs, 1992). A report demonstrated that *Ureaplasma* sp. human has an extramembranous layer of polyanions and glucosyl-like residues that bound with red ruthenium, providing cytochemical evidences (Robertson and Smook, 1976). And this helps to identify ureaplasmas by transmission electron microscopy results.

We did not find different adherence between clinical strains of biovar 1 or biovar 2; also, there were no differences about adherence and damage among reference strains and clinical strains (biotypes 1 and 2).

Scanning and transmission electron microscopy data support the hypothesis of invasiveness, for ureaplasmas is likely not related to one or a few particular serotypes. Many serotypes have antigen variability and host factors could be important determinants for ureaplasma invasive infections (Brus and Van Warde, 1991; Cassell et al., 1991; Waites et al., 2005). The damage on A549 cell showed a plane form of the cell and loss of microvillus, similar as other pathogenic microorganisms as *E. coli*, *Campylobacter* sp., *Shigella* sp., and *Haemophilus ducreyi*, using other cellular lines (Donnenberg et al., 1997).

The host cell membrane is also vulnerable to toxic materials released by the adhering mycoplasmas. Although toxins have not been associated with mycoplasmas, the production of cytotoxic metabolites and the activity of cytolytic enzymes were well established (Rottem and Naot, 1998).

Damage observed could be due to urease activity; it has been suggested like a potential virulence factor of *Ureaplasma* sp. and the ammonium has a cytotoxic effect on the cell host (Waites et al., 2005). Previous reports have suggested that some different from urogenital tract producing urea (Jernigan, 1983). Urea production in tissues could explain why ureaplasmas can colonize tissues of the respiratory tract system. Small but essential amounts of urea growth factors suggest that ureaplasma present in these tissues is different from that in the urogenital system (Kotani and McGarrity, 1986).

Ureaplasma sp. could produce damage by generation of nitric oxide radicals that stimulating release of cytokines or oxygen free radicals which are thought to induce oxidative stress in host cells, resulting in damage of the cell membrane (Waites et al., 2005). The intimate contact of the ureaplasmas with the host cell membrane might result from phospholipids changes or the potent membrane-bound to phospholipases A2 present in *Ureaplasma* sp. (Waites et al., 2005). *U. parvum* has two hemolysins in which hemolysis is mediated by H_2O_2 and may function as a virulence factor, which shows cytotoxic activity (Waites et al., 2005). Stimulation of proinflammatory cytokines (TNF-α, IL-10, IL-6 and IL-8) could be related to damage on the host cell too (Ronald et al., 1997; Viscardi et al., 2002). There are several possible pathways to explain the damage produced by *Ureaplasma* sp. on the A549 cell. More studies are necessary to explain which mechanisms are involved in pathological changes of cell host.

In conclusion, these results are the first to show the binding of *Ureaplasma* sp. human to a cellular lineA549 cell. Our work suggests that A549 cellular line is a good model for the study of adherence of *Ureaplasma* sp. We used two ways to show adherence to host cell. The first step showed that the urease test (Bertholet assay) is a good indicator of colonization. Furthermore, the Bertholet data suggested that the ureaplasma’s adhesions have proteinaceous nature, and the adherence inhibition assays by neurominidase and glucose showed that residuals of sialic acid could be related with receptor. It is possible that the multi-banded antigen may act like an adhesin.

The electronic microscopy was the second step to show that *Ureaplasma* sp. binds to the cell, producing evident morphological changes, and can even develop localized micro colonies. Clinical isolated of *U. parvum* and *U. urealyticum* showed similar adherence, suggesting that there was no differential pathogenicity between biovar 1 and biovar 2 related with adherence, and invasive to the host cell.

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Short Communication

A new alternative to treat swine influenza A virus infection: extracts from *Terminalia chebula* Retz.

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Currently, a pandemic swine influenza A virus infection causes a huge negative impact on human beings all over the world. However, the methods of treatment are not satisfactory, therefore it is urgent for us to set up new theory and practice to fight against the intractable virus. *Terminalia chebula Retz*, as a kind of traditional Chinese medicine, widely distributes and has multiple pharmacological effects. Evidences in laboratory and clinic practice confirm us to the potential of *Terminalia chebula Retz* inhibiting influenza A virus infection. We thus hypothesize that acetone extracts (tannic acids, A) of *Terminalia chebula Ret* may as a new alternative treat influenza A infection based on holistic concept of traditional Chinese medicine principle.

Key words: Swine influenza A virus, *Terminalia chebula* Retz, hypothesis.

INTRODUCTION

Beginning in March 2009, an outbreak of influenza A (Family Orthomyxoviridae, Genus Influenzavirus A) has become the greatest pandemic disease threat to humankind [Neumann et al., 2009]. The new strain of influenza virus designated Influenza H1N1 2009, is a reassortant of swine, avian and human influenza viruses [Brown, 2000; Olsen, 2002; Webby et al., 2000]. Although the influenza A infection has less mortality, compared with other companions such as HIV-1, Ebola, SARS, the current transmit will not stop until pandemic eventually involve 80 percent of population all over the world according to WHO report.

At present there are only two classes of antiviral drugs are approved to treat against influenza viruses including adamantanes and neuraminidase inhibitors such oseltamivir and zanamivir [Schnitzler and Schnitzler, 2009]. However, the effect of viral chemo-therapy that applies a single compound is limited with side effect such as diarrhea, dizziness or insomnia and this kind of therapy may cause drug resistant. *Terminaliae immaturus*, the fruitlet of *Terminalia chebula Fructus Retz*, which has been given the name of XiQingGuo, mainly distribute in Malaysia, Thailand, India, Pakistan and Yunnan, Tibet, Guangdong, Guangxi province of China [Saleem et al., 2001; Kusirisin et al., 2009; Nariya et al., 2009; Cai et al., 2008].

Its active components which refer to acetone extracts (TA) contain digallic acid, chebulinic acid, chebulagic acid, terchebin and gallic acid with multiple pharmacologic actions such as anti-virus, anti-oxidation, cardiotonic action, antibacterial effect, anti-anaphylaxis, anti-tumor growth, [Saleem et al., 2001; Kusirisin et al., 2009; Nariya et al., 2009; Cai et al., 2008; Feng et al., 2008; Kusirisin et al., 2009; Murali et al., 2007; Lee et al., 2007; Lee et al., 2005; Juang et al., 2004; Saleem et al., 2002; Malekzadeh et al., 2001; Sato et al., 1997; Verma and Raychaudhuri, 1970; Cheng et al., 2003]. Those biological activities are often in connec-tion with the high contents of tannic acids [Kim et al., 2009].

THE HYPOTHESIS

To sum up the above statements, it is wise for us to get help from nature and from ethnopharmacological record

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or traditional medicine principle handed down from ancient times all over the world to battle with current pandemic influenza A virus. We will use acetone to extract from *Terminalia chebula* Retz to get a tannic acid mixture to inhibit pandemic influenza A infection.

The theory of our hypothesis

As we all know the influenza virus subtypes have a wide host range from avian to mammals including hens, pigs, horses and dogs. The genomes of influenza A virus are segmented and negative-sense RNAs which can be translated into 11 functional proteins [Schnitzler and Schnitzler, 2009; Fitzgerald, 2009; Gatherer, 2009]. The main infective proteins contain the surface glycoproteins haemagglutinin (HA) binding virus to its purposive target cell, neuraminidase (NA) facilitating virus release from infected cells and virulence factors NS1 antagonizing host interferon. There are 16 serotypes of haemagglutinin, and 9 serotypes of neuraminidase in total and according to these differences, the virus are classified. Sialic acid is the receptor for haemagglutinin and sialyltransferases is expressed in human mucosal and respiretory tissues resulting in N-glycans with α-2,6 linked sialic acids. However in avian tissues, another structure of sialic acid is expressed and N-glycans are linked with α-2,3-sialic acid. These different structures lead to virus specifying hosts, e.g. avian viruses mainly infect bird species. Like other RNA viruses, e.g. hepatitis C virus and HIV, influenza virus is characterized by genetic variability, resulting in frequent mutations and reassortment on account of influenza virus RNA polymerase lack of proofreading abilities [Reid and Taubenberger, 2003]. Thus the genetic material of current pandemic influenza A H1N1 virus is a combination of viruses that have infected pigs, birds and humans respecting swine tissues express both forms of sialic acid and can be coinfected with human and avian viruses [Olsen, 2002].

It is reported that the rate of oseltamivir-resistant human seasonal H1N1 in the USA has increased to 98.5% [Poland et al., 2009], up from 10% in the last year. Although the instances of appearance of current pandemic influenza A H1N1 virus resistant to oseltamivir in Denmark, Japan and Hong Kong are only sporadic cases, the outbreak of large scale cases will happen inevitably if no effective actions are taken on. In addition, the supply with antiviral drugs is not sufficient for a pandemic and the cost of drugs is too expensive to afford especially for the developing countries.

Another alternative to defend virus infection is to appeal to our own immune system. On way the infected host immune system counters viral infection is with interferon, one of the principle functions of which is to interfere with viral multiplication without affecting the host cell itself.

Interferons, a group of small proteins, produced by virus-infected cells, react with plasma or nuclear membrane receptors of uninfected cells to induce synthesis of antiviral proteins. Antiviral proteins are possessed of multiple functions to prevent further infection of virus including blocking initiation of virus protein synthesis, inhibiting virus polypeptide elongation and destroying viral mRNA before translation. Even through there do exists problems partially due to short term effectiveness of interferon, it typically plays a positive role against acute and short term virus infection especially influenza. Therefore any measures that could improve our immune system response to secret sufficient interferon are suggested and approved.

In laboratory test, the water decoction of *Fructus terminaliae immaturus* exhibit obvious antibacterial effect inhibiting both Gram positive bacterium including *Staphylococcus aureus, Pneumococcus, Streptococcus hemolyticus, Bacillus diphtheriae* and Gram negative bacterium including *Escherichia coli, Bacillus dysenteriae, Pseudomonas aeruginosa, Bacillus proteus, Bacillus tphpi, Bacillus typhi murium, Helicobacter pylori.* The alcoholic extracts of *Terminalia chebula Retz* demonstrated significant anti-virus effect in 2.2.15 cell line infected with HBV, and the extracts with a certain concentration of hydrochloric acid have a more powerful inhibitory effect on bacterium and fungus growth. Chinese patent medicines containing *Terminalia chebula Retz* can inhibit acyclovir-resistant herpes simplex virus I *in vitro and in vivo*. In addition, in animal research, *Terminalia chebula Retz* was used to treat endotoxin sepsis shock because *Terminalia chebula Retz* can regulate immune response by making host cells release interferon and TNF as well as activating monocyte/macrophage system [Cai et al., 2008; Feng et al., 2008].

Feasibility and prospects

We will use acetone to extract from *Terminalia chebula* Retz to get a tannic acid mixture without purification further. Base on traditional Chinese medicine and Chinese materia medica principle, we would like to emphasize the holistic concept, which means that every thing should be considered as a whole and there is synergistic effect of each component of a plant. The synergy is not the simple sum of several components, but rather mutually reinforcing role, with a single component can not be achieved. The aim of extraction is not intended to find a single anti-viral compound, but rather to remove impurities to enhance antiviral activity of *Terminalia chebula Retz* according to guiding role of holistic concept. The extracts combined with pandemic influenza A virus were inoculated into nonimmune chick embryo, and then the chick embryo and allantoic fluid were observed to evaluate the antiviral effect of the extracts.

RESULTS

Natural medicine of the antiviral and enhancing immunity

including extract or mixture, which may fight against influenza virus through different targets.

CONCLUSION

The acetone extract (tannic acids, TA) of *Terminalia chebula Ret* may be considered as a effective method for human being fighting against pandemic swine influenza A virus on account of its low cost, easy preparation and significant therapeutic action.

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