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

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Review

Plant lectins as alternative tools against bacterial biofilms

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Plant lectins has the ability to interfere in crucial biofilm formation aspects, playing a significant role in evaluation of patients at high and low risk of disease development. However, there is limited studies on the physiological role of lectins on bacteria living in biofilms like caries process. Thus, we aimed to provide a view of lectins biotechnological potential against bacterial biofilms development. Biofilm is a structured bacterial consortium that provides essential compounds for its survivor. This microorganism organization occurs naturally, since this arrangement increases its survival possibility. Bacterial biofilms are related to human health problems and are responsible for many infectious diseases, such as oral diseases, associated with inert surfaces, including medical devices for internal and external use. Thus, lectins are a large group of heterogeneous proteins that exhibit antibacterial activity, as well as ability to interfere with microbial biofilms formation process. The lectins ability to form complexes with microbial glycoconjugates has stimulated its application as probes to the whole cell, as well as its mutants and numerous cellular constituents and metabolites. Thus, the impact of bacterial resistance provided by biofilm formation on human health encourages researches aiming to understand biofilm mechanisms as well as strategies to eradicate or minimize these communities damages.

Key words: Lectins, biofilm, bacterial resistance.

INTRODUCTION

Plant lectins have been used for diagnosis and prevention of various diseases. This is justified by property set translated by the ability to recognize structural elements of organizational surface from pathogens (Cavalcante et al., 2011). However, when it comes to interaction with bacterial biofilms, this strategy has been incipiently exploited (Lopes et al., 2005).

Given this context, this study proposes the following question: what are the potential of biotechnological application of plant lectins in diagnostics and disease

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prevention?

Since lectins, as molecules, have the ability to bind glicoconjugates (crucial function for biofilm formation), these proteins may play a significant role in the evaluation of patients at high and low risk for biofilm associated disease development.

In dentistry, there are few studies related to the use of lectins in the diagnosis and prevention of diseases. Studies on this subject also failed to respond effectively, and the physiological role of lectins are against the caries process. Some authors suggest various proposals regarding to this question and present meaningful data, especially with regard to the actions of lectins of higher plants (Teixeira et al., 2007; Cavalcante et al., 2011; Klafke et al., 2013).

Thus, this study aims to give a view of the biotechnological potential of plant lectins as a tool to deal with bacterial biofilms as a source of bacterial resistance.

MICROBIAL BIOFILMS: CHARACTERISTICS, FORMATION AND FUNCTIONS

Biofilm characteristics

The microbial word observation using different microscopy techniques over the years, has provided accurate researches of the microorganisms arranged in communities sharing nutrients, metabolites, genetic elements and thus, being able to resist even in withstand environment, causing diseases that could be difficult to eliminate. Biofilms have a crucial impact on human health in different ways, since they can be formed in natural environments, medical devices and industrial equipment (Lopez et al., 2010).

The majority of microorganism naturally aggregates and produces a self-produced polysaccharide matrix called a biofilm (McDougald et al., 2012). These communities may be established in a wide variety of surfaces (Abee et al., 2011).

Besides the ability to produce extracellular polymers, cells in communities presents a reduced pattern of growth, as well as up or down regulation of specific genes. The organization of microorganisms in biofilms occurs naturally, since these communities arrangement increases the possibility of survival of these microscopic organisms. Synthesis of extracellular polymeric substances by microorganisms is accepted as a key mechanism to facilitate irreversible cell adhesion to inanimate surfaces in wet environments, thus promoting the development of a biofilm (Beech et al., 2005). The presence of the ‘‘matrix of extracellular polymeric substance’’, which contains polysaccharides, proteins and DNA, whose formation is a consequence of the metabolism of the microbial community is one specific characteristic of bacterial biofilm (Erriu et al., 2013).

These communities also display a particular profile, since they can host different species of microorganisms in an arrangement that allows cooperation instead of competition (Bordi and Bentzmann, 2011). A communication system between bacterial species is responsible for the development and integrity of the biofilm structure. These system synthetize pheromones that allow cell-to-cell communication which induce the biofilm-forming bacteria to react as one against external stress. This Quorum Sensing (QS), chemical communication between bacterial cells, is closely involved both in biofilm formation and in surface motility in pathogens, and whose activation is linked to molecules auto-inducers (AIs) (Aparna and Yadav, 2008; Karatuna and Yagci, 2010).

Furthermore, these microbial societies have their own rules and behavior, including altruism and cooperation, which benefits the group (Shapiro, 1998; Parsek and Greenberg, 2005). Some of these subpopulations can exhibit expertise that is orchestrated by chemical communications (Weigel et al., 2007) providing a singular way of interaction among species, inducing marked changes on symbiotic relationships between their components (Hansen et al., 2007).

Biofilm formation

The knowledge of the molecular basis involved in biofilm development has been updated by improvements in methods for genetic and genomic studies, as well as the development of laboratorial technology, that reveals the processes involved on development, physiology and behavior of microorganisms in this new environment condition. For example, a plethora of systems allows the bacterial identification, appropriated surface anchoring and cell adhesion to form multicellular communities (Bordi and Bentzmann, 2011). Bacterial growth in pure media conditions has been the main approach to perform microbiological culture, from Pasteur’s studies to the present day. These experiments have been used to provide knowledge and understanding of prokaryotic genetic and metabolism, further facilitating pathogens from a variety of diseases isolation and identification of diseases (Costerton et al., 1987).

The term biofilm was introduced with evidence that bacterial behavior associated with surfaces could not be predicted by observations performed in microorganisms cultured in suspension, in their planktonic form. This is a term that describes sessile microbial populations introduced through surveys of biofilms (Jakubovics and Kolenbrander, 2010).

Biofilm formation may be considered a bacterial community protective mechanism against external injury, thus, it seems reasonable that extracellular signals regulate the activation of specific metabolic patterns that trigger its stability. Such signaling may arise from various

external sources, and can be produced and secreted by the bacterial community itself, where molecules named self-inducers accumulate in the extracellular medium with concentrations correlated with population density (Lopez et al., 2010), and may trigger signaling cascades that lead to responses in multicellular bacterial population, when in high concentrations. This mechanism of cell-cell communication (called *quorum sensing*) controls a large amount of processes including those related to biofilm formation (Camilli and Bassler, 2006). Furthermore, each bacterial species has its own apparatus to accomplish adhesion, and contains a different number of antagonic or sinergistic molecules which are cell specific and can be released depending on the situation (Hagan et al., 2010).

The process of biofilm formation (Figure 1) has been extensively described (Costerton et al., 1995; Habash and Reid, 1999; Donlan and Costerton, 2002), and involves few steps: an initial reversible connection of plaktonic cells to a surface followed by a maturation phase. This initial binding involves attractive and repulsive forces between cells and surface, which include electrostatic and hydrophobic interactions, van der Waals and hydrodynamic forces at appropriated temperature (Agarwal et al., 2010). After this surface binding, bacterial cells grow and divide to form dense clusters of cells that characterize the biofilm. This phase is associated with the polysaccharide production by bacterial cells, and become irreversibly adhered to the substrate. Temporally, these microcolonies develop into a mature biofilm, acquiring a typical architecture with projections separated by channels filled with fluid. The final stage (dispersion phase) involves the shutdown of cells or groups of cells from mature biofilm, being considered an essential step in the bacterial spread (Santos et al., 2008; Batoni et al., 2011).

As far as the cell surface hydrophobicity and the presence of fimbriae and flagella is concerned, exopolissacaride production is one of the main factors that influence the rate and degree of microbial cell adhesion on different surfaces and protects against environmental stress and dehydration (Vu et al., 2009). The extracellular material is mostly produced by the biofilm cells forming. It consists of different types of biomolecules, designated as extracellular polymeric substances (EPS), that forms the scaffold for the three-dimensional architecture of the biofilm and is linked to cell adhesion to surfaces and for cohesion in the biofilm (Flemming and Wingender, 2010). Thus, the EPS production has been the subject of several studies to impair formation and maturation of these microbial communities (Murray et al., 2009; Nagorska et al., 2010).

Correlations of biomedical interest

Bacterial biofilms are related to human health problems responsible for many infectious diseases associated with inert surfaces, including medical devices for internal and external use. They could also be formed in water pipes in hospitals, leading to infections after admission (Bordi and Bentzmann, 2011). The relevance of biofilm formation on medical devices, such as catheters or implants, can result in chronic infections difficult to treat (Hall-Stoodley et al., 2004; Donlan, 2008; Hatt and Rather, 2008). Chronic infections with biofilms involvement include periodontitis, cystic fibrosis pneumonia, and others infections associated with indwelling devices such as catheters, heart valves and prostheses (Stewart, 2002).

Confocal microscopy evidenced that live mature biofilms are not single structured layers in a microbial cell surface, but a heterogeneous entities in time and space, constantly changing due to external and internal processes (Donlan and Costerton, 2002). A biofilm may be composed by bacterial or fungal species, or several species of bacterial, fungal and even algae and protozoa (Batoni et al., 2011). Chemical compounds formed by only a micro-organism may also be present in some infectious pathologies, such as in biofilms formed in heart valves infective endocarditis consisting of *Staphylococcus epidermidis* (Butany et al., 2002). Furthermore, infections have been associated with the formation of biofilms on surfaces such as human tooth, skin and urinary tract (Hatt and Rather, 2008). This community organization provides microbial resistance to various antimicrobial, protection from protozoa and host defenses (Matz and Kjelleberg, 2005; Anderson and O'toole, 2008).

It has been recently reported that 95-99% of microorganisms occurs naturally in biofilms arrangements (Nikolaev and Plakunov, 2007). These microbial communities protect their residents not only from oxygen but also from other environmental factors (Paerl and Pinckney, 1996). Bacterial growing in biofilms causes chronic infections (Costerton et al., 2003) which are characterized by persistent inflammation and tissue damage (Bjarnsholt et al., 2009). Chronic infections, including foreign body infections, are 1) persistent despite antibiotic therapy and host innate and adaptive immune system and inflammatory response and 2) in contrast to colonization, is characterized by pathological immune response and disease persistence (Hoiby et al., 2010).

Mechanisms of antimicrobial resistance

Microorganisms belonging to these microbial communities exhibit particular properties, such as tolerance and resistance to different drugs, opsonization and phagocytosis, allowing them to survive in harsh environments and resist t selective pressures (Weitao, 2009). It seems that host immunity is ineffective in "clean" these microcommunities, since evidence shows the inability of phagocytic cells to act (Leid et al., 2002) or

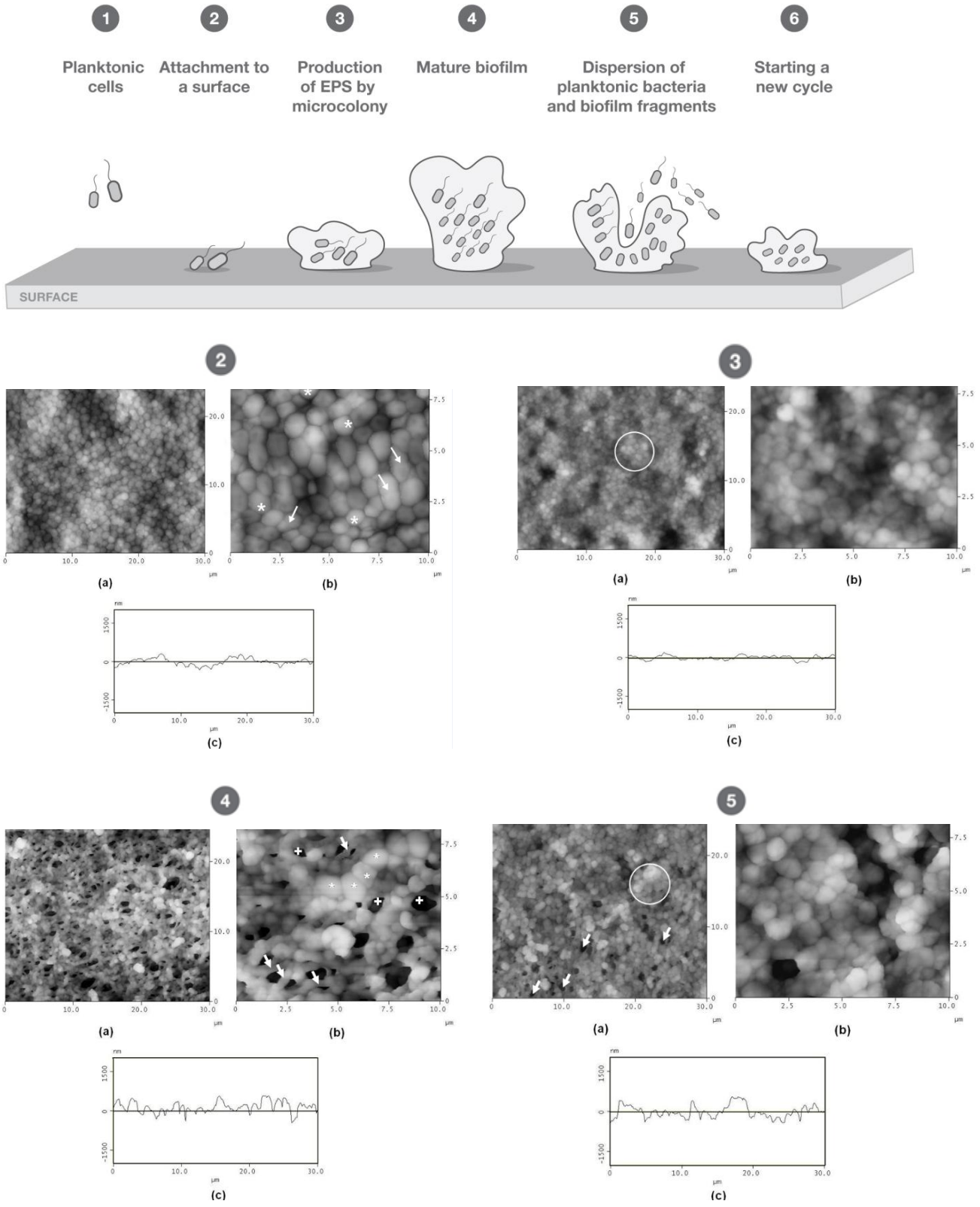

Figure 1. 2a, 3a, 4a, and 5a: Atomic Force Microscopy (AFM) 2D images with sacanning of 30x30 µm of cellulose nitrate membrane (CNM). 2b, 3b, 4b and 5b: AFM 2D images with scanning of 10x10 µm of CNM. 2c, 3c, 4c and 5c: surface roughness chart. Source: Adapted from Santos et al. (2008).

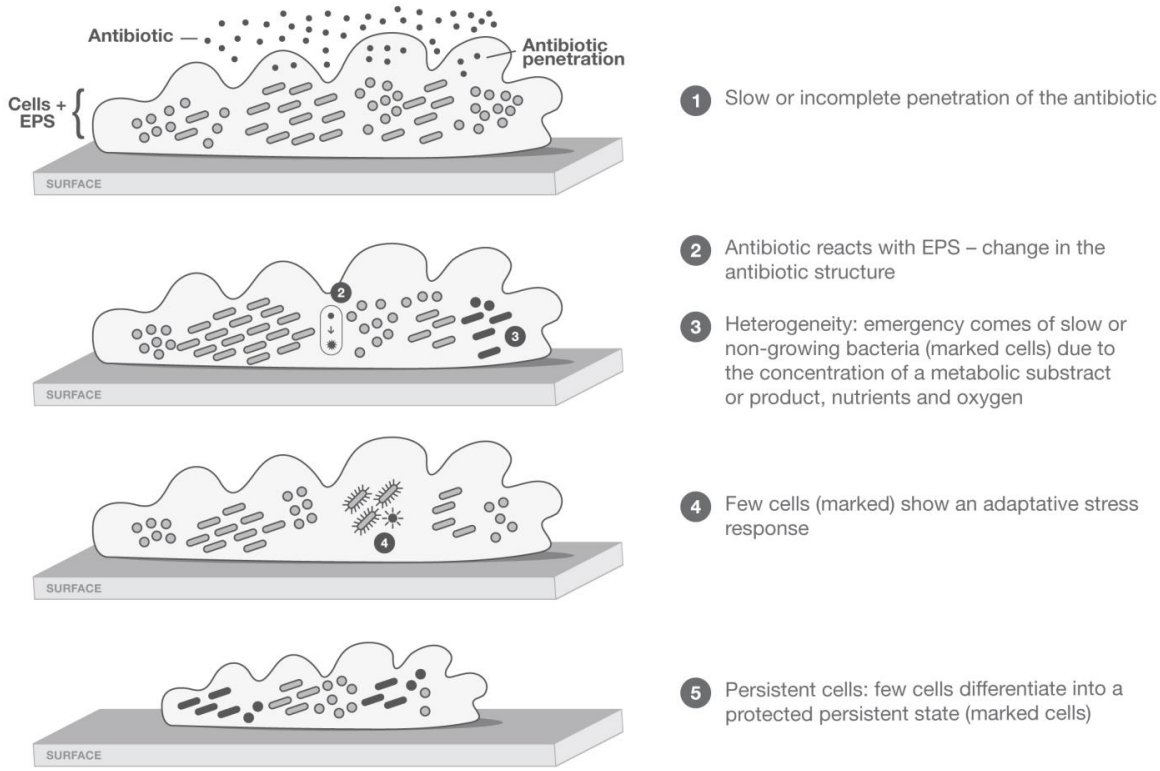

Figure 2. Mechanisms of antimicrobial resistance in bacterial biofilms.

possibly even if phagocytose occurs, macrophage production and release of reactive oxygen species is impaired (Jesaitis et al., 2003). Figure 2 shows the main mechanisms of resistance to antibacterial drugs in bacterial biofilms.

Antibiotics minimal inhibitory concentration (MIC) effective on biofilm-growing bacteria may be up to 1000-fold higher than that of planktonic bacteria (Hoiby et al., 2010). Biofilm-specific mechanisms are coordinately in a reversible and transient manner, contributing to the high levels of antibiotic resistance of these structures in a different pathway from the well-characterized intrinsic resistance mechanisms (for example, expression of anti-biotic-degrading enzymes, inducible decrease in antibiotic influx, inducible increase in antibiotic efflux and alteration in antibiotic target sites) employed by planktonic cells (Sun et al., 2013).

The biofilm EPS act as a barrier to delay the antibiotics diffusion into biofilms (Stewart, 2002) because the active substances may either react chemically with biofilm matrix components or attach to anionic polysaccharides (Sun et al., 2013).

Biofilms contain a small reversible subpopulation of so-called persister cells that adopt a slow-or nongrowing lifestyle through the emergence of small colony variants, and are highly tolerant to extracellular stresses, such as antibiotic treatment. Many antibiotics are less effective against slow- or non-growing cells when compared with fast-growing ones because these antibiotics target growth-specific factors; thereby, the slow growth rates of biofilm-growing cells will render them less susceptible to antibiotics (Sun et al., 2013). After antibiotic treatment, persister cells may survive, creating the reservoirs of cells that may regrow causing a recalcitrant chronic infection. When nutrients are limited in the media, bacteria become highly resistant to antibiotics; this phenomenon is called starvation (Nguyen et al., 2011). Starvation is found in biofilms as consequence of nutrient consumption by peripheral cells and reduced diffusion of oxygen and nutrients through biofilms. This condition induces the stringent response characterized as the repression of growth and division, with the stimulation of amino acid synthesis in order to promote bacterial survival (Chatterji and Ojha, 2001). The starvation response is a determinant of biofilm-specific antimicrobial resistance in *P. aeruginosa* (Nguyen et al., 2011).

Besides the aforementioned mechanisms, it was recently verified that inactivation of ethanol oxidation genes increases the sensitivity of *P. aeruginosa* biofilms to antibiotic treatment, indicating the contribution of ethanol oxidation to biofilm specific antibiotic resistance (Beaudoin et al., 2012).

Planktonic cells may exhibit multidrug efflux pumps that mediate antibiotic efflux leading to antibiotic resistance. Nine efflux pumps of *P. aeruginosa* have been shown to contribute to this organism's high intrinsic resistance to antibiotics (Mima et al., 2005). A *P. aeruginosa* efflux pump encoded by *PA1874-1877*, has been shown to contribute to the biofilm-specific antibiotic resistance of *P. aeruginosa* (Zhang and Mah, 2008).

A relevant factor in biofilm antibiotic resistance is the extracellular DNA within the biofilm matrix. This molecule can bind to and sequester cations, resulting in a cation-limited environment within biofilms that activates the two-component regulatory systems PhoP/Q and PmrA/B required for the expression of multiple antibiotic resistance genes in *P. aeruginosa* (Mulcahy et al., 2008).

LECTINS: CONCEPT AND CHARACTERISTICS

Based on lectins knowledge, Van Damme et al. (1996) defined it as proteins which have at least one non-catalytic domain that reversibly binds to carbohydrates, mono or oligosaccharides, and classified it into four types according to their structural characteristics: merolectins, hololectins, quimerolectins and superlectins. In 1996, the same authors introduced the class superlectins, which presents two carbohydrate binding sites, a significant different structure, and recognizes unrelated sugars. An example of this group is the lectin TxLC tulip-1 subunits, which has a specific site for mannose and one for N-acetyl-galactosamine, working completely independently (Van Damme et al., 1996).

In order to facilitate the use of lectins in glycobiology, Wu and colleagues (2009) classified the molecules according to their specificity for monosaccharides and oligosaccharides structures. These molecules can act as mediators of information in biological systems, and interact with glycoproteins, glycolipids and oligosaccharides (Gupta et al., 2010; Gomes et al., 2010).

Thus, lectins are a large group of proteins of structural heterogeneity which may differ in amino acid composition, apparent molecular weight, structure and number of subunits and also by whether or not related to metal ions or divalent cations (Cavada et al., 2001). These molecules have been used extensively in the physiology field, biochemistry and biomedical sciences. However, the true biological function of these proteins is not clear.

Some studies aimed to bring relevant issues on this topic (Rüdiger and Rouge, 1998; Lannoo and Van Damme, 2010), even for the family Leguminosae lectins with high primary sequence similarity, common functions could not be attributed, since some parameters, such specific carbohydrate, location and time of production are different (Carneiro, 2010).

Lectins in plants have important biological functions, such as protein reserves, defense and communication (Sharon, 1980; Cook, 1986; Van Damme et al., 1998). These molecules functions in plants are viewed from two perspectives: the lectin interacts with external sources, aggressors or symbionts (animals, bacteria or fungi), and another function in which the lectin plays a physiological role in the plant (Sharon and Lis, 1995).

Lectins with a high degree of similarity in amino acid sequence, secondary and three-dimensional structure are found in plants of Leguminosae family, thus revealing a well-defined taxonomic line (Cavada et al., 1993; Sharon and Lis, 1995). These lectins, generally comprise two or four subunits, that could be identical or different and with molecular weight of about 25-30 kDa. These subunits could be formed by a single polypeptide scaffold stabilized by non-covalent bonds like hydrogen bonds, electrostatic and hydrophobic interactions forming or not canonical dimmers (Vasconcelos, 2010). Lectins belonging to Diocleinae subtribe are tetramers composed by a mixture of intact subunits formed by a polypeptide chain of 237 amino acid residues and fragmented subunits, in which the same polypeptide chain is divided in two fragments (Chrispeels et al., 1986). Examples are the lectins ConA and ConBr, which have high structural similarity in amino acid sequences. The difference in crystalline structure between ConA and ConBr is only in two amino acids and neither of them is close to the carbohydrate binding site on both lectins. However, this difference makes ConBr structure more open than the Con A (Cavalcante et al., 2011).

Lectins have a variety of structural characteristics and are widely distributed in nature, been identified in fungi, bacteria, insects, animals, plants, as well as virus (Moreira et al., 1991). These molecules may be involved in various natural phenomena, among them the process of fertilization, embryogenesis, cell migration, organ formation and immune defense (Sharon and Lis, 2004). The imbalance of these processes may trigger the development of several pathologies (Sharon and Lis, 1989).

When Nowell (1960) described the mitogenic activity of *Phaseolus vulgaris* (PHA) lectin on human lymphocytes, an important new branch of research arose for the applicability of these molecules in biological systems. On cells surfaces there are carbohydrate molecules existing as glycoproteins, glycolipids and polysaccharides, and these molecules are directly involved in many cellular processes. The investigation of mechanisms involved in cell-cell interaction has emphasized the importance of carbohydrates in biochemical processes, viewed as energy-rich molecules or prosthetic elements (Carvalho, 2008).

Carbohydrates are essential elements for recognition in a wide variety of biological processes, in physiological and pathological conditions (Varki, 1993; Sharon and Lis, 1995). Thus, the fact of lectins often detect differences in

carbohydrates configuration, they would be powerful tools for this exchange of information between cells.

The use of lectins as biotechnological approaches are justified by a large number of scientific studies showing biological relevant activities related to these proteins (Kitada et al., 2010, Kimble et al., 2010; Singh et al., 2010; Cao et al., 2010). Among these biological activities, it is noted that lectins exhibit antibacterial activity (Alencar et al., 2005; Holanda et al., 2005, Wong et al., 2010), and lectins have ability to interfere with process of microbial biofilms formation (Teixeira et al., 2006, 2007, Oliveira et al., 2007; Islam et al., 2009; Cavalcante et al., 2011).

Various infections are started by lectin-carbohydrate interactions, such as cell adhesion and phagocytosis of *P. aeruginosa* (Imberty et al., 2004), *Neisseria gonorrhoeae* (Sharon, 2006), *Escherichia coli* (Firon et al., 1983), trypomastigote form of *Trypanosoma cruzi* (Silber et al., 2002) and promastigotes of *Leishmania major* (Sacks et al., 1985). Several of these pathogens establish mechanisms of a required attachment or adhesion to the host tissue or cells, otherwise, these microorganisms could be eliminated by the natural defense mechanisms of host, such as the airflow on respiratory system or urine excretory system (Sharon and Lis, 1993). In addition, proper adhesion of the pathogen provides better access to nutrient sources, facilitates the introduction of toxic substances in host tissue and even the penetration of the pathogen in these tissues (Karlsson, 1998).

Lectins action on biofilms mechanisms

The first report of inhibitory action of peptides in microorganisms, dated from 1942, refers to a protein obtained from wheat (Balls et al., 1942; Nakatsuji and Gallo, 2012). Lectins from higher plants have defense function against pathogens such as bacteria and fungi by immobilization and recognition of infectious microbial agents by binding, thus preventing the multiplication and subsequent colonization of the host plant (Etzler, 1986). Inhibition of bacteria growth and fungi by lectins, such as Amaranthus, has been previously reported in the literature (De Bolle et al., 1996). The concentrations used are considered higher than the concentration used in similar studies (Liao et al., 2003; Santi-Gadelha et al., 2006; Oliveira et al., 2007, 2008). However, Liao and colleagues (2003) tested the antimicrobial activity of plant and seaweed lectins using concentrations between 102 and 800 µg/mL and found that ConA and WGA from land plants did not inhibit any of the analyzed vibrios.

Lectins have antibacterial activity, and this effect (on Gram-positive and Gram-negative bacteria) occurs through interactions with bacterial cell wall components (Paiva et al., 2010). Santi-Gadelha and colleagues (2006), using electron microscopy, observed the presence of pores and severe disruption of bacterial membrane of Gram-positive, confirming the marked antimicrobial activity and pointing a possible mechanism of growth inhibition by lectins, since these pores formed in the membrane allows the output of the cell content (Terras et al., 1993; Oliveira et al., 2008).

By the genetic expression analysis of genes related to the *S. mutans* biofilm on *Canavalia maritime* lectin, Cavalcante et al. (2013) observed that although the mechanism of action of these lectins requires a better understanding, the results reported in that present article suggest that ConM acts by starting or interrupting intracellular signaling pathways that culminate with the lowest expression of genes associated with virulence and biofilm formation in *S. mutans*.

The carbohydrate binding sites on the bacterial surface probably have a key role in antibacterial activity, which makes it responsible for bacterial recognition. In a recent study, it was noted that differences in antimicrobial activity against *S. mutans* and *S. oralis* may be related to differences in the composition of surface carbohydrates characteristic of each bacteria. Almost all microorganisms express carbohydrates on its surface (Cavalcante et al., 2011). These carbohydrates may be covalently linked, as in teichoic acid linked to the peptidoglycan glycosylated or non-covalently linked, as in the capsular polysaccharides (Santi-Gadelha et al., 2006; Calderon et al., 1997).

The ability of lectins to form complexes with microbial glycoconjugates has stimulated its application as probes to whole cells, its mutants and numerous cellular constituents and metabolites. Microbial receptors for Concanavalin A have been described. For example, glycosylated teicoic acid found on the surface of various Gram-positive bacteria (Calderon et al., 1997) and neutral polysaccharides produced by members of the genera *Leuconostoc* and *Streptococcus* (Santi-Gadelha et al., 2006) can be sites to lectin binding. The development of high-affinity ligands able to selectively recognize a variety of different patterns in small oligosaccharides would be of significant interest as diagnostic and experimental tools for many bacterial infections. The selective binding of lectins to certain bacteria have been proposed for use in drug delivery of antimicrobial agents with the *Canavalia ensiformis* lectin having as point of action, *Streptococcus sanguis* and *Corynebacterium hofmannii;* and lectin of *Triticum vulgaris* targeting *Streptococcus epidermis* in *in vitro* experiments (Kaszuba et al., 1995).

CONCLUSIONS

Microbial biofilms formation and maintainability are directly linked to carbohydrate residues. These molecules mediate the adhesion of the bacteria to the surface

substrate (biotic or abiotic) for biofilm formation as well as, acting between microorganisms interaction to form cell aggregates. As molecules are able to bind specifically and selectively to carbohydrates, lectins have a crucial function in microbial biofilms studies, becoming a powerful tool to analyze glycidic structures of microbial origin aggregates.

The impact of bacterial resistance, provided by biofilm formation on human health encourages researches aiming to understand its mechanisms, as well as strategies to eradicate or minimize these communities' damages.

Conflict of Interests

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

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Full Length Research Paper

Production of alkaline protease from a haloalkaliphilic soil thermoactinomycete and its application in feather fibril disintegration

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Proteases have found a wide application in several industrial processes, such as laundry detergents, protein recovery or solubilization, prion degradation, meat tenderizations, and in bating of hides and skins in leather industries. In the present study, a bacterial isolate, *Thermoactinomyces* sp. RS1, isolated from soil sample was taken for enzyme production by submerged fermentation technology. The classical "one-variable-at-a-time approach" was employed. Optimum incubation time, pH and temperature were found to be 24 h; pH 9.0 and 55°C, respectively. The enzyme production was highest at salt concentration of 5%; inoculum of 4% and agitation rate of 150 rpm. RS1 could grow in the presence of all carbon sources employed and was highest with glucose. In the case of organic nitrogen sources, enzyme production was highest with peptone and in the case of inorganic nitrogen sources, enzyme production was highest in urea. Overall, 1.5 folds of production was achieved after optimization of all conditions of previously used culture media. Protease in the present study shows good feather degradation within short incubation time, presenting its utilization for poultry feed production. The study holds significance as only few reports are available on the alkaline proteases having keratinolytic property from haloalkaliphilic bacteria.

Key words: Protease, feather disintegration, haloalkaliphilic isolate, thermoactinomyces, submerged fermentation.

INTRODUCTION

Alkaline proteases are the key industrial enzymes utilized in myriad of industrial works viz. laundry detergents, protein recovery or solubilization, prion degradation, meat tenderizations, and in bating of hides and skins in leather

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industries. Extensive studies had been done on production and downstream processing to obtain purified enzyme both at laboratory and industrial scale. Various types of alkaline proteases have been characterized and their potential industrial applications have been explored (Gupta et al., 2002). Alkaline proteases are reported from various groups of bacteria but Actinomyces are not paid much attention. These groups of prokaryotes have ability to produce many bioactive compounds (Mehta et al., 2006; Gupta et al., 2002) but the production of commercial enzymes from them was totally unsighted.

Proteases of extracellular nature, having different other industrial potential are wide spread among the halo-alkaliphiles. In order to obtain commercially viable yields, it is essential to optimize fermentation media for the growth and protease production. Moreover, most of the studies on haloalkaliphilic bacteria in the past have largely focused on hyper saline environments and the exploration of relatively moderate saline and alkaline environments is only the beginning (Joshi et al., 2008)

Production of proteases was effected by various environmental factors and also the strategy of fermentation, that is, submerged or solid state. Usually, production of these enzymes was reported by submerged process due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream inspite of the cost-intensiveness for medium components.

In the present study, *Thermoactinomyces* sp. RS1, isolated from soil sample was taken for enzyme production by submerged fermentation technology and crude protease from studied isolate shows good feather fibril disintegration within short incubation time, presenting its utilization for poultry feed production.

MATERIALS AND METHODS

Microorganism and culture conditions

Alkaline protease producing thermoalkaliphilic *Thermoactinomyces* sp. RS1 used in this investigation was isolated from soil sample of Rajasthan, India and maintained on a nutrient agar medium pH 9.0, stored at 4°C and sub-cultured at monthly intervals (Verma et al., 2011).

Culture media

Protease screening medium contained (g/L) peptone 1.0, NaCl 5.0, skimmed milk 20.0, and Agar 20.0 at pH 8.0.

Protease production medium contained (g/L) peptone 7.5, glucose 5.0, salt solution 50 ml containing $MgSO_4$ 0.5%, KH_2PO_4 0.5% and $FeSO_4$ 0.01%, at pH 9.0.

Submerged fermentation

The classical “one-variable-at-a-time approach” was employed to evaluate the effect of growth conditions, including incubation time, temperature and initial pH of the medium, on cell growth and protease production. Experiments were conducted in triplicate and statistical analysis was performed.

Effect of incubation time on the production of protease

The effect of incubation time on protease production was determined by incubating the culture medium at different time intervals (24 TO 96 h) with an interval of 24 h.

Effect of initial pH on the production of protease

The effect of pH of culture media was examined by maintaining the pH 6.0 to 12.0 with increase of 1 and then inoculating the media and by incubating it at 60°C for 24 h.

Effect of temperature on the production of protease

The effect of incubation temperature on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different temperatures ranging from 40 to 70°C with increase of 5°C.

Effect of salt concentration on the production of protease

The effect of salt concentration on the growth and enzyme production was examined by supplementing the culture media with NaCl 0 to 20 % with increase of 5%.

Effect of inoculation on the production of protease

The effect of inoculation on the growth and enzyme production was examined by varying inoculation volumes 2 to 10% (v/v) with increase of 2% inoculated into culture media.

Effect of carbon sources on the production of protease

Different carbon sources (glucose (as control), fructose, maltose, sucrose, starch and lactose) were supplemented at 1% (w/v) to culture media to investigate their effect on the growth and alkaline protease production. Samples were withdrawn after 48 h for the measurement of growth and protease estimation.

Effect of organic nitrogen source on protease production

Different nitrogen source (peptone (as control), yeast extract, tryptose, tryptone, malt extract and beef extract) were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity.

Effect of inorganic nitrogen source on protease production

Different inorganic nitrogen sources, that is, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, urea and KNO_3 were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity.

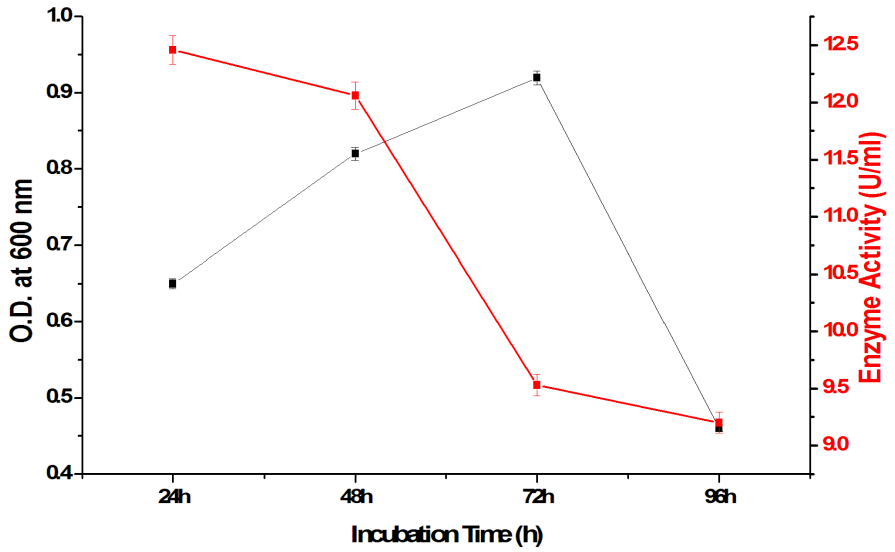

Figure 1. Effect of incubation time on growth and alkaline protease production.

Effect of agitation rate on the production of protease

The effect of agitation rate on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different shaking speeds ranging from 0 to 250 rpm with increment of 50 rpm.

Protease assay

Proteolytic activity was assayed by modified folin method; using 0.5% casein as substrate dissolved in 50 mM Glycine NaOH buffer pH 10.0. The reaction mixture was incubated at 80°C for 30 min, and reaction was stopped using 10% TCA. Tyrosine released was estimated using Folin Ciocalteau's Reagent and absorbance taken at 670 nm. One unit of protease (1 PU) was defined as amount of enzyme required to release 1 µg of tyrosine under the assay condition when reaction was incubated for 1 min.

Protein determination

The protein content in crude enzyme was determined by Lowry method (Verma et al., 2011).

Feather disintegration studies

Feather fibril detachment and hydrolysis was studied using 70% ethanol washed chicken feathers (Bockle et. al., 1995) using crude enzyme treatment along with additive βME in different combinations (feathers + buffer (control); feathers + buffer + enzyme; feathers + buffer + filtered enzyme; feathers + buffer + filtered enzyme + βME (0.1%)). Patterns of detachment were recorded by periodic observation of the treated feathers using compound light microscope at 10x magnification.

Statistical analysis

For statistical analysis, a standard deviation for each experimental result was calculated using the Excel Spreadsheets available in the Microsoft Excel. Statistical validation was performed with STPR software and graphs were prepared using ORIGIN software Version 6.

RESULTS AND DISCUSSION

Submerged fermentation

Effect of incubation time on the production of protease

The effect of incubation time on protease production was determined by incubating the culture medium at different time intervals (24 to 96 h) with an interval of 24 h. The optimum incubation time was found to be at 24 h after which there was fall in enzyme production (Figure 1). The possible reason could be denaturation and degradation by other proteases secreted along with alkaline protease, autolysis or repression of the enzyme synthesis. Earlier, similar features have been reported by Joshi et al. (2008).

Effect of initial pH on the production of protease

The effect of pH of culture media was examined by maintaining the pH 6.0 to pH 12.0 with increment of 1 and then inoculating the media and by incubating it at 60°C for 24 h. The enzyme production was highest at pH 9.0 with enzyme activity of 16.22 ± 0.02 U/ml (Figure 2). Requirement of alkaline pH for optimum growth and protease production, clearly suggested the alkaliphilic nature of the organism and enzyme.

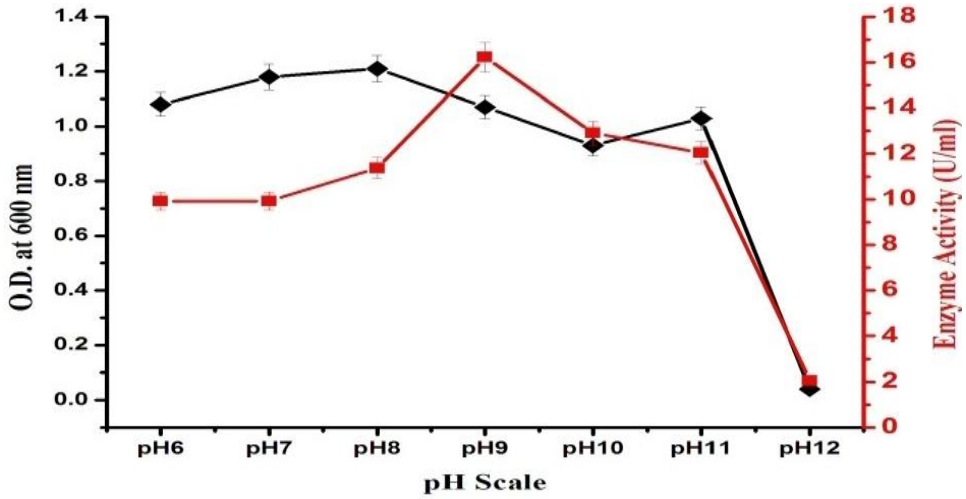

Figure 2. Effect of pH on growth and alkaline protease production.

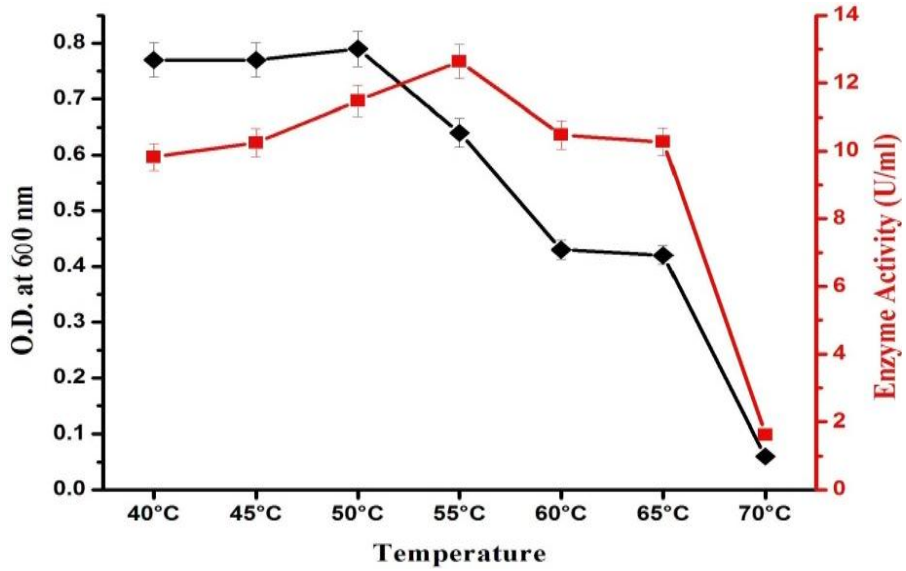

Figure 3. Effect of temperature on growth and alkaline protease production.

Effect of temperature on the production of protease

The effect of incubation temperature on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different temperatures ranging from 40 to 70°C with increase of 5°C. The enzyme production was highest at 55°C with enzyme activity of 12.63 ± 0.02 U/ml (Figure 3). Temperature is a critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood (Chaloupka, 1985). However, studies by Frankena et al. (1986) showed that a link existed between enzyme synthesis and energy metabolism in *Bacilli*, which was controlled by temperature.

Effect of salt concentration on the production of protease

The effect of salt concentration on the growth and enzyme production was examined by supplementing the culture media with NaCl 0 to 20 % with increase of 5%. The enzyme production was highest at salt concentration

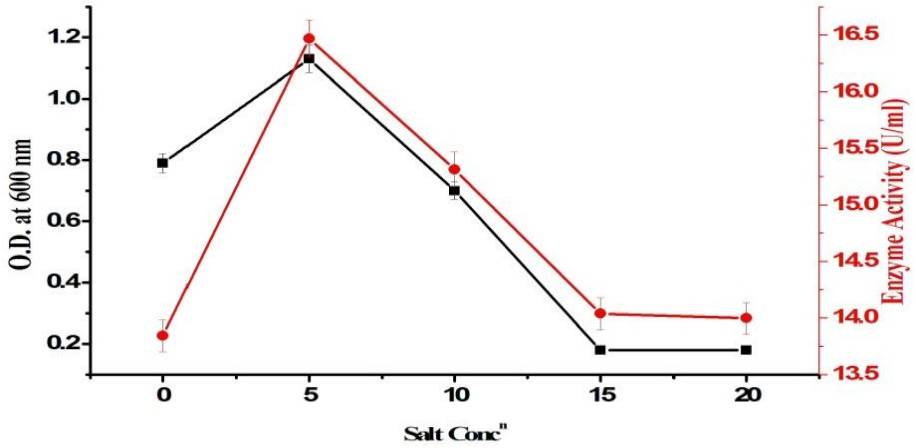

Figure 4. Effect of salt concentration on growth and alkaline protease production.

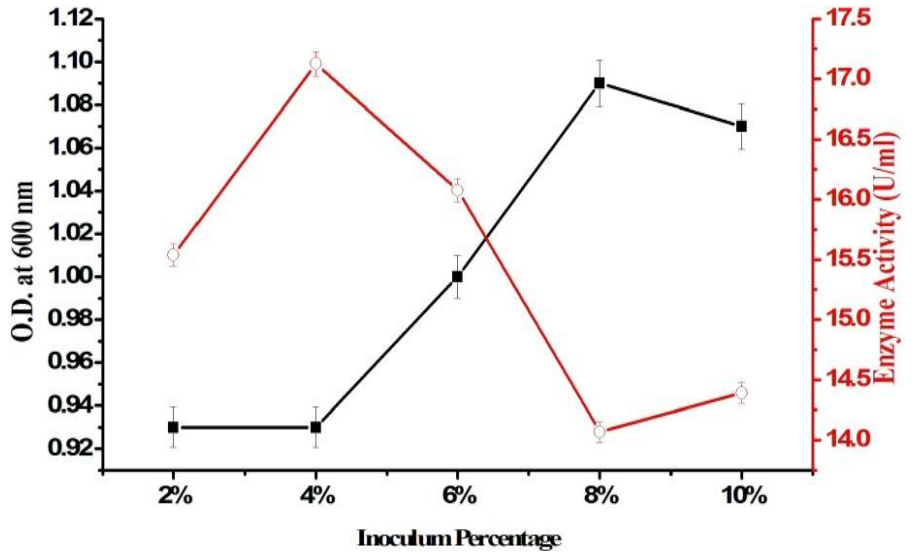

Figure 5. Effect of inoculum percentage on growth and alkaline protease production.

of 5% with enzyme activity of 16.47 ± 0.03 U/ml (Figure 4). The NaCl required for optimum growth and protease production was fairly lower as compared to other halo-alkaliphilic protease producers isolated from other saline habitats (Kostrikina et al., 1991). The optimum protease production at 15~16% NaCl for some halophilic bacteria has been reported which indicates that our protease producer was mild halophilic in nature (Horikoshi, 1971; Sen and Satyanarayana, 1993).

Effect of inoculation on the production of protease

The effect of inoculation on the growth and enzyme production was examined by varying inoculation volumes 2 to 10% (v/v) with increase of 2% was inoculated into culture media. The enzyme production was highest at inoculum percentage of 4% with enzyme activity of 17.12 ± 0.58 U/ml (Figure 5). The growth of bacterium increases with inoculum percentage; however, it had pronounced effect on protease production. The repression of protease at higher inoculation volume (>4%, v/v) was quite comparable with some of the earlier reports, where protease production was enhanced at lower inoculation volume (Mao et al., 1992).

Effect *of carbon sources on the production of protease*

Different carbon sources (glucose, fructose, maltose, sucrose, starch and lactose) were supplemented at 1%

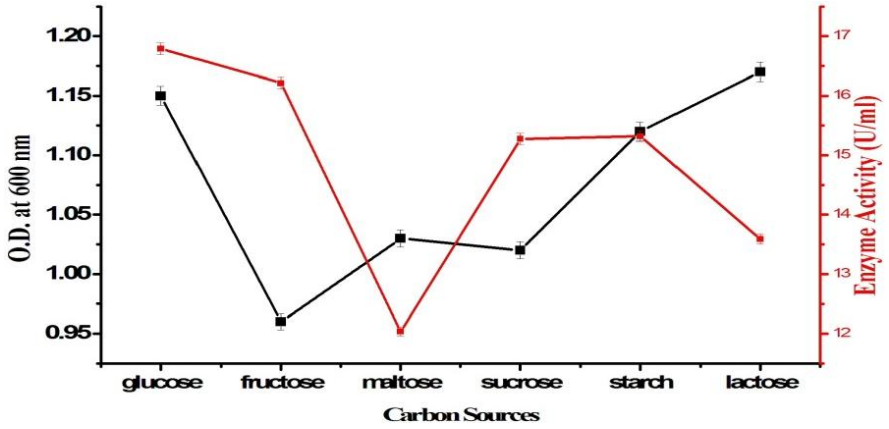

Figure 6. Effect of different carbon sources on growth and alkaline protease production.

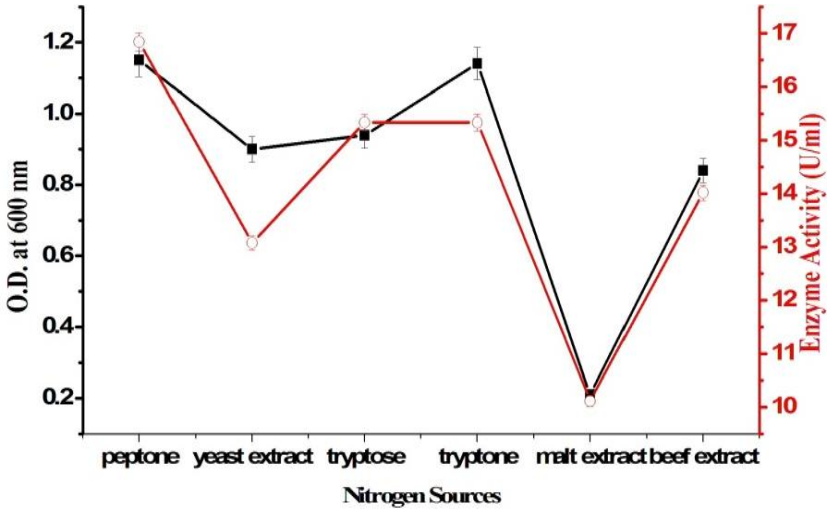

Figure 7. Effect of different organic nitrogen sources on growth and alkaline protease production.

(w/v) to culture media to investigate their effect on the growth and alkaline protease production. The enzyme production was highest with glucose (16.79 ± 0.01 U/ml) followed by fructose (16.21 ± 0.19 U/ml); starch (15.32 ± 0.57 U/ml); sucrose (15.27 ± 0.01 U/ml); lactose (13.58 ± 0.56 U/ml); maltose (12.03 ± 5.74 U/ml) (Figure 6). RS1 could grow in the presence of all carbon sources employed; the protease production was not suppressed by any of the carbon sources. But in some cases e.g. production of extracellular protease CP1 from moderately halophilic *Pseudoalteromonas sp.* strain CP76 was significantly inhibited in the presence of maltose, glucose, and lactose (Sanchez-Porro et al., 2003). Earlier, a catabolic repression mechanism for extracellular protease production has been also reported (Matsubara and Feder, 1971; Fujiwara and Yamamoto, 1987).

Effect of organic nitrogen source on protease production

Different nitrogen source (peptone, yeast extract, tryptose, tryptone, malt extract and beef extract) were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity. The enzyme production was highest with peptone (16.84 ± 0.01 U/ml) followed by tryptose (15.33 ± 0.02 U/ml); tryptone (15.33 ± 0.01 U/ml); beef extract (14.01 ± 0.01 U/ml); yeast extract (13.07 ± 0.02 U/ml); malt extract (10.11 ± 0.10 U/ml) (Figure 7). Complex nitrogen sources are usually used for alkaline protease production and the requirement for a specific nitrogen supplement differs from organism to organism.

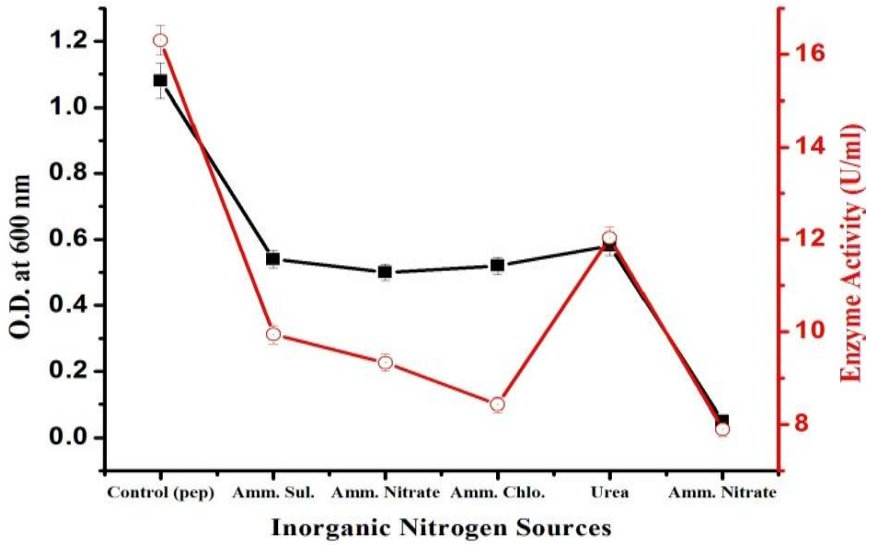

Figure 8. Effect of different inorganic nitrogen source on growth and alkaline protease production.

Peptone is one of common nitrogen source for protease production but in some cases tryptone (2%) and casein (1-2%) also serve as excellent nitrogen sources (Ong and Gaucher, 1976; Phadatare et al., 1993).

Effect of inorganic nitrogen source on protease production

Different inorganic nitrogen sources, that is, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, Urea and KNO_3 were taken to study their effect on protease production. These nitrogen source were added into production media at same concentration, enzyme were harvested and assayed for activity. The enzyme production was highest with peptone (16.30 ± 0.15 U/ml) used as control followed by urea (12.03 ± 0.54 U/ml); ammonium sulphate (9.94 ± 0.01 U/ml); ammonium nitrate (9.33 ± 0.07 U/ml); ammonium chloride (8.42 ± 0.03 U/ml); potassium nitrate (7.89 ± 0.03 U/ml) (Figure 8). Usually low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Sen and Satyanarayana, 1993; Chaphalkar and Dey, 1994). Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium (Cruegar and Cruegar, 1984), although inorganic nitrogen sources are preferred in industrial process for their low cost.

Effect of agitation rate on protease production

The effect of agitation rate on the growth and enzyme production was examined by incubating the flasks containing culture for 24 h at different shaking speeds ranging from 0 to 250 rpm with increment of 50 rpm. The enzyme production was highest at agitation rate of 150 rpm with enzyme activity of 17.77 ± 0.01 U/ml (Figure 9). The variation in the agitation speed influences the extent of mixing in the shake flasks or the bioreactor and will also affect the nutrient availability. Protease production optimizes in the range of 150 to 200 rpm e.g. optimum yields of alkaline protease are produced at 200 rpm for *Bacillus subtilis* ATCC 14416 (Chu, 1992) and *Bacillus licheniformis* (Sen and Satyanarayana, 1993). In one study, *Bacillus* sp. B21-2 produced increased enzyme production when agitated at 600 rpm (Fujiwara and Yamamoto, 1987).

Feather disintegration studies

Gradual changes in the feather upon enzymatic treatment with 0.1% β-ME could be visibly observed, and clean shaft was obtained after 18 h. Untreated control feather shows the hollow supporting shaft (rachis) of feather and its side branches (barbs) to which are attached a set of fine barbules which could be clearly observed in microscopic images. Disintegration of barbules apparently started after 2 h of enzymatic treatment along with βME and after 12 h, barbs started disintegrating and within 18 h, degradation of the barbs, leaving behind the thick basal portion of the rachis was seen (Figure 10). Efficiency of degradation in the absence of reductant was lower and took nearly 48 h to show clean shaft. The base of the shaft remained undigested even after prolonged incubation.

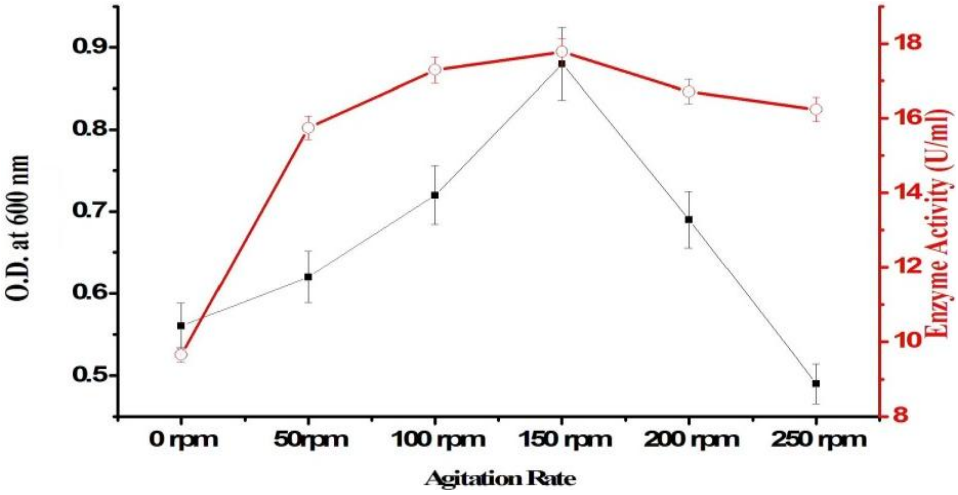

Figure 9. Effect of agitation rate on growth and alkaline protease production.

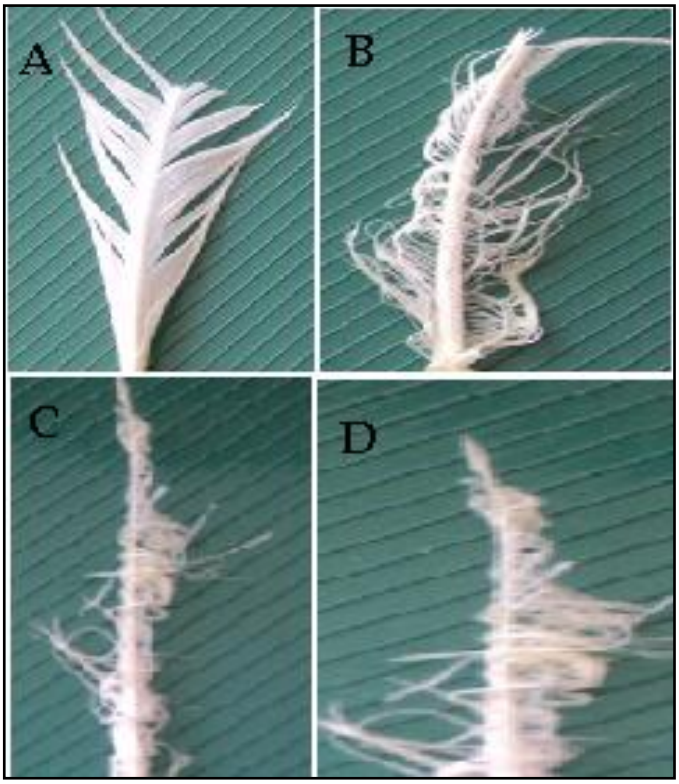

Figure 10. Feather disintegration studies: (1) Chicken feather disintegration using protease preparation from *Thermoactinomyces* sp. RS1. A) Control; B) filtered crude enzyme (20 U/ml) + buffer; C) crude enzyme (20U/ml) + buffer; D) filtered crude enzyme (20U/ml) + buffer + β ME.

Conclusions

Alkaline proteases are generally produced by submerged fermentation. In commercial practice, the optimization of medium composition is aimed to maintain a balance between the various medium components, thus minimizing

the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly toward the evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes; requirement of divalent metal ions in the fermentation medium; and optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation. In addition, no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production (Kumar and Takagi, 1999). So, in the present study, the classical “one-variable-at-a-time approach” was employed to evaluate the effect of growth conditions, including incubation time, temperature and initial pH of the medium, on cell growth and protease production. Overall, 1.5-fold production was achieved after optimization of all conditions of previously used culture media. Apart from the above, protease from the above culture was found to be keratinolytic in nature and solubilizes the keratin of chicken feathers which is beneficial for poultry feed production.

Conflict of Interests

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

ACKNOWLEDGEMENT

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Full Length Research Paper

Microbiological studies on resistance patterns of antimicrobial agents among Gram negative respiratory tract pathogens

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Respiratory tract infections (RTIs) are the most frequently-occurring infections of all human diseases and have been frequently documented. This study investigated the antimicrobial resistance patterns among Gram negative respiratory tract isolates. A total of 309 non replicate Gram negative respiratory tract isolates were collected and identified. Molecular mechanisms of antimicrobial resistance pattern were characterized by phonotypic and genotypic methods including polymerase chain reaction (PCR) amplification and DNA sequencing of isolated genes. Gram negative isolates were *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae*, *Stenotrophomonas maltophilia, Escherichia coli, Enterobacter cloacae, Serratia rubidaea, Haemophilus influenza, Citrobacter koseri, Moraxella catarrhalis, Proteus mirabilis* and *Salmonella typhimurium* with the following frequencies respectively (34.6, 26.6, 13.9, 7.7, 6.4, 5.5, 1.4, 1.3, 1, 1, 0.3 and 0.3%). *S. maltophilia* isolates were the highest that produced extended spectrum beta lactamase (ESβL) with percentage of 75% and metallo beta lactamase (MβL) with percentage of 71%, while *P. aeruginosa* isolates were the highest that produced class C beta lactamase (AmpC) with percentage of 86% and efflux pump with percentage of 73%. This study revealed two common mechanisms of antimicrobial resistance patterns, β-lactamases production and efflux pump, among Gram negative respiratory tract pathogens up to molecular level.

Key words: Resistance pattern, antimicrobial agents, respiratory, pathogens.

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) estimates that more than 100 million antibiotic prescriptions are written each year in the ambulatory care setting. With so many prescriptions written each year, inappropriate antibiotic use will promote resistance. In addition to antibiotics prescribed for upper respiratory tract infections with viral etiologies, broad-spectrum antibiotics are used too often when a narrow-spectrum

antibiotic would have been just effective (Steinman et al., 2003).

Resistance to β-lactam antibiotics occurs primarily through the production of β-lactamases, enzymes that inactivate these antibiotics by splitting the amide bond of the β-lactam ring. β-Lactamases most likely co-evolved with bacteria as mechanisms of resistance against natural antibiotics over time, and the selective pressure exerted by the widespread use of antimicrobial therapy in modern medicine may have accelerated their development and spread. β-Lactamases are encoded either by chromosomal genes or by transferable genes located on plasmids and transposons. In addition, β-lactamase genes (*bla*) frequently reside on integrons, which often carry multiple-resistance determinants. If mobilized by transposable elements, integrons can facile-tate further dissemination of multidrug resistance among different bacterial species (Weldhagen, 2004).

Four major groups of enzymes are defined by their substrate and inhibitor profiles: group 1 cephalo-sporinases that are not well inhibited by clavulanic acid; group 2 penicillinases, cephalosporinases and broad-spectrum β-lactamases that are generally inhibited by active site-directed β-lactamase inhibitors; group 3 metallo β-lactamases that hydrolyze penicillins, cephalo-sporins and carbapenems and that are poorly inhibited by almost all β-lactam-containing molecules; and group 4 oxacillin-hydrolyzing enzymes that are not inhibited by clavulanic acid (Webb, 1984).

Another important mechanism of antibiotic resistance is efflux pumps. In general, multiple antibiotic resistance in Gram-negative bacteria often starts with the relatively limited outer membrane permeability to many antibiotic agents, coupled with the over expression of multi-drug resistance (MDR) efflux pumps, which can export multiple unrelated antibiotics. In addition, by reducing the intracellular concentration of the antimicrobial agent to less than the MIC required for bacterial killing, efflux mechanisms may allow bacterial survival for longer periods, facilitating the accumulation of new antibiotic-resistance mutations (e.g., those encoding topoiso-merase IV or DNA gyrase targets, rendering fluoro-quinolones ineffective) (Piddock, 2006).

Antimicrobial agents exert strong selective pressures on bacterial populations, favoring organisms that are capable of resisting them. Genetic variability occurs through a variety of mechanisms. Point mutations may occur in a nucleotide base pair, and this is referred to as microevolutionary change. These mutations may alter enzyme substrate specificity or the target site of an antimicrobial agent, interfering with its activity **(**Medeiros, 1997). This study focused on the genetic variability among Gram negative respiratory tract isolates and its relation to antimicrobial resistance including multi-drug resistant isolates.

MATERIALS AND METHODS

Bacterial isolates

A total of 309 non replicate Gram negative respiratory tract isolates from 249 patients: 115 males, 134 females, between the ages of 3 and 50 from medical intensive care unit, MICU and surgical intensive care unit, SICU, with underlying upper and lower respiratory tract diseases with no history of antibiotic administration prior to sample acquisition for three months were collected from King Abdulaziz University Hospital, Jeddah, KSA. From September 2011 to June 2012 according to the generally accepted guidelines for specimen collection and transportation of common specimen types as illustrated in Table 1 (Murray, 2007), clinical specimens collected were isolated, identified using morphological, microscopy, biochemical tests and API kit method as well.

Characterization and molecular mechanisms of antimicrobial resistance pattern of Gram negative respiratory tract pathogens

Isolates that exhibited reduced susceptibility to one or more of ceftazidime, aztreonam, cefotaxime or ceftriaxone were considered as potential producers of ESβL. Double-disk synergy test (Figure 1) was done using ceftazidime and a ceftazidime + clavulanic acid (30 µg/10 µg) discs as confirmatory test for detection of ESβL production (Coudron et al., 1997). Isolates resistant to imipenem or meropenem were considered as suspicious for production of metallo-beta-lactamases (MβL), ethylene diamine tetraacetic acid (EDTA) disc synergy test (Figure 2) was done for detection of metallo-β-lactamases in the imipenem resistant isolates (Yong et al., 2002). Isolates resistant to one or more of cefoxitin, cefotetan, cefotaxime, ceftazidime and aztreonam were considered as suspicious for production of AmpC-beta-lactamases (AmpC-BL), combined disc test (Figure 3) using cloxacillin as inhibitor of AmpC enzymes was done as confirmatory test for detection of AmpC producing isolates (Mirelis et al., 2006). Minimum inhibitory concentration (MIC) of ciprofloxacin against the clinical isolates was determined using the two-fold serial broth dilution method with an inoculum of 1 x 10^6cells/ml. All experiments were done with and without 100 mg/L carbonyle cyanide-m-chlorophenylhydrazone (CCCP). The MIC was taken as the lowest concentration inhibiting visible growth after 18 h incubations at 37°C. CCCP inhibited multi-drug resistant (MDR) efflux pump was inferred if the MIC with CCCP was four-fold or lower than the MIC without CCCP (Omoregie et al., 2007).

Molecular identification of resistance mechanisms by PCR

Plasmid extraction was done using miniprep plasmid DNA purification kit (Sigma-Aldrich, USA), Polymerase chain reaction was carried out in PCR tubes (total volume 25 µl). The reaction mixture contained 2.5 µl 1× Taq DNA polymerase buffer containing 2.5 mM $MgCl_2$, 0.2 µl 1U Taq DNA polymerase, 200 µl mol deoxynucleoside triphosphates (2.5 µl), 15 pmol of forward and

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Table 1. Guidelines for specimen collection and transportation of common specimen types.

Specimen	Collection methods
Respiratory, Upper	
Nose	Premoistened swab was inserted 1-2 cm into nares and rotated against nasal mucosa.
Nasopharynx	Nasopharyngeal washings and swabs.
Throat or pharynx	The posterior pharynx was swabbed, avoiding saliva.
Respiratory, Lower	
Bronchial alveolar lavage	A large volume of fluid was collected; transported in sterile container.
Sputum (expectorated)	Patient was instructed to rinse or gargle with water to remove excess oral flora; then to cough deeply and expectorate secretions from lower airways; which were then collected and transported in a sterile container.
Sputum (Induced)	Induced with sterile saline using a nebulizer.
Tracheal aspirate	Same tests as expectorated sputum.

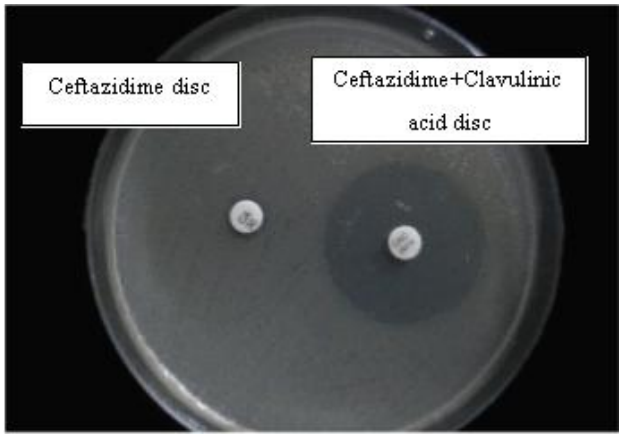

Figure 1. Double-Disc Synergy "DDS" test showing an enhancement in the zone of inhibition between a beta lactam disc and one containing the beta lactamase inhibitor.

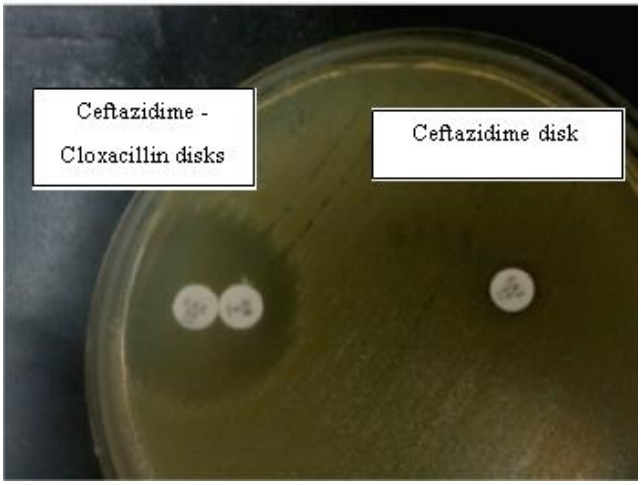

Figure 3. Combined disc test showing an enhancement in the inhibition zone around the combined antibiotic discs.

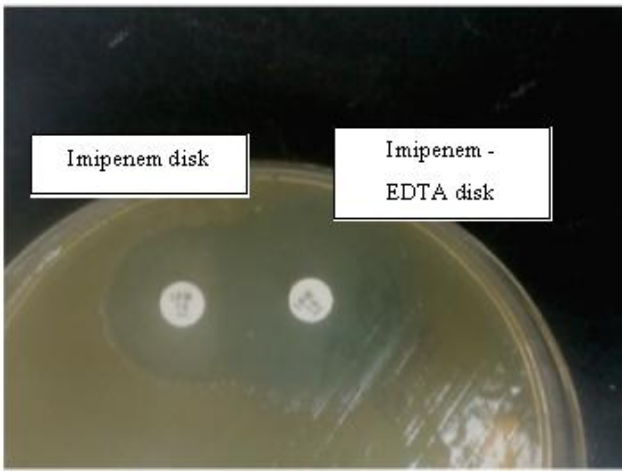

Figure 2. Ethylene diamine tetraacetic acid (EDTA) disc synergy test showing an expanded growth inhibition zone around the Imipenem - EDTA disc.

reversed primers (2 µl), 13 µl of deionized water and 5 µl of DNA template. Amplification was carried out using thermocycler (TC-5000, Techine, USA). Reaction products were separated by horizontal electrophoresis for 25 min at 100 V and varying agarose gel density within 1.2–1.7% in dependence of amplicon size. Visualization of bands was carried out after staining with ethidium bromide (0.5 µ g/ml) using an ultraviolet transilluminator and documentation system (G:Box, Syngene, UK) (NCCLS, 1997). Primers used were SHVF: 5'-GATGAACGCTTTCCCATGATG-3', SHVR: 5'-CGCTGTTATCGCTCATGGTAA-3', cycling conditions were 95°C for 5 min; 35 cycles of 95°C for 60 s, 61°C for 60 s, 72°C for 60 s; 72°C for 5 min (Kim et al., 2009), CTX-MF: 5`-TTTGCGATGCATACCAGTAA-3`, CTX-MR: 5`-CGATATCGTTGGTGCCATA-3`, cycling conditions were 95°C for 5 min; 35 cycles of 95°C for 60 s, 60°C for 30 s, 72°C for 60 s; 72°C for 5 min (Amaral et al., 2009), TEMF: 5`-ATGAGTATTCAACATTTCCG-3`, TEMR: 5`-GTCACAGTTACCAATGCTTA-3`, cycling conditions were 95°C for 5 min; 35 cycles of 95°C for 60 s, 58°C for 60 s, 72°C for 60 s; 72°C for 5 min (Kim et al., 2009), VIMF: 5`-CAGATTGCCGATGGTGTTTGG-3`, VIMR: 5`-AGGTGGGCCATTCAGCCAGA-3`, cycling conditions were 95°C

for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. IMPF: 5`-ATGAGCAAGTTATCAGTATTC-3`, IMPR: 5`-GCTGCAACGACTTGTTAG-3`, cycling conditions were 95°C for 5 min; 30 cycles of denaturation at 95°C for 1min, annealing at 57°C for 45 s, extension at 72°C for 45 s and final extension at 72°C for 7 min (Luzzaro et al., 2004), ACCF: 5`-AACAGCCTCAGCAGCCGGTTA-3`, ACCR: 5`-TTCGCCGCAATCATCCCTAGC-3`, LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1F: 5`-TGGCCAGAACTGACAGGCAAA-3`, LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1R: 5`-TTTCTCCTGAACGTGGCTGGC-3`, DHA-1, DHA-2F: 5`-AACTTTCACAGGTGTGCTGGGT-3`, DHA-1, DHA-2R: 5`-CCGTACGCATACTGGCTTTGC-3`, ACT-1F: 5`-TCGGTAAAGCCGATGTTGCGG-3`, ACT-1R: 5`-CTTCCACTGCGGCTGCCAGTT-3`, FOX-1 to FOX-5bF: 5`-AACATGGGGTATCAGGGAGATG-3`, FOX-1 to FOX-5bR: 5`-CAAAGCGCGTAACCGGATTGG-3`, MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11F: 5`-GCTGCTCAAGGAGCACAGGAT-3`, MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11R: 5`-CACATTGACATAGGTGTGGTGC-3`, cycling conditions were initial denaturation at 95°C for 2 min, 30 cycles of DNA denaturation at 94°C for 45 s, annealing at 62°C for 45 s, extension at 72°C for 1 min. Final extension at 72°C for 5 min (Perez-Perez and Hanson, 2002), AdeBF: 5`-GTATGAATTGATGCTGC-3`, AdeBR: 5`-CACTCGTAGCCAATACC-3`, AdeJF: 5`-TTCTTTGGTGGTACAACAGG-3`, AdeJR: 5`-GCTGCAATCAGTTTCTCATG-3`, AbeMF: 5`-TGCAACGCAGTTTCATTTTT-3`, AbeMR: 5`-CGATGTTTCATCGGCTTTTT-3`, MexAF: 5`-ACCTACGAGGCCGACTACCAGA-3`, MexAR: 5`-GTTGGTCACCAGGGCGCCTTC-3`, MexXF: 5`-CATCAGCGAACGCGAGTACAC-3`, MexXR: 5`-CAATTCGCGATGCGGATTG-3`, MexCF: 5`-AGCCAGCAGGACTTCGATACC-3`, MexCR: 5`-ACGTCGGCGAACTGCAACCGCTG-3`, MexEF: 5`-GTCATCGAACAACCGCTG-3`, MexER: 5`-GTCGAAGTAGGCGTAGACC-3`, cycling conditions were 10 min denaturation at 95°C, 30 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C. Final extension at 72°C for 10 min (Perez-Perez and Hanson, 2002).

DNA sequencing

After initial screening for the amplification of β-lactamases and efflux pump genes on both chromosomal and plasmid, the plasmid and chromosomal borne genes were subjected to nucleic acid sequencing. The initial PCR amplified products were purified and treated with QIAquick PCR Purification Kit (QIAgen Inc.,Valencia CA, USA).

Direct sequencing of each amplicon was carried out using the Sanger dideoxynucleotide chain termination method with the ABI Prism Big Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Inc., Foster City, CA, USA) on an ABI Prism 3500 Automated Sequencer. Using data collection software version 2.0, and sequencing analysis software 5.1.1, for each sequencing reaction, 2 µl purified PCR product were added to a final reaction volume of 10 µl containing 1× of sequencing buffer; 4 µl BigDye reaction mix; and 3.2 pM of each of the Forward and Reverse primer. The sequencing cycle was composed of two stages; stage one is denaturing at 96°C for 1 min, while stage two is composed of 25 cycles of denaturing at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min (Sabate et al., 2000).

Each cycle sequence product was purified by BigDye XTerminator Purification Kit. The purified PCR product was then placed in the DNA analyzer. The DNA sequences obtained were compared with those in the GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/).

RESULTS

Distribution of Gram negative microorganisms among respiratory tract isolates

Gram negative isolates were were *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae*, *Stenotrophomonas maltophilia, Escherichia coli, Enterobacter cloacae, Serratia rubidaea, Haemophilus influenza, Citrobacter koseri, Moraxella catarrhalis, Proteus mirabilis* and *Salmonella typhimurium* with the following frequencies respectively (34.6, 26.6, 13.9, 7.7, 6.4, 5.5, 1.4, 1.3, 1, 1, 0.3 and 0.3%. The distribution of organisms harboring β-lactamases and efflux pump among Gram negative respiratory tract isolates are illustrated in Table 2.

Detection and prevalence of beta-lactamases and efflux pump genes in Gram negative respiratory tract isolates

PCR and sequence analysis indicated the presence of bla_{SHV}, bla_{CTX-M}, bla_{TEM}, bla_{IMP}, bla_{VIM}, *ACC*, *DHA*, *AdeJ*, *MexX* and *MexE* genes in the isolated respiratory tract isolates with distribution illustrated in Table 3.

DNA sequencing results

Nucleotide composition analysis of some *A. baumannii* isolates showed that, the RND family drug transporter (*AdeJ*) gene detected was of GCwith value of 41.5 and the detailed composition was: T (30.7), C (20.8), A (27.8) and G (20.8). Among the studied 659 nucleotide bases compromising for *AdeJ* gene, 655 bases were conserved while only 4 sites were variable. Surprisingly, 3 out of the four base substitutions were transitional changes, from T→C (356 and 389) and C→T (566). Only one base substitution was transversional change from G→T (449) (Figure 4). Nucleotide composition analysis of some *P. aeruigenosa* isolates showed that, the multidrug efflux membrane fusion protein encoding gene (*MexE*) detected was of high GC htiw value of 71.1 and the detailed composition was: T (10.7), C (38.7), A (18.2) and G (32.4). Among the studied 458 nucleotide bases compromising for *MexE* gene, 455 bases were conserved while only 3 sites were variable. Two out of the three base substitutions were transitional changes, from A→G (15 and 39). Only one base substitution was transversional change from C→A (77) (Figure 5).

Table 2. Screening for ESβL, metallo β lactamase, Amp C and efflux pump production among Gram negative respiratory tract isolates.

Isolate	No. of isolates	ESβL		Metallo β lactamase		Amp C		Efflux pump	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
P. aeruginosa	107	39	68	55	52	92	15	78	29
A. baumannii	82	61	21	44	38	65	17	41	41
K. pneumoniaea	43	12	31	13	30	5	38	5	38
S. maltophilia	24	18	6	17	7	18	6	4	20
E. coli	20	10	10	9	11	5	15	8	12
E. cloacae	17	10	7	0	17	13	4	1	16
S. rubidaea	4	0	4	0	4	0	4	0	4
H. influenza	4	1	3	0	4	2	2	1	3
C. koseri.	3	0	3	0	3	1	2	1	2
M. catarhalis	3	1	2	2	1	2	1	1	2
P. mirabilis	1	1	0	1	0	1	0	1	0
S. typhimurium	1	0	1	0	1	0	1	1	0

Table 3. Prevalence of beta-lactamases and efflux pump genes in Gram negative respiratory tract isolates.

Name of organism	*bla*$_{SHV}$	*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV}$ and *bla*$_{CTX-M}$	*bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{IMP}$ and *bla*$_{VIM}$	*ACC*	*DHA-1, DHA-2*	*AdeJ*	*MexX*	*MexE*
A. baumannii	12	24	2	-	1	12	5	-	34	4	12	-	-
C. koseri	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli	1	2	5	-	-	1	1	3	-	-	-	-	-
E. cloacae	6	1	1	-	-	-	-	-	-	-	-	-	-
H. influenza	-	-	-	-	-	-	-	-	-	-	-	-	-
K. pneumoniaea	3	1	4	-	-	-	-	-	-	1	-	-	-
M. catarhalis	-	-	-	-	-	-	-	-	-	-	-	-	-
P. mirabilis	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	12	4	2	-	10	24	-	33	12	-	36	3
S. typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
S. maltophilia	-	-	6	-	-	-	-	-	-	-	-	-	-

Nucleotide composition analysis of some *P. aeruigenosa* isolates showed that, the periplasmic multidrug efflux lipoprotein encoding gene (*MexX*) detected was of high GC with value of 71 and the detailed composition was: T (13.3), C (37.4), A (15.3) and G (34). Among the studied 413

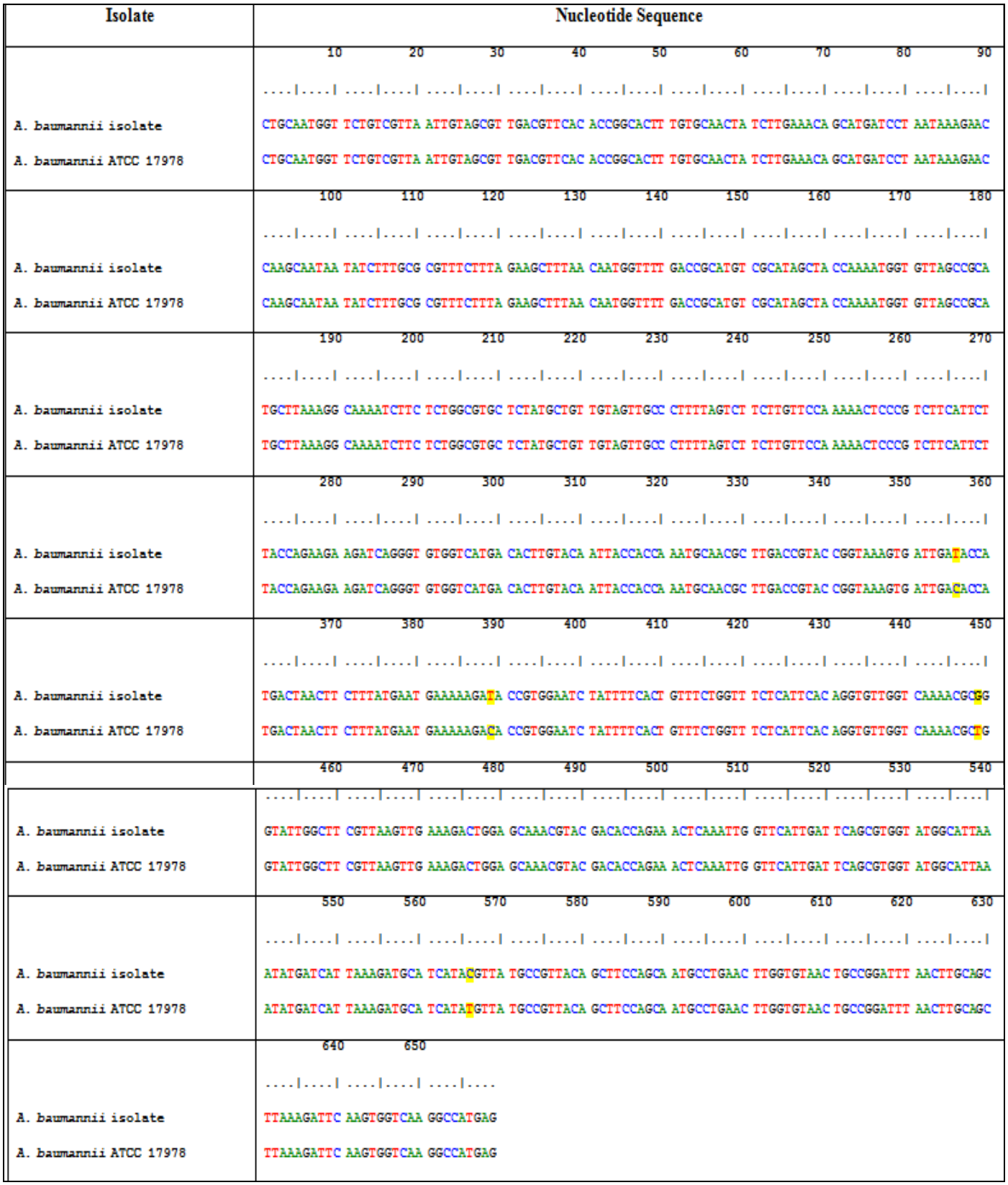

Figure 4. Multiple DNA sequence alignment of AdeJ gene isolated from *A. baummanii* tested isolate and retrieved sequences from Genbank.

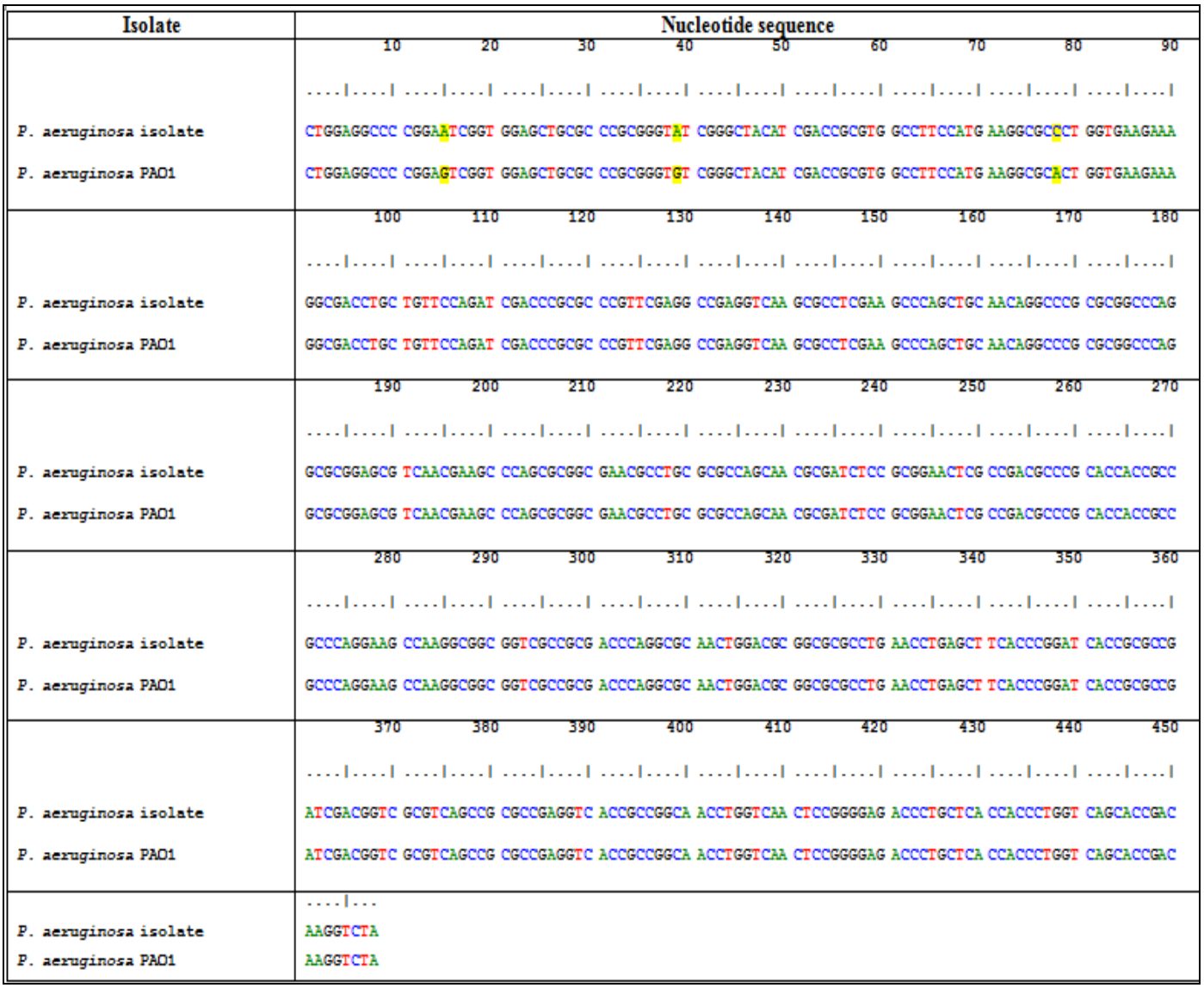

Figure 5. Multiple DNA sequence alignment of MexE gene isolated from *P. aeruginosa* tested isolate and retrieved sequences from Genbank.

nucleotide bases compromising for *MexX* gene, 411bases were conserved while only 2 sites were transitional changes, from T→C (20) and C→T (404) (Figure 6).

DISCUSSION

The present study proposes a combined phenotypic and genotypic approach for the specific diagnosis of antibiotic resistance mediated by β-lactamases and efflux pump system harboring Gram negative respiratory tract isolates.

In the present study, bla_{CTX-M} genes were predominant in *A. baumannii* and *P. aeruginosa* isolates with percentage of 39 and 31% respectively, followed by bla_{SHV} genes in *A. baumannii* and *E. cloacae* isolates with percentage of 20 and 60% respectively. bla_{TEM} genes were predominant in *E. coli*, *K. pneumoniaea* and *S. maltophilia* isolates with percentage of 50, 33 and 33%, respectively. bla_{SHV} genes were predominant in *E. cloacae* with percentage of 60%. Similar findings were found in Indian study (Gupta, 2007), from a total of 94 isolates, 50 (n = 47), 14.89 (n = 14) and 11.70 (n = 11) ESβL rates for bla_{TEM}, bla_{SHV} and bla_{CTX-M} type beta lactamases, respectively. bla_{TEM} and bla_{CTX-M} type ESβL

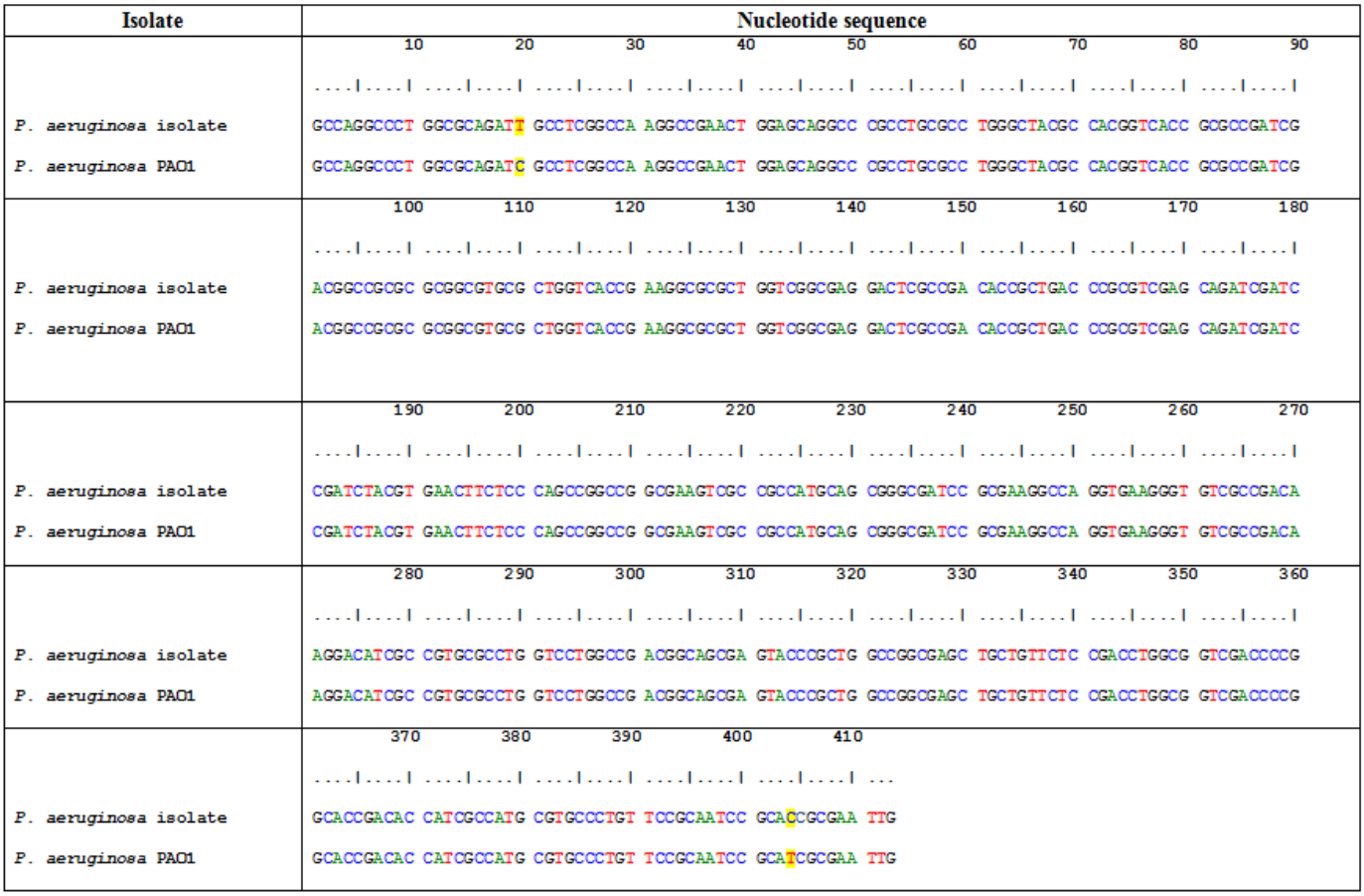

Figure 6. Multiple DNA sequence alignment of MexX gene isolated from *P. aeruginosa* tested isolate and retrieved sequences from Genbank.

were observed in 72.72 and 22.72% of *E. coli* isolates, respectively. Also, the present study revealed that bla_{IMP} gene was predominant in *A. baumannii* isolates with percentage of 27%, followed by bla_{VIM} gene 11%. bla_{VIM} gene was predominant in *P. aeruginosa* isolates with the percentage of 44%, followed by bla_{IMP} gene 18%. Both bla_{IMP} and bla_{VIM} genes were found together in *E. coli* isolates with the percentage of 33% followed by 11% of each alone. This was in accordance with Nordman and Poirel (2002), were a total of 8 pseudomonas isolates carried bla_{VIM} -type gene, these data demonstrate that bla_{VIM}-type gene are the most prevalent MβLs among clinical specimens of *P. aeruginosa*. *ACC* gene was predominant in *A. baumannii* and *P. aeruginosa* isolates with percentage of 52 and 36%, respectively, followed by *DHA-1*, *DHA-2* genes with 13 and 6% respectively. This result differs significantly from the findings of several studies were the isolation numbers of *ACC* enzymes were still significantly lower than those of *CIT (CMY)*, *FOX* and *DHA* (Philippon et al., 2002). *AdeJ* was detected in *A. baumannii* with percentage of 29.2%, while *MexX* gene was predominant in *P. aeruginosa* isolates with percentage of 46% followed by *MexE*, 3.8%. This differ from the findings of some biological observations made during a study where the basal expression level of *MexX* is much lower than that of *MexA* but that both efflux pumps are over-expressed 4 to 8 times in resistant strains, suggesting that a lower quantity of MexXY-OprM than MexAB-OprM protein may be needed for effective transport of the corresponding substrates (Llanes et al., 2004). Second, over-expression of *MexX* in clinical isolates is systematically associated with that of *MexA*. This may be related to the fact that MexXY uses OprM as a porin (Masuda et al., 2000).

Conclusion

The study figured out the most common genes responsible for the expression of β-lactamase enzymes and efflux pump system in Gram negative respiratory tract isolates. The study also revealed that, isolates

harboring more than one gene from the same class have higher resistance pattern towards antimicrobial agents than those harboring only one; also, isolates having microevolutionary changes in their nucleotide composition of the detected genes have higher resistance pattern towards antimicrobial agents than those where all bases are conserved.

Conflict of interests

The authors did not declare any conflict of interests.

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Full Length Research Paper

Antimicrobial and antioxidant activities of red onion, garlic and leek in sausage

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This study was designed to evaluate antimicrobial and antioxidant effect of ethanolic and aqueous extracts and essential oils of red onion, garlic and leek against *Escherichia coli* O_{157}:H_7, *Staphylococcus aureus*, A.F., 4, *Salmonella typhimurium*, A.F., 3, *Aspergillus niger*, H.U.B., *1*, *Aspergillus ochrecies,* H.U.B.,12 and *Fusarium oxysporum, H.U.B.,* 3 in sausage. The susceptibility of these isolates toward the extracts of these plants was compared with some antibiotics (oxytetracycline, tetracycline, ampicillin and amoxicillin) used as positive control. The phenolic contents and stable free radical 2,2-diphenyl-1-picrylhydrazyl was determined. Results show that the concentration, 60 mg/ml of ethanolic extracts and essential oils represented the optimum concentration against all microorganisms. The essential oils exhibited higher effect than ethanolic and aqueous extracts against all tested microorganisms, especially at 60 mg/ml concentration of leek essential oils, whereas red onion, garlic and leek essential oils showed a stronger antimicrobial activity for decreasing count of *E. coli* O_{157}:H_7, *S. aureus, A.F., 4* and *Salmonella typhimurium, A.F.,* 3 in sausage. The phenolic contents and antioxidant activity were higher in essential oils of garlic, leek and red onion whereas the lower contents and activity were shown in aqueous extracts. The essential oils of red onion had the highest phenolic contents and antioxidant activity in contrast to garlic essential oils.

Key words: Microorganisms, antioxidant, essential oils, ethanolic, aqueous extracts, onion, garlic, leek.

INTRODUCTION

The presence of spoilage microorganisms in food can accelerate the lipid oxidation and other oxidation processes, or can produce changes in the organoleptic properties of the foods, specially the fungi that can give some characteristic color to the food due to its growth (Saggiorato et al., 2012). Most of the foods borne bacterial pathogens are sensitive to extracts from plants such as garlic, mustard, onion and oregano. Gram-positive bacteria are more sensitive to antimicrobial compounds in spices than Gram-negative bacteria (Lawson, 1996). Essential oils extracts have been considered as natural preservatives or food additives, and can be used as additional methods of controlling pathogens (Naidu, 2000). Besides the antibacterial, antifungal and anti-inflammatory activities, many essential oils (Eos) also have been confirmed to possess antioxidant activity

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(Elaissi et al., 2011; Prakash et al., 2012; Viuda- Martos, et al., 2010).

Onion (*Allium cepa* L.), garlic (*Allium sativum* L.), leek *(Allium porrum*) and other edible *Allium* are among the oldest cultivated plants, and all belong to a family of Alliaceae. They are a rich source of several phytonutrients, and recognized to have significant and wide biological activities (Benkeblia and Lanzotti, 2007). Onion is commonly used as a spice in Turkey especially in ground beef, doner kebab, meat ball and raw meat balls-cig kofte; it may also be used to reduce pathogenic microorganisms contamination during unhygienic productions (Degirmencioglu and Irkin, 2009). Onion aqueous extracts are effective against many yeast species and several G (+) bacteria but ineffective against G (-) bacteria (Benkeblia, 2004; Ghahfarokhi- Shams et al., 2006). A strong antimicrobial effect of fresh onion homogenates was due to both methyl cysteine sulfoxide and S-n- propyl cysteine sulfoxide from which the corresponding thio- sulfinates are formed enzymatically (Kyung and Lee, 2001). Onion is an important food because it supplies various activated phytomolecules such as phenolic acid, flavonoids, copaenes, thiosulfinate, organosulfur compounds (OSCs), and anthocyanin (Slimestad et al., 2007). Among the species of onions, the red onion is abundant in polyphenols, flavonoids, flavonol and tannin (Gorinstein et al., 2010). The essential oil from *A. cepa* may be a new potential source of natural antimicrobial and antioxidant agents applied in food systems (Ye et al., 2013).

Garlic is one of the most commonly used ingredients as a flavor enhancer for sausage (Harris et al., 2001). Garlic is effective against bacteria, protozoa, fungi and some viruses (Jaber and Al-Mossawi, 2007). Thiosulfinates play a major role in the antibiotic activity of garlic (Durairaj et al., 2009). Addition of fresh garlic and garlic powder produced significant antioxidant and antimicrobial effects and extended the shelf-life during refrigerated storage (Dewi et al., 2010).

Leek extracts showed positive antioxidant activities and positive antimicrobial activities (Kyoung-Hee et al., 2012). Aqueous extract of A. *porrum* had antimicrobial activity against six bacterial species: three Gram-positive bacteria (*Bacillus subtilis, Streptococcus pneumonia* and *Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli, Proteus vulgaris* and *Pseudomonas aeruginosa*). The biologically active components include amino acid with sulphate, glycol-sides, saponin and phenol present in the aqueous extract (Naem-Rana and Hadi-Noora, 2012).

Aspergillus species are the most common fungal species which are able to produce mycotoxins in food and feed stuffs and these mycotoxins are known to be potent hepatocarcinogens in animal and humans (Soliman and Badeaa, 2002). The presence and growth of fungi may cause spoilage and result in reduction in quality and quantity of foods (Rasooli and Abyaneh, 2004).

The purpose of this study was to investigate the anti-bacterial and antifungal activities of onion, garlic and leek against some pathogenic bacteria and fungi *in vitro* and in beef sausage. Also, the total phenolic contents and antioxidant properties of studied plant materials were assessed.

MATERIALS AND METHODS

Plant materials

Fresh garlic (*A. sativum* variety), leek (*A. porrum* variety) and red onion (*Allium cepa* variety) were purchased from the local market, Cairo, Egypt. They were peeled, sliced, and dried in an air dry oven at 40°C, then ground to a fine powder using an electrical mill, and kept in polyethylene bags in a refrigerator at 4°C for further analysis.

Microorganisms

Three pathogenic bacterial strains *(E. coli* O_{157}*: H7, S. aureus***,** A.F., *4* and *S. typhimurium,* A.F., *3)* and three pathogenic fungal strains *(A. niger,* H.U.B., *1, Aspergillus ochrecies,* H.U.B., 2 and *Fusarium oxysporum***,** H.U.B., *3)* were used throughout this study. Bacterial strains were obtained from Dr Abdel-Salam. A.F., Regional Center for Food and Feed, ARC, Giza Egypt. Fungal strains were obtained from Biotechnology Department, Heliopolis University, Cairo, Egypt. The bacteria were selected because they are frequently reported in food spoilage, while the selected fungi are commonly encountered in onions and responsible for bulb diseases. The isolates were stored at 4°C. The symbols and numbers of the end of microbial syntific name are code numbers for microbial registration of each strain.

Essential oils extraction

The dried samples were subjected to steam distillation using method of Aqel and Shaheen (1996). 10 g of powdered sample were added to 200 ml distilled water and extraction was carried out by steam distillation. The process continued until about 200 ml of distillate was collected. The distillate was extracted 3 times with chloroform. After removing moisture by using anhydrous sodium sulphate, the extract was evaporated on a water bath (40°C). Stock solutions of crude essential oils were prepared by diluting the dried essential oils with 10% dimethyl sulphoxide (DMSO) solution.

Preparation of aqueous and ethanolic extracts

Ten grams of each dry powder samples were extracted with 100 ml of 80% ethanol or distilled water in a screw-capped flask and shaken at room temperature for 24 h. The extracts were filtered through Whatman paper (No. 1) and the solvent was removed using rotary vacuum evaporator at 40°C, then the concentrated extracts were restored in a freezer at -20°C until analyzed. Stock solutions of crude extracts were prepared by diluting the dried extracts with dimethyl sulphoxide (DMSO) solution.

Preparation of inoculum

Bacterial inoculum was prepared by growing in Brain-Heart Infusion broth (Merck, Darmstat, Germany) for 24 h at 37°C. All bacteria tested were enumerated by using the serial dilution method on specific selective agar for each strain.

Fungi was cultured on potato dextrose agar (PDA) medium for 7-10 days at 30°C, and then fungi suspension for each strain was prepared.

Preparation of sausage

Beef meat, animal fat, sheep casing and salt were purchased from local market. Sausages were manufactured according to the following traditional formula, 55% lean beef meat and a maximum of about 25% animal fat, 3% salt and 21% ice water. This mixture additives was added, and filling in casings using filler (Moulinex, France) according to the method of Nowak et al. (2007). Sausages were examined for presence of *E. coli* O_{157}*: H7, Saureus and Salmonella typhimurium.*

Antibacterial and antifungal activity

The plant extract (ethanolic, essential oils and aqueous) were prepared from onion, garlic and leek at different concentrations (20, 40 and 60 mg/ml) and antibiotics (oxytetracycline, 30 µg; tetracycline, 30 µg; ampicillin 10 µg and amoxicillin, 25µg) then tested for antibacterial and antifungal activity. Antibiotics were used for the comparison with the plant extractions.

Agar disc diffusion method

The agar disc diffusion method is the most widespread technique of antibacterial and antifungal activity assessment. This method is normally used as a preliminary check and to select the best efficient against pathogenic bacteria and fungi (Nedorostova et al., 2009). Nutrient Agar was used for *E. coli* O_{157}*: H7*, *S. aureus* and *S. typhimurium* after inoculated with bacterial inoculums. Potato dextrose agar (PDA) was used for *A. niger*, *A. ochecies* and *F. oxysporum* after inoculation with fungal inoculum.

The microorganisms (bacteria or fungi) and growth media were mixed thoroughly to ensure uniform distribution of the microorganisms. The sterile filter discs (Whatman No 1, Maidstone, England, 6 mm diameter) were impregnated with 30 µl of each dilution to each filter paper disc and placed on the agar surface using forceps dipped in ethanol and flamed. All Petri dishes were sealed with sterile laboratory parafilm and left for 30 min at room temperature to allow the diffusion of extractions. Antibiotics discs were placed on the agar surface with the same media mentioned before for bacteria and fungi using forceps dipped in ethanol and flamed also. Plates for antimicrobial activity test were incubated at 37°C for 24-48 h. Plates for antifungal activity test were incubated at 30°C for 7-10 days. After the incubation period, the mean diameter of inhibition halo where test microorganism did not grow (clearly visible inhibition zone) was measured in millimeter, for each disc and evaluated for susceptibility or resistance (Konman et al., 1997).

Application effect of essential oils on growth of *Escherichia coli* O_{157}*: H7*, *S. aureus* and *S. typhimurium* in beef sausage

Sausage samples were divided into nine groups (the group composed of spices with 25 g each) three groups dipped in *E. coli* O_{157}*: H7* culture (about 10^8 cfu/ml) for 5 min, three groups dipped in *S. aureus* culture (about 10^8 cfu/ml) for 5 min and another three groups dipped in *S. typhimurium* culture (about 10^8 cfu/ml) for 5 min and then left all inoculated groups with *E. coli* O_{157}*:H7* or *S. aureus* or *S. typhimurium* for 10 min under aseptic condition before initial cfu/g was determined, then dipped in different essential oils of onion, garlic and leek at 40 mg/ml concentration for 15 min and the mean cfu/g was determined for *E. coli* O_{157}*: H7, S. aureus* and *S. typhimurium*.

Determination of total phenolic contents

The total phenolics in the extracts were estimated by spectrophotometric assay (Barreira et al., 2008). One milliliter of sample (concentration 1 mg/mL) was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of sodium carbonate solution (7.5%) was added to the mixture and made up to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm in a spectrophotometer against a blank sample. Gallic acid was used for constructing the standard curve (20-100 µg/mL) and the results were expressed as µg of gallic acid equivalents (GAE)/mg of extract and the values are presented as means of triplicate analyses.

Determination of the scavenging activity by using DPPH

The radical scavenging activity of antioxidants of plant essential oils, ethanolic extracts and water extracts against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured using the method of Barros et al. (2007). 300 µl of samples were taken in different test tubes. 2.7 ml of 6×10^5 mol/l DPPH solution made up with DPPH (4.8 mg) in methanol (200 ml) was added to these tubes. The mixture was shaken and left to stand at room temperature in the dark for 90 min. Absorbance of the resulting solution was measured at 517 nm by a UV visible spectrophotometer. The readings were compared with controls, which contained 300 µl of methanol instead of the extract. Methanol was used as blank. Radical scavenging activity (RSA) was expressed as the inhibition percentage and was calculated using the following equation:

$$RSA\ (\%) = [(A_{DPPH} - A_S) / A_{DPPH} \times 100]$$

Where, A_S is the absorbance of the solution when the sample extract is added and A_{DPPH} is the absorbance of the DPPH solution.

Statistical analysis

Duncan's multiple range test was used to test significance of means of 5 replicates of samples according to IBM ® SPSS ® Statistics software (IBM, 2011).

RESULTS AND DISCUSSION

Red onion, garlic and leek extracts exhibited different inhibition levels against *E. coli*O_{157}*: H7, S. aureus*, A.F., 4*, S. typhimurium*, A.F., 3*, A. niger*, H.U.B., No.1*, A. ochrecies*, H.U.B., 2 and *F. oxysporum*, H.U.B., 3 as shown in Tables 1, 2 and 3. In the dose response study, the inhibition zone increased with increasing concentration of extracts.

Inhibition zone (mm) of red onion extracts (mg/ml) on different microorganisms

The data recorded in Table 1 obviously showed that *S. typhimurium*, A.F., 3 was more sensitive to ethanolic and

Table 1. Inhibition zone (mm) of red onion extracts (mg/ml) on different microorganisms.

Microorganism	Plant extract										
	Control	Ethanolic			Control	Essential oil			Control	Aqueous		
		20	40	60		20	40	60		20	40	60
E. coli O_{157}:H_7	5^{e}	8^{d}	10^{c}	13^{b}	7^{d}	11^{c}	13^{b}	15^{a}	0^{f}	0^{f}	8^{d}	10^{c}
S. aureus , A.F., 4	6^{e}	9^{d}	11^{cd}	14^{b}	9^{d}	12^{c}	14^{b}	16^{a}	0^{f}	0^{f}	9^{d}	11^{cd}
S. typhimurium , A.F., 3	5^{f}	10^{d}	12^{c}	15^{b}	7^{e}	13^{c}	16^{b}	18^{a}	0^{g}	0^{g}	9^{d}	12^{c}
A. niger, H.U.B., 1	5^{e}	7^{d}	9^{bc}	10^{ab}	5^{e}	8^{cd}	10^{ab}	11^{a}	0^{f}	0^{f}	0^{f}	0^{f}
A. ochrecies , H.U.B., 2	5^{e}	8^{d}	10^{bc}	11^{b}	5^{e}	9^{cd}	11^{b}	13^{a}	0^{f}	0^{f}	0^{f}	0^{f}
F. oxysporum , H.U.B., 3	5^{e}	9^{cd}	10^{bc}	12^{ab}	6^{e}	10^{bc}	11^{ab}	13^{a}	0^{f}	7^{de}	9^{cd}	10^{bc}

Values in the same row followed by the same letter (s) do not significantly differ from each other according to Duncan's at 5% level.

Table 2. Inhibition zone (mm) of garlic extracts (mg/ml) on different microorganisms.

Microorganism	Plant extract										
	Control	Ethanolic			Control	Essential oil			Control	Aqueous		
		20	40	60		20	40	60		20	40	60
E. coli O_{157}:H_7	5^{e}	7^{de}	9^{c}	11^{bc}	7^{de}	11^{bc}	13^{b}	15^{a}	0^{f}	7^{de}	7^{de}	8^{d}
S. aureus , A.F., .4	6^{f}	9^{de}	11^{d}	13^{c}	9^{de}	14^{c}	17^{b}	19^{a}	0^{g}	7^{df}	8^{df}	9^{de}
S. typhimurium , A.F., 3	5^{e}	7^{de}	10^{bc}	11^{b}	7^{de}	10^{bc}	12^{b}	14^{a}	0^{f}	7^{de}	8^{cd}	8^{cd}
A. niger, H.U.B., 1	5^{e}	7^{d}	7^{d}	9^{b}	5^{e}	8^{cd}	10^{b}	14^{a}	0^{f}	0^{f}	0^{f}	0^{f}
A. ochrecies , H.U.B., 2	5^{d}	7^{c}	8^{bc}	9^{b}	5^{d}	8^{bc}	9^{b}	12^{a}	0^{e}	0^{e}	0^{e}	0^{e}
F. oxysporum , H.U.B., 3	5^{g}	8^{efc}	10^{bc}	11^{b}	6^{fg}	9^{bce}	11^{b}	15^{a}	0^{h}	7^{efg}	8^{cef}	10^{bc}

Values in the same row followed by the same letter (s) do not significantly differ from each other according to Duncan's at 5% level.

Table 3. Inhibition zone (mm) of leek extracts (mg/ml) on different microorganisms.

Microorganism	Plant extract										
	Control	Ethanolic			Control	Essential oil			Control	Aqueous		
		20	40	60		20	40	60		20	40	60
***E.coli* O_{157}:H_7**	5^{f}	8^{de}	10^{cd}	12^{bc}	7^{ef}	12^{bc}	14^{b}	16^{a}	0^{g}	7^{ef}	9^{de}	10^{cd}
***St. aureus* , A.F., 4**	6^{h}	10^{defg}	13^{d}	15^{c}	9^{efg}	16^{c}	19^{b}	22^{a}	0^{i}	8^{fg}	11^{def}	12^{de}
***S.typhimurium* , A.F., 3**	5^{f}	9^{de}	11^{bcd}	13^{ab}	7^{ef}	12^{bc}	13^{ab}	15^{a}	0^{g}	7^{ef}	7^{ef}	10^{bcd}
***A. niger, H.U.B.,* 1**	5^{e}	8^{cd}	11^{b}	12^{b}	5^{e}	10^{bc}	11^{b}	14^{a}	0^{f}	7^{de}	8^{cd}	10^{bc}
***A. ochrecies , H.U.B.,* 2**	5^{e}	7^{de}	9^{bcd}	11^{ab}	5^{e}	10^{bc}	11^{ab}	13^{a}	0^{f}	7^{de}	8^{cd}	10^{bc}
***F. oxysporum , H.U.B.,* 3**	5^{f}	8^{de}	10^{cd}	12^{bc}	6^{ef}	10^{cd}	13^{b}	15^{a}	0^{g}	7^{ef}	8^{de}	10^{cd}

Values in the same row followed by the same letter (s) do not significantly differ from each other according to Duncan's at 5% level.

aqueous extracts and essential oils of onion than *E. coli* O_{157}*: H_7, S. aureus*, A.F., 4, *A. niger*, H.U.B., 1*, A. ochrecies*, H.U.B., 2 and *F. oxysporum*, H.U.B., No.3 as exhibited by inhibition zones 10, 12, 15, 13, 16 and 18 diameter mm for ethanolic extract and essential oils at different concentrations (20, 40, 60 mg/ml), respectively and 9 and 12 mm for aqueous extract at concentrations (40 and 60 mg/ml, respectively). *F. oxysporum* exhibited inhibition zones 7, 9 and 10 mm for aqueous at concentrations (20, 40 and 60 mg/ml) respectively.

A. niger, H.U.B., 1 was less susceptible than other microorganisms for ethanolic extract and essential oils of onion which exhibited inhibition zones of 7, 9, 10, 8, 10 and 11 mm at concentrations of 20, 40, 60 mg/ml of onion respectively.

All pathogenic bacteria (*E. coli* O_{157}*: H_7, Staph aureus*, A.F., 4 and *Salmonella typhimurium*, A.F., 3) were not sensitive to aqueous extract at 20 mg/ml concentration. *A. niger*, H.U.B., 1 and *A. ochrecies*, H.U.B., 2 were also unsusceptible to aqueous extract at different concentrations (20, 40, 60 mg/ml). The essential oils at concentration of 60 mg/ml of red onion extracts exhibited significantly higher inhibition zone than other tested extractions.

Inhibition zone (mm) of garlic extracts (mg/ml) on different microorganisms

Data in Table 2 showed that *S. aureus, A.F.,* 4 was the most sensitive of the microorganisms to ethanolic extract and essential oils of garlic such exhibiting 9, 11, 13, 14, 17 and 19 mm inhibition zones at the different concentrations (20, 40 and 60 mg/ml) respectively.

Almost all effect of the different concentrations (20, 40 and 60 mg/ml) of garlic aqueous extract on *E. coli* O_{157}*: H_7, S. aureus A.F.,* 4*, S. typhimurium A.F.,* 3 and *F. oxysporum, H.U.B.,* 3 were similar. On the other hand, *A. niger*, *H.U.B.,* 1 and *A .ochrecies, H.U.B.,* 2 were not sensitive to garlic aqueous extract at concentrations (20 and 40 mg/ml). The essential oils at concentration of 60 mg/ml of garlic extracts revealed significantly higher inhibition zone than other tested extractions.

Inhibition zone (mm) of leek extracts (mg/ml) on different microorganisms

Data reported in Table 3 clearly showed that essential oils of leek at 60 mg/ml concentration exhibited higher antimicrobial activity against *S aureus, A.F.,* 4 where the inhibition zone reached 22 mm in diameter. On the other hand, the zone of inhibition reached 16, 15, 14, 13 and 15 mm in diameter against of *E. coli* O_{157}*: H_7, Salmonella typhimurium, A.F.,* 3, *A. niger, H.U.B.,* 1*, A. ochrecies , H.U.B.,* 2 and *F .oxysporum , H.U.B.,* 3 respectively. All microorganisms were sensitive to aqueous extract of leek at different concentrations (20, 40 and 60 mg/ml), but 60 mg/ml concentration exhibited high effect against *S. aureus, A.F.,* 4 in 22 mm diameter. The maximum inhibition of ethanolic extract was recorded at 60 mg/ml concentration against *E. coli* O_{157}*: H_7* 12 mm for *S. aureus, A.F.,* 4, 15 mm, for *S. typhimurium, A.F.,* 3, 13 mm, for *A. niger , H.U.B.,*1, 12 mm, for *A. ochrecies, H.U.B.,* 2 and 11 mm for *F. oxysporum , H.U.B.,* 3 in 12 mm.

Different concentrations (20, 40 and 60 mg/ml) of onion, garlic and leek essential oils were more effective than ethanolic and aqueous extracts against *E. coli* O_{157}*: H_7, S. aureus, A.F.,* 4*, S. typhimurium, A.F.,* 3*, A. niger, H.U.B.,*1*, A. ochrecies , H.U.B.,* 2 and *F .oxysporum , H.U.B.,* 3*.* Therefore could be used as essential oils for treatment of food to get rid of some pathogenic bacteria which contaminate meat products e.g*. E. coli* O_{157}*: H_7, S. aureus* and *S. typhimurium.* The essential oils at concentration of 60 mg/ml of leek extracts had significantly higher inhibition zone than other tested extractions.

Table 4. Effect of essential oils (mg/ml) of onion, garlic and leek on the viable count growth of *E. coli* O_{157}*: H_7, S aureus, A.F.,* 4 and *Salmonella typhimurium, A.F.,* 3 in beef sausage (cfu/g x 10^3).

Microorganism	Essential oils (40 mg/ml)		
	Onion	Garlic	Leek
E. coli O_{157}*:H_7*	80^a	90^a	8^b
S. aureus , A.F., 4	50^b	70^a	4^c
S. typhimurium , A.F., 3	60^b	30^a	20^b

Values in the same row followed by the same letter(s) do not significantly differ from each other according to Duncan's at 5% level; *The used inoculum of *E. coli* O_{157}*: H_7* was 5 x10^8 cfu/ml *The used inoculum of *S. aureus , A.F.,* 4 was 6 x10^8 cfu/ml;*The used inoculum of *S. typhimurium , A.F.,* 3 was 7 x 10^8 cfu/ml.

Effect of essential oils (mg/ml) of red onion, garlic and leek on survival and growth of *E. coli*O_{157}*: H_7, S. aureus* and *S. typhimurium in beef sausage*

The obtained results (Table 4) clearly showed that the concentration of 40 mg/ml of onion essential oils was able to decrease *E. coli* O_{157}*: H_7, S. aureus, A.F.,* 4 and *S. typhimurium, A.F.,* 3 counts in beef sausage from 5x10^8 to 8x10^4, 6x10^8 to 5x10^4 and 7x10^8 to 6x10^4 cfu/g, respectively. Garlic essential oil decreased *E. coli* O_{157}*: H_7, S. aureus*, *A.F.,* 4 and *S. typhimurium, A.F.,* 3 counts in sausage from 5x10^8 to 9x10^4, 6x10^8 to 7x10^4 and 7x10^8 to 3x10^5 cfu/g, respectively. In addition, leek essential oils revealed higher antimicrobial activity in decreasing of *E. coli* O_{157}*: H_7, S. aureus, A.F.,* 4 and *S. typhimurium, A.F.,* 3 counts in sausage from 5x10^8 to 8x10^3, 6x10^8 to 4x10^3 and 7x10^8 to 2x10^4 cfu/g, respectively than other tested essential oils of onion and

Table 5. Inhibition zone (mm) of different concentrations of antibiotics (µg) against different microorganisms.

Microorganism	Antibiotic (µg)			
	Oxytetracycline	Tetracycline	Ampicillin	Amoxicillin
	30	30	10	25
E. coli O_{157}:H_7	25[a]	23[b]	0[d]	17[c]
St. aureus , A.F., 4	25[a]	20[b]	0[d]	13[c]
S. typhimurium , A.F., 3	30[a]	26[b]	0[c]	25[b]
*A. niger, H.U.B.,*1	0	0	0	0
A. ochrecies , H.U.B., 2	0	0	0	0
F. oxysporum , H.U.B., 3	0	0	0	0

Values in the same row followed by the same letter (s) do not significantly differ from each other according to Duncan's at 5% level.

leek. It could be concluded that the essential oils of leek showed significantly pronounced effect on the reduction of all counts of tested microorganisms.

The extent of the inhibitory effect of the onion extracts could be attributed to the presence of antimicrobial compounds and their dissolving ratios in the solvents and concentration doses. The same observations were reported by Jeyakumar et al. (2005) who reported the bacterial effect of onion extracts against *E. coli, S. aureus* and *S. entritids* by using agar diffusion method. Srinivasan et al. (2001) reported moderate antibacterial activity of an onion extract against *E. coli* and *S. typhimurium*. Indu et al. (2006) demonstrated that the various concentrations of an onion extract field inhibited the growth of *S. entritidis* and *S. typhimurium*. Good antibacterial activity of an onion extract on the growth of *S. entritidis* was also reported by Suresh et al. (2006). On the other hand, Elnima et al. (1983) reported that 66% of aqueous extracts of red onion inhibited the growth of *S. aureus*. In other families, the ethanol extract showed more activity than the water extracts perhaps due to the increased solubility of the active principle in ethanol (Vaijayanthimala et al., 2000). The major antimicrobial compound in garlic is allicin (Conner, 1993). Garlic extracts have been found to possess antibacterial property against *S. typhimurium, E .coli* No.1*, Staphylococcus epidermidis* and *Staphyloccoccus aureus* (Arora and Kaur, 1999). The inhibitory activity of EO extracts of *Allium* plants against mold was reported by Zaika (1988). Garlic showed highest antifungal activity against three *Aspergillus* species tested (Yin and Tsao, 1997).

Benkeblia (2004) stated that *F. oxysporum* showed the lowest sensitivity towards EO extracts of garlic and onion whereas *A. niger* No.1 was significantly inhibited particularly at low concentrations. Bandna (2013) mentioned that garlic extracts exhibited excellent antibacterial activity against *E. coli, S. typhi* and *S. aureus*. In contrast, water extracts of garlic were reported to be more potent than ethanol and chloroform extracts against the tested microbes in the study of Abubakar (2009). The ethanolic extract of garlic was more effective than the aqueous extract, inhibiting all the test organisms. While the aqueous extract was effective against *E. coli*, *P. aeruginosa* and *Klebsiella pneumonia* (Arekemase et al., 2013). Ethanolic extract of garlic significantly inhibited growth of *A. flavus* and *A. niger* (Onyeagba et al., 2004). Dankert et al. (1979) reported that garlic extracts were very effective in inhibiting the growth of *Aspergillus* species. As observed by Naem-Rana and Hadi-Noora (2012), aqueous extract of *Aporrum* (leek) had antimicrobial activity against *S. aureus, P. aeruginosa* and *E. coli*. Benkeblia et al. (2005) showed that leek inhibited the growth of *E. coli* and *S. aureus*. As explained by Breu and Dorsch (1994) aqueous and alcoholic extracts of leek leaves have a powerful antibacterial activity as they inhibit the growth of *E. coli, S. aureus, S. marcesceus, P. aeruginosa* and *S. typhi*.

Inhibition zone (mm) different concentrations of antibiotics (µg) against different microorganisms

Data recorded in Table 5 revealed that *S. typhimurium, A.F.,*3 was more susceptible than *E. coli* O_{157}:H_7 and *S. aureus, A.F.,*4 against *oxytetracycline* 30 µg, *tetracycline* 30 µg and amoxicillin 25 µg which exhibited inhibition zone diameter of 30, 26 and 25 mm for oxytetracycline 30 µg, tetracycline 30 µg and amoxicillin 25 µg, respectively, while both *E. coli* O_{157}:H_7, *S. aureus, A.F.,*4 and *S. typhimurium, A.F.,*3 were not sensitive to ampicillin 10 µg. On the other hand, both *A. niger, H.U.B.,*1*, A. ochrecies, H.U.B.,*2 and *F. oxysporum , H.U.B.,* 3 were unsusceptible to oxytetracycline 30 µg, tetracycline 30 µg, ampicillin 10 µg and amoxicillin 25 µg.

The antimicrobial activity of ethanolic and aqueous extracts and essential oils of red onion, garlic and leek were compared with that of antibiotics (oxytetracycline, tetracycline, ampicillin and amoxicillin) which is considered to be accepted in the treatment of diseases caused by types of standard bacteria and fungi species. Ampicillin is not appropriate for infections caused by *E.*

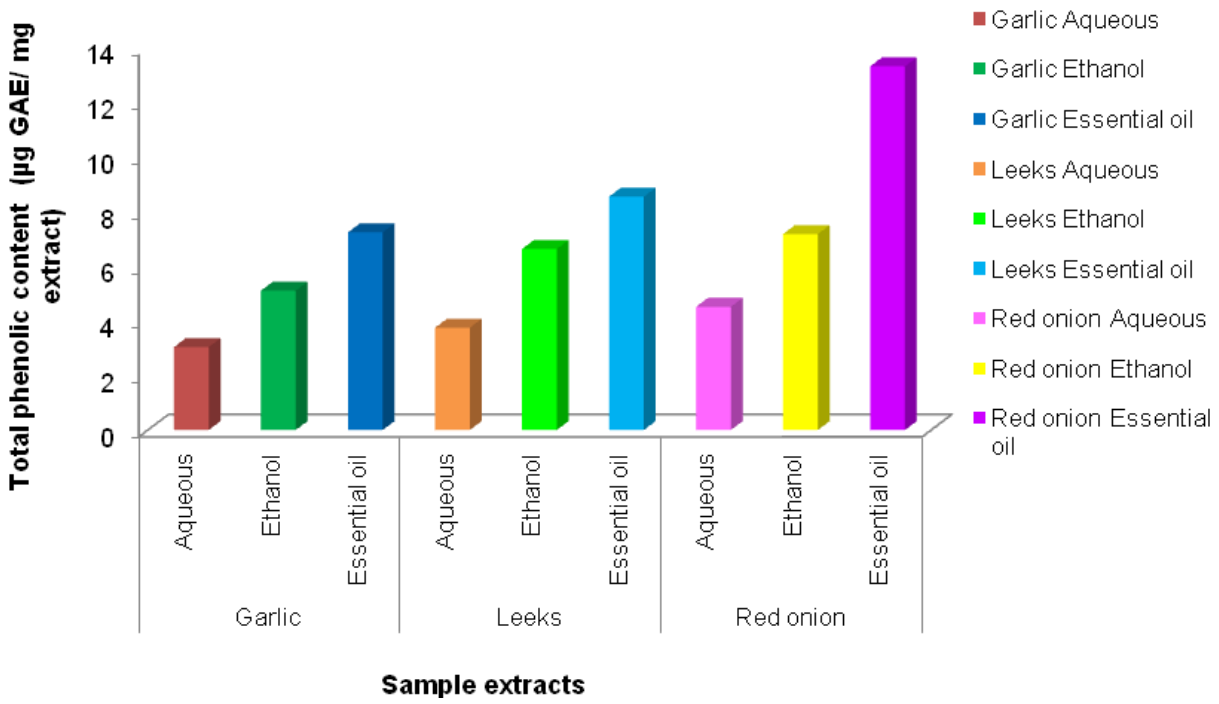

Figure 1. Total phenolic contents of the essential oils, aqueous and ethanolic extracts of red onion, garlic and leek.

coli O_{157}:H_7, *S. aureus, A.F.,*4, *S. typhimurium , A.F.,* 3, *A. niger, H.U.B.,*1, *A .ochrecies, H.U.B.,*2 and *F. oxysporum, H.U.B.,*3, but oxytetracycline, tetracycline and amoxicillin can be used as a broad spectrum drug against these pathogenic bacteria, such can be substituted for natural antimicrobial agents. Oxytetracycline showed significantly higher inhibition zone than other tested antibiotics.

Total phenolic content

Total phenolic contents of the essential oils, aqueous and ethanolic extracts of red onion, garlic and leek are presented in Figure 1. In general, the total phenolic contents were higher in essential oils extracts of red onion, garlic and leek whereas the lower contents were shown in aqueous extracts. The highest level of phenolic contents of red onion, garlic and leek were found in red onion (13.34 µg GAE/ mg extract), while the lowest contents were found in garlic (7.25 µg GAE/ mg extract). A similar observation has been reported by Gorinstein et al. (2008) who found that the total concentration of phenolic was higher in red onion than in white onion and in garlic.

DPPH radical scavenging activity

DPPH is a free radical compound that has been widely used to determine the free radical scavenging capacity of various samples because of its stability (in radical form), simplicity and fast assay. The results of DPPH free radical-scavenging ability of the essential oils aqueous and ethanolic extracts of red onion, garlic and leek are shown in Figure 2.

The present investigation depicts that the essential oils extracts have more stronger antioxidant properties than ethanol and aqueous extracts of red onion, garlic and leek used in the present study. The essential oils extracts of red onion had the strongest radical-scavenging effect (30.81%), while the essential oils extracts of garlic had the lowest radical-scavenging effect (22.04 %).These findings are in agreement with the data of Lee et al. (2012) who showed that red onion exhibited approximately six and fivefold higher DPPH radical-scavenging activity than garlic and white onion, respectively.

Conclusion

From this study, it could be recommended to apply onion, garlic and leek in controlling infection by *E. coli* O_{157}:H_7, *S. aureus , A.F.,*4, *S. typhimurium , A.F.,*3, *A. niger, H.U.B.,*1, *A. ochrecies , H.U.B.,*2 and *F. oxysporum, H.U.B.,*3, to prevent cold meat spoilage and preserve meat for longer periods against pathogenic bacteria mentioned above. The three extracts from *Allium* common plants which had higher phenolic content showed higher antioxidant activity in terms of DPPH

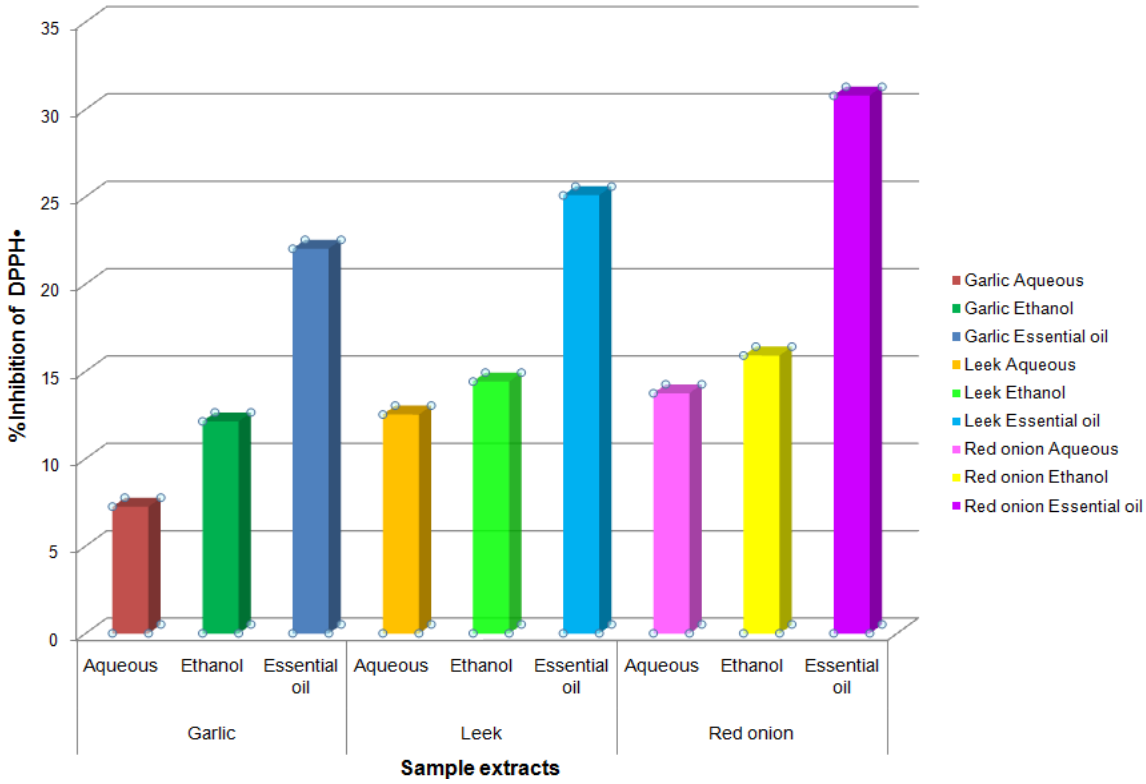

Figure 2. Inhibition effects of the essential oils, aqueous and ethanolic extracts of red onion, garlic and leek DPPH radical.

radical scavenging activity. The phenolic contents could be used as an important indicator of the antioxidant capacities.

Conflict of Interests

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

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Disease reaction studies of maize (*Zea mays* L.) against turcicum leaf blight involving indigenously identified cytosterile source

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Among biotic stresses affecting maize, the turcicum leaf blight caused by *Exserohilum turcicum* is one of the most important diseases in India. Disease reaction studies against turcicum leaf blight were done with two crosses viz., 15C (A) x I-318 (R) and I-401(A) x I-318(R) for all six generations with P_1, P_2 and F_1 having 30 plants each and F_2 (300 plants), BC_1 (180 plants) and BC_2 (180 plants). Analysis of variance of arc sin transformed data for leaf blight in the present study revealed significant variability has been exhibited by fungus to infect different generations of a particular cross. In I-15C(A) x I-318(R) cross, F_1 was moderately resistant to turcicum leaf blight but F_1 of I-401(A) x I-318(R) cross was moderately susceptible to the disease. Disease screening of both crosses indicated that the latent period was longer, suggesting presence of resistant genes in both the crosses which further can be exploited in the production of successful commercial hybridsby using these CMS sources as parents to develop turcicum leaf blight (TLB) resistant, cost effective and stable hybrids.

Key words: Maize, *Zea mays* L., turcicum leaf blight, cytosterile source.

INTRODUCTION

Maize (*Zea mays* L.) holds a unique position in world agriculture as food, feed and source of diverse Industrially important products. Maize is cultivated on nearly 100 million hectares in developing countries and about 70% of the total maize production in developing world is from low and lower middle income countries (Faostat, 2010). In sub-Saharan Africa, it provides food and income to over 300 million households (Tefera et al., 2011).

Turcicum or northern corn leaf blight (NCLB) is a serious foliar wilt disease of maize in many tropical and temperate environments. NCLB is a severe fungal disease causing yield losses worldwide, is most effectively controlled by resistant varieties. Genomic prediction could greatly aid resistance breeding efforts (Frank et al., 2013). It is caused by the ascomycete fungus *Setosphaeriaturcica* (Luttrell) Leonard and Suggs, with its conidial state *Exserohilum turcicum* (Passerini)

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Leonard and Suggs. Symptoms can range from small cigar shaped lesions to complete destruction of the foliage (Welz and Reiger, 2000). Turcicum leaf blight causes extensive defoliation during grain filling period, reduce succulence of leaves and stalk necrosis resulting in grain yield losses (Perkins and Pederson, 1987). The disease was reported as early as 1923 in India and assumed as an epiphytotic form in Kashmir valley (Koul, 1957). The disease is favoured by high humidity with moderate to high temperatures from three leaf stages to grain development of crop (Palaversic et al., 2012).Turcicum leaf blight disease in maize is particularly prevalent during *Kharief* (rainy) season in the Zones I, II and IV as delineated by the All India Coordinated Reseach Project (Maize), namely Peninsular, North eastern and Northern hill regions. Yield losses due to TLB worldwide can range from 27 to 90% in addition to predisposing plant to stalk rots and reducing forage value (Chenulu and Hora, 1962).

Turcicum leaf blight (TLB) is characterized by long elliptical, greyish green or tan leaf lesions that first appear on the lower leaves and increase in size and number until very little living tissue is left. Yield is reduced due to lack of carbohydrates for grain filling (Paliwal et al., 2000). Eight three-way and four commercial maize hybrids for yield and resistance to maize streak virus using controlled leaf hopper infestation and turcicum leaf blight under artificial inoculation was studied. The hybrid 053WH54 had multiple resistances to turcicum leaf blight and maize streak virus. The hybrids 043WH61 and 043WH07 were high-yielding even at high disease pressure while 043WH41 and 013WH03 were relatively low yielding at low disease pressure. This showed the inherent genetic diversity of the hybrids. The hybrids ZS 225, 043WH61 and 043WH07 are recommended for production in areas with high prevalence of both diseases (Karavina et al., 2014). The use of resistant cultivars is most effective, economical and environmental friendly means to control epidemics of turcicum leaf blight. Thus, in the present study, screening for TLB was carried out in all the six generations of both crosses viz., 15C (A) x I-318 (R) and I-401 (A) x I-318 (R).

MATERIALS AND METHODS

The material for study was developed by attempting the crosses 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) during *kharif* 2010 to generate F_1 generation at High Altitude Rice Research Sub-station, Larnoo. The F_2 and backcrosses generation (BC_1 and BC_2) were developed at Winter Nursery Centre (ICAR) Hyderabad, during *rabi* 2010-11. All the six basic set of generations P_1, P_2, F_1, F_2, BC_1 and BC_2 and 15C (B), I-401 (B) and restorer R-line I-318 (R) of the crosses thus obtained were raised and screened for turcicum leaf blight.

Six generations of each cross were evaluated in randomized complete block design with three replications at the Experimental Farm of Division of Plant Breeding and Genetics, Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Shalimar during *kharif* 2011. The nonsegregating (P_1, P_2 and F_1) and segregating generations (F_2, BC_1 and BC_2) were raised in four and six rows with inter and intra row spacing of 60 and 25 cm, respectively. Screening for disease was carried out with 30 plants each of P_1, P_2 and F_1 and 300 plants of F_2, 180 plants each from BC_1 and BC_2.The plants were inoculated artificially at 5-6 leaf stage with the conidial suspension of test fungus (*E. turcicum*) 4-5 x 10^4 conidia ml^{-1} (Figures 1, 2 and 4) in the evening hours and high humidity was maintained by spraying water 5-6 times in the next 3 days to ensure infection. All the leaves on infected plants were scored using 0-5 scale (Figure 3) adopted by maize pathology unit, CIMMYT as: 0 = no symptoms; 1 = one to few scattered lesions on leaves covering up to 10% leaf area; 2 = lesions on leaves covering 11-25% leaf area; 3 = lesions on leaves covering 26-50% leaf area; 4 = lesions abundant on leaves covering 51-75% leaf area; 5 = lesions abundant on almost all leaves, plants prematurely dried or killed with 76-100% leaf area covered.

Figure 1. Conidial suspension of test suspension.

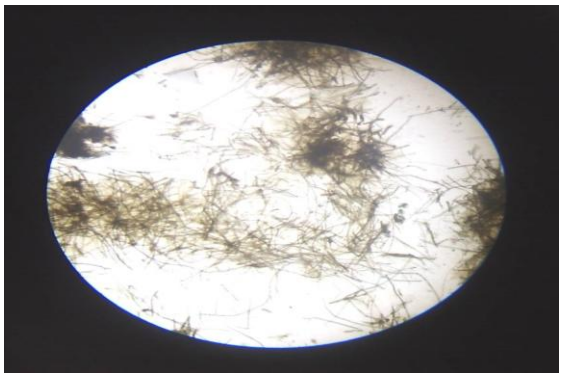

Figure 2. Microscopic view of conidial fungus of *E. turcicum*.

The per cent disease incidence and severity were calculated in each observation as per the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased leaves}}{\text{Total number of leaves assessed}} \times 100$$

$$\text{Disease Severity (\%)} = \frac{\text{Sum of all numerical ratings}}{\text{Number of leaves examined x maximum diseases rating}} \times 100$$

The data was arc sine transformed as recommended for data,

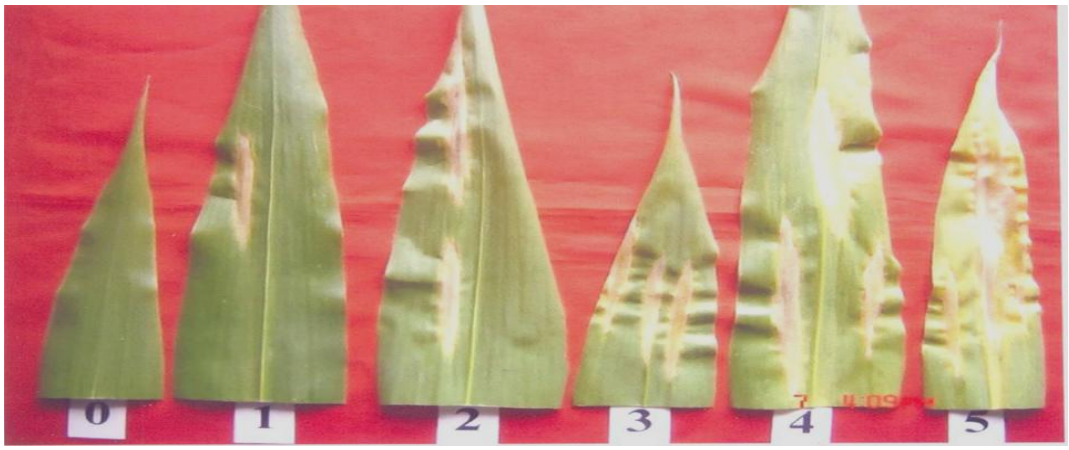

Figure 3. Scale (0-5) for assessment of Turcicum leaf blight intensity on maize.

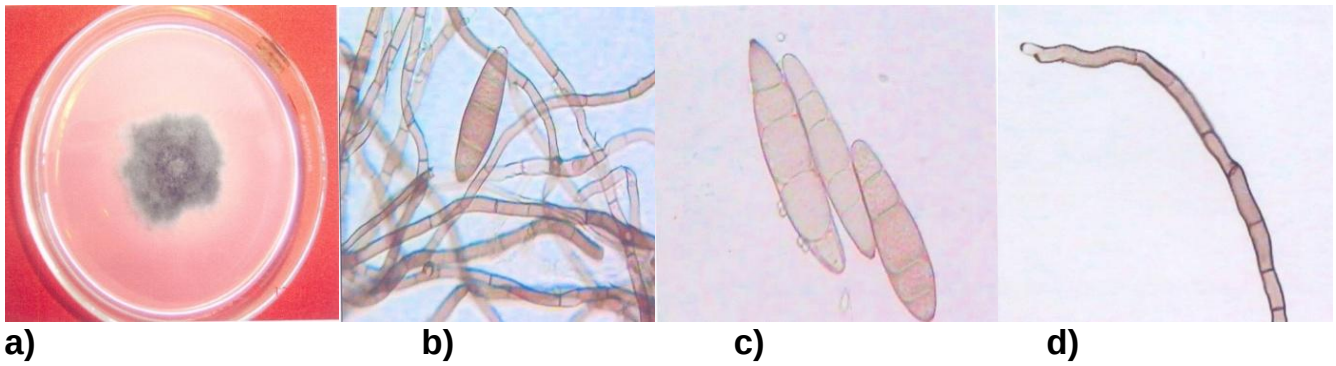

Figure 4. a) Mycelial colony of *E. turcicum*, b) mycelium of the fungus, c) conidia of the fungus, d) conidiophore with scarlet tip.

expressed as decimal fractions or percentages as per the procedure of Steel and Torrie (1980).

RESULTS AND DISCUSSION

Moderate to high disease pressure was achieved through artificial inoculations of conidial suspension of test fungus (*E. turcicum*) as was evident from the disease severity of the crosses. The six basic set of generations, P_1, P_2, F_1, F_2, BC_1 and BC_2 of two crosses viz., 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) were evaluated by recording observations on percent disease incidence and percent disease severity following (0-5) scale as described in material and methods.

Transformation of the data by arc sine method (Steel and Torrie, 1980) was done and analysis of variance in transformed data for both the crosses is presented in Table 1. Significant differences among generations of both crosses were revealed suggesting presence of sufficient variability for prevalence of diseases.

Significant critical difference of 4.11 and 4.07 with respect respect to disease incidence and significant critical difference of 1.44 and 1.52 with respect to disease severity were observed in 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) crosses for turcicum leaf blight. Responses of the two crosses viz., 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) to TLB in the trial (*Kharief*2011) are shown in Figure 5.

Subsequently, six generations of the two crosses were grouped into categories as moderately resistant (MR) and moderately susceptible (MS). The F_1 of the cross 15C (A) x I-318 (R) was moderately resistant (MR) to turcicum leaf blight with mean disease incidence of 49.24% and severity of 23.52%. But in BC_2, mean disease severity was lower, 21.54% than both F_2 and BC_1. Contrary to this, in cross I-401 (A) x I-318 (R) F_1 was moderately susceptible (MS) with mean disease incidence of 56.72% and severity of 26.77% but again BC_2 showed lower mean disease severity of 22.54% than F_2 and BC_1 as revealed in Table 2.

Dominance nature of genes was exhibited in the cross 15C (A) x I-318 (R) as F_1 of the cross was moderately resistant (MR) whereas F_1 was moderately susceptible

Table 1. Analysis of variance for arc-sine transformed generation means for reaction to Turcicum leaf blight (*Exserohilum turcicum*) in two crosses I-15C(A) x I-318(R) and I-401(A) x I-318(R) of maize.

Cross : I-15C(A) x I-318(R) [Leaf blight incidence]						**Cross : I-401(A) x I-318(R) [Leaf blight incidence]**					
S.V	**D.F**	**S.S**	**M.S**	**F**	**P**	**S.V**	**D.F**	**S.S**	**M.S**	**F**	**P**
Rep.	3	2.56	0.85	0.11	0.95	Rep.	3	11.94	3.98	0.55	0.65
Treat.	5	842.88	168.58**	22.71	0.00	Treat.	5	1053.08	210.62**	28.86	0.00
Error	15	111.35	7.42			Error	15	109.47	7.30		
Total	23	956.78				Total	23	1174.49			
$S.E_{(diff.)}$ = 1.93; C.D = 4.11**						$S.E_{(diff.)}$ = 1.91; C.D = 4.07**					
Cross : I-15C(A) x I-318(R) [Leaf blight severity]						**Cross : I-401(A) x I-318(R) [Leaf blight severity]**					
S.V	D.F	S.S	M.S	F	P	S.V	D.F	S.S	M.S	F	P
Rep.	3	11.348	3.78	4.09	0.26	Rep.	3	0.922	0.307	0.52	0.672
Treat.	5	84.68	16.93**	18.32	0.000	Treat.	5	106.505	21.301**	36.34	0.00
Error	15	13.68	0.924			Error	15	8.792	0.586		
Total	23					Total	23	116.219			
$S.E_{(diff.)}$ = 0.679; C.D = 1.446**						$S.E_{(diff.)}$ = 0.541; C.D = 1.152**					

** = Significant at 5% level.

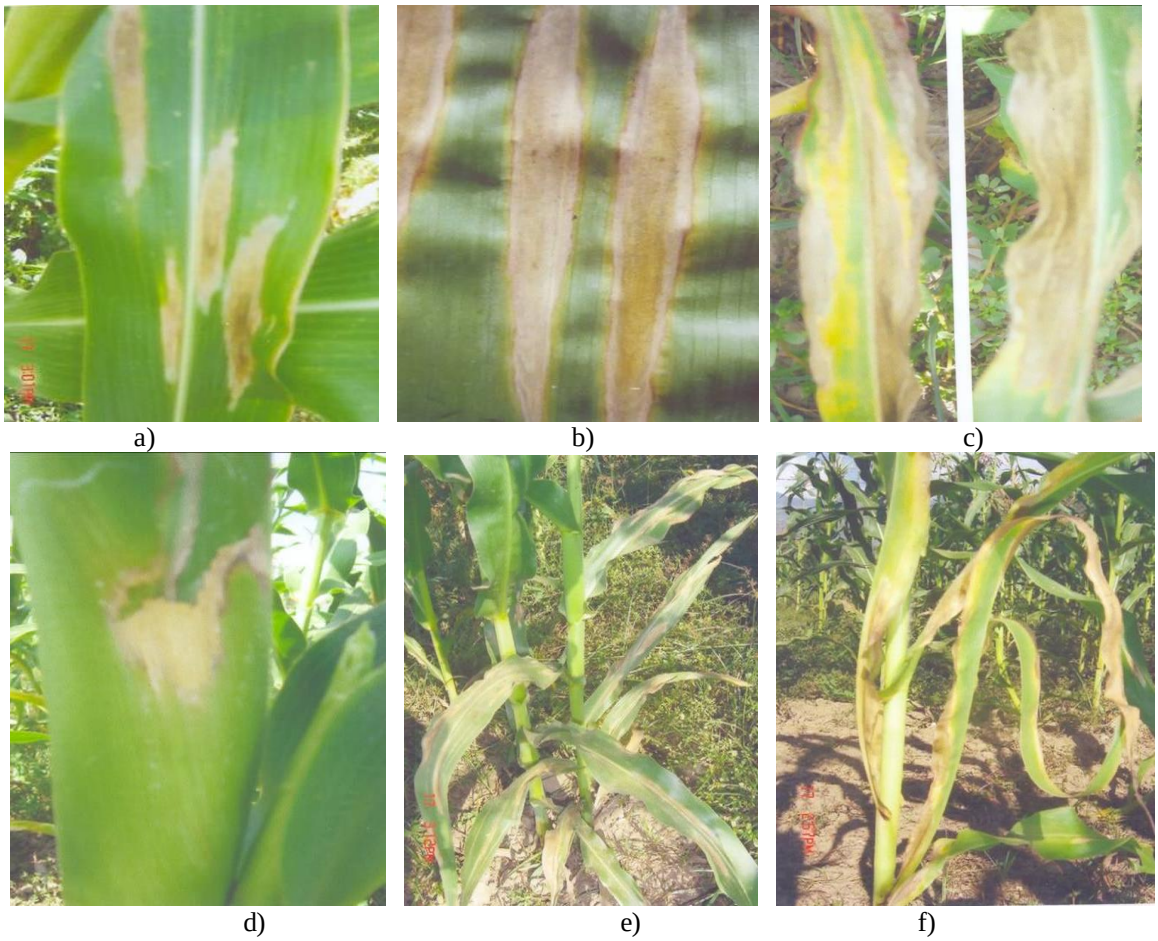

Figure 5. Turcicum leaf blight symptoms of two crosses [I-401(A) x I-318 (R) and I-15C(A) x I-318 (R)]. **a)** Elongated lesions on leaf; **b)** Mature lesions on leaf (centre covered by greyish black masses of conidia and condiophores); **c)** Leaf blight symptoms (coalescence of lesions); **d)** symptoms on cob sheath blight; **e)** Severe infection of *E. turcicum* on plant; f) plant showing leaf symptoms due to *E. turcicum.*

Table 2. Arc sine transformed mean disease incidence and severities of six generation of two crosses, I-15C(A) x I-318(R) and I-401(A) x I-318 (R) of maize for reaction to Turcicum leaf blight (*Exserohilum turcicum*).

Cross		Mean disease incidence (%)	Mean disease severity (%)	Reaction
Cross 15C(A) x I-318(R)				
P_1	I-15C(A)	61.06 (51.41)	18.13 (25.17)	MR
P_2	I-318(R)	33.29 (35.20)	14.00 (21.98)	MR
F_1	I-15C(A) x I-318(R)	57.36 (49.24)	16.26 (23.52)	MR
F_2	I-15C(A) x I-318(R)	59.32 (50.30)	20.08 (25.71)	MR
BC_1	[I-15C(A) x I-318(R)] I-15C(A)	60.15 (50.88)	20.50 (25.02)	MR
BC_2	[I-15C(A) x I-318(R)] I-318(R)	45.33 (42.31)	14.58 (21.54)	MR
Cross I-401(A) x I-318(R)				
P_1	I-401(A)	66.05 (57.33)	25.69 (28.27)	MS
P_2	I-318(R)	33.29 (35.20)	14.00 (21.98)	MR
F_1	I-401(A) x I-318(R)	67.60 (56.72)	19.96 (26.77)	MS
F_2	I-401(A) x I-318(R)	60.71 (49.24)	25.84 (27.84)	MS
BC_1	[I-401(A) x I-318(R)] x I-401(A)	65.10 (56.41)	27.91 (31.87)	MS
BC_2	[I-401(A) x I-318(R)] x I-318(R)	47.41 (42.30)	15.45 (22.54)	MR

MR = Moderately resistant (5.1-25.0% of leaf area infected); MS = moderately susceptible (25.1-50% of leaf area infected) (0-5 Scale of Maize Pathology Unit CIMMYT).

(MS) in cross I-401 (A) x I-318 (R) suggesting inheritance of resistance was governed by recessive genes. A single dominant gene in dent inbred GE 440 and in pop corn variety ladyfinger was detected by Hooker (1963) whereas mutagenic and major gene resistances for leaf blight have been reported by Jenkins and Roberts (1952) and Ullstrup (1970). Cross I-401 (A) x I-318 (R)F_1 was moderately susceptible (MS), suggesting that disease resistance was governed by additive genes in this cross. Thus, presence of resistant genes in both crosses can be exploited in the production of successful commercial hybrids by using these CMS sources as parents to develop TLB resistant, cost effective and stable hybrids. Also, further testing of these crosses through molecular markers can be helpful in identifying resistant gene in commercial hybrids.

Additive gene action was of major importance in all studies done on inheritance of corn leaf blight (Hughes and Hooker, 1975; Sigulas et al., 1988; Carson, 1998). Resistant lines had different set of genes controlling resistances operating at different levels of disease intensity across different generations as reported by Jenkins and Roberts (1952). Shankaralingam et al. (1989) also suggested that additive gene action and dominance x dominance type of epistasis with duplicate nature are important in controlling resistance to TLB. Thus, both dominance and additive gene effects seem to be governing resistance to leaf blight. Turcicum leaf blight, a ubiquitous foliar disease of maize for which diverse qualitative and quantitative sources are available need to be incorporated in the susceptible cultivars by back-crossing because of unstable nature of qualitative *Ht* genes as envisaged by Welz and Reiger (2000). Therefore, pedigree and recurrent selection methods should be used in development of high yielding and resistant cultivars.

Conflict of Interests

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

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Detection and characterization of bacteriophages attacking dairy *Streptococcus thermophilus* starter cultures

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Sixty (60) strains of *Streptococcus thermophilus* isolated from dairy traditional Egyption yoghurt samples were characterized. The yoghurt samples were collected from the local markets in Egypt. The results showed that few phages can attack *S. thermophilus* strains isolated from traditional Egyptian yoghurt (Zabady) samples, only three temperate phages were isolated. The isolated phages attacking samples were induced with mitomycin C (0.05 or 0.1 µg/ml, final concentration) and characterized by SDS-polyacrylamide gel electrophoresis, PCR and electron microscope. The characterization of phages showed that the bacteriophages belonged to the family *Siphoviridae* and they had an isometric head and long non contractile tail. SDS polyacrylamide gels and PCR amplification showed that all isolated phages belongs to *pac* phage. The PCR described in this work can detect and differentiate *S. thermophilus* phages in a short time, it is very sensitive and reveals a high limit of detection.

Key words: *Streptococcus thermophilus*, bacteriophage, SDS-polyacrylamide gel electrophoresis, PCR amplification.

INTRODUCTION

Streptococcus thermophilus is one of the most economically important lactic acid bacteria (LAB), it is a component of dairy starter cultures and used in a large amount for the manufacture of dairy products (annual market of about 40 billion USD). It is usually used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) or *Lactobacillus helveticus* for the manufacture of yoghurt and other fermented milk products like cooked cheeses (Mercanti et al., 2011; Canchaya et al., 2003; Ventura et al., 2002). *S. thermophilus* is also used alone or in combination with lactobacilli for the production of mozzarella and cheddar cheeses (Duplessis et al., 2006; Binetti et al., 2005; Hols et al., 2005). It is a Gram-positive microaerophilic, moderately thermophilic bacterium which possesses a relatively small genome of approximatley 1.8 Mb with a

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low G+C% (Roussel et al., 1997; O'Sullivan and Fitzgerald, 1998). Some insertion sequences (ISs) have been reported in its genome (Guedon et al., 1995; Bourgoin et al., 1996) and these ISs may contribute to the genetic instability in some of these strains (Roussel et al., 1997). It has been reported that many *S. thermophilus* are highly susceptible to phage infection but only 1 to 10% of them are lysogenic and contain intact temperate phages (Josephsen and Neve, 2004; Carminati and Giraffa, 1992; Neve et al., 2003). It is known that the majority of dairy phages are resistant to standard pasteurization procedures (Roussel et al., 1997). Later, other phages infecting different lactic acid species from various industerial environments were reported to exhibit extra ordinary thermal resistances (Atamer et al., 2011).

S. thermophilus phages (virulent or temperate) belong to *Siphoviridae* family and they are relatively homogenous group with the same morphology (B1 morphotype) (Ackermann, 2001). They have an isometric head (diameter, 45 to 60 nm) and a long noncontractile tail (length, 240 to 270 nm; thickness, 9 to 13 nm) (Brüssow, 2001). *S. thermophilus* phages have a linear double-stranded DNA genome ranging from 30 to 45 kb (Brüssow, 2001). According to the packaging mechanism of their double-stranded DNA and their structural proteins pattern on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), they are divided into two types, *pac*- (41, 25 and 13 kDa) and *cos*- (32 and 26 kDa) type (LeMarrec et al., 1997).

Bacteriophages attack is always a serious problem in industrial fermentation, especially in the dairy industry. It can lead to slow fermentation or even complete failure of starter with consequent loss of product leading to a high financial losses because a large dairy plant can processes more than 2 x 10^5 litres of milk per day, so phage attack could be more expensive (Sturino and Klaenhammer, 2007). However, for minimizing phage attack on LAB in dairy industry, some precautions could be followed like rotation of non-phage-related strains, using mixed starter culture, use of phage inhibiting media for culture propagation and sterile processing conditions but usually these are not enough to prevent phage infection (Binetti et al., 2005; Josphen and Neve, 2004). Using bacteriophage-resistant mutants derived from phage-sensitive strains or using genetically modified phage-resistant starter cultures harbour plasmids containing abi or R/M genes.

Due to the high economical losses that may result from a phage infection of starter cultures, a rapid and sensitive method is required for phage detection and identification in the dairy. In this work, a rapid and reliable multiplex-PCR method is described allowing the simultaneous detection of *S. thermophilus* phages and their differentiation into the two well known *pac*- and *cos*-type subgroups. Also, this work was designed to characterize the bacteriophages that attack *S. thermophilus* in Egyptian yoghurt (Zabady) that contaminate raw milk during milk collection in dairy farms or manufacture of yoghurt.

MATERIALS AND METHODS

Collection of yoghurt samples

Twenty yoghurt samples were collected from different sites in Egypt (Cairo, Assiut, and El Mansoura). Samples were stored at 4°C until used.

Isolation and identification of *S. thermophilus* from yoghurt samples

S. thermophilus was isolated from yoghurt samples by plating on LM17 agar plates at 40°C for 24 h (Krusch et al., 1987). The *Streptococcus* was examined and identified. Identifications of pure *S. thermophilus* isolates were made on the basis of their cultural, morphological and microscopic characteristics and by reference to Krusch et al. (1987). The bacterial identification was confirmed by API-test system (rapid ID 32 Strep, Biomerieux, Nuertingen, Germany) according to Freney et al. (1992). Five colonies from each yoghurt sample were used for bacteriophage induction and identification of lysogenic bacterial strains of *S. thermophilus*.

Induction and isolation of Bacteriophages

S. thermophilus bacteriophages were isolated by inducing their lysogenic strains using mitomycin C. Induction was performed on 0.5 L of LM17 broth which was inoculated with 1.5% overnight *S. thermophilus* culture and incubated at 40°C until OD_{620} 0.2 then another 0.5 L of LM17 broth containing 0.4 µg/ml Mitomycin C (final concentration 0.2 µg/ml) was added to the bacterial culture and incubated at 40°C until lysis occurred (Neve et al., 1998).

Bacteriophage concentration and purification

Phage concentration were performed according to Sambrook et al. (1989) with some modifications (Neve et al., 1998).

Multiplex colony PCR for detection of lysogenic *S. thermophilus* strains

A set of 60 artisanal *S. thermophilus* strains isolated from traditionally produced Egyptian yoghurt samples (by spontaneous fermentation of milk) were screened using colony-PCR (multiplex PCR) with pac- and cos-primers. To design primers for *pac*-type phages Tp-J34, O1205, Sfi11 and 2972 sequences were used and for *cis*-type phages, Sfi21, 2701, Sfi19 and DT1 were used. A set of sixty *S. thermophilus* strains, isolated from traditionally produced (using unidentified starter culture) Egyptian yoghurt (Zabady) samples was screened by colony-PCR (multiplex PCR) with primers for *cos*-phage YC-F/YC-R and primers YP-F/YP-R for *pac*-phage. All primers were designed with DS gene software and were manufactured by MWG-Biotech, Germany. The pac specific-primers were YP-F (GCT CGT CTT GAA GCT ATG C), YP-R (GAT AAG AGT CAA GTG ACC GTC) and the cos specific-primers were YC-F (GCT ATG CTT GAC GAT TCA GT) and YC-R (AGC AGA ATC AGC AAG CAA G).

Polymerase chain reaction (PCR)

Colony-PCR

Colony PCR was performed using 10X PCR-buffer (Fermentas). Three colonies were picked from agar plate into 1.5 ml centrifuge tube containing 100 µl 1X PCR-buffer to give a turbid solution using sterile toothpicks. The tubes were then placed in a thermoblock and heated at 95°C for 12 min with gentile shaking. Finally, the tubes were cooled, 2 µl were used for 50 µl PCR reaction volume and the rest was stored at -20°C for further use.

Polymerase chain reaction was performed as described previously by Mohamed et al. (2012). The amplified PCR products were analyzed using 1.5% agarose gel electrophoresis as reported by Sambrook et al. (1989). After adequate migration has occurred, DNA fragments were visualized on a ultraviolet transilluminator (Biometra, Germany) and photographed.

SDS-PAGE of the phage structural proteins

Sodium deodecyl sulfate polyacrylamide gel electrophoresis from bacteriophage structural proteins was performed as described by Fayard et al. (1993) with some modifications. The protein loading buffer Pack (Fermentas GMBH- Germany) was used for samples preparations. Bacteriophages particles obtained from Cesium Chloride step gradients were dialyzed against dialysis buffer (10 mM NaCl, 50 mM Tris-HCl PH 8, 10 mM $MgCl_2$) for 1 h. 30 µl of the extract were mixed with 8 µl of 5x protein loading buffer and 2 µl of 20x DTT, then heated at 95°C for 5 min. 25 µl of the mixture were loaded on 12% SDS gel. Electrophoresis was carried out at 10 mA until the samples reached resolving gel, then the electrophoresis was continued at 20 mA until the blue lane of the samples buffer was completely removed from the gel. The gel was stained by Coomassie Brilliant Blue R250 solution for overnight. The gel was de-stained until clear protein bands appeared.

Electron microscopy

For transmission electron microscopy, the detected bacteriophages were purified and concentrated using CsCl gradient centrifugation as previously descriped by Sambrook and Russell (2001). Purified phages were stained negatively with 2% (w/v) uranyl acetate on freshly prepared carbon films. Grids were analyzed in a Tecani 10 transmission electron microscope (FE1 Company, Eindhofen, Netherlands) at an acceleration voltage of 80 kV.

RESULTS AND DISCUSSION

Identification of *S. thermophilus*

In this study, sixty *S. thermophilus* strains were isolated from yoghurt samples and identified based on their cultural, morphological, microscopic characteristics and by using API-test system. Three lysogenic *S. thermophilus* strains harbouring inducible *pac*-type prophages were identified. Intact temperate phages were released from two of the three strains (strains YA1 and YA2) upon mitomycin C induction (Figure 1), while defective temperate phages (phage tails) were induced from strain YA3 (Figure 4).

Induction of phages with mitomycin C

S. thermophilus strains were grown in LM17 broth at 40°C and treated with different concentrations of mitomycin C (0.00, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 µg/ml) during the early logarithmic growth phase. Optimal induction and lysis of host cells due to the release of temperate phages were obtained either with 0.1 µg/ml mitomycin C in strains 1 and 3 or with 0.05 µg/ml mitomycin C in strains 2 (Figure 1).

Multiplex colony PCR for the detection of lysogenic *S. thermophilus* strains

A rapid and reliable multiplex-PCR method is described allowing the simultaneous detection of *S. thermophilus* phages and their differentiation into the two well known *pac*- and *cos*-type subgroups. Since *pac*- and *cos*-type phages are composed of different structural proteins, two sets of primers were designed from the internal highly conserved region of the major head protein gene of eight completely sequenced *S. thermophilus*.

The two primer sets could be used simultaneously in a multiplex PCR assay to detect and distinguish *S. thermophilus* phages, because the PCR-products differed in fragment size (432 bp product for *pac*-type phages versus 514 bp product of *cos*-type phages). The PCR results identify three new lysogenic S. thermophilus strains harbouring inducible pac-type prophages (Figure 2).

SDS-PAGE of the phage structural proteins

The reliability of the multiplex PCR protocol was validated and confirmed by SDS-PAGE analysis of the structural proteins of *pac*-type phages have three major bands and *cos*-type phages have two major bands as reported previously by LeMarrec et al. (1997). The structural proteins from the *cos*-type reference phage P53 was analysed by SDS-PAGE and compared with the structural protein profiles of the new isolates YA1, YA2 and YA3. It is shown in Figure 3 that the protein profile of the *cos*-type reference phage P53 has two main bands (32 and 26 kDa), while YA1, YA2 and YA3 phages revealed three main protein bands (41, 25 and 13 kDa). These data do also confirm the classification the new phages by multiplex PCR as *pac*-type phages.

Electron microscopic analysis of phages:

Phage particles obtained from induction were examined and photographed using transmission electron microscope. The majority of phage particles were intact showing

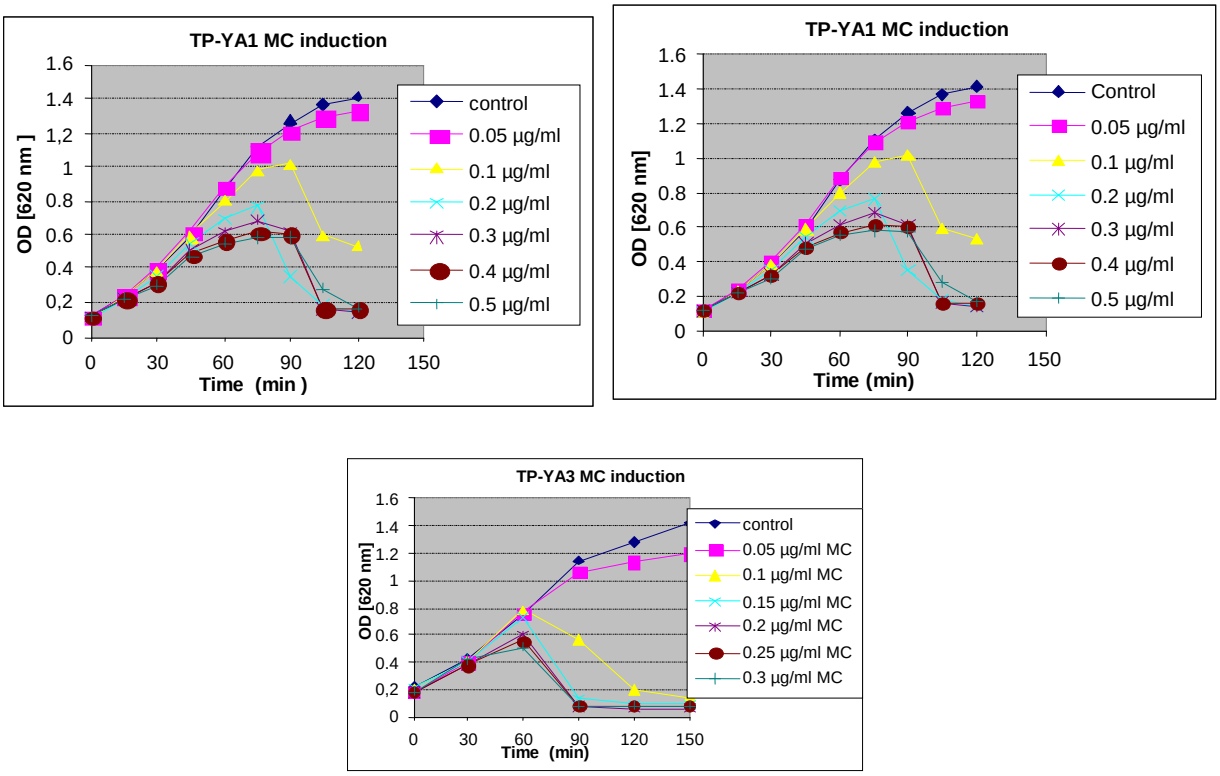

Figure 1. Mitomycin induction of TP-YA1; TP-YA2 and TP-YA3.

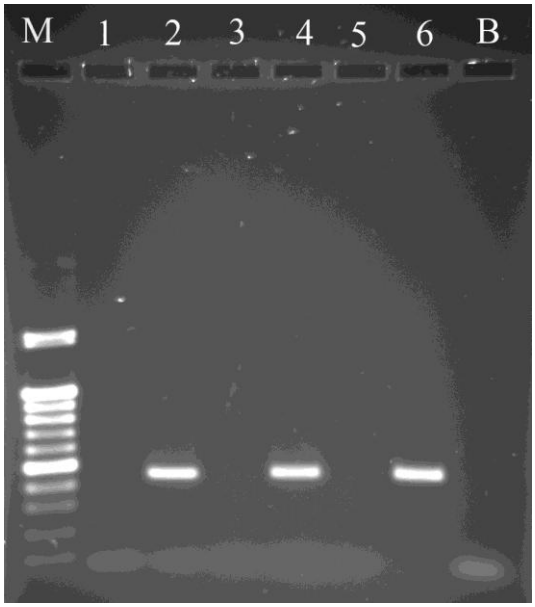

Figure 2. Agarose gel (1.5%) electrophoresis of DNA products obtained by colony multiplex-PCR for identification of three "artisanal" lysogenic *S. thermophilus* strains. Lane 2, 4 and 6 (strains: YA1, YA2, YA3) as a *pac* phage. lanes 1, 2, 3 *cos*-phage primers. Lane B: negative control. Lane M: 100 bp DNA size marker (AppliChem, Darmstadt, Germany).

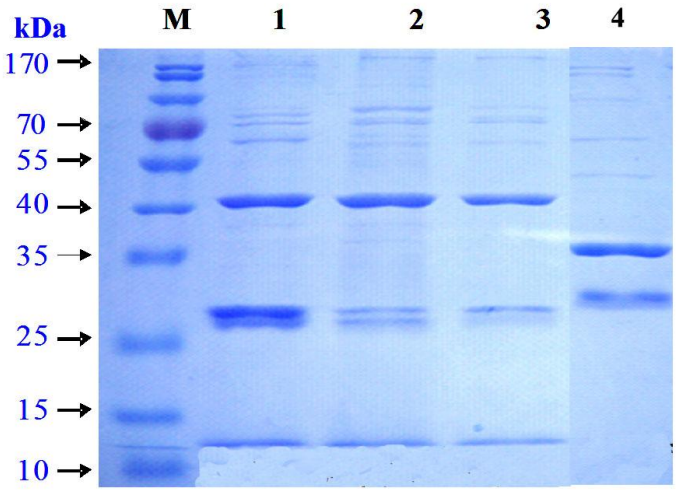

Figure 3. SDS-PAGE (12%) of the structural proteins of the isolated *pac*-type *S. thermophilus* phages YA1, YA2 and YA3 (lanes 1, 2 and 3 respectively). Lane 4 of the *cos*-type reference phage P53. Lane M: PageRuler^{TM} prestained protein marker (Fermentas, St. Leon-Rot, Germany).

isometric- headed *Siphoviridae* phages. They all revealed the same basic morphology (head diameter: 57 nm) and

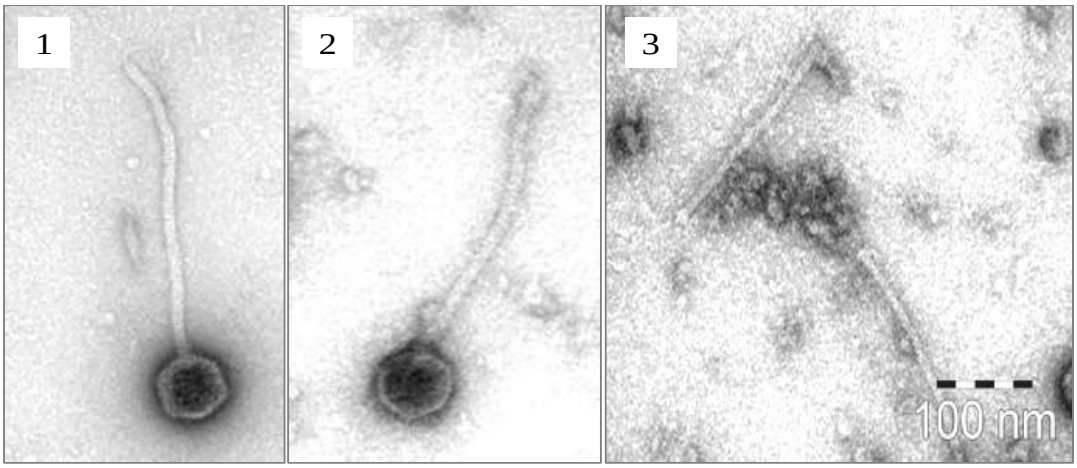

Figure 4. Electron micrographs of induced TP-YA1(1); TP-YA2 (2) and TP-YA3 (3) phage particles.

have long non contractile tails (length ca. 240 nm) (Figure 4). This corresponds with the basic morphology of other *S. thermophilus* phages (Neve et al., 1989; Brüssow, 2001; Bruttin et al., 1997). Hence, phage 1 and 2 are a member of the well described *Siphoviridae* phage family (morphotype B1) as described by Ackermann (2001) and Bradley (1967).

The data confirmed that lysogeny is a rare event in *S. thermophilus* cultures of Neve et al. (2003), who found that only 1 - 10% of the strains contain inducible prohages. However, the analysis of the traditional cultures may indicate a higher dissemination of lysogenic strains. The multiplex-colony PCR approach to detect and identify three new lysogenic *S. thermophilus* strains harbour inducible *pac*-type prophages within a set of sixty *Streptococcus thermophilus* strains isolated from traditional Egyption yoghurt (Zabady) samples.

Conclusion

From the above results, it can be concluded that the conserved DNA-regions of the structural genes for the major head proteins of *S. thermophilus* phages are reliable targets PCR for the differentiation of *pac*-type phage by multiplex PCR. The multiplex PCR described in this work can detect and differentiate *S. thermophilus* phages in a short time (2.5 h); it is very sensitive and reveals a high limit of detection. In whey samples, phages could be detected at low titers of less than 10^3 pfu per ml. Also, *S. thermophilus* starter cultures can be screened rapidly for the presence of lysogenic strains by PCR. Phage monitoring is very important in the dairy industry to avoid economical losses that may happen due to phage infection.

Conflict of Interests

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

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Full Length Research Paper

Evaluation of *Pseudomonas* isolates from wheat for some important plant growth promoting traits

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Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements. In the present study, isolation and characterization of PGPR from the rhizospheric soil of wheat was done for the enhancement of wheat growth. Rhizospheric soils samples were collected from different areas of Uttar Pradesh, India. Twenty two (22) isolates of bacteria were successfully isolated, biochemically characterized and screened for their plant growth promoting traits like production of indole acetic acid (IAA), ammonia production, siderophore production and phosphate solubilization. On the basis of multiple plant growth promoting activities among 22 isolates, 17 isolates were also evaluated for their quantitative production of IAA. The isolates Ps-AB4, Ps-BK1 and Ps-JN 2 showed highest IAA production (79.67-110.12 µg/L). Out of 22, 20 and 6 isolates produced ammonia and siderophore, respectively, while 10 isolates solubilized phosphate on the Pikovskaya's agar medium. The present study reveals that the use of *Pseudomonas* isolates Ps-AB4, Ps-BK1 and Ps-JN2 as inoculants biofertilizers might be beneficial for wheat cultivation.

Key words: Indole acetic acid, NH_3, *Pseudomonas* spp., phosphate solubilization, wheat.

INTRODUCTION

Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as plant growth-promoting rhizobacteria (PGPR). In the context of increasing international concern for food and environmental quality, the use of PGPR for reducing chemical inputs in agriculture is a potentially an important issue. PGPR have been applied to various crops to enhance growth, seed emergence and crop yield, and some have been commercialized (Herman et al., 2008; Sachdev et al., 2009, Dilfuza, 2010; Rawat et al., 2011; Minaxi et al., 2013). The *Pseudomonas* spp. isolated from the roots of graminaceous plants has been shown to colonize the roots of various plants, and to increase the height, flower number, fruit number and total fruit weight of tomato plants. Under salt stress, PGPR have shown positive

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effects in plants on such parameters as germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Kloepper et al., 2004; Kokalis-Burelle et al., 2006). Another major benefit of PGPR is to produce antibacterial compounds that are effective against certain plant pathogens and pests (Herman et al., 2008; Fatima et al., 2009). PGPR mediate biological control indirectly by eliciting induced systemic resistance against a number of plant diseases (Jetiyanon and Kloepper, 2002). In addition to improvement of plant growth, PGPR are directly involved in increased uptake of nitrogen, synthesis of phytohormones, solubilization of minerals such as phosphorus, and production of side-rophore that chelate iron and make it available to the plant root (Mohite, 2013; Schoebitz et al., 2013). Wheat is the most important staple food in several developing countries, and chemical fertilizer is the most important input required for wheat cultivation. The high-yielding wheat variety has resulted in an increase in wheat production but requires large amount of chemical fertilizers, leading to health hazards and environmental pollution. In order to make wheat cultivation sustainable and less dependent on chemical fertilizers, it is important to know how to use PGPR that can biologically fix nitrogen, solubilize phosphorus and induce some sub-stances like indole acetic acid (IAA) that can contribute to the improvement of wheat growth. Recently, there is a growing interest in PGPR due to their efficacy as biological control and growth promoting agents in many crops (Thakuria et al., 2004). There is very little infor-mation regarding the use of PGPR as biofertilizers in wheat. Therefore, the present study was undertaken to screen the PGPR strains that are compatible with wheat in agro-ecological conditions of Uttar Pradesh India.

MATERIALS AND METHODS

Isolation of *Pseudomonas* from wheat rhizosphere

Soil samples were collected from the rhizosphere of wheat plants at flowering stage from Faizabad (FZ), Kanpur (KN), Barabanki (BK), Muzzaffarnagar (MZ), Ambedkarnagar (AB), Jaunpur (JN) and Lucknow (LK) districts of Uttar Pradesh, India. The wheat plants were uprooted from the agricultural fields and the rhizosphere soil was pooled together, placed in plastic bags and stored at 4°C and immediately microbiological processing was carried out. Serial dilutions were made up to 10^{-4} for all seven soil samples and 10^{-3} and 10^{-4} dilutions were taken for spread plating on Kings B medium containing per liter of distilled water: 20.0 g peptone, 10.0 g glycerol, 1.50 g K_2HPO_4, 1.50 g $MgSO_4$:$7H_2O$, 18.0 g agar and pH 7.2. The plates were incubated at 30°C for 48 h. After incubation, plates were observed for different isolates based on morphological traits. Morphologically variable colonies were picked up and purified on Kings B medium plates. Pure cultures were maintained on the respective medium slants.

Characterization of *Pseudomonas* isolates

The bacterial isolates were characterized by their morphological (shape, size, elevation, surface, margin, color, pigmentation and Gram's reaction) and biochemical (carbohydrate fermentation, oxidase test, H_2S production, starch and gelatin hydrolysis, NO_2 reduction, citrate and catalase reactions) characteristics using standard methods (Cappuccino and Sherman, 1992). Gram reaction was determined by making a thin smear of inoculums of rhiozbacterial isolates in a separate glass slide and heat fixed. The smear was covered with crystal violet for 30 s. The slide was washed with distilled water for few seconds by using wash bottle. The smear was stained with iodine solution for 60 s. The iodine solution was washed with 95% ethyl alcohol. Ethyl alcohol was added drop by drop until no more colour flows from the smear. The slides were washed with distilled water and drained. Saffranin was applied to smear for 30 s and washed with distilled water and blot dried with absorbent paper. The slide was examined under oil immersion objective. Carbohydrate fermentation pattern of isolates was determined by medium containing specific carbohydrate source (dextrose, sucrose and lactose) along with phenol red as indicator at 37°C for 48 h. The starch hydrolysis capacity of test isolates was checked on starch nutrient agar medium. Cultures were streaked and incubated at 37°C for 24 h. After incubation, plates were flooded with Gram's iodine and zone of clearing around the growth was observed on the medium plate. For gelatin hydrolysis, Stab tubes of gelatin nutrient agar were inoculated with cultures and incubated. Tubes were chilled in ice and liquefaction of gelatin was observed. To test the presence of nitrates, nitrate broth was inoculated with test cultures and incubated at 35°C for 48 h and equal parts of sulfuric acid and alphanaphthyl amine was mixed. Pink color development was observed for the presence of nitrite. Cimmons citrate agar medium slants were inoculated by isolates and incubated at 35°C for 48 h for citrate utilization. Observations were recorded for presence of growth which indicated citrate utilization. To check the catalase reaction, a loopful of culture from freshly grown slant of nutrient agar was taken out and placed on slide. A drop of H_2O_2 (3%) was added for growth. Production of bubbles indicated presence of catalase. Oxidase reagent (Kovacs) was added to colonies growing on nutrient agar medium. Development of purple color indicated presence of oxidase.

Production of indole acetic acid

Indole acetic was detected as described by Brick et al. (1991). The bacterial cultures were grown in100 mL of conical flask containing Luria-Bertani broth for 48 h on a rotary shaker. After incubation, fully grown cultures were centrifuged at 10000 rpm for 10 min at 40°C. The supernatant 2 mL was mixed with two-three drops of Ortho-phosphoric acid and 4 mL of the Salkouski reagent (50 mL, 35% of perchloric acid 1 mL 0.5 $FeCl_2$ solution). Development of pink color indicates IAA production. Optical density was taken at 530 nm with help of UV visible spectrophotometer. Concentration of IAA produced by culture was measured with the help of standard graph of IAA obtained in the range of 20-200 µg/mL.

Phosphate solubilization

The plates were prepared with Pikovskaya's medium. All the bacterial isolates streaked on the surface of Pikovskaya's agar plate and phosphate solubilizing activity was estimated after 4 days of incubation at 28°C. Phosphate solubilization activity was determined by the development of the clear zone around the bacterial colonies (Frioni, 1990).

Production of Ammonia

All the bacterial isolates were tested for the production of ammonia in the peptone water (peptone 10 g, NaCl 5 g in litre, pH 7.0). Freshly

Table 1. Morphological and cultural characteristics of test isolates.

Biochemical characters	*Pseudomonas* spp.
Number of isolates	22
Grams reaction	Negative
Shape	Short rods
Pigment	Cream , yellow to greenish
Colony morphology	Round colonies to entire margin
Arrangement	Mostly single
Endospore Position	No Spore
Sucrose	+
Dextrose	+
Mannitol	+
H_2S production	-
Citrate Utilization	+
Starch	+
Gelatin hydrolysis	+
Catalase test	+
Nitrate reduction	+
Lipid hydrolysis	+
Casein hydrolysis	+

grown culture were inoculated in 10 mL peptone water in each test tube and incubated for four days at 30°C. Nessler reagent (1 mL) was added in each test tube. Development of brown to yellow color was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Siderophore production

Bacterial isolates were assayed for siderophore production on the chome azurol agar (CAS) medium (Husen, 2003). Bacterial isolates were spot inoculated on the chrome azurol s (CAS) ager plates and incubated at 30 for 72 h. Siderophore production was indicated by orange halos around the colonies after incubation.

RESULTS AND DISCUSSION

Isolation and biochemical characterization

Twenty two bacterial strains were successfully isolated from wheat field and identified as *Pseudomonas* spp. on the basis of cultural, morphological and biochemical characteristics as described in Bergeys manual of determinative bacteriology (Holt et al., 1994). The *Pseudomonas* spp. strains from rhizosphere of different crops were isolated and extensively studied by Fischer et al. (2007), Ahmad et al. (2008), Fatima et al. (2009), Joshi et al. (2011) and Rawat et al. (2011). Sinha and Simon (2013), characterized *Pseudomonas fluorescens* on the basis of morphological (Grams stain and colony morphology), physiological and biochemical tests (gelatin hydrolysis, IAA production test, starch hydrolysis, urease, oxidase, ammonia production, HCN, Levan production from sucrose). Similarly, Kushwaha et al. (2013) also isolated *Pseudomonas* strains from cauliflower and characterized for cultural, morphological and biochemical characters while Singh et al. (2013) isolated various strains of *Pseudomonas* from wheat rhizosphere and characterized them by morphological and biochemical characteristics like production of IAA, HCN, catalase, phosphatase enzymes etc. The general characteristics of the isolates are illustrated in Table 1.

IAA production

All 22 bacterial strains were tested for quantitative estimation of IAA, most interestingly all the test *Pseudomonas* strains showed IAA production in the range of 2.22-110.12 µg/mL (Figure 1). The strain Ps-BK1 and Ps-AB4 were able to produce 95.60 and 110.00 µg /mL IAA in the broth culture medium, respectively. The produced IAA was measured with the help of standard graph of IAA obtained in the range of 20-200 µg/mL. It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mirza et al., 2001). Ahmad et al. (2004) reported that *Pseudomonas* isolate Ps_1 produced 53.20 µg/mL IAA in culture medium supplemented with L Tryptophan @5 mg/mL. Deshwal and Kumar (2013) screened out qualitatively 55 *Pseudomonas aeruginosa*, 22 *Pseudomonas putida*, 26 *Pseudomonas cepacia* and 37 *Pseudomonas fluorescens* strains for IAA and most of the *Pseudomonas* strains showed encouraging results for IAA as 44 *P. aeruginosa*, 14 *P. putida* , 19 *P. cepacia* and *22 P. fluorescens* were found positive while Bhakthavatchalu et al. (2013) observed significant increase in IAA production (80 µg/mL) by *P.s aeruginosa* when L- tryptophan was supplied as precursor. The present investigation findings are outstanding with reference to earlier reports.

Ammonia production and phosphorus solubilization

Out of 22 *Pseudomonas* strains, 20 strains were found to produce NH_3. Few isolates like Ps-FZ2, Ps-BK1, Ps-JN1, Ps-JN3, Ps-AB1, Ps-AB2, Ps-AB4, Ps-AB5, Ps-MZ3 and Ps-LK3 which produced deep brown color indicated higher production of ammonia. Only two *Pseudomonas* isolates namely Ps-JN2 and Ps-MZ1 did not produce ammonia where as the rest isolates were found to be medium producer of ammonia (Table 2). Sinha and Simon (2013) isolated 13 *Pseudomonas* strains from wheat rhizosphere and were found positive for ammonia production, likewise Kaushal et al. (2013) reported ammonia producing *Pseudomonas* from rice rhizosphere. Only ten strains solubilized phosphate on Pikovskaya's agar plates at 30°C and the remaining strains did not

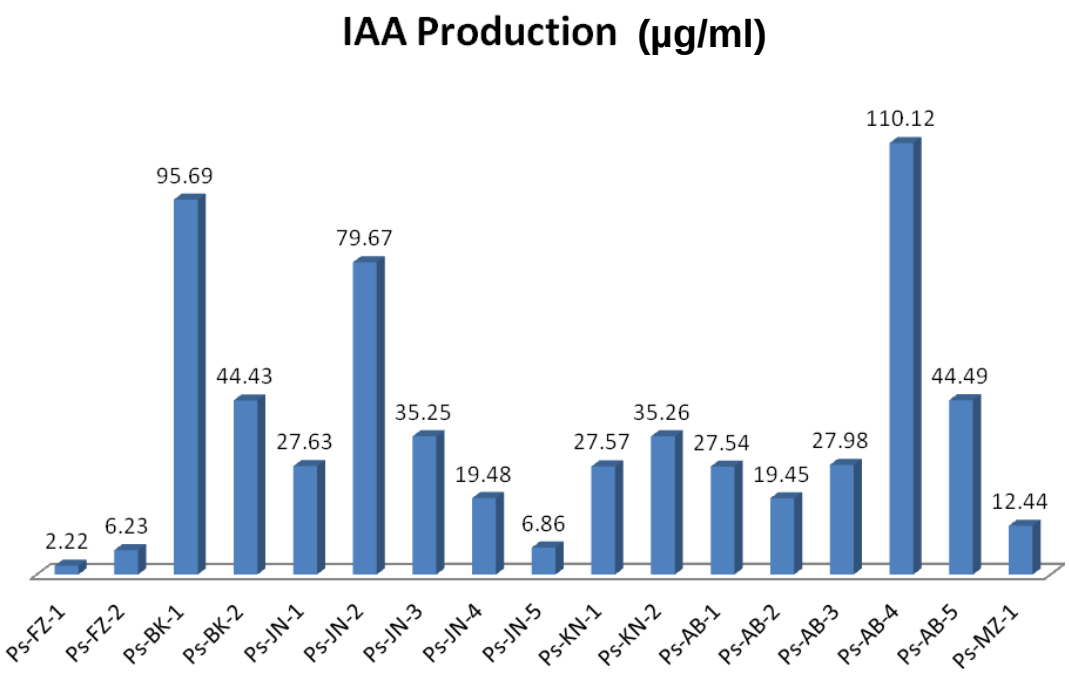

Figure 1. Indole acetic acid production by *Pseudomonas* isolates.

Table 2. Plant growth promoting characteristics of *Pseudomonas* isolates.

Isolate	Siderophore production[b]	Phosphate solubilization[c]	Ammonia production[a]
Ps-FZ-1	-	-	+
Ps-FZ-2	-	-	+++
Ps-BK-1	-	+	+++
Ps-BK-2	-	+	++
Ps-JN-1	+	++	+++
Ps-JN-2	-	-	_
Ps-JN-3	+	++	+++
Ps-JN-4	-	-	+
Ps-JN-5	-	-	++
Ps-KN-1	-	-	+
Ps-KN-2	-	-	++
Ps-AB-1	-	+++	+++
Ps-AB-2	-	-	+++
Ps-AB-3	-	++	+
Ps-AB-4	+	++	++++
Ps-AB-5	+	-	++++
Ps-MZ-1	+	-	-
Ps-MZ-2	-	-	+
Ps-MZ-3	-	++	+++
Ps-LK-1	-	-	++
Ps-LK-2	-	+++	+
Ps-LK-3	+	+++	+++

[a]; + = weak producer of NH_3, ++ = medium production of NH_3, +++ = good producer of NH_3,++++ = strong producer of NH_3, [b]; + = Siderophore Production, - = no Siderophore Production [c]; + = poor solubilization of Phosphate in Pikovskaya's medium, ++ = medium solubilization of phosphate in Pikovskaya's medium, +++ = very good solubilization of phosphate in Pikovskaya's medium, - = not solubilization of phosphate in Pikovskaya's medium.

show phosphate solubilization. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to rice that represent a possible mechanism of plant growth promotion under field conditions (Verma et al., 2001). Several other worker like, Ahmad et al. (2008), Fischer et al. (2007), Sachdev et al. (2009), Rawat et al. (2011) and Joshi et al. (2011) observed phosphate solubilization and ammonia production by *Pseudomonas* isolated from wheat and other crops while Schoebitz et al. (2013) found that *P. fluorescens* from wheat rhizosphere solubilized phosphate in the range of 89-93 µ g/mL.

Siderophore production

Out of 22 isolates, six were found to produce siderophore. All six isolate formed orange halos surrounding the colonies on CAS medium (Table 2). This study has demonstrated that some of the isolates produced siderophore which are able to suppress the phyto-pathogenic fungi. Deshwal and Kumar (2013) also found *Pseudomonas* species to be producer of siderophore which could suppress the phyto-pathogenic fungi and Bhakthavatchalu et al. (2013) confirmed the ability of *P. aeruginosa* FP6 to produce siderophore and maximum production of siderophore (85.70 µM) was recorded after 36 h of incubation.

Conflict of Interests

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

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Full Length Research Paper

Prevalence of antimicrobial resistant *Aeromonas* in chicken and fish washings

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This study was undertaken to assess prevalence of virulent and antibiotic-resistant *Aeromonas* species in chicken meat and fresh water fish washings procured from local market. Isolation was done on three selective agar viz. *Aeromonas* isolation media, ampicillin dextrin agar and *Aeromonas* starch DNA agar. Presumptive colonies were directly screened by multiplex polymerase chain reaction (PCR) targeting genus specific 16S rRNA gene, aerolysin (*aerA)* of *Aeromonas hydrophila* and hemolysin (*asa1)* gene of *Aeromonas sobria.* Of the 200 samples (100 each of chicken and fish washings), 21 isolates were confirmed as *Aeromonas* species. We could not detect *aer A,* however, *asa1* of *A. sobria* was detected in six (28.57%) fish isolates. *Aeromonas* isolates exhibited 100% resistant to amoxicillin, ampicillin and 95.23% to carbenicillin. Moderate sensitivity was observed to kanamycin (90.47%) and neomycin (71.42%). Isolates were 100% sensitive to gentamicin and ciprofloxacin. Maximum sensitivity was recorded with chloramphenicol, tobramycin (95.23% each) and amikacin (80.95%). PCR characterization revealed presence of *class 1 integron* and *Tet (C)* genes in six and 10 isolates, respectively. *PSE-1 β-lactamase* was not detected in any of the isolates. This study demonstrate the incidence of antimicrobial resistant *Aeromonas* in chicken and fish environment, which may be a potential source of spread of this enteropathogen in food chain.

Key words: Aeromonads, antimicrobial resistance, chicken, fish.

INTRODUCTION

Aeromonas species are ubiquitous Gram negative bacilli, nowadays classified within the new *Aeromonadaceae* family (Martin-Carnahan and Joseph, 2005). They are widely distributed in aquatic environments and are isolated from a wide range of food of animal and plant origin. Exotoxins are major virulence factors of aeromonads that include a cytotoxic heat-labile enterotoxin *(Act),* also known as aerolysin/hemolysin; a cytotonic heat-labile enterotoxin *(Alt),* known as lipase, extracellular lipase, or phospholipase and a cytotonic

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heat-stable enterotoxin (*Ast)* (Kingombe et al., 2010). Other than gastroenteritis, *Aeromonas* are responsible for meningitis, cellulites, otitis, endocarditis, osteomyelitis, peritonitis, bacteremia and septicemia (Albert et al., 2000). Motile aeromonads due to their ubiquitous distribution are considered as bacterial indicators of fresh water environment, especially for harboring resistance genes (Schmidt et al., 2001). In India, incidence of *Aeromonas* species is reported from various foods of animal origin viz. fish, seafood, raw and cooked meat, poultry, vegetables, milk and milk products (Khurana and Kumar, 1997; Agarwal et al., 2000), however, reports on detection of virulence and drug resistance genes are limited. Antimicrobial resistance among enteric pathogens is a serious problem in developing countries where there is a high frequency of gastroenteric illnesses and many antibiotics fall routinely into inadequate use. Acquisition of new genetic material by susceptible bacteria from resistant strains often facilitates the incorporation of the multiple resistance genes into the host's genome or plasmids (Tenover, 2006). The consequences of horizontal gene transfer are even more alarming. This mechanism can often promote the simultaneous spread of resistance to several unrelated classes of antibiotics, particularly if the genes for such resistance are co-located on the transmissible genetic element. Antimicrobial-resistant populations are present everywhere in all bacterial communities. Their expansion follows complex pathways through environmental systems, people, animals, food and water. Therefore, surveillance of antimicrobial resistance in commensal, zoonotic and pathogenic bacteria from humans, animals and food is crucial while formulating food safety measures (Acar and Moulin, 2013). Considering the high frequency of *Aeromonas* in poultry feces, poultry carcasses can be considered as a risk group for *Aeromonas* infections and dissemination (Jindal et al., 1993). *β*-Lactam antibiotics are commonly used in the treatment of bacterial infections but they are hydrolysed by *β*-lactamase enzymes produced by resistant bacteria (Li et al*.,* 2009). Also, class I integrons are usually reported to contain antibiotic-resistant gene cassettes and related with other mobile elements such as plasmids, which could contribute to the dissemination of resistance genes (Agersø and Sandvang, 2005). Antimicrobials are also used in the aquaculture environment which may create selective pressure on bacterial environment. In view of these facts, the aim of the study was to estimate prevalence of *Aeromonas hydrophila* and *Aeromonas sobria* in chicken and fish washings and to find their antimicrobial resistance pattern.

MATERIALS AND METHODS

Sample collection

Chicken meat (100) and fish washings (100) were collected aseptically during January to October, 2013 from the local market in Shirwal. Approximately 50 g chicken and 10 ml fish washing water were collected in pre-sterilized specimen tubes and transported on ice to the laboratory for bacterial isolation. Samples collected were further processed on the day of collection for isolation of *Aeromonas* species.

Isolation and identification of *Aeromonas* species

For isolation, enrichment was done in alkaline peptone water (APW). Approximately, 10 g of chicken meat sample homogenized with 90 ml sterile normal saline solution (NSS), and 1 ml homogenate was further transferred to 9 ml APW. From fish washings, 1 ml of sample was inoculated with 9 ml of APW according to the methods described by Agarwal et al. (2003) and HiMedia (2009). After enrichment at 37°C for 18 h, selective plating was done on *Aeromonas* isolation media, ampicillin dextrin agar (ADA) and *Aeromonas* Starch DNA agar. After inoculation agar plates were incubated at 37°C for 24 h. Presumptive identification of *Aeromonas* species was based on colony characteristics, morphology and Gram's staining. Presumptive colonies were further subcultured on ADA and subjected to molecular studies. *Aeromonas* colonies on ADA are yellow with dark orange centre, on *Aeromonas* isolation media are dark green colored clear to slightly opalescent and on *Aeromonas* Starch DNA agar are creamy. Candidate colonies were further kept at 4°C in brain heart infusion (BHI) broth until use. All the bacteriological media were procured from HiMedia Laboratories Private Limited, Mumbai, India.

Antimicrobial susceptibility testing

Antibiotic sensitivity was determined by single disc diffusion method of Bauer et al. (1966) using Mueller Hinton agar (MHA). Bacteria were cultivated in BHI broth and incubated for 12 h at 37°C. Further, the bacterial suspension were seeded onto MHA plates. Antimicrobial profiles of *Aeromonas* isolates were studied against 12 different antimicrobials *viz.* amikacin (AK, 30 mcg/disc), amoxicillin (AMX, 30 mcg/disc), ampicillin (AMP,10 mcg/disc), carbenicillin (CB, 100 mcg/disc), cephalothin (CEP, 30 mcg/disc), chloramphenicol (C, 30 mcg/disc), ciprofloxacin (CIP, 5 mcg/disc) gentamicin (GEN, 30 mcg/disc), kanamycin (K, 30 mcg/disc), neomycin (N, 30 mcg/disc), tobramycin (TOB, 10 mcg/disc), tetracycline (TE, 30 mcg/disc). Zones of inhibition were read after incubation at 37°C for 24 h in accordance with Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI as mentioned by the manufacturer of antimicrobial discs (HiMedia Laboratories, Mumbai).

Molecular detection

Genus and species specific multiplex PCR assay was performed for detection of *Aeromonas* species targeting conserved and virulence genes according to the methods described by Wang et al. (2003) with suitable modifications. Briefly, presumptive colonies were further subcultured on ADA. All PCR reactions, both for virulence associated gene and antimicrobial genes were performed by colony PCR in 25 µl containing 12.5 µl PCR master mix (HiMedia), 1 µl (10 picomoles) forward (F) and reverse(R) primers, 7.5 µl nuclease free water and a pure single colony as a DNA template. Bacterial colonies were picked up with sterilized tooth pick and directly transferred to PCR tubes. The program was run on a Verity 96 well thermal cycler PCR system (Applied Biosystems). The PCR products were cooled at 4°C and resolved by submarine gel electrophoresis on 1.5% agarose gel. Each run contained a 100 bp DNA molecular weight marker (GeneRuler, Fermentas). The gels were stained with ethidium bromide (0.5 µg/ml), observed under UV

Table 1. Oligonucleotide sequences used for detection of different genes in *Aeromonas* species.

Target gene	Primer sequence	Amplicon size (bp)	Reference
16S rRNA	F- GGGAGTGCCTTCGGGAATCAGA R- TCACCGCAACATTCTGATTTG	356	Wang et al. (2003)
asa1 of A. sobria	F- TAAAGGGAAATAATGACGGCG R- GGCTGTAGGTATCGGTTTTCG	249	Wang et al. (2003)
aerA of A. hydrophila	F- CAAGAACAAGTTCAAGTGGCCA R- ACGAAGGTGTGGTTCCAGT	309	Wang et al. (2003)
PSE β-lactamase	F- ACCGTA TTGAGCCTG ATT TA R- ATT GAAGCC TGT GTT TGAGC	321	Bert et al. (2002)
Class 1 integron	F- GGC ATC CAA GCA GCA AG R- GGC ATC CAA GCA GCA AG	410	Igbinosa and Okoh, (2012)
Tet C	F- GGT TGA AGG CTC TCA AGG GC R- CCT CTT GCG GGA TAT CGT CC	505	Agerso and Sandvang, (2005)

Table 2. PCR conditions used for detection of antimicrobial resistance genes in *Aeromonas* species.

Target gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension
PSE β-lactamase	96°C/5 min	96°C/30 s	55°C/30 s	72°C/60 s	72°C/5 min
Class 1 integron	94°C/5 min	95°C/45 s	56°C/60 s	72°C/90 s	72°C/10 min
Tet C	94°C/5 min	94°C/60 s	65°C/60 s	72°C/60 s	72°C/10 min

Repeated 30 cycles for each gene.

light at a λ 420 nm and images were captures through gel documentation system (G Box, Syngene UK).

For detection of *16S rRNA* gene, and two virulence associate species specific genes *viz.* aerolysin gene (*aerA)* of *A. hydrophila* and hemolysin gene (*asa1)* of *A. sobria, a* multiplex PCR assay was carried out with pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 7 min. Particulars of oligonucleotide sequences and PCR conditions used for detection of antimicrobial resistance genes are shown in Tables 1 and 2.

RESULTS

Aeromonads were isolated from 21 (10.5%) out of 200 samples (100 each of chicken and fish washings). Prevalence of *Aeromonas* was recorded higher (15%) in fish washings as compared to chicken (6%). We used three selective media viz. ADA, *Aeromonas* isolation media, and *Aeromonas* Starch DNA agar for isolation of mesophillic aeromonads. Findings revealed that ADA is the best suitable bacteriological media for recovery of *Aeromonas* species from food samples. The recorded prevalence is the actual prevalence based on molecular confirmation of the isolates targeting *16S rRNA* gene. Screening of the *Aeromonas* isolates for *aerolysin A (aerA)* of *A. hydrophila* and *asa1 hemolysin* gene of *A. sobria* revealed presence of *asa1* in six (28.57%) isolates. None of the isolates were positive for *aerolysin A* (Figure 1). All the isolates showing positivity for *asa1* were from fish source. Thus only *A. sobria* could be detected and we could not isolate virulent *A. hydrophila.*

All the *Aeromonas* isolates irrespective of the source exhibited moreover similar profile in terms of sensitivity and resistance to the antimicrobials (Table 3). They were 100% resistant to amoxicillin, ampicillin and 95.23% to carbenicillin. Moderate sensitivity was observed to kanamycin (90.47%) and neomycin (71.42%). The percent sensitivity was observed against gentamicin and ciprofloxacin. Maximum sensitivity was recorded with chloramphenicol (95.23%), tobramycin (95.23%) and amikacin (80.95%).

Antibiotic resistance profiles of all 21 *Aeromonas* isolates was assessed genotypically for association of *PSE β*-lactamase, *class 1 integron* and *Tet (C)* tetracycline genes. Genomic resistant to *PSE β*-lactamase was not detected in any of the isolates, however, distribution of *class 1 integron* and *Tet (C)* was observed. *Class 1 integron* cassette was detected in six (28.57%) aeromonads and all were from fish washings (Figure 2). Ten isolates (47.62%) showed positive amplification of *Tet (C)*, three from chicken and seven were from fish washings (Figure 3). Combination of *class 1 integron* and *Tet (C)* was also recorded in three (14.28%) aeromonads in the present study.

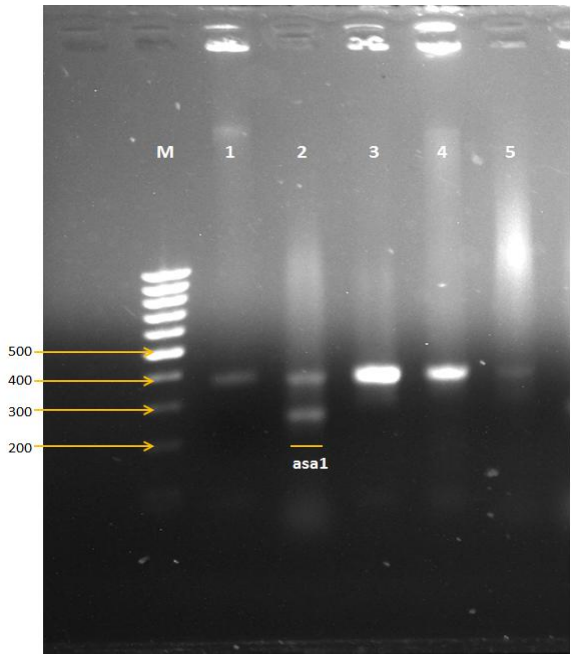

Figure 1. Detection of *Aeromonas* genus specific *16S rRNA* and *asa1* genes. M- 100 bp DNA ladder, Lanes: 1-4 (16S rRNA gene-356 bp); Lane: 2-16S rRNA and *asa1* gene (249 bp).

Table 3. Antimicrobial sensitivity test of *Aeromonas* isolates

Antibiotic	Resistant		Moderate sensitive		Sensitive	
	(No.)	(percent)	(No.)	(percent)	(No.)	(percent)
Amikacin	4	19.04	0	0.00	17	80.95
Amoxicillin	21	100.00	0	0.00	0	0.00
Ampicillin	21	100.00	0	0.00	0	0.00
Carbenicillin	20	95.23	1	4.76	0	0.00
Cephalothin	14	66.66	2	9.52	5	23.80
Chloramphenicol	0	0.00	1	4.76	20	95.23
Ciprofloxacin	0	0.00	0	0	21	100.00
Gentamicin	0	0.00	0	0	21	100
Kanamycin	0	0.00	19	90.47	2	9.52
Neomycin	0	0.00	15	71.42	6	28.57
Tetracycline	0	0.00	6	28.57	15	71.42
Tobramycin	0	0.00	1	4.76	20	95.23

DISCUSSION

Observed prevalence in the present study is comparable with previous reports from India especially from foods of animal origin (Yadav and Verma, 1998; Agarwal et al., 2000; Kumar et al., 2000; Rathore et al., 2005; Shinde et al., 2005; Smita et al., 2011). Aforementioned researchers observed the incidence of aeromonads with varying percentage in the range of 8 to 55%. Agarwal et al. (2000) isolated aeromonads from fish (22%) and goat meat (8.9%). Kumar et al. (2000) also reported close prevalence of *Aeromonas,* 16.7% in poultry meat, 12% in chevon and 7.7% in buffalo meat. Rathore et al. (2005) recorded higher prevalence of aeromonads in water and fish with *A. hydrophila* as a predominant species (43%) followed by *A. sobria* and *A. veronii* (13%, each). Shinde et al. (2005) observed moderate incidence of 24% in poultry meat, however, very high prevalence of 55% was

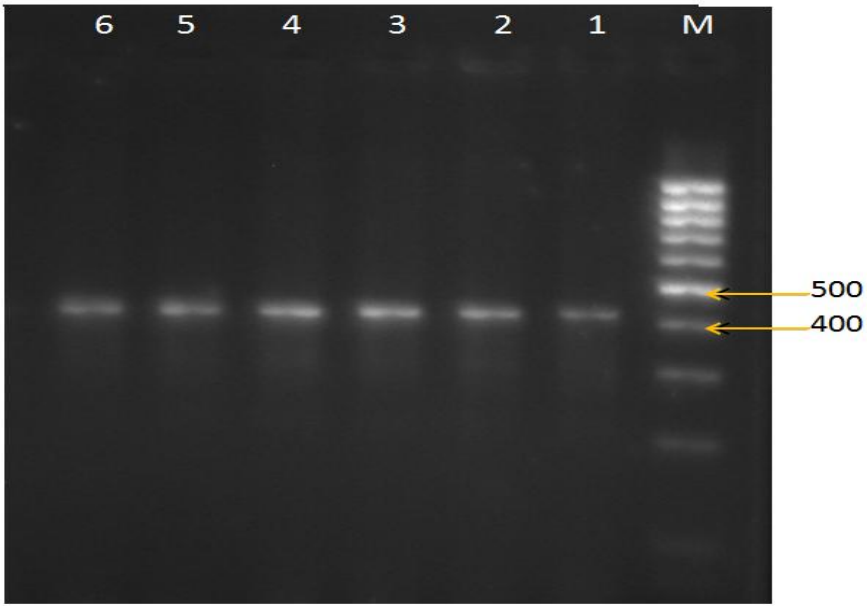

Figure 2. Detection of *Class 1 integron* gene in *Aeromonas* isolates (410 bp). M- 100 bp DNA ladder, Lanes : 1- 6 (class 1 integron genes).

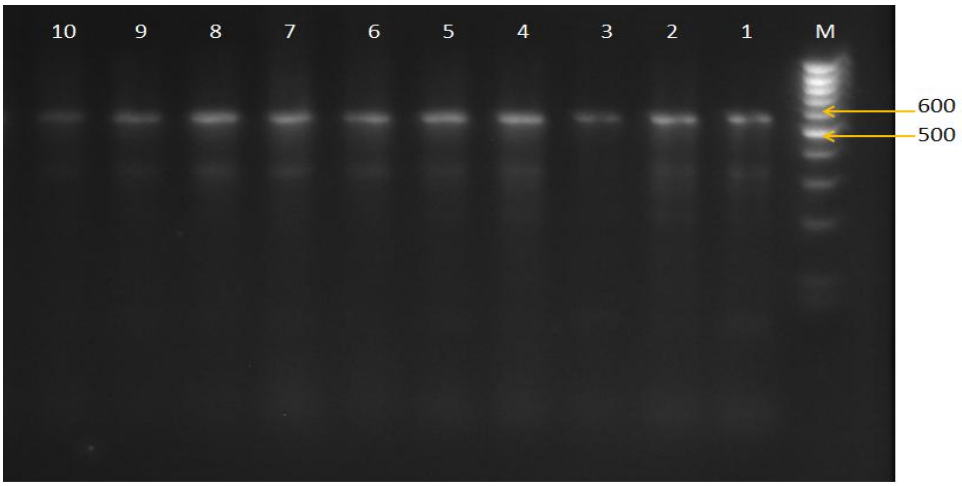

Figure 3. Detection of tetracycline *Tet (C) gene* in *Aeromonas* isolates (505 bp). M- 100 bp DNA ladder, Lanes: 1- 10 (Tet C- gene).

found in the studies of Smita et al. (2011). Variation in geographical distribution to certain extent has also been documented by Sinha et al. (2004). Literatures from other part of the world revealed widespread distribution of *Aeromonas* species in meat, fish and fresh water environment (Hanninen and Sitonen, 1995; Akan et al., 1998; Neyts et al*.,* 2000; Villari et al., 2000; Dallal et al., 2012). Presence of *Aeromonas* species in foods of animal origin, water environment and retail foods as evident from the available literatures may indicate that these products can act as possible source of vehicles for dissemination of food-borne *Aeromonas* gastroenteritis (Neyts et al., 2000; Bhowmik et al., 2009).

Present findings emphasize the need of more intensive study on distribution of aeromonads in soil, water environment and foods of animal origin considering its public health implications. Hygiene at retail chicken shop where samples were collected, was heavily compromised and water use for carcass washing may be important critical point in carcass contamination from wide number of gastrointestinal food-borne zoonotic pathogens. It was observed that water kept in bucket for washing either fish

or chicken carcass was not subject to frequent change and used for dipping of number subjects.

High level of resistance encountered against amoxicillin is alarming since this antibiotic is frequently used in aquaculture operations in order to treat fish diseases. The present findings are in tune with John and Hatha (2012) wherein amoxicillin, carbenicillin and cephalothin resistant strains were detected from fresh water fish. Antimicrobial resistance in fish could be attributed to the practice of using antimicrobials in hatcheries and aquaculture for control of bacterial diseases in fish. Our findings are not in agreement with the findings of Dallal et al. (2012) wherein, number of the aeromonads isolated from ground meat and chicken exhibited resistance to cephalothin (82.5%), tetracycline (69%), kanamycin (25%) and neomycin (4%). Results of the present study are also in tune with the findings of Sharma et al. (2010) who isolated ciprofloxacin and amikacin sensitive *Aeromonas* strains from various foods of animal origin including fish. Ghenghesh et al. (2013) recorded significantly higher tetracycline resistance in aeromonads from chicken carcass (33%) in contrast with the present study. Tetracyclines are common additives in poultry feed which can contribute to acquired resistance in poultry. We could not record resistance to tetracycline phenotypically but could detect presence of *Tet (C)* genes *Aeromonas* isolates. As tetracycline has been a widely used antibiotic because of its low toxicity and broad spectrum activity, tetracycline resistance gene was studied. The broad ecological presence of *tet*(C) is not surprising, since it has been widely distributed in different bacterial genera (Aminov et al., 2002).

Since, laboratory procedures for isolation and identification of aeromonads are laborious and time consuming, several virulence associated genes have been targeted for detection of potentially pathogenic aeromonads by PCR including *hemolysin, cytolysin, aerolysin* genes (Wang et al., 2003; Aslani and Hamzeh, 2004; Balakrishna et al., 2010). Earliest report on development of PCR assay to detect *aerolysin* gene in *A. hydrophila* is from Pollard et al. (1990) with a detection limit of 1 ng of total nucleic acid. Recently, multiplex PCR method for detection of three *Aeromonas* enterotoxin genes viz. cytotoxic *(act),* heat labile *(alt),* and heat stable *(ast)* was developed by Kingombe et al. (2010). Their finding revealed *act* and *alt* as most dominant genes in food-borne *Aeromonas* isolates. A PCR reaction was also standardized by Porteen et al. (2007) for detection of *Aeromonas* species from chicken and fish samples using primers against *16S rRNA* and aerolysin gene.

Based on the results of PCR, only *A. sobria* could be detected and we could not isolate *A. hydrophila.* However, as the strains were confirmed as aeromonads targeting *16S rRNA* genes, further studies on isolation and characterization of Aeromonas species from wide range of food and environmental sources is needed. Wang et al. (2003) detected aerolysin gene in 55% and *asa1* gene in 12.05% of the aeromonads isolated from sporadic cases of human diarrhoea. The *hly* and *aerA* positive genotype was most common genotype found in the study of Aslani and Hamzeh (2004) conducted on *Aeromonas* species isolated from diarrheal human cases and Gonzalez-Rodriguez et al. (2002) in raw and cold-smoked freshwater fish. Nagar et al. (2011) also revealed prevalence of *hly* and *aerA* positive aeromonads isolated from chicken, fish and ready to eat sprouts from Mumbai.

Genotypically, resistance to PSE1*β-lactamase* could not be detected, however, distribution of *class 1 integron* and *Tet (C)* was observed. Findings of the present study are in corroboration with Schmidt et al. (2001) and Igbinosa and Okoh (2012) in terms of detection of *class 1 integron* but contrast for *Tet (C).* Both authors could not detect *Tet (C)* in *Aeromonas* species; however *class 1 integron gene* cassette was detected. Agerso and Sandvang (2005) investigated the role of environmental bacteria as a reservoir for spread of antimicrobial resistance between the animals and human population. Their study revealed that soil bacteria may play important role in horizontal transfer of plasmid mediated resistance encoded by *class 1 integron* genes cassette and *tet* genes. Diversity in the gene cassette of *class 1 integron* was also observed in another study, wherein 13.9% *Aeromonas* isolates from human clinical cases were positive for *class 1integron* (Lee et al., 2008). The detection of *class 1 integron* indicates the potential spread of antimicrobial resistance genes within the *Enterobacteriaceae* family in the simulated environment. Presence of *β-lactamase* genes in *Aeromonas* has been reported by Igbinosa and Okoh (2012) which is an alarming public health concern.

Member of the genus *Aeromonas* readily develop single or multiple antimicrobial resistance phenotypes, and R-plasmids are commonly found, thus they are well suited for monitoring the incidence of antibiotic resistance, as well as for investigating the conjugative spread of resistance genes (Schmidt et al., 2001). Since antibiotic resistance exists in bacteria in different and potentially linked reservoirs, an integrated laboratory-based surveillance programme for monitoring resistance in all relevant reservoirs is needed.

Conflict of Interests

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

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Full Length Research Paper

Phosphate solubilization and phytohormone production by endophytic and rhizosphere *Trichoderma* isolates of guanandi (*Calophyllum brasiliense* Cambess)

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This work aimed to isolate and evaluate the phosphate solubilization and phytohormone production abilities of endophytic and rhizosphere fungi belonging to the genus *Trichoderma* isolated from guanandi (*Calophyllum brasiliense* Cambess). From the guanandi collected from the field, 12 isolates obtained were grown in potato dextrose broth and supplemented with the phosphate sources, to test their capacity to solubilize phosphates. A strain of *Trichoderma asperellum* from a commercial inoculant was also used. One isolate was able to solubilize calcium phosphate, 12 solubilized iron phosphate and two solubilized aluminum phosphate. Only the rhizosphere isolates were able to synthesize indole acetic acid (IAA) and none of the rhizosphere or endophytic isolates produced cytokinin or gibberellin. There are *Trichoderma* isolates that can benefit plant development, both for their known antagonistic ability against phytopathogenic fungi and for their ability to provide phosphates and or to produce phytohormones.

Key words: *Trichoderma*, inoculants, calcium, iron, aluminum, solubilization, plant growth promotion.

INTRODUCTION

The guanandi (*Calophyllum brasiliense* Camb. (Clusilaceae) is a tree species native to Brazil and is primarily found in the midwest, north, and southeast regions of the country. The guanandi is notable because it presents some important properties that are useful for reforestation programs, primarily the ability to grow in flooded environments (Oliveira and Joly, 2010). The microbial community, in general, acts cooperatively to enhance plant growth. Thus, the diversity and dynamics of these species is highlighted because plant-microorganism interactions may favor the propagation of plant species of interest (Singh et al., 2011).

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Fungi belonging to the genus *Trichoderma* are common in soils, consist of a wide diversity of species, and are characterized by different abilities emphasizing the promotion of growth and the development of plants that justify efforts aimed at the isolation and selection of species belonging to this genus, which can be used to increase the production of seedlings (Sofo et al., 2012).

Rhizosphere *Trichoderma* species can be selected for use as inoculants that act in the biological control of phytopathogens and stimulate plant growth and development (Avis et al., 2008). Some strains of this genus can be found in endophytic environment and cause no harm to the host, but enhances their growth and the control of pathogens in plants (Santos and Varavallo, 2011).

Although there are no significant registers of pathogens that seriously attack guanandi plants, the pathogenic fungi *Sclerotium rolfsii* (Ohto et al., 2007) and *Colletotrichum gloeosporioides* (Rosa et al., 2008) were reported in seedlings and older plants. In some *in vitro* tests, *Trichoderma* isolates were efficiently used as biocontrol agents against these pathogens (Sobowale et al., 2010; Bhuiyan et al., 2012).

Root colonization by *Trichoderma* spp. can substantially alter the plant metabolic profile, which reflects the energy supply required for the activation of the defense system and the promotion of plant growth (Brotman et al., 2012). An increase in the fresh and dry weights of the aerial part of the passion fruit tree with the use of *Trichoderma* spp. was observed by Santos et al. (2010).

Phosphate solubilizing microorganisms (PSM) can maximize plant growth by increasing the availability of nutrients, especially phosphorous (P). Plants associated with P-solubilizing microbial species have increased phosphate source use efficiencies because this combination can increase the sustainability of agricultural production in a system through the reuse of P residues that are by products of the microbial metabolism (Shrivastava et al., 2011).

The efficiency of phosphate solubilization depends on the phosphate source and the presence of different sources of carbon (C) and nitrogen for the PSM (Barroso et al., 2006). According to Altomare et al. (1999), *Trichoderma harzianum* was able to solubilize phosphates and promote the development of plants. The contribution of *Trichoderma asperellum* T34 in providing iron to cucumber plants in calcareous soil was demonstrated by Santiago et al. (2013). However, Oliveira et al. (2012) confirmed that all tested fungal isolates from this genus had the ability to solubilize calcium phosphate.

The production of auxins by microorganisms, in some circumstances, can be considered one of the major means through which they promote plant growth and depends on the growth conditions (Dastager et al., 2010). The interaction of a plant species with *T. harzianum* involves systemic modifications of the levels of different phytohormones and may have physiological implications on growth and plant resistance (Medina et al., 2011). Most of the *Trichoderma* genus isolates tested by Oliveira et al. (2012) produced the auxin indole acetic acid (IAA), with or without the L-tryptophan precursor.

This work aimed to isolate and evaluate the phosphate solubilization and phytohormone production abilities of endophytic and rhizosphere fungi belonging to the genus *Trichoderma* isolated from guanandi.

MATERIALS AND METHODS

Guanandi seedlings were grown in the nursery for 120 days, in tubes containing soil from B horizon of an Oxisol, and collected in the permanent preservation area of the Instituto Federal Goiano - Câmpus Rio Verde, GO. The seedlings were irrigated daily using an automated system. To isolate fungi belonging to the genus *Trichoderma* from the plant in the field, an individual that was located at the following coordinates obtained by using global positioning system (GPS) was chosen: latitude 17° 48' 1.692" S; and longitude 50° 53' 57.0696" W. The root systems, with soil attached, were collected from a plant in the nursery and from a plant in the field, placed in an isothermal box, and taken to the Laboratory of Agricultural Microbiology for processing.

Isolation and identification of rhizosphere and endophytic fungi

To isolate rhizosphere fungi, ten grams of root fragments with adhered soil were transferred to an Erlenmeyer flask containing 90 ml of peptone water (0.1%). This flask was left shaking (90 rpm) for 1 h to remove the soil from the rhizosphere of the guanandi, and serial dilutions were prepared, followed by the surface plating of the 10^{-2}, 10^{-3} and 10^{-4} dilutions in *Trichoderma* Selective Medium with Captan (TSMC) according to Askew and Laing (1993).

The endophytic isolation roots were washed in running water to remove the adhered soil, and subsequently left shaking with Tween-80 for 3 min, and were rinsed until the complete removal of the detergents was achieved. To eliminate the microbiota on the outer surface of the roots, the roots were treated with alcohol (70%) for 1 min, sodium hypochlorite (2%) for 3 min, 70% alcohol, and sterile distilled water to remove the previous solutions. Intact fragments were selected for further inoculation in selective medium TSMC.

For both rhizosphere and endophytic isolation the plates were incubated at 25°C. After the emergence of colony forming units (CFU) of the fungi, growing colonies were restreaked on Potato Dextrose Agar (PDA) plates. On the 13th day of incubation, the frequency of root fragment colonization was evaluated using the following formula: Frequency of colonization = No. of colonized fragments x 100/No. of total fragments.

The isolates obtained were identified by observation of their morphologic characteristics on PDA, and a micro-culture of each fungus was performed. The ANATIQUANTI program was used to measure the sizes of the spores observed under a microscope (Aguiar et al., 2007).

Determination of *in vitro* phosphate solubilization ability

Fungi belonging to the genus *Trichoderma* which was isolated from the rhizosphere and root tissues of the guanandi plants were cultured on glucose + yeast extract + peptone (GELP) medium with

$CaHPO_4$ precipitate (10%), according to Sylvester-Bradley et al. (1982), to verify the occurrence of a transparent halo around the mycelium of solubilizing isolates.

The isolates were cultured separately on potato dextrose broth (PDB) supplemented with $CaHPO_4$ (5 g l^{-1}), $FePO_4$ (3 g l^{-1}), or $AlPO_4$ (1 g l^{-1}) and incubated with shaking for 15 days. For the solubilization of $CaHPO_4$ and $AlPO_4$, the strain *T. asperellum* T22, isolated from the commercial inoculant Trichodermax EC®, was also tested.

To quantify the solubilization ability, a calibration curve was created with increasing concentrations of phosphoric acid, using a stock solution (20 mg ml^{-1}). From this stock solution 0, 100, 200, 300, 500, 700 or 900 µL was added to distilled water for a final volume of 1.0 ml. Subsequently, 1.0 ml of the working reagent [0.4 g of ascorbic acid; 100 ml of solution 725 (1.0 g of bismuth subcarbonate dissolved in 68 ml of sulfuric acid and added to 300 ml of distilled water, combined with 20 g of ammonium molybdate dissolved in 68 mL of sulfuric acid and added to 300 ml of distilled water) and 900 ml of distilled water] was added to all tubes of the standard curve.

A 1.5 ml aliquot was removed from the cultures for centrifugation at 8000 rpm for 10 min at 4°C. Then, 1.0 ml of the supernatant was transferred to test tubes, and 1.0 ml of the working reagent was added. After stirring and left to stand for 20 min, the phosphate solubilization was determined using a spectrophotometer (725 nm) according to Braga and DeFelipo (1974) modified by Reis et al. (2008).

Production of indole acetic acid (IAA)

All isolates evaluated in the phosphate solubilization tests had spore concentrations that were standardized to 10^5 CFU ml^{-1} by dilution with saline solution (0.9%). The isolates were grown on PDB medium supplemented with tryptophan (1%). Incubation was performed with shaking (90 rpm) for 13 days, in the absence of light.

For the quantitative evaluation, a calibration curve was initially created using a stock solution of IAA (300 mg ml^{-1}) that was diluted with distilled water to obtain increasing concentrations (0, 10, 20, 50, 100, 210, 430 and 500 µL ml^{-1}).

After centrifugation of the cultures (8000 rpm for 10 min at 4°C), 1.0 ml was removed from the supernatant and transferred to test tubes, and 1.0 ml of Salkowski reagent (0.62 g $FeCl_3.6H_2O$; 33 ml H_2O; and 50 ml H_2SO_4) was added. The test tubes were allowed to stand for 15 min in the dark, and then, the production of IAA was determined by colorimetric spectrophotometry (530 nm) (Gordon and Weber, 1950; Pereira et al., 2012).

Production of cytokinin and gibberellin

To evaluate the production of cytokinin and gibberellin by the *Trichoderma* isolates, the method suggested by Cattelan (1999) was used. Fungi were cultured in PDB medium for 8 days and centrifuged (6000 rpm for 10 minutes at 4°C). Radish seeds were passed through a sieve with 2 mm opening (ABNT 10) and germinated on wet filter paper in Petri dishes. These dishes were incubated at 25°C for 35 h in the dark.

After germination, the hypocotyls and cotyledons were separated and transferred to Petri dishes containing filter paper moistened with the supernatant from each isolate. Specifically, 10 smaller cotyledons and 10 hypocotyls fragments standardized to 3 mm were used. They were then incubated at 24°C for 72 h under continuous weak fluorescent light to compare the biomasses of the cotyledons and the lengths of the hypocotyls with the control, which consisted of filter paper moistened only with sterile medium.

Data were subjected to an analysis of variance, and the results of the phosphate solubilization and IAA, cytokinin, and gibberellin synthesis assays were compared by the Scott-Knott test (5%) using the SISVAR statistical software (Ferreira, 2011).

RESULTS AND DISCUSSION

Phosphate solubilization

Thirty (30) rhizosphere and 12 endophytic fungi were isolated from the seedlings of the guanandi plant grown in the nursery, whereas 83 rhizosphere and 18 endophytic fungi were isolated from the seedlings grown in the field. Among the isolates from the plant cultivated in the nursery, none were found to belong to the genus *Trichoderma*, whereas, among the fungal isolates from the guanandi plant collected in the field, 8 isolates belonging to the genus *Trichoderma* were found in the rhizosphere and 4 in the endophytic environment of the root (Figures 1 and 2).

The guanandi grown in the field allow the isolation of a higher density of microorganisms and fungi belonging to the genus *Trichoderma*, likely due to the increased concentration of organic matter and the presence of soil with a greater diversity of plant species than the soil used for growing seedlings in the nursery. Given that the nursery seedlings were grown under high stress conditions, including a low concentration of organic matter, a root system confined to tubes, and the soil of B horizon, a lower density of microbial species was expected.

The frequency of colonization for the root tissue fragments was 47.5% after 13 days of incubation. There was a higher incidence of *Trichoderma* species among the endophytic fungi, with 9.63% of the rhizosphere fungi and 22.22% of the endophytic fungi belonging to the genus *Trichoderma* (Table 1).

In the GELP solid medium, the ability to solubilize $CaHPO_4$ was not observed, due to the rapid growth of the isolates tested. Among the isolates grown in PDB that was supplemented with the phosphate sources mentioned above, 1 isolate was capable of solubilizing $CaHPO_4$, 12 isolates solubilized $FePO_4$, and 2 isolates solubilized $AlPO_4$. Thus, there are *Trichoderma* isolates that can benefit plant development, both for their known antagonist capacity against phytopathogenic fungi and for the solubilizing capacity and/or availability of phosphates.

All *Trichoderma* isolates demonstrated an ability to solubilize $FePO_4$ (Table 2). In the case of $AlPO_4$ solubilization, only isolates EC10M and EC12M demonstrated this capacity. Similarly, the same isolates and isolate RC28M were able to solubilize $CaHPO_4$. *T. asperellum* T211 demonstrated the highest ability to solubilize $AlPO_4$ but was unable to solubilize $CaHPO_4$ (Table 2).

Kapri and Tewari (2010) isolated 14 strains of *Trichoderma* spp. from the rhizosphere of different plant species and found the ability to solubilize phosphates in all of them, in different proportions. The P uptake mechanisms depend on the ability of the microorganism to

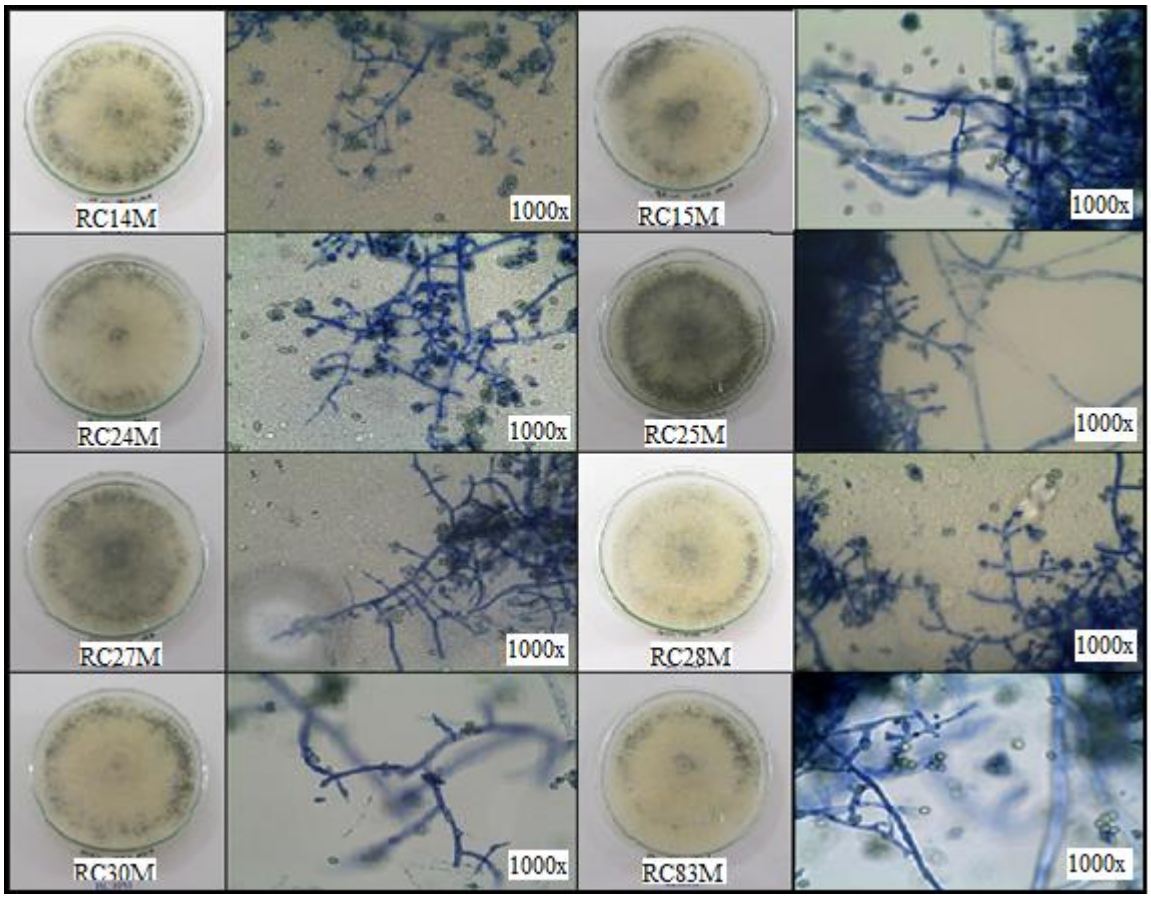

Figure 1. Isolates of *Trichoderma* isolated from the rhizosphere of a guanandi plant collected in the permanent preservation area, in Rio Verde, GO, Brazil. Spores: RC14M: L - 25.19 µ, W - 16.41 µ; RC15M: L - 25.07 µ, W - 18.05µ; RC24M: L - 24.47 µ, W - 15.89 µ; RC25M: L - 25.54µ, W - 16.89 µ; RC27M: L - 27.11 µ, W - 15.78 µ; RC28M: L - 26.91 µ, W - 16.94 µ; RC30M: L - 28.58 µ, W - 19.20 µ; RC83M: L - 28.92 µ, W - 17.57 µ (L = length, W = width).

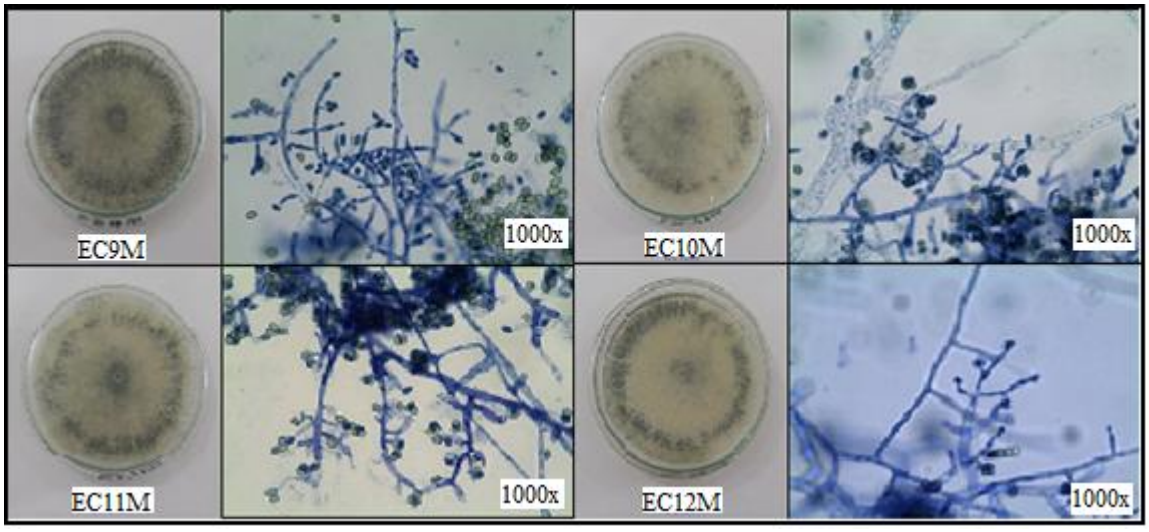

Figure 2. Isolates of *Trichoderma* isolated from the root interior of a guanandi plant collected in the permanent preservation area, in Rio Verde, GO, Brazil. Spores: EC9M: L - 28.39 µ, W - 18.81 µ; EC10M: L - 29.33 µ, W - 17.54 µ; EC11M: L - 25.77 µ, W - 16.16 µ; EC12M: L - 25.31 µ, W - 17.53 µ (L = length, W = width).

Table 1. Total of rhizospheric and endophytic fungi and *Trichoderma* isolates obtained from the root fragments of guanandi.

Fungi isolate	Number of fungi isolates	Number of *Trichoderma* isolates	Percentage (%)
Endophytic	18	4	22.22
Rhizospheric	83	8	9.63

Table 2. Contents of P solubilized by *Trichoderma* isolates of guanandi on PDB supplemented with calcium phosphate, iron phosphate and aluminum phosphate.

Trichoderma isolates	$CaHPO_4$ (µg ml^{-1})	$FePO_4$ (µg ml^{-1})	$AlPO_4$ (µg ml^{-1})
*RC14M	4.12^{d}	5.24^{b}	0.73^{e}
RC15M	4.39^{d}	5.13^{b}	0.56^{h}
RC24M	4.53^{c}	5.52^{b}	0.59^{g}
RC25M	4.33^{d}	5.38^{b}	0.41^{i}
RC27M	4.21^{d}	5.54^{b}	0.56^{h}
RC28M	5.54^{a}	6.09^{a}	0.61^{g}
RC30M	4.41^{d}	6.45^{a}	0.58^{h}
RC83M	5.43^{b}	5.99^{a}	0.75^{e}
**EC09M	5.35^{b}	5.74^{a}	0.67^{f}
EC10M	5.67^{a}	5.80^{a}	1.00^{c}
EC11M	5.20^{b}	5.67^{b}	0.67^{f}
EC12M	5.96^{a}	5.93^{a}	1.61^{b}
T. asperellum	4.19^{d}	ND	3.17^{a}
Control	5.32^{b}	2.92^{c}	0.91^{d}

Means followed by the same letter, in each column, do not differ by the Scott-Knott test (5%). *RC = rhizosphere isolate; **EC = endophytic isolate; ND = not detected.

colonize the plant roots, which demonstrates the importance of the distribution and metabolic activity of the microbial isolates for the preparation of inoculants (Behbahani, 2010).

According to John et al. (2010), soybean plants showed greater shoot and root growth when inoculated with *T. viride*. Phosphate solubilization by microorganisms plays an important role in providing P to the plants. Therefore, other fungal genera, such as *Aspergillus* and *Penicillium*, are equally efficient (Coutinho et al., 2012).

The use of phosphate solubilizing species as biofertilizers is an effective approach to replace or reduce the dependence on chemical fertilizers (Mamta et al., 2010).

The application of *Trichoderma* spp. combined with other microbial species may be a good strategy to increase growth, nutrient uptake and plant yield (Rudresh et al., 2005).

Carvajal et al. (2009) reported the potential to solubilize phosphates in 20% of 101 isolates of *Trichoderma* spp. Badawi et al. (2011) showed that *T. harzianum* had a greater ability to solubilize phosphate when compared with *Bradyrhizobium* spp. and *Serratia marscescens*.

Production of phytohormones

The capacity for IAA synthesis in PDB medium containing tryptophan was observed in 2 *Trichoderma* isolates obtained from the rhizosphere of guanandi. The production of this phytohormone by the endophytic isolates was not detected (Table 3).

The production of cytokinin and gibberellin was not detected in any of the isolates tested because only differences among the hypocotyls but not among the radish cotyledons were observed for all treatments (Table 4).

Auxins promote the growth of stems and coleoptiles and inhibit root growth (Taiz and Zeiger, 2009). The *Trichoderma* isolates tested by Oliveira et al. (2012) were able to produce IAA, and the use of the L-tryptophan precursor provided a positive effect as an inducer for the synthesis of this phytohormone. Gravel et al. (2007) observed the production of IAA by *T. atroviride*, using the precursors L-tryptophan, tryptamine and tryptophol. The species *T. harzianum* also demonstrated the ability to produce this phytohormone, according to Badawi et al. (2011).

Table 3. Indole acetic acid (IAA) contents produced by endophytic and rhizosphere *Trichoderma* isolates of guanandi on PDB supplemented with tryptophan (1%).

Trichoderma isolate	IAA(μgml^{-1})
*RC14M	1.82b
RC15M	1.30c
RC24M	1.46c
RC25M	1.38c
RC27M	1.56c
RC28M	2.18a
RC30M	1.49c
RC83M	1.32c
**EC09M	1.21c
EC10M	1.25c
EC11M	1.24c
EC12M	1.47c
T. asperellum	1.36c
Control	1.27c

Means followed by the same letter do not differ by the Scott-Knott test (5%). *RC = rhizosphere isolate; **EC = endophytic isolate.

Table 4. Cytokinin and gibberellin contents produced by endophytic and rhizosphere *Trichoderma* isolates of guanandi.

Trichoderma isolate	Cytokinin cotyledons (g)	Gibberellin	
		hypocotyls (mm)	+ cotyledons (g)
*RC14M	0.050a	3.625c	0.050a
RC15M	0.015a	3.375c	0.015a
RC24M	0.020a	3.875b	0.020a
RC25M	0.050a	4.750a	0.050a
RC27M	0.035a	3.125c	0.035a
RC28M	0.040a	5.250a	0.040a
RC30M	0.035a	3.500c	0.035a
RC83M	0.035a	4.250b	0.035a
**EC09M	0.050a	4.000b	0.050a
EC10M	0.040a	4.125b	0.040a
EC11M	0.040a	4.500b	0.040a
EC12M	0.035a	3.000c	0.035a
T. asperellum	0.040a	4.125b	0.040a
Control	0.040a	3.125c	0.040a

Means followed by the same letter in each column do not differ by the Scott-Knott test (5%). *RC = rhizosphere isolate; **EC = endophytic isolate.

Following the inoculation of cytokinin-producing microorganisms, increases in the weights of the cotyledons were observed, and for the microorganisms producing gibberellins, increases in the sizes of the hypocotyls and in the weights of the cotyledons were observed (Cattelan, 1999). In addition to playing a role in cell division in the shoots and roots, cytokinins affect other processes, such as vascular development, apical dominance and leaf senescence. The gibberellins stimulate both elongation and cell division (Taiz and Zeiger,

2009).

The *Trichoderma* spp. should be selected for the production of phytohormones, with subsequent use as inoculants to increase the growth of guanandi, given that this is not an ability found throughout the genus.

Conclusions

The *Trichoderma* isolates evaluated in the present study were able to solubilize phosphates in different proportions and have potential for use as inoculants to increase the formation of guanandi seedlings. The rhizosphere *Trichoderma* isolates RC14M and RC28M synthesized IAA and have potential for use as inoculants to promote the growth of guanandi seedlings.

The rhizosphere *Trichoderma* isolates were more efficient for IAA production than the commercial strain, *T. asperellum* T211.

The endophytic *Trichoderma* isolates did not demonstrate an ability to produce IAA, and none of the rhizosphere or endophytic isolates belonging to this genus produced neither cytokinin nor gibberellin.

Conflict of Interests

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

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Full Length Research Paper

Lipopolysaccharide isolated from *Rhizobium leguminosarum* strain P.SOM induces resistance in pea roots against *Orobanche crenata*

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Our previous studies showed that living and heat-killed cells of the *Rhizobium leguminosarum* strain P.SOM induce in pea roots systemic resistance to infection by *Orobanche crenata*. To better understand the mechanisms of induced resistance, we focused on identifying the inducing agent. Since heat stable bacterial surface carbohydrates such as exopolysaccharides (EPS) and lipopolysaccharides (LPS) are essential for recognition in the symbiotic interaction between *Rhizobium* and legumes, their role in the *R. leguminosarum*-pea interaction was studied. EPS and LPS were extracted from bacterial cultures, applied to pea roots, and tested for activity as an inducer of plant resistance to broomrape. Whereas EPS did not affect *O. crenata* infection, LPS reduced *Orobanche* infection significantly in concentrations as low as 1 and 0.5 mg ml^{-1}. In contrast these compounds did not influence parasite germination induced by the artificial stimulant, GR24. Our results show that soil treatments with LPS resulted in a highly significant reduction of *O. crenata* infection of pea roots. The results clearly showed that LPS of *R. leguminosarum* P.SOM act as the inducing agent of systemic resistance in pea roots against Orobanche.

Key words: *Rhizobium leguminosarum*, Lipopolysaccharides, Exopolysaccharides, Induced resistance, *Orobanche crenata*, Pea.

INTRODUCTION

Studies on a number of plant-microbe interactions showed that such antagonistic rhizobacteria can function directly by competition and antibiosis (Buchenauer, 1998) but also indirectly by inducing systemic resistance in the plant toward soil-borne pathogens (Hasky-Günther et al., 1998; Liu et al., 1995; van Peer et al., 1991).

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Rhizobacteria-mediated induced resistance has been demonstrated against fungi, bacteria, and viruses in Arabidopsis, bean, cucumber, and radish, under conditions in which the inducing bacteria and the challenging pathogen remained spatially separated. Furthermore, some *Rhizobium* strains can reduce pea and chickpea parasitism by *Orobanche crenata* and *Orobanche foetida* respectively (Mabrouk et al., 2007; Hemissi et al., 2013). However, bacterial compounds that induce plant defense mechanisms are highly variable depending on bacterial strain and pathosystems. For example, salicylic acid (SA) production has been observed for several bacterial strains, and exogenously applied SA can induce resistance in many plant species. In beans, enhanced defense by *Pseudomonas aeruginosa* strain 7NSK2 toward the pathogenic fungus *Botrytis cinerea* was initiated by SA synthesized by bacteria (De Meyer and Höfte, 1998). In tomato and radish, lipopolysaccharides (LPS) of nonpathogenic pseudomonads induced resistance against challenge inoculations by pathogenic bacteria (Müller et al., 1998). Similarly, LPS of *Pseudomonas fluorescens* strain WCS 417 have induced systemic resistance in carnation against *Fusarium* wilt caused by *F. oxysporum* f. sp. *dianthi* (Van Peer and Schippers, 1992). In the case of *Rhizobium etli*, LPS have been implicated in triggering the induced systemic resistance (ISR). For instance, Reitz et al. (2000, 2002) showed that LPS from *R. etli* played a major role in the elicitation/triggering of ISR in potato against cyst nematode *Globodera pallida*. Other studies showed that EPS purified from a *Rhizobacterium* strain, IN26, efficiently elicited ISR against cucumber anthracnose (Kyungseok et al., 2008). Recent data discusses the function of the genes required for EPS synthesis and the regulation of this process by several environmental signals, especially for the species *Sinorhizobium meliloti* (Janczarek, 2011).

Our previous work demonstrated that living and heat-killed cells of *Rhizobium leguminoisarum* P.SOM induced in pea roots systemic resistance against *O. crenata* infection (Mabrouk et al., 2008). The results of these studies suggested that heat-stable surface structures of *R. leguminosarum* P.SOM may be the inducing factors. Rhizobia produce several surface polysaccharides that are critical for attachment and biofilm formation; some of these polysaccharides are specific for their growth on root hairs and can considerably enhance their ability to infect their host legumes (Downie, 2010). Surface carbohydrates of *Rhizobium* consist mainly of exopolysaccharides (EPS) as additional capsular or slimy layers around the bacterial cell and LPS, which are an integral part of the outer membrane of the cell. Lipopolysaccharides contribute to the cocktail of microbe derived molecules, which plant cells are exposed to upon bacterial infection (Boller and Felix, 2009; Segonzac and Zipfel, 2011; Zhang and Zhou, 2010). LPS is major constituent of the Gram-negative outer membrane and are released from the bacterial cell wall into the plant apoplast through shedding by living cells or dissolution of dying or dead cells. In addition, LPS is important component of outer membrane vesicles, released from bacterial membranes (Sidhu et al., 2008). When plant cells perceive LPS, it can lead to the triggering of defence responses (Gerber et al., 2004; Zeidler et al., 2004), or to the priming of the plant (Madala et al., 2012; Newman et al., 2007). For the biological activity kutkowska et al. (2011) demonstrate that polysaccharide obtained by gel chromatography on Bio-Gel P-4 of the high molecular mass material from Rt120 had a toxic effect on tumor HeLa cells but was inactive against the normal human skin fibroblast cell line. Exopolysaccharides (EPSs) are a group of carbohydrates secreted by various bacterial species including pathogenic and symbiotic bacteria and fungi (Choi et al., 2007; Leigh and Coplin, 1992). For beneficial bacteria, including the rhizobia, various functions of EPSs have been reported. EPS produced by the alfalfa-symbiotic bacterium *S. meliloti* functions as a signaling molecule that triggers a developmental response in the plant or suppresses host defense responses (Mendrygal and González, 2000).

The objective of this investigation was to extract EPS and LPS from *R. leguminosarum* P.SOM strain and to determine whether these carbohydrates act as inducers of systemic resistance in pea roots to *O. crenata* infection.

Materials and methods

Bacterial strain and growth conditions

R. leguminosarum strain P.SOM was isolated from pea roots harvested from *Orobanche*-free crops (Mabrouk et al., 2007). This strain was grown at 28°C on a yeast extract mannitol medium containing 0.08% yeast extract (w/v) and 1% mannitol (w/v). The bacterial isolate were derived from single colonies. For further root inoculations, the bacterial cultures were prepared in distilled water, with several washes to remove traces of growth medium.

EPS and LPS extraction

For EPS extraction, *R. leguminosarum* P.SOM strain was cultured on 15 g agar plates with YEM at 28°C. After three days, the growth was scraped from the agar plates, resuspended in a 500 ml sterile 0.9% NaCl solution containing 5 mM EDTA, and thoroughly stirred. After centrifugation at 4,600 x *g* for 20 min at 4°C, the supernatant containing loose and bound EPS was sterile filtered (pore size, 0.2 µm) to remove any remaining bacterial cells. The EPS solution was dialyzed (12,000 Da; Serva) against demineralized water for 4 days and lyophilized.

LPS of *R. leguminosarum* P.SOM were extracted from cells grown in 1 L of YEM broth. After 72 h of growth, the cells were harvested by centrifugation at 4°C for 15 min at 4,600 x *g* and washed three times with 0.5 M NaCl containing 5 mM EDTA to remove loose and bound EPS. The bacterial pellet was then lyophilized, suspended in 50 ml of buffer L (50 mM sodium phosphate buffer [pH 7.0], 5 mM EDTA, 0.05% sodium azide), and digested by lysozyme [6 mg g^{-1} (dry weight), 50,000 U; Sigma] at

4°C for 16 h. The bacterial extract was then treated with DNase (0.3 mg g^{-1} [dry weight], 2,000 U mg^{-1} [solid]; Boehringer) and RNase (0.3 mg g^{-1} [dry weight], 37 U mg^{-1} [solid]; Sigma) at 37°C for 30 min. Remaining protein was digested overnight by incubation with proteinase K (0.3 mg g^{-1} [dry weight], 15 U mg^{-1} [solid]; Sigma) followed by incubation for 10 min at 60°C to denature the protein. Finally, LPS was purified by the hot phenol-water method (Westphal and Jann, 1965). The aqueous LPS solution was dialyzed (12,000 Da; Serva) for four days against demineralized water to remove traces of phenol and again lyophilized. Two stock solutions of EPS and LPS were prepared: 1 mg ml^{-1} of sterile one-fourth-concentrated Ringer solution (for bioassays).

Effect of EPS and LPS on *Orobanche* seeds germination

To test the effect of EPS and LPS on *O. crenata* seeds germination, 0.3 ml of either 10 ppm of the germination stimulant GR24 (optimal concentration for germination), either alone or in combination with one of rhizobial polysaccharides (EPS and LPS) was used. EPS and LPS were tested at two concentration: 0.5 and 1 mg/ml. Each rhizobial polysachharides compound was added to Petri dishes containing preconditioned seeds and then incubated at 21°C. Germination was evaluated 7 days later using a binocular microscope. Germination was expressed as a percentage of the total seeds. Seeds were considered to be germinated when the germ tube was at least 0.1 mm long.

Evaluation of EPS and LPS as resistance inducers against broomrape (*Orobanche crenata*)

Co-culture using Petri dishes experiments

Co-cultures were performed according to Labrousse et al. (2001). Pea seedlings (7-10 day old) were transferred to Petri dishes (120 X 120 X 17 mm, Greiner). Three perforations were made, in the two opposite borders of the Petri dish, one to hold the pea shoot out of the dish and the others to allow plant root feeding in culture medium. Roots were spread between the dish cover and a fibre filter paper (MN 85 ⁄ 90, 12.5 cm diameter, Macherey-Nagel). A 1 cm thick rock-wool layer (Master from Grodan, Town) was placed on the other side of the glass fibre filter paper. Petri dishes were closed, stored vertically in a sterile polypropylene tray containing sterile solution of Coïc neutrophile nutrient (Coïc and Lesaint, 1975) and the whole was covered with aluminium foil and maintained at 21°C with 100 µmol m^{-2} s^{-1} photosynthesis activity report (PAR) under a 16-h photoperiod. When 15-day-old pea plants displayed well-developed roots on the glass fibre filter paper, 3 mL of P.SOM bacteria suspension (10^7 rhizobia mL^{-1}) or 3 mL of rhizobial polysaccharides (EPS or LPS) suspensions were added to the roots. In addition, preconditioned seeds of *O. crenata* (100 seeds) were placed regularly at 1-2 mm from roots. *O. crenata* seed germination was evaluated at 15, 30 and 45 days after inoculation by rhizobia or treatments with rhizobial polysaccharides by using a binocular microscope. Four areas per Petri dish were observed and the number of germinated seeds counted and expressed as percentage of total seeds. Germination rates were expressed by taking account of the viability of the seed lot used for experiments (70%). By 45 days after treatment (DAT), necrotic tubercles and necrotic germinated seeds that did not succeed in attaching to host roots in the previously selected areas of Petri dishes were counted and expressed as the percentage of total tubercle and germinated seeds respectively. Non-inoculated pea seedlings growing in contact with Orobanche served as controls.

Pot experiments

R. leguminosarum P.SOM strain, EPS and LPS were tested in a pot experiment with five replicates per treatment. Pea seedlings were transferred to plastic pots (0.5 L) containing a sterilised mixture of local field soil and sand (1:1, v/v) and then either left as such or contaminated with *O. crenata* seeds (5 mg/pot). Five sets of pot cultures were managed simultaneously: (i) pea grown in Orobanche-free soil; (ii) pea grown in infested soil; (iii) pea grown in infested soil inoculated with *R. leguminosarum* P.SOM strain (inducer of defence; Mabrouk et al., 2007); (iv) pea grow in infected soil treated by EPS (5 mg/pot) and (v) pea grown in infected soil treated by LPS (5 mg/pot). Inoculations were performed directly following transfer of pea seedlings into culture pots by addition of 3 mL of bacterial suspension (10^7 rhizobia mL^{-1}). Treatments with rhizobial polysachharides were performed using 3 mL of suspensions of EPS or LPS per pot. Plants were supplied weekly with N-free nutrient solution (Vadez et al., 1996) and water. The impact of rhizobia, EPS and LPS on pea infection by *O. crenata* was estimated at 70 days old cultures. Pea roots were gently harvested, washed with water, and total tubercles per plant as well as the dry matter of Orobanche were recorded.

Statistical analysis

Five plants were grown per treatment in all the experiments and experiments were conducted twice. Similar results were obtained in both experimentations. In all the experiments, 10 plants were grown per treatment. Consequently, the data were means ± confidence limits (n =10, α=0.05 Student's t-test). Data were analysed by multifactorial analysis of variance (ANOVA, SPSS 13.0 for Windows) and significant differences among treatments were considered at the $P < 0.05$ level.

RESULTS

Analysis of EPS and LPS

Analysis of LPS patterns by SDS-PAGE confirmed the presence of LPS constructs in the LPS extract (data not shown). The LPS of *R. leguminosarum* P.SOM separated into seven bands. The LPS clearly possessed characteristic patterns of *Rhizobium* spp. In the EPS extract of *R. leguminosarum* P.SOM, typical LPS bands were not detected. The test for KDO verified the presence of the core region in the LPS of *R. leguminosarum* P.SOM at concentrations of 15 nmol of KDO per mg of LPS. The lack of KDO in the EPS extract excluded any contamination by LPS. The proteins are not detectable in the EPS and LPS extracts.

Evaluation of Rhizobium polysaccharides (LPS and EPS) to control of broomrape (*O. crenata*)

Effect of rhizobial polysacharides on underground stages of O. crenata

Our experiments confirmed the induction of systemic resistance in pea roots against Orobanche parasitism by pretreatment with *R. leguminosarum* P.SOM (Table 1). The EPS of *R. leguminosarum* P.SOM showed no effect on broomrape parasitism when applied on pea roots (Table 1). Treatment with the LPS extract (1 mg ml^{-1}),

Table 1. *Orobanche crenata* germination and tubercles formation in co-culture experiments on pea roots in relation to *Rhizobium leguminosarum* strain P.SOM inoculation or rhizobial polysaccharides (LPS and EPS) treatments.

Treatment	30 DAT			45 DAT		
	% of *O. crenata* seeds germination	Necrotic germinated seeds (% of total germinated seeds)	Number of tubercles/plant	% of *O. crenata* seeds germination	Necrotic germinated seeds (% of total germinated seeds)	Number of tubercles/plant
Pea	53.45±7.98	0	28.33±3.23	59.66±6.66	2.88 ±0.42	48.15± 4.98
Pea +LPS (0.5 mgmL^{-1})	48.98±5.34	0	12.99±1.44	41.87±7.35	4.67±0.85	11.22 ±0.78
Pea +LPS (1 mgmL^{-1})	38.66±3.87	0	0	28.35±4.65	52.87±4.68	0
Pea+ EPS (1 mgml^{-1})	57.34±8.55	0	27.67±2.87	61.4±5.89	3.45±0.8	46.26± 3.23
Pea + P.SOM strain	15.66±3.44	14.30±3.55	3.25±0.25	17.55±3.45	80.60±4.65	4.66 ±1.25

however, resulted in significant decrease in germination of *O. crenata* seeds (up to 50%) and none tubercle formed on pea roots after 45 of treatment (Table 1 and Figure 1F). At lowest concentration (0.5 mg ml^{-1}), the LPS caused also, the reduction in broomrape seeds germination and tubercles number formed on pea roots by 30 and 75% respectively at 45 DAT (Table 1). Changes in some *O. crenata* germinated seeds started to be visible on day 25. Radicles turned from colorless and translucid to brownish, elongation ceased, and orobanche development stopped (Figure 1B). The observation of early stages of the infection process (from attachment to tubercles formation) showed that *O. crenata* successfully invaded the pea root not treated with bacterial polysaccharides and small tubercles (10-14 per plant) were established 35 to 45 days after inoculation (Figure 1C). In seedlings treated with LPS, *O. crenata* radicles elongated and adhered to the root surface, but some of them became brown (Figure 1B). Some disease symptoms on early development stages were observed in the case of treated pea with LPS at low concentration (Figure 1D). The proportion of necrotic tubercles increased with time. Forty five days after transplanting the highest percentage was observed on pea treated with LPS (data not shown). Figures 1A and C show the compatible interaction between *O. crenata* and a susceptible legume (pea). Healthy tubercle developed on the host roots within 14 day attachment. In contrast, when *O. crenata* attacked the pea roots treated with LPS, it died at early stage of tubercle development (Figure 1D). The influence of *R. leguminosarum* P.SOM polysaccharides (EPS and LPS) on Orobanche seeds germination had to be clarified. Germination rate was shown to be high in the presence of the synthetic stimulant GR24 (84%). When added in complement to GR24 in Petri dishes, the bacterial polysacharides (EPS and LPS) did not influence broomrape germination (data not shown), as well as radicle growth.

Effect of rhizobial polysaccharides on O. crenata development in pots

In the pot experiments pea roots treated with LPS (5 mg/pot) resulted in decrease of the number of tubercles on pea roots. However, the number of tubercles formed did not differ statistically between control and pea treated with EPS (Figure 2). The total Orobanche dry matter per pot was significantly reduced by 75% in pea roots treated with LPS (Figure 3). Obviously, when plants grew in Orobanche-infested soil in pot experiments, pea roots treated with the LPS extract carried few broomrape tubercles by 70 DAT in comparison with non-treated peas (Figure 2). As observed in Petri dishes (Table 1), a relatively high percentage of these tubercles turned brown. In contrast, treatment with the EPS did not reduce pea susceptibility to broomrape in pot experiments, as demonstrated by the number of tubercles carried by these roots, which did not differ from that of the non-treated plants.

DISCUSSION

Many studies demonstrated that specific rhizobacteria reduce plant infection by various parasitic plant such as *O. crenata* and *O. foetida* (Mabrouk el al., 2007; Hemissi et al., 2013). In our studies, it was shown that the *R. leguminosarum* P.SOM strain reduced pea roots infection by *O. crenata* indirectly by inducing systemic resistance (Mabrouk et al., 2010). Since the plant defense capacity in pea roots was enhanced by both living and heat-killed cells of this strain, it was concluded that heat stable surface structures such as EPS and/or LPS act as inducing agents (Mabrouk et al., 2008).

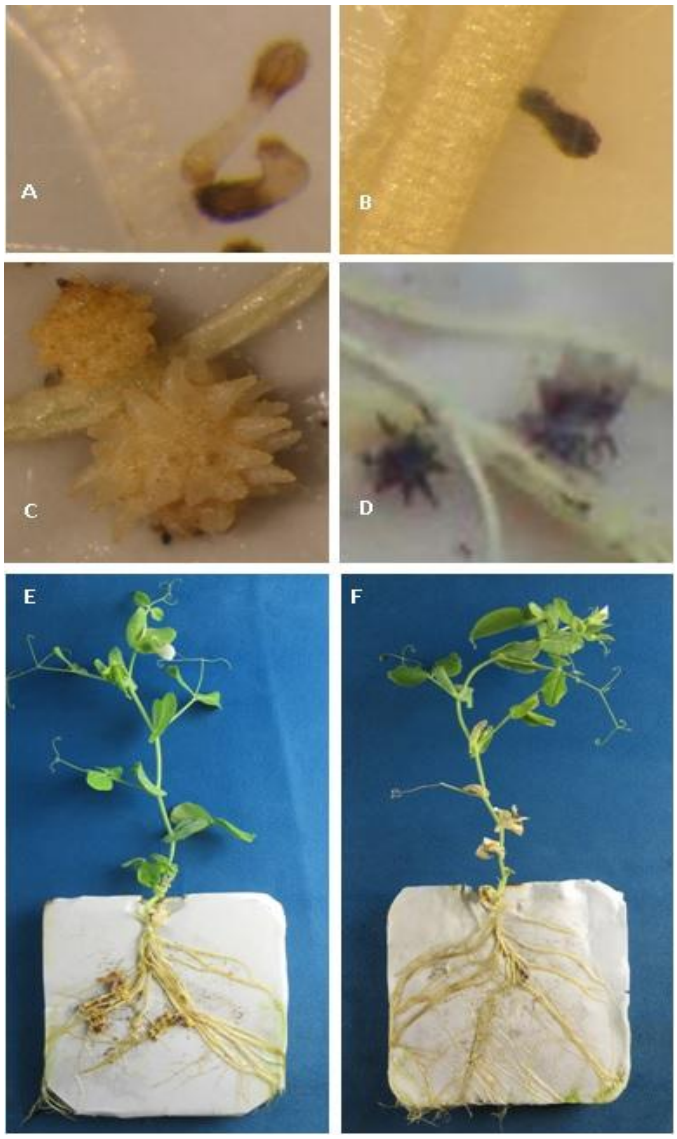

Figure 1. **(A)** Typically germinated seed. **(B)** Germinated seed showing browning symptoms. **(C)** Healthy tubercle carrying crown roots. **(D)** Failure in tubercle development, accompanied by necrotic symptoms. **(E)** Infested pea roots by Orobanche. **(F)** Pea roots treated by LPS showing no tubercles.

In this study, we demonstrated that the EPS of *R. leguminosarum* P.SOM did not affect plant defense reactions in pea roots against broomrape. Our results are in agree with those of Reitz et al. (2000), which demonstrated that EPS of *R. elti* G12 did not protect potato against *G. pallida*. In contrast Kyungseok et al. (2008) showed that EPS purified from a rhizobacterium strain, IN26, efficiently elicited ISR against cucumber anthracnose. However, a role of EPS as an inducing factor leading to a systemic reduction in broomrape infection throughout the pea root system can be ruled out.

We demonstrated that pea roots treatment with LPS extract significantly decreases their susceptibility to the parasite *O. crenata* indicating the implication of these compounds in induced resistance. The effect direct on broomrape seeds germination and development can be excluded, because LPS did not affect the germination rate of seeds induced by GR24. Induced resistance in treated peas was shown to occur throughout the infection process. Indeed, induced resistance was expressed at different developmental stages of Orobanche including germination, radicle growth, parasite attachment to pea roots and finally tubercle growth on host roots.

It has been shown that LPS of *P. fluorescens* strain WCS 417 have induced systemic resistance in carnation against *Fusarium* wilt caused by *F. oxysporum* f. sp.

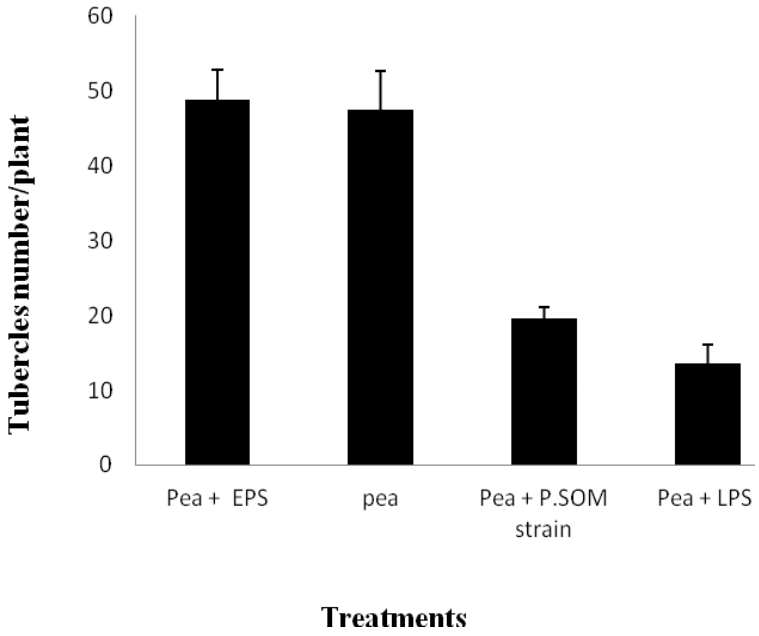

Figure 2. Impact of root inoculation with selected *Rhizobium leguminosarum* strain P.SOM or treatments by rhizobial polysaccharides (EPS and LPS) on tubercles formation in pot experiments.

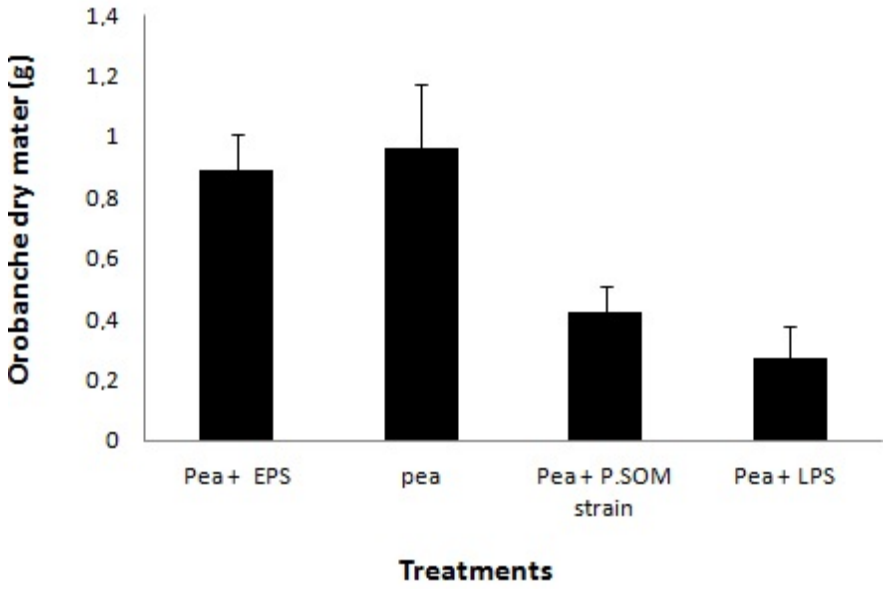

Figure 3. Impact of root inoculation with selected *Rhizobium leguminosarum* strain P.SOM or treatments by rhizobial polysaccharides on orobanche dry matter in pot experiments.

dianthi (van Peer and Schippers, 1992). Similarly, LPS of *P. fluorescens* strains WCS 374 and WCS 417 have induced systemic resistance in radish against *F. oxysporum* f. sp. *raphani* (Leeman et al., 1995). They also established that mutant of *P. fluorescens* strain WCS 417, lacking the O-antigen side chain of the LPS, has not induced resistance in radish indicating the O-antigen side chain of the LPS might have served as a signal or trigger in the induction of defence mechanism in plants. In contrast, LPS of *P. putida* strain WCS 358 having o-antigen side chain do not induce systemic resistance in radish. In another study, LPS of WCS 417r and mutant of

WCS 417r lacking O-antigen side chain of LPS elicit defence mechanism in *Arabidopsis* (van Wees et al., 1997).

We conclude that LPS could be used as inducer of defense in pea against *O. crenata*. Nevertheless, further studies are needed to identify the LPS component of *R. leguminosarum* P.SOM inducing systemic resistance of pea roots to Orobanche infestation and to characterize the mechanisms involved in this induced resistance to improve uses of LPS in broomrape control program in pea fields.

Conflict of Interests

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

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Full Length Research Paper

Detection and biovar discrimination of *Ureaplasma urealyticum* colonization in preterm neonates under ventilation and correlation with bronchopulmonary dysplasia

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Despite numerous studies, controversy regarding the association between *Ureaplasma urealyticum* colonization of premature neonates and bronchopulmonary dysplasia (BPD) still exists. The aim of the present study was to determine the colonization rate of preterm ventilated neonates with the two *U. urealyticum* biovars, parvo and T960, and to assess the correlation between colonization and the development of BPD. A prospective study was done from February 2013 to January 2014 in neonatal intensive care unit (NICU) in Mansoura University Children Hospital (MUCH) and NICU of different specialized medical centers, with 100 ventilated premature neonates (< 34 weeks) in the first 24 h of life, where tracheal secretions were aspirated, transported and cultured on 10B broth and A7 agar media. Culture was positive in 10 out of 85 samples (11.8%) as compared to 15/85 (17.6%) samples obtained by PCR technique. PCR assay demonstrated that Parva biovar (biovar 1) was the predominant, found in 9 (60%) out of 15 Ureaplasma isolates, as compared to T960 biovar (biovar 2) in 6 (40%) isolates. None of the neonates were co-colonized with both biovars. There was a statistically significant difference in the mean gestational age and mean birth weight between neonates with positive Ureaplasma colonization and neonates without colonization. BPD was significantly higher among colonized neonates than non-colonized one, 12 (80%) of 15 colonized neonates developed BPD as compared to 21 (30%) of 70 non colonized neonates. BPD was found to be correlated to decreasing gestational age (r = -0.341, p=0.001), low birth weight (r = -0.328, p= 0.002), and Ureaplasma colonization (r = 0.391, p < 0.001). Logistic regression analysis revealed that Ureaplasma colonization was a more important predictor for development of BPD than decreasing gestational age. BPD rate was higher among neonates colonized with T960 biovar (biovar 2) than those colonized with parvo biovar (biovar 1) (83.3% vs. 77.7%) with statistical significant difference (p = 0.001). colonization of respiratory tract by *U. urealyticum*, particularly biovar 2 (T960 biovar) in premature ventilated neonates was related to the development of BPD. PCR is a sensitive and specific technique for detecting Ureaplasma and for distinguishing its biovars (Parvum and T960) directly from clinical samples.

Key words: *Ureaplasma urealyticum*, bronchopulmonary dysplasia, chronic lung disease, prematurity.

INTRODUCTION

Ureaplasma spp. are members of class *Mollicutes* that colonize human mucosal surfaces of the respiratory and urogenital tracts (Waites et al., 2005). Data from 16srRNA sequencing led to classification of *Ureaplasma* serovars into two biovars; (*Ureaplasma parvum*, biovar 1, parvo) and (*Ureaplasma urealyticum*, biovar 2, T960) including a total of 14 serovars, biovar 1 included serotypes 1, 3, 6 and 14, and the remaining 10 serovars belonged to biovar

2 (Robertson et al., 2002). Characteristics of all serovars include lack of cell walls, limited biosynthetic abilities, small genome size, and mucosal association in the human host (Viscardi, 2010). The unique characteristic of *Ureaplasma* is their ability to hydrolyze urea to generate metabolic energy (Waites et al., 2005, 2009). Some debates still occur regarding whether there is a difference in pathogenicity among these 14 serovars and 2 biovars (Sung, 2010).

Although *U. urealyticum* is a common commensal microorganism in the genital tract of sexually mature women (Abele-Horn et al., 1997a; Cunliffe et al., 1996), it is strongly associated with genitourinary syndromes and pregnancy complications such as chorioamnionitis, stillbirth, preterm delivery, neonatal morbidity and perinatal death (Abele-Horn et al., 2000; Yoon et al., 2001; Waites et al., 2005; Viscardi, 2010). They are the most common perinatally acquired pathogens in preterm infants (Viscardi, 2010). It appears that in-utero infection of the fetus is common, but neonates may also be colonized initially at the time of delivery (Waites et al., 2009).

A number of studies attempted to relate *U. urealyticum* colonization to the development of respiratory diseases in premature newborns and mostly with bronchopulmonary dysplasia (BPD).

Bronchopulmonary dysplasia (BPD) or previously known as chronic lung disease (CLD) of the newborn is a serious problem among very low-birth weight infants and has become even more so due to the increased survival of more immature infants (Bancallari et al., 2003; Jobe and Bancalari, 2001). It has been suggested that development of BPD (CLD) is related to pulmonary immaturity, oxidant injury due to high levels of inspired oxygen, and volutrauma associated with mechanical ventilation. However, recent research has focused on the roles of perinatal infection and the inflammatory response as critical factors influencing chronic lung injury (De Dooy et al., 2001; Lyon, 2000). Particular attention has been given to the role of *Ureaplasma* species, found in the lower genital tracts of 40 to 80% of asymptomatic women (Cassell et al., 2001).

Since BPD etiology is multifactorial and complex, the relationship of Ureaplasma respiratory tract colonization with the development of BPD has been debated (Kallapur et al., 2013). The distinction of *U. urealyticum* and *U. parvum* species could also open new perspectives of study.

U. urealyticum is among the less frequently diagnosed respiratory pathogens in a clinical environment, mainly because of the lack of standardized and specific diagnostic tests (Blanchard et al., 1993). Routine bacterial cultures are not sufficient to recover *Ureaplasma* species. It is difficult, expensive, needs special culture media and growth conditions. Furthermore, it is time consuming, and requires repeated observations with light microscopy (Mallard et al., 2005; Petrikkos et al., 2007). In newborns, an additional problem is the necessity of antibiotic administration before the diagnostic procedures are started. Antibiotics inhibit the growth of ureaplasmas, thus limiting the reliability of the culture method (Biernat-Sudolska et al., 2006).

Currently, polymerase chain reaction (PCR) appears to be the most promising method that have replaced culture for the detection of mycoplasmas from clinical specimens (Biernat-Sudolska et al., 2006; Petrikkos et al., 2007). Several PCR methodologies for the detection of *Ureaplasma*, targeting 16S rRNA (Robertson et al., 1993), urease (Scheurlen et al., 1992, Willoughby et al., 1991), and multiple-banded antigen (MBA) (Teng et al., 1994a) gene sequences, have been described. Primers directed against MBA gene has many advantages including: (i) detection of all 14 serovars of Ureaplasma, (ii) lack of detection of product from 17 other mycoplasma species including phylogenetically closely related species such as *Mycoplasma pneumoniae*, and (iii) distinction of amplicons from biovar 1 strains (serovars 1, 3, 6, and 14) from amplicons from biovar 2 strains (serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13) (403 versus 448 bp, respectively) (Teng et al., 1994a; Nelson et al., 1998).

The aim of the present study was to determine the colonization rate of preterm ventilated neonates with the two *U. urealyticum* biovars, parvo and T960, and to assess the correlation between colonization and the development of BPD in a prospective study.

MATERIALS AND METHODS

Patients

One hundred premature newborns with gestational age less than 34 weeks, without congenital anomalies who were admitted within 24 h after birth for assisted ventilation to the neonatal intensive care unit (NICU) in Mansoura University Children Hospital (MUCH) and other specialized medical centers were prospectively enrolled into the study from February 2013 till January 2014.

Data on perinatal events were obtained from hospital records; mode of delivery, gestational age, birth weight, Apgar score, respiratory distress syndrome (RDS), maternal hypertension, premature rupture of membranes (PROM), chorioamnionitis and antenatal steroids administration. Gestational age was established by the last normal menstrual period and ultrasound examination before 20 weeks of gestation.

Bronchopulmonary dysplasia (BPD) (CLD) was considered to be present if the child needed supplemental oxygen at 28 days of age (Northway et al., 1967; Hannaford et al., 1999) or at 36 weeks gestational age accompanied by changes in chest radiographs (Bancalari et al., 2003).

Written informed consent was obtained from parents of the included infants. The protocol was accepted by the local ethical committee

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Sample collection

Tracheal aspirates were collected immediately after birth (within the first 24 h after intubation) to exclude nosocomial transmission of infectious agents. Blood cultures were obtained when septic infection was suspected.

Endotracheal aspirates (ETA) were collected aseptically; Sterile, preservative- free saline (0.5 ml) was instilled into the endotracheal tube or nostril, the infant was ventilated for 10 breaths. Using an appropriately sized catheter, the trachea was suctioned at a point 0.5 cm beyond the tip of the endotracheal tube; after another 10 ventilator breaths suctioning was repeated with a new catheter.

The suction catheter was flushed with 2 ml 10B broth, and the samples were transported on ice until processed. Aliquots were removed and frozen at -70°C for later analysis by PCR to ensure that PCR and culture would be performed on the same sample mixture.

Culture

ETA specimens were vortexed and 0.2 mL of each specimen was added to 1.8 mL of 10B broth. Tubes were incubated aerobically at 37°C. If color change occurred, 0.2 ml of inoculum was plated onto A7 agar plates, incubated at 37°C in 5% CO_2.

Liquid media were incubated for 72 h at 37°C, solid media for 5-7 days. The growth on liquid media was observed as a change of color of the medium (hydrolysis of urea with the release of ammonia, signaled by a colour change of a pH indicator), while on solid media by the presence of characteristic golden-brown colonies of ureaplasmas (magnification 125x).

Tube cultures and plates were examined daily for one week for color change (from yellow to pink) and typical colonies of Ureaplasma as described previously. A positive culture was defined as a positive broth (color change) confirmed by typical colony morphology on A7 agar.

Negative broths and plates were subcultured after 48 h to a new broth and plate. All broths were read twice daily, and the total incubation time for the cultures was 10 days.

PCR

DNA was extracted directly from samples using QIAamp DNA Blood Mini kits (QIAgen, Hilden, Germany) according to the manufacturer's protocol. Ureaplasma were detected and biotyped by PCR targeting MBA gene UMS-125 (GTA TTT GCA ATC TTT ATA TGT TTT CG) and UMA226 (CAG CTG ATG TAA GTG CAG CAT TAA ATT C) (Teng et al., 1994b).

The amplification reaction mixtures contained 50 µl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µl of each deoxynucleotide triphosphate (dATP, dCTP, dTTP and dGTP (Fermentas, Germany), 2.5 U Taq DNA polymerase (Fermentas, Germany), and 30 pmol of each primer UMS 125 (GTA TTT GCA ATC TTT ATA TGT TTT CG) and UMA 226 (CAG CTG ATG TAA GTG CAG CAT TAA ATT C) synthesized by Bio Basic Inc., Canada, and 10 µl of the sample DNA. The reaction mixtures were covered with mineral oil and subjected to an initial denaturation at 95°C for 4 min; then 40 cycles at 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s; and a final elongation at 72°C for 3 min.

Detection involved visualization after gel electrophoresis, with benefits of speciation by amplicon size of 403 bp for *U. parvum* (biovar 1) and amplicon size of 448 bp for T960 biovar (biovar 2).

Statistical analysis

Statistical analysis of the data was done by using Statistical Package for Social Science (SPSS) version 15.0. Data are expressed as mean value ± SD for quantitative data and as frequency (number/percent) for qualitative data. Comparisons between two different groups were carried out by unpaired t-test. Pearson's Chi-square or fisher exact test was used for comparisons of categorical data. Spearman's rank correlation was used to assess relations between variables. Some investigated parameters were entered into a logistic regression model to determine which factor is considered as a significant risk factor and identified its odds ratio. The sensitivity and specificity of culture was detected using ROC curve analysis. Differences were considered statistically significant when $P < 0.05$.

RESULTS

From 100 premature neonates who were enrolled in this study, only 85 met the eligibility criteria. 7 neonates had insufficient clinical data or insufficient sample collected, 8 neonates died before 28 days of age. We restricted analysis to neonates who survived beyond 28 days of age.

From 85 studied premature ventilated neonates who met the eligibility criteria, 15 (17.6%) were colonized with *U. urealyticum* as detected by culture and/or PCR. A sample was considered positive if 10B broth had color change and confirmed morphology on A7 agar and/or positive PCR.

Culture was positive in 10 out of 85 samples (11.8%), as compared to 15/85 (17.6%) samples obtained by PCR technique (P=0.001) (Table 1).

When PCR was used as a reference standard, the overall agreement between culture and PCR was 83.3% with 66.7% sensitivity and 100% specificity of culture as compared to PCR (Figure 1).

Mean ± SD of gestational age of neonates with Ureaplasma colonization were 29.7 ± 1.5 and neonates without colonization 30.94 ± 1.4 that showed a statistically significant difference (P= 0.004).

There was also a statistically significant difference in the mean birth weight between neonates with and without colonization (1279.33 ± 260.9 vs. 1573.99 ± 389.6).

Apart from spontaneous vaginal delivery (SVD), there was no statistically significant difference between colonized and non colonized neonates regarding other parameters including gender, antenatal steroids and maternal PROM (Table 2). 12 (80%) of 15 colonized neonates developed BPD as compared to 21 (30%) of 70 non colonized neonates. BPD was significantly higher among colonized neonates than non colonized ones ($P < 0.001$). Among the study 85 neonates, BPD developed in 33 (38.8%) cases. BPD was found to be correlated to decreasing gestational age ($r = -0.341$, $p=0.001$), low birth weight ($r = -0.328$, $p= 0.002$) and Ureaplasma colonization ($r = 0.391$, $p < 0.001$).

Logistic regression analysis revealed that Ureaplasma colonization was a more important predictor for development of BPD than decreasing gestational age (Table 3). PCR assay found that Parvo biovar (biovar 1) was predominant in 9 (60%) out of 15 Ureaplasma

Table 1. Comparison of Ureaplasma PCR results with Ureaplasma culture results.

PCR		Urea plasma culture		Total
		Positive	Negative	
Ureaplasma PCR	PCR positive	10	5	15
	PCR negative	0	70	70
Total		10	75	85

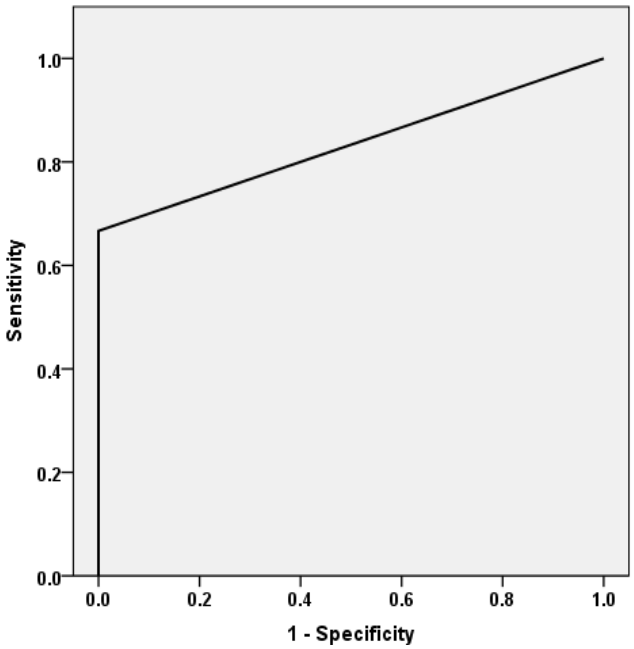

Figure 1. Roc curve.

isolates, as compared to T960 biovar (biovar 2) in 6 (40%) isolates. None of the neonates were co-colonized with both biovars (Table 4). Although, Parva biovar was more predominant than T960 biovar, BPD rate was higher among neonates colonized with T960 biovar (biovar 2) than those colonized with parvo biovar (biovar 1) (83.3 vs. 77.7%) with statistical significant difference in frequencies of BPD between neonates colonized by biovar 1 or biovar 2 (p = 0.001) (Figure 2).

DISCUSSION

The multifactorial etiology of BPD of the newborn has been reported over the last three decades (Bancalari et al., 2003; Jobe and Bancalari, 2001; Kinsella et al., 2006; Speer, 2003; Speer, 2006) With the increased survival of a greater number of more immature infants, the contribution of antenatal infection to BPD has been argued (Jobe, 2003; Lyon, 2000; Miralles et al., 2002). *Ureaplasma* spp. are associated with increased risk for preterm labor and morbidity in the preterm neonate. However, there are some controversies regarding the importance of *Ureaplasma* in the pathogenesis of BPD (Kallapur et al., 2013). We aimed to clarify the contribution of Ureaplasma biovars colonization to the development of BPD.

In the present study, 17.6% of 85 premature ventilated neonates were colonized with *U. urealyticum* as detected by culture and/or PCR. This is in agreement with the percentage of colonization of *U. urealyticum* in neonatal respiratory specimens reported in other studies from 3 to 23% (Blanchard et al., 1993; Cassell, 1993; van Waarde et al., 1997; Payne et al., 1993; Jonsson et al., 1994; Mohagheghi et al., 2013). Higher rates were reported by Nelson et al. (1998) (36%), Ollikainen et al. (2001) (33%), Pacifico et al. (1997) (40%), Ollikainen (2000) (55%) that could be explained by larger sample size, or testing multiple specimens from enrolled patients.

In the present study, 5 samples were PCR positive but culture negative for *U. urealyticum*. The sensitivity and specificity of culture when compared with PCR were 66.7 and 100%, respectively. Our results are consistent with other author's observations that culture is less sensitive (Teng et al., 1994a; Luki et al., 1998; Biernat-Sudolska et al., 2006).

The higher sensitivity of PCR may be due to the generally recognized difficulties of culturing and isolating Ureaplasma. The results of PCR amplification are less prone to being influenced by methods of sample collection, and handling. Moreover, PCR is much quicker, results can be obtained in 1-2 days, whereas it takes 5-7 days in the case of cultivation.

The ability to differentiate the Ureaplasma biovars in clinical samples has long been a challenge for investigators. Our study confirms previous observations (Abele-Horn et al., 1997a; Katz et al., 2005) that biovar 1 (parvo biovar) is the predominant species colonizing the respiratory tract of preterm infants. On the other hand, Kotecha and co-workers (2004) have described the colonization of their study patients with either biovar 1 or biovar 2 without a different distribution inside patient groups.

The detected frequency of BPD (38.8%) agrees with 17 to 60% frequencies in the previous studies (Sanchez and Regan, 1988; Illes et al., 1996). BPD rate was higher among neonates colonized with T960 biovar (biovar 2) than those colonized with parvo biovar (biovar 1). In agreement with our study, Abele-Horne et al. (1997b)

Table 2. Clinical features of 85 study neonates and Ureaplasma colonization.

Parameter		Urea plasma colonization		P
		Positive	Negative	
Gestional age (weeks) (Mean±SD)		29.7±1.5	30.94±1.4	0.004
Birth weight (g) (Mean±SD)		1279.33±260.99	1573.99±389.6	0.007
Gender	Male	7 (46.7%)	30(42.9%)	0.78
	Female	8(53.3%)	40(57.1%)	
Antenatal steroid	Yes	11(73.3%)	47(67.1%)	0.64
	No	4 (26.7%)	23(32.9%)	
Maternal PROM	Positive	7(46.7%)	23(32.9%)	0.31
	Negative	8(53.3%)	47(67.1%)	
Delivery mode	SVD	12(80%)	31(44.3%)	0.01
	CS	3(20%)	39(55.7%)	
Final outcome	Non-BPD	3(20%)	49(70%)	<0.001
	BPD	12(80%)	21(30%)	

SD: Standard deviation P: Probability CS: cesarean section SVD: spontaneous vaginal delivery; BPD: chronic lung disease.

Table 3. Logistic regression analysis with respect to BPD.

Factor	P-value	OR	95% CI
Gestational age	0.025	0.668	0.46-0.95
Ureaplasma colonization	0.008	6.748	1.64-28.0

P: Probability OR: Odds ratio CI: confidence interval

Table 4. Clinical features in relation to Ureaplasma (T960 and parvo) biovars.

Parameter		Ureaplasma biovars		P
		T 960 biovar	Parvo biovar	
Gestional age (weeks) (Mean±SD)		28.5±0.54	30.56±1.4	0.002
Birth weight (g) (Mean±SD)		1025±175.3	1448.9±138.4	0.001
gender	Male	0(0%)	7(77.8%)	0.01
	Female	6(100%)	2(22.2%)	
antenatal steroid	Yes	3(50%)	8(88.9%)	0.02
	No	3(50%)	1(11.1%)	
maternal PROM	Positive	0(0%)	7(77.8%)	0.1
	Negative	6(100%)	2(22.2%)	
Delivery mode	SVD	3(50%)	9(100%)	0.9
	CS	3(50%)	0(0%)	
final outcome	Non-BPD	1(16.7%)	2(22.2%)	0.001
	BPD	5(83.3%)	7(77.7%)	

SD: Standard deviation P: probability.

reported that the BPD rate was 2-fold higher for *U. urealyticum* (biovar 2)-colonized infants than the rate for *U. parvum* (biovar 1)-colonized infants. Heggie and coworkers (2001) found no greater risk of developing BPD among 66 Ureaplasma-colonized infants, and also found no differences between infants harboring parvo biovar as compared to those with T960 biovar. Katz et al. (2005) found no difference in BPD rates between infants colonized with either species, but a higher rate of BPD in infants positive for both species. The infants colonized

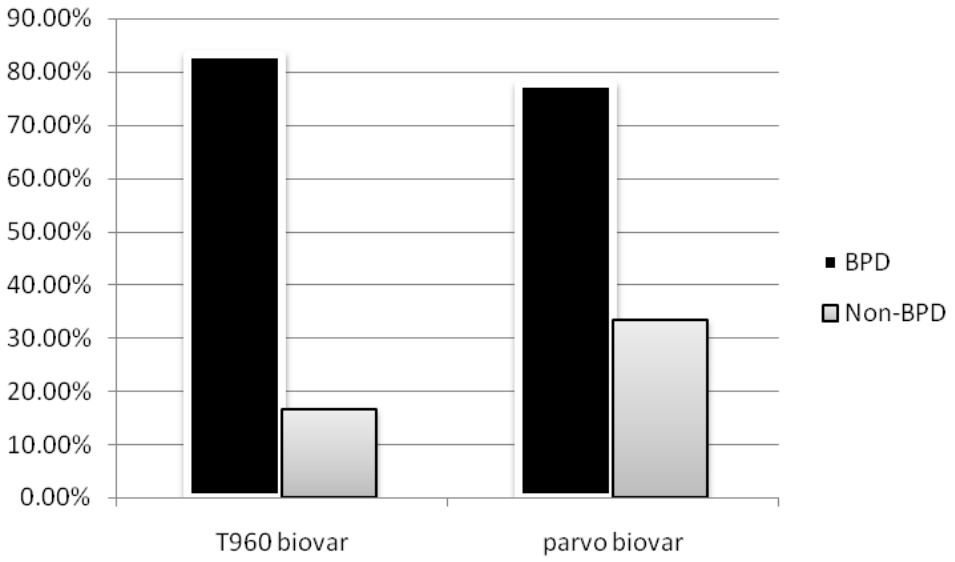

Figure 2. Frequency of BPD among neonates colonized with T960 biovar and parvo biovar.

with T960 biovar were significantly less mature and had lower birth weight than parvo biovar-colonized infants. This may explain, in part, the biovar difference in BPD rates in this study.

It was noted that neonates with positive Ureaplasma colonization had a significantly lower gestational age than non-colonized ones. This is in accordance with Pandey et al. (2007) and Mohagheghi et al. (2013). Our results are in agreement with Yada et al. (2010) and Theilen et al. (2004), Ureaplasma colonization was significantly associated with gestational age, birth weight, SVD and development of BPD.

The association between presence of ureaplasma and the development of BPD remains controversial and hotly debated. Several studies attempted to relate the possibility of an association between *U. urealyticum* respiratory colonization with the development of BPD in preterm neonates (Cassell et al., 1988; Iles et al., 1996; Garland and Bowman, 1996; Kafetzis et al., 2004; Kotecha et al., 2004). On the other hand, there have been studies that failed to detect the association (Saxen et al., 1993; Cordero et al., 1996; Van Waarde et al., 1997; Heggie et al., 2001; Ollikainen et al., 2001; Pandey et al., 2007). The great variations in the sample selection, processing, methods of identification assays applied might explain the different results observed in these studies.

Several studies declared that the most significant factor in the development of BPD was the decreasing gestational age (Smyth et al., 1993; Payne et al., 1993; Jonsson et al., 1994). The present study revealed that in addition to decreasing gestational age, Ureaplasma colonization was a more important predictor for development of BPD (OR: 6.784 and CI95%: 1.64–28.0). In 1995, a meta-analysis by Wang et al. (1995) included 1479 babies from 17 studies, reporting a significant association between BPD diagnosed at 28 days of life and ureaplasma colonization, with an overall relative risk of 1.72 (CI95%: 1.5–1.96). In a cohort of 126 preterm deliveries, Kafetzis et al. (2004) found a significant increase in BPD among ureaplasma colonized infants. Van Waarde et al. (1997) found that Ureaplasma was significantly associated with both BPD and lower gestational age, but logistic regression analysis failed to show a correlation between ureaplasma colonization and BPD. Schelonka et al. (2005) found an odds ratio (OR) of 2.83 (CI95%: 2.29–3.51) for the relationship between the presence of ureaplasma and BPD in a meta-analysis of 23 studies, and Goldenberg et al. (2008) confirmed a probable association between infection and BPD as well.

Limitations of our study

Although the BPD etiology is multifactorial and complex, our study has focused on the role of Ureaplasma colonization in development of BPD, ignoring the postnatal factors for development of BPD as oxygen toxicity, barotraumas, volutrauma, mechanical ventilation, patent ductus arteriosus (PDA) and also the role of proinflammatory cytokines.

Conclusion and future prospectives

Our finding support the evidence that Ureaplasma respiratory tract colonization in preterm ventilated neonates particularly T960 biovar (biovar 2) has been associated with the development of BPD. PCR could be a highly sensitive and specific technique for detecting

Ureaplasma and for distinguishing its biovars (Parvum and T960) directly from clinical samples. Moreover, it may be a useful tool in clinical trials studying the efficacy of early antibiotic intervention or to fully assess the benefits and risks of therapy in colonized high-risk neonates.

Furthermore, a study on large number of neonates analyzing antimicrobial therapy (macrolide) of Ureaplasma -colonized infants should be conducted in a large, multicenter, randomized clinical trial to confirm relation-ship between Ureaplasma colonization and BPD, and to determine whether these antibiotics are effective in reducing BPD rate and improving neonatal outcomes.

Conflict of Interests

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

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Full Length Research Paper

Assessment of the biological activity of kefir grains by biospeckle laser technique

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This work presents the biospeckle laser technique as a potential tool to analyse the kefir grains activity. In the present work, the kefir grains biological activity was measured from quantitative measurements by means of their speckle activity. The aim was to show that the biospeckle laser is a potential methodology to assess kefir grains viability, monitoring the kefir grains during the beverage production. The monitoring of the activity of the kefir is a key factor to guarantee the efficiency of the production of beverages, however the routine ways it is done compromise its use in the online production. The kefir grains were illuminated by a laser HeNe 17 mW 632 nm and analysed by the numerical biospeckle laser method. The results presented the statistical separation of the kefir in distinct levels of activity as expected. This can be an innovative technique to be used in the beverage industries for kefir grains inoculum control.

Key words: Kefir, speckle activity, inoculum control.

INTRODUCTION

Kefir is a culture employed to produce beverages, for example, the traditional Russian beverage also named "kefir" which is produced from milk, and has low alcohol content (Güzel-Seydim et al., 2005; Irigoyen et al., 2005; Magalhães et al., 2010; Puerari et al., 2012). The kefir is a mixed culture of various yeast species of the genus *Kluyveromyces, Candida*, *Saccharomyces* and it has lactic acid from bacteria of the genus *Lactobacillus*, and they are combined in a matrix of proteins and polysaccharide 'kefiran', which are formed during cell growth under aerobic conditions (Güzel-Seydim et al., 2005). The grains of kefir are irregularly shaped, with yellowish-white colour, and hard granules which resemble miniature cauliflower blossoms (Magalhães et al., 2011; Hamet et al., 2013).

In Brazil, the grains of kefir are used in private household for fermentation of milk (Magalhães et al., 2011), and they are added to different types of milk, such

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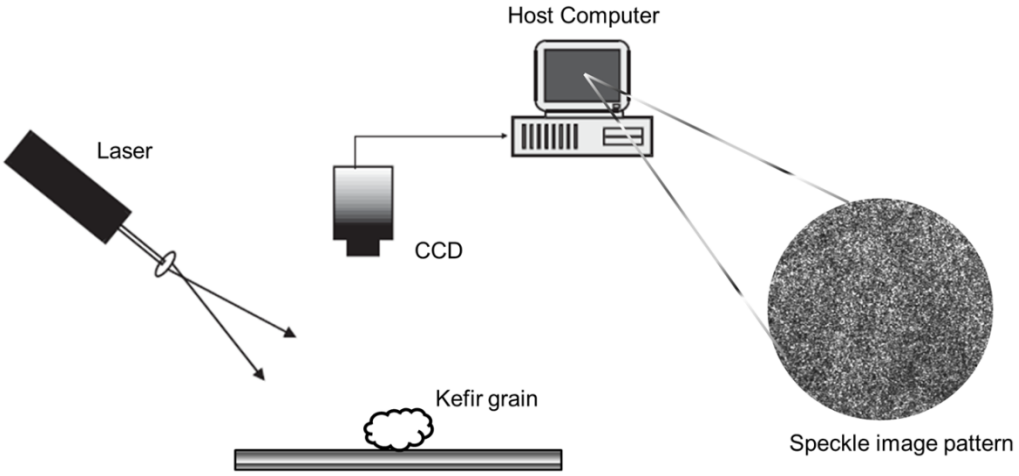

Figure 1. Experimental setup used to illuminate the kefir grain sample.

as the milk from a cow, a goat or a sheep, and as well from coconut, rice and soy. The grains are responsible for the fermentation that results in the production of numerous components in the kefir, including lactic acid, acetic acid, CO_2, alcohol (ethyl 2 alcohol) and aromatic compounds. This provides kefir's unique sensory characteristics: fizzy, acid taste, tart and refreshing flavor (Güzel-Seydim et al., 2005). The beverage contains vitamins, minerals and essential amino acids that help the body with healing and maintenance functions and also contains easily digestible complete proteins (Irigoyen et al., 2005). In accordance with Medrano et al. (2008), the benefits of consuming kefir in the diet are numerous, for instance, the antitumoral activity (Vinderola et al., 2005), the antimicrobial activity (Rodrigues et al., 2005), the antiinflammatory and the antiallergical activity (Lee et al., 2007).

The study of the biological activity of kefir grains is necessary to control the microbial stability of fermenting microorganisms. The microorganisms associated with Brazilian kefir grains in fermentative process are investigated using a combination of phenotypic and genotypic methods. Phenotypic identification of microorganisms is by Bac-Tray Kits I, II and III (Difco, S/P, Brazil) and API 50 CHL (BioMerieux, S/P, Brazil) according to the manufacturer instructions. Genotypic identification of microorganisms is by sequencing of portions of the 16S rRNA gene and Internal Tanscribed Spacer region (ITS). In addition, the visual evaluation by analysis using scanning electron microscopy (SEM) is also used to identify the microbiota of kefir grains for a fermentation process, however all these methods are time consuming and expensive (Magalhães et al., 2011).

An optical technique with potential use in biological metrology, particularly in biological activity, is the biospeckle laser (Zdunek et al., 2014). When a laser beam is scattered by a biological sample, the scattered waves generated in the illuminated sample create the speckle pattern that changes its image in accordance with the changes in the monitored material. Thus, the surface appears to be covered with tiny bright dots that fluctuate in a seemingly random way as for a boiling liquid. The intensity of the bright dots differentiates the microbial activity during fermentation process.

Many efforts have been devoted to characterise quantitatively the activity of biological material, such as in the activity of botanical specimens (Ansari and Nirala, 2013), in the evaluation of blood flow (Zakharov et al., 2009), in the viability of seeds (Braga Jr. et al., 2003) and in the maturation of meat (Amaral et al., 2013). Therefore, this work aimed to evaluate the feasibility to access the biological activity of the kefir grains fermentation in milk and its expected behaviour along the period from the substrate.

MATERIALS AND METHODS

The grains of kefir (250 g) were washed with distilled water and inoculated in 2.250 mL of milk substrate (ultra high temperature milk - UHT) and were statically incubated in a closed recipient during 24 h at room temperature. After 24 ours, that is considered as the fermentation process time, the samples of the kefir grains were taken aseptically from the milk and were evaluated by the biospeckle laser over a period of 24 h at every 3 h to assess the activity of the kefir out of the substrate (milk).

The grains of kefir were illuminated by a HeNe laser, wavelength of 632 nm, and 17mW power, enlarged by a plane concave lens in order to cover the entire sample (Figure 1). The interference patterns formed on them were captured by a CCD camera 640 × 486 pixels, with a shutter speed of 1/60 s and anacquisition rate between images of 0.08 s, creating a collection of 128 images. The analysis of the speckle images, from the laser illumination were performed by monitoring the temporal history of the speckle pattern (THSP) (Arizaga et al., 1999) and its numerical output, the absolute

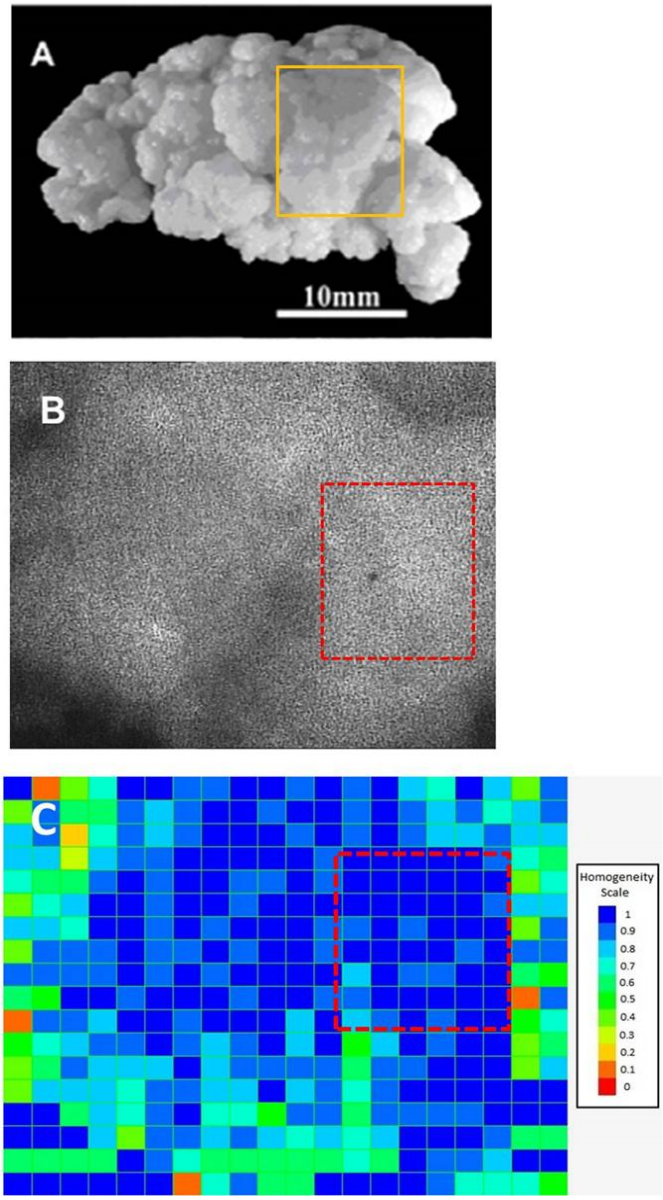

Figure 2. Analysis of kefir grains by biospeckle laser. A- Kefir grains; B- Image of kefir grains generated by biospeckle laser; C - Result of the homogeneity test and the cropped area used to process the AVD technique.

values of the differences (AVD) according to the Equation (1) described below (Braga et al., 2011):

$$AVD = \sum_{ij} \frac{OCM.|i-j|}{Normalization} \quad (1)$$

where the OCM is the occurrence matrix of the successive values in the THSP, and the i and j variables are the dimensions of the OCM matrix. The normalization provides the relation between the AVD values and the summation of all the occurrences. We performed eight sessions of illuminations in four grains of kefir with three replications at every three hours during 24 h.

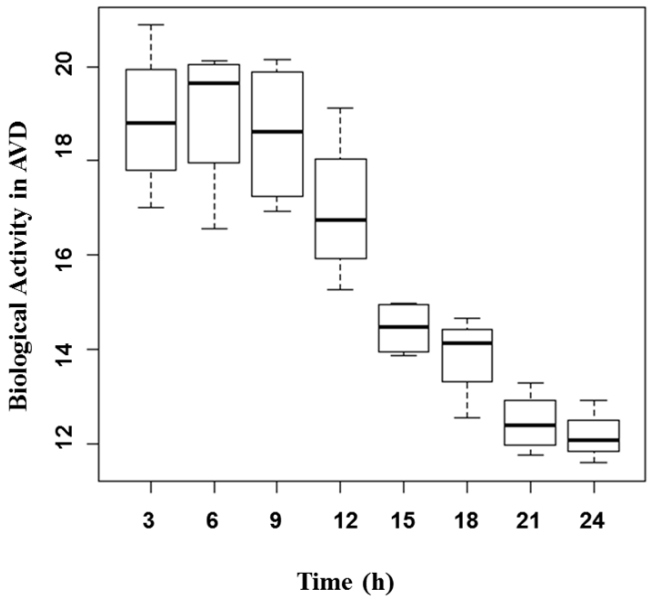

Figure 3. Evolution of the AVD values from kefir grains in time.

Each set of 128 frames was tested regarding its homogeneity (Braga et al., 2012) and the cropped area was processed by the AVD method.

RESULTS AND DISCUSSION

The test of homogeneity of the grains of kefir is shown in Figure 2, in the Figure 2a, the image of the grain is presented with an illustration of a window where the sample was illuminated and the images assembled (Figure 2b). The result of the homogeneity test is presented in Figure 2c where it is possible to see the areas with the highest homogeneity, which means the area where there is no changesof the activity, and in accordance with Braga et al. (2012), the area with high homogeneity can be processed using numerical approaches.

Therefore, the illustrated test was conducted in all the collection of images, and the numerical approach, particularly, the AVD was carried out. The results presenting the activity of the grains of kefir in time were reduced as presented in Figure 3, where it is possible to observe the sensitivity of the technique to follow the reduction of the expected activity. The kefir from the milk does not have any form to develop, therefore, reducing its activity.

An additional observation of the phenomenon presented the reduction of the dispersion of the values along the time, with a clear change of phase, which can be a useful information on the viability of the grain of kefir. The experimental results show it is possible,

however, to distinguish between viable and non-viable tissue by quantitative method. Amaral et al. (2013) also used the technique of laser bioespckle to quantify biological activity in pork meat. They describe the laser biospeckle technique combined with analysis of inertia moment to show an efficient tool for monitoring and quantifying biological activity of meat during aging process, which demonstrates the technique potential for evaluating and predicting beef quality.

The next steps to be followed are related to the research of fermentative process, by adapting kefir grains for new experiments in substrates new, and to extend the metabolic activity of the kefir grains during the fermentative process. The use of different wavelengths for illumination and alternative algorithms for the processing of the data is also to be considered. These experiments are an indication that the biospeckle technique can be used as a methodology to evaluate the kefir grains during the beverages production. The proposed technique is simple, relatively cheap and fast, easy to implement, and requires only a laser and standard digital imaging processing hardware components. The application of biospeckle methods for the kefir viability detection was the main objective of this work, but the optical technique could also be applied to characterise the kefir grains in other types of processes.

Conclusions

Laser biospeckle technique show an efficient tool for monitoring and quantifying biological activity of kefir grains, showing the viability time of these grains after a fermentation process, which demonstrates the technique potential for evaluating and monitoring of kefir grains in production of fruits beverages and fermented/distilled beverages, in addition to beer production.

Conflict of Interests

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

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Methicillin-resistant *Staphylococcus aureus* (MRSA) colonization rate among ruminant animals slaughtered for human consumption and contact persons in Maiduguri, Nigeria

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This study determined the methicillin-resistant *Staphylococcus aureus* (MRSA) colonization rate among ruminant animals slaughtered for human consumption and contact persons. Nasal and milk product samples were collected from the main abbatoir in Maiduguri and analyzed using standard bacteriological procedures. A total of 510 samples were analyzed, 87 (17.1%) *S. aureus* isolates were identified, 33 (34.6%) MRSA and 54 (65.9%) methicillin-sensitive *Staphylococcus aureus* (MSSA) isolates were detected. 19 (21.8%) MRSA and 17 (19.5) MSSA strains were recovered from cattle, 10 (12.5%) MRSA isolates were recovered from the Red Bororo cattle breed and 12 (17.1) MSSA from carmelius dromedarius. In overall antimicrobial susceptibility pattern, MRSA isolates exhibited multidrug resistance pattern, moderate susceptibility to ciprofloxacin (42.2%), tobramycin (36.4%), amikacin (36.4%), streptomycin (42.2%), while majority of MSSA isolates demonstrated high sensitivity pattern (>70%). Six (6.9%) *S. aureus* isolates (2 MRSA from cattle and 4 MSSA from sheep) exhibited inducible phenotype. In conclusion, the study findings reveal a relatively high MRSA colonization rate and unique resistance pattern, particularly to topical antimicrobial agents (fusidic acid, mupirocin) that are not routinely used in veterinary medical practice in the study area. The study findings provides a baseline epidemiological information for better understanding of MRSA infections in human and veterinary medicine including foods of animal origin.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), colonization rate, ruminant animals, contact persons, abattoir.

INTRODUCTION

Staphylococcus aureus is one of the bacterial pathogens that colonize the anterior nares of human and different animals, including farm animals (Wertheim et al., 2004; Weese and Duijkeren, 2009). The *S. aureus* pathogenicity

is attributable to the expression of wide range of extracellular toxins and virulence factors responsible for superficial and systemic infections (Jarraud et al., 2002; Francis et al., 2005). Since the first report of methicillin resistant *S. aureus* (MRSA) strain in 1961, the pathogen had attracted public health attention worldwide because it was identified as the major causative agent of hospital associated infections responsible for the significant proportion of hospital admission. Subsequently, MRSA strains was detected in the community setting termed as CA-MRSA, with distinctive predisposing risk factors and molecular characteristics (Francis et al., 2005). The epidemiological trend of MRSA continued to evolve in its phenotypic/molecular characteristics, predisposing risk factors and associated clinical conditions presentations/complications.

In the last decade, the emergence of MRSA among livestock, particularly pigs and other ruminant animals had added different epidemiological dimension to the understanding of the infection. These livestock are seen as a reservoir, capable of transmitting the pathogens to human or vice versa in the community (Vanderhaeghen et al., 2010; Graveland et al., 2011). However, the transmission of LA-MRSA will depend on the level of contact between human and animals, while the introduction into food chain will be through colonized animals (Kock et al., 2009; Lozano et al., 2011).

In Nigeria, available data on the LA-MRSA colonization rate, predisposing risk factors and transmission between humans and animals are scare. Therefore, epidemiological information on LA-MRSA pathogens is imperative, as it will provide a baseline information needed for better understanding of the possible transmission means and its overall public health implications in the community.

Maiduguri is the administrative capital of Borno state located on latitude 9°.45' and 11°.50' North and longitude 10°.05' and 13°. 05' North. It lies within the semi-arid zone, boarded by 3 republics of Niger, Chad and Cameroon. Livestock rearing, particularly ruminant animals are done at the larger scale for economic purposes, while smaller scale involves domestication of animals within the compound which allows close proximity with human population. The state remains the major source of ruminant animals, transported to other parts of Nigeria in Nigeria. In the northeastern Nigeria, few epidemiological data on MRSA in human infections has been published (Okon et al., 2013), but there are no similar data on veterinary infections. Considering the geographical location, the intra-and inter-human activities and rearing and movement of large number of ruminant animals within and outside the state, all these activities constitute major predisposing risk factors for emergence of resistant pathogens such as LA-MRSA isolates. The public health concern of LA-MRSA continued to heighten worldwide because of possible transmission to human from animals or vice versa and introduction into food chain. Based on this epidemiological information, we decided to assess the MRSA colonization rate among ruminant animals slaughtered at the major abattoir in Maiduguri, and the contact persons.

MATERIALS AND METHODS

The study samples were collected at the Maiduguri Metropolitan Council major abattoir, Maiduguri between January and June 2012. The University of Maiduguri, Faculty of Veterinary Medicine Institutional Review Board and Borno State Veterinary Department attached with abattoir approved the study and the sample collection. Demographic information of the animals were obtained from animal owners and the information entered to the study questionnaire. Demographic information, the type and breed of the animal, antibiotic/local remedy use and grazing pattern are presented in Table 1. Local remedy was defined as concoction prepared with herbs and local materials used by animals owner for treatment of animals infections. The author (IBM) was trained at the University of Maiduguri Veterinary Teaching Hospital in nasal and milk samples collection from ruminant animals and at University of Maiduguri Teaching Hospital for nasal sampling of contact persons. Contact person was defined as persons with close contact with ruminant animals, in this study abbaitor workers are classified as contact persons. A total of 510 samples were collected, the breakdown is as follows: 102 camels, 145 sheep, 113 cattle, 113 goat, 23 milk and 14 contact persons.

Sampling and bacteriological identification procedures

Sterile cotton-tipped swabs were inserted into the inner nasal septum of anterior nare of ruminant animals/contact persons, rubbed several times, removed, capped and labeled appropriately. The milk products were collected by cleaning the udder teat with 70% alcohol, the milk was expressed gently into the study labeled sterile universal bottle, and immediately transported to the laboratory for analysis. The swabs/milk products were inoculated onto blood (BA) and mannitol salt agar (MSA) plates, incubated at 37°C for 24 h. Suspected *S. aureus* colonies with heamolysis on blood agar plates and yellowish appearance on mannitol salt agar plates were further analyzed using standard procedures: colonial morphology, Gram reaction, catalase, tube coagulase and DNase test (Cheesborough, 2006).

Antibiotics susceptibility test

Antimicrobial susceptibility test was determined by disc diffusion method using Mueller-Hinton agar plates accordingly to CSLI (2006) guidelines. The following antibiotic discs (manufactured by Oxoid, UK) were tested, penicillin (PEN), ciprofloxacin (CIP), tobramycin (TOB), kanamycin (KAN), amikacin (AMK), streptomycin (S), tetracycline (TET), trimethoprim (TRIM), erythromycin (ERY), clindamycin (CLD), rifampicin (RF), fusidic acid (FA), mupirocin (MUP) (5 mg, 200 mg). The zone of inhibition around the discs were

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Table 1. Demographic variables and bacteriological data of sample analyzed.

Sample	Number analyzed (%)	Number positive for *S. aureus* (%) p-value
Milk	23 (4.5)	7 (8.0)
Cattle	113 (22.2)	36 (41.4)
Camel	102 (20.0)	18 (20.7) 0.001
Goat	113 (22.2)	7 (8.0)
Sheep	145 (28.4)	9 (10.3)
Contact persons	14 (2.7)	10 (11.5)
Exposure to antibiotic	111 (21.8)	
Local remedy	385 (75.5)	0.01
Extensive grazing	392 (76.2)	
Intensive grazing	104 (20.4)	
MRSA	33 (37.9)	0.01
MSSA	54 (62.1)	

Table 2. The Frequency of occurrence of *S. aureus* (MRSA, MSSA) strains within the sampled analyzed.

S. aureus	Milk	Cattle	Camel	Goat	Sheep	Contact persons	Total
MRSA*	2 (2.3)	19 (21.8)	2 (2.3)	3 (3.4)	4 (4.6)	3 (3.4)	33 (37.9)
MSSA*	5 (5.7)	17 (19.5)	16 (18.4)	4 (4.6)	5 (5.7)	7 (8.0)	54 (62.1)
Total	7 (8.0)	36 (41.4)	18 (20.7)	7 (8.0)	9 (10.3)	10 (11.5)	87 (100)

*Statistical significant difference was observed among the isolates and samples.

measured and interpreted as sensitive, intermediate and resistant according to CSLI breakpoint. Methicillin resistance expression was detected by disc diffusion method using oxacillin and cefoxitin discs.

The D-test for demonstration of inducible phenotype was carried out as previously described by Fiebelkorn et al. (2003), in which the erythromycin and clindamycin discs were placed at 12-14mm apart and inducible phenotype (iMLSB) was indicated by flattening of the clindamycin zone adjacent to the erythromycin discs. *S. aureus* ATCC 26923 was used as a standard control strain.

Data analysis

Data was analyzed by using SPSS version 16.0, the values were expressed as frequency of occurrence and percentages. Comparison of the demographic variables was determined by Chi-square test, p values <0.05 was considered statistically significant.

RESULTS

A total of 510 samples were analyzed, 87 (17.1%) *S. aureus* isolates were identified: 33 (37.9%) MRSA and 54 (62.1%) methicillin-sensitive *S. aureus* (MSSA) isolates. Demographic variables of the *S. aureus* isolates are presented in Table 1, 41.4% (n=36) *S. aureus* isolates were recovered from cattle, 20.7% (n=18) from camel and 11.5% (10) from contact persons (p<0.001). 111 (21.8%) ruminant animals were exposed to antibiotics as compared to 385 (75.5%) with local remedy (p<0.01). 392 (76.2%) practiced extensive grazing as compared to 104 (20.4%) intensive grazing (p<0.01).

Table 2 present the distribution of *S. aureus* (MRSA, MSSA) isolates according to samples analyzed, 19 (21.8%) MRSA isolates were recovered from cattle, 4 (4.6%) sheep, 3 (3.4%) goat , 2 (2.3%) milk and 3 (3.4%) contact persons, while the frequency of occurrence of MSSA isolates were as follows, 17 (19.5%), 5 (5.7%), 4 (4.6), 5 (5.7%) and 7 (8.0%) (p<0.001). High *S. aureus* colonization rate was recorded among two ruminant animals (Table 3), 10 (14.3%) MRSA isolates were recovered from Red bororo cattle breed as compared to 12 (17.1%) MSSA strains from Carmelius dromedarius (Table 3) (p<0.01).

The antimicrobial susceptibility pattern of *S. aureus* strain tested is Depicted in Figure 1, MRSA strains demonstrated high resistance rates to penicillin (90.9%), fusidic acid (100%), mupirocin (5 mg and 200 mg) (100%), tetracycline (81.8%), clindamycin (97.0%), erythromycin (90.9%), trimethoprim (78.8%), rifamipicin (100%), moderate resistance rate with ciprofloxacin (42.4%), tobramycin (36.4%), amikacin (36.4%) and streptomycin (42.2%), respectively. While majority of the MSSA isolates (>60%) demonstrated susceptibility to all agents tested.

Antimicrobial resistance pattern of *S. aureus* strains according to source of the samples analyzed (Figure 2), showed high susceptibility rate with *S. aureus* strains

Table 3. The *S. aureus* strains (MRSA and MSSA) colonization rate of ruminant animals breed sampled.

Ruminant animal	MRSA (%)	MSSA (%)	Total
SokotoGudali	-	2(2.9)	2(2.9)
Red Bororo	10(14.3)	5(7.1)	15(21.4)
White Fulani	7(10.0)	7(10.0)	14(20.0)
Adamawa White	3(4.3)	3(4.3)	6(8.6)
Muturu	1(1.4)	2(2.9)	3(4.2)
Carmeliusdrumedarium	2(2.9)	12(17.1)	18(25.7)
Yankasa	2(2.9)	1(1.4)	3(4.3)
Ouda	2(2.9)	4(5.7)	6(8.6)
Balami	-	-	-
Sahel	2(2.9)	4	6(8.6)
Sokoto Red	1(1.4)	0	1(1.4)
Total	30(42.9)	40(57.1)	70(100)

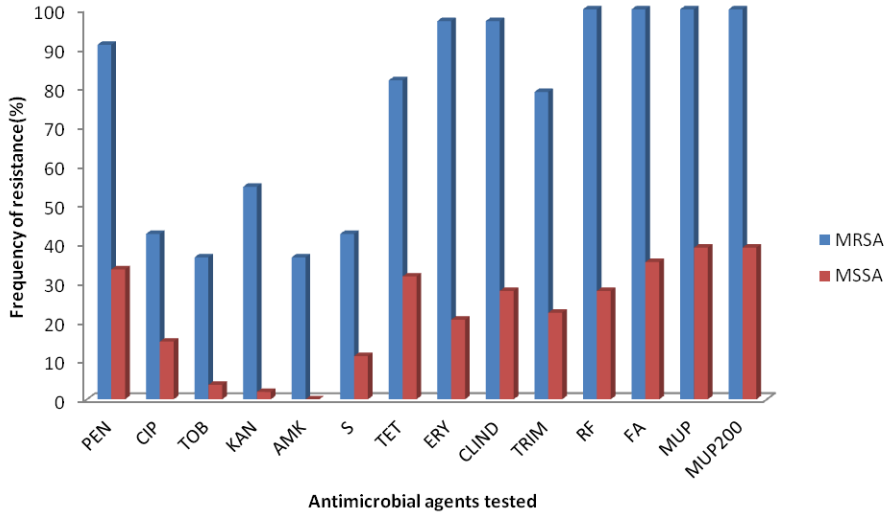

Figure 1. Antimicrobial resistance pattern of *S. aureus* (MRSA and MSSA) strains (%).

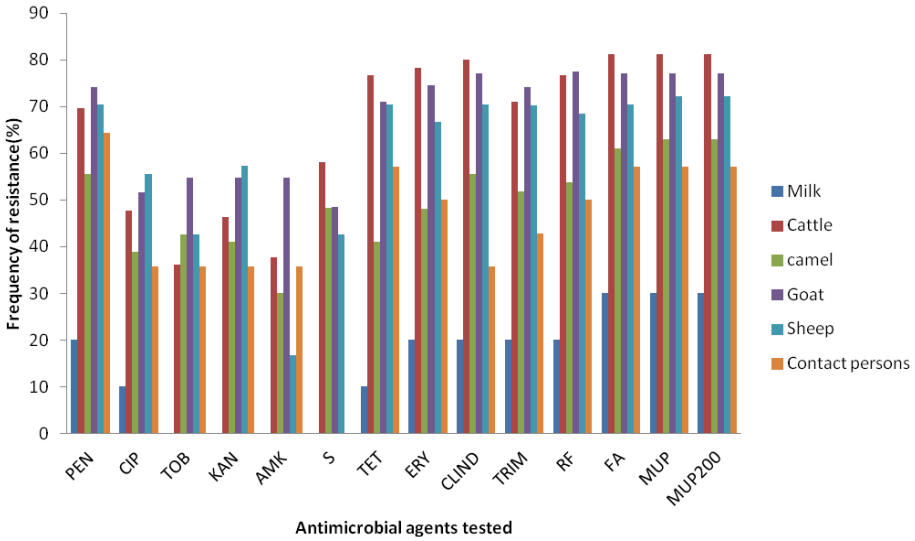

Figure 2. Antimicrobial resistance percentage of *S. aureus* from samples analyzed.

from the milk products, moderate resistance to ciprofloxacin, tobramycin, kanamycin, amikacin, trimethoprim and clindamycin for strains from contact person, camel, cattle sheep. High resistance to penicillin, tetracycline, erythromycin, clindamycin, trimethoprim, fusidic acid, rifampicin, and mupirocin was observed with *S. aureus* strains from cattle, goat and sheep. Six *S. aureus* strains (2 MRSA from cattle, and 4 MSSA from sheep) demonstrated inducible phenotype.

DISCUSSION

The epidemiology of MRSA infections continued to evolve, with different characteristic patterns and associated clinical complications are reported. Adequate knowledge on the predisposing risk factors and infection control approach within the hospital and community setting is of utmost importance. The emergence of LA-MRSA has added additional epidemiological dimension to the understanding of MRSA infections. In developing countries, particularly in sub-saharan Africa with paucity of epidemiological data on MRSA infections, future data are needed from both human and animal population. To the best of our knowledge, this is first report of MRSA colonization rate among ruminant animals slaughtered for human consumption and contact persons from Maiduguri, Nigeria. Main findings of our study are, (i) MRSA colonization rate among the ruminant animals and contact person, (ii) antimicrobial susceptibility pattern of *S. aureus* isolates and (iii) the demographic variables associated with MRSA colonization. Therefore, the findings have shed light on LA-MRSA colonization in the study area and its public health and food safety (Lee, 2003; Zschock et al., 2005).

In this study, the *S. aureus* colonization rate was 87 (17.1%), 33 (37.9%) MRSA isolates and 54 (62.1%) MSSA isolates were detected. The low MRSA colonization rate as compared to MSSA pattern is similar to the pattern reported in other studies (Alzohairy, 2011; Gharsa et al., 2012). MRSA colonization rate differs with the animals sampled and geographica location, in study conducted in Saudi Arabia high MRSA colonization rate was recorded among camels (35.5%) and cattle, 19 (21.8%) as compared to 21.8% in cattle and 4.6% in sheep recorded in our study. While varied rate had been reported in other studies, 44.8% in France and 29% in Tunisia (Vautor et al., 2005; Gharsa et al, 2012). The low MRSA colonization rate of 4.6% is similar to the level reported in Poland (Stastkova et al., 2009). Apart from the fact that high MRSA and MSSA colonization rate was recorded among the cattle in this study, it is also of public health concern, because the cattle constitute the highest number of ruminant animals reared within the community in the study area and the major source of animal proteins. Reason for public health concern are, (i) possible transmission and dissemination of the MRSA isolates could occurs through the level of contact that include close proximity through rearing and domestication, (ii) nasal dropping during movement within the community and (iii) contamination of meat and milk products by colonized handlers. Studies have reported that MRSA colonization of cattle posed a potential risk of up to 60% transmission to the contact persons (Lee, 2003; Juhasz-Kaszanyitzky et al., 2007).

In this study, the MRSA colonization rate among contact persons was 3.4%, this level is lower when compared with the level reported in other studies that assessed the level of contact as a predisposing risk factor for colonization. In these studies, the overall MRSA colonization rate was 6.5% level reported among veterinary personnel, 16% among veterinarian handling large animals and 4.4% among those handling small animals (O'Mahony et al., 2005; Simoons-Smit et al., 2000; Beth et al., 2006; Hanselman et al., 2006). Nevertheless, the level of MRSA colonization rate among contact persons varied with geographic location, type of animals and culture methods employed in the studies (Vanderhaeghen et al., 2010; Graveland et al., 2011).

The MRSA contamination of milk and dairy products are known to be through infection like masititis or the hands of the farmers. The level of milk and dairy products contamination with MRSA isolates varies with geographical location, as low contamination level is reported in European countries, the USA and Canada in contrast to high level reported in Asia and Africa (Pexara et al., 2013). In our study, the MRSA colonization rate recovered from milk product sampled was 2.3%, this level is lower as compared to the level reported in other similar studies, 17.9% in Iran (Alian et al., 2012), 60% in Ethopia (Daka et al., 2012), 36% in Jos, Nigeria (Suleiman et al., 2012) and 6% in South Africa (Ateba et al., 2010). Variation in the colonization rate might be due to the animal production systems, presence of multiple animal species within the same area that could facilitate transmission and dissemination of the pathogens and the animals handling processes particularly during the milking (Vanderhaeghen et al., 2010; Graveland et al., 2011).

We observed that the *S. aureus* isolates showed 3 distinctive patterns: low, moderate and high resistance pattern, with the MRSA exhibiting multidrug resistance pattern. The interesting finding of this study is, the MRSA isolates showed high resistance pattern to topical antibacterial agents (fusidic acid, mupirocin), that are clinically used for MRSA decolonization/decontamination in human and veterinary medicine. These topical antibacterial agents are not routinely used in veterinary practices in the study area. Therefore, the reason for such resistance pattern by the *S. aureus* isolates remain unclear. Future research studies are needed to provide insight, through molecular characterization of resistance genes. Although, some studies have reported fusidic acid and mupirocin-resistant *S. aureus* strains in human and

animals population, which varied with different geographical locations (Udo et al., 2001; Chen et al., 2010).

In Nigeria, the frontline antimicrobial agents routinely used in human and veterinary medicine are tetracycline, erythromycin, penicillin and some quinolones. In this study, of the antimicrobial agents tested, both MRSA and MSSA isolates demonstrated high degree of sensitivity to some aminoglycosides like tobramycin, amikacin and streptomycin. This pattern revealed that these agents are not only still efficacious for staphylococcal infections treatment and management, but also as alternate option for treatment of livestock infection due to multidrug resistant *S. aureus* strains in veterinary settings. In addition, 6 (6.9%) *S. aureus* strains (2 MRSA from cattle and 4 MSSA from sheep) demonstrated inducible phenotype, the pattern is consistent with other studies which predominate in MSSA AS compared to MRSA (Alzohairy, 2012; Schreckenberger et al., 2004; Levin et al., 2005). As obtainable worldwide, macrolides are frontline antibiotics widely used for the treatment of human and animal infections. Extensive usage of these antibiotics results in selection of resistant bacteria and genetic determinants of resistance can be transmitted from animals to humans via foodstuffs (Perreten et al., 1998; Schlegelova et al., 2004). The D-test is used to demonstrate the constitutive and inducible phenotype, and determination of possible chemotherapeutic failure (Levin et al., 2005).

Although, surveillance studies are encouraged worldwide to provide epidemiological data on LA-MRSA, but there are limitation, particularly in comparison with epidemiological data. These limitations include lack of standardization in the methodology employed, in addition, *Staphylococcus* spp., like *Staphylococcus intermedius*, *Staphylococcus schlieferi*, *Staphylococcus hyicus*, *Staphylococcus delphini* and *Staphylococcus pseudointermedius*, produced positive tube coagulase result that may be detected as MRSA, particularly in low-resource laboratory in which analysis are based on phenotypic characterization (Morgan, 2008).

Conclusion

Based on the findings of this study, we can state that MRSA colonization rate in ruminant animals and contact persons might be assumed to be relatively high for geographical location without no pre-existing epidemiological data for comparison and the resistance pattern is of public health concern. The reason being that these resistant strains and genes can be transmitted and disseminated between human and animals, and subsequently into the food chain. The scenario could worsen in this area if appropriate attention is not paid.

Conflict of Interests

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

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Full Length Research Paper

A survey of the microflora of the outdoor air environment of Keffi Metropolis, Nasarawa State, Nigeria

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The microbiological quality of the air environment of Keffi metropolis was assessed by determining the concentrations and composition of bacteria and fungi present in the outdoor air. Air samples were collected from ten different locations of the metropolis by the plate sedimentation methods which involved exposing media-filled Petri plates to the air for 30 min. Trypticase soy agar (TSA) was used for the enumeration of total bacterial concentrations while malt extract agar (MEA) was used for the enumeration of total fungal concentrations. Standard microbiological methods were employed for the identification of the bacterial and fungal isolates. The results obtained show that the concentrations of bacteria in the different locations of Keffi ranged from 2.8 x 10^3 to 6.4 x 10^3 CFU/m^3, while the concentrations of fungi ranged from 4.71 x $10^2$to 4.60 x 10^3 CFU/m^3. Six bacterial species belonging to six genera and nine fungal species belonging to seven genera were isolated at varying frequencies of distribution. The quantitative and qualitative analysis of the microbial flora of the outdoor air of Keffi metropolis has provided information on the airborne microorganisms. The fact that some of the bacterial and fungal species isolated are known to be pathogenic to humans has demonstrated that the microflora of Keffi metropolis has public health implication.

Key words: Bacteria, fungi, microflora, air quality, Keffi, Nigeria.

INTRODUCTION

Air quality is one of the most significant factor affecting the health and well-being of people. It has been reported that a single person inhale's an average of approximately 10 m^3 of air every day (Dacarro et al., 2003). However, the air inhaled by people is abundantly loaded with microorganisms which form part of the bioaerosol (Górny, 2004). Bioaerosol is a colloidal suspension, formed by droplets and particles of solid matter in the air, whose components can contain or have attached to them viruses, fungal spores and conidia, bacterial endospores, plant pollen and fragments of plant tissues (Karwowska, 2005). Biological contamination of air is mostly caused by

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bacteria, moulds and yeasts (Flannigan, 2001; Daisey et al., 2003; Pieckova and Kunova, 2002). They can be dangerous as pathogenic living cells but they also secrete some substances harmful to human health (Gutarowska and Jakubowska, 2001).

Airborne microorganisms are usually derived from various natural sources such as soil, animals, and humans (Posfai et al., 2003; Mouli et al., 2005; Fang et al., 2007). Human activities such as sewage treatment, plants and animal rendering, fermentation processes and agricultural activities do emit microorganisms into the air (Recer et al., 2001; Adhikari et al., 2004; Gillum and Leventin, 2008). Several studies have identified human activities like talking, sneezing and coughing (Kalogeraskis et al., 2005), while other human activities such as vehicular transportation and human movements, washing in homes and business centres, flushing of toilets and sewages, sweeping of floors and roadsides can generate bioaerosols indirectly (Kalogeraskis et al., 2005; Chen and Hildermann, 2009). Since micro-organisms can lodge in/on dust particles, dust therefore is a potential source of bioaerosols.

In recent years, monitoring of the number of airborne microorganisms has gained interest due to increasing concerns on public health, the threat of bioterrorism, surface biodeterioration and spread of plant diseases (Douwes et al., 2003; Pieckova and Jesenska, 1999; Stetzenbach et al., 2004). Exposure to bio aerosols, containing airborne microorganisms and their by-products, can result in respiratory disorders and other adverse health effects such as infections, hypersen-sitivity, pneumonitis and toxic reactions (Frachia et al., 2006; Górny et al., 2002).

Exposure to outdoor air microorganisms has been associated with allergic respiratory symptoms, asthma exacerbation, asthma related death and infections (Dales et al., 2004; Peternel et al., 2004). Several findings of epidemiological research indicate that exposure to high concentration of microorganisms frequently leads to allergies, asthma, hay fever (Björnsson et al., 1995; Newson et al., 2000), pneumonia (Siersted and Gravesen, 1993), and many other health side-effects, including infections (Renn et al., 2001). In recent years, dramatic increase in the number of allergic reactions to fungal spores has been reported, and young people do constitute a large group of allergy sufferers, whose symptoms persist throughout the year (Jain, 2000). For this reason, there is need for regular monitoring of the air in order to determine its quality as it affects the health of humans in the public as they go about their daily activities. Presently in Nigeria, attention is yet to be given to the monitoring of airborne microorganisms, whether outdoor or indoor. This study is therefore an attempt to provide some empirical data that could stimulate both outdoor and indoor bioaerosol research in Nigeria.

Keffi, a cosmopolitan settlement, is a typical example of a Nigeria town experiencing very rapid population growth and urbanization. A large proportion of the human inhabitants of Keffi are traders, small-scale entrepreneurs and menial workers, who spend most hours of their day in direct exposure to the outdoor air environment. Thus, the microbiological quality of the outdoor air in this locality is of public health significance. This study therefore aimed at evaluating the microbiological quality of the outdoor air environment of the Keffi metropolis.

MATERIALS AND METHODS

Study area

The study was carried out in Keffi, a fast growing cosmopolitan town geographically located on longitude 7° 50′E and latitude 8° 3′N, north-west of Lafia (the Capital of Nasarawa State, Nigeria), and is situated on an altitude of 850 m above sea level. Keffi, though in Nasarawa State, is about 68 km from Abuja, the Federal Capital of Nigeria (Akwa et al., 2007).

Air sampling and microbiological examination

Air samples were collected from ten different locations of Keffi metropolis by plate sedimentation methods as employed by Stryjakowska-Sekulska et al. (2007) and Ekhaise et al. (2008). The ten locations were Main Campus, Angwan Lambu, Federal Medical Centre (FMC), Emir's Palace, Pyanku Campus, Government Residential Area (GRA), Main Market, Angwan NEPA, Dadin kowa and High Court. Petri plates containing culture media suitable for bacteria and fungi were used as sampling surfaces. Trypticase Soy Agar (TSA) supplemented with cyclohexamide (which inhibits growth of fungi) was used for the determination of total number of bacteria, while Malt Extract Agar (MEA) supplemented with chloramphenicol (which inhibits growth of bacteria) was used for the determination of the total number of fungi (Kalwasińska et al., 2012). Plates in triplicates for each type of culture medium were exposed to air in each of the ten locations for 30 min in order to allow air microorganisms to settle gravitationally directly on the media surfaces of the plates. Plates with TSA were incubated for 48 h at 37°C, while the plates with MEA were incubated at 30°C for 7 days (even though colonies were counted on the 3rd day). The total number of colony forming units were enumerated and expressed as colony forming units per cubic meter of air (CFU/m^3) (Stryjakowska-Sekulska et al., 2007).

Identification of bacterial and fungal isolates

The identification of bacterial colonies was carried out according to the standard microbiological methods as described by Holt (1994), Cheesbrough (2000) and Aneja (2003), in which the colonies were characterized using macroscopic (cultural) and microscopic (morphological) features as well as biochemical tests. The API system (bio-Mérieux, Marcy-l'Etoile, France) was also used to further confirm the identity of the bacterial species.

Identification of all fungal isolates was also carried out using standard methods based on macroscopic and microscopic features as described by Ellis (1971), Domsch et al. (1980), Singh et al. (1991), Barnett et al. (2007) and Barnett and Hunter (1999).

Statistical analysis

Turkey test as recommended by Zar (1999) for analysis of data for

Table 1. Bacterial concentration in the outdoor air environment of different locations of Keffi metropolis.

Location	Mean (cfu/m^3)	Standard deviation
Main Campus	4.2x10^1	± 1.13
Angwan Lambu	4.5x10^1	± 0.83
Federal Medical Centre	4.2x10^1	± 1.13
Emir's Palace	7.6x10^1	± 2.27
Pyanku Campus	5.4x10^1	± 0.07
GRA*	3.0x10^1	± 2.33
Main Market	1.0x10^2	± 4.67
Angwan NEPA	6.0x10^1	± 0.67
Dadin Kowa	4.2x10^1	± 1.13
High Court Area	4.2x10^1	± 1.13

*****GRA, Government Reserved Area.

Table 2. Fungal concentration of the outdoor air environment of the different locations in Keffi metropolis.

Location	Mean (CFU/m^3)	Standard deviaton
Main Campus	1.2x10^1	±2.06
Angwan Lambu	1.2x10^1	±2.06
FederalMedical Centre	1.7 x10^1	±1.56
Emir's Palace	3.9 x10^1	±0.64
Pyanku Campus	1.5 x10^1	±1.76
GRA*	4.8 x10^1	±1.54
Main Market	7.0 x10^1	±3.74
Angwan NEPA	5.0 x10^1	±1.74
Dadin Kowa	2.6 x10^1	±0.66
High Court Area	3.7 x10^1	±0.44

*****GRA, Government Reserved Area.

multiple-comparison was used to determine the statistical significance of the concentrations of bacteria and fungi in the air sampled from the different locations of Keffi. Statistical Package for Social Sciences (SPSS) version 20.0 software was employed for this analysis. Percentage frequencies of occurrence of species of bacteria and fungi at the different locations were also computed according to the methods of Sampo et al. (1997).

RESULTS AND DISCUSSION

The results of the bacterial concentrations in the outdoor air environment of different locations of Keffi metropolis are shown in Table 1. The bacterial concentrations in the different locations ranged from 2.8 x 10^3 to 6.4 x 10^3 CFU/m^3. The highest bacterial concentration of 6.4 x 10^3 CFU/m^3 was recorded at the Main Market, followed by 6.2 x 10^3 CFU/m^3 and 5.7 x 10^3 CFU/m^3 recorded for Emir's Palace and Angwan NEPA, respectively. The lowest concentration of 2.8 x 10^3 CFU/m^3 was recorded at Government Reserved Area (GRA).

The results of the fungal concentrations in the outdoor air environment of different locations of Keffi metropolis are shown in Table 2. The fungal concentrations in the different locations ranged from 4.71 x 10^2 to 4.60 x 10^3 CFU/m^3. The highest fungal concentration of 4.60 x 10^3 CFU/m^3 was also recorded at the Main Market. The concentrations recorded at High Court Area, Government Reserved Area (GRA) and Dadin Kowa were 3.49 x 10^3 CFU/m^3, 2.52 x 10^3 CFU/m^3 and 2.45 x 10^3 CFU/m^3 respectively. The lowest concentration of 4.71 x 10^2 CFU/m^3 was recorded at Agwan NEPA.

The results of the distribution of the different species of bacteria and fungi isolated are presented in Tables 3 and 4, respectively. Six bacterial species were isolated at varying frequencies of occurrence. The bacterial species with their respective frequencies of occurrence were *Staphylococcus aureus* (100%), *Streptococcus pyogenes* (100%), *Escherichia coli* (90%), *Bacillus* spp. (100%), *Enterobacter aerogenes* (40%) and *Shigella* spp. (50%).

Nine species of fungi belonging to seven genera were isolated with respective percentage frequencies of 100% (*Aspergillus flavus, Aspergillus niger, Rhizopus stolonifer*), 80% (*Penicillium* spp.), 60% (*Aspergillus fumigatus, Candida albicans*) and 30% (*Mucor* spp., *Absida corymbifera, Alternaria alternata*), respectively.

Epidemiological studies have shown that a large number of people around the world are exposed to biological agents (Daisey et al., 2003; Dales et al., 2004; Golofit-Szymczak and Gorny, 2010). Unfortunately, there is no official reference limit for the microbiological quality of air in human environments, whether indoor or outdoor. The lack of quantitative health-based guideline values or thresholds for the acceptable levels of microbial contamination in the air may be due to a lack of dose-response relationship for most of the air microbiological agents (Golofit-Szymezak and Górny, 2010). Several investigators in this area had highlighted that source data on concentrations of biological agents in the air environments

Table 3. Distribution of bacterial species isolated from the outdoor air environment of the different locations in Keffi metropolis.

Bacterial isolate	Location*										Occurrence
	A	B	C	D	E	F	G	H	I	J	(%)
Staphylococcus aureus	+	+	+	+	+	+	+	+	+	+	100
Streptococcus pyogenes	+	+	+	+	+	+	+	+	+	+	100
Escherichia coli	+	+	+	+	+	-	+	+	+	+	90
Bacillus sp.	+	+	+	+	+	+	+	+	+	+	100
Enterobacter aerogenes	-	-	-	-	-	-	+	+	+	+	40
Shigella sp.	-	-	-	-	-	+	+	+	+	+	50

*Locations: A = Main Campus; B = Angwan Lambu; C = Federal Medical Center; D = Emir's Palace; E = Pyanku Campus; F = Government Reserve Area; G = Main Market; H = Angwan NEPA; I = Dadin Kowa; J = High Court Area.

Table 4. Distribution of fungal species isolated from the outdoor air environment of the different locations of Keffi metropolis.

Isolate	Location*										Occurrence
	A	B	C	D	E	F	G	H	I	J	(%)
Aspergillus flavus	-	+	-	+	+	-	+	+	+	+	70
Aspergillus niger	+	+	+	+	-	+	+	+	+	+	100
Aspergillus fumigatus	-	+	+	-	+	-	+	+	-	+	60
Penicillium sp.	-	+	+	+	-	+	+	+	+	+	80
Rhizopus stolonifer	+	+	+	+	+	+	+	+	+	+	100
Absidia corymbifera	-	-	-	+	-	-	+	+	-	-	30
Alternaria alternata	-	-	-	+	-	-	+	+	-	-	30
Mucor sp.	-	+	-	+	-	-	+	-	+	+	50
Candida albicans	+	+	+	+	-	-	+	+	-	-	60

*Locations: A = Main Campus; B = Angwan Lambu; C = Federal Medical Center; D = Emir's Palace; E = Pyanku Campus; F = Government Reserve Area; G = Main Market; H = Angwan NEPA; I = Dadin Kowa; J = High Court Area

are still insufficient (Adhikar et al., 2004; Mouli et al., 2005; Golofit-Szymczak and Gorny, 2010; Kalwasińska et al., 2012). This notwithstanding, the qualitative and quantitative information on the composition and concentrations of microorganisms in the air environment of human habitations at any point in time would help in alerting the public of possible health risk that may be encountered by vulnerable individuals.

Several researchers in this area had earlier reported that exposure to high concentrations of microorganisms in the air frequently lead to allergies, asthma (Björnsson et al., 1995; Newson et al., 2000), pueumonia (Siersted and Gravesen, 1993), and other health side-effects. In addition to public health advantage, routine monitoring of air quality can serve as a means of military surveillance for the detection of any possible biological threat of bioterrorism (Douwes et al., 2003).

Data resulting from this study revealed that the concentrations of bacteria in Keffi ranged from2.8×10^3 - 6.4×10^3 CFU/$m^3$and that of fungi ranged from4.71×10^2 - 4.60×10^3CFU/m^3. However, the concentrations for both bacteria and fungi have been shown to vary ($P<0.05$) in the different locations of the Keffi metropolis. The concentrations of bacteria at all the locations exceeded the recommended limit (10^3CFU/m^3) suggested by National Institute of Occupational Safety and Health (NIOSH). The American Conference of Governmental Industrial Hygienists (ACGIH) had suggested 500 CFU/m^3 for culturable bacteria (Kalogerakis et al., 2005). Górny and Dutkiewiez (2002) earlier presented to WHO Expert Meeting in Berlin, a proposed Residential Limit Values of 250 CFU/m^3 for bacterial concentrations.

Considering all available threshold limits for bacterial concentrations in the air environments, it is clear that the outdoor air of Keffi metropolis is heavily loaded with bacteria. The Main Market that had the highest bacterial concentration followed by the Emir's Palace is among the busiest locations in the metropolis in terms of human and vehicular movements. The high bacterial concentration recorded in these two locations is not surprising, and this

agrees with reports by several researchers (Kalogeraskis et al., 2005; Chen and Hildermann, 2009). Similarly, the concentrations of fungi in most of the locations exceeded the recommended proposal of 10^3 CFU/m3 as threshold limits for fungal concentrations in the air (Górny and Dutkiewiez, 2002).

The qualitative analysis of the microbial flora provides additional information on airborne microorganisms in the outdoor air of Keffi metropolis. In this study, six species of bacteria, *S. aureus*, *S. pyogenes, E. coli, Bacillus* spp., *E. aerogenes* and *Shigella* spp., and nine species fungi belonging to six genera, which included *A. flavus, A. niger, A. fumigatus, Penicillium* spp., *R. stolonifer, A. corymbifera, A.alternata, Mucor* spp. and *C. albicans* were isolated from the outdoor air environment of Keffi. Some of these bacteria and fungi have been shown to be amongst the most common bacterial and fungal species isolated from the air (Burge and Hoyer, 1990).

From this study, *S. aureus, S. pyogenes, Bacillus* spp. and *E. coli* are the most prevalent bacterial species isolated. *S. aureus* is known to be carried in the naso-pharynx, throat, skin, cuts, boils, nails, and such can easily contribute to the microflora in the Keffi metropolis which is always busy with activities involving in most cases human and vehicular movements. *Bacillus* spp. is spore-forming soil bacteria and the most persistent in the atmosphere (Shaffer and Lighthart, 1994). *S. pyogenes* are often found as commensals in the upper respiratory tract of human (Cheesbrough, 2000). If host defenses are weakened or a new highly virulent strain is introduced it can lead to acute suppuration infections (Brooks et al., 2001). *E. coli* is an enteric coliform which is a normal resident flora of the large intestines of mammals including humans and are used as indicators of pollution of feacal origin (Willey et al., 2008). The high prevalence of *E. coli* in the air of Keffi suggests a very low personal and environmental hygiene practice in this town.

A. niger and *Penicillium* spp. are the most predominant species isolated. Maktkovic et al. (2007) in their study reported *Aspergillus* spp. and *Penicillium* spp. as the predominant genera of organisms isolated from the air, while Ekhaise et al. (2008) reported *Aspergillus* species as the most common genus of fungi in the air environment. *Aspergillus* and other species of fungi have been implicated as pathogenic in causing several mycotic infections. The relatively high concentrations of fungi in the air environment of Keffi may pose only little health hazard to healthy individuals, but would pose serious danger and special risk to immunosuppressed persons and other severely immunocompromised individuals (Flannigan et al., 1994). Fungal spores from species of *Penicillium* have been implicated with allergies and elicit asthma in vulnerable individuals (Flannigan et al., 1991).

Conclusion

The importance of evaluating the quality of the air humans breathe whether indoor or outdoor, especially in the urban areas where there is high vehicular traffic and human activities involving rapid movements cannot be over-emphasized. The number and type of airborne microorganisms can also be used to determine the degree of cleanliness as a means of determining the source of human discomfort and certain airborne microbial infections.

Conflict of Interests

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

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Full Length Research Paper

Enhanced immune responses in mice to combined immunization with *Mycobacterium tuberculosis* Ag85A and DDA/MPL

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The tuberculosis (TB) Bacillus Calmette-Guerin (BCG) vaccine, which is based on *Mycobacterium bovis*, has variable protective efficacy in humans; therefore, safe and effective vaccines are urgently required to prevent this disease. The antigen 85A from *Mycobacterium tuberculosis* (*M. tuberculosis*) is a prime target for immunity in the early phase of tuberculosis in various animal models. The purpose of this study was to confirm whether Ag85A could elicit protective immune responses in mice. Dimethyldioctadecylammonium (DDA), an adjuvant with Th1-promoting activities and monophosphoryl lipid A (MPL), an immunostimulatory component that has strong adjuvant activity for both cellular and humoral immune responses, has been used as the adjuvant to study the biological and immunological characteristics of tuberculosis proteins in the researches previously. The gene coding Ag85A (*fbpA*), was cloned into a pET-30a(+) prokaryotic expression vector, and the induced amino acid sequence corresponds to a 33 kDa protein, which was confirmed by mass spectrometry and western blots. Mice were subcutaneously immunized with Ag85A protein emulsified with DDA and MPL and the results showed that the 85A antigen, when combined with these adjuvants, elicited strong Ag85A-specific T-cell responses and humoral responses as compared with vaccination with BCG. Based on the fact that this vaccine combination induced strong antigen-specific immune responses, it is a prime candidate as a component of a future TB vaccine.

Key words: Tuberculosis, Ag85A, prokaryotic expression, cellular immune response, humoral response.

INTRODUCTION

Tuberculosis (TB) is one of the most serious diseases that afflict humans. About one third of the world's population is currently infected with *M. tuberculosis* (Armitige et al., 2000) and approximately 3 million people die from the TB caused by it each year. Bacillus Calmette-Guerin (BCG) is the only currently licensed

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vaccine against TB (Betts et al., 2012). Although this vaccine can induce high levels of protective immunity in animal models, its efficacy in humans differs (Magalhaes et al., 2008).

More and more researchers have concentrated on the study of TB vaccines recently; TB vaccines include viral-vectored (MVA85A, AERAS-402); protein/adjuvant (M72, Hybrid-1, Hyvac 4, H56); rBCG (VPM 1002); and DNA vaccines. However, no TB vaccine can induce stable and high levels of protective immunity in adult so far.

Some studies in murine and guinea pig models of TB have shown that culture filtrate antigens play a role as targets of protective immune responses (Pal and Horwitz, 1992; Roberts et al., 1995). The Ag85 complex antigens are important exocytosis antigens of various mycobacterial species including BCG (Launois et al., 1994; Fan et al., 2009).

This complex consists of three structurally related components, Ag85A, Ag85B (Borremans et al., 1989), and Ag85C. Ag85A is a major fraction of the Ag85 complex; in terms of its immunostimulatory properties, it is the most important component of *M. tuberculosis* and has been used in numerous vaccine preparations that that been shown to induce outstanding protective efficacy (Sugawara et al., 2007; Tanghe et al., 2000; Betts et al., 2012).

The Ag85A protein of *M. bovis* BCG or *M. tb* are actually identical; it has many T-cell-epitopes and B-cell-epitopes, one of which was synthesized as the $Ag85A_{261-280}$ polypetide in this text.

When combined with adjuvant, Ag85 complex antigens can induce immunological responses sufficient for protective immunity to occur. Adjuvants can activate immune cells such as macrophages and enhance their ability to present antigen (Korsholm et al., 2007). Adjuvants can also enlarge the surface area of an antigen, and prolong its retention time, thus allowing it to remain in contact with the lymphatic system for longer than when the antigen is used on its own. Different adjuvants induce different types of immune responses. For example, aluminum salts induce Th2 differentiation and humoral immunity (Korsholm et al., 2009). To induce Th1 responses, monophosphoryl lipid A (MPL) formulated with DDA adjuvant is used. DDA can enhance antigen uptake and antigen presentation to T cells, and stimulate dendritic cells (DCs) through Toll-like receptors. Recent studies show that DDA efficacy can be enhanced by adding immunostimulatory components such as MPL (Brandt et al., 2000).

In this study, we expressed the Ag85A protein of *M. tuberculosis* in a prokaryotic expression system. The recombinant Ag85A protein was used in combination with DDA and MPL adjuvants to immunize C57BL/6 mice and investigated its immunological characteristics in such immunized mice.

Our results may make a significant contribution to the further investigation of Ag85A as a subunit vaccine against TB.

MATERIALS AND METHODS

Bacterial strains and vectors

Escherichia coli DH5α and BL21 (DE3) strains were grown in Luria-Bertani medium and used for gene cloning and expression, respectively. *M. bovi*s BCG, DH5α, BL21(DE3), and pET-30a(+) were kept by our laboratory, whereas the pMD20-T vector was purchased from TaKaRa (Dalian, China).

Experimental animals

Six-week-old female C57BL/6 mice were purchased from VITAL RIVER (Beijing, China). The mice were housed, handled and immunized at the Animal Biosafety facilities and all procedures were approved by the institutional animal experimental committee.

Construction of the pET-30a(+)-*fbpA* recombinant plasmid

The *M.tb* vaccine BCG genome was used to design primers for PCR amplification of the *fbpA* gene. The nucleotide sequences of the two primers used for PCR amplification of *fbpA* gene are as follows: forward primer, 5-AAGCGGATCCATGTTTTCCCGGCCGGGCTTG-3, and reverse primer, 5-AGTCGAATTCTGTTCGGAGCTAGGCGCCCTGGG-3. *Bam*HI and *Eco*RI restriction endonuclease sites were incorporated into the forward and reverse primers, respectively. The 891 bp *fbpA* amplicon, purified using TaKaRa mini columns according to the manufacturer's instructions, was ligated to the pMD20-T vector using T4 DNA ligase, and then transformed into DH5α competent cells. The recombinant plasmid, which we called pMD20-T-*fbpA*, was sequenced by a commercial company (genscript,Nanjing,China) after construct verification by PCR and restriction enzyme digestion.

pET-30a (+) plasmid DNA and pMD20-T-*fbpA* were digested with *Bam*HI and *Eco*RI. Prior to ligation, the linearized plasmid DNA and insert DNA were gel purified using a TaKaRa Gel extraction kit. Ligation of *fbpA* to pET-30a(+) was performed using T4 DNA ligase and the ligation products were transformed into *E. coli* DH5a cells. The transformants were screened by *Bam*HI and *Eco*RI plasmid digestion and Ag85a-recombinants were confirmed by DNA sequencing.

The pET-30a-*fbpA* plasmid, purified from an overnight culture of recombinant *E. coli* DH5a cells, was transformed into *E. coli* BL21(DE3) competent cells. LB plates containing 50 μg/ml kanamycin were used to screen for *E. coli* pET-30a-*fbpA*-positive BL21(DE3) colonies. The Ag85A protein was expressed in the same host cells by addition of 0.5 mmol/L of isopropyl-β-D-thiogalactopyranoside (IPTG), and purified by His-Bind Purified Kit (Novagen, Germany). The concentration of purified Ag85A protein was up to 7 mg/ml and the purity was 98%.

MALDI-TOF mass spectrometry (MS)

Recombinant proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. The gel containing the induced Ag85A protein was sent for secondary mass spectrometry using the MALDI-TOF-MS protein spectrum analysis technique (CISCO, Nanjng,China).

SDS-PAGE and Western blotting

Following induction of the recombinant protein, cell lysates separated by SDS-PAGE on 12% gels were analyzed by Western blotting. Total cellular proteins were transferred onto a nitrocellulose

Table 1. The immunizations of mice.

Group	The first immunity	The second immunity
Group 1	PBS+DDA-MPL	PBS+DDA-MPL
Group 2	DDA-MPL+Ag85A protein	DDA-MPL+Ag85A protein
Group 3	Ag85A protein	Ag85A protein
Group 4	BCG	BCG
Group 5	BCG	DDA-MPL+Ag85A protein

membrane. The membrane was saturated with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and then incubated with an anti-BCG mouse polyclonal antibody, followed by incubation with a goat anti-rabbit HRP-conjugated antibody diluted 1/5000 in phosphate-buffered saline containing 0.05% Tween 20, pH 7.4 (PBST). Immunoblots prepared in this manner were developed as described previously (Xie et al., 2002).

Mouse immunizations

Twenty five 6-week-old female C57BL/6 mice were randomly divided into the 5 groups: (1) the DDA-MPL+phosphate-buffered saline (PBS) group, (2) the DDA-MPL+Ag85A protein group, (3) the Ag85A protein group, (4) the BCG group and, (5) the BCG+DDA-MPL+Ag85A protein booster-immunized group. Mice were immunized two times at 2-weeks intervals subcutaneously with inoculum containing either 100 µg DDA and 25 µg MPL, 25 µg Ag85A protein, 1×10^6 colony-forming units (CFUs) BCG, or 25 µg Ag85A protein emulsified in DDA and MPL in a volume of 0.2 ml. Mice in the first group were immunized with DDA and MPL adjuvants, while mice in the second group were immunized subcutaneously with Ag85A emulsified in 100 µg of DDA adjuvant and 25 µg of MPL. Group 3 mice were immunized with Ag85A protein alone, whereas group 4 mice were immunized subcutaneously with BCG at a dose of 1×10^6 colony-forming units (CFUs). Group 5 mice were immunized with BCG at the first immunity, and then immunized with Ag85A at the second immunity (Table 1). All mice were humanely killed, dissected, and their spleens and T lymphocytes removed for immunological analysis at seven to nine days after the secondary immunization. Concurrently, serum samples were obtained from the mice for antibody detection.

Cytokine measurements

Spleen cells (2×10^7 white blood cells/ml) from five mice per group were tested individually for their cytokine responses to the $Ag85A_{261-280}$ polypeptide and the PPD of *M. tuberculosis*. 48 h later, the supernatants from at least three separate wells of a 96-well cell culture plate were pooled and stored frozen at −20°C until required. Experiments were performed at least three times, and data from one representative experiment are reported. Mouse IFN-γ and IL-4 enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, New York, New Jersey) were used to determine the IFN-γ and IL-4 expression levels of the spleen cell suspensions, following the manufacturer's instructions.

Antigen-specific IgG assays

Antigen-specific IgG antibodies responses against the purified Ag85A protein in the serum of mice from different group at 7-9 days after the second immunization were measured by ELISA. ELISA plates were coated overnight at 4°C with 10 µg of *M. tuberculosis* PPD. PPD are the purified protein derivative of *M. tuberculosis* which contains Ag85A protein. The plates were blocked with 200 µl/well of PBS containing 1% BSA for 2 h at 37°C and washed three times with PBS containing 0.05% Tween 20. Individual serum samples were added to the wells in a serial two-fold dilution series (beginning at a 1/50 dilution), incubated for 2 h at 37°C, washed, and then 100 µl/well of horseradish peroxidase-conjugated goat anti-mouse IgG (diluted at 1/10000) was added. Plates were incubated for 1 h at 37°C, washed, and then developed with 3, 3′ 5, 5′-tetramethylbenzidine substrate. Reactions were stopped by addition of 50 µl/well of 2 M H_2SO4 and the plates were read on an ELISA plate reader at 450 nm. Sera from the group of mice treated with PBS were used as the negative control (N). Sera with P/N values that were ≥ 2.1 were considered positive. We also peformed IgG2b and IgG1 assay with the method as above.

T cell proliferation assays

T lymphocytes were washed in fresh RPMI 1640 medium (Hyclone, Beijing,China) and cultured (4×10^5 cells/well) in flat-bottom 96-well plates (Roche Products, Switzerland) in 100 µl of RPMI 1640 medium supplemented with 10% FBS and a 1% solution of a broad-spectrum antibiotic-antimycotic mixture, and then incubated with the Ag85A or medium (control) at 37°C in a humidified 5% CO_2 incubator. The Ag85A polypeptide titer was determined to obtain its optimal concentration for use in the proliferation assays (that is 10 µg/ml). All stimulants were plated in triplicate and incubated for 72 h. All subsequent steps followed the manufacturer's instructions of the Cell Proliferation ELISA BrdU colorimetric kit (Roche). The mean counts for the cultures performed in triplicate and the stimulation index (SI) were obtained for each stimulant. The SI was the ratio of the mean counts in the presence of Ag85A protein to the means counts of the medium alone.

Statistical analysis

A two-way ANOVA was used to compare differences between groups and differences were considered statistically significant when the P value was less than 0.05.

RESULTS

Construction of an expression vector containing the *fbpA* gene

To obtain recombinant Ag85A protein, the *fbpA* gene was cloned into the *E. coli* expression vector pET-30a(+), which contains a C-terminal His-tag sequence to facilitate recombinant protein purification. Genomic DNA isolated from the BCG laboratory strain was used for PCR amplification of the *fbpA* gene. Agarose gel (1%) electrophoretic analysis of the PCR-amplified product revealed

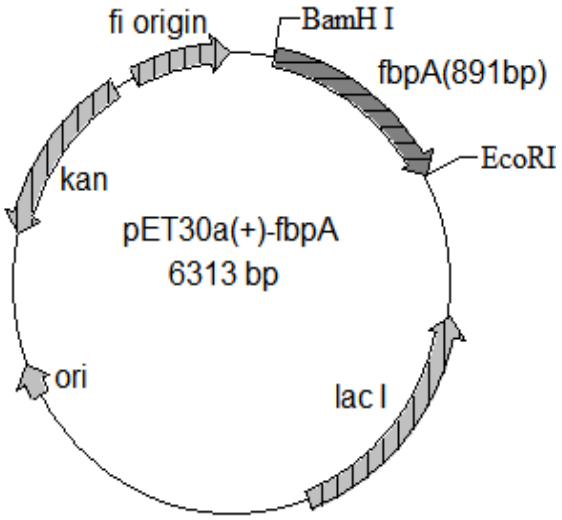

Figure 1. Construction of recombinant plasmids. The gene encoding Ag85A protein (*fbpA*) cloning from *M.bovis* genomic DNA was inserted into the pET30a(+), resulting in the recombination of plasmid pET30a-*fbpA*.

the presence of a single band of approximately 900 bp in size that was almost equal to the expected size (891 bp) of the *fbpA* gene. The PCR-amplified product was confirmed by nucleotide sequencing to be the *fbpA* gene.

During PCR amplification, *Bam*HI and *Eco*RI sites, which were incorporated into the PCR product containing the *fbpA* gene, were used to ligate the gel purified PCR product to a pMD20-T(+) plasmid containing an ampicillin resistance gene.

Following PCR and restriction digestion analysis, the recombinant plasmid, pMD20-T-*fbpA*, was commercially sequenced. After verifying that the correct sequence was present in pMD20-T-*fbpA*, the purified *fbpA* fragment was ligated to pET-30a(+) (Figure 1) and used to transform *E. coli* BL21(DE3) cells for hetero-logous expression of the protein.

The recombinant plasmids were analyzed for expression of recombinant Ag85A protein following induction with 0.5 mM IPTG. The intracellular localization of the Ag85A protein after lysis of the cells indicated that it was retained in the cytosol as an insoluble protein in the form of inclusion bodies (Figure 2a).

Identification of Ag85A protein

Because the Ag85A protein has a mass of only 33 kDa, the band could be clearly visualized by SDS-PAGE. The Ag85A protein was column-purified via its His-tag sequence under denaturing conditions. SDS-PAGE analysis of the purified samples revealed a single band with a molecular mass of about 33 kDa (Figure 2c). Western blots revealed that the recombinant Ag85A protein was specifically recognized by a mouse anti-BCG polyclonal antibody (Figure 2b).

After determining the molecular weight of the purified protein by SDS PAGE, the band was excised from the gel for analysis by MALDI-TOF-MS, a technique in which proteins with very small differences in mass can be separated. The results of the MALDI-TOF-MS analysis of the Ag85A protein is shown in Figure 3a, and the secondary mass spectrometry analysis in Figure 3b, c, d. The results confirm that the 32695 Da recombinant protein (isoelectric point of 6.04), was that of *M. tuberculosis* Ag85A.

Antibody responses to the Ag85A protein

According to the result of ELISA, mice vaccinated with DDA-MPL produced no antigen-specific antibodies. The BCG+Ag85A vaccine induced the highest IgG-specific response to PPD. The DDA-MPL+Ag85A-treated group and the BCG vaccinated group also exhibited strong IgG responses, while the Ag85A-immunized mice produced weak IgG responses. The serum antibody titer in the mice of group 2 was 1:12800 (Figure 4a). These results show that the Ag85A protein could induce production of Ag85A-specific humoral immune responses in the mice .What's more, the antibody titer of IgG2b was higher than IgG1 in group 2, which indicated that Ag85A protein had a tendency to induce Th1 type responses (Figure 4b).

Proliferative responses to mycobacterial antigens

To determine T cell reactivity to the mycobacterial antigens tested, lymphoproliferative responses were measured in the cells from the immunized mice. As shown in Figure 5, compared with the adjuvant-immunized group and the Ag85A-immunized group, the group immunized with DDA-MPL+Ag85A protein, BCG, and the booster-immunized group produced better proliferative responses (SI>2.1) upon stimulation with the synthetic Ag85A protein. Moreover, the splenocyte SI value for the DDA-MPL+Ag85A group is greater than that of the BCG group, thus indicating that the Ag85A protein induced a stronger immunological effect than that of BCG ($P<0.05$).

Cytokine profile in response to recombinant mycobacterial antigens

Splenocytes isolated from the immunized mice were stimulated with culture medium (CM), the synthetic Ag85A polypeptide, or PPD (Figure 6). Splenocytes from the DDA-MPL+PBS control group and the Ag85A group produced very low levels of IFN-γ over the whole experimental period, whether stimulated with CM, Ag85A

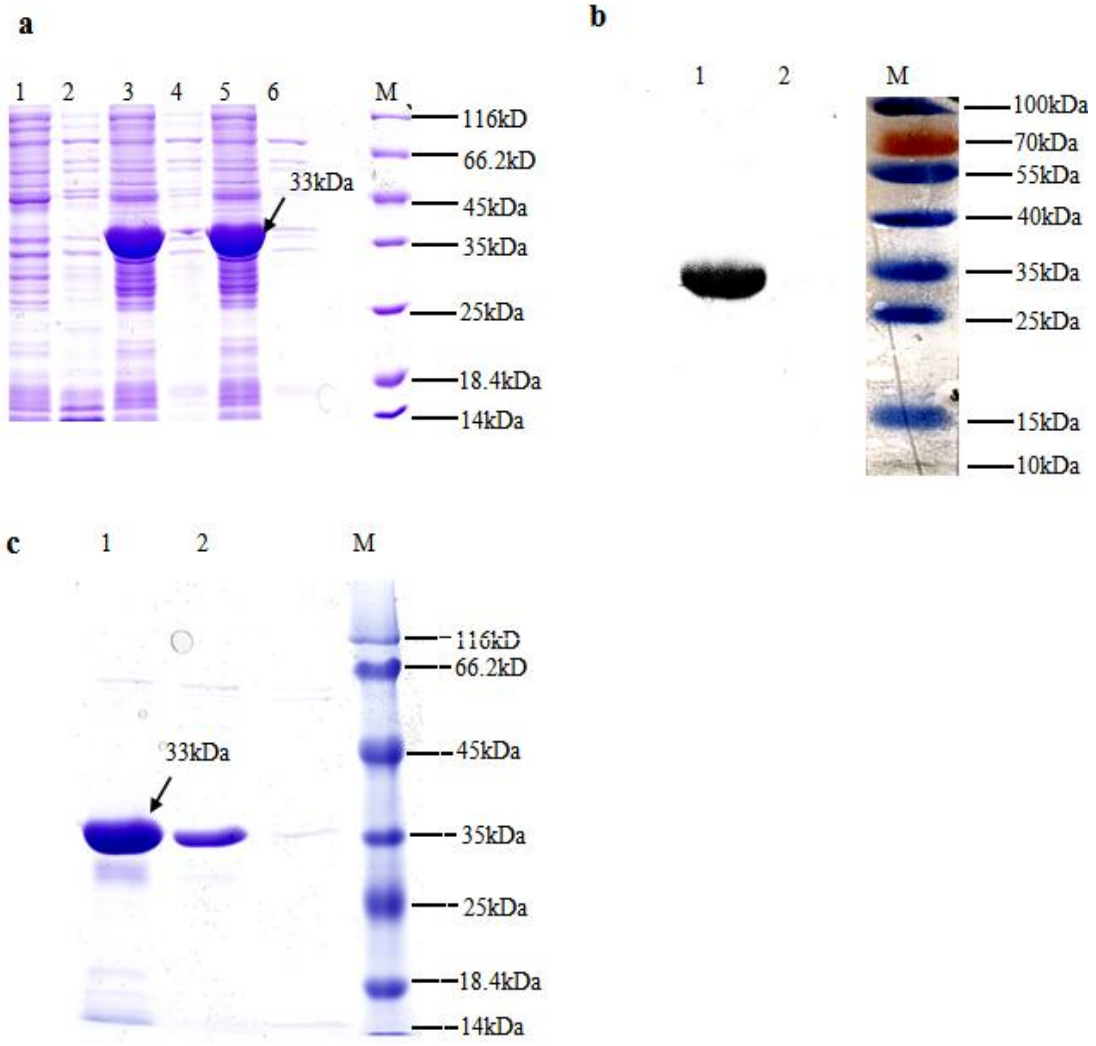

Figure 2. Expression and identification of Ag85A protein. *E.coli* BL21(DE3) harboring pET30a-fbpA was cultured with IPTG. The expression (a, c) and identification (b) of Ag85A protein was confirmed by SDS-PAGE (a, c) and Westren blotting (b). Lane a1, the precipitation of *E.coli* haboring pET30a(+); Lane a2, the supernatant of *E.coli* haoboring pET30a(+); lane a3,a5, the precipitantion of *E.coli* haboring pET30a-fbpA; lane a4,a6, the supernatant of *E.coli* haboring pET30a-fbpA; lane aM, protein marker; lane b1, the precipitantion of *E.coli* haboring pET30a-fbpA; lane b2, the supernatant of *E.coli* haboring pET30a-fbpA; lane bM, protein marker; Lane c1, c2, the purified Ag85A protein; Lane cM, protein marker.

polypeptide, or PPD. When stimulated with Ag85A polypeptide, IFN-γ levels increased significantly in the DDA-MPL+Ag85A group, the BCG group, and the booster-immunized group ($P<0.001$). The highest IFN-γ levels were observed in the booster-immunized group when stimulated with Ag85A polypeptide; these levels were much higher than those of the Ag85A and adjuvant groups ($P<0.001$). However, there were no significant differences in the IFN-γ levels between the DDA-MPL+Ag85A group and the booster immunized group. All of the groups produced lower amounts of IL-4 compared with IFN-γ regardless of whether they were stimulated with CM, Ag85A polypeptide, or PPD. Although the highest IL-4 levels were seen in the booster-immunized group, IL-4 production was lower than 200 pg/ml in all groups. Therefore, the Ag85A protein had a tendency to induce Th1 type responses in the mice, which was in accordance with the result of IgG2b and IgG1 assay.

DISCUSSION

About one third of the world's population is currently infected with *M. tuberculosis* and it is estimated that 2 million people die from TB annually (Romano et al., 2006). Vaccination is the most cost-effective strategy for TB control and an effective vaccine could eventually eliminate the disease. Currently, the only licensed

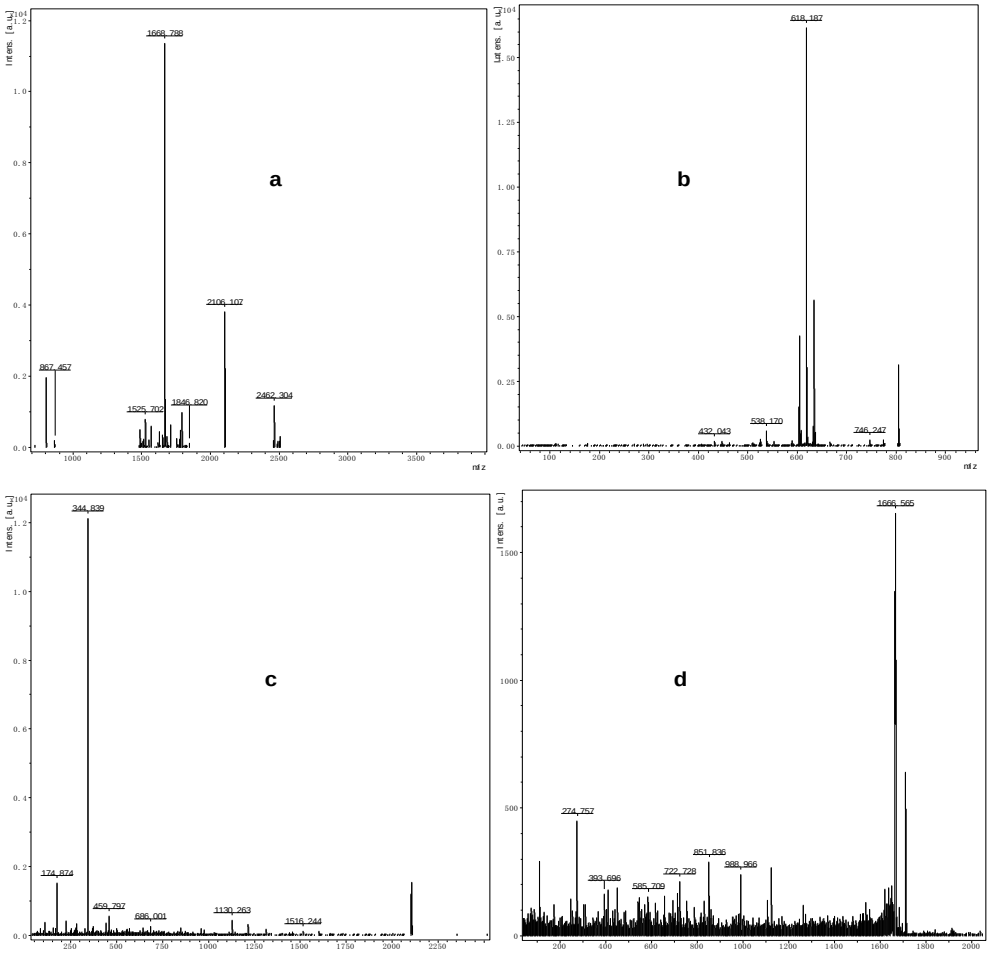

Figure 3. The result of MALDI-TOF-MS mass spectrometry. **a,** The level of mass spectrometry of Ag85A protein. **b,c,d,** The secondary mass spectrometry of Ag85A protein.

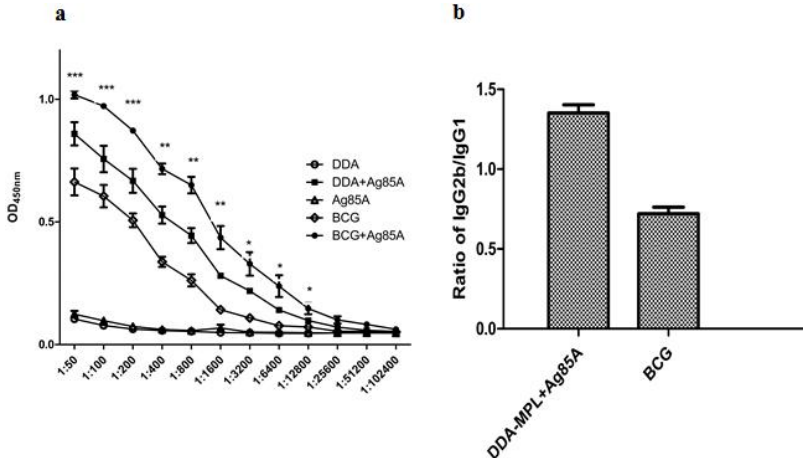

Figure 4. Antigen-specific total IgG production and the ratio of IgG2b/IgG1. C57BL/6 mice were vaccinated with different vaccines. Control groups were immunized with BCG or DDA. 8 days after the second immunization, the animals were bled and sera were obtained. PPD (10 μg/ml) were used for detection of IgG, IgG2b, IgG1 in each groups by enzyme-linked immunosorbent assay (ELISA). Detection of anti-Ag85A IgG levels in DDA-MPL+Ag85A was compared to those of other groups (a), the ratio of IgG2b/IgG1 was calculated by the titre of IgG2b and IgG1 (b).

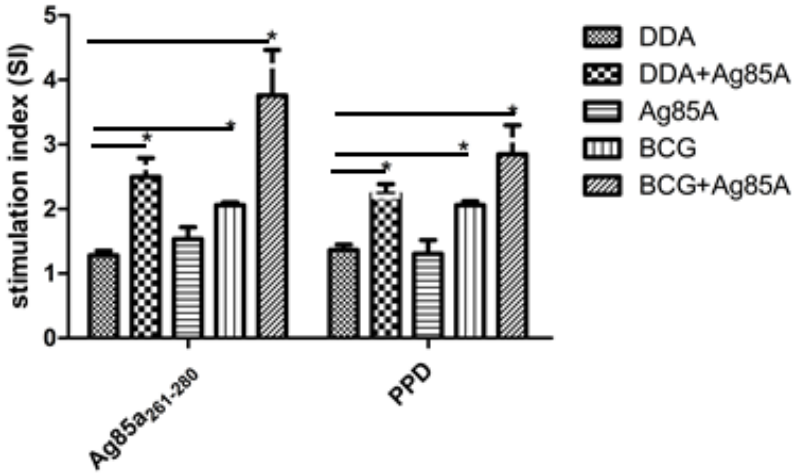

Figure 5. Lymphocyte proliferation in response to Ag85A polypeptide and PPD. Splenocytes isolated from all groups were cultured in the presence of Ag85A polypeptide and PPD(10 µg/ml) for 72 h. Results are expressed as stimulation index (SI) mean of triplicate cultures.

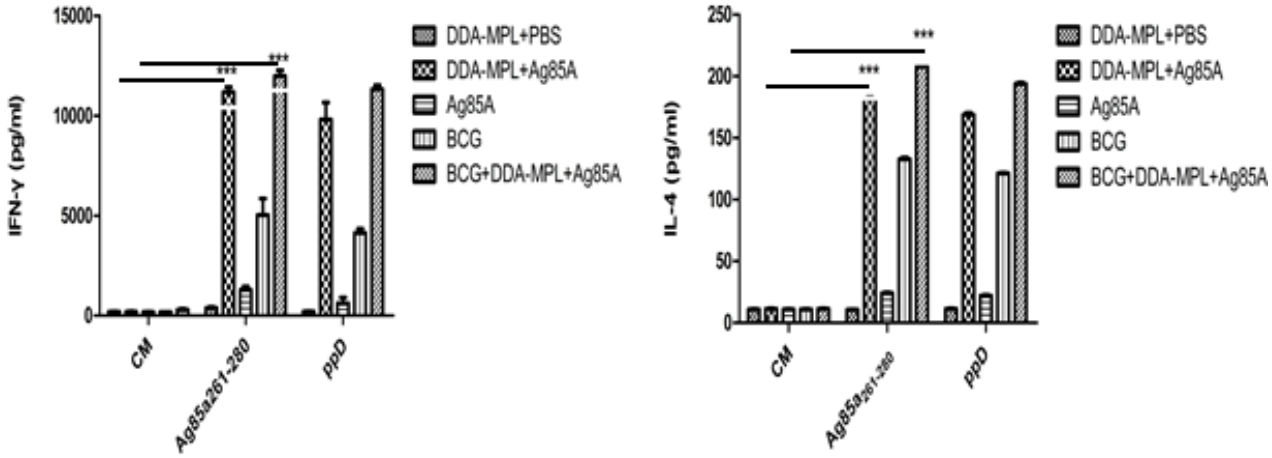

Figure 6. IFN-γ (a) and IL-4 (b) levels released by splenocytes stimulated with Ag85A polypeptide and PPD after 48 h of culture. Splenocytes were prepared and pooled from five mice per group 8 days after the last vaccination and were then stimulated in vitro with Ag85A polypeptide, PPD, and CM. The level of IFN-γ and IL-4 after 48 h incubation was analyzed. The experiments were repeated three times with similar results, and the data from one representative experiment is shown. The results are expressed as the mean (SD) amount of IFN-γ and IL-4 (pg/ml) of each group. A P value less than 0.05 was considered to be significant and is presented as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

vaccine against TB for use in humans is BCG, which has been in use since 1921 (Spencer et al., 2012); however, its efficacy is highly variable, and particularly so for protection against adult pulmonary TB. Therefore, there is an urgent need for a vaccine with better protection against adult pulmonary disease than that afforded by BCG.

M. tuberculosis secreted proteins, as opposed to cytoplasmic proteins (Fan et al., 2009), have generally been considered as strong candidates for a vaccine. Thus, much research has been focused on *M. tuberculosis* secretory antigens. Ag85A and Ag85B are the major secreted proteins in *M. tuberculosis* culture filtrates; both belong to the antigen 85 (Ag85) complex. As a major fraction of this complex, Ag85A is the most essential component involved in *M. tuberculosis* immunostimulation (Lozes et al., 1997). Recently, different gene transfer systems such as modified vaccinia

virus Ankara (Dai et al., 2013), rBCG (Rahman et al., 2012), and plasmid DNA (Tanghe et al., 2000) have been used to express Ag85A. However, compared with these systems, the Ag85A protein (with adjuvant) has the advantage that it can generate antigen-specific immune responses that are enhanced by boosting, thus offering the potential for a safe, effective and targeted vaccine against TB.

Ag85A is usually emulsified with adjuvants that enhance its ability to induce protective immunity. The adjuvants used for enhancing the immunogenicity and protective efficacy of Ag85A are DDA, CpG7909. Studies show that CpG7909 is able to enhance the immunological effects of Ag85A (Hu et al., 2013), but it does not confer significant protective efficacy against infection with *M. tuberculosis*. In contrast, Brandt's research has shown that DDA-MPL can induce similarly high levels of immunity as BCG (Brandt et al., 2000). DDA can enhance antigen uptake, antigen presentation to T cells, and stimulate DCs through Toll-like receptors. MPL can stimulate macrophages to release cytokines and enhance antigen uptake, as well as stimulating antigen processing and presentation. Recent studies show that the effect of DDA can be enhanced by the addition of MPL.

Recently, Ag85A has been expressed in different gene transfer systems. One study (Wang et al., 2012) using a recombinant BCG strain over expressing the Ag85A antigen showed that there was no significant difference between the rBCG::85A protein and the BCG group. Additionally, lower levels of IFN-γ were detected in splenocyte cultures derived from C57BL/6 mice vaccinated with the Influenza A virus expressing Ag85A, and the IFN-γ levels of the vaccinated mice were below 200pg/ml (Dai et al., 2012). Furthermore, it was found that the relative mRNA expression level of a DNA vaccine expressing Ag85A protein was lower than that obtained by BCG (Lu et al., 2011). In our study, we used the Ag85A protein emulsified with DDA-MPL adjuvants to immunized C57BL/6 mice subcutaneously, and found that IFN-γ secretion was higher than BCG, while IFN-γ production was >12000 pg/ml. The IFN-γ levels in the booster group were higher than those of the DDA-MPL+Ag85A group. Thus, the Ag85A protein, when expressed in the prokaryotic expression system used herein, induced higher levels of IFN-γ than did previously used gene transfer systems. At the same time, IL-4 levels in all of the groups of mice were relatively low. Taken together, these results indicate that Ag85A can induce Th1-type cellular immune responses in vaccinated mice. Owing to the characteristic of tuberculosis, cellular immunity, especially Th1-type cellular immunity plays a major role during the process of resisting tuberculosis. Therefore, the ability of inducing Th1-type cellular immunity may confer Ag85A protein the protective immunity for TB. Moreover, the lymphoproliferative assay shows that the Ag85a polypeptide can increase splenocyte proliferation *in vitro* (after the second immunity).

Compared with the 1:2000 serum antibody titer obtained from vaccination with plasmid DNA (Lu et al., 2011), the ELISA result in this study showed that the Ag85A protein induced a stronger humoral immune response, which was greatly depended on the B-cell epitopes of Ag85A protein. What's more, apart from the induction of strong cell-mediated immunity responses, DDA and MPL also could stimulate the host humoral immune response. The result of IgG2b and IgG1 assay indicated that Ag85A protein had a tendency to induce Th1 type response which was in accordance with the cytokine measurement assay.

Previous studies have shown that repeated vaccination with BCG may be detrimental to protection (Rodrigues et al., 2005); therefore, booster vaccinations with heterologous antigen are likely to be used to enhance the specific immunity primed by BCG (Kaufmann, 2005). Consequently, we used the Ag85A protein to boost the immunity of mice previously immunized with BCG. The results suggest that irrespective of whether it was a humoral or a cellular immune response, the booster group exhibited a stronger immune response than did the DDA-MPL+Ag85A group. Overall, the Ag85A protein expressed in the prokaryotic expression system had similar immunogenicity and immune reactivity as the natural protein, making it worth considering as a vaccine candidate. Because the Ag85A protein is only the antigen molecule of *M. tuberculosis*, it is likely to be safer than live vaccines (such as rBCG) for the immune-compromised people (for example, AIDS patients). Furthermore, the Ag85A protein and adjuvant can be combined to eliminate irrelevant or immune-suppressive components of the whole bacterium, which might enable it to induce a stronger immune response than that of the whole bacterium. Therefore, we consider that our current study serves as an important step for future investigation of a subunit vaccine against TB.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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