African Journal of Microbiology Research

Volume 10 Number 34 14 September, 2016
ISSN 1996-0808

Academic Journals

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.me/

Editors

Editorial Board Members

Editorial Board Members

Editorial Board Members

Editorial Board Members

African Journal of Microbiology Research

Table of Contents: Volume 10 Number 34 14 September, 2016

ARTICLES

academicJournals

Vol. 10(34), pp. 1371-1379, 14 September, 2016
DOI: 10.5897/AJMR2016.8178
Article Number: A69C42160504
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Importance of biofilm in medical sciences: With special reference to uropathogens

Ghanwate Niraj A.[1]*, Tiwari Anjali A.[1] and Thakare P. V.[2]

[1]Department of Microbiology, Sant Gadge Baba Amravati University, Amravati, India.
[2]Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, India.

Received 23 June 2016, Accepted 17 August, 2016.

Biofilm formation is a well organised, genetically-driven process, well characterized for numerous bacterial species which plays important role in urinary tract infections (UTIs). Adherence is a key event initiating each step in UTI pathogenesis. Such UTIs are difficult to treat owing to increased drug resistance within the biofilm cells. The review is mainly focused on biofilm-growing microorganisms because this form of growth poses a threat to chronically infected or immunocompromised patients and is difficult to eradicate from medical devices. Biofim formation process and mechanisms to its increased resistance to various antimicrobials is also discussed together with newer prophylactic and therapeutic approaches like catheters coated with hydrogels or antibiotics, nanoparticles, ionotrophoresis, biofilm enzyme inhibitors, liposomes, bacterial interference, bacteriophages, quorum sensing inhibitor, combining antimicrobial photodynamic therapy and antiadhesion agents. The review justifies the need for new antibiofilm drug.

Key words: Biofilm, uropathogens, catheter, urinary tract infection, catheter-associated UTI (CAUTI).

INTRODUCTION

Various defence mechanisms of the body prevent the infection of urinary tract. One of the most important defence mechanism is the outward flow of urine that can clear 99% of the organisms experimentally inoculated in the bladder. The acidic pH (5.5) and low osmolarity of the urine also discourage the bacterial growth. However, there are a number of factors that increase the risk of developing urinary tract infections (UTIs). Some of these are sex, age, pregnancy, catheterization, kidney stone, tumours, urethral strictures, neurological diseases, congenital anomalies of bladder, suppressed immune system diabetes mellitus and ureteric stresses (Ramzan et al., 2004).

A urinary tract infection (UTI) is a bacterial infection that affects any part of the urinary tract. Although, urine contains a variety of fluids, salts and waste products, it usually does not have bacteria in it. When bacteria get in to the bladder or kidney and multiply in the urine, they cause a UTI. The most common type of UTI is a bladder infection which is also often called cystitis. Another kind of UTI is a kidney infection, known as pyelonephritis, and is much more serious.

UTI is a serious health problem affecting millions of people each year. The recurrence rate is high and often

*Corresponding author. E-mail: nirajghanwate@gmail.com. Tel: 9922445913.

the infections tend to become chronic with many episodes. UTI usually starts as bladder infections but often evolves to encompass the kidneys and ultimately can result in renal failure or dissemination to the blood. UTI is the most common infection in patients with a chronic indwelling bladder catheter and bacteriuria is essentially unavoidable in this patient group (Foxman, 2002).

Most UTIs are thought to be caused by organisms originating from the patient's own bowel. Normally, UTIs are caused by a variety of Gram-negative and positive bacteria. The Gram-positive bacteria includes *Staphylococcus* sp, *Streptococcus* sp and *Enterococcus* sp. Gram-negative includes a large number of aerobic bacilli such as *Escherichia* sp., *Klebsiella* sp., *Enterobacter* sp., *Citrobacter* sp., *Proteus* sp., *Serratia* sp., *Salmonella* sp. and *Pseudomonas* sp. Among this, 80-90% of UTI is caused by *E. coli* (Rushton, 1997) and in ambulatory patients and nosocomial infections, *Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus* and *Enterococcus faecalis* are the most frequently isolated.

Urinary tract infections pose a serious health threat with respect to antibiotic resistance and high recurrence rates. Uropathogenic *E. coli* forms intracellular bacterial communities with many biofilm like properties within the bladder epithelium. These intracellular biofilm like pods allow bacteria to outlast a strong host immune response to establish a dormant reservoir of pathogens inside the bladder cells. Re-emergence of bacteria from this reservoir might be the source of recurrent infections (Suman et al., 2008).

A biofilm is a sessile community of microorganisms which are attached to an interface or to each other and are embedded in an exopolysaccharides matrix or to each other and alter growth rate and transcribes genes free for floating organisms (Thomas and Day, 2007). Biofilms are currently estimated to be responsible for over 65% of nosocomial infections and 80% of all microbial infections (Forster et al., 2010). Biofilm formation occurs in various stages as described below:

1) Reversible attachment of planktonic bacteria to surfaces: The first attachment of the bacteria is influenced by attractive or repelling forces that vary depending on nutrient levels, pH and the temperature of the site (Donlan, 2002). In this step, flagella (Lemon et al., 2007; Toutain et al., 2007) and chemotaxis play an important role avoiding the action of the hydrodynamic and the repulsive forces as well as selecting the surface (Schmidt and Kirsching, 2012), respectively.
2) Irreversible attachment to surfaces: In the case of *E. coli*, it is mediated by type 1 pili, curli fibres, and antigen 43 that also favours the interbacterial interactions (Danese et al., 2000; Anderson et al., 2003; Beloin et al., 2008, Cegelski et al., 2009). In the case of *P. aeruginosa* as well as other *Pseudomonas* species, transition from reversible to irreversible attachment has been well studied. It has been observed that *Pseudomonas fluorescence* requires an ATP binding cassette [ABC] encoded by the *Lap* genes for carrying out this process (Hinsa et al., 2003). On the other hand, *P. aeruginosa* requires the Sad B Protein and the two component regulatory systems; BfisR for irreversible attachment (Caiazza et al., 2012; Petrova et al., 2010).
3) Formation of a complex layer of biomolecules (Lappinscott 2001) and exopolysaccharides secretion that constitute the external matrix. The production of polysaccharides in biofilm forming strains facilitates aggregation, adherence and surface tolerance, allowing better surface colonization (Laue et al., 2006). The nucleic acids, such as DNA, proteins, surfactants, lipids, glycolipids, membrane vesicles and ions such as calcium ions can also be found forming the part of the matrix composition and may play an important role in the characteristics that biofilm structure confers to the cells.
4) When biofilm are fully matured, detachment may occur. The detachment allows cells to again take on a planktonic state and can thereby form biofilm in other settings. It has been proposed that bacterial detachment could be caused by active mechanism initiated by the bacteria themselves such as enzymatic degradation of the biofilm matrix and quorum sensing in response to environmental changes related to nutrition level and oxygen depletion (Karatan et al., 2009) and by passive mechanisms mediated by external forces and erosion (Costerton et al., 1987; Kaplan, 2010; Hong et al., 2010; Rowe et al., 2010).
Consequences of Biofilm producing infections:

a) Detachment of the cell: The cell may get detached from the biofilm. This may cause blood stream and urinary tract infections (Meluleni et al., 1995)
b) Resistance to the host immune system: Biofilm coated bacteria escape from the damaging effect of the antibodies produced by the infected host cells (Holland et al., 2000)
c) Production of endotoxins: Gram negative bacteria which are encased in biofilms, produced endotoxins (Ethers and Bouwer, 1999).
d) The generation of resistant organisms: Bacteria can transfer plasmids by conjugation with the biofilm. So resistance factors may be exchanged through a plasmid (Christensen et al., 1985).

Biofilm detection

The detection of the biofilms is done by following three methods:

1) Tube adherence method (TA) (Pramodini, 2012; Freeman et al., 1989). In this method, investigation of the biofilm production is done on the basis of the adherence

of the biofilm to borosilicate test tubes (Christensen et al., 1982). Suspensions of the tested strains are incubated in glass tubes containing trypticase soya broth aerobically at a temperature of 35°C for a period of 2 days. The tubes are decanted and stained with 0.1% crystal violet solution, washed with distilled water 3 times and dried. A positive result was interpreted as the presence of a layer of a stained material which adheres to the inner wall of the tubes.

2) Congo red agar (CRA) method (Niveditha, 2012; Tool et al., 1998). The isolate is inoculated into medium containing brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The plates are incubated aerobically for 24-48 h at 37°C. A positive result is indicated by black colonies with a dry crystalline colonial morphology.

3) Tissue culture plate (TCP) method (Pramodini, 2012; Donlan, 2001). Isolate from fresh agar plates are inoculated in trypticase soy broth with 1% glucose and incubated for 24 h at 37°C in stationary condition and diluted (1 in 100) with fresh medium. Individual wells of sterile, polystyrene, flat-bottom tissue culture plates are filled with 0.2 ml of aliquots of the diluted cultures, and only broth served as control to check for the sterility and non-specific binding of media. The tissue culture plates are incubated for 24 h at 37°C. After incubation, the content of each well is gently removed by tapping the plates. The wells are washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free floating planktonic bacteria. 1% solution of crystal violet was added to each well, rinsed thoroughly and dried. OD of each well was measured at 578 nm using ELISA reader.

BIOFILM ON MEDICAL DEVICES

When an indwelling medical device was contaminated with microorganism, several variables determine whether a biofilm develops. First, the microorganisms must adhere to the exposed surfaces of the device long enough to become irreversibly attached. The rate of cell attachment depends on the number and types of cells in the liquid to which the surface is exposed, the flow rate of liquid through the device and the characteristics of the surface. Once these cells irreversibly attach and produce extracellular polysaccharides to develop a biofilm, rate of growth is influenced by flow rate, nutrient composition of the medium, antimicrobial drug concentration and ambient temperature. These biofilms forms on different types of indwelling medical devices: central venous catheters, arterial catheters, mechanical heart valves and other surgical implants, endotracheal, nasal catheters and urinary (Foley) catheters, etc (Elliott et al., 1992). In the urinary tract, bacterial biofilms can develop on many living surfaces and virtually all artificial implants, producing chronic and often intractable infections (Warren, 2001). Bacterial biofilms were reported to affect 90% of indwelling stents in patients (Reid et al., 1992). The medical consequences of device-related infections can be disastrous; they include potentially life-threatening systemic infections and device malfunction that may require device removal, often complicated by tissue destruction. A further complication that may be associated with urological medical devices is encrustation a phenomenon that frequently results in impairment of urine patency (Gorman et al, 2003).

Urinary catheter biofilms

Urinary catheters are tubular latex or silicone devices, which when inserted may readily acquire biofilm on the inner or outer surfaces. The organisms commonly contaminating these devices and developing biofilm are *Staphylococcus epidermidis, Enterococcus faecalis, E. coli, P. mirabilis, P. aeruginosa, K. pneumoniae,* and other Gram-negative organisms (Ghanwate, 2012; Ghanwate et al., 2014). The longer the urinary catheter remains in place, the greater the tendency of these organisms to develop biofilm and result in urinary tract infections. For example, 10 to 50% of patients undergoing short-term urinary catheterization (7days) but virtually all patients undergoing long-term catheterization (>28 days) become infected (Ghanwate, 2012; Brisset et al., 1996; Balaban et al., 2004). It was found that adhesion to catheter materials was dependent on the hydrophobicity of both the organisms and the surfaces. Mack et al. (2004) stated that no single material is more effective in preventing colonization, including silicone, polyurethane, composite biomaterials or hydrogel coated materials. According to the National Institutes of Health [NIH], biofilm forming bacteria involved up to 80% of all infections (Stowe et al. 2011), with urology being one of the main fields in which biofilm can become a serious problem.

Biofilm cannot only develop into urethral stents but they can also form on catheters causing their blockage. Thus, catheter-associated UTI (CAUTI) is one of the most common catheter-associated infections around the world (Brisset et al., 1996). Commensal perineal flora is involved in most CAUTI cases. More than 90% of these infections are monomicrobial with *E. coli, Pseudomonas aeruginosa, Enterococci, Candida, Klebsiella* or *Enterobacter* spp. being the most frequently isolated pathogens (Ong et al., 2008). The environmental conditions created on the catheter surface make it an ideal site for bacterial attachment and formation of biofilm structures (Stickler et al., 1998). In this type of medical device, microorganisms producing urease, an enzyme that hydrolyzes urea to ammonium ions, can cause encrustation, formation of infected bladder calculi, and urinary obstruction. The formation of ammonium ions increases the pH of the urine; finally causing the

precipitation of magnesium and calcium phosphate crystals (Ghanwate, 2012; Jepson et al., 2001). The pH value at which precipitation occurs is called nucleation pH (Donlan 2002). These crystals can form a layer that protects bacteria from the antimicrobial effects of compounds used for coating or impregnating the catheters (Sancher et al., 2013).

Biofilm and persistent infections

Acute UTI caused by bacteria can lead to recurrent infection, which is defined as a "reinfection" when it involves a strain other than that causing the original infection or it is defined as a "relapse" when it is caused by the same strain as that involved in the original UTI. Several studies observed that most of isolates collected from patients with relapse infections were biofilm producers "*in vitro*" (Sano et al., 1999). Relapse by uropathogenic *E. coli* (UPEC) has been related to the ability of pathogenic strains to form biofilm. In these cases, biofilm production may be the key determinant for the persistence of UPEC in the vaginal reservoir, the bladder epithelial cells, or both.

Genes responsible for biofilm formation

Uropathogenic *E. coli* (UPEC) are the primary cause of urinary tract infection (UTI) in the developed world. The major factors associated with virulence of UPEC are fimbrial adhesins, which mediate attachment to specific receptors, enhance persistence and trigger innate host responses. UPEC produce a range of fimbrial adhesins, with type 1 and P fimbriae of the chaperone-usher subclass being the best characterized. The prototype UPEC strain CFT073 contains ten gene clusters that contain genes characteristic of this class of fimbriae. However, only five of these gene clusters have been characterized in detail (Nickel et al., 1985). The *f9* fimbriae-encoding genes were amplified, cloned and expressed in a K-12 background devoid of type 1 fimbriae. While *f9* fimbrial expression was not associated with any haemagglutination or cellular adherence properties, a role in biofilm formation was observed. *E. coli* K-12 cells expressing *f9* fimbriae produced a dense and uniform biofilm in both microtitre plate and continuous-flow biofilm model systems. In wild-type UPEC CFT073, expression of the *f9* major subunit-encoding gene was detected during exponential growth in M9 minimal medium.

f9 expression could also be detected following selection and enrichment for pellicle growth in a CFT073fim foc double mutant. The *f9* genes appear to be common in UPEC and other types of pathogenic *E. coli*. However, their precise contribution to disease remains to be determined (Nickel et al., 1985).

BIOFILM AND ANTIMICROBIAL RESISTANCE

In the biofilm stage, a phenotypic change occurs in which the bacteria require generally much higher concentration of antibiotics to inhibit their growth. This biofilm effect is the mechanism responsible for the frequent failure of antibiotic treatment to cure infections of medical devices and other prosthetic materials (Sepandj et al., 2003).

One of the most important advantages of biofilm status is the antimicrobial resistance shown by these structures. Biofilm can be up to 1000-fold more resistant to antibiotics than planktonic cells due to several mechanisms (Lewis, 2005; Costerton et al., 2007; Lewis, 2005, 2008; Ghanwate 2014; Ghanwate and Thakare, 2012; Morgan et al., 2009):

1. Limitation of antibiotic diffusion through the matrix- some antimicrobial agents are unable to diffuse through the matrix or sometimes the time required for the antibiotic to penetrate into biofilm is longer than the duration of treatment or the antibiotic life-time. Thus, for example, aminoglycosides penetrate more slowly through the matrix than lactams.
2. Transmission of resistance genes within the community can occur. Thus, plasmids, transposons, and other mobile genetic elements can be transmitted between cells forming biofilm by their close relationship, spreading resistance markers.
3. Expression of efflux pumps can also be considered as one of the mechanism for antimicrobial resistance not only in planktonic cells but also in biofilms structures (Van Acker et al., 2014; Lewis et al., 2001).
4. Inactivation of the antibiotic by changes in metal ion concentrations and pH values- Antibiotics able to diffuse can be inactivated by modifying the pH inside biofilm. This change in the pH could antagonize the activity of the antibiotic.
5. The persisters are dormant variants of regular cells, not mutants, which may form small colony variants that are high tolerant to extracellular stresses. They are highly tolerant to antibiotics forming a reservoir of surviving cells able to rebuild the biofilm population (Keren et al., 2004a, b 2004; Ulett et al., 2007). Persister is a problem for biofilm eradication. Proteins required for maintaining persisters may represent excellent targets for the discovery of compounds capable of effectively treating chronic infections and biofilm-related infections.

ANTIMICROBIAL TREATMENT OF BIOFILM

Several studies recommend combination therapy as the treatment of choice in biofilm-associated infections, with macrolides being one of the first antibiotics chosen (Ethers et al., 1999). Macrolides [erythromycin, clarithromycin and azithromycin] present high "*in vitro*" and "*in vivo*" antibiofilm activity against biofilm-associated

infections caused by Gram-negative bacteria inhibiting the production of a key component of the matrix, alginate (Ethers et al., 1999). The antibiotic combination, clarithromycin plus vancomycin, demonstrated the ability to eradicate both biofilm and planktonic cells (Choong et al., 2001) as well as to eradicate biofilm on the titanium washers used in animal experiments (Davenport and Keeley, 2005). Roxithromycin plus imipenem favour a higher penetration of neutrophils into biofilm structure destabilizing the biofilm.

Another approach using antimicrobials consists of coating and impregnating the catheters with these antimicrobial agents (Ghanwate et al., 2014; Morgan et al., 2009; Brisset et al., 1996).

Prophylaxis for CAUTI

CAUTIs are a major problem throughout the world. Catheter blockage is clinically important, as not only will the resulting bladder distension be painful for the patient, but a blocked catheter also increases the risk of serious clinical complications including septicaemia and pyelonephritis (Stickler et al., 1994). Management of urinary catheter encrustation is difficult, and occurrence is both unpredictable and extremely hard to prevent with existing strategies (Trautne et al., 2004). Most often, the approach used is catheter replacement once blockage has occurred (Stickler et al., 2014). Unfortunately, such treatment is often unsuccessful with frequent recurrence of blockage evident (Mathur et al., 2006). Prophylactic antibiotic use to prevent recurrence is not ideal due to the potential promotion of antibiotic resistance. One possible approach is to employ catheter materials that incorporate an antimicrobial agent that is either gradually released to the surface to inhibit colonisation or is utilised as an external catheter coating (Hooton et al., 2009; Jacobsen et al., 2010; Garibaldi et al., 1977).

Chelating agents

Metal cations, such as calcium, magnesium, and iron have been implicated in maintaining matrix integrity. Consistent with this observation, chelating agents have been shown to destabilize biofilm architecture besides interfering with bacterial membrane stability. For example, sodium citrate inhibited biofilm formation by several *Staphylococci* species *in vitro* (Shanks et al., 2006). In addition, tetrasodium-EDTA eradicated biofilms in an *in vitro* biofilm model and on explanted hemodialysis catheters, whereas disodium-EDTA, in combination with tigecyclin or gentamicin, reduced biofilm formation by *Staphylococcus* species and *P. aeruginosa.*

Antimicrobial peptides

Antimicrobial peptides are produced by the innate immune response system and have been proposed as attractive candidates for the development of novel types of antibiotics. However, their activity spectrum and mechanism of action need to be more precisely defined before they can be considered as possible therapeutic strategies (Kharidia et al., 2011). A recent work, focused on reduced biofilm formation by multidrug-resistant *P. aeruginosa* strains isolated from patients with cystic fibrosis, revealed that the bacterium was killed within preformed biofilms. Lytic peptides are another group of antimicrobial peptides assessed for their inhibitory effects on biofilm formation. Lytic peptides bind the lipopolysaccharide moieties of the bacterial cell membrane, disrupting membrane stability (Kharidia et al., 2011). Studies on *S. aureus* have shown that a lytic peptide prevented *in vitro* biofilm formation and was also capable of diffusing into the deep layer of preformed biofilm, killing 99.9% of biofilm bacteria. This peptide retained activity under highly acidic environments and in the presence of excess of metals, conditions that mimic the *S. aureus* biofilm environment.

BACTERIAL ANTIBIOFILM POLYSACCHARIDES

Polysaccharides, as sugar polymers, have the capacity to act as lectin inhibitors. Lectins are proteins that specifically recognize and bind sugars without modifying these molecules. In bacteria, the primary function of lectins is to facilitate attachment or adherence of bacteria to host cells. These proteins play an important role in biofilm formation, and are essential for bacterial colonization and infection. Lectins are mainly located on the surface of bacteria cells where they can access and bind to the glycan substrates present on the surface of host cell. By competing for the sugar binding domain of lectins, polysaccharides can inhibit lectin-dependent adhesion of pathogens and biofilm formation. In fact, several plant, microbial and milk polysaccharides have been shown to block various lectins from human pathogenic bacteria by competitive inhibition (Qin et al., 2009). Polysaccharides mediate cell-to-surface and cell-to-cell interactions that are critical for biofilm formation and stabilization. Recent evidence indicates that some bacterial exopolysaccharides inhibit or destabilize biofilm formation by other species (Qin et al., 2009). Antibiofilm properties of polysaccharides are believed to depend on their ability to: a) alter the physical characteristics of bacterial cells or abiotic surfaces; b) act as signaling molecules that impact the gene expression patterns of susceptible bacteria; or c) competitively inhibit multivalent carbohydrate-protein interactions, thereby interfering with adhesion. Many studies are reported on the ability of some bacterial polysaccharides to inhibit biofilm formation by several bacteria, including *E. coli* strains*, P. aeruginosa, K. pneumoniae, Staphylococcus* and *Enterococcus* (Rendueles et al., 2013.). Most of these antibiofilm agents are able to inhibit the biofilm formation

of a broad range of bacteria, suggesting that they may play an essential role in microbial competition and niche exclusion. Mutants unable to synthesize or export such polysaccharides are typically deficient in adherence and biofilm formation and thus are highly sensitive to killing by antibiotics and host immune defense (Maria et al., 2014).

Anti-biofilm enzymes

Enzymes that degrade biofilm extracellular matrix may play a role in biofilm dispersal and may be useful as anti-biofilm agents. N-acetyl-D-glucosamine-1-phosphate acetyl transferase is an essential peptidoglycan and lipopolysaccharide precursor in Gram-positive and negative pathogens, respectively, is among the enzymes targeted for matrix disruption (Maria et al., 2014). Treatment with such enzymes prevented *Staphylococcus* and *Enterococcus* biofilm formation and disperse preformed biofilms *in vitro* (Guiton et al., 2009) . For example, Dispersin-B is a glycoside hydrolase that cleaves β 1–6 N-acetylglucosamine polymers in the bacterial peptidoglycan layer. Dispersin-B treatment has been shown to be effective against *S. aureus* and *S. epidermidis* biofilms and bacteria (Kaplan, 2010).

Catheters coated with hydrogels or antibiotics

A high number of antimicrobial agents and other chemical compounds have been used to coat catheters. Silver alloy has been used in hydrogel coated urinary catheter observing a decrease of up to 45% of CAUTI (Devenport and Keeley, 2005; Raad et al., 2012). Minocycline rifampicin coated catheters have been shown to inhibit the biofilm formation of Gram-positive and negative pathogens, except *P. aeruginosa* and *Candida* spp. (Lellouche et al., 2012; Fisher et al., 2015).

Nanoparticles of MgF have been used for coating glass surfaces observing an inhibition of biofilm formation by both *E. coli* and *S. aureus* (Lellouche et al., 2009). Catheters have also been coated with these nanoparticles and a significant reduction of bacterial colonization was observed over a period of 1 week in comparison with the catheter uncoated catheter control. This group also demonstrated the antibacterial and antibiofilm activity of yttrium fluoride (YF3) nanoparticles which showed low solubility and provided extended protection (Roy et al., 2013).

Microwave irradiated CaO nanoparticles (CaO-NPs) have also shown the potential to inhibit biofilm formation against Gram-negative and positive bacteria (Fey, 2010). Silver nanoparticles have also been used for impregnating medical devices due to the silver antimicrobial properties (Costerton et al., 1994). Several studies have demonstrated the "*in vivo*" and "*in vitro*" inhibition of biofilm formation by numerous bacterial species and using determined nanoparticle concentrations.

Iontophoresis

Iontophoresis is a physical process in which ions flow diffusively in a medium driven by an applied electric field. This method enhances the efficacy of antibiofilm agents "*in vitro*" (Lu et al., 2007). Thus, it has been observed that low electrical currents enhance the activity of tobramycin and biocides against *P. aeruginosa* biofilm.

Enzyme inhibitors

Urease, the enzyme that allows *P. mirabilis* to hydrolyze urea to ammonium ions, has been an important target in the study of new antibiofilm compounds. In this sense, fluorofamide has been a candidate molecule because it is able to prevent the increase in pH by *P. mirabilis* "*in vitro*", thereby inhibiting the formation of urea crystal and the subsequent encrustation and catheter obstruction. Other natural compounds, such as vanillic acid, natural plum juice and germa-lactones among others, presented the ability to strongly inhibit bacterial growth as well as the formation of crystals in catheters by the inhibition of the urease enzyme.

In one study, Giwercman et al. (1991) generated a bacteriophage which expressed a biofilm-degrading enzyme during infection. The enzyme associated with the bacteriophage was DspB and it is produced by one species of Actino bacillus. DspB hydrolyses a crucial adhesion needed for biofilm formation and integrity in both *E. coli* and *Staphylococcus* (Raad et al., 1992) and attacks the bacterial cells in the biofilm and the biofilm matrix simultaneously. The percentage of eradication using this bacteriophage-enzyme combination was about 99.9% (Freeman et al., 1989). In recent years, the second messenger, c-di-GMP, has been studied in depth because it is highly conserved among bacterial species, being an important candidate for studies on biofilm inhibition. C-di-GMP is synthesized via diguanylate cyclases (DGC). Inhibition of DGC activity leads to a reduction in biofilm formation by a decrease in the intracellular levels of c-di-GMP.

Liposomes can be applied in the eradication of formed biofilm because when the antibiotic is encapsulated in a liposome carrier it does not interact with the EPS, improving its antibiofilm effect, and it is protected from degradation by antibiotic-inactivating enzymes (such as β lactamases) which can appear in the biofilm matrix (Siddiq and Darouiche, 2012).

In bacterial interference, the colonization of a surface by nonpathogenic bacteria could prevent the adherence of pathogenic bacteria thereby avoiding infection (Traunter et al., 2003). Several avirulent strains of *E. coli* have been used as a method to reduce urinary catheter

colonization by a wide variety of pathogens (Traunter et al., 2002; Anderson et al., 1991). Thus, the *E. coli* HU2117 strain, derived from *E. coli* 83972, that causes persistent colonization without symptomatic infection (Otto et al., 2001; Hull et al., 2002; Curtin and Donlan, 2006) has been used for coating urinary catheters, observing a reduction of biofilm formation by other pathogens (Trautner et al., 2002).

Bacteriophages

These phages have been incorporated into hydrogel-coating catheters, and a reduction has been observed in biofilm formation by *S. epidermidis* and *P. aeruginosa* (Carson et al., 2010; Schmidt et al., 2012). In addition, the use of lytic bacteriophages against established biofilm *of P. mirabilis* and *E. coli* caused a reduction of three to four log cycles (Hensel and Xiao, 2009). These lytic phages also prevented biofilm formation on catheters coated with hydrogel containing bacteriophages. The reduction of formation observed was about 90% (Hensel and Xiao, 2009).

Low-energy surface acoustic waves

It has been demonstrated that surface acoustic waves (SAW) interfere with adhesion of planktonic micro-organisms to cellular surfaces (Mack et al., 2004). SAW reduces biofilm bio burden on catheter segments in suspensions with several Gram-negative and positive bacteria as well as fungi, indicating its efficacy against a broad spectrum of micro-organisms.

Anti-adhesion agents

The main characteristic of an anti-adhesive compound is that it specifically interact with the adhesins of the pathogen, inhibiting the union between pathogen and eukaryotic cell (Lohr et al., 2011; Jepson et al., 2001). These anti-adhesive compounds cause a decrease in invasion or infection of host epithelial cells, also avoiding recurrence. One of the compounds most frequently studied is cranberry extract (Foo et al., 2000). The anti adherence effect of cranberry against uropathogenic *E. coli* (UPEC) is due to the presence of A-type proanthocyanidin trimers in the cranberry extract (Foo et al., 2000; Hamblin et al., 2004) that acts as an anti-adhesion agent. Salicylate is a member of a large group of pharmaceuticals referred to as non-steroidal anti-inflammatory and it is the active component of the analgesic aspirin. Salicylate has been shown to decrease biofilm formation of UPEC, inhibiting type 1 fimbriae expression (Advances in Biology vol. 2014).

Antimicrobial photodynamic therapy (Grinholc et al., 2008) in recent studies has shown that the antimicrobial effect can be obtained with the use of photosensitizers belonging to different chemical groups. Most studied PSs are phenol thiazine dyes methylene blue and toluidine blue O, porphyrin and its derivatives, fullerenes and cyanines and its derivatives (Grinholc et al., 2010, 2011; Nakonieczna et al., 2010).

Need for future research

To better understand and control biofilms on indwelling medical devices, research must progress in several key areas. More reliable techniques for collecting and sampling biofilms should be developed. Model systems should be developed and used to study biofilm processes on various indwelling medical devices. These systems should closely simulate the *in vivo* or *in situ* conditions for each device, while at the same time providing reproducible and accurate results.

Conflict of interests

The authors have not declared any conflict of interests.

REFERENCES

Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ (2003). Intracellular bacterial biofilm-like Pods in Urinary tract infections. Science 301:105-107.

Anderson P, Engberg I, Lidin-Janson G, Lincoln K, Hull R, Hull S, Svanborg C (1991). Persistence of *Escherichia coli* bacteriuria is not determined by bacterial adherence. Infec. Immun. 59:2915-2921.

Balaban N, Gov Y, Giacometti A, Cirioni O, Ghiselli R, Mocchegiani F, Orlando F, D'amato G, Saba V, Scalise G, Bernes S (2004). A chimeric pep-tide composed of a dermaseptin derivative and an RNA III-inhibiting peptide prevents graft-associated infections by antibiotic-resistant staphylococci. Antimicrob. Agents Chemother. 48:2544-2550.

Beloin C, Roux A, Ghigo JM (2008). *Escherichia coli* biofilms. Curr. Top. Microbiol. Immunol. 322:249-289.

Brisset L, Vernet-Garnier V, Carquin J, Burde A, Flament JB, Choisy C (1996). In vivo and in vitro analysis of the ability of urinary catheters to microbial colonization. Pathol. Biol. 44:397-404.

Caiazza NC, O' Toole GA (2012). Sad B is required for the transition from reversible to irreversible attachment during biofilm formation by Pseudomonas aeruginosa PA1. J. Bacteriol. 189:4476-4485.

Carson L, Gorman SP, Gilmore BF (2010). The use of lytic bacteriophages in the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli*. FEMS Immunol. Med. Microbiol. 59:447-455.

Cegelski L, Pinkner JS, Hammer ND (2009). Small- molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. Nat. chem. Biol. 51: 913-919.

Choong S, Wood S, Fry C, Whitfield H (2001). Catheter associated urinary tract infection and encrustation. Int. J. Antimicrob. Agents. 17:305-310.

Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF,Melton DM, Beachey EH. Adherence of coagulase negative Staphylococci to plastic tissue culture a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. 22:996-1006

Costerton JW, Cheng KJ, Gessey GG (1987). Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41:435-464.

Costerton JW, Ellis B, Lam K, Johnson F, Khoury AE (1994). Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. Antimicrob. Agents Chemother. 38:2803-2809.
Costerton JW, Montanaro L, Arciola CR (2007). Bacterial communications in implant infections: a target for an intelligence war. Int. J. Artif. Organs 30(9):757-763.
Curtin JJ, Donlan RM (2006). Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. 50:1268-1275.
Danese PN, Pratt LA, Kolter R (2000). Exopolysaccharide production is required for development of *Escherichia coli*. K-12 biofilm architecture. J. Bacteriol. 182:3593-3596.
Davenport K, Keeley FX (2005). Evidence for the use of silver-alloy-coated urethral catheters. J. Hosp. Inf. 60:298-303.
Donlan RM (2001). Biofilms and device-associated infections. Emerg Infect Dis. 7:277.
Donlan RM (2002). Biofilms: microbial life on Surfaces. Emerg. Infect. Dis. 9:88-890.
Elliott TS, Moss HA, Tebbs SE, Wilson IC, Bonser RS, Graham TR (1992). Novel approach to investigate a source of microbial contamination of central venous catheters. Eur. J. Clin. Microbiol. Infect. Dis. 15:13-20
Ethers LJ, Bouwer JE (1999). RP4 Plasmid transfer among species of Pseudomonas in a biofilm reacter. Water Sci. Technol. 7:163-171.
Fey PD (2010). Modality of bacterial growth presents unique targets: how do we treat biofilm-mediated infections? Curr. Opin. Microbiol. 13:610-615.
Fisher LE, Hook AL, Ashraf W, Yousef A, Barrett DA, Scurr DJ, Chen X, Smith EF, Fay M, Parmenter CDJ, *et al* (2015). Biomaterial modification of urinary catheters with antimicrobials to give long-term broadspectrum antibiofilm activity. J. Control Release 202:57-64.
Foo LY, Lu Y, Howell AB, Vorsa N (2000). A-type proantho-cyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated Escherichia coli. J Natural Prod. 63:1225-1228.
Foo LY, Lu Y, Howell AB, Vorsa N (2000). The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli in vitro*. Phyto-chemistry 54:173-181.
Foxman B (2002). Epidemiology of urinary tract infection: Incidence morbidity and economic costs. Am. J. Med. 113:5S-13S.
Freeman DJ, Falkiner FR, Keane CT (1989). A new method for the detection of the slime production by the coagulase negative *Staphylococci*. J. Clin. Pathol. 42: 872-874.
Forster T, Fu W, Mayer O, Curtin J, Lehman SM, Donlan RM (2010). Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an *in vitro* model system. Antimicrob. Agents Chemother. 54:397-404.
Lohr G, Beikler T, Podbielski A, Standar K, Redanz S, Hensel A (2011). Polyphenols from *Myrothamnus flabellifolia* Welw. Inhibit in vitro adhesion of *Porphyromonas gingivalis* and exert anti-inflammatory cytoprotective effects in KB cells. J. Clin. Periodontol. 38:457-469.
Garibaldi RA, Miller WA, Hebertson RM, Burke JP (1977). Antmicrobial prophylaxis for catheter-associated bacteriuria. Antmicrob. Agents Chemother. 11:240-243.
Ghanwate NA (2012). Biofilm eradication studies on Uropathogenic *E.coli* using Ciprofloxacin and Nitofurantion. Int. J. Pharm. Biomed. Res. pp. 127-131.
Ghanwate NA, Thakare PV, Bhise PR, Tayde S (2014). Prevention of Biofilm formation in Urinary catheters by treatment with Antibiofilm agent. Int. J. Sci. Res. 3:714-717.
Giwercman B, Jensen E T, Hoiby N, Kharazmi A, Costerton W (1991). Induction of beta-lactamase production in *Pseudomonas aeruginosa* biofilm. Antimicrob. Agents Chemother. 35:1008-1010.
Gorman SP, Jones DS (2003). Complications of urinary devices, in: Wilson, M Ed. Medical Implications of Biofilms. Cambridge University Press, Cambridge. UK. pp. 136-170.
Grinholc M, Richter M, Nakonieczna J, Fila G, Bielawski KP (2011). The connection between grand SCC mec elements of *Staphylococcus aureus* strains and their response to photodynamic inactivation. Photomed. Laser Surg. 29:413-419.
Grinholc M, Szramka B, Kurlenda J, Graczyk A, Bielawski KP (2008). Bactericidal effect of photodynamic inactivation against methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* is strain-dependent. J. Photochem. Photobiol. 90:57-63.
Grinholc M, Zawacka-Pankau J, Gwizdek-Wiśniewska A, Bielawski KP (2010). Evaluation of the role of the pharmacological inhibition of *Staphylococcus aureus* multidrug resistant pumps and the variable levels of the uptake of the sensitizer in the strain-dependent response of *Staphylococcus aureus* to PPArg2-based photo dynamic inactivation. Photochem. Photobiol. 86:1118-1126.
Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, *et al* (2009). Contribution of autolysin and Sortase A during Enterococcus faecalis DNA-dependent biofilm development. Infect. Immun. 77:3626-38.
Hamblin MR, Hasan T (2004). Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem. Photo boil. Sci. 3:436-450.
Hensel Z, Xiao J (2009). A mechanism for stochastic decision making by bacteria. Chem. BioChem. 10: 974-976.
Hinsa SM, Espinosaurgel E, Ramos JL, O' Toole GA (2003). Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescenes* WCS365 requires an ABC transporter and a large secreted protein. Mol. Micobiol. 49:905-918.
Holland SP, Mathias RG, Morck DW, Chiu J, Slade SG (2000). Diffuse lamellar keratitides related to endotoxins released from sterilize reservoir biofilm. Opthalmology 107:1227-1234.
Hong SH, Wang X, Wood TK (2010). Controlling biofilm formation, prophage excision and cell death by rewiring global regulator H-NS of *Escherichia coli*. Microb. Biotechnol. 3:344-356.
Hooton TM, Bradley SF, Cardenas DD, Colgan R, Geerlings SE, Rice JC, Saint S, Schaeer AJ, Tambayh PA, Tenke P, Nicolle LE (2010). Diagnosis, Prevention, and Treatment of Catheter Associated Urinary Tract Infection in Adults: 2009 International Clinical Practice Guidelines from the Infectious Diseases Society of America. Clin. Infect. Dis. 50(5):625-63.
Hull RA, Donovan WH, Del Terzo M, Stewart C, Rogers M, Darouiche RO (2002). Role of type 1 fimbria- and P fimbria-specific adherence in colonization of the neurogenic human bladder by *Escherichia coli*. Infect. Immun. 70:6481-6484.
Imamura Y, Higashiyama Y, Tomono K, Izumikawa K, Yanagihara K, Ohno H, Miyazaki Y, Hirakata Y, Mizuta Y, J Kadota*, et al* (2005). Azithromycin exhibits bactericidal effects on Pseudomonas aeruginosa through interaction with the outer membrane. Antimicrob. Agents Chemother. 49:1377-1380.
Jacobsen SM, Stickler DJ, Mobley HL, Shirtli ME (2008). Complicated catheter-associated urinary tract infections due to Escherichia coli and Proteus mirabilis. Clin Microbiol. Rev. 21:26-59.
Jepson RG, Mihaljevic L, Craig J (2001). Cranberries for preventing urinary tract infections. Cochrane Database of Systematic Reviews. 3: Article ID CD001321.
Kaplan JB (2010). Biofilm dispersal mechanisms, clinical implications, and potential therapeutic uses. J. Dental Res. 89:205-218.
Karatan E, Watnik P (2009). Signals, regulatory networks and materials that build and break bacterial biofilm. Microbiol. Mol. Biol. Rev. 73:310-347.
Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004). Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. 230:13-18.
Keren I, Shah D, Spoering A, Kaldalu N, Lewis K (2004). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J. Bacteriol. 186:8172-8180.
Kharidia R, Liang J (2011). The activity of a small lytic peptide PTP-7 on *Staphylococcus aureus* biofilms. J. Microbiol. 49:663-668.
Lappinscott HM, Bass C (2001). Biofilm formation: attachment, growth, and detachment of microbes from surfaces. Am. J. Infect. Control 29:250-251.
Laue H, Schenk A, Li H (2006). Contribution of alginate and levan production to biofilm formation by *Pseudomonas syrinage*. Microbiology 152(10):2909-2918.
Lellouche J, Friedman A, Gedanken A, Banin E (2012). Antibacterial and Antibiofilm properties of yttrium fluoride nanoparticles. Int. J. Nanomed. 7:5611-5624.
Lellouche J, Kahana E, Elias S, Gedanken A, Banin E (2009). Antibiofilm activity of nanosized magnesium fluoride. Biomaterials 30:5969-5978.
Lemon KP, Higgins DE, Kolter (2007). Flagellar motility is critical for

Listeria monocytogens biofilm formation. J. Bacteriol. 12:4418-4424.
Lewis K (2001). Riddle of biofilm resistance. Antimicrobial Agents and Chemother 2001. 45:999-1007.
Lewis K (2005). Persister cells and the riddle of biofilm survival. Biochemistry 70:267-274.
Lewis K (2008). Multidrug tolerance of biofilms and persister cells. Curr. Top. Microbiol. Immun. 322:107-131.
Lu TK, Collins JJ (2007). Dispersing biofilms with engineered enzymatic bacteriophage. Proc. Natl Acad. Sci. United States Am. 104:11197-1202.
Mack D, Becker P, Chatterjee I, Dobinsky S, Knobloch JK, Peters G, Rohde H, Herrmann M. (2004). Mechanisms of biofilm formation in *Staphylococcus epidermidis* and *Staphylococcus aureus*: functional molecules, regulatory circuits, and adaptive responses. Int. J. Med. Microbiol. 294:203-212.
Maria K, Maria H, Scott J (2014). Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Postantibiotic Era. Cold Spring Harbor Laboratory Press.
Mathur S, Suller MT, Stickler DJ, Feneley RC (2006). Prospective study of individuals with long-term urinary catheters colonized with Proteus species. BJU Int. 97:121-128.
Meluleni GJ, Grout M, Evans DJ, Pier GB (1995). Mucoid *Pseudomonas aeruginosa* growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharides capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients. J. Immunol. 155:2029-2038.
Morgan SD, Rigby D, Stickler DJ (2009). A study of the structure of the crystalline bacterial biofilms that can encrust and block silver Foley catheters. Urological Research. 37:89-93.
Nakonieczna J , Michta E, Rybicka M, Grinholc M, Gwizdek-Wisniewska A, Bielawski KP (2010). Superoxide dis-mutase is upregulated in *Staphylococcus aureus* following protoporphyrin mediated photodynamic inactivation and does not directly influence the response to photodynamic treatment. BMC Microbiol. 10(1):323.
Nickel JC, Ruseska I, Wright JB, Costerton JW (1985). Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. 27:619-624.
Niveditha S, Pramodhini S, Umadevi S, Shailesh Kumar, Selvaraj Stephen (2012). The isolation and biofilm formation of Uropathogens in the patients with catheter Associated urinary tract infections (UTIs). J. Clin. Diagn. Res. 6:1478-1482.
Ong CY, Ulett GC, Mabbett AN *et al* (*2008*). Identification of type 3 fimbriae in uropathogenic *Escherichia coli* reveals a role in biofilm formation. J. Bacteriol. 190: 1054-1063.
Otto G, Magnusson M, Svensson M, Braconier J, Svan-borg C (2001). Pap genotype and P fimbrial expression in *Escherichia coli* causing bacteremic and nonbacteremic febrile urinary tract infection. Clin. Infect. Dis. 32:1523-1531.
Petrova OE, Sauer K (2010). The novel two- component regulatory system BfiSR regulates biofilm development by controlling the small RNA rsm Z through CafA. J. Bacteriol. 192:5275-5288.
Pramodini S (2012). Antibiotic resistance pattern of biofilm-forming uropathogenic isolated from catheterized patients in Pondicherry. India Australas. med.J. 5:344-348.
Qin Z, Yang L, Qu D, Molin S, Tolker-Nielsen T (2009). *Pseudomonas aeruginosa* extracellular products inhibit staphylococcal growth, and disrupt established biofilms produced by *Staphylococcus epidermidis.* Microbiology. 155:2148-2156.
Raad II, Sabbagh MF, Rand KH, Sherertz RJ (1992). Quantitative tip culture methods and the diagnosis of central venous catheters related infections. Diagn. Microbiol. Infect. Dis. 15:13-20.
Ramzan M, Bakhsh S, Salam A, Khan GM, Mustafa G (2004). Risk factors in urinary tract infection. Gomal J. Med. Sci. 2:1-4.
Reid G, Penstedt JD, Kang YS, Lam D, Nause C (1992). Microbial adhesion and biofilm formation on ureteral stents in vitro and in vivo. J. Urol. 148:1592-1594.
Rendueles O, Kaplan J, Ghigo J (2013). Antibiofilm polysaccharides. Environ. Microbiol. 15:334-346.
Rowe MC, Withers HL, Wift S (1998). Uropathogenic *Escherichia coli* forms biofilm aggregates under iron restriction that disperses upon the supply of iron. FEMS Microbiol. Lett. 307:102-109.
Roy A, Gauri SS, Bhattacharya M, Bhattacharya J (2013). Antimicrobial activity of CaO nanoparticles. J. Biomed. Nanotechnol. 9:1570-1578.
Rupp ME, Fitzgerald T, Marion N, Helget V, Puumala S, Anderson JR, Fey PD (2004). Effect of silver-coated urinary catheters: efficacy, cost-effectiveness, and antimicrobial resistance. Am. J. Infect. Control. 32:445-450.
Sanchez Jr CJ, Mende K, Beckius ML Akers KS, Romano DR, Wenke JC, Murray CK (2013). Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infect. Dis. 13:47.
Sano M, Hirose T, Nishimura M, Takahashi S, Mat-sukawa M, Tsukamoto T (1999). Inhibitory action of clarithromycin on glycocalyx produced by MRSA. J. Infect. Chemother. 5:10-15.
Schmidt T, Kirsching A (2012). Total synthesis of carolacton, a highly potent biofilm inhibitor. Angew Chem. Int. Ed. 51:1063-1066.
Sepandj F, Ceri H, Gibb Read, Olson M (2003). Minimum inhibitory concentration versus minimum biofilm eliminating concentration in evaluation of antibiotic sensitivity of Gram negative bacilli causing perit. Dialysis Int. 24:65-67.
Shanks R, Sargent J, Martinez R, Graber M, O'Toole G (2006). Catheter lock solutions influence staphylococcal biofilm formation on abiotic surfaces. Nephrol. Dial. Transpl. 21:2247-2255.
Siddiq DM, Darouiche RO (2012). New strategies to prevent catheter-associated urinary tract infections. Nat. Rev. Urol. 9:305-314.
Stickler D, Morris N, Moreno M, Sabbuba N (1998). Studies on the formation of crystalline bacterial biofilms on urethral catheters. Euro. J. Clin. Microbiol. Infect. Dis. 17:649-652.
Stickler DJ, Zimako J (1994). Complications of urinary tract infections associated with devices used for long-term bladder management. J. Hosp. Infect. 28:177-94.
Stickler DJ, Morris NS, McLean RJ, Fuqua C (1998). Biofilms on indwelling urethral catheters produce quorum-sensing signal molecules in situ and in vitro. Appl. Environ. Microbiol. 64:3486-90.
Stickler DJ (2014). Clinical complications of urinary catheters caused by crystalline biofilms: something needs to be done. J Int. Med. 276:120-129.
Stowe SD, Richards JJ, Tucker AT, Thompson R, Melander C, Cavanagh J (2011). Anti-biofilm compounds derived from marine sponges. Marine Drugs 9:2010-2035.
Suman E, Jose J, Varghese S, Kotian MS (2005). Study of biofilm production in *Escherichia coli* causing urinary tract infection. Indian J. Med. Microbiol. 25:1-3.
Thomas D, Day F (2007). Biofilm formation by plant associated bacteria. Ann. Rev. Microbiol. 61:401-422.
Tool AG Kolter R (1998). Initiation of biofilm formation in *Pseudomonas fluorescence* WC5365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. 28:449.
Toutain CM, Caizza NC, Zegans ME, Toole GA (2007). Roles for flagellar stators in biofilm formation by *Pseudomonas aeruginosa*. Res. Microb. 158:471-477.
Trautner BW, Darouiche RO (2004). Role of biofilm in catheter-associated urinary tract infection. Am. J. Infect. Control. 32:177-183.
Trautner BW, Hull RA, Darouiche RO (2003). *Escherichia coli* 83972 inhibits catheter adherence by a broad spectrum of Uropathogens. Urology 6:1059-1062.
Trautner RO, Darouiche RA, Hull S, Hull JI, Thornby (2002). Pre-inoculation of urinary catheters with *Escherichia coli* 83972 inhibits catheter colonization by *Enterococcus fecalis*. J. Urol. 167:375-379.
Ulett GC, Mabbett AN, Fung KC, Webb RI Schembri MA (2007). The role of F9 fimbriae of Uropathogenic *Escherichia coli* in Biofilm formation. Microbiology 153: 2321-2331.
Van Acker H, Van Dijck P, Coenye T (2014). Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. Trends Microbiol. 22:326-333.
Warren JW (2001). Catheter associated urinary tract infection. Int. J. Antimicrob. Agent. 17:299-303.

academicJournals

Vol. 10(34), pp. 1380-1387, 14 September, 2016
DOI: 10.5897/AJMR2016.8248
Article Number: A92F1C860506
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Characterization of antifungal *Paenibacillus illinoisensis* strain UKCH21 and its chitinolytic properties

Subbanna A. R. N. S.*, Khan M. S. and Shivashankara H.

Department of Entomology, College of Agriculture, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar-263145, Uttarakhand, India.

Received 3 August 2016; Accepted 24 August, 2016.

***Paenibacillus* strain, UKCH21, isolated from Uttarakhand State, north western Indian Himalayas was found to produce high levels of extracellular chitinases. The 16s rRNA gene sequence showed 100% homology with *P. illinoisensis* (Accession No. KR856190) available in the public domain and further phylogenetic study also verified the species identity. The culture supernatants have a maximum chitinase activity of 110.8 U/ml after 3 days of culturing. The isolate showed strong antifungal activity manifested in the form of progressive mycelia degradation in dual culture plates. The pathogenicity was observed as structural deformities like uneven thickening of mycelia as a result of direct degradation of chitin. The optimum pH and temperature of UKCH21 chitinases was found to be 5 and 50°C, respectively. Partial characterization of chitinase gene also confirms the family 18 status of glycosyl hydrolase with substantial variability presented here with. Above all, percent inhibition of growth and the rapid degradation of mycelia of tested plant pathogenic fungi (*Rhizoctonia solani*, *Fusarium solani* and *Sclerotium rolfsi*) in bacteria seeded medium suggest its utilization as potent antifungal biocontrol agent.**

Key words: *Paenibacillus illinoisensis*, UKCH21, chitinase, antifungal, Indian Himalayas.

INTRODUCTION

Chitin is the second most abundant polysaccharide in nature, after cellulose (Shahidi and Abuzaytoun, 2005). It is the major structural constituent of fungal mycelium providing rigidity and protective in function. Consequently, the growth and multiplication of given fungi is highly dependent on metabolism of chitin. So, any direct damage to this vital cell wall component leads to proportionate reduction in fungal development making it as an ideal target in management of plant pathogenic fungi (PPF). Chitinases (EC 3.2.1.14) are degrading enzymes belonging to family 18 glycosyl hydrolases. They cleave β 1-4 linkages between the structural residues (N-acetyl glucoseamine) of chitin (Hamid et al., 2013). So, these enzymes have ability to directly degrade

*Corresponding author. E-mail: Subbanna.ento@gmail.com. Tel: +915962230060. Fax: +915962231539.

insoluble chitin to soluble reducing sugars. Chitinases play a vital role in chitin metabolism in all the chitin containing organisms. Many studies reported presence of chitinases in non-chitinogenic organisms as well. They include a number of prokaryotic and eukaryotic organisms including higher plants (Sharma et al., 2011). The company of chitinolytic enzymes, despite lack of chitin as a structural component in these organisms, confirms some additional functions which range from nutrition to protection (Hamid et al., 2013). Amongst different chitinolytic organisms, bacteria especially *Bacillus* were found to be well known chitinase producers (Cody et al., 1990). Besides, *Bacillus* species are well known producers of antibiotics, secondary metabolites, enzymes, plant growth promoters, etc that promotes or protects plant development. The established direct toxicity and pathogenicity of chitinases and other beneficial traits associated with *Bacillus* lead to successful worldwide exploration studies of chitinolytic *Bacillus* species with antagonistic potential against a variety of PPF (Drahos and West, 2003; Gohel et al., 2006; Prasanna et al., 2013; Brzezinska et al., 2014) with potential applications in biological control programs. Keeping this in view, a study was conducted to isolate chitinolytic *Bacillus* species with potential antagonistic activity. The study led to identification of a strain designated as UKCH21, whose characterization and molecular identification of chitinases are presented.

MATERIALS AND METHODS

Isolation and identification of bacterial strain

The bacterial isolate under investigation was isolated from soil samples collected from Kalimat village of Almora district in Uttarakhand state, India at an altitude of 1276 amsl and 29° 37' N, 79° 40' E coordinates. One gram of soil sample was mixed with 10 ml of sterile distilled water and a sample of aliquot was evenly spread on to detection agar (CHDA) plate (Kamil et al., 2007), a chemical based medium containing colloidal chitin as sole carbon source. After incubation at 30°C for 3 days, bacterial colony with clear halo around (signify utilization of colloidal chitin) was picked and purified by streaking on the same media. After confirmation of purity by microscopic observation, the strain was designated as UKCH21 and stored at -80°C as 20% glycerol stock (permanent storage) and as Luria agar slant at 4°C for further study.

The bacterial strain, UKCH21 was identified using partial sequence of 16S rRNA gene. The template DNA was extracted and purified by using CTAB solution and phenol chloroform extraction procedures, respectively (Sambrook et al., 1989). The 16S rRNA fragment was amplified in a thermal cycler using previously described *Bacillus* specific primers 16S rRNA (F) '5-CAGGCCTAACACATGCAAGTC-3' and 16S rRNA (R) '5-GGGCGGTGTGTACAAGGC-3' (Yoon et al., 2001). The reaction mixture contained 100 ng of total DNA, 0.5 mM forward and reverse primers, 3 mM $MgCl_2$, 200 nM dNTPs, 5 µl of 1X Tris-HCl buffer and 2.5 U of *Taq* DNA polymerase in a final volume of 50 µl. The reaction was performed at an initial 5 min denaturation step at 94°C followed by 30 cycles of amplification consisting of 1 min denaturation at 94°C, 45 s of annealing at 45°C, 2 min of extension at 72°C, with an extra extension step of 10 min at 72°C. The amplifications were confirmed by investigating 10 µL of PCR product by electrophoresis in a 1% agarose gel. The PCR product was sequenced at Scigenome labs, Cochin, Kerala. The obtained nucleotide sequence was BLASTN searched with the whole GenBank data base and molecular evolutionary analyses were performed using the software MEGA4 (Molecular Evolutionary Genetic Analysis version 4) (Tamura et al*.,* 2007). A phylogenetic tree was constructed using standard 16S rRNA sequences of related species by neighbor-joining method using the distance matrix from the alignment.

Preparation of colloidal chitin

Colloidal chitin was prepared from commercially available chitin flakes (Himedia) according to the procedure described by Berger and Reynolds (1988). Ten grams of chitin flakes were powdered using a mortar and pestle and added slowly to 400 ml of concentrated hydrochloric acid under continuous stirring on a magnetic stirrer at 4°C. After 30 min of stirring, the mixture was incubated at 37°C for 2 h to reduce viscosity. Then, the mixture was filtered through four layer muslin cloth (to avoid any impurities) to which 4 L of ice cold distilled water was added. After thorough mixing, the solution was allowed to stand at 4°C for overnight to allow better precipitation. Colloidal chitin was collected by centrifugation at 10000 rpm for 2 min and washed thoroughly with distilled water until neutral pH was achieved. Thus, obtained colloidal chitin was made into 20% solution, autoclaved and stored at 4°C, until used.

Purification of chitinases

Chitinases from UKCH21 was obtained from cell free culture supernatants of 3 days old culture in half strength nutrient broth supplemented with 1% colloidal chitin. The isolate was inoculated to autoclaved medium (50 ml) and incubated at 30°C and 250 rpm. After three days, the fermented broth supernatants were obtained by centrifugation at 10000 rpm for 20 min followed by 0.2 µ filtration. Then, four volumes of ice cold acetone was added and allowed to stand overnight at -20°C. Thus, precipitated proteins were collected by centrifugation at 10000 rpm for 10 min and dissolved in appropriate quantity of 15 mM Tris-HCl buffer (pH 6.8) after 80% ethanol wash. The protein content of sample was measured by standard Bradford dye binding method (Bradford, 1976) and designated as partially purified chitinases (PPC).

Estimation of chitinase activity

The enzyme activity of PPC was estimated by using natural substrate, colloidal chitin at pH 5 using 50 mM acetate buffer. The reaction mixture consists of equal volumes (250 µL each) of appropriately diluted PPC and buffer containing 1% colloidal chitin. The mixture was incubated at 50°C for 30 min followed by terminating the reaction by boiling in a water bath for 10 min. The remaining colloidal chitin was precipitated by centrifugation at 10000 rpm for 5 min and supernatant was estimated for released reducing sugars by modified Schales reagent (Imoto and Yagishita, 1971). In brief, an aliquot of 450 µL of supernatant was mixed with 600 µL of Schales reagent (0.5 g potassium fericyanide in one litter of 0.5 M sodium carbonate) and boiled for 15 min in a water bath. After cooling, absorbance was measured at 420 nm and the reducing sugar was estimated from a standard curve of N-acetylglucosamine. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar per minute.

Thermal and pH kinetics of chitinases

To study the effect of temperature and pH on chitinase activity, standard enzyme activity assays were performed at different temperatures (ranging from 30 to 80°C at intervals of 10°C) and pH (using 50 mM citrate buffer (pH 3, 4, 5 and 6), 50 mM phosphate buffer (pH 6, 7 and 8) and 50 mM borate buffer (pH 8, 9 and 10) as described. The thermal stability was assessed by incubating enzyme solutions in a microcentrifuge tube at 30 to 60°C at intervals of 10°C without substrate for 2 h followed by estimation of residual enzyme activity. The pH stability was assessed by estimating the residual activity after incubating the enzyme in different pH buffers (as described above) at 4°C overnight. All experiments were performed independently in triplicate and enzyme activities were compared with estimate percentages.

Partial characterization of *chi* gene

The purified DNA was used as template for PCR amplification of chitinase gene fragment using previously described (Williamson et al., 2000) family 18 chitinase specific primers, GA1F (5'-CGTCGACATCGACTGGGARTDBCC-3') and GA1R (5'-ACGCCGGTCCAGCCNCKNCCRTA-3'). The reaction mixture used was similar to 16S rRNA amplification modified by addition of bovine serum albumin at concentration of 3 µg/10 µL of mix. The reaction was also performed with similar conditions at an annealing temperature of 62°C. After confirming the amplification, the PCR product was purified using gel elution columns (Sigma) and sequenced from Scigenome labs, Cochin, Kerala. The obtained sequence was compared with the published sequences of chitinase gene in the GenBank databases by BLASTN nucleotide sequences aligned with the Clustal Omega (1.2.1) multiple sequence alignment (McWilliam et al., 2013). The obtained sequences were submitted to NCBI Gene Bank nucleotide sequence databases.

Estimation of antifungal activity

The antagonistic activity of Chitinolytic *Bacillus* spp. was evaluated against plant pathogenic fungi (PPF), *Sclerotium sclerotiarum, Rhizoctonia solani* and Bean *Fusarium* which were collected from local infested fields and identified according to standard protocols. Initially, the growth inhibition of PPF was evaluated in dual culture plates. A basal agar medium plate containing Luria bertain agar (LBA) and potato dextrose agar (PDA) at their half strengths was used to support the growth of both bacteria and fungi. The experimental setup consists of test bacterium streaked at the middle of plate containing solidified basal agar medium and disc inoculated PPF (0.3 mm) at both the corners of plate in parallel to streak. The dual inoculated plates were incubated at 28±2°C and the controls were devoid of bacteria. After 10 days of incubation, when the growth of PPF in control plates joined in the middle, the treatment plates were observed for growth inhibition. Further, any deformity and/or toxic effects on test mycelia were recorded using fluorescent microscope by observing contact point between test fungi and bacteria.

Further, the antagonistic activity was evaluated in bacteria seeded medium (El-Mougy et al., 2011). In brief, the test bacterium was initially grown in nutrient broth at 30°C and 250 rpm for overnight. The obtained bacterial culture was thoroughly mixed with lukewarm sterile basal medium at a rate of 10% v/v and plated in 9 mm Petri plates. After solidification, 0.5 cm diameter actively growing test fungal disc was placed at the center of plate and allowed to grow at 27±2°C. The diameter of fungal growth was measured when it reached borders in control plates with no bacteria. The *S. rolfsi* plates were further incubated to evaluate the inhibition on sclerotia formation. Triplicate plates were used to evaluate fungal growth inhibition in all the test bacteria.

RESULTS AND DISCUSSION

The present study reports a chitinolytic *Paenibacillus* strain, UKCH21 having antagonistic activity against soil inhabiting plant pathogenic fungi. Initial screening of the isolate for its chitinolytic properties in CHDA plates showed 1 cm halo around the bacterial colony of 0.4 cm after 7 days of incubation. The aerobic growth of bacterial colony was characterized by raised, creamiest white colony with smooth edges and oily appearance (Figure 1). Microscopic observation showed rod shaped vegetative cells with motility and formation of endospore confirming *Bacillus* grouping. Further species identity of UKCH21 was done using 16S rRNA gene sequencing (accession No. KX113474). The BLASTN search of the obtained 841 nucleotide sequences showed representative homology with *Paenibacillus* species with maximum homology of 100% with *P. illinoisensis* strain C1 (accession No. KR856190). The alignment and phylogenetic analysis of 16S rRNA sequences of different *Paenibacillus* species strongly suggested species status of the bacterial strain UKCH21 as *P. illinoisensis* (Figure 2). The high levels of enzyme production depicts potential degradation of chitin, in view of which, the isolate was selected to study its antagonistic potential and further identification of chitinases. Studies reported a number of *Paenibacillus* species with potential applications in biological control of variety of plant pathogenic bacteria (Budi et al., 2000; von der Weid et al., 2003; Lorentz et al., 2006; Fortes et al., 2008), fungi (Naing et al., 2015; Xu et al., 2014; Liu et al., 2008; von der Weid et al., 2003; Jung et al., 2003, 2005, 2006) and even nematodes (Jung et al., 2002). This biocidal activity of *Paenibacillus* species was supported by production of antibiotics, hydrolytic enzyme etc (Chung et al., 2000; Velázquez et al., 2004; Lorentz et al., 2006). Besides they are predominently distributed in soils and rhizosphere (Berge et al., 2002; von der Weid et al., 2002), a dynamic ecosystem. The present study also supports existence of *Paenibacillus* species in soils of Uttarakhand Himalayas as major chitinase producers.

The antifungal activity of *P. illinoisensis* strain UKCH21 in dual culture plates was manifested in the form of direct inhibition of mycelial growth upon contact with bacterial growth. Further, degradation and amputation of fungal mycelium is progressive with time. The microscopic observation of contact point between bacterial growth and test fungi showed uneven thickening of mycelia (Figure 3e) as a result of degradation and or digestion of hyphal chitin leading to loss of integrity. Senthilkumar et al. (2007) have also reported several structural deformities like hyphal lysis and bulging of the mycelium of *R. bataticola* caused by *Paenibacillus* sp. HKA-15. The bacteria seeded medium showed cent percent growth

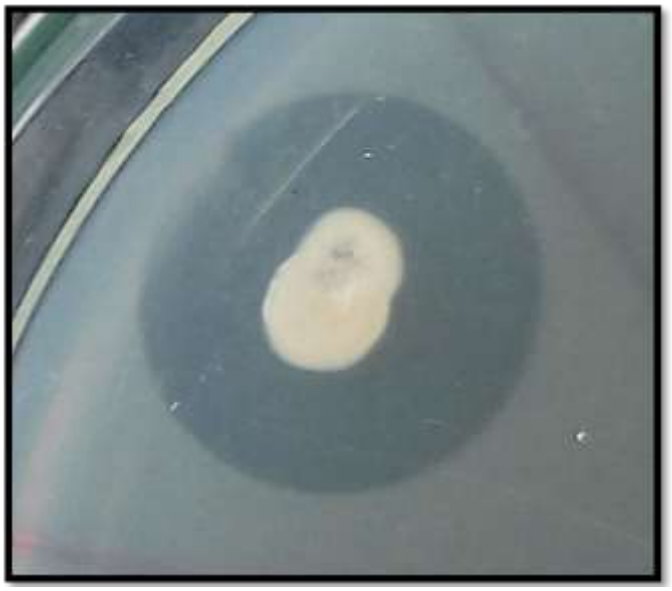


Figure 1. Chitinolytic activity of *P. illinoisensis* strain UKCH21 in CHDA plates.

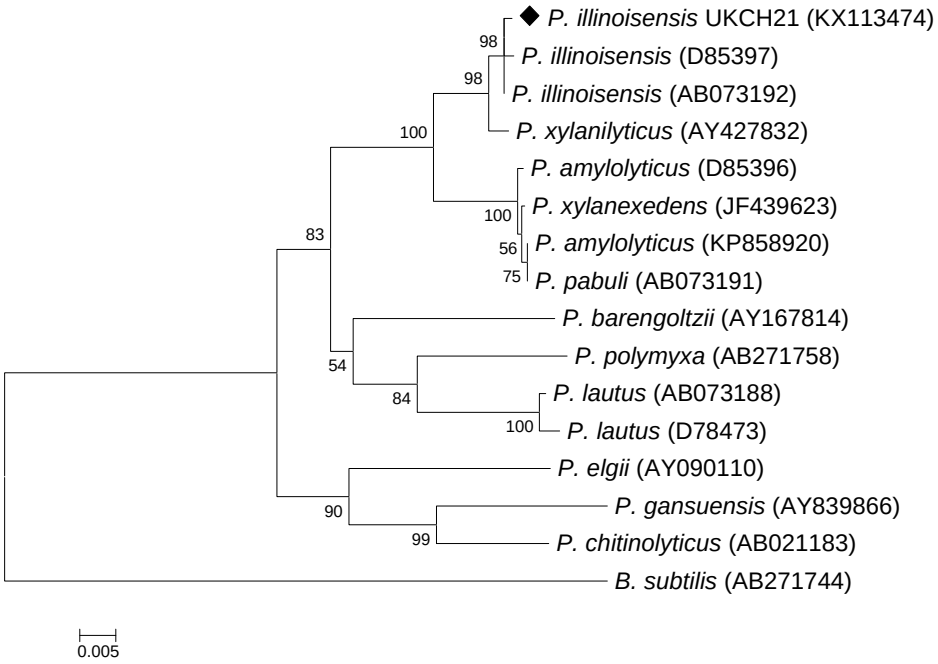


Figure 2. Phylogenetic tree constructed using 16S rRNA gene fragments by neighbor-joining method, indicating the position of the isolates UKCH21. The numbers at the nodes indicate the percentage bootstrap values for each node based on 1,000 bootstrap replicates. The tree was rooted with the 16S rRNA gene of *Bacillus subtilis* as an outgroup.

inhibition from the inoculated disc of test fungi (Figure 3). This shows that the presence of UKCH21 on given growth media completely inhibited development of tested fungi either by chitinase production or by any other secondary metabolites that act synergistically. Despite the number of *Paenibacillus* species, in particular, only minimal studies reported antagonistic potential of *P. illinoisensis*. For example *P. illinoisensis* KJA-424 against

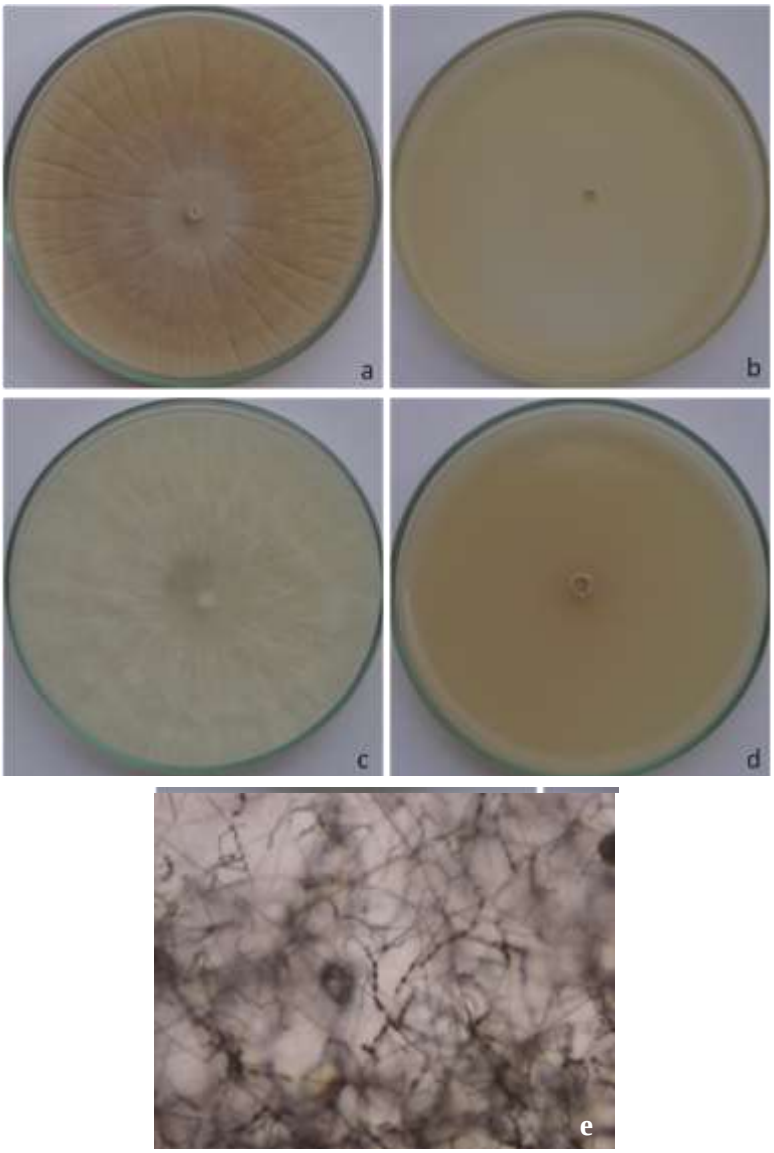


Figure 3. Antifungal activity of *P. illinoisensis* strain UKCH21 against *R. solani* and *S. rolfsi* in bacteria seeded medium. a. Control plate of *R. solani;* b. Growth inhibition of *R. solani;* c. Control plate of *S. rolfsi;* d. Growth inhibition of *S. rofsi;* e. Uneven thickening of *S. rolfsi* mycelia.

Phytophthora capsici (Jung et al., 2005; Jung et al., 2006), *Rhizoctonia solani* (Jung et al., 2003) and *Meloidogyne incognita* (Jung et al., 2002).

Partial sequencing of chitinase gene from the isolate UKCH21 resulted in 373 bp nucleotide sequence (accession no. KX446923). The nucleotide BLASTN search of obtained sequence showed maximum homology of 79% with *chiA* gene of *Paenibacillus* sp. FPU7. The sequence alignment of deduced amino acid sequence with full gene sequences from *S. marcescens* (BAA31567.1), *B. cereus* (EEK85987.1), *P. barengoltzii* (WP016312329), *P. macerans* (KFM93118.1) and *B. circulans* (AAA81528.1) showed substantial variation in amino acid composition between the species (Figure 4). Out of 121 amino acid residues, 26 amino acid were conservations (denoted by *). Especially, UKCH21 showed 17 unconserved substitutions among the tested *Paenibacillus* accessions (*P. barengoltzii* and *P. macerans*) indicating the novel characteristics of *P. illinoisensis* chitinases. However, all the sequences showed conserved characteristics of family 18 glycosyl hydrolases.

The apparent activity of any given chitinases is a function of existing temperature and pH. Studies also reported specific featured chitinases with respect to pH (Loni et al., 2014; Fu et al., 2016) and temperature

```
Sma       DKVKRDRFVGSVKEFLQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAM   345
Bce       DEKTRKVFAESTVDFLREYG-FDGVDLDWEYPGVETIPGGGSYRPEDKQNFTLLLQDVRNA   253
Pba       SQVTRETFANSAVDFLRKFN-FDGIDLDWEYPVAGGLPGNSYRPEDKQNYTKLLQEIRNK   233
Pma       DPALRENFANSAVDFLRKYQ-FDGVDLDWEYPVSGGLQGNSRRAEDKQNFTLLLQKTREK   232
Bci       TAATREVFANSAVDFLRKYN-FDGVDLDWEYPVSGGLDGNSKRPEDKQNYTLLLSKIREK   234
UKCH21    ------------------------------------------------YTLLLQKIREK   11
                                                             :. *:.. *

Sma       LDQLSVETGRKYELTSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQT   405
Bce       LNKAGAEDGKQYLLTIASGASQRYADHTELKKISQILDWINIMTYDFHGGWEATSNHNAA   313
Pba       LDSAGAVDGKRYLLTIASGASPSFVSNTELGNIASIVDWINIMTYDFNGGWQQITAHNAP   293
Pma       LDAAGAKDGKRYLLTIASGASPAFAQNTELDKISDIVDWINIMTYDFNGGWQNITAHNAP   292
Bci       LDAAGAVDGKKYLLTIASGASATYAANTELAKIAAIVDWINIMTYDFNGAWQKISAHNAP   294
UKCH21    LDAAGTADNKKYFLTIASGAGPTYAANTELGNMAKYLDWINIMTYDFNGGWQTVSAHNAP   71
          *:  ..   ::* ** * .*.     ..        :* * :*:*** *.::  .  .

Sma       ALNAP-----AWKPDTAYTTVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPF   460
Bce       LYKDPNDPAA----DTKFYVDGAIDIYTNEGVPADKLVLGVPFYGRGWKSCGKENNG-QY   368
Pba       LYYDPAAGNAGVPSASVFNADAAVQGHLNAGVPANKLVLGIPFYGRGWDGVNNAGNG-QY   352
Pma       LYLDPAAVAAGVPDSESFYVEAGVRGHLNAGVPAGKLVLGVPFYGRGWTGAAAAGNG-QY   351
Bci       LNYDPAASAAGVPDANTFNVAAGAQGHLDAGVPAAKLVLGVPFYGRGWDGCAQAGNG-QY   353
UKCH21    LYTDPAAIAAGVPNADTFNVEKGVQGHINAGVPASKIVLGLAFYGRGWTG----------   121
             *          : .  .      **   *:*:*  :***** .
```

Figure 4. Alignment of peptide sequence of chitinase from UKCH21 with *S. marcescens* (Sma, BAA31567.1), *B. cereus* (Bce, EEK85987.1), *P. barengoltzii* (Pba, WP016312329), *P. macerans* (Pma, KFM93118.1) and *B. circulans* (Bci, AAA81528.1). Conserved amino acids are with asterisk. Dashes indicate gaps left to improve alignment. All sequences are numbered from the Met-1 peptide.

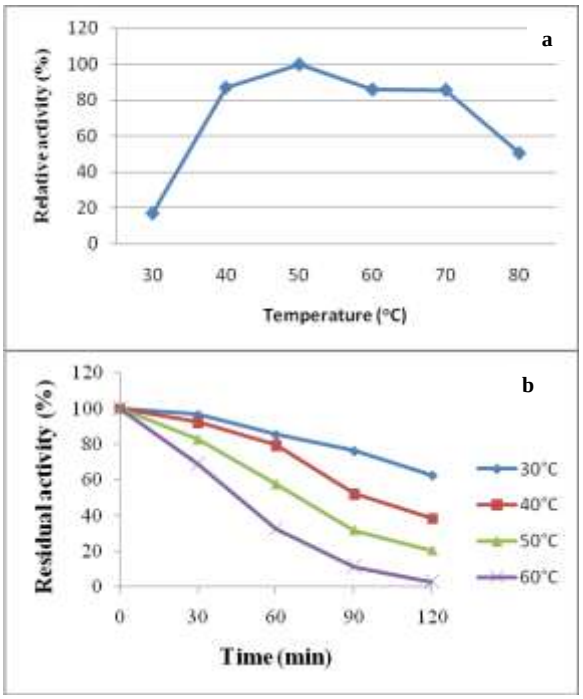


Figure 5. Effect of temperature on activity (a) and stability (b) of chitinases produced by UKCH21.

(Karthik et al., 2015) having potential in industrial applications. Besides, identification of optimum pH and temperature of any given bacterial chitinase is important to predict its activity in different environmental conditions. The culture supernatants from *P. illinoisensis* strain UKCH21 showed a maximum enzyme activity of 110.8 U/ml after 3 days of culturing with protein content of 0.69 mg/ml. Further testing of PPC from UKCH21 showed substantial enzyme activity (>80%) over a temperature range of 40 to 80°C with its peak activity at 50°C (Figure 5a). Further increase or decrease in temperature resulted in reduction in enzyme activity. However, the enzyme lacks thermal stability as revealed by the loss of more than 30% of its activity within two hours at around room temperature (30°C). At 60°C, the enzyme lost cent percent activity within 2 h of incubation is shown in Figure 5b. The enzyme showed optimum activity at pH 5 (Figure 6a). Further increase in pH showed 32% loss in activity. Interestingly, the enzyme upholds its activity between pH 6 and 9. The stability analysis showed increased enzyme activity up to pH 6 (Figure 6b).

In conclusion, the unique ecological niche presented by mountain and hill regions with associated environmental and biotic factors supports vast diversity of beneficial bacterial community with potential commercial applications.

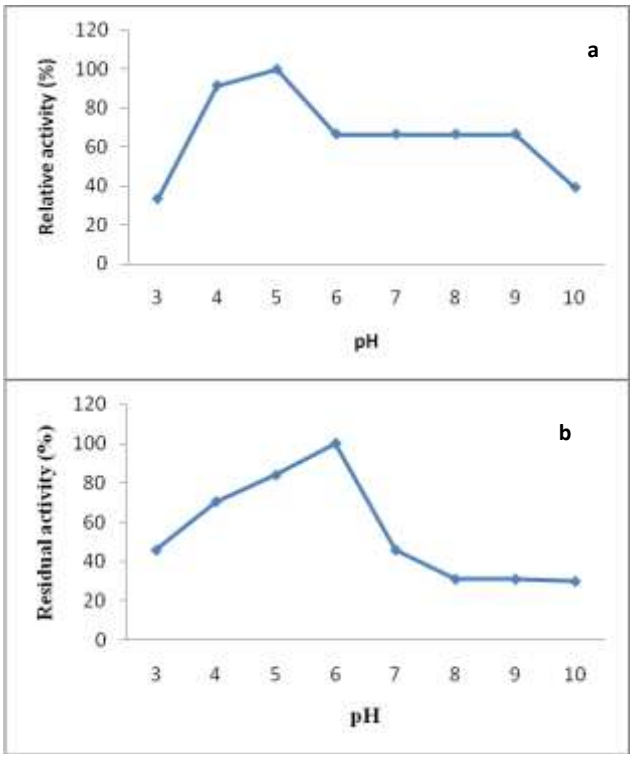


Figure 6. Effect of pH on activity (a) and stability (b) of chitnases produced by UKCH21.

To the best of the authors' knowledge, for the first time, the present study identified a strong antifungal *P. illinoisensis* strain UKCH21 from India with an extracellular chitinase production, that is, having potential applications in biological control of tested soil born plant pathogens. Keeping this in view, further studies were planned to find out the biocontrol potential of UKCH21 against other fungi and under filed conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by the Indian Council of Agricultural Research (ICAR) and College of Agriculture, GBPUA&T, Pantnagar. The present work is a part of Ph.D thesis submitted to GBPUA&T, Pantnagar.

REFERENCES

Berge O, Guinebretiere MH, Achouak W, Normand P, Heulin T (2002). *Paenibacillus graminis* sp. nov. and *Paenibacillus odorifer* sp. nov., isolated from plant roots, soil and food. Int. J. Syst. Evol. Microbiol. 52:607-616.

Berger LR, Reynolds DM (1988). Colloidal chitin preparation. Methods in Enzymol. 161:430.

Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 74:248-254.

Brzezinska MS, Jankiewicz U, Burkowska A, Walczak M (2014).Chitinolytic microorganisms and their possible application in environmental protection. Curr. Microbiol. 68(1):71-81.

Budi SW, van Tuinen D, Arnould C, Dumas-Gaudot E, Gianinazzi-Pearson V, Gianinazzi S (2000). Hydrolytic enzyme activity of *Paenibacillus* sp. strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-borne pathogenic fungi. Appl. Soil Ecol. 15(2):191-199.

Chung YR, Kim CH, Hwang I, Chun J (2000). *Paenibacillus koreensis* sp. nov., a new species that produces an iturin-like antifungal compound. Int. J. Syst. Evol. Microbiol. 50(4):1495-1500.

Cody RM, Davis ND, Lin J, Shaw D (1990). Screening microorganisms for chitin hydrolysis and production of ethanol from amino sugars. Biomass. 21(4):285-295.

Drahos DJ, West L (2003). *Bacillus licheniformis* biofungicide. United States patent US 6:569-425.

El-Mougy NS, Abdel-Kader MM, Alhabeb RS (2011). *In vitro* antifungal activity of chitinolytic enzymes produced by bio-agents against root rot pathogenic fungi. Arch. Phytopathol. Plant Protec. 44:613-622.

Fortes TO, Alviano DS, Tupinambá G (2008). Production of an antimicrobial substance against *Cryptococcus neoformans* by *Paenibacillus brasilensis* Sa3 isolated from the rhizosphere of *Kalanchoe brasoloensis*. Microbiol. Res. 163:200-207.

Fu X, Yan Q, Wang J, Yang S, Jiang Z (2016). Purification and biochemical characterization of novel acidic chitinase from *Paenicibacillus barengoltzii*. Int. J. Biological Macromol. 91:973-979.

Gohel V, Singh A, Vimal M, Ashwini P, Chhatpar HS (2006). Bioprospecting and antifungal potential of chitinolytic microorganisms. Afr. J. Biotechnol. 5:54-72.

Hamid R, Khan, MA, Ahmad M, Ahmad MM, Abdin MZ, Musarrat J, Javed S (2013). Chitinases: An update. J. Pharm. Bioall. Sci. 5: 21–29.

Imoto T, Yagishita K (1971). A simple activity measurement of lysozyme. Agric. Biol. Chem. 35:1154-1156.

Jung SJ, An KN, Jin YL, Park RD, Kim KY, Shon BK, Kim TH (2002). Effect of chitinase-producing *Paenibacillus illinoisensis* KJA-424 on egg hatching of root-knot nematode (*Meloidogyne incognita*). J. Microbiol. biotechnol. 12(6):865-871.

Jung WJ, An KN, Jin YL, Park RD, Lim KT, Kim KY, Kim TH (2003). Biological control of damping-off caused by *Rhizoctonia solani* using chitinase-producing *Paenibacillus illinoisensis* KJA-424. Soil Biol. Biochem. 35(9):1261-1264.

Jung WJ, Jin YL, Kim KY, Park RD, Kim TH (2005). Changes in pathogenesis-related proteins in pepper plants with regard to biological control of phytophthora blight with *Paenibacillus illinoisensis*. BioControl. 50(1):165-178.

Jung WJ, Jin YL, Park RD, Kim KY, Lim KT, Kim TH (2006). Treatment of *Paenibacillus illinoisensis* suppresses the activities of antioxidative enzymes in pepper roots caused by *Phytophthora capsici* infection. World J. Microbiol. Biotechnol. 22(9):901-907.

Kamil Z, Rizk M, Saleh M, Moustafa S (2007). Isolation and identification of rhizobacteria and their potential in antifungal biocontrol. Global. J. Mol. Sci. 2:57-66.

Karthik N, Binod P, Pandev A (2015). Purification and characterization of an acidic and antifungal chitinase produced by a *Streptomyces* sp. Bioresour. Technol.188:195-201.

Liu WW, Mu W, Zhu BY, Du YC, Liu F (2008). Antagonistic activities of volatiles from four strains of *Bacillus* spp. and *Paenibacillus* spp. against soil-borne plant pathogens. Agric. Sci. China 7:1104-1114.

Loni PP, Patil JU, Phugare SS, Bajekal SS (2014). Purification and characterization of alkaline chitinase from *Paenibacillus pasadenensis* NCIM 5434. J. Basic Microbiol. 54(10):1080-1089.

Lorentz RH, Artico S, Da Silveira AB, Einsfeld A, Corcao G (2006). Evaluation of antimicrobial activity in Paenibacillus spp. strains isolated from natural environment. Lett. Appl. Microbiol. 43(5):541-547.

McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, Cowley

AP, Lopez R (2013). Analysis Tool Web Services from the EMBL-EBI. Nucleic acids res. 41:W597-600.

Naing KW, Nguyen XH, Anees M, Lee YS, Kim YC, Kim SJ, Kim MH, Kim YH, Kim KY (2015). Biocontrol of Fusarium wilt disease in tomato by *Paenibacillus ehimensis* KWN38. World J. Microbiol. Biotechnol. 31(1):165-174.

Prasanna L, Eijsink VG, Meadow R, Gåseidnes S (2013). A novel strain of *Brevibacillus laterosporus* produces chitinases that contribute to its biocontrol potential. Appl. Microbiol. Biotechnol. 97(4):1601-1611.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Senthilkumar M, Govindasamy V, Annapurna K (2007). Role of antibiosis in suppression of charcoal rot disease by soybean endophyte *Paenibacillus* sp. HKA-15. Curr. Microbiol. 55:25-29.

Shahidi F, Abuzaytoun R (2005). Chitin, chitosan, and co-products: Chemistry, production, applications, and health effects. Adv. Food Nutr. Res. 49:93-135.

Sharma N, Sharma KP, Gaur RK, Gupta VK (2011). Role of chitinase in plant defense. Asian J. Biochem. 6:29-37.

Tamura K, Dudley J, Nei M, Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Velázquez E, De Miguel T, Poza M, Rivas R, Rosselló-Mora R, Villa TG (2004). *Paenibacillus favisporus* sp. nov., a xylanolytic bacterium isolated from cow faeces. Int. J. Syst. Evol. Microbiol. 54(1):59-64.

von der Weid I, Alviano DS, Santos ALS, Soares RMA, Alviano CS, Seldin L (2003). Antimicrobial activity of *Paenibacillus peoriae* strains NRRL BD-62 against a broad spectrum of phytopathogenic bacteria and fungi. J. Appl. Microbiol. 95:1143-1151.

von der Weid I, Duarte GF, van Elsas JD, Seldin L (2002). *Paenibacillus brasilensis* sp. nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil. Int. J. Syst. Evol. Microbiol. 52:2147-2153.

Williamson N, Brian P, Wellington EMH (2000). Molecular detection of bacterial and streptomycete chitinases in the environment. Antonie Van Leeuwenhoek. 78(3-4):315-321.

Xu SJ, Hong SJ, Choi W, Kim BS (2014). Antifungal activity of *Paenibacillus kribbensis* strain T-9 isolated from soils against several plant pathogenic fungi. Plant Pathol. J. 30(1):102-108.

Yoon JH, Weiss N, Lee KC, Lee IS, Kang KH, Park YH (2001). *Jeotgalibacillus alimentarius* gen. nov., sp. nov., a novel bacterium isolated from jeotgal with L-lysine in the cell wall, and reclassification of *Bacillus marinus* Ruger 1983 as *Marinibacillus marinus* gen. nov., comb. nov. Int. J. System Evol. Microbiol. 51:2087-2093.

academicJournals

Vol. 10(34), pp. 1388-1396, 14 September, 2016
DOI: 10.5897/AJMR2016.8197
Article Number: 97071CF60508
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Genetic diversity analysis of rep-PCR genomic fingerprinting of *Lysobacter* spp.

FU Li-Na, ZHANG Hai-Yan, ZHANG Xiao-Fang, WEI Lan-Fang, ZHANG Jin-Hao and JI Guang-Hai*

Key Laboratory of Agriculture Biodiversity for Plant Disease Management under the Ministry of Education, Yunnan Agricultural University, Kunming 650201, P. R. China.

Received 7 July, 2016; Accepted 24 August, 2016

Lysobacter. **spp. are considered as important biocontrol bacteria, due to their antagonistic activity against many pathogenic fungi, bacteria and nematodes. Rep-PCR was used to analyze the genetic diversity of 12 *Lysobacter* strains. These strains included *Lysobacter antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4 and LR9-3), *Lysobacter enzymogenes* (DH3, 1-T-1-4) and *Lysobacter capsici* (LG18) isolate from different regions in Yunnan province of China. Rep-PCR was performed using DNA amplification with primers based on short bacterial repetitive elements (ERIC, BOX, IS1113 and J3). The genetic diversity was analyzed through rep-PCR, molecular fingerprint clustering analysis and UPGMA to construct phylogenetic tree. The results show that when the genetic distance was 0.59, IS1113-PCR could cluster the *Lysobacter* strains as 3 species: *L. antibioticus, L. enzymogenes* and *L. capsici*. The 3 species had obvious differences between each other and the rep-PCR technique could be used to detect genetic variation between different *Lysobacter* strains, identification and strain classification.**

Key words: *Lysobacter*, Rep-PCR, genetic diversity.

INTRODUCTION

With the development of molecular biology technology, in recent years, a variety of techniques based on PCR has been widely used in genetic diversity analysis of pathogenic fungi and bacteria, especially the appearance and use of rep-PCR, it made the biodiversity research more rapid, convenience and economy (Xiang et al., 2010; Li et al., 1999). Due to the repetitive DNA sequence only in prokaryotes, but not in the eukaryotic chromosomes, rep-PCR technology can specify amplification prokaryotes DNA, and avoid the influence of eukaryotic gene, so it is especially suitable for related eukaryotes prokaryotic symbiotic bacteria and pathogenic bacteria of genetic diversity research (Li and Zhi, 2006). This technique use the highly conserved short repeat sequences in fungal or bacterial genomic DNA as target sequences of primers to amplify through PCR. Through agarose gel electrophoresis separating DNA fragments with different sizes, each species or strains can produce specific DNA fingerprints. Rep-PCR was used to analyze the genetic diversity of *Xanthomonas* strains and results indicated

*Corresponding author. E-mail: 550356818@qq.com.

rep-PCR was a useful tool for detecting genetic variation among strains of *X.o.pv.oryzicola* and identification of strains as well as classification studies (Ji et al., 2002).. REP-PCR and BOX-PCR were used to study the genetic diversity of *Ralstonia solanacearum* and also indicated that this tool was scientific and effective (Li et al.,2011).

Lysobacter. spp. belong to the *Xanthomonadaceae* family, produce yellow to brownish black pigment, rod-shaped cells, rounded ends, no flagella, the edge of the colony is clear and smooth, have the ability to slip (Ji, 2011). *Lysobacter* are widely distributed in nature and found in soil, rivers, sewers, and other extreme environment (Christensen and Cook, 1978). *Lysobacter* species are recognized as new bacterial predators with the arsenal of bioactive small molecules, the biosynthetic mechanisms and biosynthetic genes for cyclodepsipeptide lysobactin, cyclic lipodepsipeptides, cephem-type β-lactams and polycyclic tetramate macrolactams which make them as biocontrol agents and promising drug producers (Xie et al., 2012). In 1978, Christensen named 4 species of this genus of bacteria: *Lysobacter enzymogenes, Lysobacter antibioticus, Lysobacter brunescens* and *Lysobacter gummosus* (Christensen and Cook, 1978). With the development of bacterial taxonomy technology and a large number of professional database updating, this bacteria was reclassified from *Stenotrophomonas* to *Lysobacter* spp. (Sullivan et al., 2003; Islam et al., 2005). Forepart, the research mainly focused on its activity of various extracellular enzymes, they act on the pathogen cell wall and other targets, causing lysis and inhibition (Reichenbach, 2006). *Lysobacter* spp. are considered as new microbial pesticides, and produce extracellular enzymes and antibiotics that can inhibit the occurrence of several plant diseases. Anovel antifungal compound (HSAF, 1) was isolated from *L. enzymogenes* C3 and can disrupt sphingolipids important to the polarized growth of filamentous fungi, thus, *L. enzymogenes* C3 was used in the biological control of fungal diseases of plants (Lou et al., 2011). *L enzymogenes* OH11 secretes chitinases that hydrolyzed the pathogenic fungal cell wall contributing to suppression of proliferation (Postma et al., 2009); *Lysobacter* sp. strain sbk88 and *L. enzymogenes* 3.1t8 can produce antibiotics that are involved in suppression of *Pythium aphanidermatum* and *Aphanomyces cochlioides* (Kato et al., 1998). *L. enzymogenes* OH11 can control *Pseudomonas solanacearum* (Jiang et al., 2005) and *L. antibioticus* 13-1 was shown to control *Xanthomonas oryzae pv. Oryzae* and *Erwinia carotovora* subsp. *carotovor , Ecc* postulated by niche exclusion by colonizing the crop rhizosphere and competition for nutrients (Wei et al., 2014; Zhang et al., 2011; Wu et al., 2010).

Most studies on *Lysobacter* in recent years were all focused on its biological control, gene cloning and protein (Qian, 2009; Nian, 2015; Liu, 2012). Irene de Bruijn et al. (2015) studied genomics and metabolic profiling of *Lysobacter* in 2005 (de Bruijn et al., 2015). rep-PCR was utilized to analysis 12 strains of *Lysobacter* in order to investigate the diversity of this important biocontrol bacterial genome and lay the foundation for application.

MATERIALS AND METHODS

Test materials and instruments

Test strains and culture medium

L. antibioticus (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L. capsici* (LG18) were isolated from different regions in Yunnan province of China.

NA medium: sucrose 10 g, peptone 5 g, beef extract 3 g, yeast extract 1 g, agar 15 to 18 g, water after dissolving to volume 1000 mL, adjusted to a pH of 7.0, high-pressure sterilized.

R_2A medium: peptone 0.5 g, acid hydrolyzed casein 0.5 g, glucose 0.5 g, amylogen 0.5 g, yeast extract 0.5 g, $C_3H_3NaO_3$ 0.3 g, $K_2HPO_4 \cdot 3H_2O$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.005 g, agar 15 g, dissolved in water after constant volume to 1000 mL, adjusted to a pH of 7.0, high-pressure sterilized.

LB liquid medium: peptone 10 g, yeast extract 5 g, 10 g sodium chloride, dissolved in water after constant volume to 1000 mL, adjusted to a pH of 7.0, high-pressure sterilized.

Major instruments and reagents

Super clean bench, Constant temperature shaking table, Small high-speed centrifuge, Thermostatic water bath, PCR instrument (Takara), High-pressure steam sterilization pot, Image master UV imaging system , Bacterial Genomic DNA Extraction Kit (Beijing Taike Biotechnology Co., Ltd. 50 times), PCR primers by Takara Biotechnology (Dalian) Co., Ltd. synthesis; Takara SYBR premix ExTaq (perfect real time kit purchased from Takara Biotechnology (Dalian) Co., Ltd.

Isolation and identification of *Lysobacter* from soil samples

Dilution coated plate separation method was used (Wang et al., 2007), the separation is mainly based on the color and shape of colonies to screen *Lysobacter* in NA and R_2A plates, then 16S rDNA of bacterial universal PCR amplification primers 27F (AGAGTTTGATCCTGGCTCAG)/1492R (GGTTACCTTGTTACGACTT) are used. PCR reaction system (25 μL): 2 x EasyTaq PCR Supermix 12 μL, primer 27F 1 μL, primer 1492R (10 μmol / L) 1 μL, DNA template (50 ng/ μL) 1 μL, ddH_2O complement 25 μL. The initial denaturing temperature was 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min. Amplification products was by 1.0% agarose gel electrophoresis to recover. After purification, 16S rDNA fragments sequencing by Shanghai Biological Engineering Co., Ltd. sequencing primers are 27F and 1492R. Sequencing results blast in GenBank were then obtained with high similar sequence. A total of 12 strains of bacteria were isolated, purified and identified from the collected soil samples (Table 1).

Rep-PCR and genetic diversity analysis of *Lysobacter*

Lysobacter genome DNA extraction (referring to Beijing Taike Biotechnology Co., Ltd. bacterial genomic DNA extraction kit), and

Table 1. The ecological distribution of the different species of *Lysobacter.*

Strain Name	Origin Agrotype	Agrotype	Croppattern
L. ant 13-1	Kunming City, Yunnan Province	Rhizosphere soil	Rice
L. ant HY	Fuyuan County of Qujing city in Yunnan Province	Rhizosphere soil	Konjak
L. ant 13-6	Fuyuan County of Qujing city in Kunming Province	Rhizosphere soil	Konjak
L. ant 6-B-1	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. ant 13-B-1	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. ant 6-T'-4	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. ant LJ6-3	Shilin County of Kunming city in Yunnna Province	Rhizosphere soil	Panax notoginseng
L. ant LJ6-4	Shilin County of Kunming city in YunnanProvince	Rhizosphere soil	Panax notoginseng
L. ant LR9-3	Shilin County of Kunming city in Yunnan Province	Rhizosphere soil	Panax notoginseng
L. enz DH3	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Cayenne
L. enz 1-T-1-4	Fengqing County of Lincang city in Yunnan Province	Rhizosphere soil	Konjak
L. cap LG18	Fuyuan County of Qujing city in Yunnan Province	Rhizosphere soil	Potato

L. ant: *Lysobacter antibioticus*; L. enz: *Lysobacter enzymogenes* ; L. cap: *Lysobacter capsici.*

then rep-PCR was done.

DNA was used to perform rep-PCR with ERIC primers using the manufacturer's protocol. The primer pair Eric1R: 5´-ATGTAAGCTCCTGGGGATTCAC-3´, Eric2: 5'-AAGTAAGTGACTGGGGTGAGCG-3´ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 x PCR buffer, 50 ng of DNA template, 400 μM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme, ddH_2O complement 25 μL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The primer BOX: 5´-CTACGGCAAGGCGACGCTGACG-3´ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 × PCR buffer, 50 ng of DNA template, 400 μM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme, ddH_2O complement 25 μL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The primer J3: 5´-GCTCAGGTCAGGTCGCCTGG-3´ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 × PCR buffer, 50 ng of DNA template, 400 μM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme, ddH_2O complement 25 μL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 68°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The primer pair IS1113: TX1: 5´-TGTAGTGGACCTTCGAA-3´, TX2: 5´-ACGAGCGATTGATCAGG-3´ was used and composed by the Shanghai born industry and Biological Engineering Co., Ltd. synthetic. PCR reaction system: 10 x PCR buffer, 50 ng of DNA template, 400 μM dNTP, primer F 10 pmol, primer R 10 pmol, 2 U Taq enzyme and ddH_2O complement 25 μL. The initial denaturing temperature was 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 65°C for 8 min, with a final extension at 65°C for 15 min.

The end of amplification, 8 μL PCR product was taken in 0.5 * TAE buffer through the concentration of 1.5% agarosegel electrophoresis, the voltage drop to 100 V, electrophoresis was done for 5-6 h, after the completion of the electrophoresis, using ethidium bromide staining, decolorization, in gel imaging system amd the collection and preservation of image was done.

Approximately 25 mL of the post amplification reactions were separated by electrophoresis in a 1.5% agarose gel in TAE buffer, stained with an ethidium bromide solution (10 mg/mL), decolorization and photo documented under UV light. An optimized comparative study of the diversity of the obtained banding patterns showed the presence or absence of repetitive elements using photographic images.

Rep-PCR amplified products of the gel map to read the band, have band (mark "1"), no band (mark "0"), using software NTSYS for cluster analysis, and using unweighted pair group method with arithmetic mean (UPGMA) for molecular fingerprint clustering analysis and constructing system tree pattern.

RESULTS

Analysis of rep-PCR fingerprint banding patterns

The Box PCR showed 11 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates of *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L.capsici* (LG18) (Figure 1).

The Eric PCR showed 17 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates of *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4 and LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L.capsici* (LG18) (Figure 2).

The IS1113 PCR showed 5-15 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L.capsici* (LG18) (Figure 3).

The J3 PCR showed 8-16 different molecular fingerprints with bands ranging from 250 to 7000 bp for the 12 isolates of *L. antibioticus* (HY, 13-1, 13-6, 6-B-1, 13-B-1, 6-T'-4, LJ6-3, LJ6-4 and LR9-3), *L. enzymogenes* (DH3, 1-T-1-4) and *L. capsici* (LG18) (Figure 4)

Results of PCR fingerprint analysis

UPGMA cluster analysis was performed on the DNA

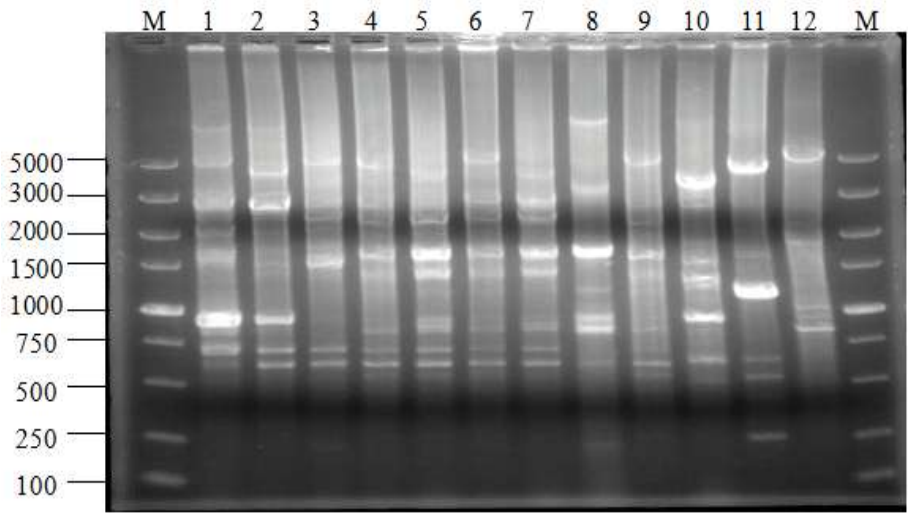


Figure 1. Box-PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M: marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.

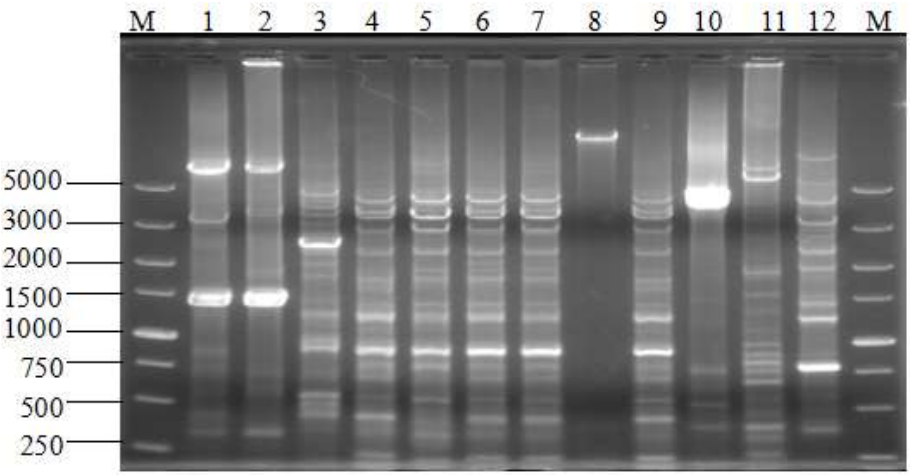


Figure 2. ERIC-PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M: marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.

fingerprints and showed that only primer IS1113 can distinguish *L. antibioticus* (r1-9), *L. enzymogenes*(r10-11) and *L. capsici L.capsici* (r12) as three groups (Figure 5). According to different geographical locations, we can reclassified *L. antibioticus* HY and *L. antibioticus* 13-6 both from Fuyuan County of Yunnan Province as the first group, *L. antibioticus* 13-1 from Kunming City of Yunnan Province as the second group, *L. antibioticus* 6-B-1, 13-B-1 and 6-T'-4 from Fengqing County of Lincang City in Yunnan Province as the third group, *L. antibioticus* LJ6-3 and LJ6-4 from Shilin County of Yunnan Province as the fourth group, *L. antibioticus* LR9-3 from Shilin County of Kunming city in Yunnan Province as the fifth group, *L. enzymogenes* 1-T-1-4 and DH3 both from Fengqing County of Lincang city in Yunnan Province as the sixth group and *L. capsici* LG18 from Fuyuan County of Qujing city in Yunnan Province as the seventh group (Table 1). The results confirmed that as compared to the similarity between species, the similarity of the isolates within in the species was higher (Table 2).

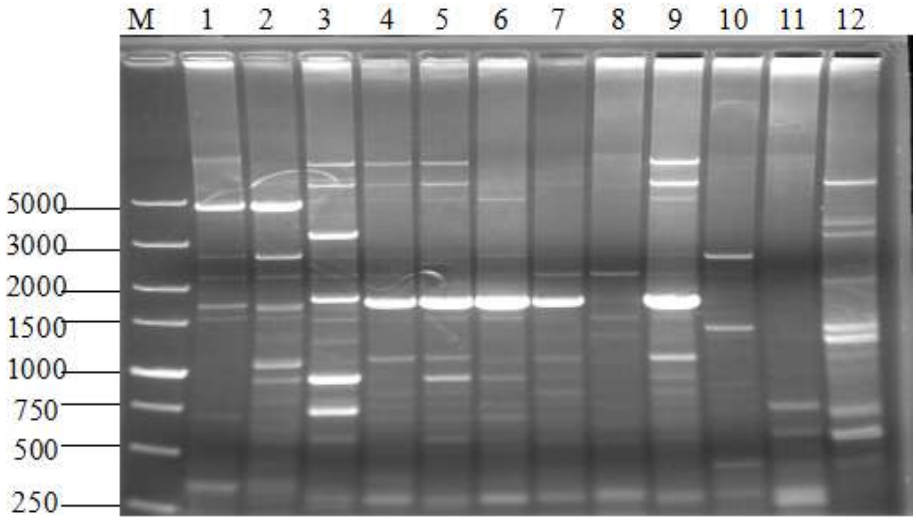


Figure 3. IS1113 -PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M:marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.

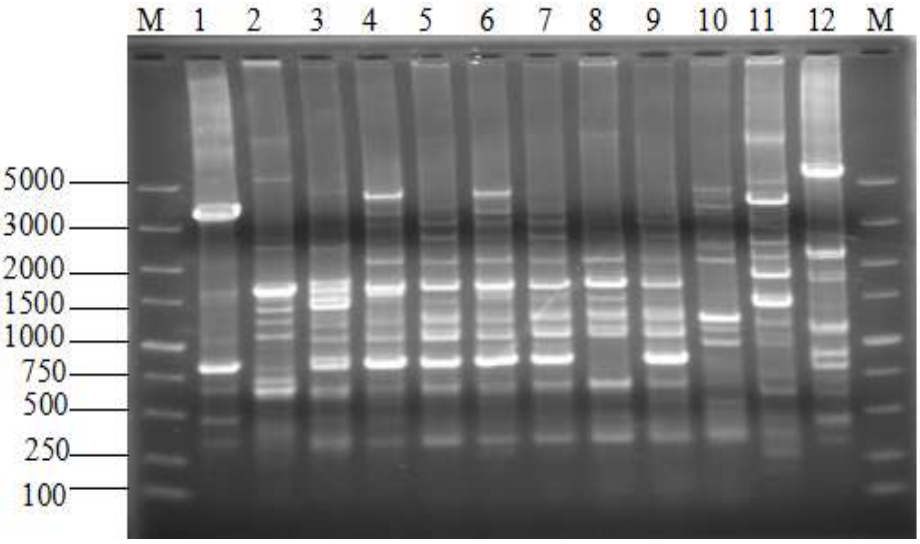
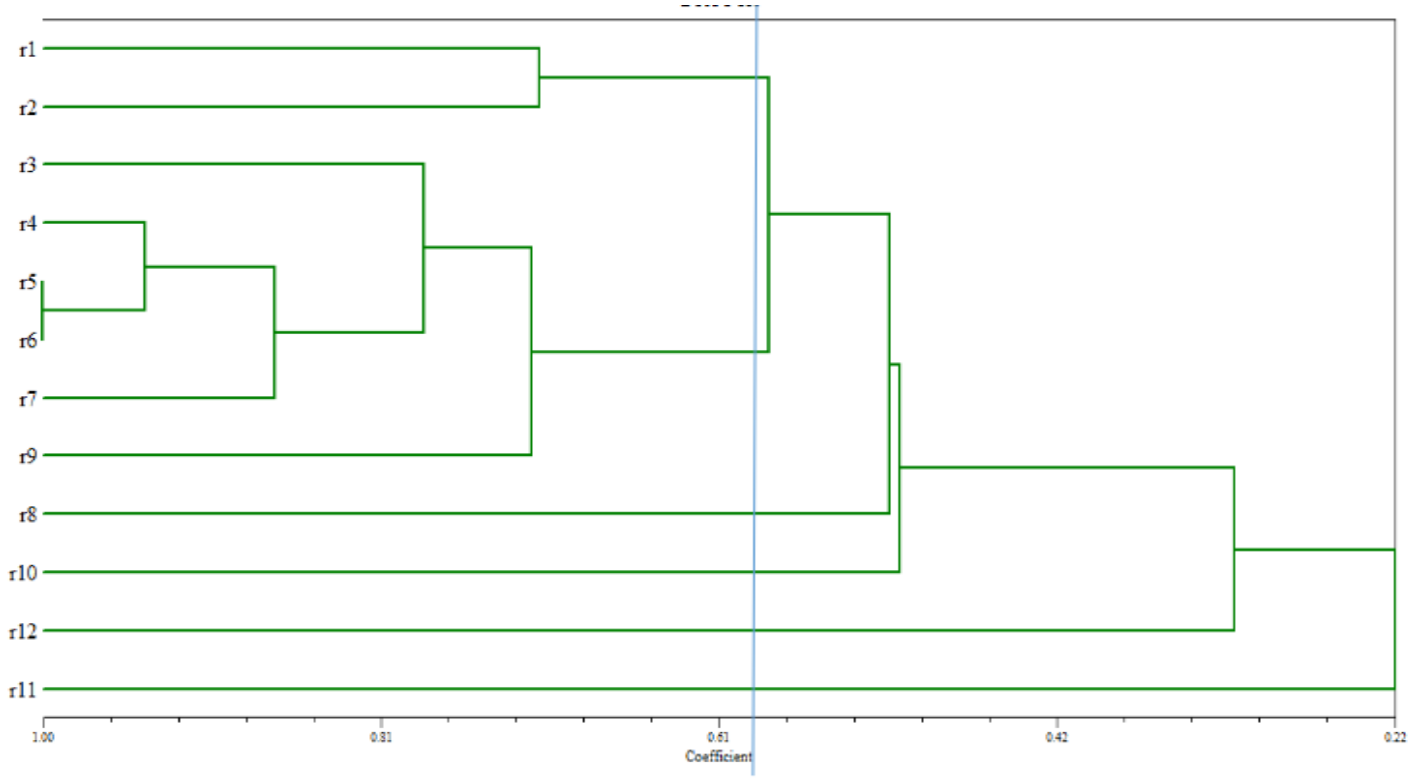


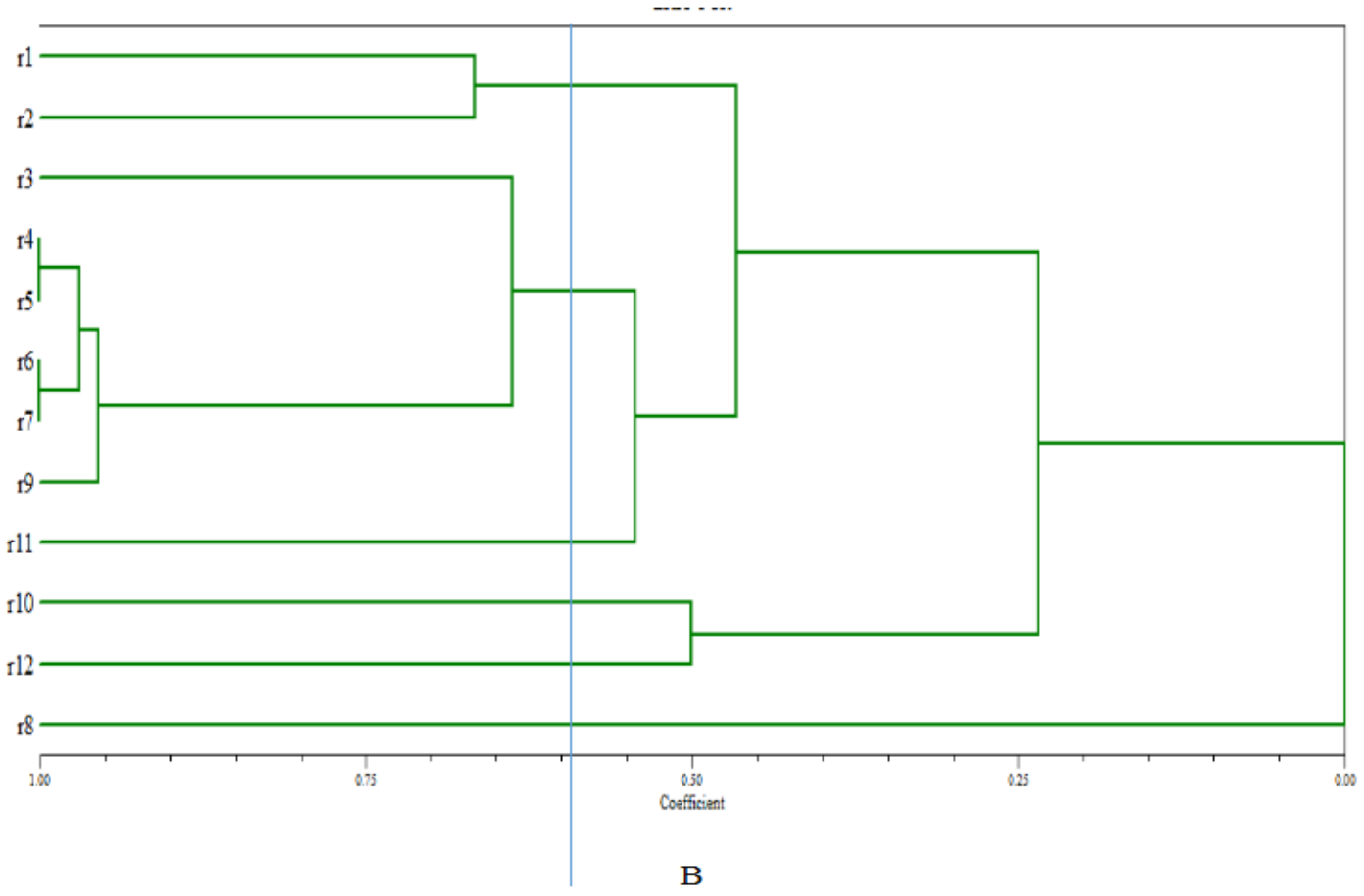
Figure 4. J3 -PCR fingerpringing patterns from genomic DNA of *Lysobacter* strains. M:marker, 1: *L. antibioticus* HY, 2: *L. antibioticus* 13-1, 3: *L. antibioticus* 13-6, 4: *L. antibioticus* 6-B-1, 5: *L. antibioticus* 13-B-1, 6: *L. antibioticus* 6-T'-4, 7: *L. antibioticus* LJ6-3, 8: *L. antibioticus* LJ6-4, 9: *L. antibioticus* LR9-3, 10: *L. enzymogenes* DH3, 11: *L. enzymogenes* 1-T-1-4, 12: *L. caisici* LG18.

DISCUSSION

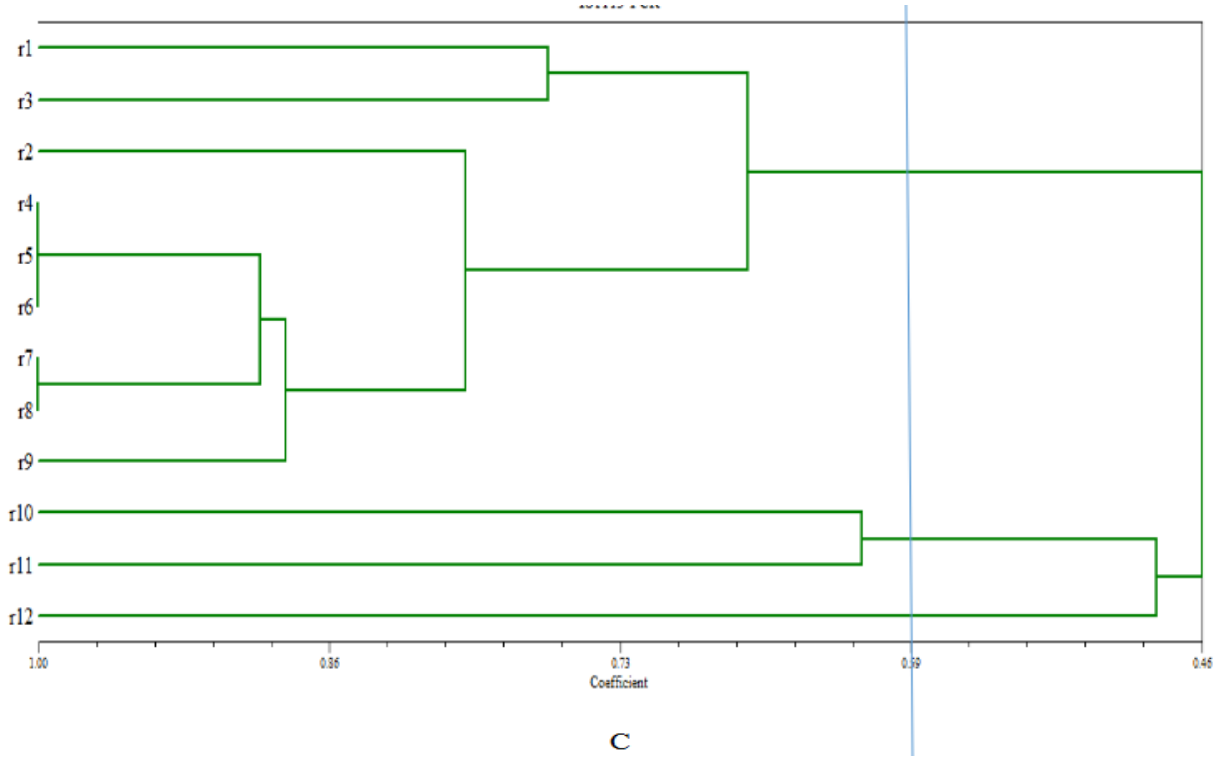
The BOX, ERIC, IS1113 and J3 repeat sequences are distributed widely in three species of *Lysobacter* genome DNA, and confirmed rep-PCR technology can distinguish different species and used for the determination of *Lysobacter* group genetic diversity. Primers of BOX, ERIC, IS1113 and J3 are used for PCR, fingerprint and clustering analysis, results show that when genetic distance was 0.59 only IS1113 primer can successfully distinguish *L. antibioticus, L. enzymogenes* and *L. capsici*. IS1113 can be used for analysis of hereditary changes in *Lysobacter* groups, except a few strains, which can be clearly divided into three main genetic


r1
r2
r3
r4
r5
r6
r7
r9
r8
r10
r12
r11
1.00
0.81
0.61
0.42
0.22
Coefficient

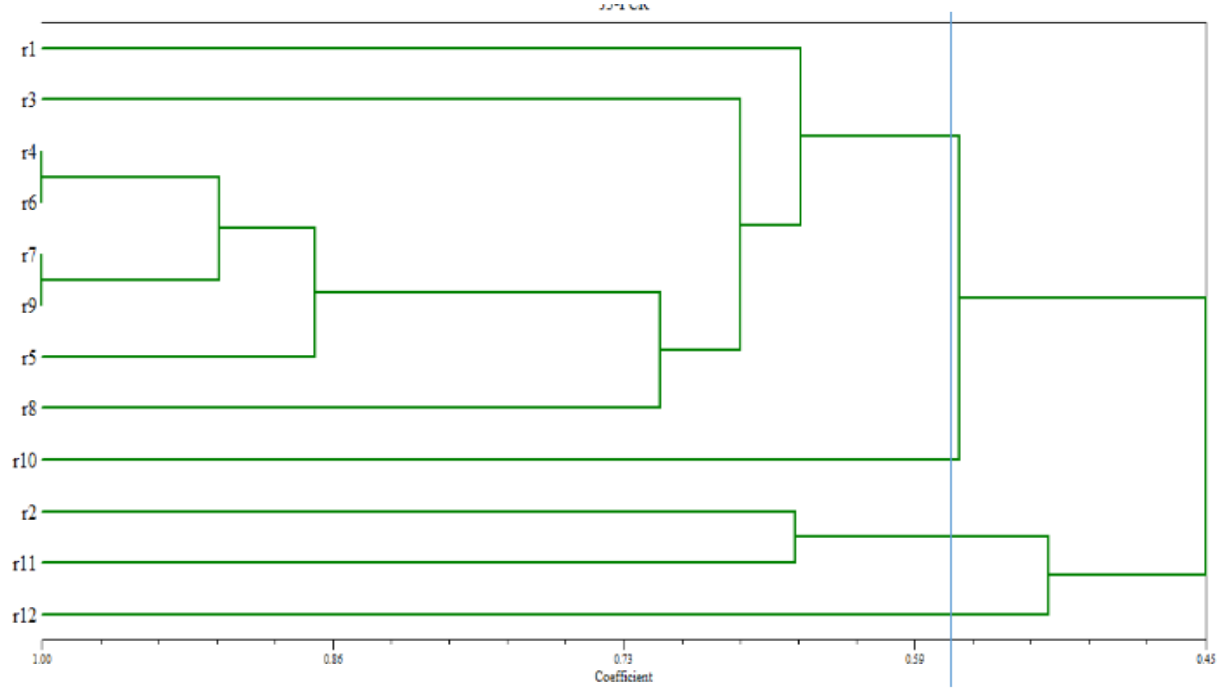
A


r1
r2
r3
r4
r5
r6
r7
r9
r11
r10
r12
r8
1.00
0.75
0.50
0.25
0.00
Coefficient

B



C



D

Figure 5. Dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. A: BOX-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains; B: ERIC-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. C: IS1113-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. D: J3-PCR of dendrograms constructed with UPGMA using DNA fingerprints patterns from *Lysobacter* strains. Strains: r1: *L. antibioticus* HY, r2: *L. antibioticus* 13-1, r3: *L. antibioticus* 13-6, r4: *L. antibioticus* 6-B-1, r5: *L. antibioticus* 13-B-1, r6: *L. antibioticus* 6-T'-4, r7: *L. antibioticus* LJ6-3, r8: *L. antibioticus* LJ6-4, r9: *L. antibioticus* LR9-3, r10: *L. enzymogenes* DH3, r11: *L. enzymogenes* 1-T-1-4, r12: *L. caisici* LG18

Table 2. Similarity of IS1113-PCR fingerprints among different strains.

Strain numbers	HY	13-1	13-6	6-B-1	13-B-1	6-T'-4	LJ6-3	LJ6-4	LR9-3	DH3	1-T-1-4	LG18
HY	100											
13-1	0.667	100										
13-6	0.762	0.769	100									
6-B-1	0.636	0.667	0.741	100								
13-B-1	0.727	0.814	0.740	1.000	100							
6-T'-4	0.636	0.815	0.741	1.000	1.000	100						
LJ6-3	0.522	0.786	0.714	0.897	0.897	0.897	100					
LJ6-4	0.522	0.786	0.714	0.897	0.897	0.897	1.000	100				
LR9-3	0.609	0.786	0.714	0.897	0.897	0.897	0.867	0.867	100			
DH3	0.500	0.571	0.571	0.546	0.546	0.546	0.609	0.609	0.522	100		
1-T-1-4	0.308	0.222	0.333	0.421	0.421	0.421	0.400	0.400	0.300	0.615	100	
LG18	0.111	0.435	0.435	0.500	0.500	0.500	0.560	0.560	0.480	0.556	0.400	100

Lysobacter antibioticus: HY, 13-1, 13-6, 6-B-1, 13-B-6-1, 6-T'-4, LJ6-3, LJ6-4, LR9-3. *Lysobacter enzymogenes:* DH3, 1-T-1-4. *Lysobacter capsici:* LG18.

clusters. *Lysobcater* have different structure, activity and biosynthetic pathways, thus the control of pathogenic bacteria is different. So we can classify species quickly through rep-PCR and then use specific biocontrol bacteria to control plant diseases.

It is the first time to apply rep-PCR to study the diversity of *Lysobacter* genome. The method of identifying *Lysobacter* usually uses 16S rDNA of bacterial universal primers to PCR and then amplified products are sequenced, with sequencing results blast in GenBank and then high similar sequence was obtained. This method is time consuming, inconvenient and the results are not accurate because the sequencing results may be inaccurate. Thus, rep-PCR was adopted, this technology has advantages of fast, handy and economic, and dispense with specific probe and southern hybridization. Due to the characteristics of electrophoresis, rep-PCR with strain level spectrum can be used for strain identification and can reflect the differences of genome of close genetic relationship between strains, but the disadvantage is that it does not reflect the differences in the plasmid DNA, and rep-PCR is affected by many factors, such as: primers from different sources or different batches, DNA polymerase or PCR instrument of different types, to an extent, these factors restrict the technology application (Laguerre et al., 1996). In spite of this, under certain experimental conditions, rep-PCR is an important and effective technology for strains identification and clustering. In the later work, Multilocus Sequence Analysis (MLSA) and Restriction Fragment Length Polymorphism Analysis (RFLP) can be used to further research on the origin of their diversity, these will be helpful to further uncover the origin, evolution and phylogeny, and provide important information and scientific basis in the production or use of *Lysobacter* for disease prevention and economic crops treatment.

Conflict of interests

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

This project was funded by National Natural Fund (31360002, 31460458), The Ministry of Agriculture Public Welfare Industry Special (201303015), Province Key New Product Plan (2014BB005) and Yunnan University Innovation Team IRTSTYN (2014-22).

REFERENCES

Christensen P, Cook F D(1978). *Lysobacter*, a new genus of nonfruiting gliding bacteria with high base ratio. Int. J. Syst. Bacteriol. 28:367-398.

de Bruijn I, Cheng X, de Jager V, Expósito RG, Watrous J, Patel N, Postma J, Dorrestein PC, Kobayashi D, Raaijmakers JM (2015). Comparative genomics and metabolic profiling of the genus *Lysobacter*. BMC Genomics 16:991.

Islam MT, Hashidoko Y, Deora A, Ito T, Tahara S (2005). Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne Peronosporomycetes. Appl. Environ. Microbiol. 71:3786-3796.

JI Guang-hai (2011). Advances in the Study on *Lysobacter* spp. Bacteria and Their Effects on Biological Control of Plant Diseases. J. Yunnan Agric. Univ. 26(1):124-130.

JI Guang-hai, XU Zhi-gang, ZHANG Shi-guang (2002). Preliminary Analysis of Genetic Diversity of *Xanthomonas oryzae* pv.*oryzicola* and *Xanthomonas leersiae* Stains in China by Rep-PCR. Acta Phytopathol. Sin. 32(1):26-32.

Jiang Ying-hua, Hu Bai-shi, Liu Feng-quan (2005). Selection and Identification of Antagonistic Bacteria against Soil-borne Plant Pathogens. Chin. J. Biol. Control 21(4):260-264.

Kato A, Nakaya S, Kokubo N, Aiba Y, Ohashi Y, Hirata H, Fujii K, Harada KI (1998). A new anti-MRSA antibiotic complex WAP-8294A. I. Taxonomy, isolation and biological activities. J. Antibiot. 51(10):929-935.

Laguerre G, Mavingui P, Allard MR, Charnay MP, Louvrier P, Mazurier SI, Rigottier-Gois L, Amarger N (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to Rhizobium leguminosarum and its different biovars. Appl. Environ. Microbiol. 62(6):2029-2036.

Li Hai-yun, Che Jian-mei, Liu Bo, XueFang Z, RongFeng X (2011). Genetic Diversity Analysis of *Ralstonia solanacearum* Based on BOX-PCR and REP-PCR. J. Agric. Biotechnol. 19(6):1099-1109.

Li Jie, Xiong Zhi (2006). Studies on Genomic Diversity of Bacteria by rep-PCR. J. Southwest For. Coll. 26(6):1-3.

Li J, Xu L, Fan H, Li L, Ge C, Yang S (1999). Use rep-PCR for Study the Diversity of Chinese Peanut Nodule Bacteria. J. Microbiol. 39(4):296-303.

Liu Yi-ru (2012). Identification and Function Characterization of Quorum Sensing System in *Lysobacter enzymogenes*. Nanjing Agricultural University pp. 1-69.

Lou L, Qian G, Xie Y, Hang J, Chen H, Zaleta-Rivera K, Li Y, Shen Y, Dussault PH, Liu F, Du L(2011). Biosynthesis of HSAF, a tetramic acid-containing macrolactam from *Lysobacter enzymogenes*. J. Am. Chem. Soc.133:643-645.

Nian Mei-yu (2015). Identification of potential antibacterial activity proteins on *Lysobacter* sp.SNNU513. Shanxi Normal University. pp. 1-63.

Postma J, Stevens LH, Wiegers GL, Davelaar E, Nijhuis EH (2009). Biological control of Pythium aphanidermatum in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan. Biol. Control 18:301-309.

Qian Guo-liang (2009). Cloning and Characterization of Biocontrol Related Genes and Construction of Engineering Strain in *Lysobacter enzymogenes* OH11. Nanjing Agricultural University pp. 1-185.

Reichenbach H (2006). The genus *Lysobacter*. Prokaryotes 6:939-957.

Sullivan RF, Holtman MA, Zylstra GJ, White JF Jr., Kobayashi DY (2003). Taxo- nomicpositioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. J. Appl. Microbiol. 94:1079-1086.

Wang P, Yang ZW, Hu HT, Wang KM, Zhang F (2007).Elementary Research on the Screening of Antagonisms and Their Antibiotic Activities Against the Phytopathogens. Hubei Agric. Sci. 46(2):236-238.

Wei Lan-Fang, Zhou Li-Hong, JI Guang-Hai, Wang Yong-Ji, WANG Shao-Xue (2014). Control of rice bacterial leaf blight by antibacterial substances from *Lysobacter antibioticus* strain 13-1. Microbiology 41(2):274-280.

Wu Ya-peng, JI Guang-hai, Chen Yun-lan, Lu Jun, Dong Kun (2010). Biocontrol Effect and Mechanisms of *Lysobacter antibioticus* 13-1 against Soft Rot Pathogen of Amorphophallus konjac. Chin. J. Biol. Control 2:193-199.

Xiang Ming-jie, Liu Jin-yan, Ni Pei-hua, Zhang Hua, Ni Yu-xing (2010). Repeat Sequence PCR (rep-PCR) Fingerprint Analysis in the Identification of Pathogenic Fungi and Classification. Chin. J. Mycol. 5(6):376-380.

Xie Y, Wright S, Shen Y, Du L (2012). Bioactive natural products from *Lysobacter*. Nat. Prod. Rep. 29:1277-1287.

Zhang Li-hui, Wang Yong-ji, Liao Lin, Ji Guang-hai (2011). Biocontrol effect of *Lysobacter antibioticus* 06−4 on soft rot pathogen of *Amorphophallus konjac* its mechanism. J. Hunan Agric. Univ. (Natural Sciences) 3:286-289.

academicJournals

Vol. 10(34), pp. 1397-1407, 14 September, 2016
DOI: 10.5897/AJMR2016.8175
Article Number: DEFE2A360510
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of a real-time PCR assay for the detection of the *Klebsiella pneumoniae* carbapenemase gene (*bla*$_{KPC}$) in enterobacteriaceae isolates from clinical samples in Menoufia University hospitals, Egypt

Amira Abdelkader Al Hindi and Abeer Hamdy El Shalakany*

Department of Clinical Pathology (Microbiology), Faculty of Medicine, Menoufia University, Egypt.

Received 22 June, 2016; Accepted 23 August, 2016.

The aim of our work is to study and evaluate a rapid method for detection of *Klebsiella pneumonia carbapenemases* genes (*blaKPC*) in enterobacteriaceae isolates from clinical samples by using real time PCR and comparison of this method with ordinary phenotypic methods. Outbreaks of carbapenem-resistant enterobacteriaceae (CRE), primarily *K. pneumoniae*, have been reported recently in several regions worldwide. The production of carbapenemases especially *K. pneumoniae* carbapenemase (KPC) is the most important mechanism of enzymatic resistance in enterobacteriaceae. One hundred and fifty clinical isolates from different departments of Menoufia university hospitals were tested by both disc diffusion method (Imipenem 10 µg, Meropenem 10 µg and Ertapenem 10 µg), and imipenem E-test for minimum inhibitory concentration (MIC) then analyzed according to cut off-points of CLSI 2014 guideline. Then all the one hundred fifty clinical isolates were tested for the presence of a *blaKPC* gene by real time PCR. We found Eighty three (83) isolates (55.3%) from 150 were resistant to one or more carbapenems by disk diffusion method, and 88 isolates (58.7%) were resistant by E test while 91 isolates (60.6%) were positive for the presence of *KPC* gene by real time PCR. There was significant difference between disk diffusion method and real-time PCR *(P < 0.001)* and E test and real-time PCR *(P < 0.001)* regarding carbapenem resistance. The highest percent of enterobacteriaceae isolates having *KPC* gene were among *K .pneumoniae* (46.1%). *KPC* positive cases were mainly (74.1%) from urology department. About (97.8%) *bla*KPC PCR positive cases had been exposed to invasive procedures such as mechanical ventilation *(P < 0.001*), and (95.6%) *blaKPC* PCR positive cases had been from hospital acquired infections *(P < 0.001).* There was a history of antimicrobial intake in 70.3% of cases infected with *KPC* PCR positive isolates. *bla*KPC PCR has sensitivity ,specificity, negative predictive value, and diagnostic accuracy (99, 87, 98 and 93%), respectively .

Key words: carbapenem-resistant enterobacteriaceae (CRE), real time PCR, *blaKPC.*

INTRODUCTION

Carbapenems are highly efficacious drugs for treating infections with extended-spectrum β-lactamase–producing gram-negative bacteria. Previously, resistance to carbapenems has been rare; however, the emergence of transmissible carbapenem resistance is now a growing concern. (Raghunathan et al., 2011).

An increasingly common mechanism of carbapenem resistance is the class-A, *Klebsiella pneumoniae* carbapenemase (KPC). KPCs have been reported in *K pneumoniae* and in *Klebsiella oxytoca, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Citrobacter freundii, Enterobacter spp, Serratia spp,* and *Salmonella spp.* (Villegas et al., 2006).

The *blaKPC* genes that encode KPCs are present on transferable plasmids and are flanked by transposable elements, thus allowing for the gene to move from plasmid to the bacterial chromosome and back (Bratu et al., 2005).

This potential to disseminate resistance has been demonstrated in several reported outbreaks with high mortality rates (Raghunathan et al., 2011).

Given the limited therapeutic options available, the accurate and timely detection of KPC-producing enterobacteriaceae is vital in order to control their spread (Nordmann et al., 2009).

The mechanisms of resistance to carbapenems may be related to the combination of decrease in bacterial outer membrane permeability, increasing production of Extended-Spectrum Beta-Lactamases (ESBLs), AmpC beta-lactamases and expression of betalactamases like Carbapenemases. The production of carbapenemases especially KPC is the most important mechanism of enzymatic resistance in isolated Enterobacteriaceae such as *K. pneumoniae* (Nordmann et al., 2012).

The detection of KPC-producing bacteria can be challenging because of heterogeneous expression of β-lactam resistance. Automated and agar diffusion methods of susceptibility testing show some inconsistencies in reliably detecting KPC-mediated resistance, and this is influenced by the carbapenem that is used for testing (Francis et al., 2012; Fallah et al., 2013).

To address these issues, confirmatory tests such as several polymerase chain reaction (PCR)-based assays have been developed to detect KPC-mediated carbapenem resistance. Real-time PCR has been employed in the rapid detection of colonization/infection with KPC-producing Enterobacteriaceae in various types of samples and clinical isolates.

These assays have demonstrated good sensitivity and specificity with favorable positive and negative predictive values (Schechner et al., 2009).

PATIENTS

This study was conducted on clinical isolates of enterobacteriaceae that were isolated from samples sent to microbiology laboratory of Menoufia University Hospitals, from July 2013 to September 2014.

Subjects

One hundred fifty clinical isolates from different departments of Menoufia university hospitals were tested. During the study period, all strains of enterobacteriaceae isolated were stored on broth glycerol at-70ºC for subsequent PCR analysis.

METHODS

Enterobacteriacae isolates were identified by conventional methods such as culture characteristics and biochemical reactions (Colle et al., 1996). Triple sugar iron agar (TSI), lysine iron agar (LIA), motility indole ornithine (MIO), Simmons citrate agar and urea agar base (Oxoid England) plus identification by API 20E (https://apiweb.biomerieux.com).

Susceptibility testing

0.5 McFarland turbidity suspension for each isolate was used to inoculate on Mueller-Hinton agar plates (Oxoid England).

Disk diffusion

By using imipenem, meropenem and ertapenem disk diffusion. Results were categorized as sensitive, intermediate and resistant as according to Clinical Laboratory Standard Institute (CLSI) guidelines 2014 (Imipenem 10 µg: S: ≤1, I: 2, R: ≥4), (Meropenem: 10 µg: S: ≤1, I: 2, R: ≥4), (Ertapenem: 10 µg: S: ≤0.5, I: 1, R: >2).

E-Test for imipenem

MIC for imipenem was determined using E-test (bioMérieux) and results were categorized as sensitive, intermediate and resistant as per Clinical Laboratory Standard Institute (CLSI) guidelines 2014 (S ≤1, I: 2, R ≥ 4) (CLSI, 2014)

Genotypic detection of KPC

All clinical isolates of enterobacteriaceae were tested for the presence of a *blaKPC* gene by real time PCR:

DNA extraction

DNA extraction using the GeneJET Genomic DNA Purification Kit (Thermo Scientific K0721, Fermentas, UE), using Gram-negative bacteria genomic DNA purification protocol**.**

Sample preparation

After an overnight pure growth on MacConkey, 2 to 3 of bacterial colonies were inoculated into 1 ml of nutrient broth water then overnight incubation.

*Corresponding author. E-mail: abeerhamdy75@gmail.com. Tel: 0966536190906.

Table 1. Reaction setup for real-time.

Component	Volume/reaction (µl)	Final concentration
5x HOT FIREPOL EvaGreen qPCR Mix plus	4	1x
Primer forward (10 pmol/µl)	0.5	0.25 uM
Primer reverse (10 pmol/µl)	0.5	0.25 uM
Template DNA	5	0.01-10 ng/µl
Nuclease free water	10	
Total reaction volume	20	

Table 2. Susceptibility testing by disk diffusion.

Disk diffusion test (DDT)	No.	%
CR	83	55.3
CS	67	44.7
Total	150	100

CR, Carbapenem resistane; CS, Carbapenem sensitive.

Table 3. Susceptibility testing by E-test.

E. test	No.	%
R	88	58.7
S	62	41.3
Total	150	100

Table 4. Detection of presence of KPC gene by PCR.

KPC gene	No.	%
Positive	91	60.6
Negative	59	39.4
Total	150	100

Real-time PCR amplification and detection

Real-time PCR was performed on Spartan RX CYP2 C 19 instrument using Syber Green with the following primers (Raghunathan et al., 2011): Forward primer, 5'-ATGTCACTGTATCGCCGTC -3' (80-250 nM final concentration), Reverse primer, 5'- CTCAGTGCTCTACAGAAAACC -3' (80-250 nM final concentration) and 5x HOTFIREPol®EvaGreen®qPCR Mix Plus (no ROX) –Solis biodyne– Cat 08-25-00001, a reaction mix was prepared according to Table 1.

Method

1. The reaction mix was mixed thoroughly, and appropriate volume was dispensed into PCR tubes or plates.
2. Template DNA was added (0.01 to 10 ng/µl) to the individual PCR tubes or wells containing the reaction mix.
3. Real-time instrument *Spartan* was programmed according to the following cycling conditions: 95C for 15 min, followed by 40 cycles of 95°C for 15 s, 57°C for 20 s, and 72°C for 20 s. And finally dissociation at 95°C for 30 min.

RESULTS

Eighty three (83) isolates (55.3%) from 150 were resistant to one or more carbapenems by disk diffusion method and sixty seven were sensitive (Table 2).

This table shows that 88 isolates (58.7%) from all enterobacteriacea isolates (150) were resistant by E test and 62 (41.3%) were sensitive (Table 3). This table shows that 91 isolates (60.6%) from all enterobactericea isolates (150) were positive for the presence of KPC gene by real-time PCR and 59 (39.4%) were negative (Table 4). In this study, the melting temperature was 81.9°C (Figure 3), the positive cases for the presence of *blaKPC* gene show melting peaks that express fluorescence exceeding the threshold line (Figure 1) while negative cases express fluorescence that does not exceed the threshold line (Figure 2).

This table shows that from 83 carbapenem resistant isolates by disk diffusion, 82 were blaKPC PCR positive and from 67 carbapenem sensitive isolates, 9 were blaKPC PCR positive, and there was highly significant difference($p<0.001$) between two methods (Table 5).

This table shows that from (88) resistant isolates by E-test, 82 (93.2%) were blaKPC PCR positive cases from 62 carbapenem sensitive isolates, 9 were blaKPC PCR positive, and there was highly significant difference ($p<0.001$) between two methods (Table 6).

Real time PCR detected 82 (98.8%) of carbapenem resistant isolates by DDT, thus the sensitivity of the PCR was 99%, specificity was 87% and diagnostic accuracy was 93%, all in relation to DDT as gold standard test (Table 7).

Real time PCR detected 82 (93.2%) of E -test resistant isolates, thus the sensitivity of the PCR was 93.2%, specificity was 85.5% and diagnostic accuracy was 90%, all in relation to E- test as gold standard test (Table 8). As regarding evaluation of the performance of the real-time PCR using disk diffusion susceptibility results, there were 2 cases which were negative for blaKPC by PCR and in the same time were resistant by disk diffusion (Table 9).

The highest percent of enterobacteriaceae isolates having KPC gene were among *K .pneumoniae* (46.1%)

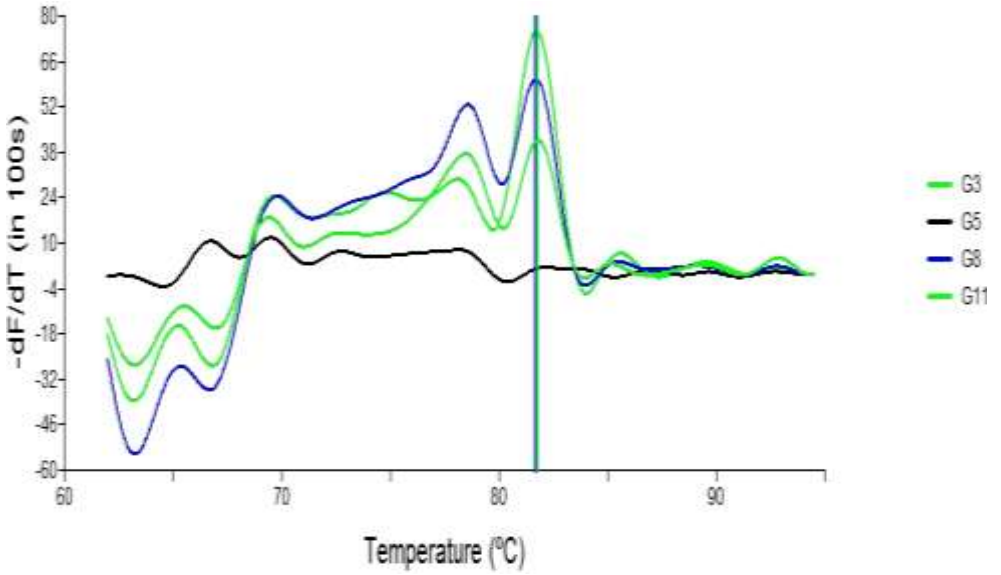


Figure 1. Real time PCR amplification curve as shown by Step 1 Applied Spartan Real time PCR equipment. Positive KPC: G3, G8 and G11 expressing fluorescence exceeding the threshold line. Negative case: G5 expressing fluorescence that does not exceed the threshold line.

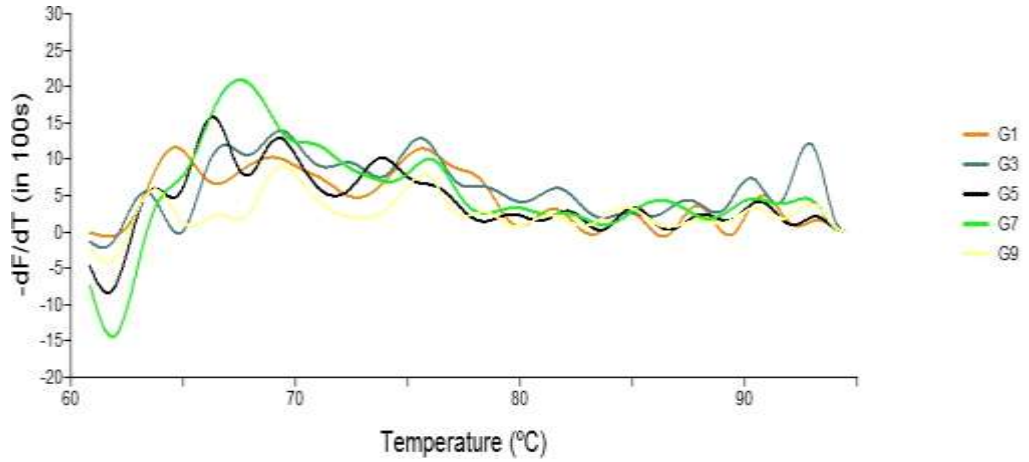


Figure 2. Negative results (G1, G3, G5, G7 and G9).

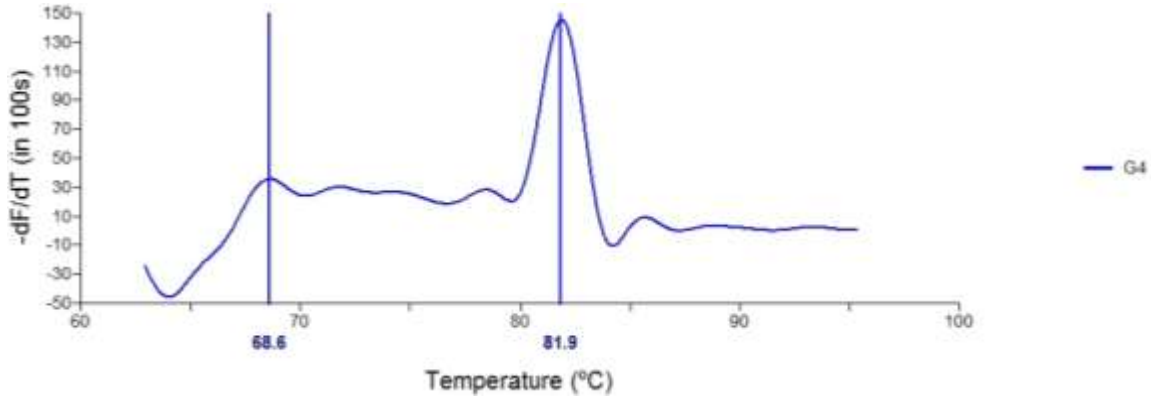


Figure 3. Melting point.

Table 5. Relation between disk diffusion and real-time PCR results in detection of carbapeneme resistance.

			DDT		Total	Symmetrical measurement
			CR	CS		
PCR	P	Count	82 (90.1)	9(9.9)	91(100%)	<0.001
		%Within DDT	98.8%	13.4%	60.6	
	N	Count	1(1.7)	58 (98.3)	59(100%)	
		%Within DDT	1.2%	86.7%	39.4	
	Total	Count	83	67	150	
		%Within	100	100	100	

*Symmetrical measurement: p< 0.05 = significant ; PCR, polymerase chain reaction; CR, Carbapenem resistane; CS, Carbapenem sensitive.

Table 6. Relation between E-test and PCR results in detection of carbapeneme resistance.

			DDT		Total	Symmetrical measurement
			CR	CS		
PCR	P	Count	82(90.1%)	9(9.9%)	91(100%)	<0.001
		%Within MIC	93.2	14.5	60.6	
	N	Count	6(10.2%)	53(89.8%)	59(100%)	
		%Within MIC	6.8	85.5	39.4	
	Total	Count	88	62	150	
		%Within MIC	100	100	100	

Table 7. Clinical performance of PCR in relation to disk diffusion test(DDT) as gold standard test .

Item	TP	FN	TN	FP	Sens.	Spec.	PPV	NPV	Accuracy
PCR	82	1	58	9	99%	87%	90%	98%	93%

TP, True positive; FN, false negative; TN, true negative; FP, false positive; Sens, sensitivity; Spe., specificity; PPV, positive predictive value; NPV, negative predictive value.

Table 8. Clinical performance of PCR in relation to E- test as gold standard test.

Item	TP	FN	TN	FP	Sens.	Spec.	PPV	NPV	Accuracy
PCR	82	6	53	9	93.2%	85.5%	90%	90%	90%

TP, True positive; FN, false negative; TN, true negative; FP, false positive; Sens, sensitivity; Spe., specificity; PPV, positive predictive value; NPV, negative predictive value.

Table 9. Comparison of disk diffusion (imipenem/meropenem or etrapenem) and KPC real-time PCR results.

KPC real- time PCR	Susceptibility by disk diffusion(imipenem/meropenem)	
	Resistant	Susceptible
Positive	83	8
Negative	2	57

Table 10. Types of KPC PCR positive isolates.

Organism	No.	%
K. pneumoniae	42	46.1
Enterobacter spp.	31	34.1
E. coli	16	17.6
Proteus	2	2.2
Total	91	100

Table 11. Distribution of KPC PCR positive isolates in hospital units.

			Unit				Total	χ^2 test	P value
			ICU	Urology	Neonatology	Others			
PCR	P	Count	34(37.4%)	20(21.9%)	4(4.4%)	33(36.3%)	91(100%)	2.70	0.44NS
		%Within unit	57.6%	74.1%	66.7%	56.9%	60.6%		
	N	Count	25(42.4%)	7(11.8%)	2(3.4%)	25(42.4%)	59(100%)		
		%Within unit	42.4%	25.9%	33.3%	43.1%	39.4%		
	Total	Count	59(39.3)	27(18.0)	6(4.0)	58(38.7)	150		
		% Within unit	100	100	100	100	100		

Table 12. Distribution of KPC PCR positive isolates in relation to exposure to invasive procedures.

			PCR results		Total	χ^2 test	P value
			P	N			
Invasive procedures	Yes	Count	82(90.1%)	9(9.9%)	91(100%)	114.73	<0.001HS
		%Within MIC	93.2	14.5	60.6		
	No	Count	6(10.2%)	53(89.8%)	59(100%)		
		%Within MIC	6.8	85.5	39.4		
	Total	Count	88	62	150		
		%Within MIC	100	100	100		

while the lowest percent were among proteus (2.2%) (Table 10) KPC positive cases were mainly (74.1%) from urology department which represented (21.9%) of their isolates, followed by ICU (57.6%) which represented (37.4%) of their isolates (Table 11). About (97.8%) blaKPC PCR positive cases had been exposed to invasive procedures such as mechanical ventilation, urinary catheterization, CVL and cannula insertion (Table 12). About (95.6%) blaKPC PCR positive cases had been isolated from cases with hospital acquired infections (Table 13).There was a history of antimicrobial intake in 70.3% of cases infected with KPC PCR positive isolates (Table 14).

DISCUSSION

The emergence and rapid dissemination of carbapenem-resistant enterobacteriaceae (CRE) worldwide is a cause for concern. Treatment options for infections due to these organisms are extremely limited and effective therapy may be delayed whilst microbiology laboratory confirmatory results are awaited (Burns and Schaffer, 2011).

The rapid detection of KPC-producing enterobacteriaceae is of great importance since these organisms have the potential to spread rapidly in hospital environments and cause nosocomial infections with high mortality rates (Samra et al., 2007; Tibbetts et al., 2008; Burns and Schaffer, 2011). The aim of our study is to study a rapid method for detection of *K. pneumoniae* carbapenemase genes (blaKPC) in enterobacteriaceae isolates in clinical samples by using real time PCR and comparing phenotypic with genotypic results.

In our study 83 (55.3%) of 150 samples had reduced susceptibility to one or more carbapenems. This is similar to a study conducted by Landman et al. (2005) in New York, where (61.5%) of lactose fermenting gram-negative bacilli were imipenem resistant by disc diffusion. In a study conducted by Patel et al. (2008) in Europe and Hindiyeh et al. (2008) in Israel, the carbapenem-resistant *K. pneumoniae* by disc diffusion was 26 and 25.1%, respectively.

Table 13. Distribution of PCR results in relation to hospital acquired infection.

			PCR results		Total	χ2 test	P value
			P	N			
Hospital acquired	Yes	Count	87(85.3%)	15(14.7%)	102(100%)	81.02	<0.001HS
		%Within PCR results	95.6%	25.4%	68.0%		
	No	Count	4(8.3%)	44(91.7%)	48(100%)		
		%Within PCR results	4.4%	74.6%	32.0%		
	Total	Count	91(60.6%)	59(39.4)	102(100%)		
		%Within PCR results	100	100	68.0%		

Table 14. Distribution of KPC PCR positive isolates in relation to antimicrobial intake.

			PCR results		Total	χ2 test	P value
			P	N			
Hospital acquired	Yes	Count	64(56.6%)	49(43.4%)	113(100%)	3.12	0.08NS
		%Within PCR results	70.3%	83.1%	75.3%		
	No	Count	27(73.0%)	10(27.0%)	37(100%)		
		%Within PCR results	29.7%	16.9%	24.7%		
	Total	Count	91(60.6%)	59(39.4)	150		
		%Within PCR results	100	100	100		

On the other hand, resistance reported by Marschall et al. (2009) was 2.9%, also in the Faculty of Medicine Vajira Hospital in University of Bangkok, Metropolis, the incidence of CRE was 0.13% and the presence of the resistance was an important public health problem (Phumisantiphong, 2011).

For certain reasons there is a wide variability in prevalence of CRE. The possible factors could be different geographical locations, variable proficiency levels of microbiology trained technical staff, different antibiotic cut offs being used, different guidelines being followed and different techniques being used for CRE detection.

In several western studies, prevalence of CRE was less than that found in our study. The higher prevalence compared to western countries can be explained by the fact that western countries had strict infection control policies and practices, efficient and effective antibiotic audit systems, shorter average hospital stay, better nursing barriers and other important health care measures that are known to substantially decrease the chances of acquisition and spread of CRE

There are several factors that make detection of CRE by susceptibility testing is challenging and make carbapenem-resistant bacteria incorrectly identified as carbapenem susceptible, resulting in inappropriate selection of therapy. One of these factors may be the heterogeneous expression of β-lactam resistance (Chen et al., 2011) or the low level of resistance that cannot be detected by ordinary susceptibility tests Thomson (2010). In addition, Meropenem and imipenem susceptibility demonstrated poor sensitivity for methods other than BMD (broth microdilution). However, the specificity of meropenem and imipenem susceptibility testing was higher than that for ertapenem susceptibility testing regardless of test method (Benenson et al., 2011).

The ertapenem disk-diffusion test has been shown to be a reliable screening method for KPC-mediated resistance (Bratu et al., 2005). Therefore, results for culture-based susceptibility to ertapenem are often used for determining carbapenem resistance in routine clinical microbiology laboratories. However, resistance to ertapenem alone is not a marker for KPC expression; it has been shown that most ertapenem resistance is related to factors such as an extended-spectrum β-lactamase (ESBL) or AmpC production in association with outer membrane porin mutations (Francis et al., 2012).

In order to overcome such shortcomings when treating infections caused by enterobacteriaceae, the Clinical and Laboratory Standards Institute (CLSI) has recently lowered the susceptibility breakpoints for meropenem, imipenem, ertapenem and doripenm (Chen et al., 2011). Also, the presence of scattered inner colonies along the inhibition zone can lead to perceived increased resistance. The presence of scattered colonies may be

due to decreased expression of the porin channel OmpK36, which has been found in isolates expressing blaKPC (Bulik et al., 2010).

Our study showed that the resistant isolates to imipenem by E-test were 88 out of 150 (58.7%). While in a study conducted by Gupta et al. (2011) in New York City, carbapenem resistance by E-Test was reported in (10.8%) of isolates that were associated with certain device-related infections.

Girlich et al. (2013) found on 133 well-characterized enterobacterial isolates, KPC and meropenem-containing MP/MPI Etest had high sensitivity (>92 %) and specificity (>97 %).

Although we included the E test method in our study, but determining resistance and susceptibility for imipenem with E test was difficult because of the colonies that were present within the zones of inhibition and make lack of consensus on reading E test method.

In Rapp and Urban (2012), most KPC-producing isolates had a carbapenem MIC ≥ 2 µg/ml, but some have been reported to be susceptible to carbapenems. The reason for this discrepancy is that full resistance to carbapenems usually requires the presence of a second mechanism of resistance such as a defect in the permeability of outer membrane. Detection of blaKPC genes by PCR has been proposed as the gold standard for detection of KPC-bearing organisms. To date, several PCR based detection methods have been described , including two real-time PCR assays, as well as a method that uses PCR in conjunction with electrospray ionization mass spectrometry (PCR/ESI-MS) (Chen et al., 2011).

Currently DNA sequencing is the definitive method for identification of blaKPC gene. However, sequencing is impractical for studies involving large sample sizes, as well as for rapid identification in clinical settings. In contrast, methods such as real-time PCR offer rapid, robust, and cost-efficient alternatives to DNA sequencing for blaKPC gene (Chen et al., 2011).

Our study showed that 91 out of 150 (60.6%) had KPC gene by real- time PCR. This result is higher than that from a United State (0.5%) (Deshpande et al., 2006). However, prevalence rates of (KPC-Kp) isolates of >30% have been recorded in the eastern United States (Nordmann et al., 2011). Another study of Brooklyn hospitals reported 38% prevalence of blaKPC (Landman et al., 2007). In this study, there were 2 cases which were negative for blaKPC by PCR and in the same time were resistant by disk diffusion. These results were not due to inhibition of the PCR reactions because the internal control target was successfully amplified.

The negative PCR result with the presence of disk diffusion resistance can be due to presence of eleven types or alleles of KPC gene (KPC1-11) while we used only single primers for detection of blaKPC (Arnold et al., 2012),or It is possible that the carbapenem resistance was due to one of several other mechanisms, including changes in outer membrane permeability, increased activity of antibiotic efflux systems, or the production of AmpC β-lactamases, ESBLs, or non-KPC carbapenemases (Queenan and Bush, 2007).

In addition, it is also possible that the amount of template DNA for these samples was inadequate or that sequence alterations in the bla KPC gene affected the binding of the primers or probes used in the assay (Francis et al., 2012).

One of the disadvantages of PCR is specificity for the particular target sequence, so they cannot be used to monitor the emergence of novel variants (Raghunathan et al., 2011).

In this study, out of the 91 blaKPC positive isolates 46.1% were *K. pneumonia*. These results are in accordance with those from a study of Landman et al. (2007), where (95%) of blaKPC positive isolates were *K. pneumoniae*. Another study conducted by Qi et al. (2011) shows that all clinical isolates of *K. pneumoniae* (100%) with carbapenem resistance were confirmed as KPC producers by PCR.

This is different from study done by (Francis et al. (2012), who showed that the most common KPC positive enterobacteriaceae was *Escherichia coli* (44.9%), and a study done by Marschall et al. (2009),where (37.0%) of isolates were *E. coli.*

K. pneumoniae remains the most prevalent bacterial species carrying KPCs .The rapid global spread of KPC-producing *K. pneumoniae* is now understood , it is a largely clonal phenomenon. A specific clone of KPC-producing *K. pneumoniae*, called ST258, is globally distributed. ST stands for sequence type, and is assigned by multilocus sequence typing, which is a nucleotide sequence-based bacterial typing method where seven genes on the chromosome are sequenced. ST258 predominates among KPC-producing *K. pneumoniae* in the United States. ST258 as well as ST512, which is closely related to ST258, has been found commonly in Israel and Italy, whereas ST11 and ST437 appear to predominate in China and Brazil, respectively (Doi and Paterson, 2015).

These STs are all closely related to ST258 suggesting the presence of a common origin, most likely in the mid-Atlantic United States. On the other hand, plasmids carrying the KPC gene are diverse in structure and often capable of self-transmission to other strains by conjugation (Doi and Paterson, 2015).

In addition, the production of carbapenemases especially KPC is the most important mechanism of enzymatic resistance in isolated enterobacteriaceae such as *K. pneumoniae*. KPCs are encoded by the gene bla-KPC, whose potential for different species and universal spreading is mainly elucidated by its location within a Tn3-type transposon, Tn4401. This transposon is able to inserting into varied plasmids of Gram-negative bacteria. Plasmids carrying bla-KPC are related to resistance factors for other antibiotics, the enzyme has been identified in several other Gram-negative bacilli (Bina et

al., 2015).

Hospital acquired infections (HAI) are defined as infections not present at the time of admission to hospital. Most infections that become clinically evident after 48 h of hospitalization are considered hospital-acquired (Edwards et al., 2008).

In our study, we found that 95.6% of KPC PCR positive isolates were from hospital acquired infection cases and this was also statistically highly significant ($P < 0.001$). This was in accordance with Aggeliki et al. (2012) who reported the recent emergence of carbapenemase-producing enterobacteriaceae strains which represented a major threat for hospitalized patients in Greek hospitals, and also show that duration of hospitalization before bacteremia was the only risk factor for multidrug bloodstream infections.

The description of outbreaks indicates that producer strains seem to benefit from selective advantages in hospitals where antimicrobial use is much higher and opportunities for transmission more frequent than in the community (Grundmann et al., 2010). According to Papadimitriou -Olivgeris et al. (2012) there was no patient positive for blaKPC without prior hospitalization or antibiotic use before ICU admission.

In our study we found that the high percentage of KPC-producing enterobacteriaceae spp. by PCR was found in patients with history of antibiotic administration (70.3%). This also coincides with Gasink et al. (2009) who found a correlation between the selective pressure of antimicrobial agents and the presence of KPC resistance genes residing on the plasmid.

According to Woodford et al. (2010) and Gasink et al. (2009) it was postulated that prior use of an extended-spectrum cephalosporin and ciprofloxacin may be selective for KPC enzymes and also it was reported by Kwak et al. (2005) that the previous use of carbapenems and cephalosporins were identified as independent risk factors for acquisition of carbapenem resistant *K. pneumonia*. In rectal and tracheal KPC- *K. pneumoniae* colonized patients, prolonged antibiotic therapy administered for non KPC-Kp infection predisposes patients to subsequent KPC-Kp ventilator associated pneumonia (VAP). Short prophylaxis of early pneumonia with amoxicillin/clavulanic acid, reducing the need for subsequent antibiotic use, may be associated with reduced risk for KPC-Kp VAP (Sbrana et al., 2016).

According to the study of Tuon et al. (2012), Fluoroquinolones were an independent risk factor for KPC production, which might be explained by the fact that plasmid-encoded qnr genes, which determine low-level fluoroquinolone resistance, have been identified in the same conjugative K. pneumonia plasmid as CP genes (specifically blaKPC-2 and qnrB4).

In our study, we found that (97.8%) of KPC PCR positive isolates had been exposed to invasive procedures such as mechanical ventilation, urinary catheterization, central venous line (CVL) and cannula insertion, and this was statistically highly significant ($P < 0.001$).

This is in accordance with Lee (2012) who reported that mechanical ventilation is a risk factor for infection with KPC producing organisms, and Kwak et al. (2005) who reported that catheterization is a risk factor for carbapenem-resistant acquisition.

KPC positive cases were mainly from urology department (74.1%) followed by ICU (57.6%). This is similar to that reported by Lee (2012) who showed that KPC positive cases were mainly isolated from patients who had high ICU admission status (72%) this may be due to that most of ICU patients were immunocompromised and may be due to the selective pressure imposed by extensive use of antimicrobials and the potential for patient-to-patient transmission of organisms was greatest.

Our study showed that 82 (98.8%) of carbapenem resistant isolates by using disk diffusion method, were blaKPC PCR positive cases. We found that the sensitivity of the PCR was 99%, specificity was 87%, PPV of 90%, NPP of 98% and diagnostic accuracy was 93%, all in relation to DDT as gold standard test.

This is similar to Hindiyeh et al. (2011) who reported that real-time PCR assay is sensitive and specific compared with culture-based methods of detecting carbapenem resistance attributable to KPC.

Also, Cole et al. (2009) reported that direct detection of blaKPC by PCR shorten the time to identify patients colonized or infected with carbapenem resistant organisms and is more sensitive than culture.

Conclusion

Real time PCR for detection CRE through detection of *bla*KPC gene in enerobacteriacea, is a sensitive, accurate, and rapid method with a shorter turnaround time than those with culture based protocols. Beside it has high negative predictive value to rule out the resistance to cabapenems.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Aggeliki EV, Georgia V, Grigorios X, Aris P, Vassiliki C, Fani M, Athanassios T (2012). Imported KPC-Producing Klebsiella pneumoniae Clones in a Greek Hospital: Impact of infection control measures to restrain their dissemination. J. Clin. Microbiol. 50(8):2618-2623.

Arnold R, Thom K, Sharma S, Phillips M, Johnson K, and Morgan D (2012). Emergence of Klebsiella pneumoniae Carbapenemase (KPC)-Producing Bacteria South. Med. J. 104(1):40-45.

Benenson S, Temper V, Cohen MJ, Schwartz C, Hidalgo-Grass C, Block C (2011).Imipenem Disc for Detection of KPC Carbapenemase-Producing Enterobacteriaceae in Clinical Practice. J. Clin. Microbiol. 49(4):1617-1620.

Bina M , Pournajaf A , Mirkalantari SH , Talebi M , Irajian GH (2015). Detection of the *Klebsiella pneumoniae* Carbapenemase (KPC) in K. pneumoniae Isolated from the Clinical Samples by the Phenotypic and Genotypic Methods. Summer 10(3):199-205.

Bratu S, Landman D, Haag R (2005). Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City: a new threat to our antibiotic armamentarium. Arch. Int. Med. 165(12):1430-1435.

Bulik C, Fauntleroy K , Jenkins S, La Bombardi M, Nicolau D, and Kuti J (2010).Comparison of meropenem MICs and susceptibilities for Carbapenemase-producing *Klebsiella pneumoniae* isolates by various testing methods. J. Clin. Microbiol. 48(7):2402-2406.

Burns K and Schaffer K (2011). CRE update issued on laboratory detection and infection control measures. Disease surveillance report of HPSC, Ireland. Epi Insight 12(5):33-47.

Chen L, Mediavilla JR, Endimiani A, Rosenthal ME, Zhao Y, Bonomo RA, Kreiswirth BN (2011). Multiplex Real-Time PCR Assay for Detection and Classification of *Klebsiella pneumoniae* Carbapenemase Gene (blaKPC) Variants. J. Clin. Microbiol. 49(2):579-585

Clinical Laboratory Standards Institute (CLSI) (2014). Performance standards for antimicrobial susceptibility testing; 24th informational supplements. CLSI document M100-S21, Wayne PA.

Cole JM, Schuetz AN, Hill CE, and Nolte FS (2009). Development and Evaluation of a Real-Time PCR Assay for Detection of Klebsiella pneumonia Carbapenemase Genes. J. Clin. Microbiol. 47(2):322-326.

Collee JG, Miles RS, Watt B (1996). Tests for the identification of bacteria In: Mackie & MacCartney, Practical medical microbiology. 14th edition, ch.7. by Collee JG, Fraser AG, Marmion BP and Simmons A (eds). Churchill Livingstone New York, USA. 1:131-149.

Deshpande LM, Jones RN, Fritsche TR, Sader HS (2006). Occurrence and characterization of carbapenemase-producing Enterobacteriaceae: Report from the SENTRY antimicrobial surveillance program (2000–2004). Microb. Drug. Resist. 12:223-230.

Doi Y, Paterson DL. (2015). Carbapenemase-Producing Enterobacteriaceae Semin. Respir. Crit. Care Med. 36:74-84.

Edwards JR, Peterson KD, Andrus ML, Dudeck MA, and Pollock DA, Horan TC (2008).National Healthcare Safety Network (NHSN) Report, data summary for 2006 through 2007, issued November 2008. Am. J. Infect. Control. 36(9):609-626.

Fallah F, Hakemi Vala M, Hashemi A, Shams S (2013):Emergence of Novel Plasmid-mediated Beta-lactamase in *Klebsiella pneumoniae*. Qom. Univ. Med. Sci. J. 6(4):104-116.

Francis R, Wu F, Della-Latta P, Shi J, and Whittier S (2012). Rapid detection of *Klebsiella pneumoniae* carbapenemase genes in Enterobacteriaceae directly from blood culture bottles by Real-Time PCR. Am. J. Clin. Pathol. 137:627-632.

Gasink LB, Edelstein PH, Lautenbach E, Synnestvedt M, Fishman NO (2009).Risk factors and clinical impact of *Klebsiella pneumoniae* carbapenemase-producing K. pneumoniae. Infect. Control Hosp. Epidemiol. 30:1180-1185.

Girlich D, Halimi D, Zambardi G, Nordmann P (2013): Evaluation of Etest® strips for detection of KPC and metallo-carbapenemases in Enterobacteriaceae. Diagn. Microbiol. Infect .Dis. 77(3):200-201.

Grundmann H, Livermore DM, Giske CG, Canton R, Rossolini GM, Campos J, Vatopoulos A, Gniadkowski M, Toth A, Pfeifer Y, Jarlier V, Carmeli Y (2010). The CNSE Working Group. Carbapenem- non - susceptible Enterobacteriaceae in Europe: Conclusions from a meeting of national experts. Euro. Surveill. 15(46):19711.

Gupta N, Limbago B, Patel J, and Kallen A (2011): Carbapenem-Resistant Enterobacteriaceae: Epidemiology and Prevention Clin. Infect. Diseases 53(1):60-67.

Hindiyeh M, Smollan G, Grossman Z, Ram D, Robinov J, Belausov N, Ben-David D, Tal I, Davidson Y, Shamiss A, Mendelson E (2011). Rapid detection of blaKPC carbapenemase genes by internally controlled real-time PCR assay using bactec blood culture bottles. J. Clinical Microbiol. 49(7):2480-2484.

Hindiyeh M, Smollen G, Grossman Z, Ram D, Davidson Y, Mileguir F, Vax M, David D, Tal I, Rahav G, Shamiss A, Mendelson E, and Keller N (2008). Rapid Detection of blaKPC Carbapenemase Genes by Real- Time PCR. J. Clin. Microbiol. 46(9):2879-2883.

Kwak YG, Choi SH, Choo EJ, Chung JW, Jeong JY, Kim NJ, Woo JH, Ryu J, Kim YS (2005). Risk factors for the acquisition of carbapenem-resistant Klebsiella pneumoniae among hospitalized patients. Microbial Drug Resistance 11(2):165-169.

Landman D, Bratu S, Kochar S, Panwar M, Trehan M, Doymaz M, and Quale J (2007). Evolution of antimicrobial resistance , among Pseudomonas aeruginos, Acinetobacter baumannii and Klebsiella pneumoniae in Brooklyn. J. Antimicrob. Chemother. 60:78-82.

Landman D, Salvani J, Bratu S, Quale J (2005). Evaluation of techniques for detection of carbapenem-resistant Klebsiella pneumoniae in stool surveillance cultures. J. Clin. Microbiol. 43:5639-5641.

Lee GC (2012). Epidemiology and treatment of KPC'S…What'S the news? Review of Current Evidence. Pharmacotherapy Rounds 2012 https://flutrackers.com/forum/forum/antibiotic-resistant-bacteria-including-mrsa-beta-lactamases/125137-epidemiology-and-treatment-of-kpc%C2%92s-klebsiella-pneumoniae-carbapenemase-%C2%85what%C2%92s-the-news.

Marschall J, Robert J, Tibbetts W, Dunne M, Frye J, Fraser V, and Warren D (2009). Presence of the KPC carbapenemase gene in enterobacteriacea causing bacteremia and its correlation with in vitro carbapenem susceptibility. J. Clin. Microbiol. 47(1):239-241.

Nordmann P, Poirel L, Dortet L (2012). Rapid Detection of Carbapenemase producing Enterobacteriaceae. Emerg. Infec. Dis.18:1503-1510.

Nordmann P, Naas T, and Poirel L (2011). Global spread of carbapenemase producing enterobacteriaceae. Emerg. Infect. Dis.17(10):105-109.

Papadimitriou-Olivgeris M, Fligou F, Marangos M, Bartzavali C, Vamvakopoulou S, Sklavou C, Anastassiou ED, Christofidou M, Filos K (2012). Risk factors for KPC-producing *Klebsiella pneumoniae* faecal carriage of patients admitted at an intensive care unit. 22nd Europ. Congress Clin. Microbiol. Infect. Dis. (ECCMID) 31.03.2012 - 03.04.2012 .

Patel G, Huprikar S, Factor SH, Jenkins S, and Calfee DP (2008): Outcomes of Carbapenem-Resistant *Klebsiella pneumoniae* Infection and the Impact of Antimicrobial and Adjunctive Therapies. Infect. Control Hosp. Epidemiol. 29(12):1099-1106.

Phumisantiphong U (2011). Incidence of carbapenem resistant Enterobacteriaceae. Vajera Med. J. 55(3):265-272.

Qi Y, Wei Z, Ji S, Du Z, Shen P, Yu Y (2011). ST11, the dominant clone of KPC-producing Klebsiella pneumoniae in China. J. Antimicrob. Chemother. 66:307-312.

Queenan A, Bush K (2007). Carbapenemases: the Versatile β-Lactamases. Clin. Microbiol. Rev. 20:440-458.

Raghunathan A, Samuel L, and Tibbetts RL (2011). Evaluation of a Real-Time PCR Assay for the Detection of the *Klebsiella pneumoniae* Carbapenemase Genes in Microbiological Samples in Comparison With the Modified Hodge Test Am. J. Clin. Pathol. 135:566-571.

Rapp RP, Urban C (2012). *Klebsiella pneumoniae* carbapenemases in Enterobacteriaceae: history, evolution, and microbiology concerns. Pharmacotherapy. 32(5):399-407.

Samra Z, Ofir O, Lishtzinsky Y, Madar-Shapiro L, Bishara J (2007). Outbreak of carbapenem-resistant *Klebsiella pneumoniae* producing KPC-3 in a tertiary medical centre in Israel. Int. J. Antimicrob. Agents. 30:525-534.

Sbrana F, Malacarne P, Bassetti M, Tascini C, Vegnuti L, Della Siega P, Ripoli A, Ansaldi F, Menichetti F (2016). Risk factors for ventilator associated pneumonia due to KPC-producing *Klebsiella pneumoniae* in mechanically ventilated patients with tracheal and rectal colonization. Minerva Anestesiol. 8 Jan 2016.

Schechner V, Straus-Robinson K, Schwartz D, Pfeffer I, Tarabeia J, Moskovich R, Chmelnitsky I, Schwaber MJ, Carmeli Y, Navon-Venezia S (2009). Evaluation of PCR-based testing for surveillance of KPC-producing carbapenem-resistant members of the Enterobacteriaceae family. J. Clinical Microbiol. 47(10):3261-3265.

Thomson K (2010). Extended-Spectrum-β-Lactamase, AmpC, and carbapenemase Issues. J. Clin. Microbiol. 48(4):1019-1025.

Tibbetts R, Frye JG, Marschall J, Warren D, Dunne W (2008). Detection of KPC-2 in a clinical isolate of *Proteus mirabilis* and first reported description of carbapenemase resistance caused by a KPC β-lactamase in P. mirabilis. J. Clinical Microbiol. 46(9):3080-3083.

Tuon FF, Rocha JL, Toledo P, Arend LN, Dias CH, Leite TM,

Penteado-Filho SR, Pilonetto M, Zavascki AP (2012) . Risk factors for KPC-producing *Klebsiella pneumoniae* bacteremia .Braz. J. Infect. Dis. 16(5):416-419.

Villegas MV, Lolans K, Correa A, Suarez CJ, Lopez JA, Vallejo M, Quinn JP (2006). Colombian Nosocomial Resistance Study Group, First detection of the plasmid-mediated class A carbapenemase KPC-2 in clinical isolates of *Klebsiella pneumoniae* from South America. Antimicrobial Agents Chemother. 50(8):2880-2882.

Woodford N, Zhang J, Warner M, Kaufmann M, Matos J, Yang Q, Wang H, Sun H, Chen H, Xu Y, Chen M (2010). Phenotypic and genotypic characterization of Enterobacteriaceae with decreased susceptibility to carbapenems: Results from large hospital-based surveillance studies in China. J. Antimicrob. Chemother. 54(1):573-377.

academicJournals

Vol. 10(34), pp. 1408-1416, 14 September, 2016
DOI: 10.5897/AJMR2016.8179
Article Number: 627BEFF60512
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of antibacterial activity of some non-steroidal anti-inflammatory drugs against *Escherichia coli* causing urinary tract infection

Eman Farouk Ahmed[1], Rehab Mahmoud Abd El-Baky[2], Abo Bakr F Ahmed[2,3], Nancy Gameel Fawzy[2], Neveen Abdel Aziz[4]* and Gamal Fadl Mahmoud Gad[2]

[1]Department of Microbiology and Immunology, Faculty of Pharmacy, Deraya University, Minia, Egypt.
[2]Department of Microbiology and Immunology, Faculty of Pharmacy, Minia University, Minia, Egypt.
[3]Department of Microbiology and Immunology Faculty of Pharmacy, Hail University, KSA, Saudi Arabia.
[4]Department of Microbiology and Immunology, Faculty of Pharmacy, Ahram Canadian University, 12451- Sixth of October City, Egypt.

Received 23 June 2016, Accepted 19 August, 2016.

Extensive use of antibiotics for urinary tract infections has led to the emergence of drug-resistant microorganisms and one solution to this problem is to search for non-antibiotic compounds that exert anti-bacterial activity through different mechanisms such as non-steroidal anti-inflammatory drugs (NSAIDs). In this study, out of 100 urine samples; 48 *Escherichia coli* strains were detected, 47.9% were multi-drug resistant. The antibiogram resistance pattern of the strains was carried out by agar dilution method. Diclofenac sodium, indomethacin, aspirin and ibuprofen were tested against the *E. coli* isolates. Diclofenac sodium showed the lowest MIC_{50} and MIC_{90}; 8 and 256 μg/ml, respectively. Aspirin showed MIC_{50} of 64 μg/ml, while both indomethacin and ibuprofen showed MIC_{50} of 256 μg/ml. Indomethacin, aspirin and ibuprofen showed the same MIC_{90} of 1024 μg/ml. The combined effects of the four NSAIDs and five antibiotics (Amoxicillin, Augmentin, Cefotaxime, Ciprofloxacin and Gentamicin) were tested on five resistant clinical *E. coli* strains by checkerboard dilution technique. All the tested NSAIDs significantly reduced the minimum inhibitory concentrations (MICs) of antibiotics against the tested bacteria and fractional inhibitory concentration indices (FICIs) for this combination ranged from 0.03 to 0.5. In this study, leakage of intracellular components suggests that the effect of NSAIDs on *E. coli* could be the formation of pores in the plasma membrane and scanning electron microscopy (SEM) observations confirmed the damage to the structural integrity of the tested bacteria. In conclusion, NSAIDs showed antibacterial activity against *E. coli* causing urinary tract infections (UTIs), a combination of them and antibiotics exhibited good synergism and the mechanism of their action was by damaging the bacterial cell membrane.

Key words: Urinary tract infection (UTI), *Escherichia coli*, NSAIDs, antibacterial resistance, antibacterial activity, synergism.

INTRODUCTION

Urinary tract infections (UTIs) are the most common hospital acquired infection with a percentage of 35% of

nosocomial infections (Stamm, 2002; Weinstein et al., 1997). *Escherichia coli* is the major pathogen causing urinary tract infections (UTIs) and represents more than 85% of recurrent cystitis and about 35% of recurrent pyelonephritis (Barnett and Stephens, 1997). UTIs remain the most common human bacterial infection, despite the high spread of antibiotics. The massive and irrational use of antibiotics and antibacterial agents for long periods has led to the emergence of multi drug resistant (MDR) microorganisms, and it is currently advised that the clinical administration of antibiotics against the pathogenic bacteria be gradually prohibited (Ray and Rice, 2004; Chowdhary et al., 1994). Another solution for this problem is to search for non-antibiotic compounds that have antibacterial activity through different mechanisms (Mazumdar et al., 2009). Recent studies have shown that some medicines have antibacterial activity in addition to their main function such as antihistamines, antipsychotics, tranquillizers, anti-hypertensives and local anesthetics (Rani et al., 2005; Dastidar et al., 1995). All these drugs with moderate to powerful anti-microbial activities have been known as “non-antibiotics” (Dastidar et al., 2000). Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used medicines for pain and inflammation management and previous studies have revealed that some NSAIDs have antibacterial activity (Wang et al., 2003; Hersh et al., 1991). NSAIDs exhibited strong antimicrobial activity when tested against a large number of Gram-positive and negative bacteria and the MIC ranged from 50-200 µg/mL in most of the cases and even lower in some cases (Annadurai et al., 1998; Sukul et al., 2015; Obad et al., 2015). The antibacterial agents, whether bacteriostatic or bactericidal, might act by inhibition of microbial cell wall synthesis, alteration of membrane function or membrane damage, inhibition of nucleic acid synthesis or inhibition of protein synthesis (Mazumdar et al., 2006). The aim of this study was to detect antibacterial activity of some NSAIDs (diclofenac sodium, aspirin, indomethacin and ibuprofen) against *E. coli* isolates causing UTIs, examine the effect of their combination with different antibiotics and finally detect the possible mechanism of antibacterial action of these NSAIDs if present.

MATERIALS AND METHODS

Isolation of bacterial strains

One hundred urine samples were collected from UTI patients in Minia University Hospitals (MUH) in Minia, Egypt during the study period, from May 2014 to December 2015. Informed consent was obtained from all the subjects. Urine samples were inoculated on cysteine lactose electrolyte deficient (CLED) media (Lab, UK) (Winn and Koneman, 2006). All the samples were examined for the presence of *E. coli* by streaking them onto MacConkey agar (Lab, UK), EMB agar (Himedia, India) and incubating the plates at 37°C for 24 h. Identification of *E. coli* was based on fermentation of lactose giving pinkish colonies. Further identification was done by biochemical (citrate and triple sugar iron) tests. Bacteria were maintained by storage at -70°C on tryptone soy broth (TSB) medium (Himedia, India) enriched with 20% glycerol (Rusu et al., 2014; Nobmannn et al., 2010).

Drugs

The following NSAIDs were used: Diclofenac sodium (Glaxo, Egypt), Ibuprofen (Kahira/Abbott, Egypt), Aspirin and Indomethacin (Kahira, Egypt). The following antibiotics were used: Ampicillin, Amoxicillin (EIPCO, Egypt), Augmentin (Sedico, Egypt), Cephalexin (Glaxo, Egypt), Cephradin (Smithkline, Egypt), Cefotaxime (EIPCO, Egypt), Ciprofloxacin (Amriya, Egypt) and Gentamicin (Memphis, Egypt). Working solution concentrations ranged from 5-1.6 mg/ml. All the drugs were obtained as pure dry powder and stored at 4°C.

Susceptibility testing

Bacterial cultures were tested against some NSAIDS (diclofenac sodium, aspirin, indomethacin and ibuprofen) by agar dilution method (CLSI, 2005). Mueller-Hinton agar (MHA) plates (Lab, UK) contained two fold serial dilutions of NSAIDS from 0.25 to 1024 µg/mL. Bacterial suspensions of isolated bacteria were made in sterile saline and matched with McFarland index 0.5 tubes. Each bacterial suspension (1 µl) was inoculated (3×10^5 CFU/spot) on drug containing plates and incubated at 37°C for 24 h.

Determination of interaction between NSAIDs and antibiotics by checkerboard dilution technique

Two drugs combined effects were determined by the Checkerboard dilution technique to determine the fractional inhibitory concentration (FIC) indices. Definition of FIC is as follows: MIC of $substance_A$ tested in combination/MIC of $substance_A$ tested alone + MIC of $substance_B$ tested in combination/MIC of $substance_B$ tested alone. The FIC index (FICI) was calculated using the following formula:

$FIC\ index = FIC_A + FIC_B = [A]/\ MIC_A + [B]/MIC_B$.

Synergism is shown as FIC index of ≤0.5, while indifference is showed as an FIC index of >0.5 ≤4 and antagonism is shown as an FIC index of>4. FIC index was an average of two independent experiments (Lorian, 2005).

Membrane-permeability assay

Membrane-disruptive activity of Amoxicillin, diclofenac sodium, aspirin, indomethacin, ibuprofen and amoxicillin/aspirin on *in vitro* grown *E. coli* (ATCC 8739) was determined by measuring the fluorescence enhancement of ethidium bromide (Sigma) (Paixão et al., 2009). To this end, *E. coli* were grown in tryptone broth medium in the presence or absence of amoxicillin as positive control, NSAIDs and Amoxicillin combined with aspirin for 24 h. The bacterial culture was incubated with ethidium bromide for 20 min at room temperature in the dark. Membrane permeability was

*Corresponding author. E-mail: neveen.abdelaziz@gmail.com. Tel: 02001005202136.

Table 1. Distribution of minimum inhibitory concentrations and the prevalence of antibiotics resistance among the isolated *E. coli.*

Antibiotics	MIC (μg/ml)													MIC_{50}	MIC_{90}	R	%
	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024				
Ampicillin	0	0	0	4	3	13	1	0	2	4	7	3	11	128	1024	27	56.3
Amoxicillin	0	0	0	10	1	5	0	2	2	1	1	3	23	512	1024	32	66.7
Augmentin	0	0	3	10	2	2	11	7	4	2	1	1	5	32	512	20	41.7
Cephalexin	0	0	0	7	6	11	3	7	2	2	2	4	4	8	512	21	43.8
Cephradin	0	0	2	7	1	6	12	2	4	6	3	1	4	16	256	20	41.7
Cefotaxime	0	2	19	17	5	0	3	0	0	0	0	1	1	2	4	10	20.8
Ciprofloxacin	2	11	23	3	3	4	0	0	0	0	1	1	0	1	8	9	18.8
Gentamicin	0	0	18	13	7	7	1	1	1	0	0	0	0	2	8	3	6.3

determined by measuring the ethidium bromide fluorescence (excitation at 518 nm, emission at 605 nm). Fluorescence values presented are corrected with those obtained from untreated bacteria (Bink et al., 2012).

Loss of 260 nm absorbing material

The release of UV-absorbing material concentrations were determined by UV spectrophotometer (Zhou et al., 2008). Loss of 260 nm absorbing material released from bacteria was measured by the technique performed by Devi et al. (2010).Overnight broth cultures of *E. coli* ATCC 43889 in tryptone broth medium were adjusted to OD 600. Cells were harvested by centrifugation at 400 rpm for 15 min, supernatant was discarded, and pellet was washed twice and re-suspended in phosphate buffer saline (PBS) at pH 7.4. Different concentrations of NSAIDs [1\2MIC, MIC, 2MIC and 4MIC] were added to the cell suspension. Amoxicillin (1.6 mg/ml) was used as positive control. The experiment was done in triplicates. Cells without NSAIDs treatment were used as control. All the samples were incubated at 37°C for 60 min. After treatment, the cell suspension was centrifuged at 13,400 rpm for 15 min and OD 260 value of the supernatant was taken as a percentage of the released extracellular UV-absorbing materials. All the measurements were done in triplicates in Jenway 7305 UV spectrophotometer (UK).

Scanning electron microscopy (SEM)

E. coli (ATCC 8739) cells were suspended in saline solution containing 0.2% Tween-80 and incubated at 37°C with Amoxicillin, diclofenac sodium, aspirin, indomethacin, ibuprofen and Amoxicillin/aspirin at 2× MIC at room temperature. After 24 h, the bacterial cells were centrifuged at 8000 rpm for 15 min. The bacterial cells were then washed with 0.1 mol/l tris-acetate buffer (PH 7.1), fixed in tris-acetate buffer containing 1.5% glutaraldehyde, and then freeze-dried. Each bacterial culture was observed by SEM (Hitachi, Japan) at magnifications of 10000, 7500 and 15000x. The bacterial cell suspension in saline with no NSAIDs treatment served as a negative control (Soboh et al., 1995).

Statistical analysis

Statistical analysis was performed using one way Anova test. P values of <0.05 were considered indicative of statistically significant differences.

RESULTS

Antibiogram pattern of the isolates

A total of 100 clinical samples were examined. All of these were urine samples from patients with UTI. Among the 100 patients samples, 48 *E. coli* strains were isolated (48%) and out of them; 23 strains (47.9%) were normally resistant to most of the antibiotics showing multi drug resistance (MDR). The antibiogram resistance pattern of the isolates, as shown in Table 1 was: amoxicillin (66.7%), ampicillin (56.3%), cephalexin (43.8%), augmentin (41.7%), cephradin (41.7%), cefotaxime (20.8%), ciprofloxacin (18.8%) and gentamicin (6.3%). *E. coli* (ATCC 8739) showed sensitive antibiogram pattern as illustrated in Table 1.

In vitro antimicrobial action of NSAIDs

NSAIDs were tested against a total of 48 isolates of *E. coli* as shown in Table 2. Diclofenac sodium showed the lowest MIC_{50} and MIC_{90}: 8 and 256 μg/ml, respectively. Aspirin showed MIC_{50} of 64 μg/ml, while both indomethacin and ibuprofen showed MIC_{50} of 256 μg/ml. Indomethacin, aspirin and ibuprofen showed the same MIC_{90} of 1024 μg/ml. But for the standard strain as illustrated in Table 3, indomethacin showed the lowest MIC: 128 μg/ml, followed by aspirin: 256 μg/ml. Diclofenac sodium and ibuprofen showed the same MIC: 1024 μg/ml.

Determination of interaction between NSAIDs and antibiotics by checkerboard dilution technique

The combined effects of the four NSAIDs (diclofenac sodium, indomethacin, aspirin and ibuprofen) and five antibiotics (amoxicillin, augmentin, cefotaxime, ciprofloxacin and gentamicin) were tested on five resistant clinical *E. coli* strains. All the tested NSAIDs significantly lowered the MICs of antibiotics against the tested bacteria. The synergistic effects of NSAIDs and five antibiotics combination are shown in Tables 4, 5, 6 and 7.

Table 2. Distribution of minimum inhibitory concentrations of NSAIDs among the isolated *E. coli.*

Drug	MIC (µg/ml)													MIC_{50}	MIC_{90}
	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		
Diclofenac sodium	4	2	1	1	3	16	2	1	2	5	7	1	3	8	256
Indomethacin	0	0	0	5	4	6	2	2	1	2	3	9	14	256	1024
Aspirin	0	0	1	8	2	2	2	1	2	2	2	3	23	64	1024
Ibuprofen	0	1	1	5	3	8	2	0	1	2	2	13	10	256	1024

Table 3. Distribution of MICs of NSAIDS and antibiotics against the standard strain.

Drug	MIC (µg/ml)
Diclofenac sodium	1024
Indomethacin	128
Aspirin	256
Ibuprofen	1024
Ampicillin	8
Amoxicillin	8
Augmentin	16
Cephalexin	16
Cefotaxime	1
Gentamicin	≤0.25
Ciprofloxacin	≤0.25

FICIs for this combination ranged from 0.03 to 0.5 against the tested bacteria. All the examined *E. coli* showed high reduction in MIC values with NSAIDs and the five antibiotics. On the other hand, the combined effects of the four NSAIDs and the five antibiotics on standard *E. coli* strain are shown in Table 8. These results showed that NSAIDs have a synergistic effect when combined with antibiotics and this combination could effectively inhibit UTIs causing bacteria.

Membrane-permeability assay

These results suggested an effect of pretreatment of NSAIDs on the *E. coli* activity. It is hypothesized that NSAIDs affect membrane permeability of the tested *E. coli* cells, exhibited by the use of the fluorescent dye ethidium bromide. It is revealed that *E. coli* treated with different concentrations of NSAIDs during the growth phase resulted in a significantly increased membrane permeability of *E. coli* cells compared to the untreated ones as found by the significant increase in fluorescence of NSAIDS-treated cells (Figure 1). The presented fluorescence values are corrected with those obtained from untreated bacteria.

Effect of NSAIDs on leakage of 260 nm absorbing materials from *E. coli*

The measurement of release of UV-absorbing materials is an index of cell lysis (Zhou et al., 2008). The leakage of cytoplasmic membrane was analyzed by determining the release of cellular materials including nucleic acids, metabolites and ions, which were absorbed at 260 nm into the bacterial suspensions (Bajpai et al., 2014). After treatment with different concentrations of NSAIDs, the OD significantly increased up to 1.87 from 0.00 (P value < 0.05) as shown in Table 9. These results suggest that NSAIDs damage cytoplasmic membrane and cause subsequent leakage of intracellular constituents.

Scanning electron microscopy (SEM)

SEM images showed differences in cell structures between NSAIDs-treated bacteria and the non-treated control bacteria. Non-treated bacteria were intact (regular rod shaped) and showed smooth surfaces as seen in Figure 2A, while bacterial cells treated with the individual NSAIDs underwent considerable structural changes as shown in Figures 2B to G. SEM observations confirmed the damage to the structural integrity of the cells and considerable morphological alteration to the tested bacteria. In Figure 2G, combined NSAIDs treatments altered the outer membrane, the structures of the cells and made them more permeable.

DISCUSSION

E. coli is the major bacterial uropathogen in the world (Miragliotta et al., 2008). In the study on 100 urine samples, 48 (48%) *E. coli* strains were detected. This is similar to findings from studies done in other countries such as India (50, 59 and 68%) (Ranjini et al., 2015; Kothari and Sagar, 2008; Tambekar et al., 2006) and Madagascar (67%) (Randrianirina et al., 2007). Another study performed in Egypt reported that *E. coli* was in 36% of UTIs patients (Alabsi et al., 2014). A study performed in South Africa revealed that *E. coli* was present in 75% of uncomplicated and 59% of complicated UTIs and it was similar to this study (Agpaoa et al., 2015). In this study, 23 strains (47.9%) of *E. coli* isolates were normally resistant to most of the antibiotics showing multi-drug resistance (MDR). The antibiogram resistance pattern of the strains was: Amoxicillin (66.7%), Ampicillin (56.3%),

Table 4. Synergistic effect of diclofenac sodium combination with five antibiotics on resistant clinical *E. coli* strains.

Antibiotics	FIC_A	FIC_B	FIC_{index}	Synergistic
Amoxicillin	0.004	0.5	0.5	S
Augmentin	0.02	0.01	0.03	S
Cefotaxime	0.008	0.06	0.07	S
Gentamicin	0.25	0.01	0.3	S
Ciprofloxacin	0.004	0.5	0.5	S

S = Synergistic effect.

Table 5. Synergistic effect of indomethacin combination with five antibiotics on resistant clinical *E. coli* strains.

Antibiotics	FIC_A	FIC_B	FIC_{index}	Synergistic
Amoxicillin	0.1	0.1	0.2	S
Augmentin	0.03	0.1	0.1	S
Cefotaxime	0.03	0.3	0.3	S
Gentamicin	0.13	0.02	0.2	S
Ciprofloxacin	0.5	0.004	0.5	S

S = Synergistic effect.

Table 6. Synergistic effect of aspirin combination with five antibiotics on resistant clinical *E. coli* strains.

Antibiotics	FIC_A	FIC_B	FIC_{index}	Synergistic
Amoxicillin	0.02	0.01	0.03	S
Augmentin	0.02	0.06	0.1	S
Cefotaxime	0.004	0.03	0.03	S
Gentamicin	0.1	0.001	0.1	S
Ciprofloxacin	0.1	0.001	0.1	S

S = Synergistic effect.

Table 7. Synergistic effect of ibuprofen combination with five antibiotics on resistant clinical *E. coli* strains.

Antibiotics	FIC_A	FIC_B	FIC_{index}	Synergistic
Amoxicillin	0.01	0.3	0.3	S
Augmentin	0.03	0.02	0.05	S
Cefotaxime	0.1	0.02	0.1	S
Gentamicin	0.1	0.002	0.1	S
Ciprofloxacin	0.13	0.001	0.1	S

S = Synergistic effect.

Cephalexin (43.8%), Augmentin (41.7%), Cephradin (41.7%), Cefotaxime (20.8%), Ciprofloxacin (18.8%) and Gentamicin (6.3%). A study done in Egypt revealed the same percentage of MDR *E. coli*: 40% (Alabsi et al., 2014). A very high degree of MDR of 82.5% among *E. coli* isolates was reported by Ranjini et al. (2015). This

Table 8. Distribution of NSAIDs/antibiotics FIC_{index}against the *E. coli* standard strain.

	Diclofenac sodium	Indomethacin	Aspirin	Ibuprofen
Amoxicillin	0.03 (S)	2 (I)	0.03 (S)	0.03 (S)
Augmentin	0.3 (S)	0.5 (S)	0.02 (S)	0.02 (S)
Cefotaxime	0.3 (S)	0.3 (S)	0.3 (S)	0.3 (S)
Gentamicin	1 (I)	1 (I)	1 (I)	1 (I)
Ciprofloxacin	1 (I)	1 (I)	1 (I)	1 (I)

S = Synergistic effect, I = additive effect, A = antagonistic effect.

Table 9. Effects of NSAIDs at different concentrations on membrane integrity in *E. coli* standard strain measured by release of UV absorbing components at 260 nm.

Drug	1/4MIC		MIC		2MIC		4MIC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Amoxicillin	0.592	±0.001	0.989	±0.002	1.199	±0.004	1.433	±0.001
Aspirin	0.422	±0.008	0.840	±0.012	1.335	±0.001	1.625	±0.010
Indomethacin	0.030	±0.015	1.103	±0.006	1.400	±0.000	1.662	±0.008
Diclofenac sodium	1.468	±0.005	1.565	±0.012	1.583	±0.003	1.746	±0.026
Ibuprofen	0.531	±0.000	1.083	±0.002	1.356	±0.002	1.529	±0.002
Aspirin/Amoxicillin	0.963	±0.003	1.342	±0.001	1.589	±0.018	1.642	±0.002
Indomethacin/Amoxicillin	1.644	±0.000	1.719	±0.017	1.714	±0.001	1.867	±0.017
Diclofenac/Amoxicillin	1.338	±0.003	1.548	±0.005	1.649	±0.027	1.671	±0.015
Ibuprofen/Amoxicillin	0.277	±0.027	0.807	±0.031	1.023	±0.006	1.536	±0.001

SD = Standard deviation.

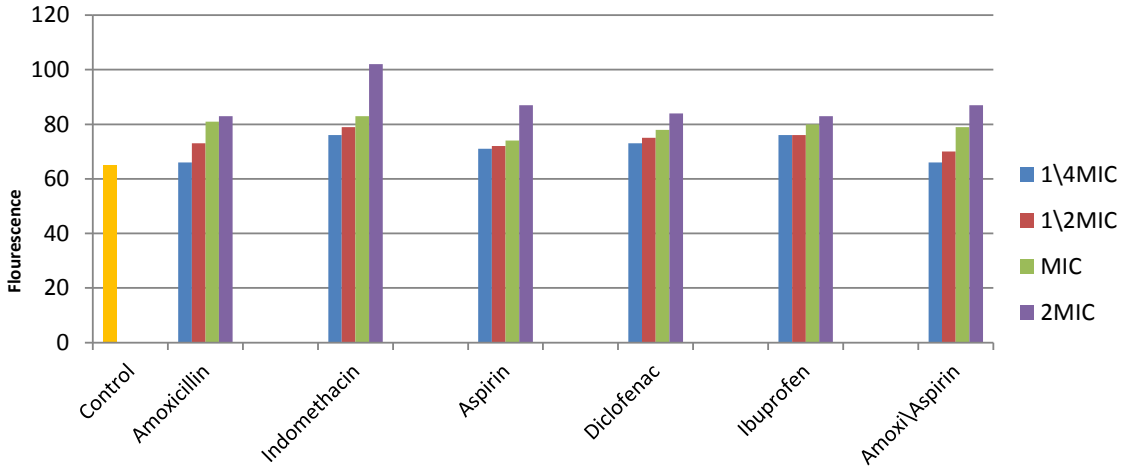


Figure 1. Membrane-disruptive activity of NSAIDs on *in vitro* grown *E. coli*. Data presented are the mean and standard error of the mean of 3 independent biological repeats. Statistical analysis was performed using one way ANOVA test and relative to untreated bacteria ($P < 0.05$).

study is in accordance with the study of Mazumdar et al. (2006) who reported the antibiogram resistance pattern of the *E. coli* strains as: ampicillin (74.4%), augmentin (59%), cefotaxime (38%), and these findings were similar to the results of Samsygina et al. (2000) and Khan et al. (2002). Alabsi et al. (2014) from Egypt reported 89 and 57% resistance among urinary *E. coli* isolates to ampicillin and gentamicin, respectively. NSAIDs are commonly used medicines for the treatment of pain and inflammation. Many studies found that some NSAIDs

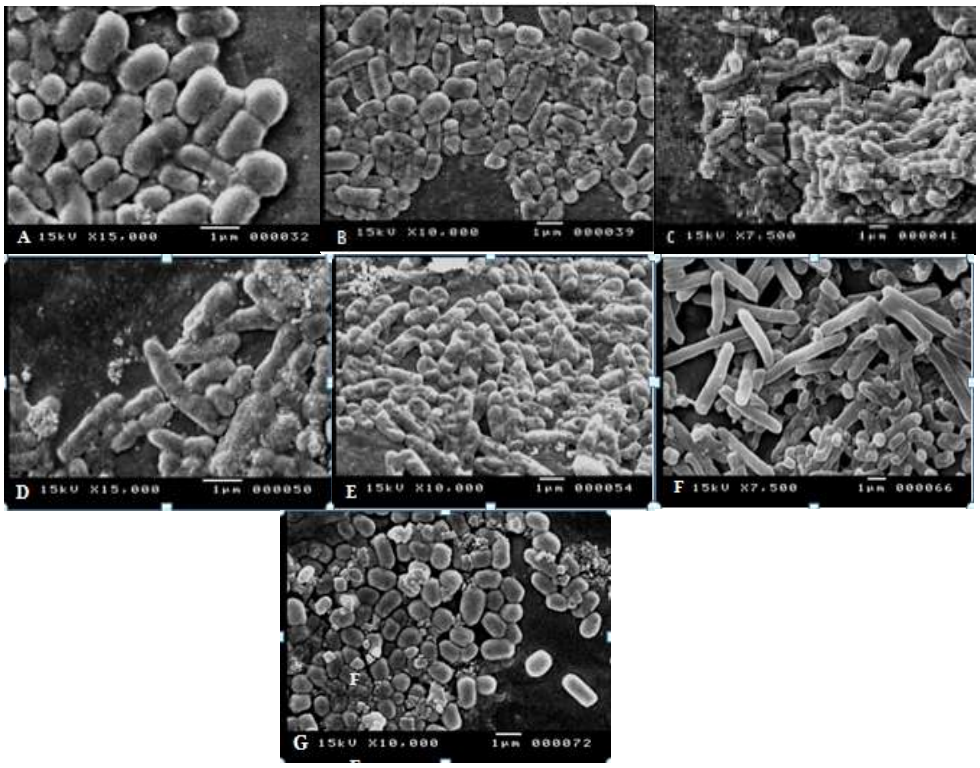


Figure 2. SEM images of (A) control, (B) amoxicillin treated cells, (C) aspirin treated cells, (D) indomethacin treated cells, (E) diclofenac sodium treated cells, (F) ibuprofen treated cells and (G) aspirin/amoxicillin treated cells.

have good antibacterial activity especially diclofenac sodium (Wang et al., 2003). In this study, some NSAIDs (diclofenac sodium, aspirin, indomethacin and ibuprofen) were tested against a total of 48 isolates of *E. coli*. Diclofenac sodium showed the lowest MIC_{50} and MIC_{90}; 8 and 256 µg/ml, respectively. Annadurai et al. (1998) reported that the MIC in most of the cases ranged from 50-200 µg/ml and even lower in some cases and diclofenac was bactericidal in its action. Dutta et al. (2007a) studied 32 isolates of *E. coli*, 8 were inhibited at 50 µg/ml diclofenac, 9 at 100 µg/ml, 5 at 400 µg/ml and the remaining isolates of *E. coli* were resistant to diclofenac (MIC≥ 800 µg/ml). In this study, aspirin showed MIC_{50} of 64 µg/ml, while both indomethacin and ibuprofen showed MIC_{50} of 256 µg/ml. Indomethacin, aspirin and ibuprofen showed the same MIC_{90} of 1024 µg/ml. Wang et al. (2003) tested the MICs of aspirin for 66 *H. pylori* isolates and the MIC_{50} of aspirin was 256 µg/ml, MIC_{90} was 512 µg/ml, and the range of MIC values was 256 to 512 µg/ml and this finding is close to the current study results. Activity of ibuprofen on *E. coli* was proximally studied by Al-Janabi (2010) and showed susceptibility to the tested agent at MIC of 2.5 mg/ml, which is higher than this results. There is an ongoing trial in Germany evaluating reduction of the use of antibiotics for uncomplicated UTI by giving initial management with ibuprofen (Gágyor et al., 2012). NSAID is equally effective as an antibiotic, and this may lead to a reduction in the use of antibiotics and reduce antibiotic resistance. This is good to the environment and will reduce the costs in health services internationally (Vik et al., 2014). The combined effects of the four NSAIDs (diclofenac sodium, indomethacin, aspirin and ibuprofen) and five antibiotics (amoxicillin, augmentin, cefotaxime, ciprofloxacin and gentamicin) were tested on five resistant clinical *E. coli* strains by checkerboard dilution technique. All the tested NSAIDs significantly reduced the MICs of antibiotics against the tested bacteria and FICIs for this combination ranged from 0.03 to 0.5 with respect to synergism. Dutta et al. (2007a) used the checkerboard technique giving a FIC index for *E. coli* of 0.49 for diclofenac and streptomycin, thereby showing a synergistic effect and another study showed that the combination effect of diclofenac with gentamicin/ampicillin which was examined by using checkerboard technique yielded FIC index ranging from 0.4 to 0.5 for diclofenac + gentamicin and values >1 for diclofenac + ampicillin (Dutta et al., 2009). In the present study, NSAIDs alone recorded antimicrobial activity, but NSAIDs in combination with antibiotics exhibited significant synergistic effect and the drugs were bactericidal. These data suggested that NSAIDs in combination with antibiotics could be useful for the treatment of complicated bacterial infections. In addition

to yielding these synergistic effects, the combinations of two or more compounds are important to prevent or suppress the developing of resistant strains, to decrease dose toxicity and to perform a broad spectrum activity (Eliopoulos and Moellering, 1996). The bacterial membrane is a structural component which may be damaged during a bactericidal challenge. Therefore, release of intracellular components is an indicator of membrane integrity. Small ions such as potassium and phosphate when treated with a suitable antimicrobial agent leach out first, followed by large molecules such as DNA, RNA and other materials. These substances have strong UV absorption at 260 nm, they are known as "260-nm absorbing materials" and this method is widely used in the determination of membrane integrity parameters (Denyer, 1990; Hugo and Snow, 1981). In this study, leakage of intracellular components suggests that the NSAIDs effect on *E. coli* can be through pores formation in the bacterial plasma membrane. The bacterial surface morphology alteration and cell damage could be confirmed thoroughly by SEM (Benli et al., 2008). In this study, SEM images showed differences in cell structures between NSAIDs-treated bacteria and the non-treated control bacteria. In addition, combined NSAIDs treatments altered the outer membrane as the structures of the cells made them more permeable. Thus, the mode of bactericidal action of NSAIDs against *E. coli* is through membrane disruption and so blocking the bacterial growth. The exact mechanism of antibacterial activity of diclofenac and ibuprofen is unclear. However, studies have suggested inhibition of bacterial DNA synthesis (Dutta et al., 2004) or impairment of membrane activity that agree with results obtained by SEM in this study (Hersh et al., 1991; Dutta et al., 2007a, b; Mohsen et al., 2015; Sikkema et al., 1995).

In conclusion, diclofenac sodium, aspirin, indomethacin and ibuprofen showed antibacterial activity against *E. coli* causing UTIs. This study results indicate that a combination of these NSAIDs and antibiotics exhibited good synergism against *E. coli* associated with UTIs, and the mechanism of their action was by damaging the bacterial cell membrane. This new finding of combination treatment with NSAIDS and antibiotics might provide an alternative way to overcome antibacterial drug resistance. However, further *in vivo* and clinical studies will be required to support this suggestion.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

Agpaoa VV, Mendoza BJ, Fernandezi AJM, Veloso JD, Bhatnagar S (2015). Predict Urinary Tract Infection and to Estimate Causative Bacterial Class in a Philippine Subspecialty Hospital. J. Nephrol Ther. 5:194.

Alabsi MS, Ghazal A, Sabry SA, Alasaly MM (2014). Association of some virulence genes with antibiotic resistance among uropathogenic *Escherichia coli* isolated from urinary tract infection patients in Alexandria, Egypt: A hospital-based study. J. Glob. Antimicrob. Resist. 2(2):83-86.

Al-Janabi AA (2010). In vitro antibacterial activity of Ibuprofen and acetaminophen. J. Glob. Infect. Dis. 2(2):105-108.

Annadurai S, Basu S, Ray S, Dastidar S, Chakrabarty A (1998). Antibacterial activity of the antiinflammatory agent diclofenac sodium. Indian J. Exp. Biol. 36(1):86-90.

Bajpai VK, Sharma A, and Baek KH (2014). Antibacterial mode of action of the essential oil obtained from Chamaecyparis obtusa sawdust on the membrane integrity of selected foodborne pathogens. Food Technol. Biotechnol. 52(1):109.

Barnett BJ and Stephens DS (1997). Urinary tract infection: an overview. Am J. Med. Sci.314(4):245-249.

Benli M, Yiğit N, Geven F, Güney K, Bingöl U (2008). Antimicrobial activity of endemic Crataegus tanacetifolia (Lam.) Pers and observation of the inhibition effect on bacterial cells. Cell Biochem. Funct. 26(8):844-851.

Bink A, Kucharikova S, Neirinck B, Vleugels J, Van Dijck P, Cammue BP, Thevissen K (2012). The nonsteroidal antiinflammatory drug diclofenac potentiates the in vivo activity of caspofungin against *Candida albicans* biofilms. J. Infect. Dis. 206(11):1790-1797.

Chowdhary M, Rehman K, Haq J (1994). Transferable drug resistance among the Enterobacteriaceae in urinary tract infection: A study at an urban hospital in Bangladesh. J. Trop. Med. Hyg. 97:161-166.

Clinical and Laboratory Standards Institute (CLSI) (2005). Performance Standards for Antimicrobial Susceptibility Testing: Fifteenth Informational Supplement M100-S15.; CLSI, Wayne, PA, USA.

Dastidar SG, Chaudhury A, Annadurai S, Roy S, Mookerjee M, Chakrabarty AN (1995). In vitro and in vivo antimicrobial action of fluphenazine. J. Chemother. 7(3):201-206.

Dastidar SG, Ganguly K, Chaudhuri K, Chakrabarty AN (2000). The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. Int. J. Antimicrob. Agents.14(3):249-251.

Denyer SP (1990). Mechanisms of action of biocides. Int Biodeter. 26: 89-100.

Devi KP, Nisha SA, Sakthivel R, Pandian SK (2010) Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. J. Ethnopharmacol.130(1):107-115.

Dutta N, Annadurai S, Mazumdar K, Dastidar S G, Kristiansen JE, Molnar J, et al.(2007)[a] Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. Int. J. Antimicrob. Agents. 30(3):242-249.

Dutta N, Kumar K A, Kaushiki Mazumdar K ,Dastidar SG (2004) In vitro and in vivo antimycobacterial activity of anti-inflammatory drug, diclofenac sodium. Indian J. Exp. Biol. 42(9):922-927.

Dutta N, Mazumdar K, Park JH (2009). In vitro synergistic effect of gentamicin with the anti-inflammatory agent diclofenac against *Listeria monocytogenes*. Lett. Appl. Microbiol.48(6):783-785.

Dutta N, Mazumdar K, Dastidar SG, Park JH (2007)[b] Activity of diclofenac used alone and in combination with streptomycin against *Mycobacterium tuberculosis* in mice. Int. J. Antimicrob. Agents. 30(4):336-340.

Eliopoulos GM and Moellering R (1996). Antimicrobial combinations. Antibiot. lab. med. 4:330-396.

Gágyor I, Hummers-Pradier E, Kochen MM, Schmiemann G, Wegscheider K, Bleidorn J (2012). Immediate versus conditional treatment of uncomplicated urinary tract infection-a randomized-controlled comparative effectiveness study in general practices. BMC Infect. Dis. 12(1):146.

Hersh EV, Hammond BF, Fleury AA (1991) Antimicrobial activity of flurbiprofen and ibuprofen in vitro against six common periodontal pathogens. J. Clin Dent. 3(1):1-5.

Hugo W, Snow G, (1981). Biochemistry of Antibacterial Action. Chapman & Hall: London, UK.

Khan NA, Saba N, Abdus S, Ali AQ (2002) Incidence and antibiogram patterns of *Escherichia coli* isolated from various clinical samples from patients at NIH Islamabad. Pak. J. Biol. Sci. 5:111-113.

Kothari A, Sagar V (2008). Antibiotic resistance in pathogens causing

community-acquired urinary tract infections in India: a multicenter study. J. Infect. Dev. Ctries. 2(5):354-358.
Lorian V (2005). Antibiotics in laboratory medicine. Lippincott Williams & Wilkins.
Mazumdar K, Dastidar SG, Park JH, Dutta NK (2009). The anti-inflammatory non-antibiotic helper compound diclofenac: an antibacterial drug target. Euro. J. Clin. Microbiol. Infect. Dis. 28(8):881-91.
Mazumdar K, Dutta NK, Dastidar SG, Motohashi N, Shirataki Y (2006). Diclofenac in the management of *E. coli* urinary tract infections. In Vivo. 20(5):613-619.
Miragliotta G, Di Pierro MN, Miragliotta L, Mosca A (2008). Antimicrobial resistance among uropathogens responsible for community-acquired urinary tract infections in an Italian community. J. Chemother. 20(6):721-727.
Mohsen A, Gomaa A , Mohamed F, Ragab R, Eid M, Ahmed AH, Khalaf A, Kamal M, Mokhtar S, Mohamed H, Salah I, Abbas R (2015). Antibacterial, anti-biofilm activity of some non-steroidal anti-inflammatory drugs and N-acetyl cysteine against some biofilm producing uropathogens. Am. J. Epidemiol. Infect. Dis. 3(1):1-9.
Nobmann P, Bourke P, Dunne J, Henehan G (2010). In vitro antimicrobial activity and mechanism of action of novel carbohydrate fatty acid derivatives against *Staphylococcus aureus* and MRSA. J. Appl. Microbiol. 108(6):2152-2161.
Obad J, Šušković J, Kos B (2015). Antimicrobial activity of ibuprofen: new perspectives on an “Old” non-antibiotic drug. Eur. J. Pharm. Sci. 71:93-98.
Paixão L, Rodrigues L, Couto I, Martins M, Fernandes p, Carvalho CC, Monteiro GA, Sansonetty F, Amaral L, Viveiros M (2009). Fluorometric determination of ethidium bromide efflux kinetics in *Escherichia coli.* J. Biol. Eng. 3(1):1.
Randrianirina F, Soares JL, Carod JF, Ratsima E, Thonnier V, Combe P, Grosjean P, Talarmin A (2007). Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in Antananarivo, Madagascar. J. Antimicrob. Chemother. 59(2):309-312.
Rani Basu L, Mazumdar K, Dutta NK, Karak P, Dastidar SG (2005). Antibacterial property of the antipsychotic agent prochlorperazine, and its synergism with methdilazine. Microbiol Res. 160(1): 95-100.
Ranjini CY, Kasukurthi LR, Madhumati B, Rajendran R (2015). Prevalence of multidrug resistance and extended spectrum beta-lactamases among uropathogenic *Escherichia coli* isolates in a tertiary care hospital in South India: An alarming trend. Comm Acq Infect. 2(1):19.
Ray A, Rice LB (2004). Wildcatters welcome: the need for new antimicrobial agents. Therapy 1(1):1-5.
Rusu E,Sarbu L , Pelinescu D , Nedelcu L , Vassu T , Cristescu C, Musat S, Cojocaru M (2014). Influence of associating nonsteroidal anti-inflammatory drugs with antifungal compounds on viability of some *candida* strains. Romanian J. Infect. Dis. 17(2).
Samsygina GA, Dudina TT, Korniushin MA, Overhkina NV (2000). The structure and antibiotic sensitivity of the causative agent of community-aquired infection diseases of bacterial origin in children. Antibiot. Khimioter. 45:15-19.
Sikkema J, De Bont J, Poolman B (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev. 59(2):201-222.
Soboh F, Khoury AE, Zamboni AC, Davidson D, Mittelman MW(1995). Effects of ciprofloxacin and protamine sulfate combinations against catheter-associated *Pseudomonas aeruginosa* biofilms. Antimic Agents Chemother. 39(6):1281-1286.
Stamm WE (2002). Scientific and clinical challenges in the management of urinary tract infections. Am. J. Med. 113(1):1S-4S.
Sukul A, Das SC, Saha SK, Rahman SA (2015). Screening of Analgesic, Antimicrobial, Cytotoxic and Antioxidant Activities of Metal Complexes of Indomethacin. Dhaka Univ. J. Pharm. Sci. 13(2):175-180.
Tambekar DH, Dhanorkar DV, Gulhane SR, Khandelwal VK, Dudhane MN (2006). Antibacterial susceptibility of some urinary tract pathogens to commonly used antibiotics. Afr. J. Biotechnol. 5(17).
Vik I, Bollestad, M, Grude, N,Bærheim, A, Mölstad, S, Bjerrum L, Lindbæk M (2014). Ibuprofen versus mecillinam for uncomplicated cystitis-a randomized controlled trial study protocol. BMC infect. dis. 14(1):1.
Wang WH, Wong WM, Dailidiene D, Berg DE, Gu Q, Lai KC (2003). Aspirin inhibits the growth of *Helicobacter pylori* and enhances its susceptibility to antimicrobial agents. Gut. 52(4):490-495.
Weinstein MP, Towns ML, Quartey SM, Mirrett S, Reimer LG, Parmigiani G (1997). The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. Clin. Infect. Dis. 24(4):584-602.
Winn WC, Koneman EW (2006). Koneman's color atlas and textbook of diagnostic microbiology. Lippincott williams & wilkins.
Zhou K, Zhou W, Liu P Li, Zhang J, Dai Y (2008). Mode of action of pentocin 31-1: an antilisteria bacteriocin produced by *Lactobacillus pentosus* from Chinese traditional ham. Food Control. 19(8):817-822.

academicJournals

Vol. 10(34), pp. 1417-1421, 14 September, 2016
DOI: 10.5897/AJMR2016.7995
Article Number: F7D66B560514
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Increasing trend of methicillin-resistant *Staphylococcus aureus* in Jaipur, Rajasthan, India

Sachin Kumar and Seema Bhadauria*

Department of Microbiology, JECRC University, Jaipur, Rajasthan, India.

Received 7 March 2016, Accepted 10 May, 2016.

Multidrug resistant strains of *Staphylococcus aureus* have become a serious threat to hospitalized patients and health workers. Recently, this bacterium has emerged as methicillin-resistant *S. aureus* (MRSA) and this bacterial strain has turned into a superbug in the health domain. The main objective of the present study was to find out the prevalence of MRSA in different clinical samples. A total number of 155 clinical samples were collected from Sawai Maan Singh hospital in Jaipur from June, 2014 to January, 2015 and subjected to MRSA screening using biochemical and microbiological methods. An antibiotic sensitivity test was performed for the confirmation of MRSA. Out of 147 strains of Gram positive cocci isolated from clinical samples, 79 (53.74%) were found to be methicillin-resistant. Moreover, this study revealed that the major MRSA isolates were from pus swabs (37.70%) followed by wound swabs (30.40%), hand swabs (8.90%), surgical wound swab (7.60%), axilla swabs (6.32%) and nasal swabs (10.12%).

Key words: Methicillin, *Staphylococcus aureus*, penicillin, oxacillin, cefoxitin.

INTRODUCTION

MRSA stands for methicillin-resistant *Staphylococcus aureus*, which is a general skin bacterium that is resistant to a series of antibiotics such as methicillin, cefoxitin, oxacillin, amoxicillin and penicillin. MRSA strains were primarily described in 1961 and emerged in the last decade as one of the most important nosocomial pathogens which were reported a year after the launch of methicillin (Maple et al., 1989). *S. aureus* has been long recognized as the most important pathogen of hospital acquired infections. Over the past decade, methicillin-resistant *S. aureus* (MRSA) strains have become very common in hospitals worldwide. In accumulation, it is now a developing community pathogen in many geographical regions (Lowy, 1998). MRSA, in addition to being methicillin-resistant, is also resistant to other β-lactam antibiotics, with the exclusion of glycopeptides antibiotics (Chambers, 1997; Brumfitt and Hamilton, 1989). MRSA is associated with high morbidity and mortality rates because of the development of multidrug antibiotic resistance. Rapid and accurate detection of

*Corresponding author. E-mail: drseema299@gmail.com. Tel: +91-8003214654.

Table 1. Isolation of MRSA in different clinical samples from SMS Hospital, Jaipur, Rajasthan, India.

Clinical sample	Sample quantity	Gram positive cocci seen	*Staphylococcus* species	*S. aureus*	MRSA	MRSA (%)
Pus swabs	57	57	20	37	29	37.70
Wound swabs	44	40	10	30	24	30.40
Hand swabs	17	16	6	10	7	8.90
Nasal swabs	16	15	5	10	8	10.12
Surgical wound swabs	12	12	3	09	6	7.60
Axilla swabs	09	07	01	06	5	6.32
Total	155	147	45	102	79	53.74

MRSA is an important role of clinical microbiology laboratories to avoid treatment failure. Prolonged hospital stay, indiscriminate and irregular use of antibiotics, lack of awareness, treating with antibiotics before coming to the hospital, etc., are the factors of MRSA infections appearance (Anupurba et al., 2003). Serious endemic and epidemic MRSA infections occur worldwide as infected and colonized patients in hospitals mediate the dissemination of these isolates and hospital staffs promote transmission.

Currently, the treatment options for MRSA infections are limited to very few and costly drugs like teicoplanin, vancomycin, linezolid and Daptomicin. Thus, control of MRSA is essential to curtail the introduction and spread of infection (Siddiqui et al., 2002). The main aim of the study was to find out the occurrence of MRSA in different clinical samples.

MATERIALS AND METHODS

Study design

A total of 155 clinical samples such as pus swabs, axilla swabs, wound swabs, surgical wound swabs, hand swabs and nasal swabs were collected over a period of eight months from June 2014 to January 2015 for research purpose from Sawai Maan Singh (SMS) Hospital in Jaipur district (Rajasthan, India). Sterile dry screw cap cotton swabs (Hi media) were used for the collection of sample. For a collection of axilla swab, nasal swab, hand swab, and the swabs were rubbed, very well by rotating 5 to 7 times over the surface and inner wall of ala and nasal septum. Before sample collection for the isolation of *S. aureus* proper explanation about this study was given to all these patients and consent was taken from them. Detailed history, including age, gender, profession, site of lesion, periods of illness and associated symptoms were also recorded from the patients.

Isolation and biochemical identification of *S. aureus*

A preliminary gram staining was performed to find out the likely organism present. The samples were inoculated on 5% blood agar plates and incubated at 37°C for 18 to 24 h. Morphological examinations were carried out to differentiate *S. aureus* from the other related organisms. Nonetheless, the specimens collected from the patients were subjected to culture on blood agar medium to observe β-haemolysis a defining feature of *S. aureus*. Moreover, biochemical tests, for example catalase and coagulase were performed to confirm the bacteria (Dubey and Padhy, 2012).

Antibiotic sensitivity test

A suspension of each *S. aureus* isolate was prepared to a 0.5 McFarland standard and swabbed on Mueller Hinton Agar (MHA) by lawn streaking. Antibiotic discs were then placed on the streaked agar surface and the plates were incubated overnight at 37°C. Antibiotic sensitivity testing was performed for the following antibiotics: penicillin (10 units/disc), cefoxitin (30 µg/disc), oxacillin (1 µg/disc). These antibiotic discs were obtained from commercial sources (Hi- Media, Mumbai). *S. aureus* ATCC 25923 was used as control strain. The results were interpreted according to the guidelines of the Clinical Laboratory Standards (CLSI, 2009).

RESULTS

A total number of 147 (94.83%) Gram positive cocci reported out of 155 clinical samples. Out of 147 Gram positive bacteria, 57 were from pus swab, 40 from wound swab, 16 from hand swab, 15 from nasal swab, 12 from surgical wound swab, and 7 from axilla swab. Out of a total of 147 Gram positive cocci, 102 (69.39%) were coagulase positive and 45 (30.61%) coagulase negative. Out of 102 *S. aureus*, 37 were from pus swabs, 30 from wound swabs, 10 from hand swabs, 10 from nasal swabs, 09 from surgical wound swabs, and 06 from axilla swabs (Table 1 and Figure 1).

By using Kirby-Bauer's disc diffusion method out of 102 isolates, 79 (53.74%) isolates were MRSA. The MRSA was the most common in pus swabs (37.70%), followed by wound swab (30.40%), hand swabs (8.90%), nasal swabs (10.12%), surgical wound swabs (7.60%), and axilla swabs (6.32%) (Table 1 and Figure 1). All these three antibiotics, that is, penicillin (10 units/disc), cefoxitin (30 µg/disc) and oxacillin (1 µg/disc) are 100% resistant

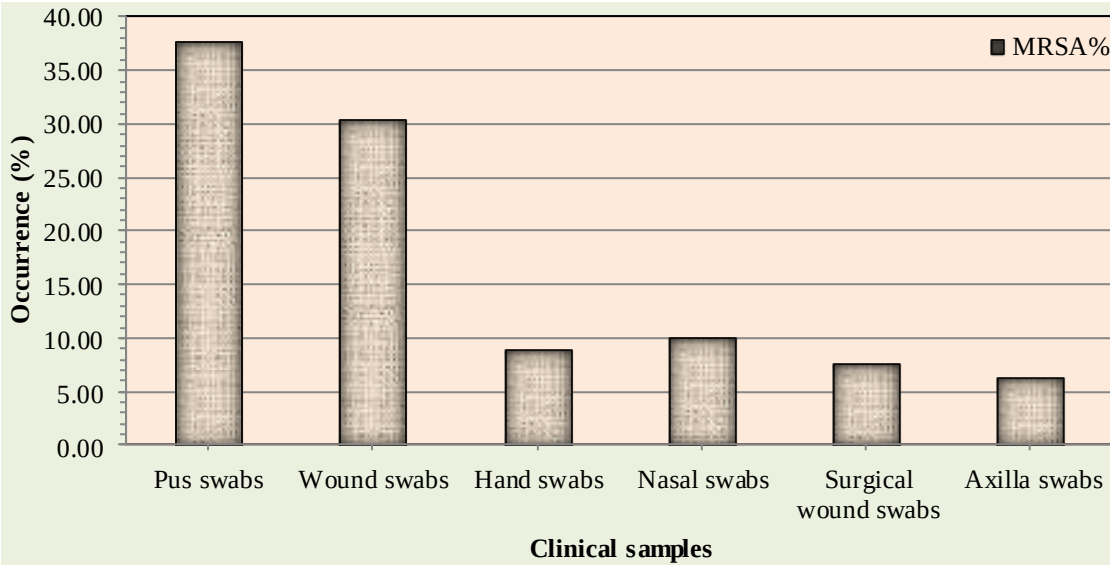


Figure 1. The prevalence of MRSA in different clinical samples.

against 79 (53.74%) caogulase positive isolates.

DISCUSSION

S. aureus is a major human pathogen that is very general and highly virulent. Increased antimicrobial resistance for such an organism is, therefore a cause of concern. In recent years, there has been an alarming increase in the *S. aureus* strains showing resistance to methicillin and reduced susceptibility to vancomycin. The potential reservoirs of MRSA include infectious patients, hospital personnel and hospital environment.

During the earlier period of 15 years, the emergence and world-wide spread of many of such clones have caused major therapeutic problems in many hospitals (Rajaduraipandi et al., 2006). The prevalence of MRSA varies from hospital to hospital in various countries and is constantly high in many countries. In many American and European hospitals, the percentage of MRSA ranged from 29 to 35% (Tahnkiwale et al., 2002; Chaudhary et al., 2009). The incidence of MRSA in India ranges from 30 to 70% (Rajaduraipandi et al., 2006; Verma et al., 2000). In the present study, we have isolated 79 (53.74%) MRSA among 102 *S. aureus* strains from clinical samples. This is an agreement with the study of Assadullah et al. (2003) who also reported prevalence of methicillin-resistant *S. aureus* (52.90%) in Assam. The prevalence of MRSA was reported by the other authors in India. Bandaru et al. (2012) also reported prevalence of MRSA (52%) in Andhra Pradesh. Anupurba et al. (2003) conducted a study of prevalence of MRSA in tertiary care referral hospital in Eastern Uttar Pradesh reported 54.8% occurrence of MRSA. Sasirekha et al. (2012) conduct a study in Bangalore reported out of 198 clinical samples, 153 *S. aureus* were isolated. MRSA screening by phenotypic methods using E-test MIC as standard. Subsequently, biotyping and biofilm production was performed for confirming MRSA isolates. Antibiotic susceptibility test by disc diffusion was also performed for all *S. aureus* isolates. Out of 153 *S. aureus* isolates, 42 (57.7%) were found to be methicillin-resistant. Pandya et al. (2014) worked on characterization of MRSA from various clinical samples at a tertiary care hospital of rural Gujarat, reported among 200 isolates of *S. aureus,* 117 (58.5%) were methicillin-resistant. Dar et al. (2006) studied the molecular epidemiology of clinical and carrier strains of MRSA in the hospital settings of North India reported 54.85% of MRSA from Aligarh Muslim University, Aligarh, India. Bala et al. (2014) conducted a study of prevalence of MRSA and its antibiotic susceptibility pattern in a tertiary health care reported 69.2% prevalence of MRSA from PGI Rohtak. 41% prevalence of MRSA was reported by INSAR in 2013. Rajduraipandi et al. (2006) reported 37.9% of MRSA from Tamilnadu, India. Shinde et al. (2016) also reported 34.61% prevalence of methicillin-resistant from South India.

The prevalence of MRSA was reported by other authors in abroad. Susethira et al. (2015) conducted a study on *S. aureus* nasal carriage among health care workers in a Nepal Hospital reported 46.2% prevalence of MRSA from Nepal. Okon et al. (2013) conducted a study on epidemiology and antibiotic susceptibility pattern

of MRSA recovered from tertiary hospitals reported 12.5% occurrence of MRSA in Nigeria. Hafiz et al. (2002) reported 42% occurrence of MRSA in Pakistan.

In the present study, the high occurrence of MRSA was observed in pus Swab samples (37.70%). Similar prevalence rate of MRSA in pus samples was observed by Pandya et al. (2014) in Gujarat. On the other hand, the prevalence of MRSA in Nasal swabs was found to be 10.12%, followed by surgical wound infection (7.60%). Nasal carriage of *S. aureus* is an important risk factor for developing a surgical site infection as it is a normal flora in the nostrils and the skin. Risk factors for surgical site infection are divided into patient related (preoperative), procedure related (pre-operative), and postoperative categories. Patient related factors again categorized into modifiable (diabetes mellitus, obesity, immuno suppressive drugs, prolonged pre-operative stay) and unmodifiable (age) factors. The preoperative, procedure related factors are class of wound type (clean, clean-contaminated, contaminated, dirty and infected), length of surgery, hair removal, hypoxia and hypothermia. In the immediate postoperative period, glucose control, oxygenation, hypothermia and wound care are the major risk factors (Vidhani et al., 1998).

Conclusion

The present study highlights the prevalence of MRSA and this is a problem for the healthcare sector in India. A high number of MRSA isolates were from pus swabs. So, there is a need to make a strict antibiotic policy and maintaining strict hand hygiene practices in medical staff to avoid cross contamination among patients and to prevent MRSA spread. The regular surveillance of hospital related infections, including monitoring antibiotic sensitivity patterns of MRSA and formulation of definite antibiotic course of action may be helpful in reducing the incidence of MRSA infections.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Nagendra Singh from Surgical Unit, Sawai Maan Singh Hospital, Jaipur for helping them during sample collection of MRSA.

REFERENCES

Anupurba S, Sin MR, Nath G, Sharma BM, Gulati AK, Mohapatra TM (2003). Prevalence of Methicillin resistant *Staphylococcus aureus* in a Tertiary care Referral Hospital in Eastern Uttar Pradesh. Indian J. Med. microbial. 21:49-51.

Assadullah S, Kakru DK, Thoker MA, Bhat FA, Hussain N, Shah A (2003). Emergence of low level vancomycin resistance in MRSA. Indian J. Med. Microbiol. 21:196-198.

Bala K, Mittal S, Deep A, Chaudhary U, Sharma A, Yadav P, Griwan A (2014). Prevalence of methecillin resistant *Staphylococcus aureus* and its antibiotic susceptibility pattern in a tertiary health care. Intr J Pharma. Med. Bio Sci. 3(1):28-33.

Bandaru NR (2012). A prospective Study of Methicillin Resistnt *Staphylococcus aureus* (MRSA) in a Teaching hospital of rural setup. J. Pharm. Sci. Innov. 1(2):37-41.

Brumfitt W, Hamilton JM (1989). Methicillin resistant *Staphylococcus aureus*. New Engl. J. Med. 320:1188-1196.

Chambers HF (1997). Methicillin resistance in *Staphylococci:* molecular and biochemical basis and clinical implications. Clin. Microbiol. Rev. 10:781-791.

Chaudhary A, Nagaraja M, Kumar AG (2009). Potential of biofilm formation by *Staphylococci* on polymer surface and its correlation with methicillin susceptibility. Indian J. Med. Microbiol. 27:377-378.

Clinical and Laboratory Standards Institute (CLSI) (2009). Performance standard for Antimicrobial Susceptibility testing: Nineteenth Informational Supplement. Clinical Laboratory Standards Institute, Wayne, PA.

Dar JA, Thoker MA, Khan JA, Ali A, Khan MA, Rizwan M, Bhat KH, Dar MJ, Ahmed N, Ahmad S (2006). Molecular epidemiology of clinical and carrier strains of methicillin resistant Staphylococcus aureus (MRSA) in the hospital settings of north India. Ann. Clin. Microbial. Antimicrob. 5(1):1.

Dubey D, Padhy RN (2012). Surveillance of multidrug resistance of two Gram- positive pathogenic bacteria in a teaching hospital and in vitro efficacy of 30 ethnomedicinal plants used by an aborigine of India. Asian Pac. J. Trop. Dis. 2(4):273-281.

Hafiz S, Hafiz AN, Ali L, Chughtai AS, Memon B, Ahmed A, Hussain S, Sarwar G, Mughal T, Awan A (2002). Methicillin resistant Staphylococcus aureus: a multicentre study. J. Pak. Med. Assoc. 52(7):312-4.

Indian Network for Surveillance of Antimicrobial Resistance (INSAR) group (2013). Methicillin resistant Staphylococcus aureus (MRSA) in India: Prevalence & susceptibility pattern. Indian J. Med. Res. 137(2):363-369.

Lowy FD (1998). *Staphylococcus aureus* infections. New Engl. J. Med. 339:520-532.

Maple PAC, Hamilton-Miller JMT, Brumfitt W (1989). Worldwide antibiotic resistance in methicillin resistant *Staphylococcus aureus*. Lancet. 1:537-540.

Okon KO, Shittu AO, Usman H, Adamu N, Balogun ST, Adesina OO (2013). Epidermiology and antibiotic susceptibility pattern of Methicillin- Resistnt *Staphylococcus aureus* recovered from tertiary hospitals in Northeastern, Nigeria. J. Med. Med. Sci. 4(5):214-220.

Pandya N, Chaudhary A, Mehta S, Parmar R (2014). Methicillin resistant staphylococcus aureus pattern at tertiary care hospital, Gujarat. J. Res. Med. Dent. Sci. 2(3):49-53.

Rajaduraipandi K, Mani KR., Panneerselvam K, Mani M, Manikandan P (2006). Prevalence and antimicrobial susceptibility pattern of methicillin resistant *S. aureus*: A multicentre study. Ind. J. Med. Microbiol. 26:34-38.

Sasirekha B, Usha MS, Amruta AJ, Ankita S, Brinda N, Divya R (2012). Evaluation and comparision of different phenotypic test to detect methicillin resistant *Staphylococcus aureus* and their biofilm production. Int. J. Pharma Tech. Res. 4(2):532-541.

Shinde RV, Pawar SK, Mohite RV, Shinde AR, Duggu P (2016). Study of Nasal Carriage of Staphylococcus aureus with Special Reference to Methicillin Resistance among Nursing Staff. Arch. Clin. Microbiol. 7:1.

Siddiqui F, Madahiah-bint-e-Masood, Noor-us-Saba, Samad A, Quayyum M, Qazilbash, AA (2002). Antibiogram sensitivity pattern of Methicillin resistant *Staphylococcus aureus* isolates from pus sample. Pak. J. Biol. Sci. 5:491-493.

Susethira AR, Saiprasanna N, Vigneshwari RS, Uma A, Prabhu N, Thiramalaikulundusubramanian P (2015). Merthicillin Resistant *S. aureus* Nasal Carriage among health care workers. J. Global Biosci. 4(6):2518-2524.

Tahnkiwale SS, Roy S, Jalgaonkar SV (2002). Methicillin resistance among isolates of *Staphylococcus aureus*: Antibiotic sensitivity pattern and phage typing. Indian J. Med. Sci. 56:330-334.

Verma S, Joshi S, Chitnis V, Hemwani N, Chitnis D (2000). Growing problem of methicillin resistant *Staphylococci:* Indian Scenario. Ind. J. Med. Sci. 54:535-540.

Vidhani S, Mehndiratta PL, Mathur MD (1998). Study of MRSA from high risk patients. Indian J. Med. Microbiol. 16:31-34.

academicJournals

Vol. 10(34), pp. 1422-1429, 14 September, 2016
DOI: 10.5897/AJMR2016.8251
Article Number: 14ED3AF60517
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparison of the prevalence of antibiotic-resistant *Escherichia coli* isolates from commercial-layer and free-range chickens in Arusha district, Tanzania

Bernadether T. Rugumisa[1,2], Douglas R. Call[1,3]*, Gaspary O. Mwanyika[1,2], Murugan Subbiah[3] and Joram Buza[1]

[1]Department of Health and Biomedical Sciences, School of Life Science and Bioengineering, Nelson Mandela African Institution of Science and Technology (NM-AIST), 447 Arusha, Tanzania.
[2]Mbeya University of Science and Technology, 131 Mbeya, Tanzania.
[3]Paul G. Allen School of Global Animal Health, Washington State University, Pullman, WA, USA.

Received 4 August 2016; Accepted 31 August, 2016

The antibiotic susceptibility of fecal *Escherichia coli* isolates from commercial-layer and free-range chickens in Arusha district, Tanzania were compared. All the chickens were raised by individual households, but commercial-layer chickens were purchased from commercial vendors, whereas no systematic breeding system was used to produce free-range chickens. A total of 1,800 *E. coli* isolates (1,200 from commercial-layer chickens and 600 from free-range chickens) were tested for susceptibility to 11 antibiotics by breakpoint assays. All *E. coli* isolates were susceptible to gentamicin, ceftazidime and cefotaxime. Isolates from commercial-layer chickens had a high prevalence of resistance (32.4-74.5%) for amoxicillin, ampicillin, ciprofloxacin, tetracycline, streptomycin, trimethoprim and sulfamethoxazole, while the prevalence of resistance to these antibiotics was lower (7-31.5%) for free-range chickens (*P*<0.05). Both groups had a similar prevalence of resistance to chloramphenicol (1.17-1.5%; *P*>0.05). For antibiotic resistant strains, 64.1 and 91.5% of free-range and commercial-layer isolates, respectively, were resistant to ≥ 2 antibiotics. Commercial-layer chickens harbored significantly more resistant *E. coli* isolates (*P*<0.001) than free-range chickens, consistent with more exposure to antibiotics when compared with free-range chickens. Efforts should be directed towards motivating household owners to limit the use of antibiotics when they are investing in these breeds.

Key words: Antibiotic resistance, free-range, commercial-layer, *Escherichia coli*, Arusha, Tanzania.

INTRODUCTION

Escherichia coli is a commensal bacterium in the gastrointestinal tract of humans and animals. Although most *E. coli* strains are harmless, there are pathogenic strains capable of causing infectious disease including

*Corresponding author. E-mail: drcall@vetmed.wsu.edu. Tel: (509) 335-6313.

diarrhea, neonatal meningitis, blood stream infections and urinary tract infections (UTIs) (Nakazato et al., 2009; Jakobsen et al., 2010). Avian pathogenic *E. coli* causes yolk-sac infections, respiratory-tract infections, bloodstream infections and colibacillosis (Yang et al., 2004; Horn et al., 2012). Transmission of these diseases accounts for significant losses for poultry producers (Ewers et al., 2009).

Antibiotics are used in poultry production particularly for commercial production to treat and prevent diseases. The demand for antibiotics is generally correlated with increased flock size both due to the number of animals and associated increases in the incidence of disease (Mathew et al., 2007). Antibiotics are also used as growth promoters. For example, tetracyclines are used in low concentrations as feed additives to enhance growth whereas higher concentrations are used to prevent or treat disease (Stead et al., 2007). In developing countries, there is an increased demand for chicken and chicken products as a result of population increase, urbanization and improved economic status. Responding to this increased demand, chicken farmers tend to shift to increasingly intensive production systems and antibiotics are often used to manage diseases in these operations (Hao et al., 2014). At a household level, free-range chickens likely forage for their food rather than receive commercially prepared feeds. Furthermore they may be more resistant to diseases and may be exposed to fewer diseases simply due to lower population densities (Hamisi et al., 2014).

Resistant bacteria from food animals can spread to humans directly or indirectly (Adenipekun et al., 2015). Direct transmission involves contact with reservoir animals, their feces or consumption of contaminated animal food products such as meat and eggs. Indirect transmission can be through contaminated water, food and environments. This is a significant public health concern when animal husbandry practices promote resistance to medically important antibiotics (Anderson et al., 2003). If antibiotics are being used in commercial-source poultry production, then *E. coli* from these sources should exhibit a significantly greater prevalence of resistance as compared to free-range chickens that are not likely to be exposed to antibiotics. To test this hypothesis, *E. coli* collected from poultry in the Arusha district of Tanzania were evaluated.

MATERIALS AND METHODS

Sampling details

In Arusha, commercial-layer chickens are purchased as day-old chicks from different commercial producers and are raised by farmers adjacent to their houses (typically up to 200 birds). These chickens are raised in enclosed structures and are given feed and water that may contain antibiotics (tetracycline for growth promotion and enrofloxacin and sulfa-trimethoprim for prophylaxis and treatment) and vaccines recommended by the commercial vendors. In addition to commercial layers, these farmers and others in the same area also raise free-range, indigenous chickens (usually in small numbers up to 20 chickens) that are used for egg and meat production. The free-range chickens used in this study were those that were neither treated with antibiotics nor fed commercial feeds but instead scavenge freely without strict physical constraints.

Fecal samples from commercial-layer and free-range chickens were obtained through convenience sampling method between April and July 2015. Briefly, five wards in Arusha (Mifugo, Nambala, Njiro, Sakina and Sansi) were selected for sampling. From each ward, one household that exclusively raised commercial-layer and one that exclusively raised free-range chickens were identified and distinct, spatially discreet fecal samples (n = 5 or 10 for free-range or commercial layers, respectively) were collected.

Sample collection and preparation

A total of 50 commercial-layer and 25 free-range chicken fecal samples were collected in individual sterile plastic bags and were transported to the laboratory at NM-AIST (the Nelson Mandela African Institution of Science and Technology) at ice cold temperature. In the laboratory, samples were mixed with sterile distilled water (approximately 1:9 ratio, feces: water) to make suspensions. An aliquot of 1 ml of each fecal suspension was added with glycerol (15% final concentration) and stored at -80°C for long-term preservation of original samples. The fecal suspensions were further diluted (1:10) with sterile distilled water. Sterile glass beads were then used to spread 30 uL of diluted fecal suspension onto 100 mm diameter MacConkey (MAC; Becton, Dickinson Company, Sparks, MD) agar plates that were then incubated overnight at 37°C.

E. coli isolation

After incubation, the plates were examined for the growth of morphologically distinct *E. coli* colonies (pink to reddish, lactose-fermenting colonies surrounded by bile salt precipitate). If the growth was numerous and individual isolates unavailable, the frozen fecal suspensions were thawed and serially diluted (10-fold) and higher dilutions were plated to obtain distinct *E. coli* colonies. Presumptive, *E. coli* colonies (n=24) were picked for each sample from the MAC agar plates and inoculated into wells containing 150 µl of LB broth1 (Luria-Bertani broth) in 96-well micro-titre plates using autoclaved tooth picks. After inoculating, 96 colonies (4 fecal samples per plate), the 96-well plates were wrapped in cling-wrap to minimize evaporation and were incubated overnight (16 to 18 h) at 37°C. Isolation of *E. coli* based on colony morphology alone yields >95% accurate identification in our hands (Liu et al., 2016), but for the current study, we further confirmed *E. coli* identification by re-growing isolates on HiChrome coliform agar (SIGMA-ALDRICH Co., St. Luis, MO). This media contains two chromogenic substrates that allow simple differentiation of *E. coli* (dark blue to violet colored colonies). Only presumptive *E. coli* from both agar media were analyzed for this study. All strains where stored at -80°C in sterile phosphate-buffered glycerol (15% final concentration).

*Determin*ation of *the antibiotic resistance profile*

To determine the prevalence of antibiotic resistance, *E. coli* isolates were tested against a panel of 11 antibiotics that belonged to seven different classes (β-lactams, cephalosporins, amphenicols, tetracyclines, sulfonamides, aminoglycosides and fluoroquinolones) by using a breakpoint assay (Subbiah et al., 2011). Briefly, MAC agar plates (150 mm, diameter) were prepared with each antibiotic at a fixed concentration (given below) that was guided by the Clinical Laboratory Standard Institute (CLSI) recommended

minimum inhibitory concentration for *E. coli* (NCCLS, 2007). The 96-well plates containing *E. coli* cultures were thawed at room temperature and stamped simultaneously on MAC agar plates containing antibiotics using a sterile 96-pin replicator. After stamping, the plates were left open at room temperature for a few minutes until the cultures were dried and then incubated overnight at 37°C. On every culture-stamped MAC agar plate a susceptible (*E. coli* K-12) and two resistant (*E. coli* NM-1 and E. coli NM-2) strains were used as negative and positive controls, respectively. The NM-1 strain was resistant to ampicillin, ciprofloxacin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline and trimethoprim. The NM-2 strain was resistant to amoxicillin, ceftazidime, cefotaxime and gentamicin. After incubation, the MAC agar plates were examined for the growth of resistant isolates and the antibiotic resistant patterns for each isolate were recorded.

The concentration for antibiotics and vendor information were as follows: ampicillin VWR International LLC, Sanborn, NY (Amp, 32 µg/ml), cefotaxime Chem-Impex International Inc, Wood Dale, IL (Ctx, 8 µg/ml), chloramphenicol Mediatech Inc., Manassas, VA (Chm, 32 µg/ml), tetracycline MP Biomedicals, LLC, Solon, OH (Tet, 16 µg/ml), trimethoprim (Tri, 8 µg/ml), ceftazidime (Cfd,8 µg/ml), sulfamethoxazole (Sul, 512 µg/ml), streptomycin Amresco Inc., Solon, OH (Str, 16 µg/ml), ciprofloxacin Enzo Life Sciences Inc., Farming Dale, NY (Cip, 4 µg/ml), amoxicillin (Amx, 32 µg/ml) and gentamicin above (Gen, 64 µg/ml).

Data analysis

Antibiotic resistance data for each isolate (coded '1' or '0' if resistant or susceptible, respectively) was managed using Microsoft Excel and Microsoft Access (ver. 2007) for descriptive analysis. A Bartlett test was used to compare variances across data and a logit transformation ($y = \ln[x/(1-x)]$) was used to meet the homogeneity of variance assumption when comparing proportions between commercial-layer and free-range chickens. Analysis of variance (ANOVA) and a Tukey-Kramer post-comparison test were used to evaluate differences between the prevalence of resistant *E. coli* isolates and identify which antibiotic resistance phenotypes differed between commercial-layer and free-range chickens. A Wilcoxon rank-sum test was used to compare the number of *E. coli* isolates resistant to at least two or more antibiotics between commercial layer and free-range chickens. Values of $P < 0.05$ were considered statistically significant.

RESULTS

A total of 1,800 *E. coli* isolates (n=600 free-range and n=1,200 commercial chickens) were collected from fecal samples. *E. coli* resistant to >1 antibiotic accounted for 47.5 and 90.7% of the *E. coli* isolates collected from free-range chickens and commercial-layer chickens, respectively. For free-range chickens the most common resistance phenotypes included sulfamethoxazole (31.5%) and trimethoprim (28.17%). The rank order of resistance was Sul, Tri, Str, Tet, Amx, Amp, Cip and Chm (Table 1). A very similar pattern was found for *E. coli* isolates from commercial-layer chickens where resistance was most prevalent for sulfamethoxazole (74.56%) and trimethoprim (68.83%). In fact, the rank order of prevalence was remarkably similar to free-range chickens with the exception that Amx and Amp were reversed (Table 1). Furthermore, the ratio of resistance to sulfamethoxazole, trimethoprim, streptomycin and tetracycline for commercial source vs. free-range isolates was remarkably constant (0.38 to 0.42), which is consistent with the presence of one or more similar populations of multidrug resistant isolates in both poultry populations. Importantly, no resistance was detected for ceftazidime, cefotaxime and gentamycin.

There were differences in the proportion of resistant isolates based on chicken type (greater for commercial-layer vs. free-range; $P<0.001$), and antibiotic type ($P<0.001$). There was also a significant interaction between the proportion of resistant isolates and antibiotic type ($P<0.01$). A plot of the interaction effect demonstrated that this was caused by the rank-order change for Amp and Amx between commercial-layer and free-range chickens. Among the antibiotic resistant free-range chicken isolates, 64.1 and 39% were resistant to ≥2 and ≥3 antibiotics (Table 2). For commercial-layer chicken isolates, 91.5 and 73.4% were resistant to ≥2 and ≥3 antibiotics, respectively. The frequency of multidrug resistance was significantly higher among *E. coli* isolates from commercial-layer chickens as compared to free-range chickens ($P<0.05$). Resistance phenotypes were diverse. For example, if we limit the analysis to seven antibiotics (excluding chloramphenicol) there were $2^7 = 128$ possible combinations of resistance phenotypes of which was observed as 111 (Table 2). The broadest resistance phenotypes were AmpChmStrSulTetTri 1 (0.5%) and AmpAmxStrSulTetTri 3 (1.5%) for free-range chickens and AmpAmxChmCipStrSulTetTri 3 (0.3%) for commercial-layer chickens.

DISCUSSION

In studies by Carraminana et al. (2004) in Spain and Kilonzo-Nthenge et al. (2008) in Cameron, all *E. coli* isolates from poultry were susceptible to cefotaxime and gentamicin. Comparable results including susceptibility to third-generation cephalosporins (ceftazidime and cefotaxime) were reported. Hamisi et al. (2014) also sampled free-range chickens in the Arusha area but found resistant strains of *E. coli* for cefotaxime and ceftazidime (29.9 and 6.5%, respectively). Hamisi et al. (2014) also reported higher resistance (54.5%) among *E. coli* isolates to a fluoroquinolone drug (ofloxacin) whereas relatively limited resistance to ciprofloxacin (3.5%) was found. This difference might be explained, in part, by published observations that ciprofloxacin is more active than ofloxacin for most bacteria (Lautzenhiser et al., 2001). Comparisons across studies, however, may be complicated when different methodologies and definitions of resistance and susceptibility are employed by the investigators.

Although, chloramphenicol is not used in Tanzanian food animals and it is not available in local veterinary

Table 1. Prevalence (%) of antibiotic resistant *E. coli* obtained from fecal samples of free-range chickens and commercial layer chickens from Arusha District, Tanzania.

Location	Amp	Amx	Chm	Cip	Str	Sul	Tet	Tri
Free-range chickens								
Mifugo	8.33	8.33	0.83	0.00	33.33	26.67	28.33	26.67
Nambala	12.50	13.33	0.83	0.00	8.33	17.50	5.00	16.67
Sakina	1.67	14.17	0.00	8.33	23.33	25.00	14.17	15.83
Sansi	11.67	6.67	0.83	0.83	9.17	42.50	23.33	40.83
Njiro	0.83	12.50	5.00	8.33	30.00	45.83	11.67	40.83
Mean (SE)	7.0 (2.45)	11.0 (1.48)	1.5 (0.89)	3.5 (1.98)	20.8 (5.19)	31.5 (5.42)	16.5 (4.17)	28.2 (5.51)
Commercial chickens								
Mifugo	39.17	36.67	1.25	17.92	50.42	83.33	31.25	75.42
Nambala	38.75	22.08	0.42	13.75	52.92	67.92	45.42	59.58
Sakina	37.92	28.33	0.42	13.75	53.75	75.00	25.42	67.50
Sansi	27.08	28.75	0.83	20.42	58.33	70.29	58.33	70.83
Njiro	47.50	46.25	2.92	26.25	50.00	76.25	57.92	70.83
Mean (SE)	38.1(3.3)	32.4(4.2)	1.2(0.5)	18.4(2.3)	53.1(1.5)	74.6(2.7)	43.7(6.7)	68.8(2.6)
P values from Tukey-Kramer post comparison test showing antibiotic effects on the prevalence of resistance between commercial-layer and free-range chickens.	<0.0001	0.15	0.99	0.0003	0.0381	0.0042	0.07	0.0097

Amp = Ampicillin (32 µg/ml), Amx = amoxicillin (32 µg/ml), Chm = chloramphenicol (32 µg/ml), Cip = ciprofloxacin (4 µg/ml), Str = streptomycin (16 µg/ml), Sul = sulfamethoxazole (512 µg/ml), Tet = tetracycline (16 µg/ml), and Tri = trimethoprim (8 µg/ml). No resistance was detected forCfd = ceftazidime (8 µg/ml), Ctx = cefotaxime (8 µg/ml) or Gen = gentamicin (64 µg/ml).

medicine outlets, low-level resistance was observed for *E. coli* isolates from both chicken populations. The presence of this trait might be explained by chance alone. It is also possible that unintentional chloramphenicol exposure occurs infrequently (Levy and Marshall, 2004). For example, Berendsen et al. (2010) reported natural occurrence of chloramphenicol in plants in Mongolia and the Netherlands. No farmer was found to use chloramphenicol for the current study.

In this study, *E. coli* isolates from free-range chickens exhibited antibiotic resistance although lower as compared to those from layer chickens. It is possible that these resistant strains spilled over from commercial-source flocks, or these animals might be exposed to selection pressure or populations of resistant bacteria in the environment (Finley et al., 2013; Wellington et al., 2013). Furthermore, environments often harbor non-pathogenic and opportunistic bacteria that are resistant to antibiotics (Wright, 2010). In this study, both chicken populations were obtained from the same wards and were located <1,000 m apart and therefore some exposure to bacteria is likely to happen between commercial-layer and free-range chickens. Regardless of chicken origin, the most frequent resistance phenotypes were for sulfonamide and trimethoprim followed by resistance to streptomycin and beta-lactams. Sulfonamide resistance genes have been linked with spread of multiple antibiotic resistance genes in *E. coli* (Bean et al., 2005). Streptomycin, trimethoprim and ampicillin resistance are the common resistances associated with sulfonamide resistance (Wu et al., 2010). Other studies assayed isolates using sulfamethoxazole in

Table 2. Prevalence (%) of antibiotic resistant phenotypes of *E. coli* isolates from fecal samples of free-range chickens and commercial-layer chickens from Arusha district, Tanzania.

Antibiotic resistance phenotypes[1]	Commercial-layer chickens (%)	Free-range chickens (%)
Susceptible	9.8	41.8
Amp	0.3	0.03
AmpAmx	0.3	-
AmpAmxChmCipStrSulTetTri	0.3	-
AmpAmxChmStrSulTetTri	0.3	-
AmpAmxChmStrSulTri	0.1	-
AmpAmxChmSul	-	0.2
AmpAmxChmStrTri	-	0.3
AmpAmxCipStrSul	0.1	-
AmpAmxCipStrSulTet	0.3	-
AmpAmxCipStrSulTetTri	5.2	-
AmpAmxCipStrSulTri	0.5	-
AmpAmxCipStrTet	0.1	0.2
AmpAmxCipSulTet	0.1	-
AmpAmxCipSulTetTri	0.8	-
AmpAmxCipSulTri	0.2	-
AmpAmxStrSul	0.6	-
AmpAmxStrSulTetTri	3.6	0.5
AmpAmxStrSulTri	5.1	1.5
AmpAmxStrTetTri	0.4	0.2
AmpAmxStrTri	0.2	0.2
AmpAmxSul	1.1	0.2
AmpAmxSulTet	0.2	0.2
AmpAmxSulTetTri	1.8	0.5
AmpAmxSulTri	3.0	0.2
AmpAmxTet	0.2	-
AmpAmxTetTri	0.2	-
AmpAmxTri	0.3	0.2
AmpChm	-	0.2
AmpChmStr	-	0.2
AmpChmStrSulTetTri	-	0.2
AmpChmStrTri	-	0.2
AmpCipStr	0.1	-
AmpCipStrSul	0.1	-
AmpCipStrSulTet	0.1	-
AmpCipStrSulTetTri	0.3	0.3
AmpCipStrSulTri	0.3	0.5
AmpCipSulTetTri	0.5	0.5
AmpCipSulTri	0.1	-
AmpCipTet	0.2	-
AmpCipTri	-	0.2
AmpStr	0.8	0.2
AmpStrSul	1.0	-
AmpStrSulTet	0.6	-
AmpStrSulTetTri	1.3	0.3
AmpStrSulTri	2.0	1.5
AmpStrTetTri	0.1	-
AmpStrTri	0.1	0.5
AmpSul	0.3	-
AmpSulTet	0.7	-

Table 2. Contd.

AmpSulTetTri	0.3	0.2
AmpSulTri	1.9	0.2
AmpTet	0.1	-
AmpTri	0.3	0.3
Amx	0.3	3.5
AmxChmStrSulTetTri	0.1	-
AmxChmSulTri	-	0.2
AmxCip	0.2	0.3
AmxCipStr	-	0.2
AmxCipStrSulTetTri	0.1	0.2
AmxCipStrSulTri	0.2	-
AmxCipSul	-	0.2
AmxCipSulTetTri	0.1	-
AmxStr	-	0.2
AmxStrSul	0.3	0.2
AmxStrSulTetTri	0.3	1.3
AmxStrSulTri	0.9	0.2
AmxStrTet	-	0.2
AmxStrTri	0.1	-
AmxSul	0.2	0.3
AmxSulTet	0.1	-
AmxSulTetTri	0.2	3.8
AmxSulTri	0.6	3.5
AmxTet	0.1	0.3
AmxTetTri	0.2	-
AmxTri	0.3	-
ChmCipStrSulTetTri	0.1	-
ChmCipSulTetTri	0.1	-
ChmStr	-	0.2
ChmStrSulTetTri	0.1	-
ChmStrSulTri	0.1	-
ChmSulTetTri	0.1	-
ChmSulTri	0.1	-
Cip	0.8	2.0
CipStr	-	0.3
CipStrSulTet	1.1	-
CipStrSulTetTri	2.2	0.2
CipStrSulTri	0.5	-
CipStrTet	0.6	-
CipStrTri	0.1	-
CipSul	0.2	-
CipSulTet	0.1	-
CipSulTetTri	0.8	-
CipSulTri	0.7	0.2
CipTet	0.3	-
CipTetTri	0.1	0.2
CipTri	0.6	-
Str	2.1	5.7
StrSul	1.8	1.3
StrSulTet	1.7	-
StrSulTetTri	7.9	1.3
StrSulTri	7.3	1.5

Table 2. Contd.

StrTet	0.5	0.3
StrTetTri	0.7	0.2
StrTri	1.6	0.8
Sul	1.8	4.0
SulTet	0.8	0.2
SulTetTri	4.0	2.5
SulTri	7.9	3.7
Tet	1.7	5.2
TetTri	1.2	0.5
Tri	1.5	3.3

[1]Amp = Ampicillin (32 µg/ml), Amx = amoxicillin (32 µg/ml), Chm = chloramphenicol (32 µg/ml), Cip = ciprofloxacin (4 µg/ml), Str = streptomycin (16 µg/ml), Sul = sulfamethoxazole (512 µg/ml), Tet = tetracycline (16 µg/ml), and Tri = trimethoprim (8 µg/ml).

combination with trimethoprim (Arslan and Eyi, 2010; Chiu et al., 2010). Adenipekun et al. (2015) reported lower resistance to sulfamethoxazole-trimethoprim (39.8%) in food producing animals in Nigeria. While resistance to these two antibiotics is conveyed by different genetic traits (Blahna et al., 2006; Hu et al., 2011), our data showed a strong correlation between these two resistance phenotypes (r = 0.99) that is consistent with closely-linked resistance traits.

Producers reported that they frequently treated commercial-layer chickens with antibiotics, including enrofloxacin, amoxicillin, oxytetracycline, chlortetracycline, sulfamethazine+trimethoprim and sulfadiazine. Farmers also reported using a coccidiostat called amprolium. Farmers specifically reported using antibiotics to treat Newcastle disease (a viral infection). We observed use of expired drugs in part because these commodities are purchased in large volumes and are simply used until gone. Farmers reported that when sick animals were observed, these were isolated and the entire flock was treated immediately to prevent a large disease outbreak. Farmers also reported using higher than recommended doses with hopes that this would lead to a shorter period of infection. We surmise that these antibiotic use practices drive the difference in prevalence of antibiotic resistant *E. coli* between the commercial-layer and free-range chickens. This also indicates that more investment is needed to help small-scale producers raise healthy animals through the use of better husbandry practices and vaccines. Such efforts are likely to help farmers reduce their reliance on antibiotics while increasing the success of their production efforts (Palmer and Call, 2013).

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was funded in part by the Government of Tanzania through NM-AIST, and by the Paul G. Allen School for Global Animal Health and the National Science Foundation (DEB1216040).

REFERENCES

Adenipekun EO, Jackson CR, Oluwadun A, Iwalokun BA, Frye JG, Barrett JB, Hiott LM, Woodley TA (2015). Prevalence and antimicrobial resistance in *Escherichia coli* from food animals in Lagos, Nigeria. Microb. Drug Resist. 21:358-365.

Anderson AD, Nelson JM, Rossiter S, Angulo FJ (2003). Public health consequences of use of antimicrobial agents in food animals in the United States. Microb. Drug Resist. 9:373-379.

Arslan S, Eyi A (2010). Occurrence and antimicrobial resistance profiles of *Salmonella* species in retail meat products. J. Food Prot. 73:1613-1617.

Bean DC, Livermore DM, Papa I, Hall LMC (2005). Resistance among *Escherichia coli* to sulphonamides and other antimicrobials now little used in man. J. Antimicrob. Chemother. 56:962-964.

Berendsen B, Stolker L, De Jong J, Nielen M, Tserendorj E, Sodnomdarjaa R, Cannavan A, Elliott C (2010). Evidence of natural occurrence of the banned antibiotic chloramphenicol in herbs and grass. Anal. Bioanal. Chem. 397:1955-1963.

Blahna MT, Zalewski CA, Reuer J, Kahlmeter G, Foxman B, Marrs CF (2006). The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* in Europe and Canada. J. Antimicrob. Chemother. 57:666-672.

Carraminana JJ, Rota C, Agustín I, Herrera A (2004). High prevalence of multiple resistance to antibiotics in *Salmonella* serovars isolated from a poultry slaughterhouse in Spain. Vet. Microbiol. 104:133-139.

Chiu LH, Chiu CH, Horn YM, Chiou CS, Lee CY, Yeh CM, Yu CY, Wu CP, Chang, CC, Chu C (2010). Characterization of 13 multi-drug resistant *Salmonella* serovars from different broiler chickens associated with those of human isolates. BMC Microbiol. 10:86.

Ewers C, Antão EM, Diehl I, Philipp HC, Wieler LH (2009). Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Appl. Environ. Microbiol. 75:184-192.

Finley RL, Collignon P, Larsson DGJ, Mcewen SA, Li XZ, Gaze WH, Reid-Smith R, Timinouni M, Graham DW, Topp E (2013). The scourge of antibiotic resistance: the important role of the environment. Clin. Infect. Dis. 57:704-710.

Hamisi Z, Tuntufye H, Shahada F (2014). Antimicrobial resistance phenotypes of *Escherichia coli* isolated from tropical free range chickens. Int. J. Sci. Res. 3:2012-2015.

Hao H, Cheng G, Iqbal Z, Ai X, Hussain HI, Huang L, Dai M, Wang Y, Liu Z, Yuan Z (2014). Benefits and risks of antimicrobial use in food-producing animals. Front. Microbiol. 5:1-11.

Horn F, Correa AMR, Barbieri NL, Glodde S, Weyrauch KD, Kaspers B, Driemeier D, Ewers C, Wieler LH (2012). Infections with avian pathogenic and fecal *Escherichia coli* strains display similar lung histopathology and macrophage apoptosis. PLoS One 7.

Hu LF, Chang X, Ye Y, Wang ZX, Shao YB, Shi W, Li X, Li J (2011). *Stenotrophomonas maltophilia* resistance to trimethoprim/sulfamethoxazole mediated by acquisition of sul and *dfrA* genes in a plasmid-mediated class 1 integron. Int. J. Antimicrob. Agents 37:230-234.

Jakobsen L, Kurbasic A, Skjøt-Rasmussen L, Ejrnaes K, Porsbo LJ, Pedersen K, Jensen LB, Emborg HD, Agersø Y, Olsen KEP (2010). *Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. Foodborne Pathog. Dis. 7:537-547.

Kilonzo-Nthenge A, Nahashon SN, Chen F, Adefope N (2008). Prevalence and antimicrobial resistance of pathogenic bacteria in chicken and guinea fowl. Poult. Sci. 87:1841-1848.

Lautzenhiser SJ, Fialkowski JP, Bjorling D, Rosin E (2001). In vitro antibacterial activity of enrofloxacin and ciprofloxacin in combination against *Escherichia coli* and staphylococcal clinical isolates from dogs. Res. Vet. Sci. 70:239-241.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10:122-129.

Liu J, Zhao A, Orfe L, Subbiah M, Call DR (2016) Soil-borne reservoirs of antibiotic-resistant bacteria are established following therapeutic treatment of dairy calves. Environ. Microbiol. 18:557-564.

Mathew AG, Cissell R, Liamthong S (2007). Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. Foodborne Pathog. Dis. 4:115-133.

Nakazato G, Campos TA, Stehling EG, Brocchi M, Silveira WD (2009). Virulence factors of avian pathogenic *Escherichia coli* (APEC). Pesqui. Veterinária Bras. 29:479-486.

NCCLS (2007). Performance standards for antimicrobial susceptibility testing.

Palmer GH, Call DR (2013). Antimicrobial resistance : A global public health challenge requiring a global one health strategy.

Stead SL, Caldow M, Sharma A, Ashwin HM, Sharman M, De-Rijk A, Stark J (2007). New method for the rapid identification of tetracycline residues in foods of animal origin - using the PremiTest in combination with a metal ion chelation assay. Food Addit. Contam. 24:583-589.

Subbiah M, Mitchell SM, Ullman JL, Call DR (2011). B-lactams and florfenicol antibiotics remain bioactive in soils while ciprofloxacin, neomycin, and tetracycline are neutralized. Appl. Environ. Microbiol. 77:7255-7260.

Wellington EMH, Boxall ABA, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W (2013). The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. Lancet Infect. Dis. 13:155-165.

Wright GD (2010). Antibiotic resistance in the environment: a link to the clinic? Curr. Opin. Microbiol. 13:589-594.

Wu S, Dalsgaard A, Hammerum AM, Porsb LJ, Jensen LB (2010). Prevalence and characterization of plasmids carrying sulfonamide resistance genes among *Escherichia coli* from pigs, pig carcasses and human. Acta Vet. Scand. 52(1):1-47.

Yang H, Chen S, White DG, Zha, S, McDermott P, Walker R, Meng J (2004). Characterization of multiple-antimicrobial-resistant *Escherichia coli* isolates from diseased chickens and swine in China. J. Clin. Microbiol. 42:3483-3489.

African Journal of Microbiology Research

Related Journals Published by Academic Journals

- *African Journal of Biotechnology*
- *African Journal of Biochemistry Research*
- *Journal of Bacteriology Research*
- *Journal of Evolutionary Biology Research*
- *Journal of Yeast and Fungal Research*
- *Journal of Brewing and Distilling*

academicJournals