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

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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African Journal of Microbiology Research

Full Length Research Paper

Identification of *Salmonella* isolated from pork sausage and evaluation of thermal and antimicrobial resistance of isolates

Francesca Silva Dias[1]*, Cíntia Lacerda Ramos[2], Amanda Rejane Alves de Ávila[2], Marianna Rabelo Rios Martins Santos[2] and Rosane Freitas Schwan[2]

[1]College of Veterinary Medicine, Federal University of San Francisco Valley, Rod. BR 407, Km 12, CEP 56.300-990, Petrolina, Pernambuco, Brazil.
[2]Biology Department, Federal University of Lavras, CEP: 37.200-000, Lavras, Minas Gerais, Brazil.

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Pork products are important reservoir of *Salmonella*. In Brazil, the consumption of pork sausage is high and the occurrence of *Salmonella* in product is usual. *Salmonella* occurrence was studied in twenty samples of fresh pork sausage. Pulsed-Field Gel Electrophoresis (PFGE) was performed to characterize *Salmonella* isolates. Their antimicrobial and heat resistance were also evaluated. *Salmonella* was detected in five samples of industrial pork sausage and six isolates were obtained and identified by phenotypic and genotypic methods: *Salmonella enterica* subsp. *houtenae* (two isolates) and serovars of *S. enterica* subsp. *enterica:* Bareilly, Typhimurium, Paratyphi C and Paratyphi B. *Salmonella houtenae* and the serovars Paratyphi B and Typhimurium were resistant to three or more of antimicrobial agents tested. The *Salmonella* cocktail presented high heat resistance in pork sausage with *D*-values at 58, 62 and 65°C of 10.99, 5.29 and 2.16 min, respectively, and a *z*-value of 10.1°C. The evaluation of thermal and antimicrobial resistance of *Salmonella* can be useful for researchers and food industry involved in the management of pork product quality and resulting in improvements in microbiological safety. According to our results, the binomial effect of time and temperature can be utilized to pork industry in designing and estimating thermal processes specific for sausage. To consumers, a longer heating time ensures the microbiological quality of sausage and reduces the risk of salmonellosis.

Key words: Pulsed-field gel electrophoresis, pork product, food safety, salmonellosis.

INTRODUCTION

Swine is an important reservoir of *Salmonella* and considered asymptomatic carriers of microorganism (Berends et al., 1996; Vieira-Pinto et al., 2006). Contamination of pork products during slaughter represents an important vehicle for *Salmonella* spp. dissemination to humans (Oliveira et al., 2012). During further processing of meat such as cutting and mincing, *Salmonella* from contaminated pork cuts may then spread into meat preparations.

*Corresponding author. E-mail: francesca.nobre@univasf.edu.br .Tel: +55 87 - 2101-4839, Fax: +55 87 -2101-4808.

Abbreviations: PFGE, Pulsed-Field Gel Electrophoresis; **TSI,** agar triple sugar iron; **LIA,** lysine iron agar; **TSB,** tryptone soy broth; **TSBP,** tryptone soy broth with 0.3% yeast extract; **MPN,** most probable number; CFU, colony-forming units.

At retail and at consumer level, cross contamination, improper storage and insufficient cooking can additionally increase the risk to consumers (Gonzales-Barron et al., 2012). In Brazil, the consumption of pork sausage is high and the occurrence of *Salmonella* in product is usual (Marques et al., 2006; Borowsky et al., 2007; Mürmann et al., 2009), which increases the population's exposure to the pathogen. Thus, the periodic outbreaks of salmonellosis caused by pork clearly demonstrate the need for improved tracking and tracing of *Salmonella* spp. in the pork production chain (Pielaat, 2011). For genotype characterization, the Pulsed-Field Gel Electrophoresis (PFGE) technique has been shown to be highly effective for epidemiological studies of some serovars of *Salmonella* (Willford et al., 2007). Also, other factors need be evaluated such as the multidrug and heat resistance. The antimicrobial resistance in *Salmonella* has lead to failure of treatment of salmonellosis and other diseases caused by bacterial pathogens (Travers and Barza, 2002) and there are few data in the literature on the inactivation of the pathogen in pork sausage by heat treatment.

The study of the *Salmonella* profiles circulating in production chain may contribute to better preventive measures to control meat products and a better protection of public health. In this sense, the objective of this study was to detect and identify *Salmonella* serovars in industrial pork sausage and evaluate antimicrobial resistance profiles and heat resistance of these isolates when challenged in a pork sausage model system.

MATERIALS AND METHODS

Sample collection

Twenty samples of fresh pork sausage industrial registered in the Ministry of Agriculture Livestock and Supply of Brazil were collected into sealed and refrigerated packages in commercial establishments from four cities from Minas Gerais state. The samples were directly transported in refrigerated isothermal boxes to the laboratory and immediately analyzed.

Isolation and phenotypic identification of *Salmonella* spp.

Detection of the *Salmonella* spp. was conducted as previously described by Pignato et al. (1995). For isolation and identification, 25 g of each sample were aseptically transferred to 225 ml of pre-enrichment broth base "Salmosyst" (Merck), homogenized in stomacher for 4 min and incubated at 37°C/6 h. For the selective Salmosyst enrichment, 10 ml of pre-enrichment broth base was supplemented with one selective supplement tablet (Merck) and incubated for 18 h at 37°C. From each tube, a loopful of culture was streaked on Rambach agar (Merck), and the plates were incubated at 37°C/24 h. Typical colonies on Rambach agar were selected and transferred to tubes containing agar triple sugar iron (TSI) (Himedia) and lysine iron agar (LIA) (Himedia), and incubated at 37°C/24 h. Characteristic strains of *Salmonella* spp. were tested for differential staining of gram, catalase and oxidase. API20E kit (BioMérieux) was used to complement these biochemical tests and the final identification was performed using the API LAB plus software (BioMérieux).

Bacterial DNA extraction and PCR analyses

DNA sequences from six isolated and phenotypically identified strains of *Salmonella* spp. were analyzed. The DNA of strains was extracted using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The PCR analyses, reaction was carried out in a final volume of 50 µl containing 25 µl of TopTaq master mix (Qiagen), 1 µl of each primer (27f /1512r), 2 µl of DNA and 21 µl of free water RNase. The amplification was carried out as follows: template DNA was denatured for 5 min at 95°C followed for 30 cycles, then denatured at 92°C for 60 s, and annealed at 55°C for 60 s, and primer extension was carried out at 72°C for 60 s. The tubes were then incubated for 10 min at 72°C for the final extension. After amplification the samples were stored at 4°C. The unpurified PCR products were sequenced by Macrogen Inc. (Seoul, South Korea), and sequences were then compared to those available in the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

Pulsed field gel electrophoresis (PFGE)

The methods previously described by Pang et al. (2005) were used to perform a restriction digestion with *Not*I (Invitrogen). The conditions used were 6 V/cm for 25 h at 12°C with pulsed time ranging from 2 to 10 s. The gels were stained with Sybr Green (Invitrogen) and photographed. The similarity between *Salmonella* strains was determined based on the presence or absence of amplicons detected by PFGE. The gels were analyzed determining the diversity of amplicons. The hierarchical clustering was performed using the program Systat 8.0, based on similarity matrices that were generated via the agreement method (simple matching) using the Ward algorithm and Euclidean distance.

Antimicrobial susceptibility testing

The antimicrobials used in this test were the following: amikacin (30 µg/disc), tetracycline (30 µg/disc), cephalothin (30 µg/disc), cefotaxime (30 µg/disc), ceftazidime (30 µg/disc), aztreonam (30 µg/disc), cefoxitin (30 µg/disc), ceftriaxone (30 µg/disc), chloramphenicol (30 µg/disc), sulphazotrin (25 µg/disc), gentamycin (10 µg/disc) and ampicillin (10 µg/disc). Isolates of *Salmonella* were grown on Case agar (Merck) for 18 h at 37°C. The bacterial population was inoculated in 4 ml of sterile distilled water to achieve the n° 0.5 McFarland turbidity standard (Probac, Brazil). A swab was used to spread the inoculum across the surface of Muller Hinton Agar (Merck), and antimicrobials disks (DME Polisensidisc® 4 × 6 - Specialized Diagnostic Microbiology, São Paulo, Brazil) were applied to the plate. Isolates resistance was assessed by measuring the inhibition of bacterial growth after incubation for 18 h at 37°C. The breakpoints to antimicrobial agents considered for *Salmonella* were those recommended by the CLSI (2011, Table 2) for *Enterobacteriaceae*. *Escherichia coli* ATCC 25922 was used as positive control.

Heat resistance tests in pork sausage

The thermo-tolerance of *Salmonella* strains was tested in fresh pork sausages. In this test, the inoculum contained a cocktail of the 6 isolated serovars of *Salmonella* in stationary phase. They were re-

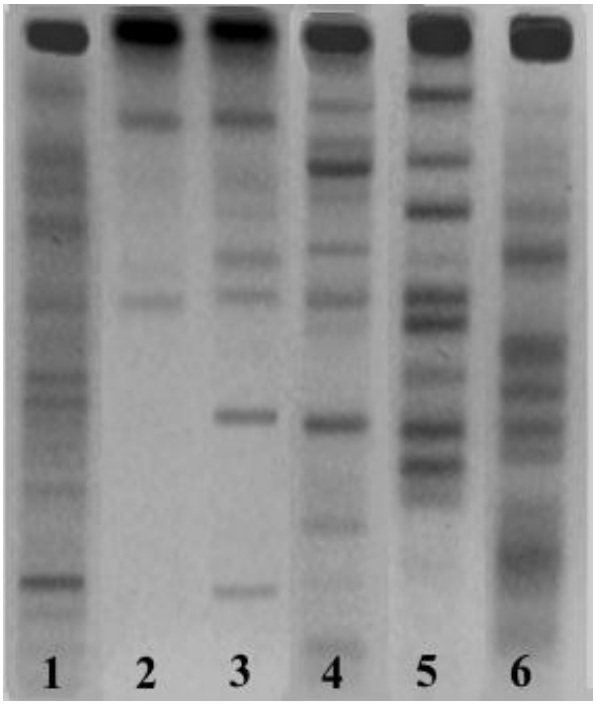

Figure 1. Pulsed field gel electrophoresis (PFGE) patterns for *Not*I-digested genomic DNA of *Salmonella* strains from sausage.
1. *Salmonella* Paratyphi B; 2. *Salmonella houtenae;* 3. *Salmonella houtenae;* 4. *Salmonella* Typhimurium; 5. *Salmonella* Bareilly*;* 6. *Salmonella* Paratyphi C.

suspended in 2 ml of Tryptone Soy Broth (TSB) (Merck) with a concentration around 10^{10} CFU/ml. The cocktail was added in 80 g of pork sausage. The sausages were prepared under aseptic conditions using the following formula: 74% lean pork ham, 20% fat pork, 2.0% NaCl, 0.5% Antioxidant Ibracor L600® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% Cure LF® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% garlic paste, 0.5% chili pepper and 1% cold water. The inoculated mixture was filled into a natural casing of 26 mm diameter. The sausages were kept in a sterile and closed plastic packet. Heat resistance trials were performed by completely submerging of these packets in a circulating water bath kept in the temperatures: 58, 62 and 65°C and in the times: 0, 5, 10 and 15, in triplicate for each time/temperature. After each time, the packets were removed and immediately cooled in water and ice. They were then stored at refrigeration temperature and survival (cell vialbility of *Salmonella* strains) counts were performed within 2 h.

Enumeration of bacteria surviving heat-treatment

Survivors counts were performed by the most probable number (MPN) dilution technique (De Man, 1983), using a series of three tubes per dilution in media TSB with the addition of 0.3% yeast extract (Himedia) (TSBP). Bacterial growth was evaluated by turbidity of the broth TSBP after 48 h of incubation at 37°C. The presumptive *Salmonella* survival was confirmed by their recovering on Rambach Agar (Merck) at 37°C for 24 h. Thus, counts were performed.

Statistical analysis

The number of *Salmonella* isolates which were survivors as a function of time was evaluated by regression analysis using SISVAR® (Lavras, Brazil) software, version 4.5. Survival curves of *Salmonella* isolates were obtained by plotting $\log_{10}$ of the surviving cells number/g versus heating time as suggested by Quintavalla et al. (2001). The line for survival plots was determined by linear regression analysis and the *D*-values (decimal reduction time) were calculated from the resulting regression equations. The *z*-values were evaluated by the linear regression of $\log_{10}$ *D*-values versus heating temperatures. The counts were subjected to analysis of variance (ANOVA) and the means were compared by Scott-Knott test, with $P < 0.01$.

RESULTS AND DISCUSSION

A total of 20 samples of industrial sausages were investigated, *Salmonella* was detected in five samples. Six isolates were obtained and identified by phenotypic testes. These isolates were also identified by comparative analysis of 16S rRNA gene sequences and identified with a similarity of 99 to 100% to *Salmonella enterica* subsp. *houtenae* (AB273733.1) and serovars of *S. enterica* subsp. *enterica*: Bareilly (U92196.1), Typhimurium (AP011957.1), Paratyphi C (EU118097.1) and Paratyphi B (DQ344539.1). *S. houtenae* was detected in two samples of industrial sausage. Two subspecies of *Salmonella*: *houtenae* and *enterica* serovar Typhimurium were detected in the same sample. In this study, all *Salmonella* isolates from pork sausage identified are described in scientific literature as human pathogenic. The PFGE characterization identified 6 different profiles for 5 different serovars, displaying 3 to 10 bands (Figure 1). The lines 2 and 3 (Figure 1) show 2 chromosomal patterns for a same serovar identified as *S. houtenae* by 16 S sequencing, showing genetic differences even into the serovar level. In this survey, it was possible observed that *Salmonella* Typhimurium and *Salmonella* Bareilly were more distant from others *Salmonella* species by chromosomal pattern analysis obtained from PFGE method. Cluster analysis (Figure 2) grouped *S. houtenae* strains with *Salmonella* Paratyphi B and *Salmonella* Paratyphi C. The *S. houtenae* strains, *Salmonella* Paratyphi B and *Salmonella* Paratyphi C are more genotypic related to each other that *Salmonella* Typhimurium and *Salmonella* Bareilly strains.

The PFGE is an approach to measure the genetic diversity of the entire genome, which may have arisen as a result of mutation. Such mutation may remove or create recognition sites through insertion, deletion, translocation and inversion or by mobile genetic elements. The assumption is that as time passes and organisms spread, divergence may occur (Bakeri et al., 2003; Abassi et al., 2010). Laconcha et al. (2000) studied genotypic characterization by PFGE of *Salmonella* strains and concluded that this technique represents a suitable tool for the

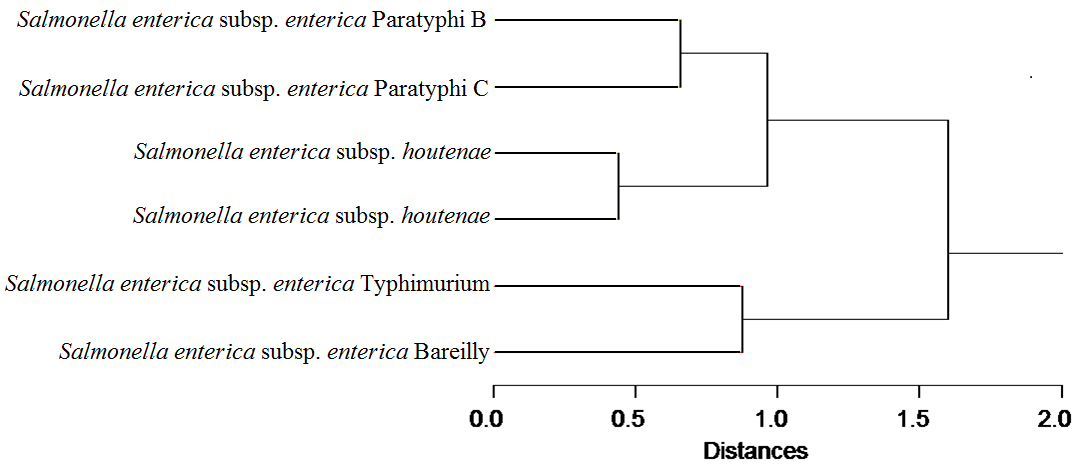

Figure 2. Cluster analysis of chromosomal pattern of *Salmonella* strains isolated from commercial Brazilian sausage.

Table 1. Antimicrobial susceptibility[a] of *Salmonella* isolates from pork sausages.

Antimicrobials	*Salmonella* isolates					
	Paratyphi B	*Houtenae*	*Houtenae*	Typhimurium	Bareilly	Paratyphi C
Cefotaxime	R	R	I	R	I	R
Ampicillin	R	R	R	R	R	I
Amikacin	R	R	R	R	R	R
Ceftazidime	I	R	S	R	I	I
Cephalothin	R	R	R	R	I	R
Sulfazothrim	S	R	S	S	S	S
Cefoxitin	R	R	S	R	S	R
Gentamicin	R	S	R	R	R	R
Tetracycline	I	S	R	S	S	S
Ceftriaxone	R	I	S	S	S	I
Chloramphenicol	R	S	I	R	S	S
Aztreonam	I	I	S	R	I	I

[a] R: Resistant; I: intermediary; S: susceptible.

epidemiological typing of *Salmonella* strains.

In the antimicrobial susceptibility test, all six *Salmonella* isolates in this study were resistant to amikacin (Table 1). Sulfazothrim was the antimicrobial in which all the serovars of *S. enterica* subsp. *enterica* were sensitive. The two isolates of *S. houtenae* showed different susceptibility to the following antimicrobial agents: ceftazidime, sulfazothrim, cefoxitin, gentamicin and tetracycline. According to Schwarz et al. (2010), the resistance of three or more classes of antimicrobial agents can be referred to as multi-resistance. In our studies, *S. houtenae* and the serovars Paratyphi B and Typhimurium isolated from pork sausage were multidrug-resistant (MDR). The serovar Typhimurium was resistant to five classes of antimicrobial agents. The large profile of antimicrobial resistance for these *Salmonella* isolates from pork sausage might be a great risk for public health. Kim et al. (2011) reported that *Salmonella* spp. isolated from pigs at slaughterhouses in Korea demonstrated an appreciable broad-spectrum, (multi)-antimicrobial resistance. Thus, a continuous antibiotic surveillance program may be worthwhile.

To determine the heat resistance, a cocktail of *Salmonella* isolated and identified in this study was inoculated into fresh prepared pork sausage. The thermal inactivation curves were linear in all temperatures evaluated (58, 62 and 65°C). The determination of R^2 coefficient of the regression curves was always higher

Table 2. Enumeration using log_{10} (CFU/g) at time points (minutes) and heat-resistance (expressed in *D*-values) of a cocktail of six *Salmonella* isolates in fresh pork sausage.

Temperature (°C)	Time (min)					Equation	*D*-values (min)
	0	5	10	15	Average		
58	10.173[a]	9.903[a]	9.586[a]	8.756[a]	9.605[a]	y = -0.091x + 10.29 R^2 = 0.92	10.99
62	10.196[a]	9.873[b]	8.836[b]	7.386[b]	9.073[b]	y = -0.189x + 10.49 R^2 = 0.93	5.29
65	10.190[a]	9.860[b]	6.396[c]	3.623[c]	7.517[c]	y = -0.463x + 10.99 R^2 = 0.92	2.16
Average	10.186	9.878	8.273	6.588	8.731		

For each column, mean values with different letters are significant ($P < 0.001$) according to the Scott–Knott test.
Standard error medium (SEM) = 0.007.

than 0.90 (Table 2). The regression curves of temperatures 58, 62 and 65°C presented a reduction of (CFU/g) 0.091, 0.189 and 0.463 log/min of microorganism, respectively. Thus, the decimal reduction times (*D*-values) of the *Salmonella* cocktails in the sausage decreased substantially with an increase in temperature (Table 2). The *D*-values for 58, 62 and 65°C were 10.99, 5.29 and 2.16 min, respectively. The *z*-value was 10.1°C. There is limited information about thermal inactivation of *Salmonella* in pork sausage (Mattick et al., 2002). In pork meat containing curing additives, Quintavalla et al. (2001) reported that for six different strains of *Salmonella* inoculated in product, the D_{58}-value ranged from 2.79 to 4.8 min and the *z*-value ranged from 4.1 to 4.8°C. Juneja et al. (2001) inoculated a cocktail of eight serovars of *Salmonella* in pork meat (8.5% fat) and determined that D_{58}, $D_{62.5}$ and D_{65} values were 6.68, 1.62 and 0.87 min, respectively, and the *z*-value was 7.1°C. The D-value and *z*-value determined in this work were higher than the values calculated for pork meat in previous studies. This difference may be due to the fat content of the sausage. The protective effects of fat may be due to the lowest heat conductivity or reduced water activity in the fat portion (Senhaji, 1977). In general, high fat content results in high thermal resistance (Juneja et al., 2001). Typically, fresh pork sausage contains between 10 to 40% of fat (according to manufacturing industries in Brazil).

In the enumeration of cell viability of *Salmonella*, there was an interaction ($p<0.01$) between heated sausages at different temperatures and their heating time (Table 2). For each temperature, the reduction (log_{10} CFU/g) of *Salmonella* was significant at each time point. At 5 min, there was no difference between the temperatures of 62 and 65°C for inactivation of the microorganism. At this time, the reduction of the microorganism was still low, only 0.32 and 0.33 log units from the initial time for 62 and 65°C, respectively. From 10 min, the temperature of 65°C was more effective in reducing of *Salmonella*, with population decline to 3.794 log units in relation to the initial time. In the pork meat chain, the specific time and temperature binomial is also valid for other processes and stages. In animal slaughter, there is a wide variation in the scalding process; Brazilian legislation on the technical standards for slaughtering pigs cites between 62 to 72°C and 2 to 5 min (Brazil, 1995). These standards may not be effective for pathogen inactivation.

Conclusion

Brazilian industrial pork sausage may be vehicles for transmitting *S. enterica*. *S. houtenae* and the serovars Paratyphi B and Typhimurium. The isolates obtained were genotypically different and resistant to three or more classes of antimicrobial agents. According to the results of this study, the time and temperature binomial of the inactivation of microorganisms in sausages is higher than the binomial typically employed for meat, probably due to the fat content in the product. Thus, heating times of 10.99, 5.29 and 2.16 min with internal temperature of 58, 62 and 65°C, respectively, in fresh sausage, ensure a higher level of microbiological quality and offer less risk of salmonellosis to consumers of product. The resulting kinetic parameter can be useful to pork industry in designing and estimating thermal processes specific for sausage.

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Full Length Research Paper

Effect of lipopolysaccharide on nitric oxide (NO) production in culture supernatants exposed to Bacillus Calmette-Guerin (BCG) and supplemented with 1, 25(OH)$_2$D3

Aurora Martínez-Romero[1], José Luis Ortega-Sánchez[2], José Ramón Hernández-Salgado[2], Norma Urtiz-Estrada[3], Maribel Cervantes-Flores[3], Estela Ruíz-Baca[3] and José de Jesús Alba-Romero[1]*

[1] División de Estudios de Postgrado e Investigación. Facultad de Ciencias Químicas, Universidad Juárez del Estado de Durango. AP No. 51 Gómez Palacio, Durango. México.
[2]Unidad Regional Universitaria de Zonas Áridas, Universidad Autónoma Chapingo, AP No. 8 Bermejillo, Durango México.
[3]Laboratorio de Biología Celular y Molecular, Facultad de Ciencias Químicas, Unidad Durango. UJED, México.

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Recently, there has been a striking increase in the number of patients with mycobacterial disease, and this is an important health problem around the world. One essential component of tubercular host defense includes nitric oxide production. The objective of this study was to investigate *in vitro* the effect of lipopolysaccharide on nitric oxide production in supernatants of mononuclear cell cultures from *Mycobacterium bovis* BCG-infected goats supplemented with 1, 25 Dihydroxyvitamin D3 [1,25(OH)$_2$ D3]. Twelve animals of goat cattle 20-to 24-month-old *sannen* selected themselves. Five samplings were made, previous to the inoculation (zero days), 3, 7, 14 and 21 days after applying the treatments. The mononuclear cells by the ficoll-hypaque method were obtained. The nitric oxide concentrations were measured directly by griess method with ELISA reader. The treatment with the 1,25(OH)$_2$D3 stimulated the nitric oxide synthesis indicating, that by itself it is a good modulator of the micobacterial replication and in the treatment with *M. bovis*-BCG vaccine increased as a result to the treatment with 1,25(OH)$_2$D3. The exhibition to *M. bovis*-BCG vaccine with 1,25(OH)$_2$D3 was able to increase answer NO_2^- in exposed animals. The LPS has effect *in vitro* in the production of nitric oxide in goats exposed to *M. bovis* BCG vaccine and supplemented with 1,25(OH)$_2$D3.

Key words: Nitric oxide, lipopolysaccharide, *M. bovis*-BCG vaccine, 1, 25 Dihydroxyvitamin D3, goats.

INTRODUCTION

Tuberculosis (TB) still remains as a major, global public health problem (Varghese et al., 2013). In 2011, an estimated 8.7 million new cases of TB arose from the approximately one-third of the world's population infected with *Mycobacterium tuberculosis* (*Mtb*) (WHO 2012). It is estimated that one-third of the world's population is

*Corresponding author. E-mail: jalbar_1@hotmail.com.

Abbreviations: TB, Tuberculosis; ***Mtb*,** *Mycobacterium tuberculosis*; **TNF,** tumor necrosis factor alpha; **PBMC,** peripheral blood mononuclear cells; **BCG,** bacillus Calmette-Guerin; **GM-CSF,** granulocyte-macrophage colony-stimulating factor; **ROI,** reactive oxygen intermediates; **RNI,** reactive nitrogen intermediates; **LPS,** lipopolysaccharide; **MNCC,** mononuclear cell cultures.

infected with *Mtb*. Evaluation of NO response may prove useful for diagnosis of bovine TB; nitrite (NO_2^-) is the stable oxidation product of NO, and the amount of NO_2^- within culture supernatants is indicative of the amount of NO produced by cells in culture (Waters et al., 2003). Waters et al. (2003) determined that there are two essential components of tubercular host defense: NO and tumor necrosis factor alpha (TNF-α). NO is readily produced by *Mycobacterium*-induced peripheral blood mononuclear cells (PBMC) from *Mycobacterium bovis*-infected cattle. NO responses play an important role in organism and host defense. In recent reports, inflammatory responses increase NO levels through increased expression of an inducible form of NOS (iNOS) in inflammatory cells such as macrophages. This is gaining wide acceptance for use in pulmonary TB diagnosis (Van Beek et al., 2011). Macrophages play an important role in the first and essential line of defense against mycobacterial disease. Stimulation of iNOS in macrophages and subsequent generation of reactive nitrogen intermediates (RNI) are potent mechanisms for mycobacterial killing (MacMicking et al., 1997; Morris et al., 2003).

1,25-dihydroxyvitamin D3 [1,25-$(OH)_2$D3] is a powerful stimulus for the production of NO by macrophages, which defend the host against TB (Rook, 1988). It has been considered that the active principle of vitamin D, 1,25-$(OH)_2$D3, is a potent modulator of immune responses and may be beneficial in the treatment of TB (Waters et al., 2003). Additionally, it has been observed that 1,25-$(OH)_2$D3 suppresses the growth of *Mycobacterium* in the macrophages through a dependent mechanism of NO (Rockett et al., 1998). An important point is that 25-OH-D3 is turned to 1,25-$(OH)_2$D3 within the macrophage, and the proportion of conversion increases within the alveolar macrophages and in human monocytes of the stimulation with IFN-γ (Koeffler et al., 1985; Rook et al., 1986). Probably, 1,25-$(OH)_2$D3 triggers a dependent mechanism of NO that increases the destruction of *Mycobacterium* (Palmer et al., 2004; Waters et al., 2004).

M. bovis bacillus Calmette-Guerin (BCG) vaccine, an attenuated strain of *M. bovis*, was developed for control of human TB more than 70 years ago and is still the only TB vaccine available. Characteristic changes in RNI metabolism are an integral part of the cellular immune assays. Weatherby et al. (2003) confirmed that macrophages are among the first cells in innate resistance to intracellular microbial pathogens. They have determined that cytokines *gamma*-interferon (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α activate macrophages to resist the growth of intracellular pathogens during improvement in the production of antimicrobials molecules, including the RNI and the reactive oxygen intermediates (ROI) (Chan et al., 2001; Weatherby et al., 2003). Proinflammatory cytokines such as interleukin (IL-12) play critical roles in the induction of host resistance to *Mycobacterium* (Bafica et al., 2005). In addition, the activated macrophages can turn L-arginine to NO_2^- in the presence of iNOS enzyme, with the development of a cytotoxic activity against tumor cells (Hibbs, 1991) and in front of bacterial infections (Adams et al., 1991). Protection against TB requires the induction of Th1 immune response, but studies with new-born animal have shown that they preferentially develop Th2-type responses following immunization and are defi-cient in Th1 responses (Buddle et al., 2003). As a result, the bacterial transcription changes of the Th1-mediated immune response are likely induced, directly or indirectly, by NO generated by infected macrophages (Shi et al., 2003).

BCG vaccination at birth induced a high level of immunity and the sensitization of very young animals (six weeks old) to antigens of environmental mycobacteria did not affect the effectiveness of BCG (Adams et al., 1991; Franzblau et al., 1991; Buddle et al., 2003; Wedlock et al., 2003). Different studies have been raised that the exhibition of the *M. bovis* BCG vaccine alters the specific immune response. It causes an increase of the immune response mediated by Th1 cells and a stimulation of the immune response Th2. In addition, Flynn and Chan (2003) demonstrated that *Mycobacterium* reactivation occurs if the production of RNI is inhibited in a murine model of latency (Flynn and Chan 2003).

Concerning lipopolysaccharide (LPS), there are evidences of endogenous NO_3^- production, especially in answer to inflammatory stimuli where NO_3^- production *in vitro* could be induced by the macrophages in the presence of LPS (Stuehr et al., 2005). It has been studied that, synthesis of prostaglandins and thromboxanes increased in LPS-stimulated macrophages results from selective expression of mitogen-inducible cyclooxygenase (Lee, Soyoola et al., 1992). Also, LPS-stimulated astrocytes produced TNF as demonstrated by Northern blot analysis using a mouse TNF probe and by functional assay (Lieberman et al., 1989). In another research, it was obtained that activation of macrophages by bacterial LPS induces transcription of genes that encode for pro-inflammatory regulators of the immune response (Hambleton et al., 1996). The combination of IFN-γ and LPS induced a much higher rate of transcription of the macrophage NO synthase gene than did stimulation with LPS alone. These results provide one explanation of why priming and triggering stimuli, such as IFN-γ and LPS, respectively, synergistically activate macrophages and may be applicable to explaining how IFN-gamma augments NO-dependent microbicidal activity in macrophages as well (Lorsbach et al., 1993). Furthermore, complement receptor type 3 CR3 molecule acts in a CD14-independent signaling pathway, and contributes to NO production by macrophages stimulated with high doses of LPS (Matsuno et al., 1994). However, recently, little is known about the role of these intermediates during latent infections and there is thus an urgent need to improve immune response increase in host and NO measurements for detecting latent TB patients.

It is so important to analyze the increased inflammatory

responses of NO levels in macrophages. As a result, the aim of this study is to evaluate *in vitro* the effect of LPS on NO production in supernatants (macrophages) of mononuclear cell cultures (MNCC) from *M. bovis* BCG-infected goats supplemented with 1,25(OH)$_2$D3.

MATERIALS AND METHODS

Animals and immunizations

Twelve goats (n=12; *sannen*) of 20-to 24-month-old were maintained with a diet supplemented with grain and supplied with water *ad libitum*. Four treatments were carried out: control, supplemented with *M. bovis* BCG vaccine (Pasteur Merieux®, Lyon France), 1,25 (OH) $_2$D3 and *M. bovis* BCG vaccine plus 1,25 (OH)$_2$D3. Three goats were injected intramuscularly with 0.1 ml of saline solution, three goats were vaccinated intradermal with 0.1 ml (10^3 viable units) of *M. bovis* BCG vaccine, three goats with 0.25 μg de 1,25(OH)$_2$D3 (CALCITRIOL –alpha 25-dihydroxycolecalcitrol GELDEX-GELPHARMA®), and three with *M. bovis* BCG vaccine plus 1,25 (OH) $_2$D3. Whole blood was collected from the jugular vein of antigen-primed goats. Samples of peripheral blood were collected in tubes (BD Vacutainer^{TM} K_3 EDTA; Becton Dickinson, Franklin Lakes, N. J.).

Peripheral blood mononuclear cells (PBMC)

Whole blood samples were collected and blood mononuclear cells were obtained immediately before stimulation [day 0, 3, 7, 14, and 21 days post challenge. Whole blood was centrifuged at 3000 x g for 15 min, and separated leucocytes layer in a 15 ml screw cap polypropylene centrifuge tube (Corning® 430790). Mixtures containing goat blood were overlaid on ficoll-hypaque (Sigma® H8889 d=1.077 g/ml) at a radio of 6 ml of calcium and magnesium free phosphate buffered saline (PBS), it is designed to maintain a physiological pH in an open system, to 3 ml of ficoll in a 15-ml polypropylene centrifuge tube. The resulting gradient was centrifuged at 1500 x g for 15 min. Goat PBMC were then collected, washed one time in PBS solution, and suspended in RPMI 1640 medium supplemented (Waters et al., 2003).

Cell viability

Viability of the samples was measured by trypan blue (derived from toluidine, that is, any of several isomeric bases, $C_{14}H_{16}N_2$, derived from toluene) dye exclusion method where the viable (unstained) cells were counted in a hemocytometer. Samples of at least three independent experiments were analyzed in duplicate (Huttunen et al., 2004). The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable. Trypan blue staining of cells was place 0.5 ml of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of 1 x 10^5 to 2 x 10^5 cells per ml) in a screw cap test tube; add 10 μl of 0.4% trypan blue stain. Mix thoroughly; allow standing 5 min at 15 to 30°C.

Cell culture and administration of lipopolysaccharide (LPS)

The wells of 24-well round-bottom microtiter plate (Corning Costar®24-well T-C Treated microplates, Individually Wrapped No. 3526) were seeded with 2 X 10^5 PBMC in a total volume of 200 μl per well. The goat macrophage cell was obtained from a cell culture. The cells were cultured at 37°C in 5% carbon dioxide atmosphere in RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units of penicillin per ml, 0.1 mg of streptomycin per ml, 1% nonessential amino acids (Sigma), 2% essential amino acids (MP Biomedicals®), 50 μM 2-mercapto-ethanol (Sigma), and 10% (vol/vol) heat-inactivated fetal bovine serum (Gretzer and Thomsen, 2000). The macrophages (5X10^5 cells/ml) were dispensed to six-well plates, 2 ml/well. The cells were allowed to adhere for 48 h, was removed the supernatant from adherent cells and were suspended in cool PBS by scraping, the viability of the cells was assessed, and the cell suspension was obtained in a micro centrifuged tube (Eppendorf Axygen®), centrifuged (5 min; 1,500 rpm) to separate the cells from the PBS. The cells were suspended in an adequate cryoprotection it was constituted by 90% of fetal bovine serum and 10% of dimethyl sulfoxide (DMS) and later were stored at –70°C for the subsequent analyses. Sterilize DMS was by filtration through a 0.2 μm cellulose membrane and was stored in small quantities (2 ml). The *E. coli* LPS (serotype O111:B4; Sigma) was reconstituted by adding 1 ml of sterile balanced salt solution 100 ng LPS/ml, and was added 10 μl to each well.

Measurement of nitrites

The NO_2^- concentrations were obtained prior and after vaccination and challenge. Since NO is unstable and rapidly converted to NO_3^- /NO_2^-, it is necessary to determine both NO_3^-/NO_2^- concentrations in the samples. NO_2^- optical densities were measurement in culture supernatants with ELISA microplate reader (Organon Tecknika, Microwell system), according to the griess method (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2% phosphoric acid) (Rhoades and Orme, 1997). The NO_2^- is the stable oxidation product of NO, and the amount of NO_2^- within culture supernatants is indicative of the amount of NO produced. Stock solution of sodium nitrite (Baker) at 100 Mm in phosphates buffer was stored at 4°C. Samples of at least four independent experiments were analyzed in duplicate. The assay was performed in non-sterile 8-well plates. Briefly, 50 μl of supernatant was added in duplicate to an 8-well plate and mixed with an equal volume of griess reagent (Jeevan et al., 2006) and left for 5 min at room temperature. All the plates were read at 620 nm (reference) and 540 nm (test). NO_2^- concentrations were calculated directly from the nitrite standard curve (Rockett et al., 1998).

Statistical analysis

A random design with repeat measures in the periods was used completely (Steel et al., 1980). The following statistical model was used: Version 8.0; SAS Institute Inc., Cary, North, Carolina,

$$Y_{ijkl} = \mu + \zeta_i + C\zeta_{j(i)} + P_k + \zeta P_{ik} + \beta\,(x_i. - x..) + \varepsilon_{ijkl}$$

(Version 8.0 SAS. Institute Inc. Cary, North, Carolina).

RESULTS AND DISCUSSION

Control animals' NO_2^- concentrations were between 7.9-18.64 μM/l. The supplemented treatment with *M. bovis* BCG vaccine was 10.84-17.06 μM/l; the next treatment, 1,25(OH)$_2$D3 was 13.93-25.38 μM/l and the last treatment,

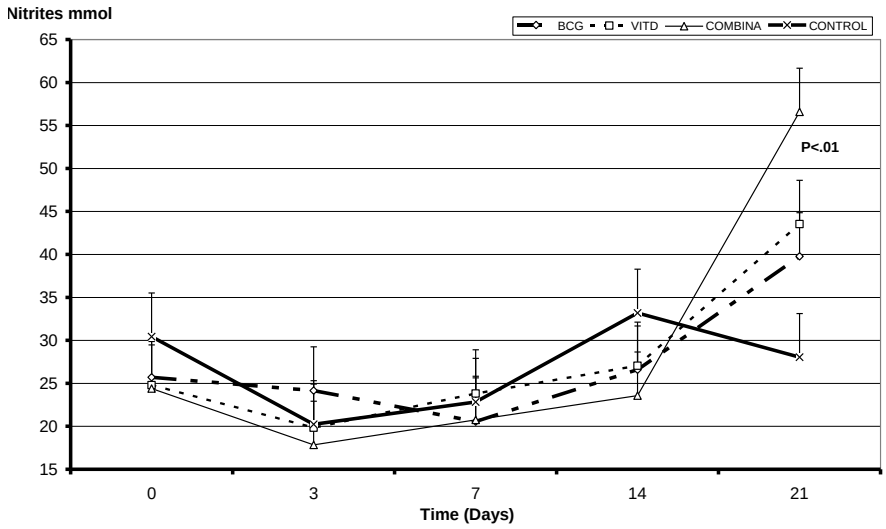

Figure 1. The measurements (n=12) were prechallenge (day 0) and 3, 7, 14, and 21 days postchallenge for Nitrites.

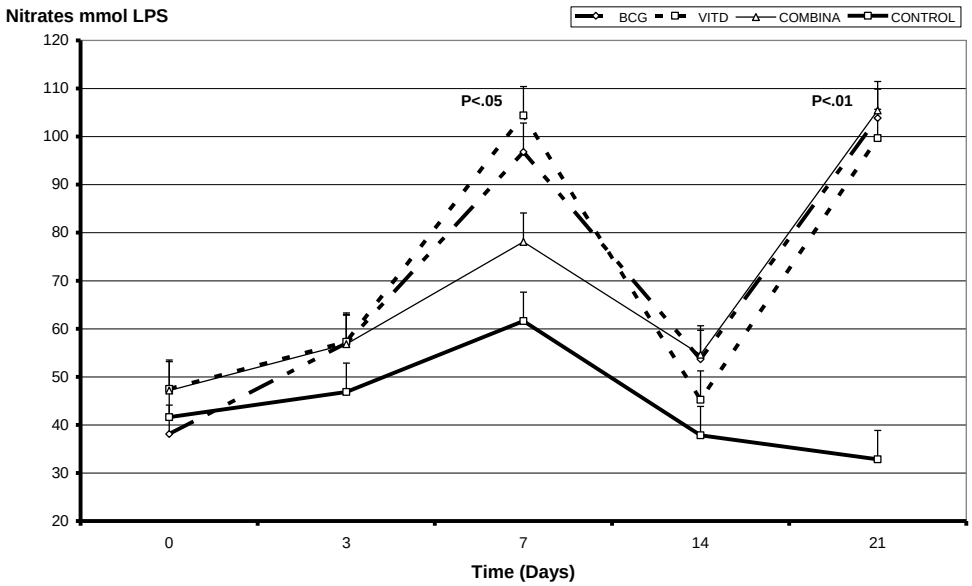

Figure 2. The measurements (n=12) were prechallenge (day 0) and 3, 7, 14, and 21 days postchallenge for Nitrates.

M. bovis BCG vaccine plus 1,25$(OH)_2$D3 was 12.64-28.29 μM/l. The two groups vaccinated with BCG produced significantly higher mean peripheral blood NO_2^- and NO_3^- responses to BCG and BCG plus 1,25$(OH)_2$D3 than those for the non-vaccinated group ($P < 0.05$). The group vaccinated with BCG plus 1,25$(OH)_2$D3 produced significantly higher mean NO_3^- response than those for the BCG only group ($P < 0.05$). There were no significant differences between the mean NO_2^-. The concentrations differences between NO_2^- and NO_3^- were considered significant at $P < 0.01$. However, the effect of the treatments was not significant ($P > 0.9$). The effect of stimulation (Figure 1) with LPS was similar during the period of 0 to 14 days; in every case twelve determinations were realized; a sample of twelve goats (n=12) was worked with. We observed high production of NO_2^- until the 21 days of treatment (average 56.55 μmol) and obtained higher production of NO_2^- with the treatment combined with 1.25 $(OH)_2$D3 and vaccine BCG. The effect of the treatments with LPS in front to NO_3^- was different during the period of 0 to 21 days (Figure 2); an increase was observed in 7° and 21° days of stimulation (average: 90.11 μmol and 100.94 μmol,

respectively); differences between treatments were significant ($P < 0.01$).

In this investigation, we observed the effect of LPS on cell culture and was examined by *in vitro* stimulation with *E. coli* LPS. The NO has an antimicrobial activity and plays a vital role in host defense and immunity, including the regulation of inflammatory responses. The addition of LPS was second activation in the production of RNI, because the first immunological signal was BCG infection, $1,25(OH)_2D3$ alone, and a combined treatment of BCG plus $1,25(OH)_2D3$. NO was significantly higher in culture supernatants from LPS-stimulated cultures *in vitro* than in culture supernatants from samples with the first challenge. $1,25(OH)_2D3$ treatment stimulated NO_3^- synthesis, indicating, that it is a good modulator of the mycobacterial replication; and *M. bovis*-BCG vaccine increased treatment as a result of $1,25(OH)_2$ D3 treatment. The exhibition of *M. bovis*-BCG vaccine with $1,25(OH)_2D3$ treatment was able to increase NO_3^- in exposed animals. NO is an unstable molecule in the biological systems and quickly turns NO_2^- and NO_3^-. However, the amount of NO_2^- within culture supernatants is indicative of the amount of NO produced by cells in culture (Waters et al., 2003). It was reported for the first time that *Mycobacterium* induces mRNA for iNOS, iNOS protein, NO, and peroxinitrite in human monocyte/macrophage cultures (Gretzer and Thomsen, 2000). However, in culture supernatants exists IFN-γ that increases macrophage NO_2^- production. In addition, IFN-γ was found to be the critical mediator of NO production (Roy et al., 2004). The aim of this work is to investigate *in vitro* the effect of LPS on NO production MNCC from *M. bovis* BCG-infected goats supplemented with $1,25(OH)_2D3$. The results of the BCG experiment further support that T lymphocytes may play a role in NO_3^- biosynthesis. BCG infection causes a pronounced activation of the host goat's macrophages. It is possible that the NO_3^- generated during BCG infection is produced by macrophages that have been activated by T-cell lymphokines. The observed after 48 h of culture in BCG-induced NO_3^- production is consistent with T-cell involvement and does not support a direct activation of macrophage NO_3^- synthesis by BCG. The time course of NO_3^- production during BCG infection parallels other immunological changes that occur, such as NO_2^- production. This observation agrees with Stuehr and Marletta (1985) who suggest that the magnitude of NO_3^-/NO_2^- biosynthesis may be directly related to the state of activation of the immune system.

In this research we obtained an increase in NO_3^-/NO_2^- (NO) concentrations in the supernatants of MNCC stimulated with LPS and $1,25(OH)_2$ D3. Similar results were got in another work. The macrophages were exposed to LPS in culture, the macrophages were activated during BCG infection and the first stimulation increased the amounts of NO_3^-/NO_2^-. However, macrophages exposed to LPS in culture showed higher production of NO_3^-/NO_2^- because in the culture, there are macrophages in the rest that were not stimulated with the first immunological signal.

In conclusion, we obtained an increase in NO_3^-/NO_2^- (NO) concentrations in the supernatants of MNCC; LPS has effect *in vitro* on the production of NO in goats exposed to *M. bovis* BCG vaccine and supplemented with $1,25(OH)_2D3$. *In vitro*-based cellular immune assays are gaining wide acceptance for use in TB diagnosis. Of relevance to cattle, an NO responses may prove useful for diagnosis of bovine TB; the nonspecific production of nitrite in MNCC cultures from infected cattle may be problematic for development of a useful NO based diagnostic assay of infection. It is necessary to enhance the practicality and specificity of that assay to realize adaptation of NO with another *M. bovis* BCG-induced inflamematory cytokines. Consequently, whether NO measurement would be capable of detecting TB patients would be a fabulous finding to lower this global public health problem.

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Isolation of salt tolerant endophytic and rhizospheric bacteria by natural selection and screening for promising plant growth-promoting rhizobacteria (PGPR) and growth vigour in tomato under sodic environment

T. Damodaran[1]*, V. Sah[2], R. B. Rai[2], D. K. Sharma[1], V.K.Mishra[1], S. K. Jha[1] and R. Kannan[1]

[1]Central Soil Salinity Research Institute, Regional Research station, Lucknow- 226005 India.
[2]Indian Veterinary Research Institute, Bareilly - 243122 India.

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The importance of plant growth promoting rhizobacteria in growth promotion and their ability to elicit 'induced systemic tolerance' against abiotic stresses has been documented. However, the performance of these microbes under various abiotic stresses especially saline-sodic conditions will be of great importance in the current agricultural scenario. In this study, we isolated 16 rhizobacteria through natural selection from saline sodic soils, and characterized them using morphological and biochemical parameters. These bacteria were assessed for their plant growth-promoting rhizobacteria (PGPR) traits like indole-3-acetic acid (IAA) production, ammonia and hydrogen cyanide (HCN) production, phosphate solubilization, etc. Furthermore, they were screened for *in-vitro* salt (NaCl) tolerance and Na^+ uptake pattern, where two stress tolerant rhizobacteria B-1 and B-3 identified as *Bacillus pumilus* and *Bacillus subtilis* showed all PGPR traits with tolerance to salinity. These isolates also elicited significantly higher vigor index in tomato seedlings grown in pot culture experiments under saline sodic soils of pH 9.35 and EC 4.2.

Key words: Rhizobacteria, salt tolerant, natural selection, PGPR.

INTRODUCTION

Rhizosphere is centre to microbial and nutrient dynamics and describes the zone of soil surrounding roots of plant species which release organic substances. Bacteria that are present in the rhizosphere and enhance plant growth by any mechanism are referred to as plant growth promoting rhizobacteria (PGPR) (Arnou et al., 1953). In both natural and man-made agro ecosystems, interactions between plants and soil micro-organisms have a profound effect on adaptation of plant to changing environment and plant growth (Kloepper et al., 1989; Bashan et al., 2004). Selections of microbial isolates from naturally stressed environment or rhizosphere are considered as possible measures for improving crop health which can control diseases and also promote plant growth (Lugtenberg and Kamilova, 2004; Mayak et al., 2004).

Worldwide, salinity is one of the most severe abiotic stresses that limit crop growth and productivity. Around 20% of worlds irrigated land is salt affected, with 2,500-5,000 km^2 of production lost every year as a result of

*Corresponding author. E-mail: damhort2002@yahoo.com.

salinity (UNEP, 2009). About 60% of salt affected soils are of sodic and saline sodic in nature which has increased steadily over decades in the northwest plains of the Indo-Gangetic basin and in China's Yellow River basin (Gupta and Abrol, 2000). High alkalinity (pH > 8.5) and high exchangeable sodium percentage (ESP>15) of the soil render it inhospitable for normal crop production and there is minimal bioproductivity in such soil (Chhabra, 1995). The utilization of salt-affected soil for agriculture has become necessary to meet the rise in food demand. One possible strategy to counteract the adverse effect of salinity is to exploit the avenues of bio-agents or bio-inoculants (Egamberdieva, 2012). Under salt stress, PGPR have shown positive effects in plants on parameters like germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Raju et al., 1999). In an era of sustainable agricultural production, the interactions in the rhizosphere by soil micro-organisms with soil and plant plays a vital role in mobilization of nutrients from the limited pool (Mantelin and Touraine, 2004).

The combination of IAA production ability (Goldstein, 1995), phosphorous solubilization (Gyaneshwar et al., 1998) and siderophore production (Dulfy, 1994) of bacteria aid the plant rhizosphere in enhan-cing the nutrient absorption potential under sodic environ-ment for enabling economic production of commercial horticultural crops (Damodaran et al., 2013). This has been extensively attracting attention due to their efficacy as biological control and growth promoting ability in many crops. Though researchers earlier, have worked on isolation of salt (NaCl) tolerant rhizobacteria from halophytic environment where the conductivity (EC) of the soils is > 4 dsm^{-1}, little is known about their tolerance to saline sodic environment where the soils are severely affected by high pH characterized by high Na^+ in the soil solution phase as well as on cation exchange complex (Qadir and Schubert, 2002), exhibiting unique structural problems (slaking, swelling, and dispersion of clay). Therefore the present study is focused to survey plants habitat in the sodic soils and isolate rhizobacteria from sodic environment, to characterize and screen the isolates for PGPR and salt tolerant traits and further, to assess the growth vigor index of the tomato seeds primed with salt tolerant isolates in saline sodic soils.

MATERIALS AND METHODS

Survey of sodic soils

For the present experiment, a survey was conducted in 2009 in the Sharda Sahayak Canal Command areas of Rae Bareily district, Uttar Pradesh, India as it harbours major sodic belt. Collection of the rhizospheric soils and roots were made from halophylic plants grown in wild.

Physicochemical analysis of soil

The collected soil sample was analyzed for physicochemical parameters like pH and conductivity. The pH of the soil extract was determined potentio-metrically by an ORION ion analyzer (5 star series) using a pH electrode.

Isolation of rhizospheric and endophytic bacteria

The rhizospheric microbes were isolated from roots of phyto-ameliorant grasses collected from survey of undisturbed sodic site with prominent salt efflorescence as described by Quadt-Hallman et al. (1997). The soil samples were collected by fine brushing in sterile Petri dish for diluting and plating. Soils sample from rhizosphere of plants (10 g) were thoroughly mixed in 90 ml of autoclaved distilled water to make suspension. Soil suspension was kept for 30-60 min with periodic shaking. 1 ml of this suspension was added to 10 ml dilution vial and shaken. Serial dilution technique was performed up to 10^{-7} dilution. An aliquot of this suspension was spread on nutreint agar (NA) plate and incubated for 24-48 h at 28-30°C for observing colonies developed on it. Fine isolated colonies were picked up and streaked again on fresh NA plate and incubated similarly. This process was carried out thrice to get pure single colony.

Standard plate counting method

To enumerate the bacterial and fungal cultures, standard plate count method was used. The number of viable bacterial cells per unit volume of a sample using agar plate media was enumerated. The inoculum sample was spread across the plate and the colonies that were formed after incubation were counted. The colonies are referred to as colony forming units (CFU). Once the CFUs are counted on the plate, they were divided by the volume plated to determine the concentration of cells in the sample.

Bacterial identification

Bacteria were identified based on different morphological and staining characteristics. Based on the Gram staining property and cell morphology, the bacteria may be tentatively placed in four groups, that is, Gram +ve rods, Gram +ve cocci, Gram -ve cocci and Gram -ve rods. Usually, the predominant bacteria in rhizosphere of crop plants are Gram negative rods belonging to Gram -ve *Pseudomonas* and Gram +ve *Bacillus*. Further identification was done with specific biochemical tests (Cappunccino and Sherman, 1992).

In vitro analysis for the identification of PGPR strains

Production of indole acetic acid (IAA)

Indole acetic acid (IAA) production was detected as described by Brick et al. (1991). Bacterial cultures were grown for 72 h in nutrient broth media at 36- 38°C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production.

HCN production

Production of HCN was detected according to the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine / L and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate and 0.5% picric

Table 1. Enumeration of Rhizobacteria from soil samples by SPC method.

S/N	Location	Dilution	Amount of sample (ml)	Dilution factor (D)	Number of colony	Mean cfu per 100 mg soil	pH	Ece (dsm^{-1})
1	Kasrawa	10^{-3}	0.1	10^3	35	$45X10^3$	9.70	1.50
2	Hardoi	10^{-3}	0.1	10^3	5	$05X10^3$	10.2	4.55
3	Thakurenda	10^{-3}	0.1	10^3	45	$25X10^3$	10.0	1.24
4	Paschim Gau	10^{-3}	0.1	10^3	90	$90X10^3$	9.65	0.75
5	Gurbakshganj	10^{-3}	0.1	10^3	5	$35X10^3$	9.88	4.30

acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36 ± 2°C for 4 days. Development of orange to red colour indicated HCN production.

Ammonia production

Bacterial isolates were screened for the production of ammonia in peptone water. Freshly grown culture were inoculated in 10 ml peptone water in each tube and incubated for 48-72 h at 28±2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive for ammonia production (Cappucino and Sherman, 1992).

Siderophore production

Bacterial culture (48 h) was streaked on nutrient agar medium amended with an indicator dye. The tertiary complex chrome-azurol-S(CAS) / Fe^{3+} / hexadecyl trimethyl ammonium bromide served as an indicator. Change of blue color of the medium surrounding the bacterial growth to fluorescent yellow indicated production of siderophore. The reaction of each bacterial strain was scored either positive or negative to the assay (Schwyn and Neilands, 1987).

Phosphate solubilization

Phosphate solubilization of isolates was evaluated from the ability to solubilize inorganic phosphate. Pikovskaya's agar medium containing calcium phosphate as the inorganic form of phosphate was used in assay. A loopful of bacterial culture were streaked on the plates and kept for incubation at 28°C for 4-5 days. The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

Salinity tolerance

For determining salt tolerance of the isolated bacteria, they were streaked on nutrient agar supplemented with 0.5, 5, 7.5 and 10% NaCl which acts as a selective medium. After the appearance of colonies, bacteria's were marked positive or negative for their ability to grow in different concentration of NaCl.

Sodium uptake

Potential isolates growing luxuriantly in 7.5% NaCl were screened for sodium uptake pattern. The isolates were grown overnight at 37°C in L-broth containing different NaCl concentration (0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 M). After 24 h, cells were harvested by centrifugation and bacterial pellet was washed with sterilized distilled water to remove the traces of medium. Washed pellet was digested overnight with 0.1 N HCl at room temperature. Samples were centrifuged and supernatant was taken for the estimation of uptake by bacterial cells. Sodium contents were measured by Flame photometer.

Determination of PGPR in sodic conditions

The rhizosphere and endophytic bacteria grown on nutrient broth with constant shaking on rotary shaker at 150 rpm for 48 h at room temperature (28±2°C) were harvested by centrifugation at 6000 rpm for 15 min. The bacterial cells were re-suspended in PB (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 10^8 CFU (OD_{595}=0.3) and used as inoculums for treating rice seeds (Thompson, 1996). Plant growth promoting activities of bacterial strains were assessed based on the seedling vigor index of tomato seed under pot culture studies in soil of pH 9.35, ECe of 4.2, Na^+ of 23.50 meq / l and sodium adsorption ratio (SAR) of 19.36. Sodium (Na^+) was determined by flame photometer (Richards, 1954) while sodium adsorption ratio (SAR) was determined by following generic equation:

$$SAR = \frac{Na}{\sqrt{(Ca + Mg)/2}}$$

The vigor index was calculated by using the formula as described by Abdul Baki and Anderson (1973):

Vigor index= Percent germination x Seedling length (shoot length + root length).

Statistical analysis

The pot culture experiment on assessing vigor index in tomato seeds treated with bacterial isolates was conducted in completely randomized design (CRD) with three replications and the data was analyzed using SAS 9.2 version. Prior to analysis of variance, the percentage values of germination were arcsine transformed.

RESULT

A transect survey was carried out in sodic lands of Rae Bareily district in Uttar Pradesh, India. The locations surveyed included Kasrawa, Hardoi, Thakurenkeda, Paschim Gau and Gurubakshganj (Table 1). These locations were observed to have extended patches of barren

Table 2. Morphological characteristics of isolated bacteria.

S/N	Experiment procedure	Observations And Result														
		G-1	G-2	-G-3	G-4	G-5	G-6	G-7	G-8	G-9	G-10	G-11	B-1	B-2	B-3	P-1	P-2
1	Colour	W	W	W	LW	Y	OW	LY	C	W	W	Y	CW	CW	C	Y	LY
2	Shape	I	S	Co	S	R	R	S	R	I	R	R	R	R	R	R	R
3	Elevation	Cv	Ra	Cv	Ra	Ra	Ra	Ra	Ra	F	Ra	Ra	Ra	Ra	Ra	Ra	Ra
4	Pigmentation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	B	B

W=White, LW= light white, Y= yellow, O=off white, LY= light yellow, C=creamy off white, CW= creamy white, I= irregular, S= spherical, Co= cocci, R= round, Ro= rod, Cv= convex, Ra= raised, F= flat, B= bright, N= none.

sodic soils with pH ranging from 9.65 (Paschim Gau) to 10.2 (Hardoi). The population (cfu gm^{-1}) of bacteria ranged from 90 $\times 10^3$ (Paschim Gau) to 5 $\times 10^3$ (Hardoi). About 32 bacterial isolates were collected and among them 16 were selected and sorted out into pure different colonies, exhibiting morphological and staining characteristics (Tables 2 and 3). Among the 16 isolates, five (G-5, G-7, G-11, P-1 and P-2) were yellowish to light yellowish colour while the others were white to creamy white in colour. The isolates were of irregular, spherical, cocci, round and rod shaped. Pigmentation was absent in most of the isolates except P-1 and P-2 which displayed bright pigments.

Further, they were screened for Gram nature, motility, pigmentation, colony and morphological characteristic. Among the 16 isolates, six were gram positive and 10 were Gram negative (Table 3). Results have indicated that 10 out of 16 isolates were able to assimilate starch, glucose and fructose, while seven were able to assimilate nitrate and 8 were positive for indole production.

PGPR traits

Sixteen isolates were further screened for PGPR traits (Table 4) like IAA, siderophore, ammonia and HCN production and also phosphate mobilization ability. Among them, seven showed IAA and HCN production. The isolates B-1 and B-3 had extensive zone formation for IAA (>1cm). Four isolates showed siderophore production with two of them (B-1 and B-3) belonging to genus *Bacillus* having much higher zone ranging from 0.6 - 0.9 cm.

Production of ammonia was detected in nine isolates and phosphate solubilization zone was observed in eight isolates. Four isolates B-1, B-3, P-1 and P-2 showed higher phosphate soulubilization (0.6- 0.9 cm). Among the 16 isolates screened, two (B-1 and B-3) of them exhibited positive response to all the *in-vitro* PGPR characteristics studied.

Salt tolerance traits

On screening all the 16 bacterial isolates for growth in different NaCl concentrations; five isolates (B-1, B-2, B-3, P-1 and P-2) growing luxuriantly in 7.5 % NaCl concentration were selected for further evaluations (Table 5). These five isolates also exhibited halo formation when grown on Mannitol salt agar medium containing 7.5% NaCl. Furthermore, analysis of these isolates for sodium uptake pattern (Figure 1) at different molar (M) concentration of NaCl showed an increasing sodium (Na^+) uptake up to 1 M NaCl in all the isolates beyond which there was a significant decline. However, among them, two isolates B-3 and B-1 identified as *Bacillus pumilus* and *Bacillus subtilis* showed higher uptake of Na^+ (1.272 meq / L and 1.122 meq / L respectively) at 1 M NaCl concentration.

Screening of the salt tolerant isolates for plant growth potential (PGP) in saline-sodic soils of pH 9.35 and EC 4.2 dsm^{-1} under pot culture experiment of tomato var. Himsona showed that among the five, B-1 and B-3 had plant growth enhancing activities with the germination percentage of 95.0 and 93.0% (Table 6). Significantly higher shoot and root growth were observed in B-3 followed by B-1 with higher vigour index (1900.0 and 1525.2 respectively).

DISCUSSION

A detailed survey of the natural population in the rhizosphere of grasses grown in sodic soils was carried out in the present study to isolate and identify salt tolerant bacteria that could express plant growth promotion (PGP) traits at high salt concentrations. Our studies establish that the bacterial diversity reduce with increase in soil pH under natural selection sites. It has been reported

Table 3. Identification of potential bacterial isolates based on bio-chemical tests.

S/N	Parameter	G-1	G-2	G-3	G-4	G-5	G-6	G-7	G-8	G-9	G-10	G-11	B-1	B-2	B-3	P-1	P-2
1	Gram staining	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
2	Gelatin liquefaction	-	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+
3	Catalase test	-	+	_	+	+	-	-	+	+		-	+	+	+	+	+
4	Oxidase test	-	_	_	-	-	-	+	-	-		+	-	-	-	+	+
5	Starch hydrolysis	-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-
6	Fluorescent pigment	-	_		-	-	-	-	+	-	-	-	-	-	-	+	+
7	Indole	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	+
8	Methyl red	+	-	_	-	-	-	+	+	-	+	-	-	-	-	+	+
9	VP	+	+	+	+	+	+	-	-	+	-		+	+	+	-	-
10	Citrate	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	-
11	Nitrate	_	_	_	+	+	-	-	-	+	+	-	+	+	+	-	-
12	Glucose	A^+	A^-	A^+	A^+	A^+	A^+	A^+	A^+	A^-	A^-	A^-	A^+	A^+	A^+	A^-	A^-
13	Fructose	A^-	A^+	A^+	A^+	A^+	A^+	A^+	A^+	AP	A^-	A^-	A^+	A^+	A^+	A^-	A^-
Identification of the isolates		A	B	C	D	E	F	G	H	I	J	K	M	L	N	O	P

A = *Serratia* sp.; B = *Micrococcus* sp.; C = *Azotobacter* sp.; D = *Bacillus safensis;* E = *Bacillus subtilis;* F = *Rhizobium;* G = *Bacillus* sp.; H = *Pseudomonas* sp.; I = *Brevibacillus* sp.; J = *Azospirillum* sp.; K = *Uncultured bacterium;* L = *Bacillus pumilus*; M = *Bacillus cereus*; N = *Bacillus subtilis*; O = *Pseudomonas putida*; P = *Psuedomonas* sp.; A^+ = Acid producers; A^- = No acid producers.

Table 4. Assessment of plant growth promoting bacteria for different growth promotion traits.

S/N	Experiment procedure	Observations and result															
		G-1	G-2	-G-3	G-4	G-5	G-6	G-7	G-8	G-9	G-10	G-11	B-1	B-2	B-3	P-1	P-2
1	IAA production	+	-	-	-	-	-	-	+	-	+	-	+++	-	+++	++	++
2	HCN	+	-	-	-	-	-	-		+	+	-	+	+	+	-	+
3	Siderophore	_	+	-	-	-	-	-	-	-	-	-	++	-	++	-	+
4	Ammonia	+	+	+	+	+	-	-	-	-	+	-	+	-	+	+	-
5	P- solubilization	+	-	-	-	-	+	+	-	-	+	-	++	-	++	++	++

IAA, Indole-3-acetic acid; HCN, hydrogen cyanide; -, no production; +, 0.3-0.5 cm; ++, 0.6-0.9 cm; +++, >1 cm.

earlier that soil salinity plays a prominent role in the microbial selection process as environmental stress leads to reduce bacterial diversity (Borneman et al., 1996). In our study, we have isolated 32 isolates from natural selection in the rhizopshere of grasses grown in sodic soils and sorted them into 16 different pure colonies. Majority of the bacterial isolates are identified as *Bacillus* spp. based on biochemical and morpholo-gical observations. Earlier studies show that genera such as *Bacillus* and *Pseudomonas* tend to be pre-dominant in saline soils (Tank and

Table 5. Screening of isolates for tolerance to salinity.

S/N	NaCl concentration	Observations and result (48 h)															
		G-1	G-2	-G-3	G-4	G-5	G-6	G-7	G-8	G-9	G-10	G-11	B-1	B-2	B-3	P-1	P-2
1	Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	0.5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	5%	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
4	7.5%	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
5	10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Luxiriant, - = no growth

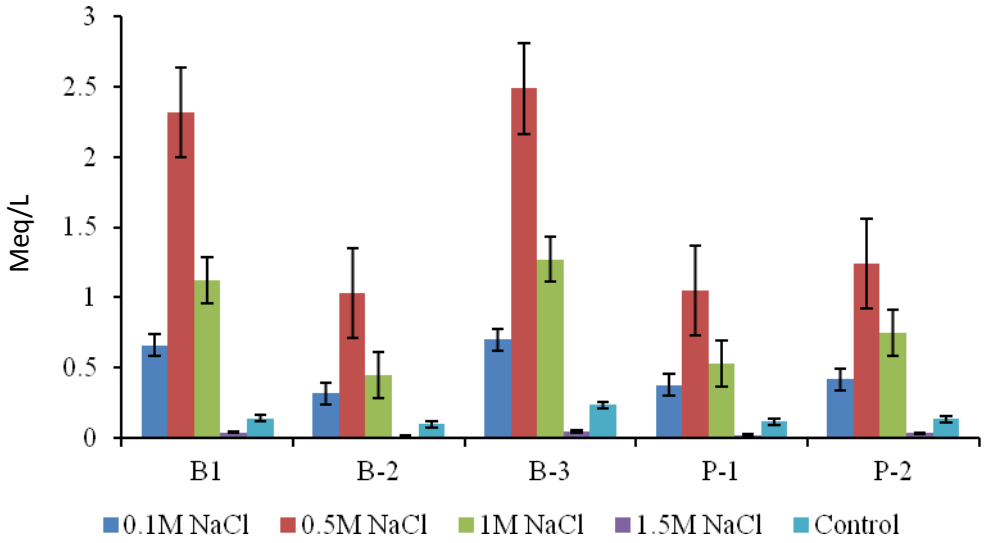

Figure 1. Sodium uptake pattern of the elite PGPR strains of sodic soils. Vertical bars indicate ±standard error

Saraf, 2010).

PGP activity of the bacteria present in the rhizosphere is found to exert beneficial effects on plant growth mechanism. Several mechanisms such as production of phytohormones, suppression of deleterious organisms, production of IAA, activation of phosphate solubilization and promotion of the mineral nutrient uptake are believed to be involved in plant growth promotion by PGPR (Glick, 1995). IAA, the most common auxin function as important signal molecule in the regulation of plant development (Usha Rani et al., 2012). Out of 16 isolates in our present study seven exhibited IAA production which can attribute significant growth enhancement in plants.

Phosphorous (P) is an essential nutrient for plant growth, development and is typically insoluble or poorly soluble in soils under salt stressed conditions (Harrison et al., 2002). Some of the bacteria are known to improve the solubilization of the fixed soil phosphorous and applied phosphates, resulting in higher yields even under stress conditions (Banerjee et al., 2010). In our experiment, eight rhizobacterial isolates showed *in-vitro* phosphate solubilizing efficiency and has been tested in plant growth. Ability to solubilize various insoluble phosphates is always desirable attribute for a competent PGPR. Phosphate solubilization by *Bacillus sp.* isolated from salt stressed environment had been observed by earlier researchers (Son et al., 2006).

Siderophore chelates iron and other metals contributing to disease suppression and acquisition of Fe^{2+} to plants for increasing the crop growth under stressed conditions (Hofte et al., 1992; Duffy, 1994). Our study shows four isolates with siderophore production ability which will be a productive PGPR trait for selection. Production of ammonia (Wani et al., 2007) and HCN (Schippers et al., 1990) is an important attribute of PGPR that influences plant growth indirectly and strengthen the host disease resistance mechanism respectively. In our present study, nine isolates produce ammonia and seven produce HCN. Majority of the ammonia producing bacteria were identified to be of genus *Bacillus* and *Pseudomonas* spp. Production of ammonia was commonly detected in the isolates of *Bacillus* and *Pseudomonas* (Joseph et al., 2007).

Out of the 16 bacteria isolated from sodic rhizospheres, five showed tolerance to high salt concentration (7.5 % NaCl) and among them isolate B-1 and B-3 had higher uptake of sodium when cultured under *in-vitro* conditions in 1 M NaCl solution. It has also been reported previously that bacteria isolated from saline soil are more likely to withstand saline conditions (Upadhyay et al., 2009). On the other hand, if such bacteria also possess plant growth promoting traits, they would be ideal for use in sustainable agriculture (Egamberdiyeva and Islam, 2008). Therefore, the two salt tolerant bacterial isolates B-1 and B-3 identified as *B. pumilus* and *B. subtilis* also exhibited positive response for *PGPR* characteristics like IAA, HCN, siderophore, HCN and ACC deaminase production. Production of IAA, siderophore, phosphate solubilization had been observed in *Bacillus* and *Pseudomonas sp*. in earlier studies (Xie et al., 1996; Loper and Henkels, 1997). Furthermore, in the current experiment, the assessment of vigor index of tomato seeds treated with five salt tolerant isolates (B-1, b-2, B-3, P-1 and P-2) showed that the isolates B-1 and B-3 are potential growth promoter with higher vigour index even under saline-sodic conditions apart from salinity. Though PGPR are more commonly known to induce resistance against pathogen infection, reports are now available on their ability to elicit 'induced systemic tolerance' against abiotic stresses (Usha et al., 2011).

In this study, we have shown that two strains B-3 and B-1 identified as *B. pumilus* and *B. subtilis* isolated from the rhizosphere of grasses in sodic soils of high pH are efficient for the tested salt tolerant traits under saline and sodic conditions. They also showed positive response for PGPR traits like IAA production, phosphate solubilizaiton, etc. These two isolates show potential as plant growth beneficial inoculants in alkaline soil regions suggesting further studies on rhizocompetence in commercial crops grown under salt stressed conditions.

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Full Length Research Paper

Preparation of scFv against HrpA of *Pseudomonas syringae* pv. tomato DC3000

Yanling Yang, Hairong Liu, Ziqin Zheng, Rongzhi Wang, Shihua Wang and Zhenhong Zhuang*

Key Laboratory of Biopesticide and Chemical Biology, Ministry of Education, and School of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China.

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Hrp pilus is a pathogenic bacterial surface appendage which serves as a tool for the transformation of bacterial effecter proteins into the plant cell. Hrp pilus is assembled by HrpA protein. In this study, a HrpA protein was successfully expressed, purified and a phage library single-chain fragment variable (scFv) was constructed. After six rounds of bio-panning, a specific scFv against HrpA protein named scFv-FE5 was screened by phage display. The results of enzyme-linked immunosorbent assay (ELISA) and western blotting analysis showed that scFv-FE5 could recognize HrpA protein specifically. Current work demonstrates the preparation of the antibody anti-HrpA scFv-FE5 using phage display technology, a very useful tool for generation of a specific scFv to a certain antigen.

Key words: HrpA, expression, scFv, phage display, bio-panning.

INTRODUCTION

Pseudomonas syringae pv. tomato DC3000, a Gram-negative bacteria causing the defense-associated hyper-sensitive response (HR) in plant (Fouts et al., 2003), is one of the major plant pathogenic bacteria that elicits plant disease through the translocation of effecter proteins into plant cells (Alfano et al., 2000). *Hrp* genes are present in almost all Gram-negative pathogenic bacteria, encoding hairpin which is the main component of type III secretion system (Hrp system) (Fu et al., 2006). Many gram-negative phytopathogenic bacteria use type III secretion system to inject type III effector proteins into plant cells to promote pathogenicity (Block et al., 2010). In *P. syringae,* few virulence effectors are translocated into plant cells via type III secretion system encoded by *hrp* genes (Oh et al., 2007). Hence, *hrp* genes may play an important role in pathogenic bacteria colonization and plant defense response induction.

Hrp pilus is formed on the surface of *P. syringae,* which is required for pathogenicity and allergic reactions, and normal function of Hrp type III secretion system depends on Hrp pilus. It is speculated that Hrp pilus on pathogenic bacteria grows through the plant cell wall. Bacterial cell proteins and other virulence factors are secreted through the Hrp pilus, and absorbed by endocytosis of plant cell. The major subunit of the Hrp plius of *P. syringae* pv. tomato DC3000 is the HrpA protein, an important component of the type III secretion system (Deng et al., 1998; He et al., 2003). Hence, HrpA has been suggested as a promising target for prevention and control of diseases.

Single-chain fragment variable (scFv) based on phage display is a versatile technology to generate single-chain antibody for interested antigen (Wang et al., 2006), and provides a useful tool that allows the selection of single-chain antibody that is specific for certain antigen. The technique of scFv has been developed for recognizing molecular target of cancer (Sakai et al., 2010; Zhang et al., 2010), and has the power to mimic the feature of immune diversity and selection, and to synthesize and express unlimited quantities of antibodies. By the tool, we

*Corresponding author. E-mail: xzhzhenhong@163.com.

are able to characterize the binding property of scFv and investigate the potential use of scFv as a diagnostic tool or therapeutic agent (Eisenhardt et al., 2007; Doppalapudia et al., 2010).

To better understand the role of HrpA in the type III secretion system of *P. syringae* pv. tomato DC3000, we successfully amplified *hrpA* gene and expressed the HrpA protein. A phage antibody library was constructed, and the specific scFv clones against HrpA were screened by bio-panning. Finally, a phage clone named scFv-FE5 was successfully obtained by showing the strongest positive signal in reaction to recognize HrpA protein.

MATERIALS AND METHODS

Bacterial strains and chemicals

P. syringae pv. tomato DC3000 was donated by Prof Zonghua Wang (Fujian Agriculture and Forsetry Universtiy, China). Plasmid pET28a(+) were purchased from Novagen. *E. coli* BL21 (DE3), plasmid pCANTAB-5E, *E. coli* TG1 and HB2151, M13K07 helper phage was stored in our laboratory. DNA restriction enzymes, RNA isolation kits and reverse transcription kits were purchased from Promega. Taq DNA polymerase and T4 DNA ligase were purchased from Takara (Dalian, China). HRP-labeled goat anti-mouse IgG was from Boster Biological Technology Co. (Wuhan, China).

Expression and purification of HrpA protein

To construct the expression vectors pET28a(+)-*hrpA*, the genomic DNA of *P. syringae* pv. tomato DC3000 was extracted by the CTAB DNA Isolation Technique (Current Protocols in Molecular Biology, 2001). The *hrpA* gene was amplified by PCR with the forward primer P1 (AGAAGAATTC ATGGTCGCATTTGCAGGAT, *EcoR* I) and reverse primer P2 (CGGTCTCGAGTTAGTAACTGA TACCTTTAGCGT, *Xho* I). For cloning the *hrpA* gene into pET28a(+) vectors, restriction enzymatic sites, *EcoR* I and *Xho* I, were designed into the primers. Before protein expression, the sequenced plasmid was transformed into *E. coli* BL21 by electroporation, and a single positive colony from the selection plate was inoculated in 5 mL LB liquid media containing 100 μg/mL ampicillin. The culture was incubated overnight with shaking at 37°C, and then transferred to a larger-scale LB media (500 μL culture was transferred into 50 mL fresh LB). Expression of the target protein was induced by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD_{600} of the culture reached 0.8. Cells were harvested by centrifugation after grown for an additional 6 h at 28°C. Then, target protein was purified by Ni^{2+} affinity chromatography.

Immunization and total RNA extraction

Four female Balb/c mice were immunized with the purified HrpA protein by subcutaneous injection. The mixture of HrpA and the same volume of complete Freund's adjuvant was used to immunize mice for the first time, and the later injections were taken every 10 days for further three times with the mixture of HrpA and the same amount of incomplete Freund's adjuvant. The anti-HrpA antibody titer was determined by indirectly ELISA. Once high serum titers were obtained, animals were sacrificed at the 5th day after the last immunization. Total mRNA was extracted from the isolated spleens by Trizol method (Promega Biotech).

Construction of a phage-displayed anti-HrpA scFv library

The first cDNA was amplified with the oligo dT15 primer using the above extracted mRNA as template. The phage-displayed anti-HrpA scFv library was prepared using a recombinant phage antibody system (RPAS) according to the protocols supplied by the manufacturer. Briefly, the amplified V_H and V_L genes were assembled into a scFv gene using a linker sequence. The assembly was re-amplified to incorporate flanking *Sfi* I and *Not* I cloning sites at its 5'- and 3'-ends, respectively. The amplified products were purified, digested with *Sfi* I and *Not* I, and cloned into the phagemid pCANTAB-5E containing E-tag sequence in frame. The recombinant phagemid was then transformed into competent *E. coli* TG1 cells by electroporation, and the transformed cells were spread onto the SOB-AG plates to calculate the capacity of the library.

Bio-panning of phage display library

A 96-well microtiter plate was coated with the purified 10 μg HrpA/ml (100 μl/well) at 4°C overnight, and the plates were washed 3 times with PBS and blocked with 4% PBSM (PBS containing 4% fat free milk) for 2 h at 37°C. Then, the prepared phages were added to the reaction wells and incubated for 2 h at 37°C. Unbound phages were removed by washing 10 times with PBST (PBS containing 0.05% Tween) and 10 times with PBS. Antigen-bound phages were eluted by adding 100 μL of 1.0 M triethylamine for 10 min and then neutralized with 100 μL of 1.0 M Tris-HCl (pH 7.4). The eluted phages were used to infect the *E. coli* TG1 in logarithmic growth phase and plated on SOB-AG medium to determine the titer (Wang et al., 2006).

Screening specific binding clones by phage-ELISA

The picked phage clones were cultured and infected with the M13KO7 for preparation of phage scFv. ELISA was used to determine the binding activity of individual clone. First, the HrpA coated plates were washed and then blocked with 4% PBSM at room temperature for 1 h. Phages scFv derived from individual clone were added into the reaction wells, and incubated at 37°C for 2 h, respectively. The binding activity was detected with HRP conjugated anti-M13 monoclonal antibody by ELISA. Absorbance at 450 nm was measured with a microplate reader.

Soluble expression and extraction of scFv

To obtain the soluble scFv protein, HB2151 was infected with the positive phage, and grown in 2×YT-AG media at 37°C overnight. The culture was then diluted at 1:100 ratio into 100 ml fresh 2×YT media containing 100 μg ampicillin/ml, and continued to grow at 37°C until the OD_{600} reached 0.8. IPTG (final concentration is 1 mM) was added to induce the expression of scFv, and the cultures were further incubated overnight at 28°C. After expression, the cell culture pellet was resuspended in 10 ml ice-cold 1× Tris-HCl, EDTA, sucrose (TES) buffer. After centrifugation, the culture pellet was resuspended by adding ice-cold 1/5×TES buffer, and incubated on ice for 30 min. The contents was transferred to a microcentrifuge tube, and centrifuged at full speed at 4°C for 10 min. The supernatant was carefully collected and analyzed for the presence of soluble scFv by ELISA and SDS-PAGE.
(Tris-HCl, EDTA, sucrose

ScFv specificity analysis

Specificity analysis of the anti-HrpA scFv was carried out by phage ELISA according to Wang et al. (2012). Associated antigens HrpA,

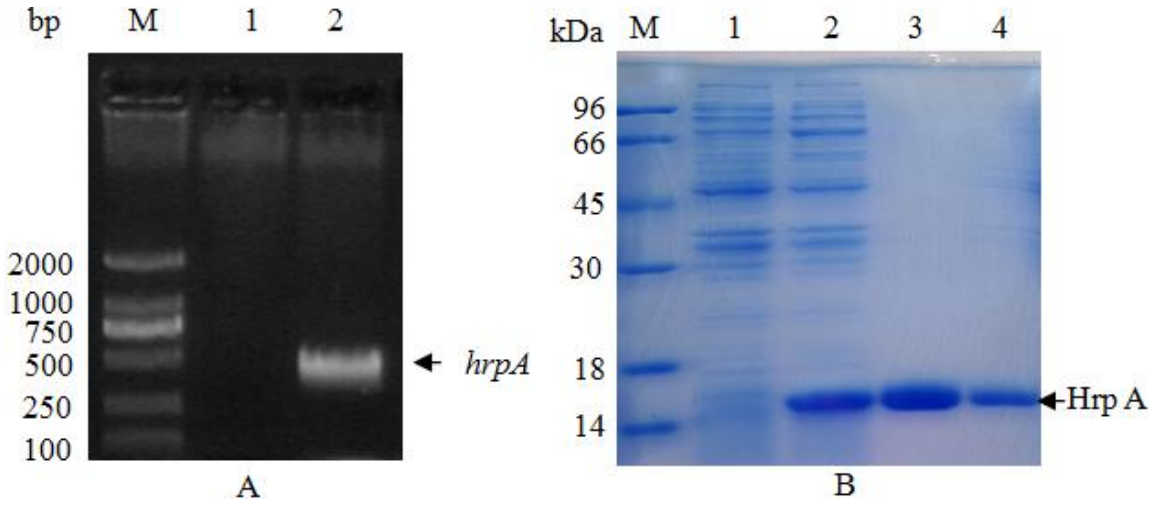

Figure 1. Amplification of *hrpA* gene and expression of HrpA protein. **A.** The amplified fragment of *hrp A* gene. Lane M, DL-2000 Marker; Lane 1, negative control; Lane 2, the amplified fragment of *hrp A* gene. **B.** SDS-PAGE analysis of expression and purification of HrpA protein. Lane M, Mid-Range Protein Molecular Weight Markers; Lane 1, control (induced *E. coli* BL21 containing empty vector pET28a(+); Lane 2, induced *E. coli* BL21 containing recombinant plasmid pET28a(+)-*hrpA*; Lane3-4, the purified HrpA protein by Ni^{2+}-NTA.

HrpJ, HrpZ, 6His, BSA, KLH, and OVA were coated on 96-well plates. After blocking with 4% PBSM, secreted recombinant phage scFv were added to the reaction wells. The specificity of the scFv clone was detected with the HRP conjugated anti-M13 antibody. The enzyme reaction was then performed with TMB as a substrate.

Western blotting and far western blotting

Far western blotting was used to analysis the binding ability of the selected scFv to HrpA. Antigen HrpA with different concentration (10, 20, 40, 80 µg/ml) and BSA (control) were loaded onto PVDF membrane for overnight at 4°C. After blocking and washing, extracted soluble scFv was added for 2 h at room temperature. After washing, the membrane was subsequently incubated with anti-M13 antibody and AP-conjugated goat anti-mouse IgG antibody.

Signals were visualized with substrate BCIP/NBT. To further identify the specificity between the selected positive scFv and HrpA antigen, western blot was performed. The total protein of *P. syringae* pv. tomato DC3000 and the purified HrpA antigen protein was transferred from a SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane, and the membrane was treated with soluble scFv according to standard protocol. The last step was the same as that given above.

RESULTS

Expression and purification of Hrp A

Genomic DNA of *P. syringae* pv. tomato DC3000 was isolated and used as the PCR template. The intact DNA fragment of *hrpA* was 342 bp, and a PCR product about 342 bp shown in Figure 1A was amplified. PCR product was cloned into TA vector and sequenced. The result shows that it was identical to *hrpA* gene. The PCR product was digested with restriction enzmyes *EcoR* I and *Xho* I, and was ligated to digested plasmid pET28a(+) to form the recombinant plasmids named pET28a-*hrpA*. To express the target protein effectively, the *E. coli* BL21 containing recombinant plasmid pET28a(+)-*hrpA* was induced by IPTG. The size of fusion protein expressed by pET28a(+)-*hrpA* was about 14 kDa. The results in Figure 1B (Lane 2) show that HrpA was expressed correctly, and the purified target protein was successfully obtained (Figure 1B) (Lanes 3 and 4).

Immunization and extraction of total RNA

To obtain the specific anti-HrpA antibody and higher serum titer, the purified target protein HrpA was used for immunization for four Balb/c mice by subcutaneous inject-tion. The mixture of HrpA and complete Freund's adju-vant was used for immunization for the first time, and the later injections were administered with the mixture of HrpA and incomplete Freund's adjuvant for a further three times. Blood were collected from animals and used for serum titer detection by ELISA. The detection results showed that all four mice had a relatively high titer (Figure 2) and all of them fully met the requirements. Total RNA was extracted from the spleen cells of Balb/c mice that had a higher anti-HrpA serum titer.

Construction of phage-displayed anti-HrpA scFv library

Total RNA was used to generate the cDNA by reverse transcription reaction. The cDNAs were then used as

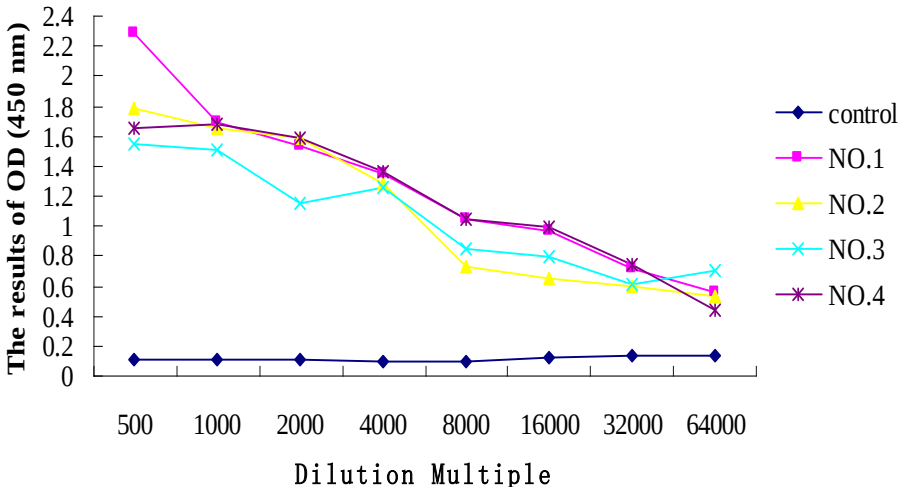

Figure 2. The result of titer assay of serum. The serum from the HrpA immunized mice were used for titer detection by ELISA. No. 1, 2, 3, 4 represent the four immunized mice with high titer respectively, while control represent non-immunized mice as negative control.

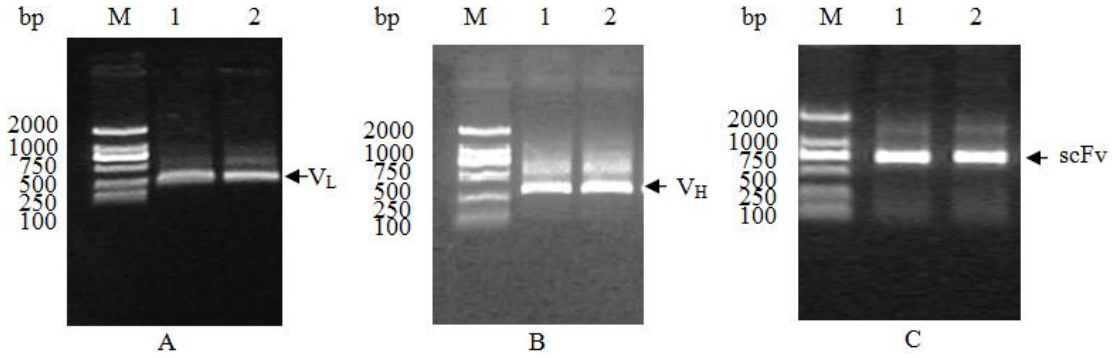

Figure 3. The amplified fragments of V_L, V_H and scFv genes. **A.** The amplified fragment of V_L gene. Lane M: DL-2000 Marker; Lane 1-2: the amplified fragment of V_L gene. **B.** The amplified fragment of V_H gene. Lane M, DL-2000 Marker; Lane 1-2, the amplified fragment of V_H gene. **C.** The amplified fragment of scFv. Lane M, DL-2000 Marker; Lane 1-2, the assemble fragment of scFv.

template to amplify the V_H (340 bp) and V_L (325 bp) genes. As shown in Figure 3, the V_H and V_L genes were amplified successfully (Figure 3A and B). Besides, a scFv DNA fragments with an expected length of 750 bp was obtained (Figure 3C) by overlap PCR with the V_H and V_L genes as template. The phage displayed scFv library was constructed with a transformed rate of 5.6×10^7 (CFU/ml). Fifty (50) clones were randomly selected from the plates for plasmid extraction and analysis, and the result showed that the recombinant rate was 85%. So the size of the phage displayed scFv library was 4.76×10^7(CFU/ml).

Bio-panning and selection of specific HrpA-binding scFv clones

The input and output of the library during bio-panning are shown in Figure 4A, and about 5×10^7 CFU/ml phage clones were checked (input) in each panning round. The elution phage clones (output) were very low at the first two rounds, but the output was kept in a stable level of approximately 4×10^6 CFU/ml after the third round. Lastly, 100 clones from the third round to the six rounds were randomly selected to test the binding ability to HrpA. Finally, 5 scFv clones showing relatively stronger binding ability to HrpA protein were isolated from the library (Figure 4B), and the phage clone with the strongest positive signal designated scFv-FE5 (Figure 4B) was chosen for further study.

Soluble expression and far western blotting

E. coli TG1 was a suppressor strain, allowing the expres-

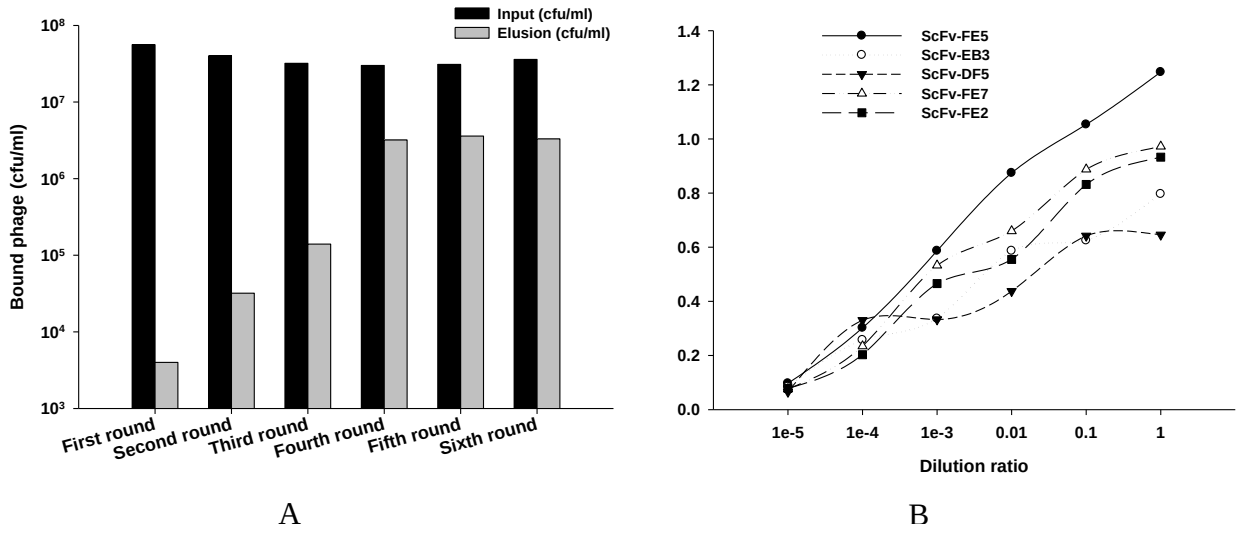

Figure 4. Biopanning and selection of specific HrpA-binding scFv clones. **A.** Biopanning result of the phage-scFv library. The number of input phages in each round were about 5×10^7 CFU/ml. After three rounds biopanning, the number of elution phages was observed 4×10^6 CFU/ml constantly. **B.** ELISA analysis of the binding activity of 5 different anti-HrpA scFv clones. The graph showed the relationship between OD450 value and HrpA concentration.

sion of a fusion protein of the pIII and the scFv. In contrast, *E. coli* strain HB2151 was a non-suppressor strain, so it was used for production of soluble scFv. Soluble expressed scFv was extracted from periplasm, and were evaluated by 14% SDS-PAGE. The protein was detected by staining with Coomassie Brilliant Blue R-250. A 29 kDa protein band was found, corresponding to scFv (Figure 5A). The binding ability of scFv-FE5 to HrpA was firstly analyzed by far western blot. HrpA antigens with different concentrations were loaded onto the PVDF member (BSA was used as control). After the extracted scFv was added, the enzyme-labeled antibody and the substrate was used to develop color. The result shows that the scFv-FE5 could bind to HrpA specifically, but not bind to BSA (Figure 5B).

Specificity analysis of scFv and western blotting

To further identity the specificity of scFv-FE5, some related antigen HrpA, HrpJ, HrpZ, 6His, BSA, KLH, and OVA were used to detect the specificity of scFv-FE5 by indirectly ELISA. As seen in Figure 6A, the scFv-FE5 antibody had stronger signal when detected in ELISA for HrpA antigen compared to other antigens. The result demonstrates that the selected scFv-FE5 could specifically recognize HrpA antigen with no cross-reaction to other related antigen proteins. Besides, the binding activity of the soluble scFv with HrpA was further evaluated by western blotting. As shown in Figure 6B, the scFv-FE5 could recognize the HrpA from *P. syringae* pv. tomato DC3000 and the purified HrpA at the same time, the size of HrpA matched the detected band on the position of 14 kDa. The result demonstrated that the selected scFv-FE5 could recognize HrpA specifically.

DISCUSSION

The type III system (hrp T3SS) in plant pathogens is capable of delivering various effectors into the interior of host cells (Deng et al., 1999). The Hrp pilus has been identified previously from preparations of bacteria recovered from agar or liquid culture (Roine et al., 1997). A major problem with both methods is the fragmentation of the delicate pili and detachment from bacterial cells before examination, and only a low level of constitutive HrpA was expressed in bacteria grown in rich media prior to transfer to inducing conditions (Brown et al., 2001). As it is not easy to get large scale of pure HrpA pilus, we cloned *hrpA* gene of *P. syringae* pv. tomato DC3000 from its genome. The DNA fragment encoding the HrpA was cloned into expression vector pET28a(+); target HrpA was successfully expressed in *E. coli*. The recombinant protein was expressed with a 6His-tag at C-terminal to facilitate the purification of the desired protein. It was showed that expressed HrpA could be easily prepared in large scale, and used for further research.

To gain scFv against HrpA, female Balb/c mice were immunized with purified HrpA, and cDNA was prepared from spleen mRNA of the immunized mice. With a linker molecule, scFv was assembled through joining the amplified V_H and V_L genes together by overlap PCR (Heng et al., 2003). To obtain a large size of phage scFv library, the scFv gene was cloned into pCANTAB-5E vector, and the recombinant vector was transformed into *E. coli*

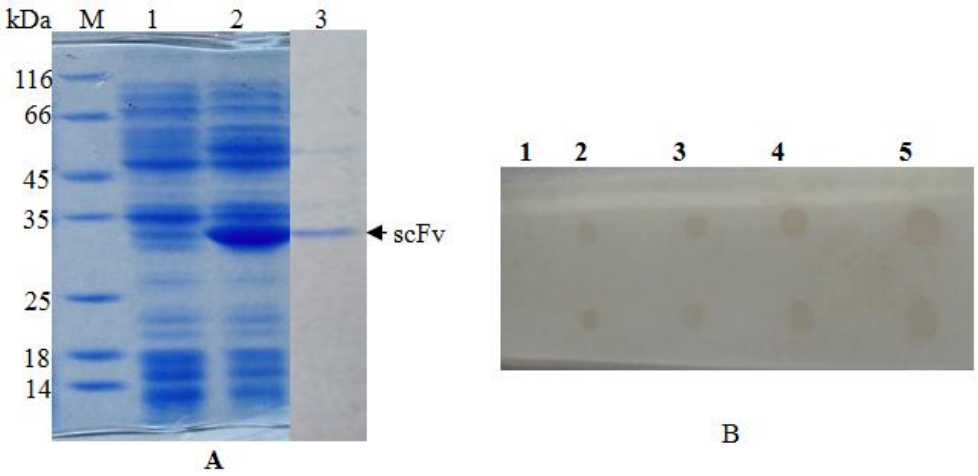

Figure 5. Soluble expression and far western blotting. **A.** SDS-PAGE analysis of soluble expressed scFv. Lane M: protein marker; Lane 1, induced *E. coli* HB2151 containing empty plasmid pCANTAB-5E; Lane 2, induced *E. coli* HB2151 containing recombinant plasmid pCANTAB5E-*scFv*; Lane 3, extracted periplasmic protein containing soluble *scFv.* **B.** Detection of scFv binding ability by far western blot. Antigen HrpA with different concentration and BSA (control) were loaded on to PVDF membrane, and extracted soluble scFv was added. Lane 1, BSA as control (25 ng); Lane 2-5, purified HrpA protein with 25, 50, 100, and 200 ng, respectively.

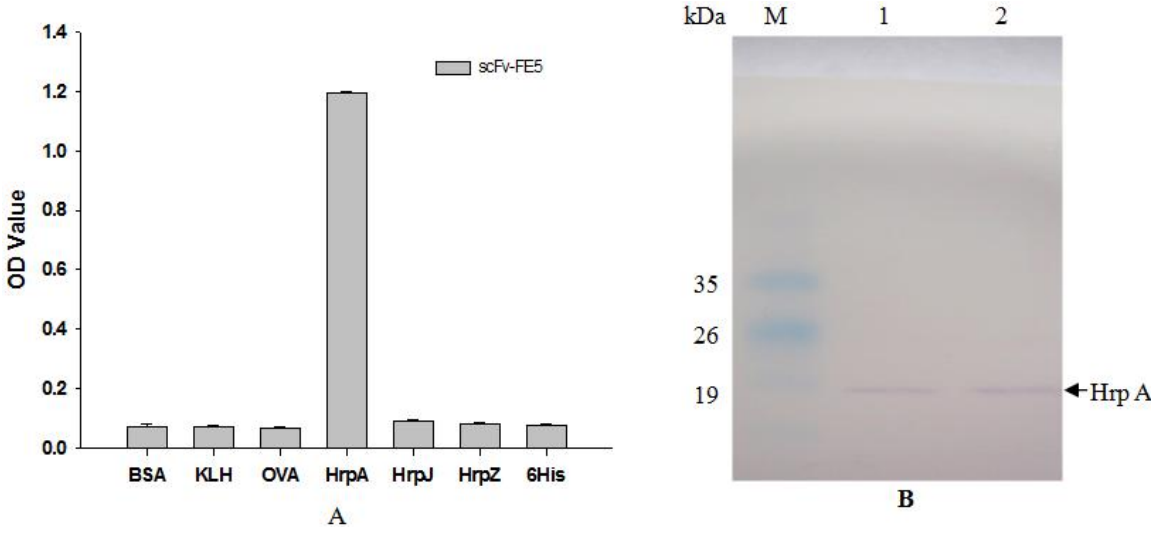

Figure 6. Specificity analysis of scFv and western blotting**. A.** scFv-FE5 specifically binds the HrpA antigen. Associated antigen HrpA, HrpJ, HrpZ, 6His, BSA, KLH, and OVA were coated on 96-well plates in 5 µg /ml (100 µl/well, triplicate), the soluble scFv-FE5 were added to the reaction wells and incubated for 2 h at 37°C. Specificity of the scFv-FE5 clone was determined using an anti-M13 HRP-conjugated antibody. **B.** Western blotting. The total protein of *P. syringae* pv. tomato DC3000 and the purified HrpA antigen protein was transferred from a SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane, and the membrane was treated with soluble scFv-FE5 using standard protocol. The last step were the same to far western blotting. Lane M: pre-stained protein marker; Lane 1: the total protein of *P. syringae* pv. tomato DC3000; Lane1-2: the purified HrpA antigen protein.

TG1 by electronic transformation. The size of the library depends on the transformation efficiency, which is the major limitation for phage display technology (Azzazy et al., 2002; Singh et al., 2010). By using electroporation, the size of the phage displayed antibodies library was 4.76×10^{7} (CFU/ml), which met the needs for scFv bio-

panning.

After six rounds panning, one phage clone which gave the strongest positive signal designated scFv-FE5 was selected. The result of soluble expression of scFv-FE5 indicated that the size of the scFv-FE5 is about 29 KDa. Further western blot analysis showed that the scFv could specifically recognize the Hrp A. All these results show that anti-HrpA scFv was successfully prepared, and the scFv could be used as a tool to study the function of the HrpA in the type III system in plant pathogens in further research. Shahryari et al. (2013) have developed a single-chain variable fragment antibody (scFvIMP6) against the immunodominant membrane protein (IMP) of witches' broom phytoplasma and expressed it in different plant cell compartments. They found that scFvIMP6 binds with high activity and can be used for the detection of Ca. Phytoplasma aurantifolia and is also a suitable candidate for stable expression in lime trees to suppress witches' broom of lime (Shahryari et al., 2013).

The anti-HrpA scFv-FE5 obtained in the study by phage display technology was a very useful tool to develop a kit to detect HrpA antigen, or to be used as a therapy for plant defense-associated hypersensitive response caused by bacteria type III secretion system.

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Full Length Research Paper

Antibacterial activity of leaves extracts of *Jatropha curcas* and *Euphorbia heterophylla*

E.O. Ekundayo* and J. N. Ekekwe

Department of Microbiology, College of Natural and Applied Sciences, Michael Okpara University of Agriculture, Umudike, PMB 7267, Umuahia 440001, Abia State, Nigeria.

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The antibacterial activity of aqueous and ethanolic extracts of two Nigerian medicinal plants, *Euphorbia heterophylla* and *Jatropha curcas* was investigated using disc agar diffusion and broth dilution assays against four clinical isolates of bacteria consisting of two gram positive (*Staphylococcus aureus and Enterococcus faecalis)* and two gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa)*. The antibacterial activity was measured by the diameter zone of inhibition and minimum inhibitory concentration (MIC). The extracts exhibited broad spectrum antibacterial activities against the microorganisms. The diameter of zone of inhibition ranged from 10-15 mm at 2000 µg of crude extract per disc. The MIC values were between 125 -1000 µg/ml. The results of this study indicate that these plants contain compounds with antibacterial activity which validates their use for treatment of various microbial infections in traditional medicine. The findings in this study provide the basis for further study on the plants with the aim of isolating and identifying the active substances. The plants could also be standardized to develop cheap, culturally acceptable herbal medicines.

Key words: Antimicrobial activity, medicinal plants, *Jatropha curcas*, *Euphorbia heterophylla*, minimum inhibitory concentration.

INTRODUCTION

Medicinal plants serve as the major source of medicines for treatment of various ailments in the primary health care for majority of the rural populace in Nigeria as in other parts of Africa. According to the World Health Organization (WHO, 2002), over 80% of the world's population especially in the developing world relies on medicinal plants as sources of medicines for their primary healthcare. Traditional system of medicine which depends mainly on medicinal plants is rich in ethnomedical knowledge of the uses of medicinal plants in the treatment of infectious conditions (Iwu, 1993). These medicinal plants employed in traditional medicine represent potential sources of cheap and effective standardized herbal medicines (phytomedicine) and leads in the discovery of novel molecules for the development of new chemotherapeutic agents (Farnsworth and Morris, 1976). Several infectious diseases including malaria, diarrhea, dysentery, gonorrhea and fungal infections have been successfully managed in traditional medical practice employing medicinal plants (Sofowora, 1993).

Jatropha curcas Linn and *Euphorbia heterophylla* Linn are two plants in the family Euphorbiaceae that have been identified as plants widely used in traditional medicine in various parts of Africa (Iwu, 1993, Burkill, 2008).

J. curcas variously known as physic nut, purging nut or pig nut (Uche and Aprioku, 2008; Igbinosa et al., 2009) and "*Lapalapa*" in Yoruba Language (Burkill, 1994) is used in folklore remedies for treatment of various ailments such as skin infections, gonorrhea, jaundice and fever (Akinpelu et al., 2008). In Akwa Ibom State, the oil from

*Corresponding author. E-mail: ekundayo.emmanuel@mouau.edu.ng.

the crushed seeds is used for treatment of skin diseases and as a laxative (Ajibesin et al., 2008). *E. heterophylla* with the common name “spurge weed” grows in semi-humid places especially in cassava, cowpea and soya beans plantations (Falodun et al., 2006). *E. heterophylla* has been used in the traditional medicine for the treatment of constipation, bronchitis and asthma. The plant has also been reported to be used as purgative (Erdem et al., 1999, Ajibesin et al., 2008).

The desire to scientifically validate the medicinal properties of these plants has resulted in the investigation of their various biological activities. The antibacterial activity of the methanolic extract of the leaves of *J. curcas* was investigated against 13 bacterial species including *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.* The extract showed appreciable inhibitory activity against these organisms (Akinpelu et al., 2009).

Although *E*. *heterophylla* is reputed to be widely used in folklore medicine, reports on the previous pharmacological studies are few. Falodun et al. (2003) reported the antibacterial activity of the petroleum ether, butanolic and ethanolic extracts of the leaves against strains of typed culture organisms. Only the butanolic extract showed antibacterial activity.

The aim of this study was to further evaluate the antibacterial activity of the aqueous and ethanolic extracts of the leaves of *J. curcas* and *E. heterophylla* against four clinical isolates of common bacterial pathogens namely, *S. aureus, S. faecalis, E. coli and P. aeruginosa.*

Collection of plant materials

Fresh leaves of *J. curcas* and *E. heterophylla* were collected during the rainy season in the month of August from Ibinaukwu Igbere, Bende L.G.A. of Abia State, Nigeria. Dr. Osuagwu of the Department of Plant Sciences and Biotechnology, Michael Okpara University of Agriculture, Umudike, taxonomically authenticated the plants and voucher samples were deposited in the Department herbarium. The leaves were air dried at ambient temperature for several days until well dried. The dried leaves were reduced to fine powder using laboratory mortar and the powder stored in an air-tight container until needed.

Extraction of plant materials

20 g amount of the powdered leaves was weighed and percolated in 200 ml of 96% ethanol contained in 500 ml conical flask. The flask was agitated manually several times over a period of 24 h. The extract was filtered using Whatman No. 1 filter paper and the filtrate collected in a clean beaker was concentrated to dryness by evaporation over a steam bath at 80°C. The aqueous extract was similarly prepared using 20 g of the powdered leaves material in 200 ml of distilled water.

Test organisms

The four test bacteria used in the study were *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*. The bacteria were clinical isolates obtained from the Microbiology Laboratory of the Federal Medical Centre (FMC), Umuahia, Abia State. The microorganisms were maintained at 4°C on Nutrient Agar slant in the Department of Microbiology, Michael Okpara University of Agriculture, Umudike and fresh subcultures were made before use.

Preparation of extract impregnated paper discs

The extract impregnated paper discs were prepared as described by Ekundayo and Ezeogu (2006). Whatman No. 1 filter paper was cut into discs of 6 mm diameter using an office perforator. The discs were placed in glass Petri dish and sterilized in hot air oven at 160°C for 1 hour. Each disc was impregnated with 20 µl portion of stock solution of the extract (100 mg/ml) to give a concentration of 2000 µg of crude extract per disc. The discs were dried in an incubator at 35-37°C for 2 h. Discs of Ampicillin and Tetracycline used as control antibiotic discs were similarly prepared.

Antimicrobial activity by zone of inhibition

The antimicrobial activity of the extracts was assessed using the disc diffusion and broth dilution methods. A cell suspension of each test bacterial strain was prepared by transferring 4-5 isolated colonies on Nutrient agar plate into sterile normal saline in a bijou bottle. The turbidity was adjusted to McFarland turbidity standard tube No. 0.5 by adding sterile normal saline. The surface of Mueller Hinton Agar (Fluka BioChemika, Fluka Chemie GmbH,Buchs) plate was inoculated by swabbing the surface with a sterile swab stick dipped into the bottle containing the standardized cell suspension. The prepared disc was aseptically transferred unto the inoculated culture plate using a pair of flame sterilized forceps. The plates were incubated aerobically at 37°C for 18-24 h. The diameter zone of inhibition was measured using a transparent plastic ruler. The tests were carried out in duplicate.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of each of the extracts was determined using the tube serial dilution method. Extracts (100 mg/ml) were dissolved in water or ethanol and diluted with Nutrient broth in two fold serial dilutions in test tubes to obtain the following concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.82, 3.91, 1.96 µg/ml. An overnight broth culture of the test organism was adjusted to McFarland turbidity standard No. 0.5 (10^6 CFUs/ml) and 50 µl (0.05 ml) of the cell suspension added to each of the tubes. The tubes were incubated aerobically at 37°C for 18 h. The MIC was defined as the lowest extract concentration that inhibited the growth of the test organism as indicated by absence of visible turbidity in the tube compared with the control tubes.

RESULTS

The antimicrobial activities of the leaves extracts of *J. curcas* and *E. heterophylla* measured by diameter of the zone of inhibition against the test organisms are shown in Table 1. The aqueous extract of *J. curcas* inhibited *S. aureus* and *E. coli* with diameters of zone of inhibition of 10 and 14 mm, respectively. The ethanol extract of the plant had no activity against all the test organisms. Similarly, the aqueous extract did not show activity against *E. faecalis* and *P. aeruginosa.* The aqueous extract of the leaves of *E. heterophylla* had antimicrobial activity

Table 1. Diameter of zone of inhibition (mm) of *J. curcas* and *E. heterophylla* extracts against clinical isolates of four bacterial pathogens.

Plant specie	Extract type	Bacterial isolate			
		S. aureus	***E. faecalis***	***P. aeruginosa***	***E. coli***
J. curcas	Ethanol (2000 µg)	No inhibition	No inhibition	No inhibition	No inhibition
	Aqueous (2000 µg)	10	No inhibition	No inhibition	14
E. heterophylla	Ethano l (2000 µg)	12	No inhibition	No inhibition	12
	Aqueous (2000 µg)	14	13	11	15
Antibiotics	Ampicilin (10 µg)	20	12	15	No inhibition
	Tetracycline (30 µg)	34	17	20	No inhibition

Table 2. Minimum inhibitory concentrations (MIC, µg/l) of *J. curcas* and *E. heterophylla* extracts against clinical isolates of four bacterial pathogens.

Plant extract		Bacterial isolates			
		S. aureus	***E. faecalis***	***P. aeruginosa***	***E. coli***
J. curcas Aqueous		1000	>1000*	>1000*	500
E. heterophylla	Aqueous	125	250	250	125
	Ethanol	250	>1000*	>1000*	250

*Extracts showed no inhibitory activity in disc agar diffusion assay

against all the test organisms with mean diameter of zone of inhibition ranging from 11 to 15 mm. The activity was highest against *E. coli* and least against *P. aeruginosa.* The ethanol extract was active only against *S. aureus* and *E. coli* but not against *E. faecalis* and *P. aeruginosa.* Table 2 shows the MIC values of the two plant extracts against the test organisms. The MIC values ranged from 125 to 1000 µg/ml of crude extract. The aqueous extract of *E. heterophylla* exhibited the highest activity against *S. aureus* and *E. coli* with MIC of 125 µg/ml, respectively.

DISCUSSION

The aqueous extract of *E. heterophylla* leaves produced diameters of zone of inhibition of 14 and 15 mm against *S. aureus* and *E. coli*, respectively, 13 mm against *E. faecalis* and 11 mm against *P. aeroginosa*. Thus, the aqueous extract of the leaves showed a broad spectrum of antimicrobial activity against the test organisms. Falodun et al. (2003), had previously investigated antibacterial activity of the petroleum ether, butanolic and ethanolic extracts of the leaves of *E. heterophylla* against *E. coli*, *Klebsiella pneumonia*, *S. aureus*, *P. aeruginosa* and *Bacillus subtilis*. The butanolic extract showed a broad spectrum of antibacterial activity against the test organisms at concentration of 100, 150 and 200 mg/ml. The petroleum ether and ethanolic extracts did not show any antibacterial activity against any of the test organisms. Similar to the results of Falodun et al. (2003), the ethanolic extract of the leaves at 2000 µg/20 µl/disc (equivalent to 100 mg/ml) did not show antibacterial activity against the test organisms in our study. In traditional medical practice, it is the aqueous extract or decoction of the leaves that is used to prepare food such as yam porridge or taken directly to “wash out the bowels” or as a purgative (Oksuz et al., 1994, Falodun et al., 2003). According to Falodun and Agbakwuru (2004), the leaves of *E. heterophylla* contain quercetin, saponins, tannins and flavonoids. Antimicrobial activity of medicinal plants has been linked with the presence of these chemical substances in medicinal plants (Cowan, 1999; Cushnie and Lamb, 2005; Ebana et al., 2005). The antibacterial activity of *E. heterophylla* may be due to the presence of these chemical substances in the leaves (Falodun et al., 2006).

The aqueous and ethanolic extracts of *J. curcas* tested in this study showed moderate antibacterial activity against *S. aureus* and *E. coli* with diameter of zone of inhibition of 10 and 14 mm, respectively. The results of our study are similar to those of Akinpelu et al. (2009) who reported strong inhibitory activity against *S. aureus* and *E. coli* in the methanolic extract of the leaves of *J. curcas*. Aiyelaagbe et al. (2007) reported the antimicrobial activities of some secondary metabolites from the root extract of the plant against some microorganisms associated with sexually transmitted diseases. Igbinosa et al. (2009) also reported the antimicrobial activity of the stem bark extracts against 12 bacterial species consisting both gram positive and gram negative organisms while Igbinosa et al. (2009) found little or no antibacterial activity in the aqueous extract of the stem bark of *J. curcas*; in our study, both aqueous and ethanolic extracts of the leaves showed moderate antibacterial activity against both *S. aureus* and *E. coli*. While the differences in results

may be due to the varying concentrations of active substances in the stem bark and leaves, it may also be due to the different concentrations used. The concentration in our study was 2000 µg/20 µl/disc (100 mg/ml) but the highest concentration used in their study was 10 mg/ml. However, the actual concentration of the extract used in agar well diffusion method employed by Igbinosa et al. (2009) may be difficult to determine as the stated concentration is nominal and does not represent the actual amount of substance present in the volume of extract dispensed into agar well. The work of Kisangau et al. (2007) showed the possibility of discrepancy in the results of antibacterial activity tested by agar well diffusion method and the paper disc diffusion method. Kisangau et al. (2007) found no activity in the water extract of *J. curcas* when agar well was used but strong inhibitory activity of the extract was seen in the disc diffusion method. The effect of these two methods of antibacterial activity screening of plant extracts may require further study.

It is interesting to note that the extracts of the leaves of *J. curcas* and *E. heterophylla* had activity against the *E. coli* isolate which was resistant to Ampicillin and Tetracycline. A major goal of the antimicrobial screening of medicinal plants is to find substances with novel mechanism of action against drug resistant strains. The aqueous extract of *E. heterophylla* was also active against *P. aeruginosa*, a gram negative bacterium commonly resistant to many antibiotics.

The results obtained in this study contribute to the scientific validation for the use of these medicinal plants in traditional medicine and serve as a guide for selection of plants with antimicrobial activity for further phytochemical work on isolation and identification of the active compounds. Furthermore, these results show the potential of some of these medicinal plants for development of standardized culturally acceptable herbal medicines for local use as broad spectrum antimicrobial agents.

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Full Length Research Paper

Molecular epidemiology and vaccine matching study on foot-and-mouth disease virus circulating in Ethiopia

Haileleul Negussie[1]*, Gelagay Ayelet[2], Shiferaw Jenberie[2], Sintayehu Minda[3] and Liyuwork Tesfaw[1]

[1]College of Veterinary Medicine and Agriculture, Addis Ababa University, P. O. Box 34, Debre Zeit, Ethiopia.
[2]National Veterinary Institute, P. O. Box 19, Debre Zeit, Ethiopia.
[3]College of Agriculture, Debremarkos University, P. O. Box: 269, Debremarkos, Ethiopia.

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The study was conducted in three regional states of Ethiopia: Amhara, Oromia, and Addis Ababa with the aims of identifying the molecular epidemiology of Foot and Mouth Disease (FMD) virus in Ethiopia and to determine the appropriate vaccine strains of FMD virus. From the total of 33 bovine epithelial tissue cultured samples, 19 (57.57%) samples were showed cytopatic effect for FMD virus. Of these, three samples were found serotype A and Africa topotype and 16 samples were found serotype O and East Africa-3 topotype. Certain FMD isolates were characterized by two dimensional virus neutralization test (2dmVNT) and liquid phase block ELISA (LPBE) in order to choose an appropriate vaccine strain found at World Reference Laboratory for FMD (WRLFMD). The result indicates that most vaccine strains found at WRLFMD can protect against serotype O of Ethiopian isolates, while serotype A has highest antigenic variation and only few vaccine strains found at WRLFMD can provide protection. Various strains of foot and mouth disease virus (FMDV) were isolated in Ethiopia and therefore, continuous monitoring of newly emerging strains is necessary to perform vaccine matching studies to support the efficacy of actual vaccine formulations.

Key words: Ethiopia, foot and mouth disease virus (FMD), phylogeny, vaccine match, serotype, topotype.

INTRODUCTION

Foot and Mouth Disease (FMD) is a highly contagious viral disease of cloven-hoof animals and is one of the most important economic diseases of livestock (Bronsvoort et al., 2004) and the disease is characterized by fever, and vesicular eruptions in the mouth, feet and teats. It is caused by a virus of the genus *Aphthovirus*, in the family *Picornaviridae*, of which there are seven immunologically district serotypes; A, O, C, South African Territories (SAT) 1, SAT2, SAT3 and Asia1 (OIE, 2004). FMD serotypes O and C were first recorded in Ethiopia in 1957 (Martel, 1974). Serotypes A and SAT 2 were not identified until 1969 and 1989, respectively (Roeder et al., 1994). From 1988 to 1991, serotype O and serotype SAT- 2 were identified from outbreaks in Ethiopia (Roeder et al., 1994). The existence of SAT-1 in Ethiopia has also been reported in the first time in 2007 (Yoseph et al., 2013). The occurrence of FMD in Ethiopia has apparently increased since 1990; outbreaks throughout the country are reported frequently (Asfaw and Sintaro, 2000). FMD remains largely uncontrolled in the country because vaccination for prophylactic purpose is not being practiced except for a few dairy herds containing exotic animals (Sahle, 2004). With no control and preventive measures in place, FMD causes substantial economic loss to farmers and to the nation from embargoes of livestock and livestock product trade (Sahle et al., 2004; Megersa et al., 2009). Therefore, livestock are at risk from endemic strains as well as from antigenic variants prevailing in neighboring countries.

The official data may not exhibit the reality of the

*Corresponding author. E-mail: haileleul2011@yahoo.com.

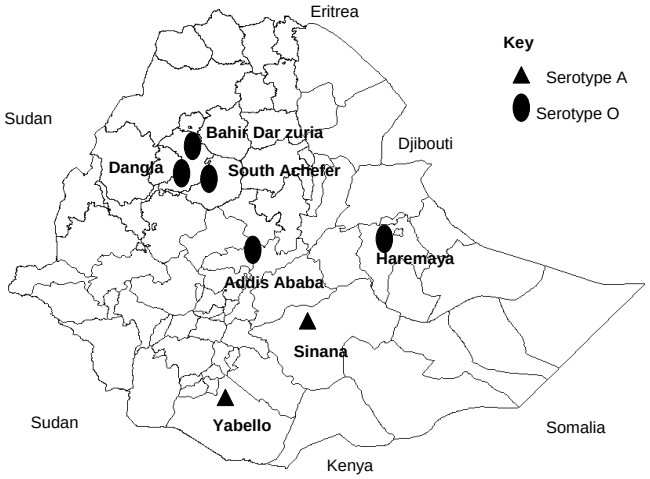

Figure 1. Map of Ethiopia showing the distribution of FMD virus serotype O and A isolated in Ethiopia during study period.

disease, due to the insidious nature of the disease, the unreported cases by farmers, as well as the few samples submitted to WRLFMD, Pirbright, for identification. Therefore, the present study was designed with the aims of identifying the molecular epidemiology of FMDV circulating in the three regions of Ethiopia and to determine the appropriate vaccine strains for the country.

MATERIALS AND METHODS

Study areas, study design and sampling methods

The study was conducted from August 2008 to April 2009 in three national regional states of Ethiopia: Amhara (Bahirdar zuria, Yilmana Densa, South Achefer, Dangela), Oromia (Haremaya, Sinana, Yabello) and Addis Ababa city council (Akaki sub city) as shown in Figure 1. The study design was purposive sampling. Animals were clinically examined for presence of FMD lesions on the mouth, teats, nostril, vagina, and feet and samples were collected. Accordingly, a total of 33 epithelial tissue samples were collected from animals that showed typical clinical signs of FMD. Epithelial tissue was collected from unruptured or freshly ruptured vesicles and placed in a bottle with transport medium composed of equal amount of glycerol and 0.04 M of phosphate-buffered saline solution pH 7.2 to 7.6 with antibiotics (OIE, 2004). Breed, identification number, sex, age, village, and type of tissue were labeled and samples were transported in cold-chain and stored at -20°C until processed.

Cell culture for virus isolation

About 1 g of epithelial tissue sample was grounded using sterile mortar and pestle by adding 10 ml of sterile phosphate-buffered saline containing antibiotics. The tissue suspension was centrifuged at 1,500 rpm for 15 min. The supernatant was collected and filtered by Millipore filter of 0.22 µm pore size. About 1 ml of filtered tissue suspension was inoculated on baby hamster kidney (BHK-21) monolayer cells grown on 25 cm^2 tissue culture flask and then flashed with MEM media (2%) and incubated at 37°C and 5% CO_2 for 48 h. Cytopathic effects (CPE) were noted after 24 to 48 h in positive samples (OIE, 2004).

Serotype and topotype identification

Tissue-cultured FMDV samples that showed CPE were submitted to World Reference Laboratory for FMD (WRLFMD), Pirbright, UK, according to recommended international standards for serotype and topotype characterization, phylogenic analysis and vaccine matching (r1-value) study.

Phylogenetic analysis

The VP1 gene characterization was used to study phylogenetic relationships of FMD viruses. FMD viruses that differ between 2 to 7% from each other are generally believed to originate from the same epizootic (Samuel et al., 1997, 1999).

Vaccine matching study (r_1-value determination)

Serotype A and O FMDV isolated strains within this study were matched with some of reference vaccine strains of serotype A and serotype O FMDV isolated from different countries of the world and archived in the WRLFMD in order to select an appropriate vaccine strains to control the disease. Two serotype A viruses (A/ETH/9/2008 and A/ETH/7/2008 strains in Yabello and Sinana districts, respectively) and four serotype O FMDV (O/ETH/15/2008 strain from South Achefer, O/ETH/24/2008 strain from Yilmana Densa, as well as O/ETH/24/2009 and O/ETH/28/2009 strain from Haromaya University dairy farm) were used for vaccine matching study.
The formula for r1 determination was:

$$r1 = \frac{\text{Reciprocal titre of reference serum against field virus}}{\text{Reciprocal titre of reference serum against vaccine virus}}$$

In case of liquid phase blocking ELISA (LPBE) test, r_1-values were interpreted as proposed by Samuel et al. (1990) and OIE (2004), where values between 0 to 0.19 indicated highly significant antigenic variation from the vaccine strains that the vaccine is unlikely to protect; values of 0.20 to 0.39 suggested the field strain were genetically related to the vaccine strain; while r_1-values of 0.40 to 1.0 demonstrated that the vaccine and field strains were similar and the vaccine would provide good protection. In case of neutralization, the r_1-values were interpreted as values ≥ 0.3 suggested that there was a close relationship between field strain and vaccine strain and likely conferring protection, but < 0.3 suggested that the field strain was so different from the vaccine strain that the vaccine is unlikely to protect.

Data management and analysis

The antigenic relationship between a field strain and a vaccine virus (r_1-values) was determined by 2dmVNT and LPBE test. The r_1-values were calculated as followed: (r_1 = serum titre against heterologous virus/ serum titre against homologous virus). The molecular sequences generated in this study were done at WRLFMD, Pirbright, UK. Neighbor-joining method included in the MEGA 4 program was used to construct gene trees and the confidence levels were assessed by 1000 bootstrap replications.

Table 1. Serotype and topotype identified in eight districts of the three regional states of Ethiopia.

No.	Site of outbreaks	No. of samples	Showing CPE	Serotype identified	Topotype identified	Strain
1	Bahirdar zuria	5	2	O	EA-3	-
2	Yilmana Densa	5	3	O	EA-3	-
3	South Achefer	7	3	O	EA-3	-
4	Dangela	4	1	O	EA-3	-
5	Akaki	4	2	O	EA-3	-
6	Haremaya	5	5	O	EA-3	-
7	Sinana	2	2	A	Africa	G-VII
8	Yabello	1	1	A	Africa	G-VII

Table 2. Antigenic characterization of FMD field isolates of serotype A by matching with vaccine strains.

Filed isolate	r1-value by 2dVNT							r1 value by LPBE	
	A/Eritrea RZ pool	A/Tur06 Arriah 2nd	A22 Irq23-32 pool	A/Sau41 91 SI94	A/sau95 bvs	A/Irn87 VQ pool	A/Irn 96 UZ pool	A/Eri3/98	A/Sau95
A/ETH/9/08	012	0.46	0.07	0.04	0.3	0.04	0.07	0.1	0.25
A/ETH/7/08	-	-	-	-	-	-	-	0.31	-

- Not done.

RESULTS

FMD virus isolation and serotype and topotype identification

From the total of 33 bovine epithelial tissue cultured samples, 19 (57.57%) samples were showed CPE on BHK-21 monolayer cells culture for FMDV (Table 1). The CPE was characterized by a fast destruction of the monolayer cell and infected cells were round and formed singly. Complete destruction of the cell sheet was mostly seen within 48 h of inoculation.

Out of the 19 samples that showed CPE, three samples were serotype A, Africa topotype, and G-VII strain, while 16 tissue cultured sam-ples were found serotype O and East Africa-3 topotype.

Vaccine matching

The vaccine matching result revealed that A/ETH/9/2008 was antigenetically close to A/Tur06/Arriah2nd, and A/Sau95/Bvs strain with r_1-values of 0.46 and 0.3, respectively, using 2dmVNT. Although, this field strain was related with A/Sau95 in which r_1-value was 0.25 using LPBE. Highly significant antigenic differences were observed to A/Eritrea RZ pool, A22/Irq23-32 pool, A/Sau41/91/SI94, A/Irn87/VQ pool, and A/Irn96/UZ pool with r_1-values of 0.12, 0.07, 0.04, 0.04 and 0.07, respectively, using 2dmVNT. A/ETH/7/2008 was antigenically similar with A/Eri3/98 where r_1-value was 0.31 as shown in Table 2. Field strain O/ETH/15/2008 was antigenetically close to O1/Manisa, O/BFS/VP pool, O/Ind/R2/75, and O/Kaufbeuren (O/Kauf/VQ) in which r_1-values were 0.46, 0.70, 0.69 and 0.58, respectively, using 2dmVNT. This was also close related with O/3039 and O/4174 in which r_1-values were 0.75 and 0.25, respectively, using LPBE. Field strain O/ETH/24/2008 strain from Yilmana Densa was antigenetically related to O1/Manisa, O/BFS/VP pool, and O/Ind/R2/75 in which r_1 values were 0.44, 0.70 and 0.65, respectively, but there was no antigenic relation with O/Kauf/VQ in which r_1-value was 0.23 using 2dmVNT. In addition, it was also antigenetically matched with O/3039 and O/4174, in which r_1- values were of 1.00 and 0.25, respectively, using LPBE.

O/ETH/24/2009 was antigenetically related with O/manisa, O/Bfs, and O/Ind/R2/75, in which r_1-values were 0.32, 0.43, and more than 1, respectively, using 2dmVNT. In addition, this strain was antigenetically close to O/manisa (r_1-value = 0.75), but different from O/Bfs/1860, O/4174, O/Hkn/6/83, and O/k77/78 in which r_1-values were 0.13, 0.11, 0.25, and 0.13, respectively, using LPBE. O/ETH/28/2009 was also antigenetically matched with O/manisa, O/Bfs, and O/Ind/R2/75 in which r_1-values were 0.32, 0.74, and more than one, respectively, using 2dmVNT. Moreover, this strain was antigenetically related with O/manisa (r_1-value = 0.46), but there was no antigenic similarity with O/Bfs/1860, O/4174, O/Hkn/6/83, and O/k/77/78 in which r_1-values were 0.13, 0.16 and 0.50, respectively, using LPBE as shown in Table 3.

Table 3. Antigenic characterization of FMD field isolates of serotype O by matching with vaccine strains.

Filed isolate	r1-value by 2dVNT				r1 value by LPBE					
	O/Manisa	O/Bfs	O/Ind R2/75	O/Kauf/ VQ pool	O/Manisa	O/Bfs	O/4174	O/3039	O/Hkn/6/83	O/k/77/78
O/ETH/15/08	0.46	0.70	0.69	0.58	-		0.25	0.75	-	-
O/ETH/24/08	0.44	0.70	0.65	0.23	-		0.25	1	-	-
O/ETH/24/09	0.32	0.43	>1.0	-	0.75	0.13	0.11	-	0.25	0.13
O/ETH/28/09	0.32	0.74	>1.0	-	0.46	0.13	0.16	-	0.50	-

Key: - Not done.

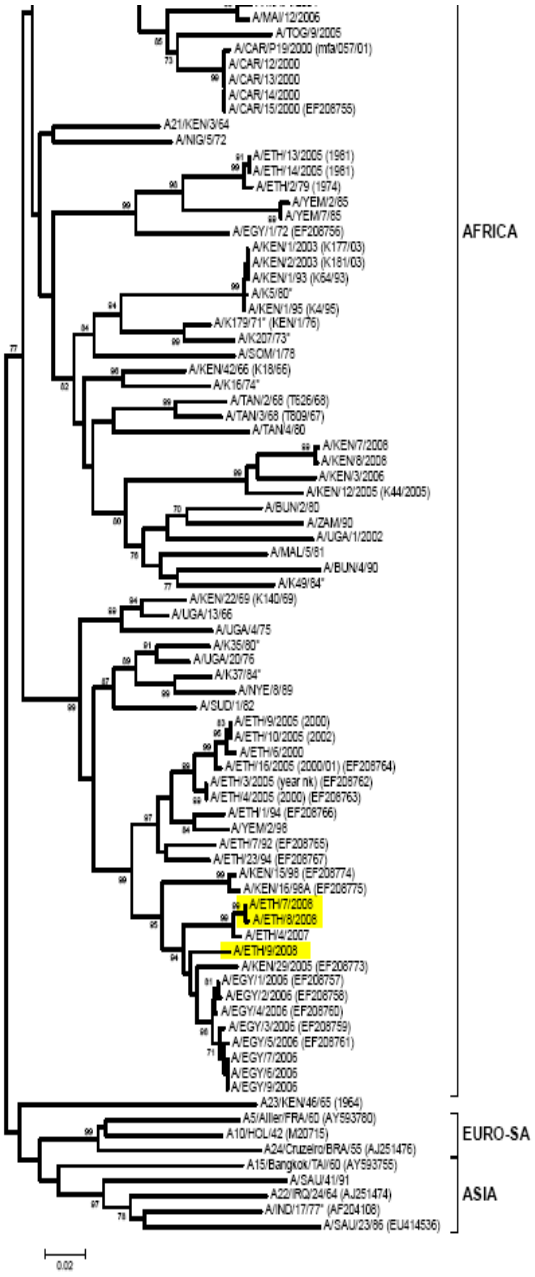

Figure 2. phylogenetic tree of FMDV serotype A.

Phylogenetic analysis

The 1D gene characterization was used to study phylogenetic relationships between 16 serotype O and 3 serotype A FMD viruses in Ethiopia as well as with other O-type and A-type isolates from other countries of the world. Serotype O isolated in this study falls within East Africa-3 topotype while serotype A was identified as Africa topotype and G-VII strains. Serotype A recovered from Sinana (A/ETH/7/2008 and A/ETH/8/2008) and Yabello (A/ETH/9/2008) districts were compared based on the complete 1D sequence (639 nuclotide) data of VP1 gene. Viruses isolated at Sinana A/ETH/7/2008 and A/ETH/8/2008 were shared > 99% identity with each other and > 94% identity with Yabello isolate A/ETH/9/2008. Also the serotype A isolates were antigenetically homologues (> 94% identity at 630 nt. sequence level) with viruses isolated in Egypt in 2006 (A/EGY/1/2006, A/EGY/2/2006, A/EGY/3/2006, A/EGY/4/2006, A/EGY/7/2006 and A/EGY/9/2006) and Kenya isolated in 2005 (A/KEN/29/2005) as shown in Figure 2. Serotype O from Yilmana Densa O/ETH/24/2008, O/ETH/25/2008, and O/ETH/26/2008 were showed > 99% nucleotide sequence similarity. Isolates recovered from Bahirdar zuria O/ETH/20/2008 and O/ETH/21/2008 were 100% nucleotide sequence similarity. Similarly, O/ETH/19/2008, O/ETH/13/2008 and O/ETH/15/2008 isolated from South Achefer showed 100% nt. sequence similarity. In addition, O/ETH/24/2009, O/ETH/25/2009, O/ETH/26/2009, O/ETH/27/2009 and O/ETH/28/2009 isolated from Haromaya University dairy farm were shared more than 99% antigenetic identity with each other.

These isolates were also antigenetically homologues (more than 98% nt. identity) with virus isolated in Yemen in 2009 (O/YEM/6/2009, O/YEM/16/2009, O/YEM/18/2009, O/YEM/19/2009, O/YEM/20/2009 and O/YEM/21/2009) at 92% bootstrap support as shown in Figure 3.

DISCUSSION

Serotype A (Africa topotype) and serotype O (East Africa-

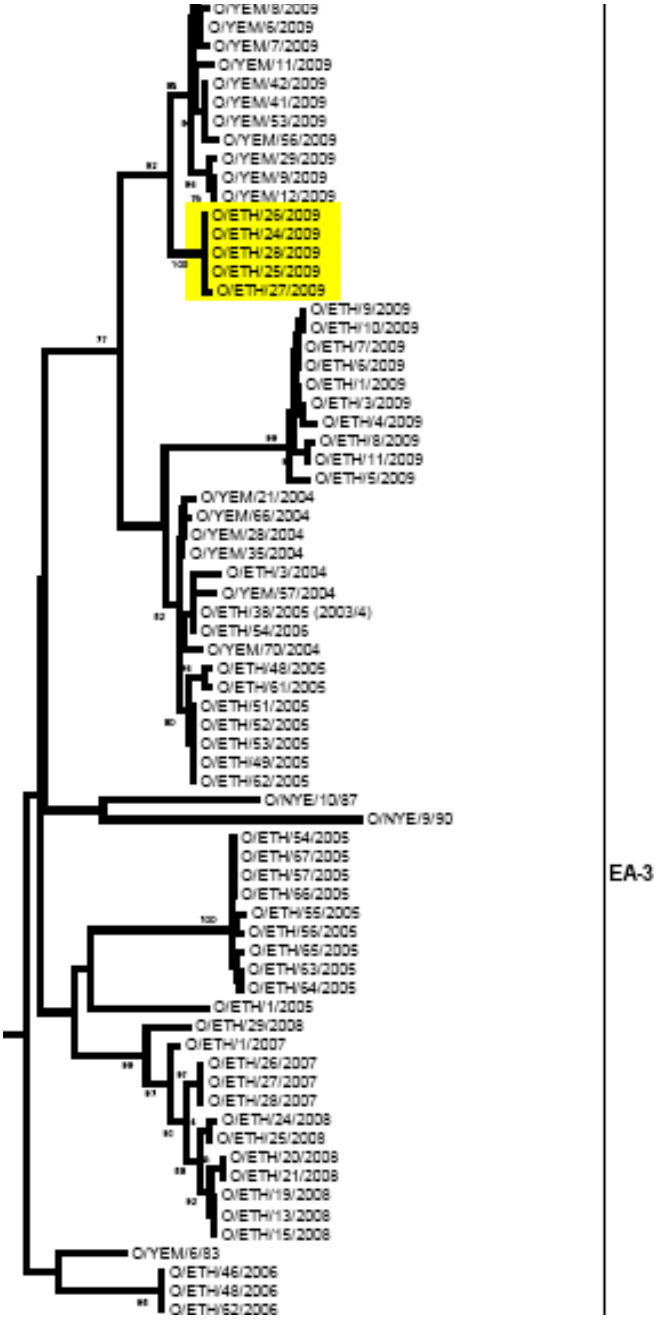

Figure 3. Phylogenetic tree of FMDV serotype O.

3 topotype) of FMDV was isolated in this study and serotype O was the dominant serotype circulating in Ethiopia. Most of the outbreaks were responsible by serotype O followed by serotype A. This showed that serotype O was highly prevalent and a dominant serotype causing outbreaks in Ethiopia. Gelaye et al. (2005), Ayelet et al. (2009) and Klein (2009) reported that serotype O was a dominant FMD virus serotype circulating in Ethiopia. Serotype A isolated from Sinana district (A/ETH/7/2008 and A/ETH/8/2008) and Yabello districts (A/ETH/9/2008) were closely related (< 6% nt. difference) and this indicated that they are antigentically related. In addition, these isolates were antigenetically related (< 6% nt. difference) with viruses isolated in Egypt in 2006 and in Kenya in 2005, indicating that they share a common ancestor. This similarity might be due to the presence of uncontrolled transboundary animal movement. This statement is supported by Samuel et al. (1999) who demonstrated that closely related viruses could either be from the same outbreak or from viruses temporally closely related. All serotype O strains had falls within East Africa-3 topotypes. These indicated that EA-3 topotype has wider distribution and highly prevalent in Ethiopia. This is in agreement with Ayelet et al. (2009) who demonstrated the existence of topotypes EA-3 and EA-4 in Ethiopia with the highest rate of EA-3 topotype. African topotype of serotype A and G-VII strains were also recorded in this study.

Serotype O isolated from Yilmana Densa (O/ETH/24/2008, O/ETH/25/2008 and O/ETH/26/2008), Bahirdar zuria (O/ETH/20/2008 and O/ETH/21/2008), and South Achefer (O/ETH/19/2008, O/ETH/13/2008, and O/ETH/15/2008) were closely related and < 2% nt. sequence difference. This indicated that outbreaks due to these isolates were from the same origin. These might be due to free movement of livestock and livestock products among various markets in different regions and states and this plays an important role in the dissemination of the virus. Furthermore, serotype O isolated from Haromaya University dairy farm (O/ETH/24/2009, O/ETH/25/2009, O/ETH/26/2009, O/ETH/27/2009, and O/ETH/28/2009) were closely related with each other (> 98% nt. sequence similarity), which indicated that these viruses isolated from the same outbreaks. These isolates were also antigenetically more closely related (< 2% nt. sequence) with viruses isolated from Yemen in 2009 and it indicated that they belong to the same epizootics (common origin). The antigenic relationship of serotype A isolated during this study revealed that serotype A (A/ETH/9/2008) isolated from Yabello had an antigenic similarity and could provide protection to A/Tur06/Arriah2nd, (A/Sau95/Bvs), and A/Sau95 isolates where r_1-values were 0.46, 0.3 and 0.25, respectively. However, highly significant antigenic differences were observed with A/Eritrea RZ pool and A/Eri3/98, A22/Irq23-32 pool, A/Sau41/91/SI94, and A/Irn87/VQ pool and A/Irn96/UZ pool in which r_1-values were less than 0.12. This suggested that the vaccine strain might be suitable for use if no closer match could be found provided that a potent vaccine was used and animals were preferably immunized more than once.

Serotype O/ETH/15/2008 was highly significant antigenic similarity to reference vaccine strain of Turkey (O1/Manisa), United Kingdom (O/BFS/VP pool), Indian (O/Ind/R2/75), and Germany (O/Kauf/VQ) where r1-values were 0.46, 0.70, 0.69 and 0.58, respectively. Field isolated strain O/ETH/24/2008 was antigenetically related

to O1/Manisa, O/BFS/VP pool, and O/Ind/R2/75 where r_1 values were 0.44, 0.70 and 0.65, respectively. These suggested that there was a close relationship between field isolate and vaccine strain and a potent vaccine containing the vaccine strains were likely to confer protection. O/ETH/24/2009 was antigenetically related with O/manisa, O/Bfs, and O/Ind/R2/75, in which r_1-values were 0.32, 0.43, and more than 1, respectively. O/ETH/28/2009 was also antigenetically matched with O/manisa, O/Bfs/ and O/Ind/R2/75 in which r_1-values were 0.32, 0.74, and more than one, respectively, using 2dmVNT. Generally, despite the genetic variation observed for serotype O virus worldwide, the antigenic variation is not extensive and the current vaccine strains can protect against most outbreaks.

In conclusion, serotype O and A was identified with highest prevalence of serotype O. The O serotype isolated in Ethiopia lies on East Africa-3 topotype, while serotype A was laid in Africa topotype. The antigenic variation was not diverse for serotype O and most vaccine strains found at WRLFMD can protect against this strain in the study areas while serotype A has highest antigenic variation and only few vaccine strains found at WRLFMD can provide protection against this strain.

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Full Length Research Paper

Diagnostic assessment of Xpert MTB/RIF in a sample of *Mycobacterium tuberculosis* Egyptian patients

Manal Darwish[1], Magda Abd El Wadood[1]* and Hazem ALnagdi[2]

[1]Medical Microbiology and Immunology Department, Ain Shams University, Egypt.
[2]Chest Department, Ain Shams University, Egypt.

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Global *Mycobacterium tuberculosis* (MTB*)* control efforts have been severely hampered by the lack of diagnostic tests that are accurate, simple to use and can be applied at the point of clinical care. This compounded by the widespread inability to test for drug resistance. The newly developed Xpert *MTB*/RIF assay utilizes real-time PCR technology to both diagnose *TB* and detect rifampicin (RIF) resistance concurrently using unprocessed clinical specimens, regardless of their smear status. The study was designed to assess the diagnostic accuracy of Xpert MTB/RIF assay in detecting *MTB* and MDR-*TB* in comparison to the conventional methods in a sample of *MTB* Egyptian patients. Forty (40) sputum specimens were collected from adult patients having pulmonary tuberculosis from Chest hospital, Egypt. Ten (10) external control patients were enrolled. The conventional method, including Ziehl-Neelsen staining showed the presence of *MTB* in 77.5% and bacterial culture in 85%. Whereas, the Xpert *MTB*/RIF test provided detection of 82.5%, in addition it correctly identified five out of six cases of RIF resistant *MTB* with sensitivity and specificity (83 and 100%) respectively, the resistant cases were all previously treated with RIF. Sensitivity, specificity, positive and negative predictive value of Xpert *MTB*/RIF in comparison to conventional culture was 97.14, 100, 100 and 85.7%, respectively. The control group showed no positive results with the Xpert *MTB*/RIF. The sensitivity of Xpert for smear positive, culture positive *TB* was 100% and in smear negative, culture positive *TB* was 66.6% while its specificity in both was 100%. Comparing processed and unprocessed samples, the sensitivity of Xpert *MTB*/RIF was 94 and 97%, respectively while its specificity was 100% in both conditions. Thus, Xpert *MTB* /RIF outperformed smear microscopy, establishing a diagnosis in a proportion of patients with smear negative *MTB*, which detected many highly likely *MTB* by culture, and accurately ruled out rifampicin resistant *TB*.

Key words: Multi Drug Resistance Tuberculosis (MDR-TB), polymerase chain reaction (PCR), *Mycobacteruim tuberculosis* (MTB), extensively drug-resistant TB (XDR-TB).

INTRODUCTION

MDR-TB essentially means that the organism is resistant to both Isoniazid (ISN) and RIF drugs which is considered most effective in treatment of tuberculosis. Patients may be infected by already drug resistant strain or the resistance may develop in erstwhile susceptible strain in the course of treatment. XDR-TB is a form of *TB* caused by organisms that are resistant to ISN and RIF (that is, MDR-TB) as well as any fluoroquinolone and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin). About 3.7% of new *TB* patients in the world have MDRTB strains. Levels are much higher in those previously treated - about 20%. The frequency of MDR-TB varies substantially between countries. About 9% of MDR-TB cases also have resistance to two other classes of drugs, and hence fall into the XDR-TB category. By March 2013, eighty four

*Corresponding author. E-mail: ramadan_magda@yahoo.com.

countries had reported at least one XDR-TB case (WHO updates, 2013). In a nationwide survey in 2011, MDR-TB was found in 5.2 and 40.8% of patients with new and previously treated TB, respectively. These levels of drug resistance are among the highest ever documented in Africa and the Middle East. This finding presents a serious challenge for *TB* control (Sindani et al., 2013). Worldwide, substantial percentages (˜35%) of patients with drug-susceptible TB remain undiagnosed and a staggering percentage (˜85%) of patients with MDR-TB remains undiagnosed (WHO Global Report, 2011). Of the people diagnosed with *TB*, less than 3% are tested to determine the pattern of drug resistance (Chaisson, 2012). In addition to drug resistance, another major challenge is the accurate detection of smear-negative disease which disproportionately occurs in HIV-positive people with TB (Harries, 2004).

Egypt is ranked as a country with middle/ low level of tuberculosis incidence. It is estimated that 11 cases per 100 000 population develop active pulmonary smear positive *TB* annually, while 24 per 100 000 develop all types of *TB* annually. According to WHO TB profile for Egypt (2011), it reported that the new case detection rate of *TB* were as follows: smear positive (52%), smear negative (12%), smear not done (1%), extra pulmonary cases (36%) and as regards the retreatment cases were as follows: relapse (50%) and treatment failure (17%). In addition, WHO (2011) estimated MDR-TB burden in Egypt to be 3.4% for the new cases and 32% for the retreatment cases. Safwat et al. (2011) reported MDR-TB prevalence in Egypt to be around 0.5%, which is much less than that reported by WHO (2011). They posited that this difference underscores the need for better collection and analysis of data, transparency in information gathering, more departments for MDR-TB isolation in other governorates and better notification policy from private sector of health service. Global control of tuberculosis is hampered by slow, insensitive diagnostic methods, particularly for the detection of drug-resistant forms.

Early detection is essential to reduce the death rate and interrupt transmission, but the complexity and infrastructural needs of sensitive methods limit their accessibility and effect (Boehme et al., 2010). Conventional diagnostic methods for *MTB* are slow and/or lack sensitivity. It requires skilled technicians and tools, and lacked either timeliness or sensitivity. Culture methods are highly sensitive, but they take as long as two to six weeks to produce results and demand special materials to support the virulent micro bacteria in the culture. Although, sputum smear test is quicker and produces results in about 30 min, it can only detect 10 to 75% of TB cases and also requires trained persons. In developing countries, the technical expertise and tools needed to perform these tests are limited, and TB is often not diagnosed or treated early, which allows the disease to spread quickly in crowded living quarters and to build resistance to the drugs used in the treatment of the infection.

Current nucleic acid amplification methods to detect *MTB* are complex, labor-intensive and technically challenging. A number of new diagnostic approaches have brought incremental improvements to detection and drug susceptibility testing; however, the technical complexity of these assays and their dependence on sophisticated laboratory infrastructure have limited their adoption, especially in low-resource, high-burden settings (Balasingham et al., 2009; Migliori et al., 2008). The recently introduced Xpert MTB/RIF (manufactured and marketed by Cepheid, Sunnyvale, CA) assay simultaneously detects the presence of *M. tuberculosis* and its susceptibility to the important first-line drug RIF (Helb et al., 2010). It is unique because sample processing and PCR amplification and detection are integrated into a single self-enclosed test unit, the GeneXpert cartridge.

Following sample loading, all steps in the assay are completely automated and self-contained. In addition, the assay's sample reagent, used to liquefy sputum, has potent tuberculocidal (the ability to kill TB bacteria) properties and so largely eliminates biosafety concerns during the test procedure (Banada, 2010). The assay can be performed directly from a clinical sputum sample or from a decontaminated sputum pellet and can generally be completed in less than 2 h (Boehme et al., 2010). These features allow the technology to be taken out of a reference laboratory and used nearer to the patient (Small and Pai, 2011). Thus, investment in the tuberculosis diagnostics pipeline should remain a major priority for funders and researchers in various countries.

That is why the aim of our work was to evaluate the efficiency of Xpert *MTB*/ RIF assay in detecting *MTB* and MDR-*TB* as a point of care test in a sample of mycobacterium tuberculosis Egyptian patients in comparison to the conventional methods of *MTB* detection hopefully that Xpert *MTB*/RIF be used as a simple accurate system in detecting *M. tuberculosis* directly from sputum in less than 2 h thus controlling the spread of *MTB* and consequently the resistant strains in Egypt.

MATERIALS AND METHODS

Forty sputum specimens from adult patients with age from 33 to 58 years old strongly suspected by clinical parameters of having pulmonary tuberculosis as cough, night sweat, weight loss or fever, were studied from Abbassaia Chest Hospital, Cairo, Egypt. Ten external control patients with chest infection confirmed by culture to be bacterial other than *MTB* to exclude the cross reaction of Xpert *MTB* /RIF with other bacterial organisms. They were enrolled in the study in the period from January 2013 to August 2013. Verbal approval was taken from the patients. The following variables were collected through a questionnaire administered during sputum collection: patient sex, age, treatment history (new or previously treated), residence in Egypt. Cases were fallen in three categories: 1) not received anti-tuberculosis therapy, 2) had < 7 days of therapy, or 3) have not received therapy in the last 60 days. Xpert MTB/RIF assay was compared with conventional culture method for detecting *TB* and with conventional phenotypic drug susceptibility

Table 1. Comparative detection of *MTB* in sputum samples using three alternative test methods.

No. of specimens examined	Observed outcome per test Method					
	Microscopy (ZN staining)		Culture		Xpert *MTB*/RIF	
	No. positive (%)	No. negative (%)	No. positive (%)	No. negative (%)	No. positive (%)	No. negative (%)
40	31 (77.5)	9 (22.5)	34 (85)	6 (15)	33 (82.5)	7 (17.5)

testing for detecting RIF resistance. Eligible patients provided three sputum specimens each. Two specimens were processed with N-acetyl-L-cysteine and sodium hydroxide before microscopy, solid culture, and the *MTB*/RIF test, and one specimen was used for direct testing with the Xpert *MTB*/RIF test. Children were excluded from the study and specimens obtained by gastric aspiration were equally not included.

Processing of specimens

Specimens were processed within 24 h after collection. Modified Petroff's method using double the volume of NaOH (4%) was adopted. The specimens were kept in a shaker for homogenization and then decontaminated for 20 and 10 min respectively. The processing was stopped by the addition of distilled water up to the brim and centrifuging in a shielded centrifuge (3000 g) for 15 min. The supernatant fluid was then discarded and the sediment was used for inoculation by conventional methods. All specimens were subjected to Ziehl-Neelsen staining and smear grading as per the guidelines under the Revised National Tuberculosis Control Program (Rieder et al., 2008). The samples were cultivated on solid Lowenstein Jensen (LJ) medium and were subjected to species identification by a macroscopic analysis of colonies on LJ medium and microscopic analysis. In addition, complementary niacin, nitrate-reduction assay which is based on the ability of *MTB* to reduce nitrate to nitrite, which can easily be detected with specific reagents producing a color change were done. Initially, the test was performed on solid Löwenstein-Jensen medium with the addition of a NO_3 source. Antibiotics were added to the medium as per the classical proportion method. Reading of the results after induction of the color change performed within 7 to 14 days of incubation (Golyshevskia et al., 2006; Kristian et al., 2002). Etest (bioMerieux) is a predefined, stable gradient of 15 antibiotic concentrations on a plastic strip was used for MIC determination for a variety of antibiotic. RIF E test was used and was done based on BioMerieux application guide (2010). Results obtained within 5 to 15 days. This was compared with the Xpert *MTB*/RIF assay. The Xpert MTB/RIF assay and the GeneXpert instrument have been described in detail by Helb et al. (2010). In brief, the assay consists of a single-use multi chambered plastic cartridge preloaded with the liquid buffers and lyophilized reagent beads necessary for sample processing, DNA extraction and hemi nested real-time PCR. Clinical sputum samples or decontaminated sputum pellets that were treated with a NaOH and isopropanol-containing sample reagent (SR).

The SR is added to the sample at a 3:1 ratio for sputum pellets and 2:1 ratio for unprocessed sputum samples) and incubated at room temperature for 15 min. This step is designed to reduce the viability of *MTB* in sputum at least 10^6-fold to reduce risk of infection. The treated sample is transferred into the cartridge, the cartridge is loaded into the Gene Xpert instrument, and an automatic process completes the remaining assay steps. Following sample loading, all steps in the assay are completely automated and self-contained. The test material is combined with the assay sample reagent, mixed by hand or vortex, and incubated at room temperature for 15 min. After the incubation step, 2 ml of the treated sample are transferred to the cartridge and the run initiated (Helb, 2010). The assay cartridge also contains lyophilized *Bacillus globigii* spores which serve as an internal sample processing and PCR control. The spores are automatically resuspended and processed during the sample processing step, and the resulting *B. globigii* DNA is amplified during the PCR step. The standard user interface indicates the presence or absence of *MTB*, the presence or absence of RIF resistance, and a semi quantitative estimate of *MTB* concentration (high, medium, low and very low). Assays that are negative for *MTB* and also negative for the *B. globigii* internal control are reported as invalid.

The PCR assay amplifies a 192-bp segment of the *MTB rpoB* gene in a hemi nested real-time PCR. The internal control heminested *B. globigii* assay is multiplexed with the *MTB* assay. *MTB* is detected using five overlapping molecular beacon probes (probes A to E) that are complementary to the entire 81-bp RIF's resistance-determining "core" region of the wild-type *rpoB* gene (El-Hajj et al., 2001; Helb et al., 2010).

RESULTS

The study was performed on 40 sputum samples from patients presented with symptoms and signs of pulmonary tuberculosis. As shown in Table 1, conventional analyses, including Ziehl-Neelsen staining showed the presence of *MTB* infection in 31 samples (77.5%) and bacterial culture, showed the presence of *MTB* infection in 34 samples (85%). The performance of Xpert *MTB*/RIF for *MTB* and resistance to RIF were assessed with fully integrated sample processing in patients with suspected drug-sensitive or multidrug-resistant pulmonary tuberculosis. The *MTB*/RIF test provided detection of 33 cases of tuberculosis (82.5%) and correctly identified five out of six cases of RIF resistant *MTB* infection with sensitivity and specificity of 83 and 100%, respectively (Table 2). The control group showed no positive cases for *MTB.* Sensitivity, specificity, positive predictive value and negative predictive value of Xpert *MTB*/RIF in comparison to conventional culture technique were found to be 97.14, 100, 100 and 85.7%, respectively (Table 3). Among culture-positive patients, Xpert *MTB*/RIF test detected all 31 cases with smear-positive tuberculosis (100%) and an additional two cases out of nine with smear-negative tuberculosis (22.2%) thus the sensitivity of Xpert for smear positive, culture positive *TB* cases was 100% and its sensitivity in smear negative, culture positive *TB* cases was 66.6% (two out of three cases) and specificity

Table 2. Comparison of *MTB* detection by conventional culture and molecular techniques Xpert *MTB*/RIF.

		Conventional culture technique		Total
		Positive	Negative	
Molecular technique Xpert MTB/RIF	Positive	33	0	33
	Negative	1	6	7
	Total	34	6	40

Table 3. Sensitivity, specificity, positive predictive value and negative predictive value of Xpert *MTB*/RIF in comparison to conventional culture technique.

Sensitivity	TP/TP + FN 34/34 + 1	97.14%
Specificity	TN/FP + TN 7/0 + 7	100%
Positive predictive value (PPV)	TP/TP + FP 34/34 + 0	100%
Negative predictive value (NPV)	TN/TN + FN 6/6 + 1	85.6%

TP: True positive, FP: false positive, TN: true negative, FN: false negative; positive predictive value (PPV), negative predictive value (NPV).

Table 4. Comparison of the sensitivity and specificity of Xpert *MTB*/RIF when dealing with processed and unprocessed samples.

Type of analysis of the specimen	Sensitivity	Specificity
Unprocessed specimens	33/34 (97%)	6/6 (100%)
Processed Specimens	32/34 (94%)	6/6(100%)

is 100% in both cases. Among the 31 smear positive, culture positive cases, the study found that 6 cases were resistant to RIF by the conventional method (19.4%).

On revising the demographic and socioeconomic characteristics of these patients, it was found that they were males; aged 33 to 55 years and they were manual workers, heavy smokers that were previously treated from *TB* (acquired resistance).

Compared with the phenotypic drug susceptibility testing, Xpert *MTB*/RIF testing correctly identified five out of six cases (83.3%) RIF resistant *MTB* with sensitivity and specificity of 83 and 100%, respectively. On comparing the sensitivity and specificity of Xpert *MTB*/RIF when dealing with processed and unprocessed samples, the study showed that the sensitivity of Xpert *MTB*/RIF was 94 and 97% respectively while its specificity was 100% in both the processed and unprocessed samples (Table 4).

DISCUSSION

In this study, comparative detection of *MTB* in sputum samples from patients with pulmonary tuberculosis using three alternative test methods (ZN staining, conventional culture methods and Xpert MTB/RIF) were done and revealed 77.5, 85 and 82.5% of *MTB* respectively. The control group showed no positive cases by Xpert *MTB*/RIF. This is in line with Theron et al. (2011) who reported that Xpert *MTB*/RIF outperformed smear microscopy, established a diagnosis in a significant proportion of patients with smear-negative *TB*, detected many highly likely *TB* cases missed by culture. As well as Lawn et al. (2013) concluded that Xpert *MTB*/RIF assay is a rapid, accurate point-of-care diagnostic test that is affordable and can be readily implemented in urgently needed conditions. Helb et al. (2010) defined Xpert's limit of detection by "the lowest number of colony forming units per sample that can be reproducibly distinguished from negative samples with 95% confidence" (Cepheid, 2009), is 5 genome copies of purified DNA per reaction or 131 colony forming units per ml in *M. tuberculosis* spiked sputum. Toman (2004) previously mentioned that to see *TB* bacilli by microscopic examination, it requires at least 10,000 bacilli per ml of sputum. In addition, Xpert detects

both live and dead bacteria (Miotto, 2012). In addition, Lawn and Zumla (2011) laid stress that over 90% of *TB* cases develop among people living in low- and middle-income countries where diagnosis still relies heavily on the use of sputum smear microscopy and chest radiology. Despite microscopy being the diagnostic test most widely used worldwide, only 45% of *TB* cases that were notified in 2009 were sputum smear-positive, and these represented just 28% of the estimated total burden of incident disease globally.

WHO Global tuberculosis control 2010 focused attention on the lack of rapid and accurate diagnostics of *T.B*, which is undermining progress towards the 2015 millennium development goals for *TB* control. Such low rates of case ascertainment reflect the critical deficiency in diagnostic laboratory capacity. The study recorded that among the 31 smear positive, culture positive cases of *TB*, there were 6 cases rifampicin resistant (19.4%) by the conventional methods and E test. The studying of these cases showed that they were all previously rifampicin treated. Although, according to the WHO tuberculosis profile for Egypt 2011, the incidence of new cases of MDR-*TB* was 3.4%, while that of previously treated *TB* cases which was discovered to be MDR-*TB* was 32%. The difference in the values between this study and the WHO profile may be attributed to the small studied volume population, the methodology used and the type of samples collected weather they are pulmonary or extra pulmonary, more departments for MDR-TB isolation were needed. The present study is in line with a previous study done by Ali et al. (2011) in Egypt who reported that 19.5% of the 72 tested *mycobacterium* strains were resistant to each of ISN and RIF (MDR-TB), whereas 26.4% of these strains were susceptible. This indicated that these MDR-TB strains are initially resistant strains.

This recent study identified six cases as being RIF resistant MTB by the conventional method as well as by the Etest. On comparing this with Xpert MTB/RIF, it revealed five out of the six cases to be RIF resistant MTB with sensitivity 83% and specificity with 100%. This is in accordance with Blakemore et al. (2010) who spotlight that the *Xpert MTB*/RIF assay detects *MTB* and RIF's resistance by PCR amplification of the rifampin resistance-determining region (RRDR) of the *MTB rpoB* gene and subsequent probing of this region for mutations that are associated with RIF resistance. Approximately, 95% of RIF-resistant tuberculosis cases contain mutations in this 81-bp region (Van Der Zanden et al., 2003). Steingart et al. (2013) emphasized that Xpert can be used as an initial diagnostic test for TB detection and rifampicin resistance in patients suspected of having *TB*, MDR-*TB* or HIV-associated *TB*.

Xpert may also be valuable as an add-on test following microscopy for patients who have previously been found to be smear-negative as well as they stated that Xpert *MTB*/RIF when used replacing the conventional drug susceptibility, it can detect 94% of RIF resistant *T.B* with high specificity of 98%. The difference in this study and Steingart study could be attributed to volume of studied population, sputum processed method. Regarding the only case which is reported as rifampicin resistant by conventional method and rifampicin sensitive by Xpert *MTB/* RIF was revised and E test was repeated and recorded as borderline. This may clarify the difference in the results between the conventional and the Xpert *MTB*/ RIF method. In addition, this result can also be clarified by Lawn and Nickol (2011) who reported that to enable detection of rifampicin resistance by the Xpert, there must be present between 65 and 100% of the DNA from the rifampicin-resistant isolate depending on the mutation. They suggested that in patients with mixed infections, the Xpert *MTB*/RIF assay might only detect the resistant strain if it is the predominant one present. However, selection of resistant strains during the course of standard *TB* treatment might lead to an apparent switch from a susceptible to a resistant phenotype when comparing baseline testing with repeat testing during treatment. This may be the difference between the conventional phenoltypic drug susceptibility E test method and the Xpert assay. Thus, WHO recommended that if Xpert detects rifampicin resistance in patients considered at risk of MDR-TB, an appropriate MDR-TB regimen should be started while additional sputum specimens are obtained for culture and drug susceptibility testing.

Subsequent testing will confirm the presence of rifampicin resistance and enable testing for drug resistance to isoniazid and other first-line drugs and second-line drugs. Thus ideally, Xpert should be used at the district or sub district health facility level (WHO Policy Xpert, 2011). In the present study, sensitivity, specificity, positive predictive value and negative predictive value of Xpert *MTB*/RIF to detect *MTB* in comparison to conventional culture technique was found to be 97.14, 100, 100 and 85.7%, respectively. Thus, the *MTB/*RIF assay has a sensitivity that approximately approaches that of culture. This is with agreement with Bodmer et al. (2012) who stated that the Xpert *MTB*/RIF assay's overall clinical sensitivity of detecting MTB in sputum of patients with suspected pulmonary TB was 97.6% when compared to culture as the reference. There were also the findings of a prospective multi-center study that involved five study sites (Lima, Peru; Baku, Azerbaijan; Cape Town and Durban, South Africa; Mumbai, India) and a total of 1462 patients. Steingart et al. (2013) found that Xpert sensitivity for smear positive, culture positive *TB* was very high and consistent (98%), while Xpert sensitivity for smear negative, culture positive *TB* was lower and more variable (68%); this was in line to this study who reported the sensitivity of Xpert for smear positive, culture positive *TB* cases was very high (100%) and Xpert *MT*B/RIF sensitivity in smear negative, culture positive *TB* cases was lower (66.6%), two out of three cases and specificity for both is 100%. This present result can also be sup-

ported by study of pulmonary *TB* done by Vadwai et al. (2011) who reported that sensitivity was higher for smear-positive specimens (96%) compared with smear-negative specimens (64%). Thus, Boehme et al. (2011) drew atten-tion that patients with smear-negative *TB*, can make use of these Xpert assay results to reduce the time to start of treatment from 56 days [interquartile range (IQR) 39 to 81) to 5 days (IQR, 2 to 8). Rates of untreated smear-negative culture-positive TB decreased from 39.3% without Xpert to 14.7% using the assay to direct treatment initiation.

In this study, comparison in the sensitivity and specificity of Xpert *MTB*/RIF when dealing with processed and unprocessed samples was done and reported that the sensitivity of Xpert *MTB*/RIF was 94 and 97%, respectively. And as regards the specificity was 100% in both the processed and unprocessed samples. This is nearly in concordance with the study done by Steingart et al. (2013) who reported that the sensitivity of the Xpert between the processed and unprocessed samples was 85 and 91%, respectively and the specificity was 98 and 99%. The difference can be attributed to the difference in size of the studied population between the two studies, as well as to the sputum processing methods like time, rpm centrifugation as well as the condition of the sputum weather they are fresh or frozen. In addition, Boehme et al. (2010) reported that Xpert *MTB*/RIF test provided sensitive detection of tuberculosis and rifampin resis-tance directly from untreated sputum in less than 2 h. Helb et al. (2010) reported that Xpert *MTB*/RIF assay detect *MTB* complex DNA in sputum or concentrated sputum sediments. Thus, finally this present study under-lined the ability of the Xpert *MTB*/RIF assay to rapidly and reliably detect *TB* cases with sensitivity 97.14% and specificity 100% including nearly 66.6% of smear-nega-tive cases. Moreover, Xpert *MTB*/RIF achieved sensitivity of 88% and specificity 100% to RIF resistant *MTB.* In spite that it was limited by the small number of the studied cases and smear negative / culture positive *TB* cases.

Conclusively, the high sensitivity in smear positive and modest sensitivity in smear negative *TB*, along with high specificity of Xpert *MTB*/RIF mean that it may be used as the initial diagnostic test for *TB* detection in individuals suspected of having *TB* and MDR-*TB*. Xpert *MTB*/RIF may also be valuable as add on test following a negative smear microscopy result in patients suspected of having *TB*. In addition, the high sensitivity and specificity of Xpert *MTB*/RIF for RIF resistance detection mean that it may be used as an initial diagnostic test for RIF resistance. These results can be considered as an initial step to use Xpert *MTB*/RIF to control the spread of *TB* and MDR-*TB* in Egypt. Taking in consideration the obstacles faced by this study which is the small studied volume as it is not funded work as well the lack of previous studies done in Egypt by the same method to be comparable by our results in the same country. Lastly, the study suggests that future governmental funded researches should be done in Egypt to assess the diagnostic accuracy of Xpert *MTB*/RIF in peripheral laboratories and clinical settings, especially settings where the test is performed at the point of care on a large studied population.

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Full Length Research Paper

Antimicrobial activity of leaf and fruit extracts of Jordanian *Rubus sanguineus* Friv. (Rosaceae)

Rana Zeidan[1], Sawsan Oran[1], Khaled Khleifat[2] and Suzan Matar[1]*

[1]Department of Biological Sciences, Faculty of Science, The University of Jordan, Amman 11942, Jordan.
[2]Department of Biology, College of Science, Mutah University, Mutah, Karak, 61710, Jordan.

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***In vitro* antimicrobial activity of ethanolic and methanolic extracts of the leaf and fruit of *Rubus sanguineus* were investigated against pathogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans* using agar well diffusion and microdilution broth assays. This study showed that leaf ethanolic extract exhibited the best antimicrobial activity with zone of inhibition ranging from 20 to 22 mm. The minimum inhibitory concentration (MIC) of the ethanolic leaf extract range was 1.56 and 12.5 mg/mL while that of methanolic extract range was 0.78 to 12.5 mg/mL. The ethanolic leaf extract exhibited appreciable activity against *Candida albicans* with zone of inhibition of 20 mm. The anticandida activity was support by MIC tests. In conclusion, the methanolic and ethanolic leaf and fruit extracts of *Rubus sanguineus* have a significant activity against Gram positive bacteria and Candida but have not shown any significant activity against Gram negative bacteria investigated in this work. Results showed that there is a basis for the traditional use of this plant as a healthy remedy in Jordanian culture.**

Key words: Antimicrobial activity, Gram positive bacteria, Gram negative bacteria, *Candida albicans*, *Rubus saguineus*, Jordan.

INTRODUCTION

Plants have been playing an important role in alternative medicine since ancient times (Oran and Al-Eisawi, 1998). Many of these plants are used as chemical feed stocks or as raw material for many scientific investigations, also they are commercially important especially in pharmaceutical industry (Joy et al., 1998). *Rubus* species belong to the family Rosacea and in use as alternative medicine to cure diarrhea, intestinal disorders and its fresh juice is used for treating tuberculosis (Oran and Al-Eisawi, 1998). *Rubus*, latin name for holy bramble or blackberry and *sanguineus* is blood colored. This plant is a wild shrub with edible fruits found near river banks, by .prings and swamps (Zohary, 1972). *Rubus sanguineus* disperse their seeds via frugivores, change fruit color from green to red with very sour taste while still unripe and then to black or dark blue upon ripening. The antimicrobial effect of this plant has not been extensively studied, and there is little information about the medicinal uses of different species of *Rubus*. However; few studies showed that some species of *Rubus* have antimicrobial capacities and they have been investigated using different techniques (Panizzi et al., 2001; Thiem and Goślińska, 2004).

In Jordan, this plant is reputed traditionally for its use to

*Corresponding author. E-Mail: s.mattar@ju.edu.jo. Tel.: +96-26-535-5000/22238. Fax: +96-26-530-0253.

treat different infections. This necessitated our attention to investigate the role of ethanol and methanol extracts of *R. sanguineus* leaf and fruit, which is found in the upper and lower Jordan valley, for its antibacterial activity against human pathogenic Gram positive bacteria that is, *Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) and Gram negative bacteria that is, *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and also to determine the antifungal activity of these extracts against *Candida albicans*.

MATERIALS AND METHODS

Plant materials

The plants leaves and fruits of *R. sanguineus* were collected from different areas along Jordan valley and later identified by Sawsan Oran (Professor of plant biosystematics at the University of Jordan, Faculty of Science, Department of Biological Sciences, Amman, Jordan).

Microorganisms and growth conditions

Microorganisms were obtained from American Type Culture Collection. Pathogenic organisms were two Gram positive bacteria*, S. aureus ATCC 29213* and *B. cereus ATCC14579*, and two Gram-negative bacteria, *E. coli ATCC35218* and *P. aeruginosa ATCC10145,* and the yeast *C. albicans ATCC 90028*. The bacterial cultures were maintained on Mueller Hinton Agar (MHA) (Oxoid). Overnight cultures were prepared by inoculating 5 mL of Mueller Hinton Broth (MHB, Oxoid) with 5 colonies of each microorganism taken from MHA. Broths were incubated overnight at 37°C. However, *Candida* strain was maintained on Sabouraud Dextrose Agar (SDA, Oxoid). Suspensions were prepared using Sabouraud Dextrose broth. Bacterial and yeast suspensions were prepared by diluting overnight cultures in PBS to 0.5 *McFarland* standard. These suspensions were further diluted with PBS as required.

Preparation of plant extracts

Collected plant materials (leaves and fruits separated) were air dried for approximately two weeks. Dried plant samples were grinded using a grinder (Ambar, Liban) and then 50 g of the dried powdered plant were soaked separately in 1 L of ethanol and methanol. After soaking for two weeks they were filtered using Whattman no. 1 filter paper. All filtrates were evaporated using rotary evaporator (Janke and Kunkel, Germany) and left to dry at room temperature for 24 h and weighed. The air dried stock extracts were then reconstituted in 25% dimethylsulphoxide (DMSO) solution to get 25 mg/ml concentrations and sterilized by filtration (mini pore filter 0.22 µm) and stored in refrigerator at 4°C prior to determination of antimicrobial activities of the extracts (Othman et al., 2011; Rawani et al., 2011).

Microbiological screening

Antimicrobial activities of different extracts were evaluated by the agar well-diffusion method, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (Murray et al., 1995). MIC of each extract was expressed as the lowest dilution level of the extract needed to inhibit bacterial growth, while MBC is the lowest dilution level which completely kills the bacteria.

Determination of antimicrobial activity of the extracts

The antimicrobial activity was determined by the well diffusion method according to NCCLS M2-A5 (NCCLS, 1993). Mueller-Hinton and Sabouraud Dextrose plates were inoculated by streaking the swab over the entire agar surface using bacterial suspensions containing 10^8 CFU/mL and yeast suspensions containing 10^7 CFU/mL. The plates were allowed to dry at room temperature. Using a sterile agar cutter, 6 mm diameter wells were bored in the agar. The antimicrobial activity of the extracts was checked by introducing 50 µL of 25 mg/mL concentrations into triplicate wells. An additional well in each plate was filled with the solvent DMSO 25% v/v as a control. Commercially prepared gentamicin susceptibility discs 5 µg from Oxoid and 50 µg fluconazole (Sigma) discs were prepared by pipetting 12.5 µl volumes of stock fluconazole (4 mg/mL) onto sterile blank discs were used as positive controls. The culture plates were allowed to stand on the bench for 30 min at room temperature and were incubated at 35°C for 24 h. After 24 h, the antimicrobial activity of the extracts and the antibiotics were determined. Zones of the inhibition around each of the extracts and the antibiotics were measured to the nearest millimeter (Lino and Deogracios, 2006; Wendakoon et al., 2012). The experiment was repeated at least three times for each microorganism.

Determination of minimum inhibitory concentration (MIC)

The MIC test was carried out on the plant extract which showed inhibition zones in the antimicrobial screening. The MIC of the extracts were determined for each microorgansim in triplicates by double fold serial microdilution assay using 96-well microliter plates (Nunc) according to NCCLS M7-A5 guidelines (NCCLS, 2000). The different plant extracts were taken (25 mg/mL) and serially diluted with Mueller-Hinton broth for bacterial culture and Sabouraud Dextrose broth for yeast with their respective inocula were used (Sarker et al., 2007). The final concentrations ranged from 12.5 to 0.097 mg/mL when reconstituted with bacterial and yeast suspension. The wells were inoculated with 5×10^5 CFU/mL of the test bacterial strain according to NCCLS M7-A5 and with 1×10^5 CFU/mL candida strain according to NCCLS M-27 (NCCLS, 1997). The microplates were incubated for 24 h at 35°C. One of the 12 columns served as growth control (bacterial suspension or yeast without plant extract) and another one for the sterility control with only broths in them. The lowest concentration without visible growth was defined as MIC. The readings were compared with gentamicin for flucanazole for bacteria and *Candida* respectively used as control.

Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The MBCs were determined by sub-cultivation of loopful (0.001 mL) the test dilution from 96 well plates used to determine MIC previously onto Mueller –Hinton and Sabouraud Dextrose agar plates. The plates were incubated at 35°C overnight and the lowest concentration, with no visible growth, was defined as MBC or MFC (Minimum Fungicidal Concentration), indicating 99.5% killing of the original inoculum (Wendakoon et al., 2012).

Table 1. Antimicrobial activity of methanol and ethanol extracts of *R. sanguineus* fruit and leaf by well diffusion method.

Organism	Antibiotic/antifungal	Ethanol extract		Methanol extract	
		Leaf	Fruit	Leaf	Fruit
	Zone of Inhibition (mm)				
Yeast	Flucanazole 25 µg				
C. albicans	20 ± 2*	20 ± 1	15 ± 1	18 ± 1	16 ± 1
Gram Positive bacteria	Gentamicin 5 µg				
S. aureus	20 ±1	20 ± 0.5	18 ± 0.5	18 ± 0.5	18 ± 0.5
B. cereus	12 ± 1	22 ± 0.5	19 ± 1	19 ± 1	20 ± 0.5
Gram Negative Bacteria	Gentamicin 5 µg				
E. coli	10 ± 1	7 ± 0.5	7 ± 0.5	7 ± 0.5	7 ± 0.5
P. aeruginosa	7 ± 2	7± 0.5	7 ± 0.5	7 ± 0.5	7 ± 0.5

Values indicate average zone of inhibition in (mm). * Standard errors for three experiments.

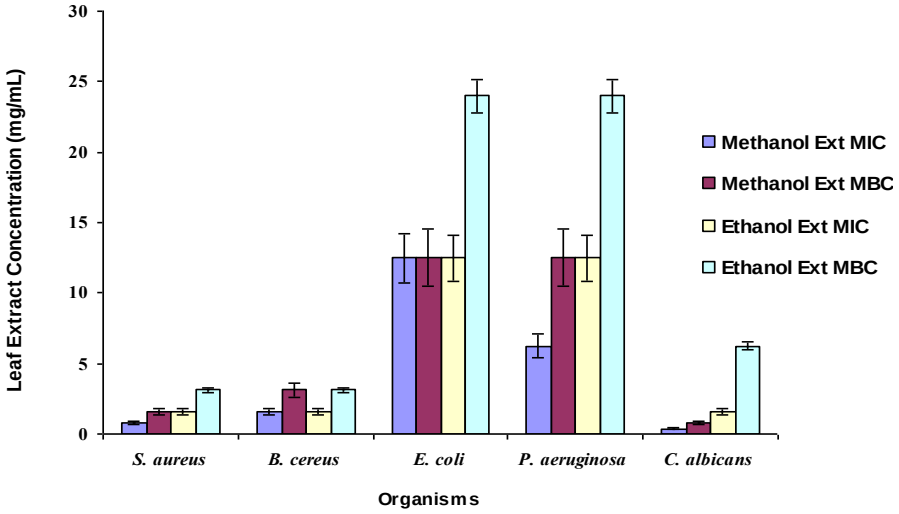

Figure 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of leaf ethanol and methanol extracts of *Rubus sanguineus* against the test organisms.

RESULTS

The results obtained on methanol and ethanol extracts of *R. sanguineus* fruit and leaf by agar well diffusion method are presented in Table 1. Both extracts showed antimicrobial activity against Gram positive bacteria and *C. albicans.* The eaf ethanol extract exerted highest activity on bacteria and *Candida* tested when compared with others. The leaf ethanol extract (1.25 mg) in 6 mm well, 22 mm was recorded as diameter of the zone of inhibition against *B. cereus,* this was followed 20 mm zone of inhibition against *C. albicans* and *S. aureus*. Methanolic fruit extract exerted the highest activity against *B. cereus* (20 mm) when compared with the other extracts. The lowest antimicrobial activity was for ethanolic fruit extract against *C. albicans* (15 mm). The lowest activities (7 mm zone of inhibition) were recorded by DMSO (25 v/v %) used as a solvents, and leaf/fruit ethanol/methanol extracts against *E. coli* and *P. aeruginosa*.

The antimicrobial activity and the potency of the extracts were quantitatively assessed by MIC and MBC as given in Figures 1 and 2. Wherever low MIC and MBC values observed against the test organism means that the plant has the potential to treat any ailments associated

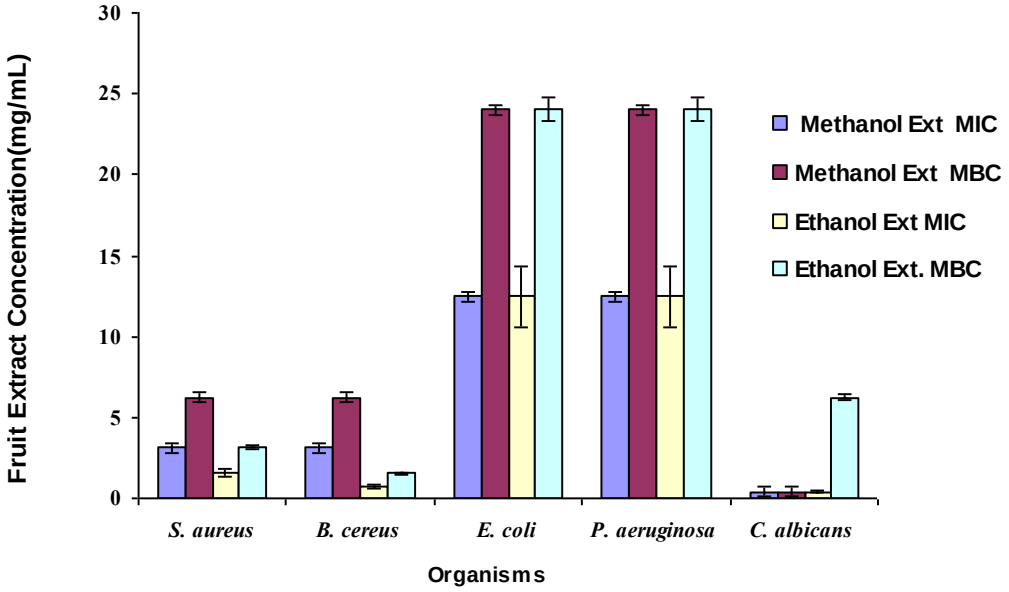

Figure 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fruit ethanol and methanol extracts of *Rubus sanguineus* against the test organisms.

with these pathogens effectively. At the same time high MIC and MBC values however, is an indication of lack of efficacy of the plant extracts against the test organism.

The lowest MIC and MBC values were recorded for methanol leaf extracts with 0.37 and 0.78 mg/mL, respectively against *C. albicans.* Low MIC and MBC values were read when the methanol leaf extracts were tested against Gram positive bacteria (*S. aureus* and *B. cereus).* The MIC readings were in range of (0.78 to 1.56 mg/mL) and MBC readings were in range of (1.56 to 3.13 mg/mL). Whereas high MIC and MBC values were recorded when the extracts were tested against Gram negative bacteria (*E. coli* and *P. aeruginosa*). This result is in accordance with the poor activity noticed in agar diffusion tests. However, the MIC and MBC for the ethanol leaf extracts showed the best activity against Gram positive bacteria and the values were 1.56 and 3.13 mg/mL, respectively (Figure 1).

In Figure 2 the MIC and MBC of fruit ethanol and methanol extracts of *R. sanguineus* are presented against the test microorganisms. Based on the results showed in Figure 2, the MIC value of 0.39 mg/mL is equal to MBC value of the methanol fruit extracts when tested against *C. albicans*. Interestingly, the same MIC value was recorded for the ethanol fruit extract against the yeast; however the MBC was 6.25 mg/mL. Whereas the ethanol fruit extract showed good activity against *B. cereus* with MIC and MBC values 0.78 and 1.56 mg/ml, respectively. Furthermore, MIC and MBC were of 1.56 and 3.13 mg/mL, respectively when the extract was screened for its activity against *S. aureus*.

DISCUSSION

Plant extracts are considered to be valuable source of biologically active compounds showing significant antimicrobial in several cases. In this study, the antimicrobial activity of *R. sanguineus* leaf and fruit ethanol and methanol extracts was assessed against different bacteria and *C. albicans*. These organisms are associated with different types of infections including urinary tract infections, wound infections, gastroenteritis, food poisoning, pneumonia and meningitis (Jawetz et al., 2010).

Results recorded by agar well diffusion method indicated that, the strongest antibacterial activity was obtained for the ethanol extract of *R. sanguineus* leaf against *B. cereus* (zone of 22 mm), followed by methanol extract of the fruit (zone of 20 mm) and by both the ethanol fruit and methanol leaf extracts (zone of 18 mm).

The leaf ethanol extract of *R. sanguineus* showed the highest antibacterial activity against *S. aureus* (zone of 20 mm) and the other extracts had the same activity of 18 mm. Whereas the Gram negative bacteria had showed resistance to all the studied extracts and that was manifested with no inhibition zones in agar well diffusion experiments. The extracts' activities were recorded same as DMSO (25 v/v %) activity which was used as a solvent control in the study.

Among the leaf and fruit methanol and ethanol extracts of *R. sanguineus*, the ethanolic extract of the leaf showed high zone of inhibition with diameter of 20 mm against *C. albicans*. This result was not in full support according to microdilution broth experiments. The lowest MIC value

was recorded for the ethanol and methanol fruit extracts and methanol leaf extracts (MIC = 0.39 mg/mL), this was followed by the ethanol leaf extract activity with MIC equals to 1.56 mg/mL against *C. albicans* . The differences in the observed activity of the various extracts may be caused by the varying solubilities of the active ingredients in the primary solvents (ethanol and methanol) and the secondary solvent which was DMSO (25 v/v %). It is well known that different solvents have diverse solubilities capacities for different phytoconstituents (Marjoriue, 1999). The other explanation might be due to the presence/ absence of one or more ingredient(s) different in dextrose sabouraud broth and dextrose sabouraud agar which influence or blocks the active phytoconstituents.

Results from microdilution experiments showed that the MIC and MBC values of the ethanol extract of *R. sanguineus* leaf against *B. cereus, and S. aureus* were 1.56 and 3.13 mg/mL, respectively. While the MIC and MBC values of the fruit methanol extract were 3.13 and 6.25 mg/mL, respectively. At the same time high MIC and MBC values for the extracts against *P. aeruginosa,* and *E. coli* is an indication of lack of efficacy of the plant extracts against the test bacteria and/or the possibility that the bacteria may possess the capacity to develop resistance against the plant extracts. However, the observed low MIC and MBC values against candida and Gram positive bacteria means that the plant has the potential to treat any ailments associated with these pathogens effectively.

Conclusion

Demonstrating the antimicrobial activity of leaf and fruit extracts of *R. sanguineus* against some pathogenic Gram positive bacteria and *C. albicans* is an indication that this plant might be considered as an alternative therapy to antibiotics for developing novel antimicrobial agents.

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Full Length Research Paper

Isolation and characterization of a *Bacillus* strain for alkaline wastewater treatment

Kun Chen[1, 2], Jing Yang[1, 3] and Hua Zhao[1, 3]*

[1]Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, College of Bioengineering, Tianjin University of Science and Technology, Tianjin, China.
[2]School of Pharmaceutical, Liaocheng University, Shandong, China.
[3]Tianjin Key Laboratory of Industrial Microbiology, Tianjin, China.

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Biological treatment is one of the considerable choices for removing of organic pollutants present in petrochemical wastewaters. In this study, five strains, named as BS1, BS2, BS3, BS4 and BS5, were isolated from sludge close to a petroleum smelter. BS5, the isolate with the highest chemical oxygen demand (COD) removal rate, was identified as *Bacillus flexus*, based on 16S rDNA sequences. Subsequently, the optimized COD removal conditions of BS5 were investigated. It was indicated that the optimal conditions were 0.5% corn starch, 1% corn steep liquor, 35°C pH 7.5, and 10% (v:v) inoculation size. Under such circumstance, the removal rate of COD can reach 81.04%. The isolation of *Bacillus flexus* strain BS5 provided an alternative for the bioremediation of alkaline wastewater. Lastly, the study showed that consecutive disposal process may help to reducing COD of wastewater effectively.

Key words: Alkaline wastewater, *Bacillus flexus*, chemical oxygen demand (COD).

INTRODUCTION

Petrochemical production technology had became more sophisticated in recent years, in turns, organic substances in the sewage tends to be prosperous, which caused a great burden to traditional treatment process (Rao et al., 2007). Sewage in petroleum processing industry was mainly resulted from liquid hydrocarbon alkali refining, diesel alkaline cleaning, and ethane pyrolysis gas alkaline cleaning process and so on (Garcia et al., 2000). Such sewage contained neutral oil, volatile phenol, sulfide and other toxic and detrimental organic substance in abundance (Xie et al., 2007), which endowed it with a high chemical oxygen demand (COD), high total dissolved solids, high levels of volatile phenol and sulfide, and high alkalinity (Ma et al., 2006).

The sewage discharged contaminates the environment gravely and also has seriously impacts on the subsequent disposal process (Prisciandaro et al., 2005).

Nowadays, most of sewage disposal techniques based on the recovery of the naphthenic acid and phenol (Choe et al., 2005), while the alkaline residue wastewater should be processed after the recovery (David and Peter, 1995).

Modern biotechnology, especially molecular techniques, to select and cultivate the dominant strains so to enhance the biological systems removal capability to refractory organics (Richard, 1983). Comparing with other methods, this method has many advantages, such as lower cost, higher efficiency, easier to be handled and without secondary pollution (Loh and Liu, 2001). All those characteristics attracted a great attention from all over the world (Leal et al., 1998). Many relevant studies had been carried out, including printing and dyeing, chemical waste water and removal characteristics (García Becerra et al., 2010).

In this study, bacteria with high efficiency of degrading alkaline wastewater had been screened from sludge

*Corresponding author. E-mail: zhaohua@tust.edu.cn. Tel.: +86 22 60601369. Fax: +86 22 60601369.

Table 1. Composition of the sublayer liquid.

Property	pH	COD (g/L)	Sulfide (mg/L)	Phenol (mg/L)
Value	6.73	58	996	41.39

nearby a petroleum smelter. Furthermore, a strain with the highest COD removal rate was identified and characterized for the possible applications for environmental management.

MATERIALS AND METHODS

Samples

Sludge samples were collected from a petroleum smelter.

Bacteria screening culture medium

Screening culture medium was LB medium which containing a different proportion of pretreated alkaline wastewater.

Alkaline wastewater obtained from the oil refinery should be pretreated. Firstly, 5.5% sulfate acids was added. 24 h later, the sublayer liquid was collected and used as experimental material. The main composition of the sublayer liquid obtained showed as Table 1.

Degrading medium

The degrading medium was consisted of pretreated alkaline wastewater, necessary salts for growth, extra carbon and nitrogen sources. The pH value was adjusted to 7.0. The medium was sterilized at 121°C for 20 min.

Isolation of strains degrading alkaline wastewater

Enrichment and isolation of bacteria degrading alkaline wastewater were done using bacteria screening culture medium.

Identification of the bacteria isolated

DNA extraction was adapted from the protocol previously (Hoisington et al., 1997). The 16S rDNA was amplified by PCR using the following universal primers pairs, 5'-AGTTTGATCCTGGCTCA-3' and 5'-ACGGCTACCTTGTTACGACTT GCA-3' (David and Peter, 1995). The protocol consisted of 30–33 cycles of incubation at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by extension for 5 min at 72°C. The 16S rDNA amplified was sequenced and blast before it was submitted to NCBI GenBank.

Method of chemical oxygen demand (COD) determination

The COD was determined by potassium dichromate method (Rocenkery, 1993). Wash culture tubes and caps with 20% H_2SO_4 before using to prevent contamination. Place sample in culture tube or ampule and add digestion solution. Carefully run sulfuric acid reagent down inside of vessel so an acid layer is formed under the sample-digestion solution layer and tightly cap tubes or seal ampules, and invert each several times to mix completely. Place tubes or ampules in block digester preheated to 150°C and reflux for 2 h behind a protective shield. Cool to room temperature and place vessels in test tube rack. Some mercuric sulfate may precipitate out but this will not affect the analysis. Remove culture tube caps and add small TFE-covered magnetic stirring bar. Add 0.05 to 0.10 mL (1 to 2 drops) ferroin indicator and stir rapidly on magnetic stirrer while titrating with standardized 0.10 M FAS (ferrous ammonium sulfate titrant). The end point is a sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample.

Determination of the optimized alkaline-degrading condition

The effects of different carbon sources, nitrogen sources, pH, temperature, inoculum concentration and consecutive treatment process on COD removal rate were investigated.

RESULTS AND DISCUSSION

Isolation of strains with high alkaline wastewater degrading activity

Five bacteria exhibiting high alkaline wastewater degrading activity were isolated after serial enrichment, which called BS1, BS2, BS3, BS4 and BS5 respectively.

The COD removal rate of the five optimized strains was compared as Figure 1. It was showed that BS5 had the highest COD removal rate, which was up to 73.42%.

Identification of BS5

The genome DNA of BS5 was extracted and its 16S rDNA was amplified by PCR. The 1067 bp product was sequenced and analyzed. It was identified as *Bacillus flexus*. The 16S rDNA sequence was submitted to NCBI GenBank and the accession number was JX677863.

Effects of nutrient substances on the chemical oxygen demand (COD) removal rate of BS5

In the processes of alkaline wastewater treatments, bacteria make use of all kinds of organic pollutant as nutrition for their growth and proliferation. But these nutrients in industry wastewater cannot fully meet the need of these demands. So, some extra nutrition should be added.

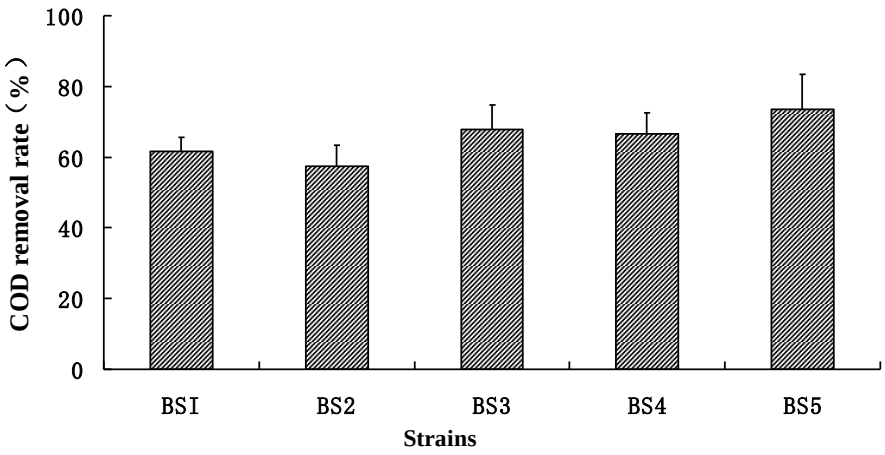

Figure 1. COD removal rate of five strains. The strains were inoculated into pretreated alkaline wastewater, respectively, and then cultured at 35°C at 150 r/min, the pH was control at 7.0. Fifty hours later, the COD value was measured.

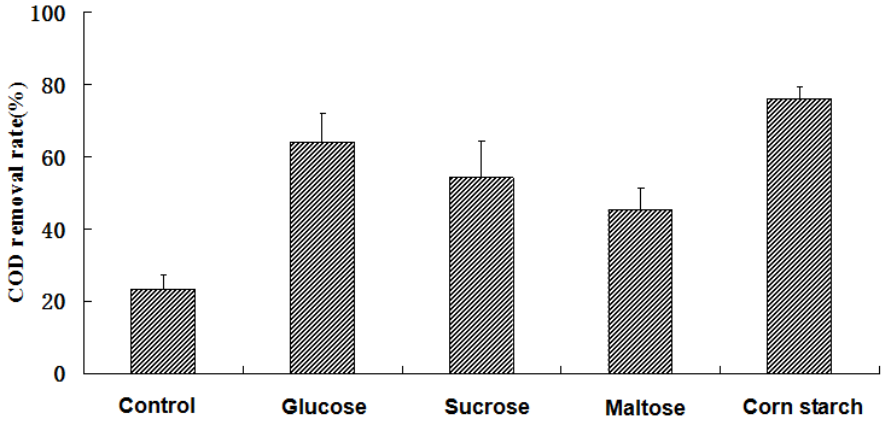

Figure 2. Effects of different carbon sources on the COD removal rate. BS5 was inoculated into degrading medium. 0.5% (mm) glucose, sucrose, maltose and corn starch were added respectively, inoculum size 10%, then cultured at 35°C at 150 r/min, the pH was control at 7.0. Fifty hours later, the COD value was measured.

Effects of different carbon sources on the COD removal rate

Glucose, sucrose, maltose and corn starch were chosen as carbon source, respectively. The group without extra carbon source was used as control. As shown in Figure 2, the addition of carbon source remarkably increased the COD removal rate of the alkaline wastewater. It was proved that the best carbon source was corn starch.

Effects of different nitrogen sources on the chemical oxygen demand (COD) removal rate

Ammonium nitrate, ammonium sulfate, bran, yeast powder and corn steep liquor was selected as extra nitrogen source, respectively. The group without additional nitrogen source was used as control. It was showed that these nitrogen sources can increase the COD removal rate dramatically (Figure 3). Corn steep liquor was performed as the optimal nitrogen source.

Besides nutrition, the physical culture conditions can also have dramatically influences on the wastewater disposal process.

Effects of temperature on the chemical oxygen demand (COD) removal rate

The effects of temperature on the COD removal rate of

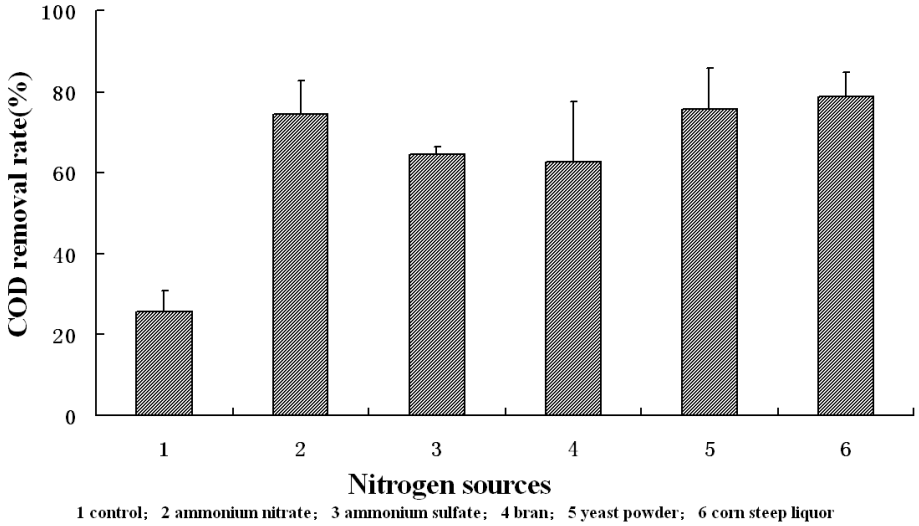

Figure 3. Effects of different nitrogen sources on the COD removal rate. BS5 was inoculated into degrading medium. 1% (m:m) ammonium nitrate, ammonium sulfate, bran, yeast powder and corn steep liquor was added, respectively. Corn starch (0.5%) was used as carbon source. The culture condition were inoculum size 10%, 35°C, pH 7.0, and agitation rate 150 r/min. Fifty hours later, the COD value was measured.

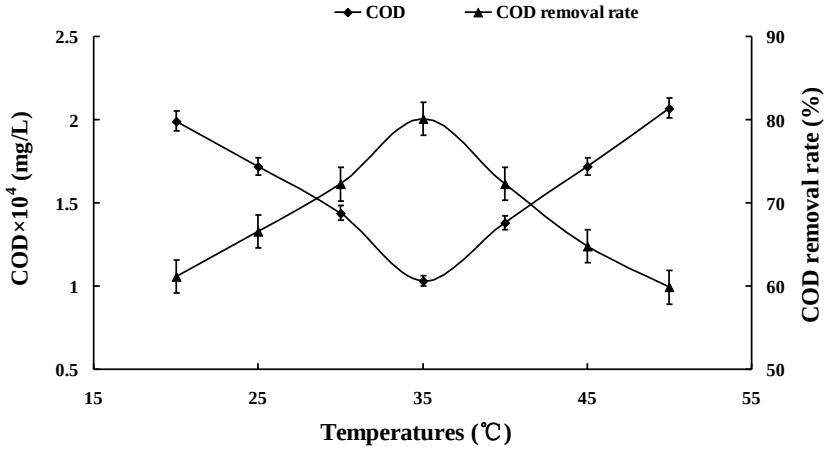

Figure 4. Effects of temperature on the COD removal rate. BS5 was inoculated into degrading medium and cultured at temperatures ranging from 20°C to 50℃. The culture condition were 0.5% corn starch, 1% corn steep liquor, inoculum size 10%, pH 7.0, and agitation rate 150 r/min. Fifty hours later, the COD value was measured.

BS5 in degrading medium were studied. It was showed that the optimum growth temperature for higher degrading rate was 35°C. When the temperature was less than 25°C or more than 40°C, the COD removal rate decreased significantly (Figure 4).

Effects of pH on the chemical oxygen demand (COD) removal rate

The effects of pH on the COD removal rate were tested. It was obviously presented that the optimum pH value for COD removal was 7.5 (Figure 5). When pH < 6.0, COD removal rate was less than 50%. If pH was higher than 6.0, the COD removal rate increased rapidly while pH increased. Until pH was 7.5, COD removal rate reached its peak. If pH>7.5, the COD removal rate decreased dramatically.

Effects of inoculum size on the chemical oxygen demand (COD) removal rate

The influence of inoculum size on the COD removal rate

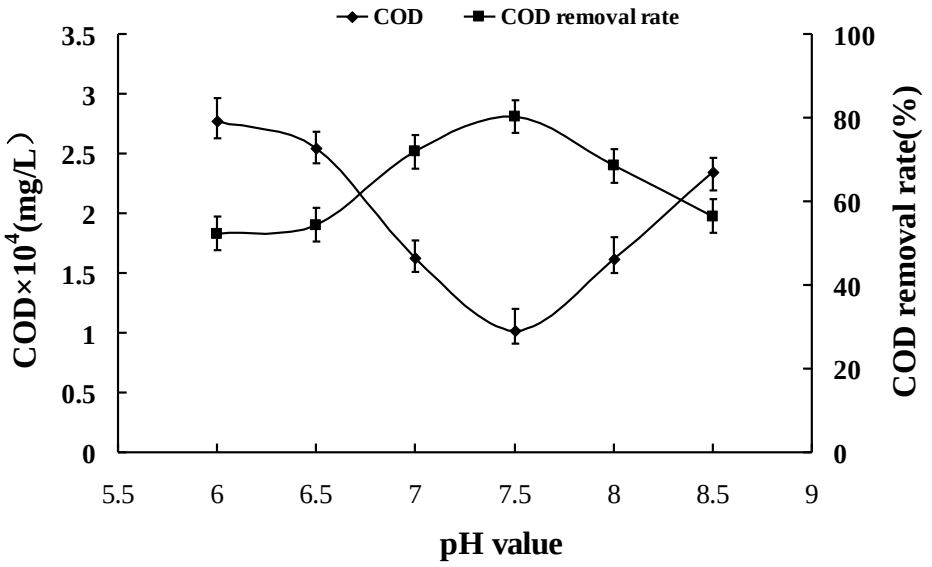

Figure 5. Effects of pH on the COD removal rate. BS5 was inoculated into degrading medium at pH 6.0, 6.5, 7.0, 7.5, 8, and 8.5, respectively. The condition of culture was as following, the volume of the alkaline wastewater was 30 mL/250mL, 0.5% corn starch, 1% corn steep liquor, 10% inoculum size, then cultured at 35°C at 150 r/min. Fifty hours later, the COD value was measured.

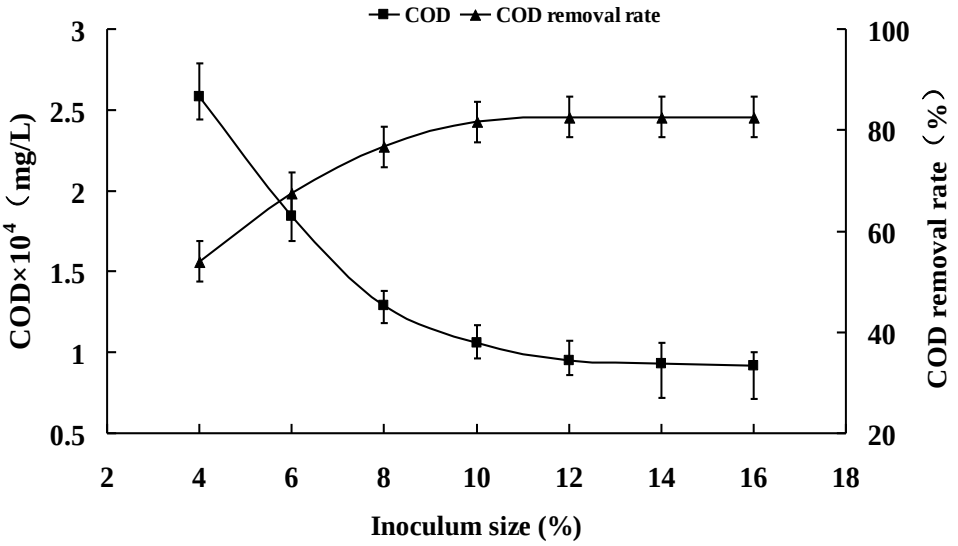

Figure 6. Effects of inoculum size on the COD removal rate. BS5 was inoculated into degrading medium at six different inoculum size (4, 6, 8, 10, 12, 14 and 16%). The condition of culture was as following, the volume of the alkaline wastewater 30 mL/250mL, 0.5% corn starch, 1% corn steep liquor, then cultured at 35°C, pH 7.5, at 150 r/min. Fifty hours later, the COD value was measured.

was investigated. Six different inoculum sizes (4, 6, 8, 10, 12, 14 and 16%) were test. It could be concluded from the results presented in Figure 6 that inoculum size would have a major influence on bacteria breeding and COD removal rate. When inoculum size was low, the degradation was lower obviously. When the inoculum size increased, the COD removal rate increased gradually. The COD removal rate reached 81.04% while the inoculum size was 10%. But the increment of COD removal rate was not obvious if inoculum size continued to increase.

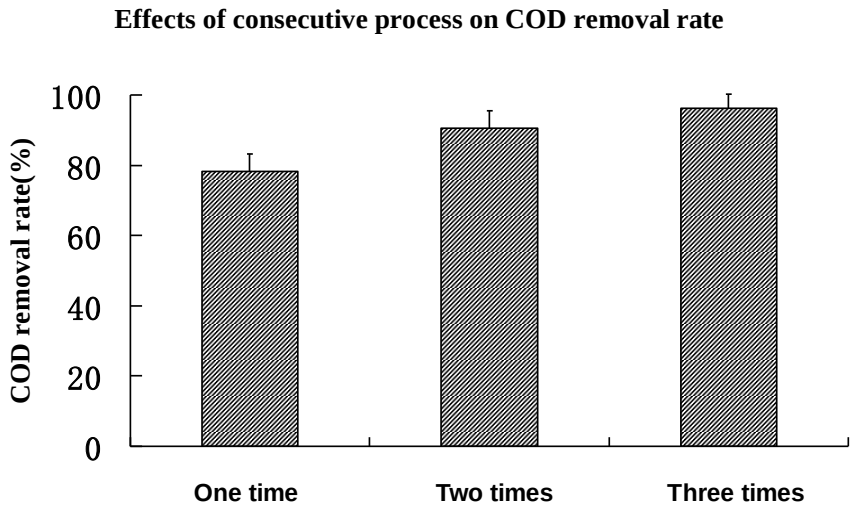

Figure 7. Effects of consecutive process on COD removal rate. Firstly, treated alkaline wastewater at the speed 4000r/min, 10 min, and take 30 mL clear liquid to 250 mL flask. The culture condition were 0.5% corn mill, 1% corn steep liquor, 35°C, pH 7.5, 10% inoculum size and agitation rate 150 r/min. Fifty hours later, the COD value was measured. The wastewater disposed by one, two, three times process, and they correspond COD removal rate was showed.

So the optimum inoculum size was set as 10%.

Effects of consecutive treatment on the COD removal rate

The performance of optimized strain BS5 was stable by taming.TheCODofalkalinewastewaterobviouslydecreased under the optimized condition after 50 h treatment with the bacteria. But when the initial COD of wastewater was very high, the COD of treated wastewater cannot satisfy the demand of discharge or utilization standards. Take it into account, consecutive treatment processes may be necessary.

The wastewater disposed for one, two or three times process, and the corresponding COD removal rate was showed. According to the data presented in Figure 7, the aim of consecutive treatment on the COD removal rate was achieved. After the first round treatment, the COD removal rate was up to 78. 3%. Whereas, the second round treatment culture enabled the COD removal rate reached 90.5%. Through a third disposal process, the COD removal rate was 96.2%. It was considered that consecutive disposal process may help to reducing COD of wastewater effectively.

Conclusion

The organic pollutants in petrochemical and oil refining which are resistant to degradation can be dreadfully hazardous to human health. As they persist in the environment, they are capable of long range transportation, bioaccumulation in human and animal tissue and biomagnification in food chain (Robles et al., 2000). Thus treatment of alkaline wastewaters is necessary and biological methods are the most appropriate techniques due to mineralization of toxic organic compounds and inexpensiveness (Prieto et al., 2002).

The use of microbial catalysts in the biodegradation of organic compounds has advanced significantly during the past days. It has been found that large numbers of microbes co-exist in almost all natural environments. Identification of effective microbial species is considered as one of the important priorities for production of the biomass in order to achieve desirable kinetic of biological reactions (Liu et al., 2002).

At the same time, several external factors can limit the rate of biodegradation of organic compounds. These factors may include temperature, pH, oxygen content and availability, substrate concentration and physical properties of contaminants. Each of these factors should be optimized for the selected organism for the maximum degradation of the organic compound of choice.

In this study, strains degrading alkaline wastewater were isolated from the sludge nearby petroleum smelter and enriched, among which BS5 performed the highest degradation ability. Furthermore, BS5 was identified as *Bacillus flexus* through 16S r DNA and some other data (not showed in this paper).

Data presented in this study demonstrated that the strain's optimum disposal condition should be 0.5% corn starch, 1% corn steep liquor, temperature 35°C, initial pH 7.5, 10% inoculation size. In such conditions, the removal

rate of COD can be up to 81.04% and almost 10.38% higher than before. Meanwhile, successive process was developed to enhance the degradation efficiency.

In conclusion, a *Bacillus flexus* strain characterized stable performance and inexpensive cost in alkaline wastewater treatment was isolated and identified, which has prospective application values in this area.

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Full Length Research Paper

Seasonal variation of citrinin in traditionally brewed African beer

B. K. Ikalafeng[1], E.J. Pool[2], R. Lues[1] and W. H. Groenewald[1]*

[1]Unit for Applied Food Science and Biotechnology, Central University of Technology, 20 President Brand Street, Bloemfontein 9300, South Africa.
[2]Department of Medical Bioscience, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.

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A seasonal study on citrinin concentrations in traditional beer was conducted using a standardised enzyme-linked immunosorbent assay. Representative samples of indigenous South African beer were collected during the hot, mild and cold seasons from marginal-urban settlements in the Kimberley area, South Africa. The mean temperature and humidity readings were 37°C and 29% for respectively summer, 19.4°C and 7.1% for spring, and 16.3°C and 6.5% for winter. Samples were analysed for the mycotoxincitrinin using a commercial Ridascreen (citrinin) ELISA kit, validated for the purpose of the study. The recovery level achieved for citrinin in beer samples using the kit was 117 ± 15%. The inter-assay and intra-assay variations were 17.8 and 14.8% respectively. Standard curves prepared using traditional beer samples were similar to the standard curves prepared using the kit standards. The mean citrinin concentrations found in the various samples were 257.6 µg/kg, 99.0 and 185.7 µg/kg for the summer, spring and winter seasons respectively and the levels ranged between 35.6 µg/kg (min) and 924.2 µg/kg (max). Statistically significant differences (p ≤0.05) were found between the citrinin levels of the summer and spring samples and also between citrinin levels of the spring and winter samples (p ≤0.001). The relatively high levels of citrinin in selected samples may, with prolonged exposure, have adverse impacts on the health of consumers. Thus further studies into the origin and levels of this mycotoxin should be a priority.

Key words: Citrinin, traditional beer, seasonal variation.

INTRODUCTION

Citrinin is a fungal metabolite and a common food contaminant (Flajs and Peraica, 2009) which can cause the deterioration of liver or kidney function in animals (Chan and Shiao, 2007; Sweeney and Dobson, 1998). Citrinin has been reported in foods by several authors and its presence, particularly in fermentation products, is thought to be a potential threat to public health (Bennett and Klich, 2003; Pitt et al., 1996). Krejci et al. (1996) found that food contaminated with *Penicillium citrinum* caused functional and morphological renal damage and resulted in increased urination. Ingestion of these mycotoxins by humans occurs mainly through plant-based foods, but also as residues and metabolites present in animal-derived foods.

Citrinin is produced by moulds such as *Aspergillus* and *Penicillium* and the species most widely recognised for producing this mycotoxin are *Penicillium citrinin*and *Penicillium expansion,* although *Penicillium verrucosum* is also known to produce citrinin (Watanabe, 2008). Fungal species that produce citrinin are predominantly mesophilic and grow at a temperature range of 5 to 40°C with an optimum of between 26 and 30°C.

*Corresponding author. E-mail: wgroenewald@cut.ac.za.

The production of citrinin occurs from 15 to 37°C (Roberts et al., 1996). Environmental factors such as temperature, humidity and rainfall also play a role in fungal growth which may result in mycotoxin production. Citrinin levels varying from 0.28 and 6.29 μg/g in *Monascus* fermentation products have been reported by Liu et al. (2005). In countries such as Denmark samples of barley and oats have been reported to be contaminated with 160 to 20000 $\mu g.kg^{-1}$ of Citrinin (Lillehoj and Goransson, 1980). Whilst several studies have also reported on the occurrence of mycotoxins in traditional beer produced from malt, as well as several other cereal-based commodities (Zinedine and Manes, 2009; Samar et al., 2007; Wolf-Hall, 2007; Odhav and Naicker, 2002; Scott, 1996). The maize used in the brewing process has frequently been reported to be contaminated with fungi (Hazel and Patel, 2004; Bullerman and Bianchini, 2007). Apart from maize, mycotoxin-producing fungi have been reported to also infect barley, wheat, corn, fruit and sorghum (Zinedine and Manes, 2009; Larsen et al., 2004). Most mycotoxins are chemically stable and as a result tend to survive storage and processing even at high temperature.

The aim of this study was to validate the Ridascreencitrinin enzyme-linked immunosorbent assay (ELISA) for screening indigenous South African beer for the occurrence of citrinin as well as to investigate possible seasonal variations of citrinin concentrations in traditional beer, brewed in the greater Kimberley area.

MATERIALS AND METHODS

Sampling Protocol

Seventy beer samples were collected from various traditional beer brewers during spring, summer, and winter in the greater Kimberley area, South Africa. Brewers of indigenous South African beer were selected using the stratified method and the samples were collected in the early hours of the morning from freshly produced supplies using sterile sampling bags (Whirl-pack, NASCO). The samples were transported on ice to the laboratory, mixed thoroughly and centrifuged for 5 minutes at 1000 x g to separate the supernatants from the cells. The supernatants were aliquoted and stored in 96 well trays.

Quantification of citrinin in beer samples using enzyme-linked immunosorbent assay (ELISA)

Ridascreencitrinin ELISA kits (AECI Amersham, South Africa) were used to analyse the mycotoxins and the manufacture`s protocol was followed. The sample or standard (50 μl per well) and 50 μl per well of anti-citrinin antibody were added to citrinin coated plates, agitated and incubated for 10 min at room temperature. After incubation the plates were washed four times with 250 μl per well deionized H_20. The plates were then dried and 100 μl of the secondary antibody conjugate solution was added to the wells. The reaction was allowed to proceed for 10 minutes at room temperature and unreacted antibodies were removed by washing three times with 250 μl of deionized H_20 per well. Two drops of chromogen substrate solution were added to each well, mixed and incubated in the dark for 5 minutes at room temperature after which the reaction was terminated by the addition of a stopping solution. The plate was read on a plate spectrophotometer at 450nm and the percentage absorbance was calculated for all the wells using the 0 ppb citrinin standard as the reference. The kit standards were used for the construction of a standard curve obtained by plotting percentage absorbance against citrinin concentrations. The resulting values were read from this standard curve.

Validation of the Ridascreen enzyme-linked immunosorbent assay (ELISA) for citrinin

The inter- and intra-assay variation of the Ridascreen ELISA for citrinin was determined to ascertain whether assays were reproducible. Samples were assayed in triplicate. The mean and standard deviations for the samples were calculated and the results were used to determine inter- and intra-assay variations.

RESULTS

Validation of the Ridascreen enzyme-linked immunosorbent assay (ELISA) citrinin for traditional beer

The citrinin recovery level was 117 ± 15% and the inter- and intra- assay variations were 17.9% and 14.9% respectively (data not shown). Figure 1 shows the results obtained using kit standard and beer standard. The curve obtained using beer samples is parallel to the curve obtained using the standards provided with the kit.

The screening of traditional beer for citrinin using Ridascreen enzyme-linked immunosorbent assay (ELISA) kits

Citrinin was detected in all the samples, but in varying concentrations during the different seasons. The mean citrinin concentrations found in the various samples were 99.0, 257.6 and 185.7 μg/kg for the spring, summer, and winter seasons respectively and the levels ranged between 35.6 μg/kg (min) and 924.2 μg/kg (max). Citrinin concentrations during spring and summer were found to differ significantly ($P \leq 0.05$). This was also the case between the spring and winter ($P \leq 001$) sample sets (Figure 2).

DISCUSSION

The results obtained from this study differed from those obtained in a previous study on South African beer which reported that citrinin was not detectable in beer, as it is destroyed during the mashing step (Odhav and Naicker, 2002). The levels of citrinin detected in the present study have likely been determined by the climatic conditions as well as the more sensitive Ridascreen method used for detection. Because of the difference in humidity between the different collection periods, it would be expected that

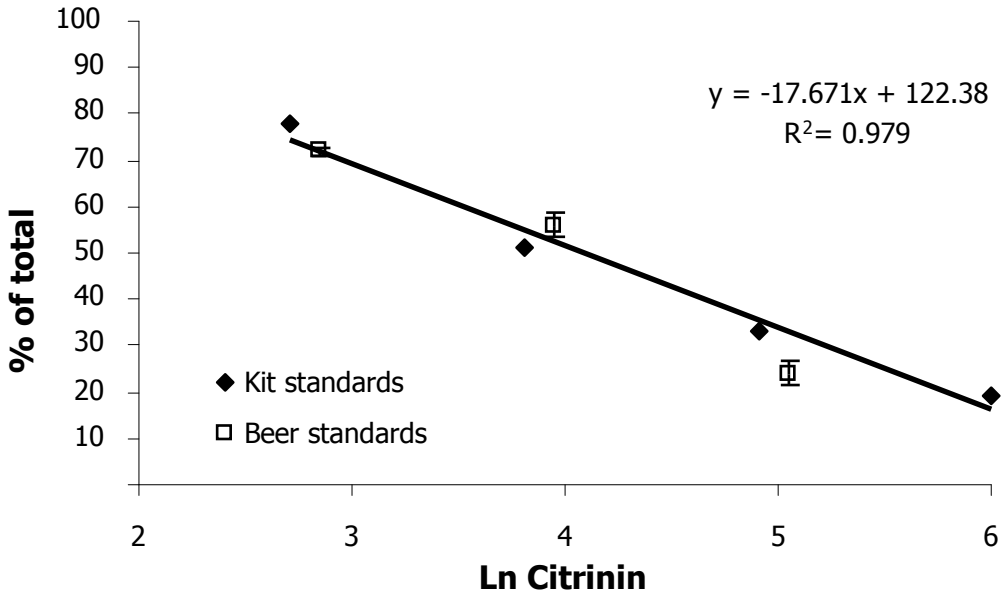

Figure 1. A comparison between the citrinin kit standard curve and standard curve using dilution series of indigenous traditional beer (Ln transformation).

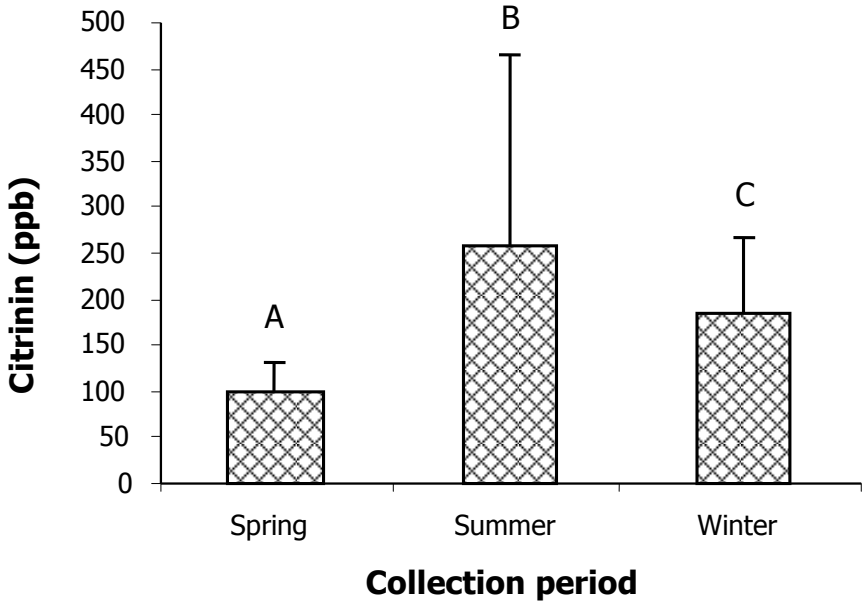

Figure 2. A comparison of the citrinin concentrations (mean) in samples collected at different seasons of the year (A/B significant $P \leq 0.05$; A/C significant $P \leq 0.001$).

there would be differences between the citrinin levels of the samples collected at these intervals. The citrinin levels in the samples collected during summer and spring, when the humidity and temperature were high, were found to be significantly higher than during the other seasons. The growth of fungi is known to be enhanced by high temperatures and humidity (Schrodter, 2004) and the findings of this study thus substantiate reports that countries with warmer climates are prone to mycotoxin-contaminated grains and consequently to beers containing toxins (Magan et al., 2011; Raghavender and Reddy, 2009). The levels of citrinin detected in this study could pose serious health risks to consumers, especially considering citrinin can accumulate in the liver (Gao et al., 2010). Habitual consumers of these brewers therefore have greater exposure to the harmful effects of this myco-

toxin. Prevention of and/or monitoring of the production process in areas where traditional beer is brewed and consumed in large quantities are thus important.

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UPCOMING CONFERENCES

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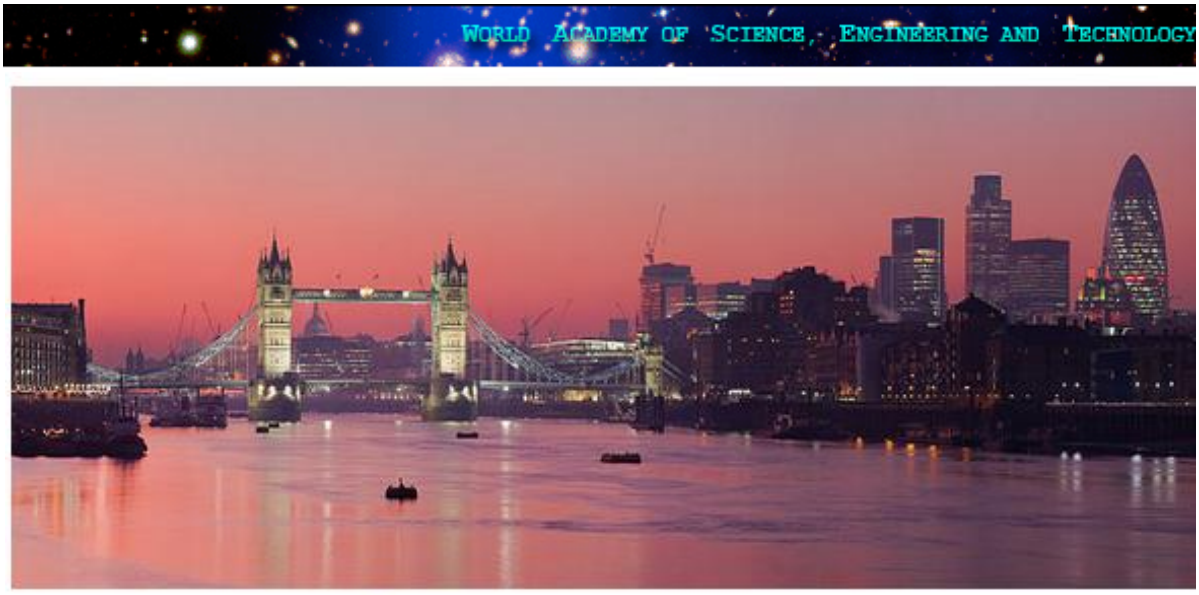

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