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Full Length Research Paper

Profile of bacterial and parasitic urinary infections in Saint Louis Senegal between 2000 and 2010

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Infections of the urinary tract are important part of infectious diseases that mainly involve bacteria. In parasite endemic areas, bacterial and parasitic co-infections may occur, however in Saint-Louis region which is a Schistosoma-endemic area, little is known about this association. Here this study aim to investigate the spectrum of bacterial and parasitic urinary infections as well as co-infections with both species. Concordantly to the current algorithm at the biomedical laboratory at Saint Louis Hospital, bacterial and parasitic investigation has been performed using the conventional method of bacteriological and parasitological examination of urine. Data were collected from register records from 2000 to 2010, recorded and analysed using Epi Info 7. 17107 urines samples were recorded and among which, 2352 (14%) were positive bacterial cultures including mainly *Escherichia coli* (54%) and *Klebsiella* spp (19%). Seven hundred and forty-three parasites have been identified including *Trichomonas vaginalis* (64%), Schistosoma *haematobium* (34%) and *Schistosoma mansoni* (2%). Both parasites and bacteria were found in 55 samples with *T. vaginalis/E. coli* (79%) as main combination, followed by *T. vaginalis /Klebsiella* spp. (11%). Regarding *S. haematobium,* it was found to be associated with *E. coli* in 7 samples and *Klebsiella* spp. in 4 samples. In the region of Saint-Louis, the urinary tract infections are dominated by bacterial infections including mainly *E. coli* and *Klebsiella* spp and may, for lesser extent, be caused by *Schistosoma haematobium* and *Trichomonas vaginalis*. Thus, the evaluation of the impact bacterial and parasitic co-infection in the urinary tract should have a particular interest especially in Schistosoma endemic area and need further study to better understand the potential interactions.

Key words: Urines, bacteria, parasites, co-infection, Saint-Louis, Senegal.

INTRODUCTION

Infections of the urinary tract are important part in infectious diseases among which bacteria remain far the main causes. Among the bacteria, the Gram negative bacilli, especially the enterobacteria, represent 85% with *Escherichia coli* in first line (75 to 90%), followed by *Klebsiella spp* and *Pseudomonas spp* (Ferjani et al., 2011). The parasitic strains reaching the urinary system are mainly the schistosomes; however, other parasites

such as *Trichomonas vaginalis* or filaria can be involved in urinary tract infections, leading to renal disturbances (Bourée et al., 2007).

Urinary schistosomiasis is endemic in 76 countries, affecting 207 million harbouring the Schistosomiases and about 600 million people at risk of contracting urinary diseases (Organisation Mondiale de la Santé (OMS), 2011). Eighty percent of infected people are from sub Saharian Africa and Senegal is not unscathed from that pathology with a prevalence of 44% for *Schistosoma haematobium* (Meurs et al., 2013).

Furthermore, in certain sites of northern Senegal, the prevalence of *S. mansoni* is slightly above that of *S. haematobium* which are 61 and 50% respectively (Meurs et al., 2012a). Thus, co-infection with bacteria and parasites may occur in the urinary tract of the same individual and can lead to interactions that might have important implications on the morbidity of urinary tract infections.

However in the study context, few data regarding the co-infection prevalence in the urinary tract are available. Thus, this is a retrospective study that aims to assess the bacterial and parasitic profile in urine samples collected from 2000 to 2010 at the regional hospital center (RHC) of Saint Louis in Senegal as well identifying possible co-infections.

METHODOLOGY

Study site

This retrospective study was conducted at the Regional Hospital Center (RHC) of Saint-Louis region. Located in the Nord-East of Senegal in the outfall of the Senegal River, Saint-Louis is part of the Ferlo region and possesses a Sahalian climate. The main activities in that region are fishing and agriculture that both promote permanent water contact. Moreover, an important ecologic changes resulting from the implementation of the dam of Diama in 1989 has widespread in the distribution of schistosomes and markedly increased the prevalence of *schistosoma* infections (Meurs et al., 2012b; Southgate et al., 2001). Indeed, *Schistosoma* strains possess an intermediate life cycle that requires sweet water and involving molluscs from *Bulinus* genus (Bourée et al., 2007).

Urine collection period

Collection of urine samples used for parasitic and bacterial examination was seasonal and has been performed in two different seasons of the year: from October to June corresponding to the dry season and from June to September that coincides to the raining season.

Bacterial examinations

Urine tests were performed using the routine procedure of bacterial examination at the laboratory of the Saint-Louis hospital. It consists of macroscopic and microscopic examination and culture in cystine-lactose-electrolyte-deficient agar (CLED). Bacterial infection was defined with a positive threshold 10^5 colonies per milliliter of urine associated with leukocyte reaction. Bacteria were identified through their morphologic, biochemistry and antigenic characteristics. Gram negative bacilli have been identified on the basis of biochemical characteristics such as oxidase, fermentation of sugars, possession of an urease, indole prodcution and the presence of tryptophan desaminase. Concerning Gram positive cocci, the study used the test to catalase, the type of hemolysis and specific agglutination tests for their identification. Antimicrobial susceptibility was performed for samples that were positive by diffusion technique on ordinary blood agar, according to the recommendations of the "Comité de l'Antibiogramme de la Société Française de Microbiologie" (CA-SFM 2010).

Parasitological examinations

After centrifuging at 3000 rpm for five minutes, specimens were tested under optical microscope in order to detect Schistosoma haematobium eggs by a terminal spine, Schistosoma mansoni by a lateral spine and Trichomonas vaginalis with the motility.

Data analysis

Recoded data arising from bacteria and parasite tests performed at the medical biology laboratory from 2000 to 2010 were analysed using Epi info version 7.

RESULTS

Overall, 17107 urine samples were analysed at the medical biology laboratory of Regional Hospital Center of Saint-Loui,s from 2000 to 2010. The prevalence of bacterial and parasitic infections were 13.7 (n=2346) and 4.3% (n=743) respectively.

Profile of bacterial infections in the urinary tract

Among identified bacteria, 84.67% were enterobacteria including *Escherichia coli* that represented 54% of them, followed by *Klebsiella spp* (18.98%), *Enterobacter spp* (5%) and *Proteus* spp (3.6%). Other bacteria such as *Pseudomonas* spp, *Enterococcus* spp and *Staphylococcus aureus* were also found (Figure 1).

Profile of parasitic infections

Parasites found in urine samples were mainly *Trichomonas vaginalis* representing 64% (n= 477) and *Schistosoma* species with 35% (n= 261) mainly *S. haematobium* (n=257). The prevalence of these parasites

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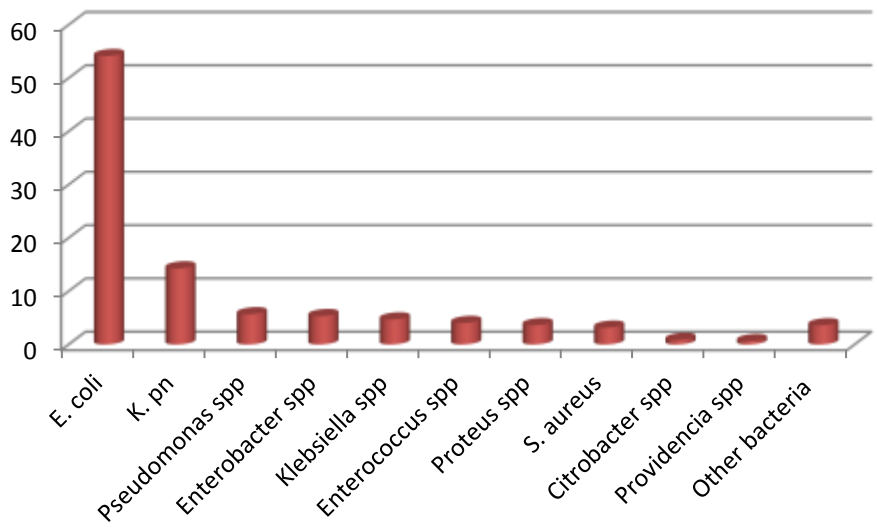

Figure 1. Distribution of bacteria according to species.

Table 1. Distribution of parasites and bacteriuria according to the season and sex.

Variable	*T. vaginalis*	*S. haematobium*	*S. mansoni*	*S. haematobium/ mansoni*	Positive cultures	Negative cultures
Saison 1	340	193	2	3	37	507
Saison 2	137	61	1	1	18	181
Total	477	254	3	4	55	688
Female	436	105	1	1	48	500
Male	35	149	2	3	7	182
Sex NP	6	0	0	0	0	6
Total	477	254	3	4	55	688

was 74% in female subjects compared to 26% in male. 73% of parasites were found in the dry season and 27% in the raining season (Table 1).

Bacterial and parasitic co-infection

Simultaneous presence of bacteria and parasites were found in 55 samples. The bacteria found in association with parasites were *E. coli* (62%), *K. pneumoniae* (16%), *Enterobacter spp* (7%), *Enterococcus* spp (5%), *and Streptococcus spp* (4%). Other bacteria such as *Klebsiella spp*, *Pseudomonas* spp., *Staphylococcus* spp., *Moraxella spp* and *Proteus vulgaris* were also associated once with parasites (Table 2).

Regarding parasites, *T. vaginalis* was found in association with *E. coli* in 27 subjects (49%) and *Klebsiella* spp. in six patients (10.9%). *S. haematobium* was found in association with *E. coli* in seven urine samples and with *Klebsiella* spp. in four patients. Presence of both *Enterobacter* spp. and *T. vaginalis* was found in four subjects, all female. *Enterococcus spp* strains were found associated to *T. vaginalis* in three positive cultures of which two were polybacterial (*E. coli* for the one and *Klebsiella* the other). *S. haematobium* and ectopic *S. mansoni* eggs was found in four samples, all free from bacteria.

Table 2. Co-infections bacteria/parasites.

Bacteria	Parasites		Total
	T. vaginalis	*S. haematobium*	
E. coli	27	7	34
Klebsiella spp.	6	4	10
Enterococcus spp.	3	-	3
Enterobacter spp.	4	-	4
Pseudomonas spp.	-	1	1
Streptococcus spp.	-	2	2
Moraxella spp.	-	1	1
Total	40	15	55

Table 3. Percentage of susceptibility to antimicrobial agents.

Bacteria	C3G	Fluoroquinolones	Trimethoprim/Sufamethoxazole
E. coli	100	12	0
Klebsiella spp	90	90	70
Enterobacter spp	100	100	50
Pseudomonas spp	100	100	-
Enterococcus spp	67	67	0

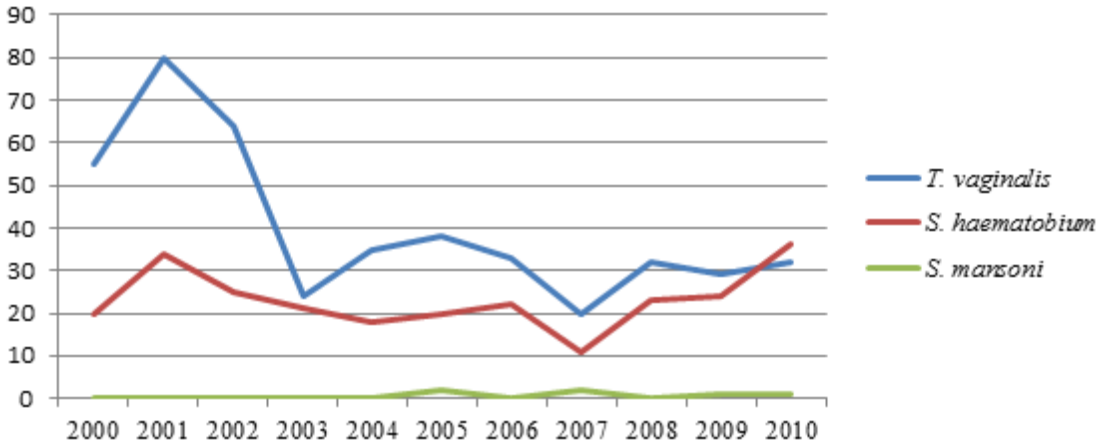

Figure 2. Annual distribution of parasites.

Antibiogram

The results of susceptibility testing are summarized in Table 3.

DISCUSSION

Bacterial and parasitic infections are important part of infectious disease. However in this context, the possible interactions between the microorganisms in the urinary tract remain unknown. Among 17107 microbilogical urines tests that have been performed between 2000 and 2010, the study found a frequency of 13.7% for bacteria. The study data along with higher prevalence of bacteria in urines reported in Ethiopia between 2003 and 2010 (22,7%), and the study of Kibret and Abera (2014) suggested quite a high prevalence of bacterial urinary infection in tropical and sub-tropical settings.

As previously shown (Daza et al., 2001; Kibret and Abera, 2014), the enterobacteria were largely predominant in the urines with *E. coli* reprensenting more than half of isolated bacteria. Indeed, several studies reported that *E. coli* remain the main enterobacteria identified in urinary tract followed by *K. pneumoniae* and *Pseudomonas spp* or *P. mirabilis* (Daza et al., 2001; Kibret and Abera, 2014; Meiland et al., 2004). This high prevalence might be related to the proximity of the cutaneous and mucosal microbiata to the genital mucosa as well as the ascendant physiopathology of urinary tract microorganism deriving from uretral microbiota. Regarding parasitic infections the study found a prevalence of 4.3 (including mainly *T. vaginalis* and *S. haematobium)* and 0.94% for *S. mansoni*. This ectopic release of *S. mansoni* eggs through the urines takes particular importance because of its association with the severity of urinary schistosomiasis. Despite a similar trend, the frequency of *T. vaginalis* was higher than those of *S. haematobium* from 2000 to 2009. However from 2010, a slight increase of the frequency of Schistosomes compared to *Trichomonas* has been observed (Figure 2).

Several studies have shown that the prevalence and infection intensity are function of environmental conditions and age of the hosts (Ibikounlé et al., 2013; Meiland et al., 2004; Sy et al., 2011). In South Benin, a *S. haematobium* prevalence of 32.78% along with high infection intensity has been reported in school children 7 to 8 years old, especially in males (Ibikounlé et al., 2013). We were not able to analyse the data according to age which was missing from the study data. According to the season, Sy et al. (2011) have reported that *S. haematobium* was not detected at the beginning of the raining season in the Eastern part of Senegal whilst its prevalence was 7.6%; this prevalence was higher in

school children compared to adults (Sy et al., 2011). The same study has shown that in the dry season, *S. haematobium* was associated to females compared to males (Sy et al., 2011). However, other investigations did not show any difference in the prevalence of schistosomiasis according to the sex (Dabo et al., 2011). It appears that the prevalence of S. haematobium changes following the season and would be higher the raining season because of ecoclimatic conditions promoting parasite transmission (Clements et al., 2008).

The study found a *T. vaginalis* prevalence of 4.3% with 91% in female; this prevalence was lower than what has been reported by Schwebke et al. (2003) in men having sex with men. We found that prevalence of schistosomes were higher in males compared to females. These results are not consistent with others showing higher prevalence in male school children compared to females; however reverse phenomenon was observed in adults (Schwebke et al., 2003).

Regarding bacteria and parasite co-infection, the study data have shown that *E. coli* was the main bacteria associated to parasites in over 50%, followed by *K. pneumoniae*, *Enterobacter* spp, *Enterococcus* spp and *Streptococcus spp. S. haematobium* was associated to *E. coli* in 12.7% of urine samples and *Klebsiella* spp in 7%. However, we did not find any co-infection with *Neisseria* and *Trichomonas* though Jane et al. () have reported such association in 9.4% of urinary infections (Schwebke et al., 2003). Good sensitivity was obtained with the third generation of cephalosporin for all isolates and fluoroquinolones (except *E. coli*). The high resistance of *E. coli* with quinolones was obtained by Kibret and Abera (2014), unlike Daza et al. (2001) who showed acceptable levels of activities for quinolones with gram negative bacilli (Daza et al., 2001).

To the best of the study knowledge, any study investigating the co-infection with bacteria and parasites has been conducted in Senegal. Interactions between these microorganisms may result in potentialization of transmission of one or other microorganisms; inversely, such interaction may lead to suppression of virulence or proliferation of one species at the expense of the other (Hoffman et al., 2006). This thus justify the need of conduction prospective study on parasitic and bacterial infections in co-endemic settings.

A limitation in this study is that the analysis of the age of which prevalence depend and morbidity of urinary schistosomiasis is missing.

Conclusion

In Saint Louis region, bacteria, particulary the enterobacteria, remain far the main causes of urinary tract infections. Nevertheless, because of its geographical specificity promoting schistosomiasis infections, the prevalence of *S. haematobium* remain high in such setting. The bacterial and parasitic co-infection might lead to interactions able to influence the pathology of urinary tract infection. Further investigations need to be conducted for better understanding of the interactions between bacteria and parasites in the urinary tract infections.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Prevalence and antimicrobial susceptibility pattern of *Staphylococcus aureus* from raw camel and goat milk from somali region of Ethiopia

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The study was carried out with the aim to isolate *Staphylococcus aureus* from camel and goat raw milk and determine their antimicrobial susceptibility pattern. 204 raw milk samples were collected from randomly selected lactating camels (n=62) and lactating goats (n=142) in live-stock producing pastoralists' areas of Somali region for isolation and identification of *S. aureus*. Antibiotic susceptibility tests were performed on all *S. aureus* isolates to 16 antibiotics by Kirby-Bauer disk diffusion method. Twenty three (11.27% of the total) *S. aureus* strains were isolated. Four (6.45%) strains were isolated from camel raw milk samples and 19 (13.34%) from goat raw milk samples. *S. aureus* isolates showed resistance to Nalidixic acid, Polymixin B, and Penicillin G. *S. aureus* isolates were sensitive to vancomycin, ciprofloxacin, cefoxitin, cephalotin, gentamycin, Doxycycline, kanamycin, trimethoprim-sulfamethoxazole, chloramphenicol, norfloxacin, and erythromycin. Multi drug resistance was detected in 69.2% of the isolates. The present study has demonstrated the existence of alarmingly high level of multiple antimicrobial resistances of *S. aureus* among camel and goat milks.

Key words: *Staphylococcus aureus,* camel and goat milk, antimicrobial susceptibility test, Ethiopia.

INTRODUCTION

Staphylococcus aureus is a ubiquitous human and animal pathogen and a common cause of invasive and life threatening infections. Human illness through *S. aureus* range from minor skin infection such as pimples, boils, cellulites, toxic shock syndrome, impetigo, and abscesses to life threatening disease such as pneumonia, meningitis, endocarditis, and septicaemia (Daka et al., 2012; Thaker et al., 2013; Mekuria et al., 2013). *S. aureus* is also a major causative pathogen of clinical and subclinical mastitis in animals (Adwan et al., 2005; Mekuria et al., 2013). On top of it, it is also an important food borne pathogen. Milk has been reported as a common food which is a source of staphylococcal infections and a cause of staphylococcal poisoning (Lowy, 1998; Le loir et al., 2003). About 50% strain of *S. aureus* are able to produce enterotoxins associated with food poisoning (Payne and Wood, 1974).

The natural ecological niches of *S. aureus* are the

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nasal cavity and the skin of warm-blooded animals. The skin, mucosa membranes, teats and udders of milking animals are the most important reservoir of this contaminant. *S. aureus* is frequently isolated from raw milk manually draw from individual animals, bulk raw milk and naturally from milk of dairy cattle suffering from mastitis. (Medveďová and Valík, 2012).

Contamination of milk and milk products with pathogenic bacteria is mainly due to processing, handling and unhygienic environment. The occurrence of these pathogenic bacteria in milk and milk products can cause severe health hazards to people as they are highly susceptible to variety of microorganism because of high nutritive value and toxic nature caused by, or thought to be caused by the complex chemical composition (Thaker et al., 2013).

The indiscriminate use of antibiotics for the treatment of animal and human diseases as well as preservatives for milk has led to the development of multiple antibiotic resistances thereby rendering the antibiotic treatment ineffective. *S. aureus* has been reported to frequently show multiple antimicrobial resistance patterns (Enright, 2003). There is a limited number of studies on prevalence and antimicrobial resistance of *S. aureus* originated from camel and goat milk in the pastoralist areas of Ethiopia. Thus, this study aimed to isolate *S. aureus* from milk of healthy lactating camel and goat in Somali region of Ethiopia and found out its prevalence and antimicrobial resistance pattern.

MATERIALS AND METHODS

Study design and area description

A cross-sectional study was conducted from November 2013 to April 2014 in Somali region which is located in Eastern part of Ethiopia. The area was selected for its potential production of milk by pastoralist and semi-pastoralists. Pastoralists/semi- pastoralists were included using systematic random sampling methods to meet our total sample number requirement. The areas are geographically found at a latitude of 9°21'N and a longitude of 42°48' E and characterized by unreliable and erratic rainfall with a precipitation ranging from 300 to 600 mm per annum, high ambient temperature of 30°C, sparsely distributed vegetation dominated by Acacia species, cactus and bushy woodlands. These are arid and semi-arid lowlands lying at an elevation of 500 to 1500 m above sea level and are not suitable for crop production (Tafesse, 2001).

Dairy pastoralists/semi-pastoralists' household selection

Zonal and woreda agriculture bureau were contacted to obtain the important base-line data about the potential milk production of dairy pastoralists/semi-pastoralists living in each kebeles (the smallest administrative divisions in each region of Ethiopia) and get guidance during sample collection. Based on this base-line data, rural *Kebeles* with milk production potentials were selected using purposive sampling. Thirteen kebeles were included in the study from four woredas (Larger administrative division in Ethiopia and comprises around 20-25 kebeles) and, pastoralist/semi- pastoralist households who own local camel and goat breeds from each rural *Kebeles* were selected. The local camel breeds were one humped African camels (*Bos indices*) and the goat breeds were Short-eared Somali and Long-eared Somali Milk samples were collected from pastoralist/semi- pastoralist households based on availability. Housedhods which have one lactating camels and/or goats were taken as milk sources.

Study population and sampling techniques

A total of 204 raw milk samples were collected from randomly selected lactating camels (n=62) and lactating goats (n=142) in live-stock producing pastoralists' areas of Somali region that were kept under traditional management from different woredas (Larger administrative division in Ethiopia and comprises around 20-25 kebeles) in Somali, eastern Ethiopia. The total number of samples were determined based on previously reported prevalence of *S. aureus* which were 4.2% in camel milk of Jijiga town (Husein et al., 2013) and 12.8% in goats milk of Adami Tulu areas (Molla et al., 2006) in Ethiopia, respectively.

The study included major local camel and goat breeds of Somali region of Ethiopia for sources of raw milk. The lactaing camels and goats were included from the selected zones and woredas of the region based on randomly selected households.

Sample collection and transportation methods

From the randomly selected dairy pastoralist/semi-pastoralist' households, milk samples were collected based on availability after gathering information about the local breed types they own. Raw milk was directly drawn from udder of lactating camels and goats which were found in the selected pastoralist/semi-pastoralist' households in each kebeles. A total of 204 raw milk samples were collected using sterile plastic test tubes. For collection of raw milk samples, the udder was washed with antiseptic solution, wiped dry with clean cloth and then disinfected with 70% alcohol, the foremilk was discarded and 20-40 ml of pooled milk were collected. Milk samples were transported in an ice box with ice to Ethiopian Biodiversity Institute, Addis Ababa, Ethiopia for further bacterial identification.

Isolation and identification of *S. aureus*

About 0.1 ml of milk sample was streaked on Mannitol Salt Agar (MSA) (Hi-Media Laboratories, Mumbai, India) plate and incubated at 37°C for 24-48 h for the isolation of *S. aureus*. Suspected colonies of *S. aureus* that were yellow in colour on each MSA plate were further purified by sub-culturing onto MSA plates and incubated aerobically at 37°C for 18–24 h (Daka et al., 2012; Sharma et al., 2011).

Bacterial identification was performed by gram staining, microscopic examination of the morphology and biochemical tests. Gram-positive cocci that occurred in clusters under the microscope were subjected to preliminary biochemical tests (the catalase and oxidase tests). The identities of the isolates were confirmed based on positive results for the DNase test, beta-haemolytic patterns on blood agar enriched with 5% sheep blood and the coagulase slide and coagulase tube test for *S. aureus* (Bergey's Manual of Systematic Bacteriology, 2009; Daka et al., 2012).

Antimicrobial susceptibility test

Antibiotic susceptibility tests were performed by the Kirby-Bauer disk diffusion method (CLSI, 2008) on all *S. aureus* isolates (n=23) using Mueller Hinton agar (supplied by Oxoid, UK). Fresh overnight cultures were prepared and used for antibiotic sensitivity tests. An

Table 1. Prevalence of *Staphylococcus aureus* in camel and goat milk samples.

Source	Number examined	Number of positive (%)	Number of negative
Goat milk	142	19(13.34)	123(86.62)
Camel milk	62	4(6.45)	58(93.55)
Total	204	23(11.27)	181(88.73)

aliquot (100 µL) from each isolate suspension was spread plated on Mueller Hinton agar (supplied by Oxoid Company). Susceptibilities of the isolates to a panel of sixteen different antibiotic discs (6 µm in diameter, Mast group LTD MERSEY SIDE, UK) were determined. The following antimicrobial disks (Oxoid disks) with their corresponding concentration were used: Penicillin G (10 units), Amoxicillin (25 mcg), Cephalothin (30 mcg), Cefoxitin (30 mcg), Gentamicin (10 mcg), Kanamycin (30mcg), Nalidixic acid (30 mcg), Ciprofloxacin (5 mcg), Norfloxacin (10mcg), Trimethoprim-Sulfamethoxazole (25 mcg), Polymixin B (300unit), Erythromycin (15 mcg), Vancomycin (30 mcg), Chloramphenicol (30 mcg), Doxycycline (30 mcg) and Tetracycline (30 mcg). Antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar to ensure intimate contact with the surface and the plates were incubated aerobically at 37°C for 18h-24 h (CLSI, 2008). Inhibition zone diameters were measured and value obtained from Clinical and Laboratory Standards Institute (CLSI, 2008) was used to interpret the results obtained. *S. aureus* isolates were then classified as resistant, intermediate-resistant or susceptible to a particular antibiotic. Multiple drug resistant (MDR) phenotypes were recorded for isolates showing resistance to three and more antibiotics (CLSI, 2008; Rota et al., 1996).

Data analysis

Statistical analyses were carried out using STATA version 11 and WHONETs version 5.6 statistical software packages after exporting the raw data which were entered into excel spread sheet. Descriptive statistics such as percentages and frequency distribution was used to describe bacterial isolates and antimicrobial susceptibility which was expressed as percent of resistant, intermediate and susceptible. In addition, the proportion of bacteria resistant to at least one of the sixteen antibiotics and resistant two or more were calculated.

RESULTS

Prevalence of *S. aureus* in camel and goat milk samples

Of the total 204 examined goat and camel raw milk samples, 23 (11.27%) were positive for *S. aureus.* Four (6.45%) *S. aureus* were isolated from the 62 camel raw milk samples, and 19 (13.34%) were isolated from 142 goat raw milk samples (Table 1). The highest isolation of *S. aureus* was from goat milk (13.34%) while less was from camel milk (6.45%) (P value=0.15).

Antibiotic susceptibility test results

All 23 *S. aureus* isolates were subjected to antibiotic susceptibility tests. Sixteen different antibiotics were used. *In vitro* antimicrobial resistance pattern of *S. aureus* isolates to the 16 antibiotics was investigated.

Antimicrobial susceptibility pattern of *S. aureus* isolated from goat milk samples

Results of antimicrobial susceptibility test against *S.aureus* showed high susceptibility to ciprofloxacin (89.5%) and vancomycin (89.5%) followed by cephalothin (84.2%), cefoxitin (84.2%), kanamycin (84.2%), gentamicin (84.2%), trimethoprim-sulfamethoxazole (78.9%), erythromycin (78.9%), doxycycline (78.9%), and chloramphenicol (78.9%) (Table 2) whereas high level of resistance was recorded against Penicillin G (78.9%), nalidixic acid (78.9%), polymixin B (78.9%), and tetracycline (36.8%) (Table 2).

Antimicrobial susceptibility pattern of *S. aureus* isolated from camel milk samples

Results of antimicrobial susceptibility test against *S.aureus* showed high susceptibility to vancomycin (100%), cephalothin (100%), cefoxitin (100%), norfloxacin (100%), doxycycline (100%) followed by gentamicin (75%), trimethoprim-sulfamethoxazole (75%), penicillin G (75%), Amoxicillin (75%), ciprofloxacin (75%), and Chloramphenicol (75%) (Table 3) whereas high level of resistance was recorded against Nalidixic acid (100%), Polymixin B (75%), and Tetracycline (50%) (Table 3).

Multiple drug resistant phenotypes of *S.aureus*

The present study demonstrated multiple drug resistance of *S.aureus* isolates (Table 4). All of the 23 confirmed *S. aureus* isolates showed resistance to one or more antimicrobial agents. Three isolates (13.0%) were resistant to single antibiotic and four isolates (17.4%) showed resistance to two antimicrobial agents. Multiple drug resistance (MDR) which is defined as resistance to three or more antimicrobial agents was found in 69.6% of *S. aureus* isolates. The predominant MDR phenotypes of *S. aureus* isolated from this study area was POL-PEN-NAL in 34.8% of the isolates. Furthermore, MDR phenotypes POL-PEN-NOR-NAL, POL-KAN-ERY-NAL, POL-PEN-ERY-GEN-NAL, POL-KAN-ERY-GEN-NAL, POL-KAN-PEN-NOR-NAL, POL-PEN-NOR-VAN-ERY- NAL,

Table 2. Antimicrobial susceptibility pattern of *S. aureus* isolated from goat milk samples.

Antibiotic name	Number	%R	%I	%S
Penicillin G	19	78.9	0	21.1
Amoxicillin	19	42.1	0	57.9
Cephalothin	19	15.8	0	84.2
Cefoxitin	19	10.5	5.3	84.2
Gentamicin	19	5.3	10.5	84.2
Kanamycin	19	10.5	5.3	84.2
Nalidixic acid	19	78.9	21.1	0
Ciprofloxacin	19	5.3	5.3	89.5
Norfloxacin	19	15.8	10.5	73.7
Trimethoprim/Sulfamethoxazole	19	15.8	5.3	78.9
Polymixin B	19	78.9	0	21.1
Erythromycin	19	10.5	10.5	78.9
Vancomycin	19	5.3	5.3	89.5
Chloramphenicol	19	15.8	5.3	78.9
Doxycycline	19	10.5	10.5	78.9
Tetracycline	19	36.8	10.5	52.6

Table 3. Antimicrobial susceptibility pattern of *S.aureus* isolated from camel milk samples.

Antibiotics	Number	%R	%I	%S
Penicillin G	4	25	0	75
Amoxicillin	4	25	0	75
Cephalothin	4	0	0	100
Cefoxitin	3	0	0	100
Gentamicin	4	25	0	75
Kanamycin	4	25	25	50
Nalidixic acid	4	100	0	0
Ciprofloxacin	4	0	25	75
Norfloxacin	4	0	0	100
Trimethoprim/Sulfamethoxazole	4	25	0	75
Polymixin B	4	75	0	25
Erythromycin	4	25	25	50
Vancomycin	4	0	0	100
Chloramphenicol	4	0	25	75
Doxycycline	4	0	0	100
Tetracycline	4	50	0	50

Table 4. Multiple drug resistant phenotype of *S.aureus* isolated from goat and camel milk samples (n= 23).

Resistance profile (Phenotypes)	Number of resistant isolates	% of isolates
NAL	3	13
PEN NAL	2	8.7
POL NAL	2	8.7
POL PEN NAL	8	34.8
POL PEN NOR NAL	1	4.3
POL KAN ERY NAL	1	4.3
POL PEN ERY GEN NAL	1	4.3
POL KAN ERY GEN NAL	1	4.3

Table 4. Contd.

POL KAN PEN NOR NAL	1	4.3
POL PEN NOR VAN ERY NAL	1	4.3
POL KAN PEN NOR ERY GEN NAL	1	4.3
POL KAN PEN NOR VAN ERY GEN NAL	1	4.3

NAL, Nalidixic acid; PEN, Penicillin G; POL, Polymixin B; NOR, Norfloxacin; KAN, Kanamycin; ERY, Erythromycin; GEN, Gentamicin; VAN, Vancomycin.

POL-KAN-PEN-NOR-ERY-GEN-NAL, and POL-KAN-PEN-NOR-VAN-ERY-GEN-NAL were obtained in 4.3% of the isolates each.

DISCUSSION

The observed prevalence of *S. aureus* in camel and goat milk found in the present study is in line with the findings of Husein et al. (2013) who found 4.2% isolation rate in camel milk in and around Jijiga town and Molla et al. (2006) who isolated 12.8% from goat milk in Adami Tulu town in Ethiopia. Similarly, it was closely comparable with the findings of Ebrahim et al. (2013) who reported 3.4 and 7.5% prevalence of *S. aureus* in camel and goat milk in Iran, respectively. However, the present findings are lower than that of Abdurahman et al. (2006) who reported 12.7% in Errer valley of eastern Ethiopia and Abera et al. (2010) who showed a 26.3% prevalence of *S. aureus* from mastitis milk of camel at Jijiga, eastern Ethiopia. This high isolation rate of *S.aureus* from camel milk is due to the fact that the collected milk unlike ours is from clinicaly diseased camel suffering from mastitis which is mainly caused by *S.aureus.*

Studies show that susceptibility patterns of *Staphylococcus aureus* to antimicrobial agent have varied worldwide, but isolates were usually susceptible to kanamycin, ciprofloxacin, vancomycin, and gentamicin (Alian et al., 2012; Daka et al., 2012; Thaker et al., 2013; Mekuria et al., 2013). *S. aureus* isolates in the present study also showed high sensitivity towards vancomycin, ciprofloxacin, cefoxitin, cephalotin, gentamycin, chloramphenicol and kanamycin.

The antimicrobial susceptibility result in the present study is comparable with the results obtained in Alamin et al. (2013) where *S.aureus* isolates were found to be sensitive for the tested antibiotics in the following percentage: ciprofloxacin (77.8%), gentamycin (88.8%), tetracycline (77.8%), amoxicillin (66.7%). In Mekuria et al., (2013) study *S. aureus* isolates were sensitive to vancomycin (88.2%) and Trimethoprim-Sulfamethoxazole (66.7%). High antibiotic susceptibility of *S. aureus* to some of the drugs is also reported in Tofaily et al. (2011). Tofaily et al. (2011) showed in their findings that *S. aureus* isolates were sensitive to amoxicillin (83.3%), tetracycline (83.3%), chloramphenicol (83.3%), ciprofloxacin (100%), doxycycline (100%), trimethoprim-sulfamethoxazole (83.3%) which has much similarity with the present study result.

In contrast to the present study, the reported sensitivity of *S. aureus to* some antibiotics is much different. For instance, Mekuria et al. (2013) reporetd that the susceptibility rate of *S. aureus* isolates to Erythromycin was 21.6%. Alamin et al. (2013) reported a susceptibility rate of 33.3% to Trimethoprim-Sulfamethoxazole and Tofaily et al. (2011) cited a sensitivity percentage of 16.6% to Erythromycin. *S. aureus* isolated in the present study found to be highly susceptible to these antibiotics. This might be that these antibiotics are not frequently used in the study area in veterinary services and perhaps in human medicine.

The high percent of antimicrobial resitance exihibited to Nalidixic acid, Polymixin B , and Penicillin G. in this study is in line with the findings of Tariku et al. (2011) who reported 87.2% resistance to penicillin in Ethiopia, 64.3% of resistance to penicillin G (Daka et al., 2012) in Hawassa area of Ethiopia and 80% resistance to penicillin which is reported in Sweden by Landin (2006). This is in contrast to findings observed by Adesiyun (1994) who reported 23% of resistance to pencillin G in West India and Alian et al. (2012) who reported 17.4% of resistance to pencillin G in Iran.

The probable explanation to the presence of high antibiotic resistant *S. aureus* to nalidixic acid, polymixin B, penicillin and tetracyclin is the indescriminate and repeated use of these antibiotics in animal and human health facilities of the present study area. Penicillin and tetracycline are the most commonly used antimicrobials in the treatment of infections in the livestock sector in Ethiopia. Moreover, tetracycline is widely used as growth factors in Veterinary Medicine for livestock rearing in addition to the treatment of bacterial infections.

The present study has demonstrated the existence of alarmingly high level of multiple antimicrobial resistances of *S. aureus.* 69.2% of the isolated *S. aureus* developed MDR. This result is in line with Daka et al. (2012) who reported 62.8% multidrug resistance rate of *S. aureus* isolates from cow's milk in Hawassa town which is located in southern Ethiopia. Chao et al. (2007) similarly reported a higher multidrug resistance rate (79%) of *S. aureus*. This is also comparable with findings of Sharma et al. (2011) who reported 70% MDR *S. aureus* from

raw milk of dairy cattle in India. A special attention must be given to the high prevalence of multidrug resistant *S.aureus* indicated in the present study area among dairy goat and camel which has great risk for consumers and individuals who have contact with animals. If these strains do cause diseases, treatment with the antibiotics that they are resistant to might not be useful. It is also a concern if those isolates get transmitted to humans and cause disease (Zunita et al., 2008).

Conclusion

The prevalence of *S. aureus* in camel and milk is found to be 6.45 and 13.34% in Somali region of Ethipia. A large proportion of the isolates were resistant to three or more antibiotics which showed the existence of MDR *S. aureus* in the husbandry. Resistance to Vancomycin is shown in some *S. aureus* isolates. Thus, it needs an attention since Vancomycin is one of the last choice in the treatment of *S. aureus* infections. The antimicrobial resistance tests carried out in this study indicated the high resistance of *Staphylococcus* species to nalidistic acid, polymixin B and penicillin G. The high level of MDR *S. aureus* needs further investigation based on molecular methods to study its impacts and dynamics of genetic antibiotic determinants.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Role of bacteriological investigation of endotracheal aspirate in diagnosis of ventilator-associated pneumonia

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Ventilator associated pneumonia (VAP) is a common complication of ventilatory support for patients with acute respiratory failure, currently related to high mortality rate. Therefore, this complication of mechanical ventilation requires a prompt diagnosis and adequate antibiotic treatment. The study aimed to investigate the role of endotracheal aspirate (ETA) surveillance cultures in identifying the aetiology of VAP earlier. The study was conducted over a period of 12 months and included 152 patients under mechanical ventilation for >48 h from different ICUs of Assiut University Hospitals. Quantitative cultures of ETA at threshold of 10^5cfu/ml were performed. The organisms were primarily identified by colony morphology, microscopy of Gram stain and standard biochemical tests. The antibiotic resistance pattern of the isolated bacteria was determined by Kirby-Bauer disk diffusion method. VAP was suspected in 92/152 patients (60.53%). Microbiological support for VAP was obtained by ETA in 90 patients. Positive cultures occurred in 88 patients, the infection was polymicrobial in 50 (54.34%) of cultures. Major isolated pathogenic bacteria were gram negative (54.79%); *Klebsiella sp*ecies was the commonest organism (23.29 %). Gram positive bacteria were detected in 42.47% of the cultures; methicillin resistant *Staphylococcus aureus* (MRSA) was the predominant organism (24.40%). Gram negative bacteria showed high resistance to penicillins, cephalosporins and quinolones, the least resistance was to imipenem. Mortality was higher in VAP group (47.8%) than non VAP (30%). It is indicated that quantitative cultures of ETA is a useful method for early diagnosis of VAP with subsequent proper selection of adequate therapy.

Key words: Endotracheal aspirate, quantitative cultures, ventilator-associated pneumonia.

INTRODUCTION

Ventilator associated pneumonia (VAP) is defined as infection of lung parenchyma which develops 48 h or more after mechanical ventilation and not present or incubating at the time of initiation of mechanical

ventilation (Ali et al., 2015).

VAP is associated with prolonged mechanical ventilation and increased hospital costs; mortality ranges from 17 to 50% (Yang et al., 2009). Inadequate antibiotic treatment has led to an increase in the incidence of the multi-drug resistant (MDR) strains of pathogen (American Thoracic Society, ATS 2005; Golia, 2013). Early appropriate antibiotic therapy is associated with better outcomes including reduction in mortality (Muscedere et al., 2012). The diagnosis of VAP remains challenging and there is a lack of diagnostic standardization. One of the major stumbling blocks to improving diagnosis of VAP is that there is no diagnostic gold for diagnostic techniques against which to compare (Guidelines for the management of adult with VAP, 2005).

This has paved the way for less invasive tests such as endotracheal aspirates (ETA) and quantitative ETA cultures with a threshold of 10^5 to 10^6 bacteria per milliliter of exudates that is considered as optimal for the microbiological confirmation of VAP (Nair et al., 2008). Routine endotracheal aspirate cultures of critically ill patients in intensive care units (ICUs) may be predictive of patients who are at high risk of invasive disease, and may guide the selection of appropriate empirical therapy based on the predominant pathogens identified in these cultures in the event of the development of VAP (Joseph et al., 2010). An ETA sample is more readily obtainable from mechanically ventilated patients, and is more frequently a component of microbiological surveillance (Brusselaers et al., 2013).

The American thoracic society (ATS) guidelines recommended quantitative/ semi quantitative culture of ETA or bronchoscopic aspirates from the infected lung segments for the diagnosis of VAP (Rajasekhar et al., 2006). The majority of VAP guidelines recommend the use of ETA or bronchoalveolar lavage fluid (BALF) analysis to diagnose VAP. These guidelines thereby suggest that the results of ETA and BALF analysis are in accordance (Muscedere et al., 2008; Raoof and Baumann, 2014).

The present study is undertaken to bacteriologically confirm the clinical diagnosis of VAP using quantitative culture of endotracheal aspirate.

MATERIALS AND METHODS

A prospective, single-center, observational, clinical study enrolling 152 mechanically ventilated patients selected from different intensive care units (ICUs) of Assiut University Hospitals, Egypt; including medical ICU, respiratory ICU and trauma ICU over a 12 months period from July 2013 to July 2014. The study was approved by faculty research ethics committee. Informed written consent was obtained from a close relative of all subjects.

Study population

Inclusion criteria

Patients were eligible for the study if placed on mechanical ventilation (MV) for ≥48 h.

Exclusion criteria

All patients with clinical and radiological signs suggestive of pneumonia or acute respiratory distress syndrome (ARDS) secondary to pneumonia on admission were excluded. For each patient, the following data are collected: age, gender, admission diagnosis, date and duration of mechanical ventilation.

The enrolled patients were carefully followed up for signs of VAP. This included apart from clinical examination, regular recording of body temperature, observance of tracheal aspirate appearance, leukocyte count and chest radiograph. The diagnosis of VAP was based on the American College of Chest Physicians criteria and was defined as the occurance of new or progressive pulmonary infilterates on chest X-rays along with the presence of at least two of the following criteria: (a) fever ≥38°C, (b) leukocytosis ≥ 10,000 cells/mm^3, or leukopenia≤4,000 cells/mm^3 (c), purulent tracheal secretions (AST, 2005). Patients with clinical suspicion of VAP (based on the above criteria) underwent endotracheal aspiration (ETA).

Procedure of ETA

Endotracheal aspirate (≥1 ml) was collected under aseptic precaution after 48 h of intubation whenever patient was suspected to have developed VAP. The ETA was collected using a 22-inch, 12 F suction catheters, which was gently introduced through the endotracheal tube for a distance of approximately 25 to 26 cm. Chest vibration or percussion for 10 min was used to increase the retrieved volume (1 ml) in case the patient produced very little secretions. Only one ETA sample was collected from each patient and was immediately taken to the laboratory for processing. The aspirate specimens showing the presence of <10 squamous epithelial cells per low power field or organisms seen under oil immersion in the entire field on Gram stain by direct microscopy were included in the study (Forbes et al.,2007).

Method of quantitative analysis

Quantitative analysis of ETA was done according to gram stain smear interpretation. Depending on the number of organisms seen on direct smear, the clinical sample was diluted in 1 in 100 or 1 in 1000 and subsequently 10 µl of diluted sample was uniformly inoculated on to blood agar, chocolate agar and McConkey agar. If no organism was seen on direct smear, an undiluted sample was inoculated on the agar plates. After overnight incubation, the

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Table 1. Monomicrobial and polymicrobial growth in VAP.

Type of infection	Number of percentage (%)
Monomicrobial	38 (41.30)
Polymicrobial	50 (54.34)

number of colonies were counted on each plate and multiplied by the appropriate dilution factor to express the colony count as cfu/ml. Samples with large mucus plugs were liquefied and homogenized by vortexing for one minute with glass beads followed by centrifuging at 3000 rotations per minute (rpm) for 10 min. The cfu/ml considered as significant helps discriminate colonization from infection, with thresholds of $>10^5$ cfu /ml being suggestive of infection rather than colonization (Nair et al., 2008). Organisms were primarily identified by colony morphology, microscopy of Gram stain and standard biochemical tests (Harvey et al., 2012). The antibiotic resistance patterns of the isolated microorganisms were determined by Kirby-Bauer disk diffusion method using commercially available discs (HiMedia, Mumbai, India) and interpreted as recommended by Clinical Laboratory Standard Institute (CLSI, 2010). Resistance to three or more classes of antibiotics was recorded as multidrug resistance (MDR).

Statistical analysis

Date entry and analysis were done using Statistical Package for Social Science Statistical Package for Social Science version 14 (SPSS). Results were presented as number, percentage, mean, standard deviation for quantitave variables and number (percentage) for qualitative variables. Difference in proportion was assessed by using Chi-square test. P. value was considered statistically significant when $P < 0.05$.

RESULTS

During the 12 months study period, 152 mechanically ventilated patients for > 48 h were enrolled. Their ages ranges from 18 to 79 years with a mean age of 54.15±15 years,100 (65.79%) were males and 52 (34.21%) were females. VAP was diagnosed clinically and rardiologically in 92 (60.53%) patients. Quantitative culture of ETA was done in 90 patients and positive culture occurred in 88 patients yielding 146 isolates. Fifty (54.34%) of the positive cultures were polymicrobial and 38(41.30%) were monomicrobial (Table 1). Major pathogenic isolated bacteria were gram negative (54.79%), among this group *Klebsiella* species,was the predominant pathogen (23.29%) followed by *Escherichia coli* (*E. coli*) (8.22%),then *Acinetobacter* and *Proteus* species (6.85%) for each. Gram positive bacteria were detected in (42.47%) of the cultures, methicillin resistant *Staphylococcus aureus* (MRSA) was the major organism (27.4%) (Table 2).

Gram-positive isolates in the present study were highly resistant to penicillins, cephalosporins and macrolides while lower resistance was detected to chloramphenicol, tetracyclines, linezolid and vancomycin (Table 3). Gram negative bacteria showed high resistance to many groups of antimicrobials as penicillins (except for *E.coli* that showed 0% resistance to piperacillin –tazobactam), cephalosporins and quinolones (50-100%), and the least resistance was reported to imipenem and meropenem (0 to 50%) (Table 4). Mortlity rate was higher in VAP group (47.8%) than non VAP group (30%) (Table 5).

DISCUSSION

VAP is a common complication of mechanical ventilation (MV), with a significant mortality rate, especially when associated with potentially antibiotic-resistant microorganisms (Melsen et al., 2009).

VAP is one of the most important causes of mortality in patients treated with invasive mechanical ventilation (IMV) in intensive care unit (ICU). Microbiological examinations are required as clinical and radiological findings are usually insufficient in the diagnosis (Gurgun et al., 2013). The microbiological diagnosis of VAP can be reached by invasive methods, such as fiberoptic bronchoscopic protected specimen brush (PSB) and brochoalveolar lavage (BAL), or by non-invasive methods, such as endotracheal aspiration (EA). The latter methods can be readily performed, being also cost effective and less invasive. Ideally, both specimens can be quantitavely cultured, aiming at reducing inappropriate treatment and the selection of multi resistant organisms (Corrêa et al., 2014). Quantitative EA culture is a useful noninvasive tool for the diagnosis of pneumonia pathogens in critically ill patients.

Additionally, the results of quantitative EA cultures were comparable to the results of using invasive methods and were helpful in limiting the prescription of broad-spectrum antibiotics (Yagmurdur et al., 2016).

This study was conducted from July 2013 to July 2014 and included 152 mechanically ventilated adult patients for≥ 48h admitted to different ICUs in Assuit University Hospitals.

VAP was suspected clinically and radiologically in 92 patients (60.53%). This percentage is higher than the reported by Francois et al., 2013 as VAP was detected in 9 to 40 % of intubated patients. VAP was confirmed by quantitative culture of ETA which was done in 90 patients and yield positive result in 88 patients giving 146 isolates as the infection was polymicrobial in almost 54% of VAP patients. In a study by Ahmed et al. (2014) among 48 VAP cases 32 (66.67%) were monomicrbial and 16 (33.33%) were polymicrobial. Charles et al. (2013) reported that 72.2% of VAP patients had monomicrobial and 27.8% had polymicrobial infection.

Gram negative organisms had been recovered in 80 (54.79%) isolates while gram positive organisms were

Table 2. Aetiological agents of VAP.

Individual Isolated organism	Number of percentage (%)
Total No. of isolates	146
Gram negative organisms	80 (54.79)
Klebsiella *sp.*	34 (23.29)
E. coli	12 (8.22)
Acinetobacter *sp.*	10 (6.85)
Proteus *sp.*	10 (6.85)
Heamophilus *sp.*	4 (2.74)
Pseudomonas *sp.*	4 (2.74)
Burkholderia *sp.*	2 (1.37)
Enterobacter	4 (2.74)
Gram positive organims	62 (42.47)
MRSA	40 (27.40)
MSSA	12 (8.22)
Enterococci	10 (6.85)
Candida *sp.*	4 (2.74)

MRSA: methicillin resistant *S. aureus;* MSSA:methicillin sensitive *S. aureus.*

Table 3. Antibiotic susceptibility pattern of Gram positive bacteria.

Variable	Percentage of resistant gram positive bacteria		
Antibiotics	MRSA	MSSA	Enterococci
Penicillins			
Penicillin G	100	100	100
Extended penicillins			
Amoxicillin	100	100	100
Ampicillin	97.6	100	100
Cloxacillin	97.4	40	100
Pencillins+beta lactam inhibitors			
Amoxicillin/clavulonic acid	100	100	80
Cephalosporins			
Cefazoline	89.6	66.7	100
Ceftriaxone	100	100	77
Quinolones			
Ciprofloxacin	97.1	66.7	100
Lomifloxacin	92.5	66.7	100
Norfloxacin	90	50	91.7
Aminoglycosides			
Amikacin	64.7	100	33.3
Gentamycin	100	83.3	81.8
Glycopeptides			
Vancomycin	33.3	10.9	40
Ticoplanin	66.7	38.8	43.8

Table 3. Contd.

Lincosamides			
Clindamycin	74.5	66.7	100
Macrolides			
Azithromycin	85	100	100
Erythromycin	73.3	66.7	84.6
Others			
Chloramphenicol	44.4	33.3	46.2
Tetracyclin	67.3	66.7	58.3
Oxytetracyclin	100	80	66.7
Trimethoprim/Sulphamethoxazole	100	55	66.7
Linezolid	11.8	0	33.3

MRSA: methicillin resistant *S. aureus;* MSSA:methicillin sensitive *S. aureus.*

responsible for 42.47% of the isolates. In agreement with the current study**,** Arabi et al. (2008) reported the range for gram negative bacilli and gram positive cocci as 41 to 92% and 9 to 52%, respectively. In this work, *Klebsiella sp.* was the most common gram negative bacteria (23.29%), followed by *E. coli* (8.22%). This was in accordance with Lagamayo (2008) who reported that both organisms have been responsible for nosocomial outbreaks of infection.

Acinetobacter sp. was detected in 6.85% of pathogens. This is in agreement with the world wide surveillance system that reported *Acinetobacter baumannii* to cause 7% of isolates in hospital acquired pneumonia and VAP (Jones, 2010**).** Nosocomial *A. baumannii infection* is commonly acquired through cross-transmission because of its propensity to survive in the hospital environment. Data from published studies have shown that *A. baumannii* can survive for long periods of time on inanimate surfaces (Borer et al., 2005). Daef et al. (2014) recorded an outbreak caused by 51 strains of multi-drug resistant *Acinetobacter baumannii*. Mokhless et al. (2010) used ETA as a diagnostic sample but he reported different frequencies for causative organisms; *Acinetobacter sp.* 51.5%, *P. aeruginosa* 18.2% and *Klebsiella sp.* 15.1%.

MRSA was identified in 27.40% of isolates. This is in agreement with Lee et al. (2013) who identified MRSA (27.9%) in a multicenter study. The microbiological spectrum in this study was in accordance to that had been reached by Daef and Elsherbiny **(**2012) in which the most frequently isolated microorganisms were gram negative bacteria (54.2%) amongest which, *Klebsiella sp.* was the most common while gram positive bacteria accounted for 45.8% with MRSA being the predominant (23.6%). C*andida sp.* was detected in 2.74% of isolates. In many studies of VAP in developing countries *Candida sp.* accounted for 0 to 7% of VAP episodes (Arabi et al., 2008).

The present study showed that gram negative bacteria had high resistance to many groups of antimicrobials as penicillins (except for *E.coli* that had 0% of resistance to piperacillin –tazobactam), cephalosporins and quinolones (50 to 100%). In agreement with this, Ashour and El-Sharif (2009)**,** reported high resistance to many groups of antibiotics in Egypt.

Extended spectrum beta-lactamases (ESBLs) are defined as enzymes produced by certain bacteria that are able to hydrolyze extended spectrum cephalosporin. They are therefore effective against beta-lactam antibiotics such as ceftazidime, ceftriaxone, cefotaxime and oxyiminomonobactam (Ghafourian et al., 2015). In recent years, ESBL production in *Enterobacteriaceae*, particularly *Escherichia coli*, has significantly increased in several countries (Eckert et al**.,** 2004**).** Regarding the current study, all gram negative isolated pathogens had high resistance to cefotaxime, ceftazidime and ceftriaxone. These results suggest a high prevalence of extended spectrum B lactamase producing strains. Similar results found by Mukhopadhya et al. (2010) as all the enterobacterial isolates in their study were ESBL producing.

Looking at individual organisms, *Acinetobacter s*howed 100% resistance to cephalosporins but much lower resistance to imipenem (42.9%) while all isolates were sensitive to tigecyclin and azithromycin (0 % resistance). Varun et al., 2012 found that (100%) of isolates of *Acinetobacter baumannii* were multidrug resistant (MDR) that is, resistant to three or more class of antibiotics.

E. coli resistance to cephalosporins ranged from 66 to 100% and to quinolones from 75 to 80%, this is in accordance with Karlowsky et al. (2006) who reported that *E. coli* isolates showed high resistance to

Table 4. Antibiotic susceptibility pattern of Gram negative bacteria.

Antibiotics	Percentage of resistant gram negative bacteria				
	Klebsiella sp.	***E.coli***	**Acinetobacter sp.**	**Enterobacter**	**Pseudomonas *sp.***
Extended penicillins					
Ampicillin	100	100	100	100	100
Piperacillin / Tazobactam	80	0	70	75	75
Penicillins+ beta lactam inhibitors					
Amoxicillin/calvulonic acid	100	93.3	64.3	100	76.2
Cephalosporins					
Cefazoline	98.4	100	100	83.3	100
Cefaclor	98.1	100	100	100	100
Cefoperazone	90.9	92.9	100	100	93.3
Ceftriaxone	86.7	88.9	100	80	84.6
Cefotaxtime	80	50	65	80	100
Ceftazidime	100	70	100	75	100
Cefixime	100	66	100	50	100
Cefopodoxime	100	66.7	100	100	60
Quinolones					
Lomifloxacin	74.2	80	90	83.3	72.2
Ciprofloxacin	67.9	76.9	90	83.3	72.2
Norfloxacin	80	80	85.7	83.3	81.3
Levofloxacin	58.1	75	64.3	80	70
Glycopeptides					
Amikacin	47.2	46.7	45.5	50	65
Macrolides					
Azithromycin	66.7	100	0	40	100
Others					
Imipenem	19	0	42.9	50	24
Tobramycin	81	87.5	90	80	77.8
Aztreonam	91.3	100	71.4	80	76.2
Chloramphenicol	61.6	45.5	83.3	0	72.7
Nalidixic Acid	73.5	66.7	100	100	85.7
Oxytetracyclin	90.2	100	71.4	100	100
Trimethoprim/ Sulphamethoxazole	85.7	75	66.7	83.3	90
Tigecyclin	48.1	100	0	40	66.7
Meropenem	23.7	0	50	0	32

Table 5. Outcomes of the study population.

Variable	VAP No. (92)	Non (VAP) No. (60)	
Death	44 (47.8%)	18 (30%)	*P* value 0.03*
Discharge	48 (52.2%)	42 (70%)	

*P < 0.05 is statistically significant.

cephalosporins and quinolones.Pseudomonas *sp.* was resistant to piperacillin- tazobactam (75%), ceftazidime (100%), ciprofloxacin (72.2%), amikacin (65%) and imipenem (24%). In comparison, Varun et al. (2012) reported the following resistance rates for *Pseudomonas*, piperacillin-tazobactam (23.53%), ceftazidime (35.29%), ciprofloxacin and amikacin (82.35%) and imipenem (47.06%).

Gram-positive isolates in the present study were highly resistant to penicillins, cephalosporins and quinolones. These antibiotics are commonly prescribed empirically in the ICUs. Lower resistance was detected to chloramphenicol and tetracyclines which may reflect the reduction in their use. Some studies noticed a positive correlation between resistance rates of hospital isolates and the utilization rates of ciprofloxacin, cephalosporin, carbapenems, piperacillin/tazobactam, or all of these (Willemsen et al., 2009). Resistance of gram positive organisms to macrolides (azithromycin and erythromycin) was 50 to 100%. Ahmed et al. (2011) reported the resistance of gram positive bacteria to macrolides were 64.3 and 66.4%.

The high rates of antimicrobial resistance identified in the present study is similar to what has been found by Daef and Elsherbiny (2012). They reported gram negative bacteria with high resistance (50 to 100%) to many groups of antimicrobials, as penicillins, cephalosporins, quinolones and aminoglycosides. Mortality was higher in VAP patients (47.8%) than non VAP and this is in agreement with Gupta et al. (2011) who reported mortality in 46.67% of VAP patients.

Conclusion

VAP is a common and serious hospital aquired infection. The bacteriological approach for the management of VAP helps choosing the appropriate antibiotics. This study showed that quantitatve culture of ETA is helpful for early diagnosis and management of VAP.

Conflicts of interest

The authors have not declared any conflict of interest

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Full Length Research Paper

Isolation of heavy metal-resistant fungi from contaminated soil and co-culturing with rice seedlings

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Environmental pollution from toxic heavy metals is a growing problem throughout the world due to the expansion of industrialisation. The use of microbes and biotechnological processes can provide alternative or complementary methods for the removal or recovery of heavy metals through bioremediation. The present study isolated, identified and characterised heavy metal-resistant fungi from cadmium- and chromium-contaminated paddy soil from Daye City, Hubei Province, China. Eight metal-resistant fungal species were obtained using an acclimation to concentration gradient approach. Five strains tolerated cadmium (Cd^{2+}) to a maximum concentration of 16 mM, four strains could endure 1000 mg/L chromium (Cr^{2+}), and *Fusarium oxysporum* could withstand high levels of both metals. Morphological examination and 18S rDNA sequence analysis identified the strains. Metal-resistant fungi were co-cultured with 5-day-old rice (*Oryza sativa*) seedlings in 1/2 MS medium for 7 days and growth parameters were compared with control rice not incubated with microbes. In the obtained strains, *Metarhizium anisopliae* had no influence on plant height or root length, and *Saccharomyces cerevisiae* had no effect on these parameters or on fresh weight. Heavy metal-resistant fungi such as those identified in this study could prove useful for the bioremediation of heavy metal-contaminated environments.

Key words: Heavy metal resistance, co-cultivation, filamentous fungi, acclimation of concentration gradient, rice, bioremediation.

INTRODUCTION

Heavy metals are released from urban areas, metalliferous mines and major road systems, and pollution is an increasing problem due to the rapid growth of populations, industrial activities and technological development (Alloway et al., 1995). Industrial activity in particular continually discharges heavy metals such as cadmium, lead, chromium, copper and nickel (Hemambika et al., 2011), eventually causing toxic contamination to soils, sediments and surface and ground water, which presents a serious threat to human health.

Heavy metal pollution has been widely reported in arable fields in South China in recent years, due to the

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rapid expansion of mining and metallurgical activities, sewage irrigation, and the application of pesticides and fertilizers (Chen et al., 2014; Gong et al., 2009; Zeng et al., 2013). Around 2500 hm^2 of farmland has been contaminated by Hg and Cd, and by As, Cr and Cu to a slightly lesser extent (Xu et al., 2014). There are more than 329000 tons of chromic salt produced by only 25 companies every year, accompanied by 450000 tons of Cr residue. Approximately 5000 tons of Cr residue has been deposited along the riverside of Nanpanjiang in Yunnan Province each year for the last 15 years, which has resulted in concentrations of Cr (VI) in the river itself that are 2000 times higher than acceptable national standards (Gao et al., 2011). In 2002, the Ministry of Agriculture found very high levels of cadmium in 10% of rice samples, while the Guangdong provincial government found that 44% of rice sample contained excessive cadmium in a 2013 investigation. However, in general research on cadmium contamination has been minimal (Kevin et al., 2014).

Several conventional physicochemical approaches have been used for the removal and treatment of heavy metal pollution sites, including electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Kadirvelu et al., 2002; Luo et al., 2010). However, these methods are uneconomical due to high reagent and energy requirements, or ineffective at removing all metal contamination, and they can generate large quantities of toxic sludge (Hemambika et al., 2011). In contrast, bioremediation offers a cleaner and more economical option for treating heavy metals in contaminated sites (Iskandar et al., 2011). The use of microorganisms and solid-liquid separation (Yang et al., 2012) has several advantages, not least the highly efficient removal of heavy metals from dilute solutions (Kapoor et al., 1999; Cruz et al., 2004; Fan et al., 2008).

Some fungi are known to be particularly tolerant of heavy metals (Gavrilesca, 2004; Baldrian, 2003), and many species can adapt and grow under conditions of extreme pH, temperature, nutrient availability and high metal concentrations (Anand et al., 2006). Fungi tolerate and detoxify metals through various mechanisms involving valence transformation, extra- and intracellular precipitation or active uptake (Mala et al., 2006; Turnau et al., 2006). Scientists are currently exploring the use of microbes and associated biota residing in ecosystems for the bioremediation of pollutants through degradation or accumulation (Khan et al., 2000), and numerous strains isolated from contaminated sites possess such abilities. Vadkertiova and Slavikova (2006) studied metal tolerance in yeasts isolated from polluted environments and found inter- and intraspecific variation in metal tolerance among tested strains. Similarly, Zafar et al. (2007) reported promising biosorption of Cd and Cr using *Aspergillus* sp. and *Rhizopus* sp., filamentous fungi isolated from metal-contaminated agricultural soil. Given the types of mechanisms underpinning metal resistance, screening of metal-tolerant fungi is likely to identify strains with improved capacity for metal accumulation (Bai and Abraham, 2003).

The present work reports the identification and characterisation of metal-resistant microorganisms isolated from polluted environments and the selection of strains with improved heavy metal resistance. Contaminated fields are the ideal environment in which to find organisms that have evolved survival mechanisms to adapt to polluted conditions that could be used for bioremediation of heavy metals in other contaminated locations.

MATERIALS AND METHODS

Soil sampling and analysis of heavy metal content

Soil samples were collected from rice paddies near Chenjiazuiwan village Luojiaqiao Towns Daye City, Hubei Province, China (30°08′47.63''N, 114°57′23.20''E, 21 m a.s.l.). Soil samples (1000 g) were taken from the surface down to a depth of 20 cm using a wooden spatula and half was stored at 4°C for fungi isolation, while the other half was air-dried for physicochemical analysis. Plant residues and stones were removed by hand, and soil samples were passed through a 1 mm sieve prior to analysis of heavy metal content (Shazia et al., 2013). Cr and Cd concentrations were determined using atomic absorption spectrometry (AAS 6200, SHIMADZU).

Screening and selection of heavy metal-resistant fungi

Purified isolates were screened on the basis of their tolerance to Cr^{6+}, Pb^{2+}, Hg^{2+}, Cd^{2+} and Cu^{2+}. A disk of mycelium was inoculated aseptically on Martin medium plates supplemented with different concentrations of individual heavy metal potassium dichromate, lead carbonate, mercury bichloride, cadmium sulfate and cupric sulfate salts, respectively. Inoculated plates were incubated at 25°C for at least 7 days. The effect of heavy metals on growth was estimated by measuring the radius of the colony extension (mm) compared with controls (medium lacking heavy metals) and determination of the index of tolerance (the ratio of the extension radius of treated vs. untreated colonies). Isolates showing resistance to Cr^{6+}, Pb^{2+}, Hg^{2+}, Cd^{2+} and Cu^{2+} were selected for subsequent experiments.

Isolation of heavy metal-resistant fungi

Martin media was used to culture fungi for isolation experiments (Iram et al., 2011). Soil samples were processed and fungi isolated using the soil dilution plate method (Waksman, 1922). After incubation, individual colonies were counted and species identified on the basis of macroscopic (colony morphology, colour, texture, shape, diameter and appearance) and microscopic (septation of mycelia, presence of specific reproductive structures, shape and structure of conidia, presence of sterile mycelia) characteristics. Pure cultures were identified with the help of the existing literature (Domsch et al., 1980, Barnett and Hunter, 1999).

Molecular identification of heavy metal-resistant fungi

Fungal DNA was extracted for amplification using the thermolysis

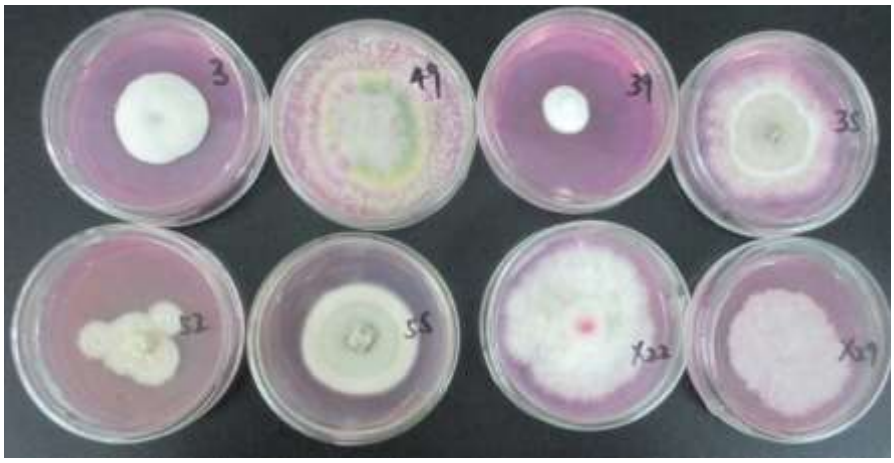

Figure 1. Colony morphology of the eight identified fungal strains.

method devised by Zhang et al. (2010), and amplification was performed as described previously (Onn, 2012). Primers (18S-1, 5'-GTAGTCATATGCTTGTCTC-3'; 18S-2, 5'-TCCGCAGG TTCACCTACGG A-3') were designed and used with the following cycling conditions: An initial denaturing step at 94°C for 2 min, followed by 25 cycles of amplification at 94°C for 30 s, 45°C for 30 s and 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were separated by 1% agarose gel electrophoresis and a band of ~1700 bp was gel-extracted, ligated, transformed, sequenced and aligned with sequences in GenBank.

Co-cultivation of heavy metal-resistant fungi with rice tube seedlings

Rice seed (Guangliangyou, 272) was provided from the Hubei Academy of Agriculture, and sterile rice tube seedlings were obtained as previously described (Li et al., 2008). Sterile seedlings were grown for five days to a height of ~1 cm in 10 cm diameter petri dishes. Square culture bottles were filled with 1/2 MS medium and autoclaved at 121°C for 15 min. A quarter of the culture medium was cut vertically after cooling, and five fungi blocks of 0.5 cm in diameter were inoculated ~2 cm from the bottom of the culture bottle. The rice seedlings were then inoculated above the corresponding fungi blocks and incubated at 28°C with a 16/8 h (day/night) photoperiod (2000 lux light intensity). Rice plant height, root length and fresh weight were recorded after seven days. Control plants were inoculated onto Martin media agar blocks without fungal strains.

Statistical analysis

All experiments were performed with three replicates. Individual isolates were subjected to analysis of variance using SPSS 17 statistical software to compare resistance to metals.

RESULTS AND DISCUSSION

Detection of the heavy metal content

According to the evaluation standards for heavy metal pollution in soil in China (GB15618-1995), the concentration of Cd in the soil sample was 0.94 mg/kg, which corresponded to primary standard levels, while Cr was 60.2 mg/kg, which corresponded to second grade standard levels. The introduction of heavy metal compounds into the environment generally induces morphological and physiological changes in microbial communities (Vadkertiova and Slavikova, 2006), and exerts a strong selective pressure on the microbiota (Verma et al., 2001), making contaminated sites an ideal source of metal-resistant microorganisms (Gadd, 1993). Sampling polluted sites therefore increases the probability of isolating cadmium- and chromium-resistant strains that have evolved to adapt to heavy metal-rich niches.

Screening and selection of heavy metal-resistant microorganisms

We identified eight resistant fungal strains through screening of cadmium- and chromium-contaminated paddy soil using an acclimation of concentration gradient approach. Following purification, the colony morphology of individual strains resistant to different heavy metals was assessed (Figure 1 and Table 1). Five strains could endure a maximum concentration of 16 mM Cd^{2+}, four strains could tolerate Cr^{2+} up to 1000 mg/L, and strain X29 could withstand these maximum concentrations of both heavy metals.

Identification of metal-resistant fungal species

The identity strains was confirmed using 18S rDNA sequence analysis following comparison with sequences in the NCBI, which also afforded their registration numbers (Table 2). The raw data have been submitted to a public repository (NCBI) under accession number KU350742-KU350749.The eight metal-resistant filamentous fungi were identified as *Metarhizium anisopliae*, *Phomopsis* sp., *Aspergillus* sp., *Trichoderma*

Table 1. Maximum concentration of heavy metals tolerated by resistant fungi.

Strain No.	Cd^{2+} (mM)	Cr^{6+} (mg/L)	Pb^{2+} (mM)	Hg^{2+} (mg/L)	Cu^{2+} (mM)
3	16	800	30	450	4.0
X22	12	1000	15	200	5.6
X29	16	1000	20	150	3.0
35	10	1000	10	120	3.0
39	16	800	50	300	5.0
49	8	1000	30	250	4.0
52	16	800	40	300	3.0
55	16	800	40	120	7.4

Table 2. GenBank accession numbers for the eight identified strains.

Strain no.	Generic name	Accession number	Strain no.	Generic name	Accession number
3	*Metarhizium anisopliae*	KU350742	X22	*Aspergillus* sp.	KU350749
39	*Saccharomyces cerevisiae*	KU350743	49	*Trichoderma* sp.	KU350745
X29	*Fusarium oxysporum*	KU350748	52	*Penicillium* sp.	KU350746
35	*Phomopsis* sp.	KU350744	55	*Penicillium* sp.	KU350747

sp., *Fusarium oxysporum*, *Saccharomyces cerevisiae* and *Penicillium* sp., while Strain 35 and Strain 55 belonged to *Aspergillus* sp. Soils contaminated long-term with both organic compounds and heavy metals contain microbial communities that are structurally and functionally adapted to grow under the polluted conditions. These adapted microorganisms could in principal be used for bioremediation of organic compounds and heavy metals into non-toxic products at other polluted sites. Adaptation involves evolving catabolic activities that enables the organisms to utilize the contaminants as nutrients and energy sources (Atlas and Unterman, 1999; Boopathy, 2000). However, when allochthonous microorganisms are incorporated into new soils, they may not be fully capable of participating in and complementing the existing microbial community, unlike indigenous microorganisms that are more likely to be better adapted to the local conditions and hence more useful for bioremediation. Ezzouhri et al. (2009) identified 36 microorganisms in isolates from heavy metal-contaminated sites in Tangier, Morocco, belonging to *Aspergillus, Penicillium, Fusarium, Alternaria* and *Geotrichum* genera that demonstrated high levels of resistance to all metals tested. This and previous studies reveal fungi as promising agents for bioremediation of heavy metal-polluted soils, and biosorption of cadmium and chromium in particular.

Co-cultivation of heavy metal-resistant fungi with rice seedlings

Heavy metal-resistant strains were co-cultured with rice seedlings in 1/2 MS medium for 7 days (Figure 2), and plant height, fresh weight, root length and root number were analyzed (Table 3). Plants co-cultured with strains 3 and 39 grew better than controls, whereas strains X22, X29, 35, 49, 52 and 55 grew significantly worse than controls.

Increasing attention is being paid to the use of growth-promoting soil microbes for bioremediation of heavy metal pollution in soils, and to the nature and benefits of specific plant-microbe interactions. Rice is the most widely planted grain crop grown in heavy metal-polluted farmland in Hubei province and other areas of China, and this important food crop can accumulate high levels of toxic Cd from contaminated soil. Reducing the transfer of heavy metals from the soil into plant material is a major research goal, and selection of appropriate functional microbes can facilitate phytoremediation of mining-contaminated soils. If such microbes are present or introduced, the slow or non-existent growth and low biomass often associated with mining wastelands can be overcome, and soil fertility and heavy metal toxicity can be improved and reduced, respectively (Wong, 2003). In order to survive in heavy metal-contaminated soils, functional microbes (Drake and Liu, 2008) have undergone adaptation of their metabolic activities to suit their particular environment (Kumar et al., 2009; Turgut et al., 2010). In this study, although plant height and root length were not enhanced by co-culturing with strain 3, the number and fresh weight of rice roots was significantly improved, compared to control plants. Similarly, strain 39 did not improve plant height, root length or fresh weight, but significantly increased root number. Strain 52 had the largest positive impact on rice

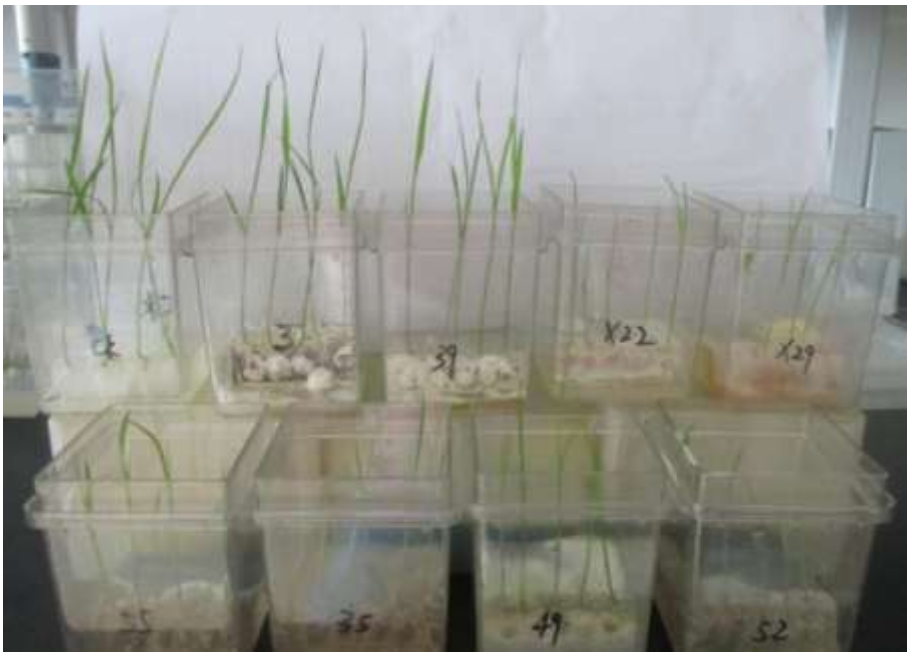

Figure 2. Co-cultivation of rice tube seedlings with heavy metal-resistant fungal strains.

Table 3. Biological characteristics of rice seedlings co-cultivated with heavy metal-resistant fungi for 7 days.

Strain No.	Biological characteristics			
	Plant height (cm)	Root number (pcs)	Root length (cm)	Fresh weight (g)
3	13.56 ± 0.47^{a}	5.47 ± 0.47^{b}	2.53 ± 0.16^{a}	0.11 ± 0.005^{b}
22	7.97 ± 0.32^{c}	5.27 ± 0.56^{b}	1.44 ± 0.10^{bc}	0.06 ± 0.002^{c}
29	6.20 ± 0.25^{de}	3.27 ± 0.44^{c}	1.02 ± 0.11^{d}	0.04 ± 0.003^{d}
35	7.21 ± 0.52^{cd}	1.67 ± 0.23^{d}	1.56 ± 0.12^{b}	0.06 ± 0.004^{c}
39	12.97 ± 0.48^{a}	6.33 ± 0.46^{b}	2.60 ± 0.14^{a}	0.12 ± 0.007^{a}
49	9.57 ± 0.44^{b}	2.00 ± 0.22^{d}	1.45 ± 0.17^{bc}	0.08 ± 0.004^{c}
52	5.93 ± 0.29^{e}	2.20 ± 0.30^{cd}	1.65 ± 0.15^{b}	0.06 ± 0.004^{c}
55	7.39 ± 0.36^{c}	1.80 ± 0.37^{d}	1.37 ± 0.14^{bc}	0.06 ± 0.003^{c}
ck	14.61 ± 0.30^{a}	9.00 ± 0.54^{a}	2.33 ± 0.10^{a}	0.12 ± 0.003^{a}

Values followed by different letter among the different treatment differ at p=0.05 (Tukey's HSD multiple comparison).

growth. The results suggest the combined use of heavy metal-adapted soil microbes and functional plants could be an effective approach for improving plant growth and lowering assimilation of heavy metals in crops grown on contaminated land.

Conclusion

Our preliminary findings indicated that native fungi by screening from cadmium and chromium contaminated paddy soil could grow at high heavy metal concentrations. Five fungi (strain 3, strain X29, strain 39, strain 52 and strain 55) that showed high resistance to Cd^{2+} (up to 16 mmol/L), four fungi (strains X22, X29, 35 and 49) that showed high resistance to Cr^{6+} (up to 1000 mg/L), strain 39 showed high resistance to Pb^{2+} (up to 50 mmol/L), strain 3 showed high resistance to Hg^{2+} (up to 450 mg/L), and strain 55 showed high resistance to Cu^{2+} (up to 7.4 mmol/L). Findings of the present study indicate that fungi populations isolated from heavy metal-contaminated sites have the ability to resist higher concentrations of metals.

Strains 3 and 39 growing on the surface was similar to contrast with co-cultured rice seedlings. Further investigations are needed to assess their biosorption capabilities and determine such things as the proteins

involved in the biosorption process, the most efficient contact time and optimum growth conditions. Microbes can affect the availability of heavy metals through direct or indirect metabolic activities, which play a decisive role in maintaining soil function. The results indicate that native fungi play a more important role in the process of phytoremediation of heavy metal-contaminated soils.

Conflict of interests

The authors have not declared any conflict of interest.

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