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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

Genetic diversity and phosphate solubilizing ability of *Triticum aestivum* rhizobacteria isolated from Meknes region, Morocco

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The objective of this work was to isolate, screen and evaluate *in vitro*, the phosphate solubilization activity of rhizobacteria isolated from *Triticum aestivum* rhizosphere soil in the Meknes region of Morocco. Five best isolates from 240 ones were selected based on the solubilization of insoluble phosphates (Ca_3 $(PO_4)_2$) in both agar plate and broth assays using National Botanical Research Institute's phosphate (NBRIP) medium. The bacterial isolates were identified based on their phenotypic and 16S rRNA genes sequencing data as *Variovorax paradoxus* (BT1), *Pseudomonas reinekei* (BT4), *Pseudomonas libanensis* (BT10), *Pseudomonas lurida* (BT11) and *Pantoea agglomerans* (BT12). P solubilization index of these isolates ranged from 2.88 to 3.48, amount of phosphate solubilized ranged from 24.23 to 56.95 mg P L^{-1} and drop in pH of the medium ranged from 7 to 3.2. All the isolated PSB were efficient phosphate solubilizers and can be used as bioinoculants to increase the available phosphorus in the soil for wheat plant growth.

Key words: *Triticum aestivum*, rhizobacteria, rhizosphere, phosphate, solubilizing capacity.

INTRODUCTION

Phosphorus is one of the major plant nutrients limiting plant growth. Most agricultural soils contain large reserves of P, a considerable part of which has accumulated as a consequence of regular applications of chemical fertilizers. A deficiency in soluble P for many agricultural soils is one of the major factors hampering crop production worldwide (Arcand and Schneider, 2006). It is now becoming urgent to reduce the environmental impact of agriculture and, for example by replacing the expensive soluble chemical P fertilizers by novel, cheaper, more ecological but nevertheless efficient P fertilizers (Macias et al., 2003). It is well known that a considerable number of phosphate solubilizing bacteria (PSB) have the ability to solubilize insoluble mineral P by producing various organic acids, siderophores, mineral acids, protons, humic substances, CO_2 and H_2S, and release soluble P (Illmer and Schinner, 1995; Ivanova et al., 2006; Song et al., 2008). Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s (Kudashev, 1956; Krasilinikov, 1957). The maintenance

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of high level of soil phosphorus has been a major challenge to agricultural scientists, ecologists and farm managers because in most of the soils, phosphate is present in unavailable form due to complex formation with Ca^{2+}, Al^{3+}, Fe^{2+} or Mn^{2+} depending on soil pH and organic matter. The main problem of phosphorus in soil is its rapid fixation and the efficiency of P solubilization rarely exceeding 10–20%. The fixed forms of P in acidic soils are aluminium and iron phosphates while in neutral to alkaline soils as calcium phosphates (Kuhad et al., 2011). Release of P by PSB from insoluble and fixed / adsorbed forms is an import aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P, and prevents it from adsorption or fixation (Khan and Joergesen, 2009). Accordingly, it is reported that the phosphate-solubilizing bacteria (PSB) when applied with PGPR could reduce P fertilizer application by 50% without any significant reduction in crop yields (Jilani et al., 2007; Yazdani et al., 2009). The aim of this study was to isolate and identify efficient phosphate solubilizing bacteria (PSB) from *Triticum aestivum* rhizosphere soils of different regions of Meknes.

MATERIALS AND METHODS

Soil samples and isolation of PSB

During the fall of 2012, in Meknes area (33°53'42" North, 5°33'17" West), the soils were classified as alkaline, vertisols, according to the Provincial Directorate of Agriculture of Meknes (http://www.dramt.e-makane.net/site/dpameknes.htm). Soil samples were collected from rhizosphere of wheat variety Marchouch (National Institute of Agronomic Research, Morocco, selected in 1984). The samples were stored at 4°C in sterile containers. Soil samples of each soil were mixed thoroughly. Phosphate solubilizing bacteria were isolated from soil samples by serial dilution using spread plating on NBRIP medium (Nautiyal, 1999) supplemented with tricalcium phosphate as insoluble inorganic phosphate source, and incubated at 27°C for 24-48 h. Colonies showing clear zone of P-solubilization were counted as PSB (Gyaneshwar et al., 1999). Different types of single, well separated colonies, from each sample site, which grew on plates showing clear zones were picked and restreaked on to fresh NBRIP solid medium. This procedure was repeated until pure culture with high P solubilization was obtained. Once purified, each isolate was stored as a glycerol 40% stock at -80°C.

Qualitative evaluation of phosphate solubilization in agar assay

All bacterial strains were tested by an agar assay using National Botanical Research Institute's phosphate (NBRIP) medium supplemented with tricalcium phosphate. Each isolate was assayed by spotting 10 µl of cultures on the media plates. The halo and colony diameters were measured after 10, 20 and 30 days of incubation of the plates at 27°C. The ability of the bacteria to solubilize insoluble phosphate was described by the solubilization index (SI) = the ratio of the total diameter (colony + halo zone) to the colony diameter (Edi-Premono et al., 1996).

Quantitative estimation of phosphate solubilization in broth assay

The quantitative bioassay was carried out using Erlenmeyer flasks (250ml) containing 50 ml of NBRIP broth medium supplemented with (Ca_3 $(PO_4)_2$) and inoculated by 200 µL of bacteria (5×10^8 CFU ml^{-1}). Autoclaved uninoculated NBRIP medium served as control. The flasks were incubated on rotary shaker (180 rpm) at 30°C. After 168, 336 and 504 h of incubation, the growth medium was centrifuged at 10,000 rpm for 20 min. Supernatant was decanted and autoclaved at 121°C for 20 min. Autoclaved samples were then filtered through Whatman paper no. 42 followed by 0.2 µm millipore membrane and were used for the determination of the pH and the soluble P released into the solution. P was measured with molybdenum blue method as described by Murphy and Riley (1962). The pH of the supernatant was measured in each case by pH meter (Metrohm 620 pH meter, swiss made). All the data were an average of three replicates.

Phenotypic characterization

Morphological and biochemical identification of PSB were characterized for colony morphology, Gram staining and biochemical analysis (Holt et al., 1994). Isolates were also tested for catalase (Graham and Parker, 1964) and oxidase (Kovacs, 1956). The tolerance of strains in extrinsic and intrinsic environmental was tested by the ability of the PSB isolates to grow on media at several values of stresses.

Salt tolerance test

PSB were examined for their tolerance of salinity on yeast extract mannitol (YEM) agar plate (Vincent, 1970), medium supplemented with 0.5, 1, 1.5 and 2 gL^{-1} NaCl (Ben Romdhane et al., 2006).

Temperature tolerance

Temperature tolerance was tested by incubating the inoculated plates with PSB at 4, 20, 30, 40 and 50°C for 48 h (Hung et al., 2005).

pH tolerance test

The ability of the PSB isolates to grow on media at several pH values was tested by streaking cultures on the YMA plates at different pH values, the pH values were adjusted to 4.8, 5.8, 6.8 and 8.8 with either NaOH or HCl (Kucuk et al., 2006). Isolates were incubated at 27°C for 48 h.

Intrinsic antibiotic resistance

The bacteria were spread out on the YMA medium containing antibiotics discs of tetracycline (30 µg), streptomycin (25 µg), kanamycin (30 µg) and ampicillin (10 µg). The control to YMA medium without antibiotics discs. PSB were incubated at 27°C for 24 h.

Genotypic identification

The identification of PSB was done on the basis of 16S rRNA gene sequencing. The genomic DNA of PSB isolates was extracted by the kit of the platform "GenElute Bacterial Genomic DNA kit"

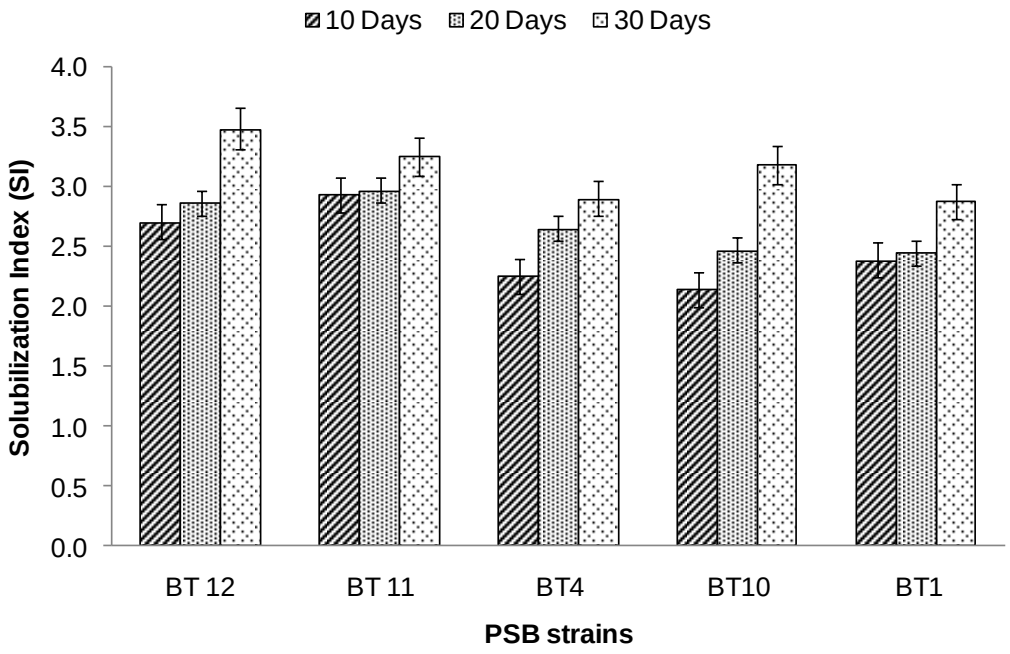

Figure 1. Solubilization index (SI) of PSB isolated from the rhizosphere of *Triticum aestivum* after 10, 20 and 30 days of incubation at 27°C.

(Sigma-Aldrich Corp., St. Louis, MO) according to the manufacturer's protocol. The primers fD1 (5'AGAGTTTGATCCTGGCTCAG-3') and rP2 (3'ACGGCTACCTTGTTACGACTT-5') were used for amplification of 16S rRNA gene (Weisburg et al., 1991). The total PCR reaction mixture was 25.0 µl in each tube comprising 2 µl dNTPs (10 mM), 0.125 µl each primer (100 mM), 2.5 µl PCR buffer (10x), 0.2 Taq DNA polymerase (5 U/µl), and 5 µl genomic DNA (30 ng/µl). The thermocycling conditions involved an initial denaturation at 96°C for 4 min, followed by 35 cycles of 96°C for 10 s, 52°C for 40 s, and 72°C for 2 min and final extension at 72°C for 4 min. Successful amplification of a 1500 bp DNA fragment was confirmed by running 5 µl of the PCR product on 1% agarose gel and sequenced by ABI 3130 XL (Applied Biosystems, CA, USA). Sequence data were aligned and compared with available standard sequences of bacterial lineage in the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST.

Statistical analysis

Simple correlation was run to determine correlation coefficients (r) by the method of Ordinary Least Squares (OLS). The result means were depicted diagrammatically using Microsoft Excel version 2010.

RESULTS

Isolation and qualitative evaluation of PSB in agar assay

A total of 240 bacterial strains were isolated out of which 52 strains (21.6%) showed clear zones but after repeated plating on NBRIP solid medium only five strains (2.08% of the total) preserved P-solubilization function. Fluctuations in solubilization index based on colony diameter and holozone for each PSB were observed during the thirty days observation period as presented in Figure 1. Solubilization index showed that among PSB, BT12 was most efficient phosphate solubilizer on NBRIP agar plate with SI = 3.48. BT12 followed by BT11 (3.25) and BT10 (3.18). Moderate solubilization index was observed in BT1 (2.88) and BT4 (2.90) isolated strains (Figure 1).

Quantification of P-solubilizing activity by PSB in broth assay

The PSB strains forming clear zones in NBRIP agar medium were able to release P from tricalcium phosphate in NBRIP broth medium. P release was observed from first week onwards and gradually increased until after 21 days (Figure 2). The final pH of the culture filtrate ranged from 3.7 to 3.1 starting at initial pH of 6.8 - 7.0 after 504 h, indicating acidic nature (Figure 3). PSB strains BT10 (56.95 mg P L^{-1}), BT12 (54.55 mg P L^{-1}) and BT1 (47.5 mg P L^{-1}) released high amount of P on 21 day, while BT4 (37.43 mg P L^{-1}) and BT11 (24.23 mg P L^{-1}) released low amount of P. Maximum P release was seen in BT10 and BT12 strains (56.95 and 54.55 mg P L^{-1}) as compared to all the other PSB strains (24.23 to 47.5mg P L^{-1}) (Figure 2). The relationship between final culture pH/solubilization index and concentrations of solubilized P in the culture released by PSB was analyzed using the method of ordinary least squares (OLS). From the

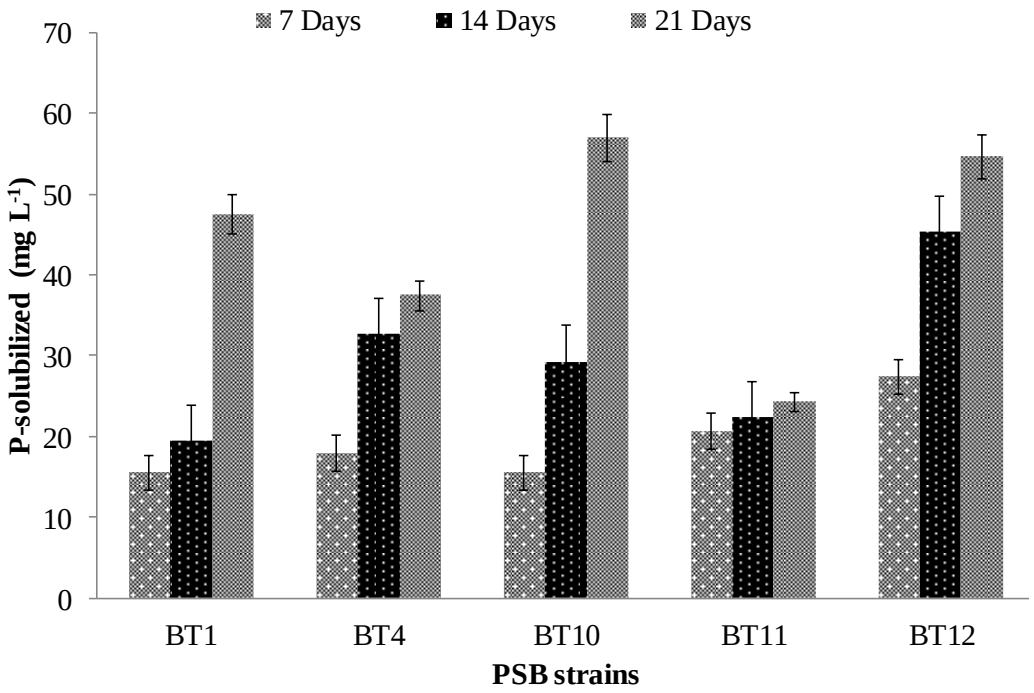

Figure 2. Change in P-solubilized in NBRIP liquid medium by the PSB strains isolated from the rhizosphere of *Triticum aestivum* after 7, 14 and 21 days of incubation.

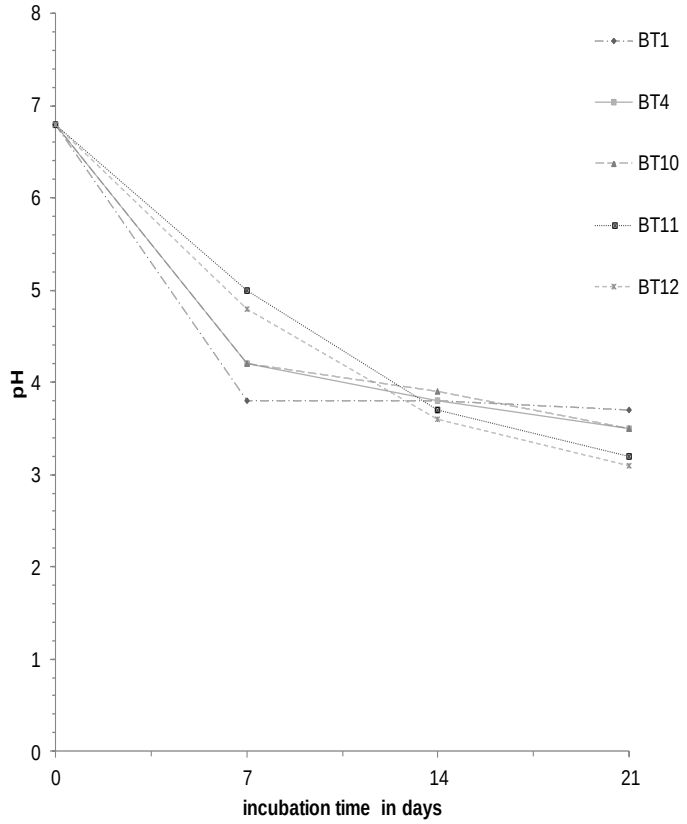

Figure 3. Change in pH values in NBRIP liquid medium by five selected PSB after 7, 14 and 21 days of incubation.

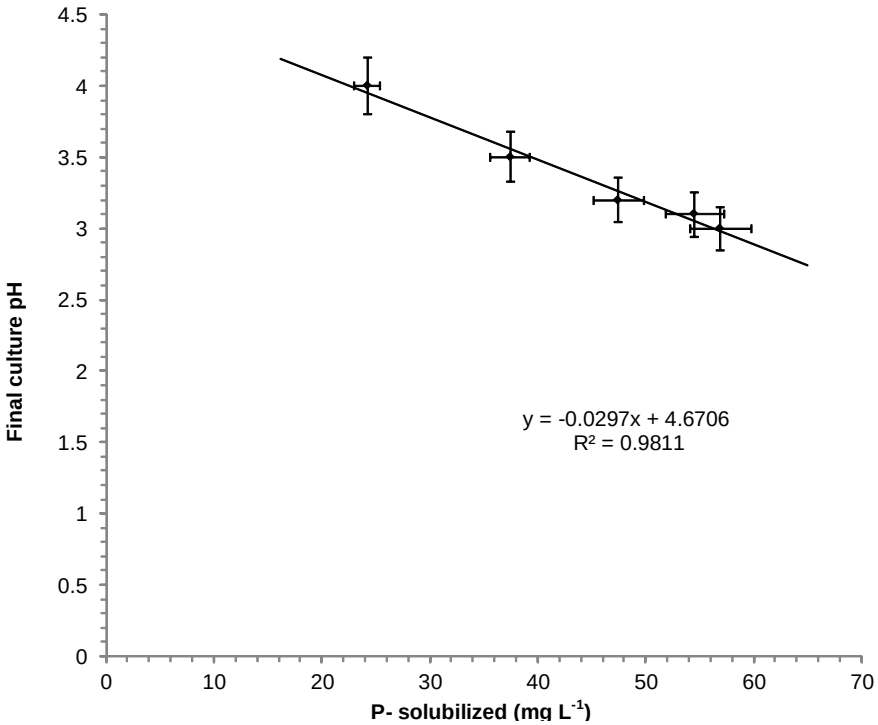

Figure 4. Correlation analysis between final culture pH and tricalcium phosphate solubilizing ability of phosphate solubilizing bacteria.

statistical analysis, it is clear that no significant positive correlation between the solubilization index (SI) and concentrations of solubilized P was found (r = 0.188) (Figure 5). For example, the isolate BT11 had a good SI (3.25) on NBRIP agar medium, but its solubilized P released into the liquid culture was 24.23 mg L^{-1}, contrarily, the isolate BT1 had a small SI (2.88) but released the highest amount of solubilized P of 47.50 mg L^{-1}. However, significant negative correlation between final culture pH and concentrations of solubilized P was found (r = - 0.99) (Figure 4). If the final culture pH decreased concentrations of solubilized P increased.

Identification of PSB isolates

PSB selected in the previous steps were all Gram-negative, some differences were observed in the phenoltypic study of colonies (Table 1). Others biochemical and physiological characteristics were also studied as shown in Table 1. Nucleotide sequencing of 16S rRNA genes amplified by PCR and sequence comparison with the data available in GenBank using the BLAST (Altschul et al., 1997) algorithm has enabled us to identify the isolates (Table 2). The tolerance of strains in extrinsic and intrisic environmental stress is shown in Table 3.

DISCUSSION

This study provides the first clear characterization of phosphate solubilizing bacteria in Meknes. Phenotypic and genotypic methods were used to evaluate these PSB isolates. Studies on agar plates revealed that solubilization index (SI) increased with increase in the total diameter (colony + halo zone), these results are in accordance with that of Baig et al. (2010) and Yang et al. (2012). Most of PSB isolated strains in this study lost their ability to form halozone on agar medium on repeated subculturing. This result is in accordance with that of Kucey et al., (1989); Illmer and Schinner (1995) and Rodriguez and Fraga (1999). From the observed results, it is clear that P released by BT12 strain was higher as compared to other PSB strains. All the PSB strains isolated were efficient in solubilizing tricalcium phosphate. From the statistical analysis by method of ordinary least squares (OLS), no significant positive correlation between the solubilization index (SI) and concentrations of solubilized P was found (r = 0.188) (Figure 5). These results are in accordance with those obtained by Yang et al. (2012), which reported that no significant positive correlation was found between halo diameter and solubilized P (r = 0.03). However others reports show no correspondence between solubilization ability in plate and in liquid culture (Baig et al., 2010). It has been reported that solubilization halo measurements is not always the best way to discriminate those isolates that would eventually have the greatest solubilization potential. Thereby, significant negative correlation between final culture pH and concentrations of solubilized P was found (r = - 0.99) in our study (Figure 4). On the contrary, Yang et al. (2012) reported no significant negative correlation

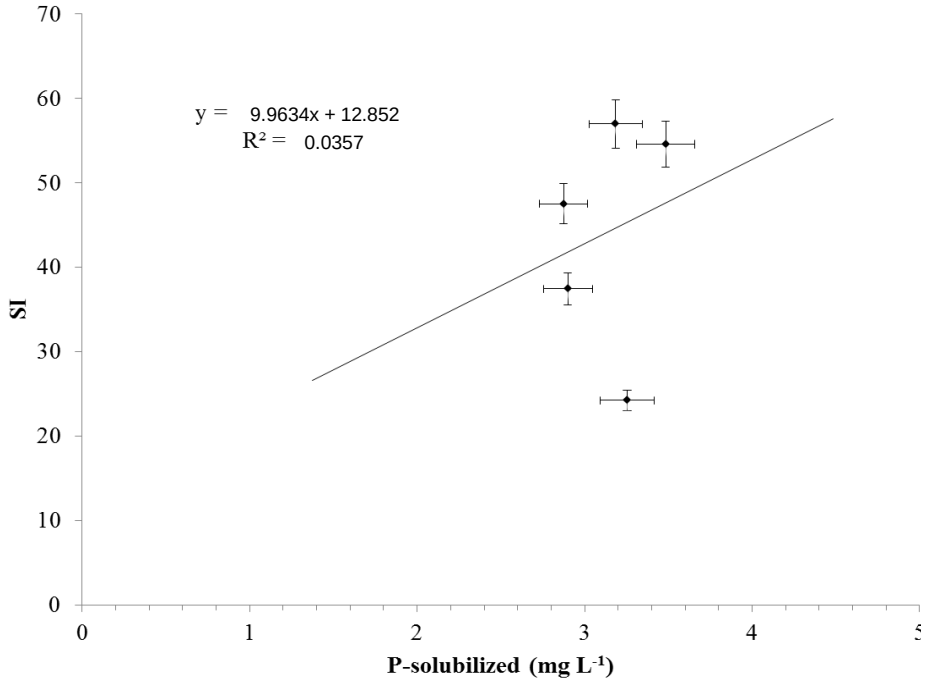

Figure 5. Correlation analysis between the solubilization index (SI) of the PSB strains in the NBRIP medium and their tricalcium phosphate solubilizing abilities.

Table 1. Phenotypic and biochemical characteristics of the PSB isolates[a].

Isolate	Colony morphology[b]	Oxidase	Motility	utilization				
				Lactose	Mannitol	Mannose	Arabinose	Maltose
BT1	C, E, R, Y, Cy	-	+	+	+	+	+	+
BT4	C, I, R, W, Cy	+	+	+	+	+	-	+
BT10	C, E, R, OW, Cy	+	+	-	+	-	+	-
BT11	C, I, R, W, Cy	-	+	-	+	+	+	+
BT12	C, E, R, OW, V	+	-	-	-	-	+	+

+, Tested positive/utilized as substrate; -, tested negative/non-utilized as substrate. [a]All PSB isolates were shown to be Gram-negative, reacted positive to catalase, glucose utilization, and negative for H_2S production and citrate (Simmons) utilization. [b]Colony morphology in YMA medium: C: circular; E/I: entire/irregular edge; R/Cr: raised/crateriform; Y/W/OW: yellow/white/off-white; V/Cy: viscous/creamy.

Table 2. Identification of PSB isolates by 16S rDNA sequencing.

Isolate	Length of 16S rDNA gene sequenced (bp)	GenBank accession number	Most closely related organism		
			Specie (strain)	Accession number	Gene identity (%)
BT1	1524	CP001635.1	*Variovorax paradoxus S110*	NR_074654	94
BT4	1492	AM293565.1	*Pseudomonas reinekei MT1*	NR_042541.1	99
BT10	760	AF057645.1	*Pseudomonas libanensis CIP 105460*	NR_024901.1	97
BT11	514	AJ581999.1	*Pseudomonas lurida DSM 15835T*	NR_042199.1	98
BT12	1473	AJ233423.1	*Pantoea agglomerans DSM 3493*	NR_041978.1	96

between the final culture pH and tricalcium phosphate solubilization activity (r = -0.15). Others studies support the idea that there is a strong negative correlation between the concentration of soluble P and the final pH value of the PSB culture (Hwangbo et al., 2003; Perez et al., 2007); these studies are in accordance with our results. The values of P in solution obtained for our isolates are in accordance with those of other studies which

Table 3. Phenotypic characteristics of the strains under environmental stresses.

Isolate	Growth pH				Growth salinity (g/l)				Growth temperature (°C)					Antibiotic resistance[a]			
	4.8	5.8	6.8	8.8	0.5	1	1.5	2	4	20	30	40	50	AMP	TE	S	K
BT1	-	+	+	+	+	+	+	±	-	+	±	-	-	+	-	-	+
BT4	+	+	+	+	+	+	+	+	±	+	+	+	±	+	-	-	-
BT10	+	+	±	-	±	+	+	±	-	+	±	-	-	-	±	-	+
BT11	+	+	±	±	+	+	±	±	-	+	±	-	-	+	-	-	-
BT12	+	+	±	-	+	+	+	±	±	+	-	-	-	+	±	±	±

+: Growth, ±: weak growth, -: no growth. [a]AMP: Ampicillin (10 µg), TE: tetracycline (30 µg), S: Streptomycin (25 µg), K: kanamycin (30 µg).

obtained concentrations ranging between 60 and 450 mg L^{-1} of soluble P in liquid medium (Yang et al., 2012; Baig et al., 2010; Son et al., 2006).The data of this study showed that the isolates studied are globally tolerant of alkalinity and neutrality. They were able to grow at an initial pH of 8.8 and 4.8 (Table 3). The isolates which can survive on a wide pH range are candidates for further strain improvement to highly acidic or alkaline conditions. Extremes of pH can be a major factor limiting microorganisms in soil (Rodriguez and Fraga, 1999). The ability to adapt to temperature stress may be important in the survival of the microorganisms during drought. All the isolates in this study could tolerate temperatures ranging between 20 and 30°C, but some of them were also able to grow at 4 (BT4 and BT12) and at 50°C (BT4) (Table 3). The results of this test are in concordance with previous studies (Chaiharn and Lumyong, 2009; Thakuria et al., 2004). Acid tolerance is important in the growth and survival of microorganisms in wheat soil. The bacteria tested in this study tolerated 2 g L^{-1} salt concentration (Table 3). These results are in accordance with those obtained by Chaiharn and Lumyong (2009). Regarding the intrinsic resistance, all PSB isolated strains in this study showed a good resistance against different antibiotics (Table 3). In general, the phenotypic study showed physiological and biochemical differences between isolates. Indeed, the studied strains showed a variable resistance against stress factors, namely, temperature, pH, salinity and resistance to antibiotics, which allowed the selection of good candidates as biofertilizers for wheat crops. Five isolates with the greatest solubilization potential were selected to determine their 16S rDNA sequences. When those sequences were compared with the GenBank (NCBI) database, three out of the five isolates belong to the genus Pseudomonas (Table 2). The genus Pseudomonas has been extensively studied as a plant growth promoting rhizobacteria (PGPR) (Walsh et al., 2001), and it is known for having members able to synthesize phytohormones, act as biocontrol agents, and solubilize phosphate (Jha et al., 2009). Nevertheless, Pseudomonas appears as a very promising group for its proven suitability as a potential inoculant. Most of the PSB obtained in our study were affiliated with groups previously identified in other soils. However, novel PSB, *Variovorax paradoxus* S110, was identified for the first time in this investigation. Application of bacterial inoculants as biofertilizers has been reported to result in improved plant growth and increased yield (Bashan and Holguin, 1998; Vessey, 2003). The results of this study make these isolates attractive as phosphate solubilizers. It requires further in depth studies based on the plant growth promoting activities of these isolates under pot culture of wheat as well as field conditions in Meknes area before recommended as biofertilizers for wheat crops.

Conclusions

In our study, the characterization and screening of rhizobacteria from wheat rhizosphere soil helped in the selection of various phosphate-solubilizing bacteria, have the potential to increase the available phosphate in the soil, which in turn will help to minimize the P-fertilizer application, reduce environmental pollution, promote sustainable agriculture and increase yields of wheat in Meknes area and other similar areas. The studied phosphate solubilizing bacteria tolerate high concentrations of NaCl. Hence, these isolates may be the candidates for use in the saline soil. The selected isolates were tolerant to temperatures ranging from 4 and 40°C, and to extreme pH from 4.8 to 8.8. The antibiotic resistance of the isolated strains showed a high level of resistance against streptomycin, kanamycin, ampicillin and tetracycline. The advantage of using native PSB isolated from *T. aestivum* rhizosphere soil in the Meknes region of Morocco is the ability to more easily adapt and go through succession when inoculated into soil. Further investigation is required on the bacterial strains to exploit these strains as biofertilizers for wheat field soils to enhance the growth of wheat crops.

Conflict of Interests

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

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Full Length Research Paper

Comparative evaluation of cellulase activity in *Trichoderma harzianum* and *Trichoderma reesei*

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Cellulase activity of two promising species of *Trichoderma harzianum* and *Trichoderma reesei* were assessed for agro-industrially important cellulase (E.C.3.2.1.4) production. Both species were used for media optimization studies and effect of pH, temperatures and incubation periods on cellulase activity. The maximum cellulase activity was found to be 1.76 U/ml (EXG) in *T. reesei* at pH 4 in comparison with *T. harzianum* with a maximum of 0.76 U/ml (EXG). The optimum temperature for increased cellulase activity was 35°C in *T. reesei and* incubation period of 112h was found ideal for increased cellulase activity in *T. harzianum* and *T. reesei.* Similarly, with 1% sucrose (w/v), maximum cellulase activity was achieved in *T. reesei* was 0.76 U/ml (EXG,EG). 1% yeast (w/v) was found most suitable nitrogen source for increased cellulase activity in *T. reesei*, that is, 1.96 U/ml (EXG) in comparison with *T. harzianum* where it was found to be maximum (1.29 U/ml (EG)). The potential of these lignocellulytic fungi for industrial cellulase production was tested through cellulase activity assay.

Key words: Cellulase, *Trichoderma harzianum*, *Trichoderma reesei*, enzyme activity.

INTRODUCTION

Plant cell walls are majorly comprised of cellulose, hemicellulose and lignin, where cellulose is the most abundant component (Han et al., 2003). Plant biomass comprises of an average of 23% lignin, 40% cellulose and 33% hemicellulose by dry weight (Sa-Pereira et al., 2003). Rauscher et al. (2006) showed that about 830 Gt of renewable plant biomass is formed annually consisting mainly of cellulose and hemicelluloses. Cellulases are industrially important enzymes (Schulein, 2000). They are known for their role in exhibiting high substrate specificity and less side chain products formation. These have created an interest in the market because of their wide-spread applications mainly in textile industries and biorefineries because a large amount of consumption of cellulase for biomass saccharification (Zhang et al., 2006; Zhu et al., 2009).

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Abbrevations: EXG, Exoglucanase; **EG,** endoglucanase; **PDA,** potato dextrose agar; **CMC,** carboxymethylcellulose; **CBH,** cellobiohydrolase.

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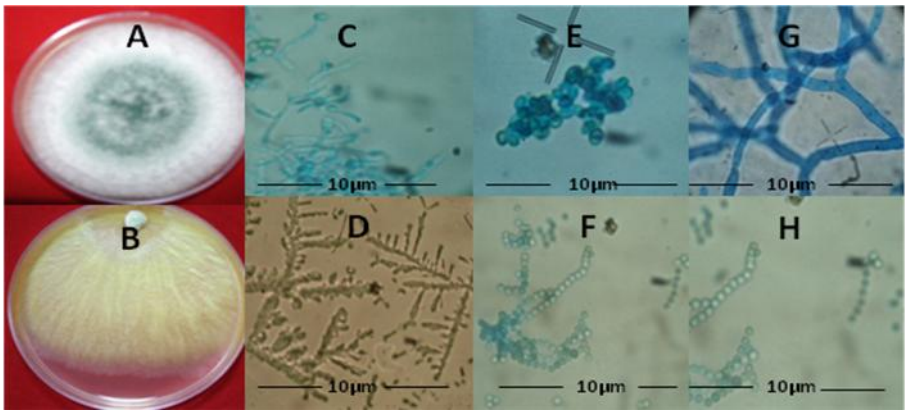

Figure 1. Morphological characteristics of *T. harzianum* and *T. reesei* (A and B) on PDA plates. Spore morphology and mycelia bearing phialides after staining with lactophenol blue. (C and D) Branched mycelia ribbons; (E and F) Conidiospres. Scale bar = 10 µm

Many fungal strains are known for their secretion of higher amounts of cellulases than bacterial strains, where *Trichoderma* is a leading one (Amouri and Gargouri, 2006). The complex structure of lignocellulose and its heterogeneous substrate hampers an efficient conversion to simple sugars and presents a number of technical and economic challenges in bringing cellulosic biofuels to the market. One of the major economical barriers for the production of biofuels is the intrinsic recalcitrance of lignocellulosic plant matter (Himmel et al., 2007). The synergestic decomposition power of mesophilic enzymes and uncomplicated multi-enzyme complex of filamentous fungi like *Trichoderma* spp. particularly *Trichoderma reesei* and *Aspergillus niger* made them effective agents for cellulase production. Cellulases produced by *Trichoderma harzianum*, is the most efficient enzyme system for the complete hydrolysis of cellulosic substrates into its monomeric glucose, which is a fermentable sugar. Mainly filamentous fungus, *T. reesei* and *T. harzianum* are commercially explored now-a-days for the large scale production of different cellulases and hemicellulases in bioreactor cultivations and many scientific groups are working for the improvisation of other strains of *Trichoderma* for the over-production of cellulases and cellulose degrading property of the biocontrol organism (Tiwari et al., 2013). Besides, with well established applications of these enzymes in pulp, paper, food, feed or textile processing industries, these plant cell wall degrading enzymes are now-a-days also employed for the saccharification of cellulosic plant biomass to simple sugars for biofuel production (Bouws et al., 2008; Harman and Kubicek, 1998; Kumar et al., 2008). A cellulosic enzyme system of *Trichoderma* spp. comprises of three major components: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). The exo-β-glucanase causes disruption in cellulose hydrogen bonding, which was later followed by hydrolysis of the accessible cellulose with endo-β-glucanase (Reese et al., 1950). The whole process occurs simultaneously and the rate limiting step is the depolymerisation of the insoluble cellulose by the CBHs and EGs. It is the synergestic action of both types of enzymes exoglucnases and endoglucanases which are involved in degradation of cellulase (Beguin and Aubert, 1994; Tomme et al., 1995).

The main objective of the present study was to comparatively evaluate two potential strains of *Trichoderma*, that is, *T. harzianum* and *T. reesei* by comparing crude cellulase activity, anticipating their possible fruitful role in the production of commercially important cellulases.

MATERIALS AND METHODS

Microorganisms for the study

Fungal cultures of laboratory developed strain of *Trichoderma harzianum* strain Th3 obtained from Indian Type Culture Collection (ITCC: 5593) isolated from carnation rhizosphere from IARI field submitted by the author to ITCC in 2005 and *T. reesei* (ITCC:4026) submitted to ITCC by S. Verma were taken for this study from Biocontrol Laboratory, Division of Plant pathology, IARI, New Delhi. The experimental *Trichoderma* spp. were multiplied in potato dextrose agar media, with the combination of peeled potato: 250 g, dextrose: 20 g, agar: 15 g and distilled water: 1000 mL. It was multiplied at 30°C (Barnett and Hunter, 1972) in a BOD incubator.

Morphological characterization of *T. harzianum* and *T. reesei*

T. harzianum was fast growing and produced branched mycelia on PDA plate after 24 h, whereas *T. reesei* was quite sluggish in growth. Mycelial stage of *T. harzianum* was whitish after sporulation, it changed to greenish. Sporulation phase appeared after 4 days of incubation at 30°C.The mycelia in both *T. harzianum* and *T. reesei* were profusely branched with fruiting bodies (phialides). The greenish spores were spherical and biconcave in the case of *T. harzianum* while in *T. reesei*, spores were whitish and form chains as shown in Figure 1.

Enzyme production

The culture was grown in 250 ml Erlenmeyer flask that contained 50 mL of the medium. Concentrations of the nutrients were 5 g/L trisodium citrate, 5 g/L KH_2PO_4, 2 g/L NH_4NO_3, 4 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4$ (Ahmed et al., 2007) supplemeted with different carbon and nitrogen sources. After adjusting pH 5 of the medium prior to sterilization, the flasks were then inoculated with 2 agar discs (2 mm in diameter) of 5 days old culture grown on PDA plates and later incubated under stationary condition at 25, 30 and 35°C for up to 5 days. The crude enzyme was filtered and centrifuged at 12000 xg for 20 min.

Enzyme assay

Cellulase (exoglucanase activity) was assayed by measuring the release of reducing sugar by DNS (Miller, 1959). The assay mixture contained 1 ml of 0.5% cellulose (Sigma Co.) suspended in 50 mM citrate phosphate buffer (pH 4.8) and 1 ml of culture filtrates of different *T. harzianum* and *T. reesei* strains, respectively. The reaction mixture was incubated for 30 min at 50°C and then centri-fuged at 12000 rpm for 15 min at 4°C. The reaction was arrested by adding 3 ml of 1% DNS (dinitrosalicylate) reagent in 1 M NaOH and followed by 1 mL of 40% Rochelle salt (potassium sodium tartarate) which was added to stabilize the colour. Endoglucanase activity (CMCase) was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) made in 0.5 M citrate acetate buffer (pH 5.0) into the culture filtrates. The blanks were made in the same way using distilled water and absorbance was measured at 540 nm. One unit of cellulase activity was defined as the amount of enzyme in 1 ml of the reaction mixture that released 1 µmol of reducing sugar under assay condition.

Optimization of parameters for cellulase production

Effect of temperature and incubation period on enzyme activity

In this study, the cellulase activity of fungal isolates grown under different temperature conditions of 25, 30 and 35°C and at time interval of 96, 112, 128, 134, 150, 166, 172h and 188 h was monitored.

Effect of pH on enzyme production

The most suitable pH optimum for the growth of *Trichoderma* sp. was obtained by adjusting the pH of the growth medium from 2-5 using 50 mM sodium phosphate and 50 Mm citrate buffers and the cellulase activity was measured at 540 nm at each pH to know the ideal condition.

Effect of carbon sources on enzyme production

Effect of various carbon compounds viz., cellulase, CMC, glucose, sucrose and maltose were used for the study. The broth was distributed into different flasks and 1% of each carbon sources were then added before inoculation of the strain at 28°C.

Effect of nitrogen sources on enzyme production

In the present study, the whole idea was to detect the appropriate nitrogen source for getting maximum cellulase enzyme activity by *T. harzianum* and *T. reesei*. The influence of peptone, beef extract, ammonium nitrate and yeast extract procured from HIMEDIA, India, by supplementing the growth medium with the organic and inorganic compounds was studied.

Statistical analysis

Average value of cellulase activity was determined for multiple mean comparisons obtained through three separated experiments and to use the values for analyzing data for calculating standard deviation (±SD) from there independent experiments ranging between ±0.01 to ±0.05, respectively.

RESULTS

Effect of incubation period on enzyme production

Time of incubation has great bearing with the production of enzyme and operation of other metabolic systems. To a certain extent, *Trichoderma* sp. showed most active cellulolytic species along different incubation period (96, 112, 128, 134, 150, 166, 172 and 188 h, respectively). Cellulase activity values ranges from 0.1 to 0.47 U/ml (EXG) and 0.13 to 0.46 U/ml (EG) in *T. harzianum* with peak cellulolytic (EXG) activity of 0.7U/ml achieved at 112 h whereas activity value ranges from 1.30 to 1.76 U/ml (EXG) and 1.30 to 1.76 U/ml (EG) at different hours of incubation with the peak value of 1.96 U/ml at 112h in *T. reesei* shown in Figure 2.

Effect of pH on enzyme production

Cellulase yield by *Trichoderma* sp. depends on the pH value. Results illustrated in Figure 3 indicate that cellulase activity increased gradually as pH increase from 2 to 4 with the increase of 0.02 to 0.76 U/ml (EXG) and 0.03 to 0.07 U/ml (EG) in *T. harzianum* and remain maximum (0.76U/ml) for *T. harzianum* at pH 5. Almost, similar trend was observed in *T. reesei* from pH 2 to 5 with the maximum value of cellulose activity, 1.76 U/ml (EXG) at pH 4. Effect of pH on cellulase production by *Trichoderma* supports the findings of Lee et al. (2002) who reported that CMCase exhibit the pH optimum of 4 and β-glucosidase ranges between pH 4-5.

Effect of temperature on enzyme activity

Like pH, temperature is also an important factor that influences the cellulase activity. It was found to be 0.45 U/ml at 25°C and 0.57 U/ml at 30°C and maximum (EXG) activity of *T. harzianum* was found to be 0.95 U/ml. In the case of *T. reesei*, it was 1.22 U/ml at 25°C and 1.44 U/ml at 30°C. If we see the EG activity of *T. harzianum,* it was found to be 0.33 U/ml at 25°C and 0.47U/ml at 30°C which was low in comparison with *T. reesei* with EG activity of 1.22 and 1.33 U/ml activities at 25 and 30°C as shown in Figure 4. Maximum activity in *T. harzianum* was found at 35°C that is, 0.94 U/ml (EXG), 0.77 U/ml

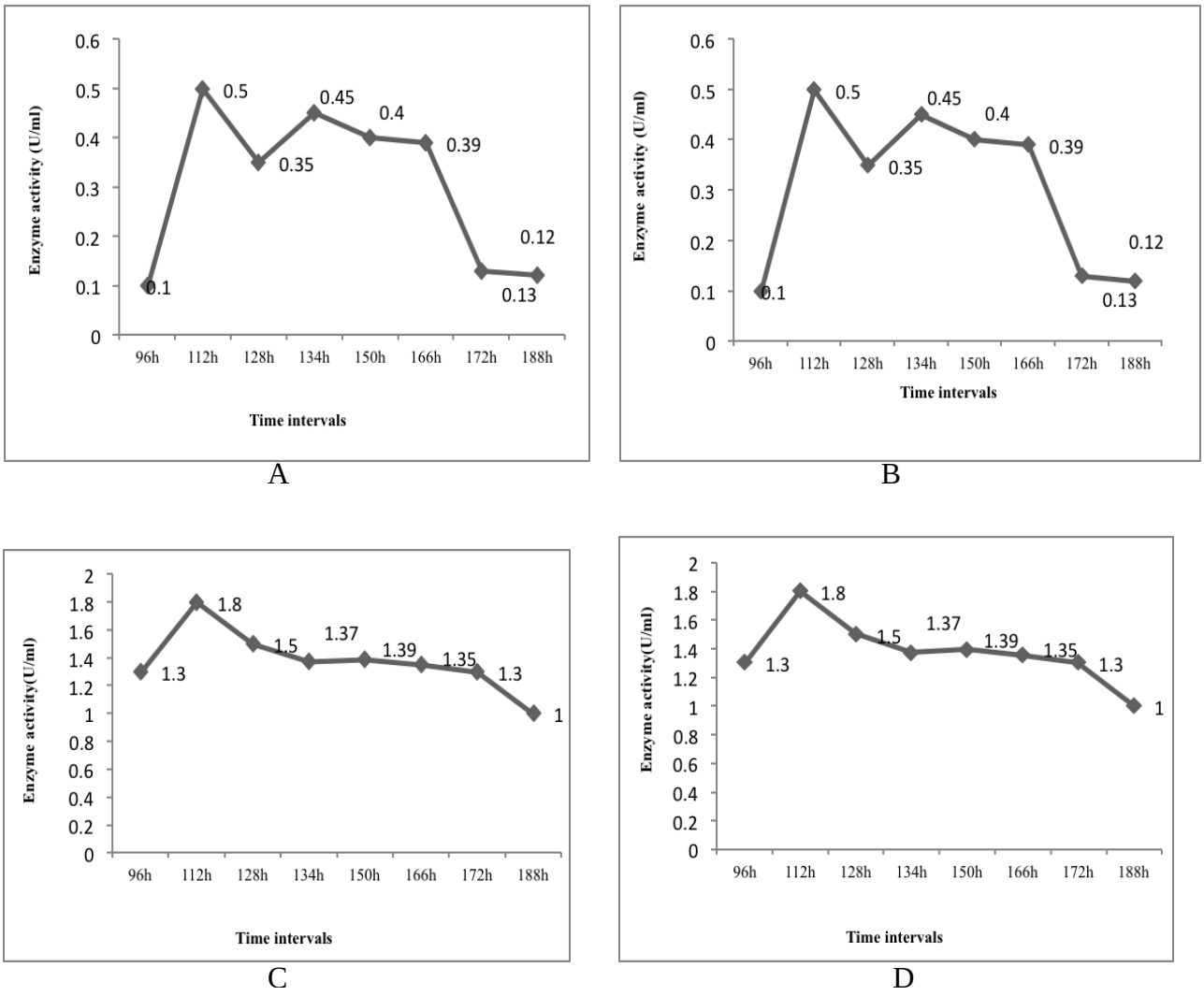

Figure 2. (A) Effect of incubation period on cellulase (EXG) activity (U/ml) in *T. harzianum* (B) cellulase (EG) activity (U/ml) in *T. harzianum*; (C) Effect of incubation on cellulase (EXG) activity (U/ml) in *T. reesei* (D) cellulase (EG) activity (U/ml) in *T. reesei.*

(EG) and in *T. reesei* it was 1.83 U/ml (EXG) and 1.78 U/ml (EG) at 35°C.

Effect of carbon source on enzyme activity

Data presented in Figure 5 showed that cellulase activity by *Trichoderma* sp. under test was significantly influenced by the type of carbon source in the medium. Sucrose was found to be most effective as a sole carbon source for cellulase enzyme production, which results in increased enzyme activity, being 1.16 U/ml (EXG), 1.24U/ml (EG) in *T.harzianum* while 1.76U/ml (EXG) and 1.76U/ml (EG) were obtained in *T. reesei* when grown in Vogel growth medium containing 1.0% sucrose followed by cellulose, glucose and maltose as shown in Figure 5. It was also reported that maximum yields of cellulase were obtained on 1% different carbon substrate using *T. viride.* Cellulase production reached nitrogen limiting conditions and the yield of cellulase decreased when excess peptone was presented, various inorganic nitrogen sources have been optimized by different workers for cellulase production.

Effect of nitrogen sources on enzyme activity

Various inorganic nitrogen sources have been optimized by different workers for hemicellulase production to evaluate the effect of nitrogen source on cellulase formation. In this study, different nitrogen sources were supplemented in the growth medium to optimize cellulase activity. Data revealed in Figure 6 showed that the supplementation of organic and inorganic nitrogen sources of 1% (w/v) of peptone, beef extract, yeast extract, ammonium nitrate, stimulated the cellulase yield and activity.

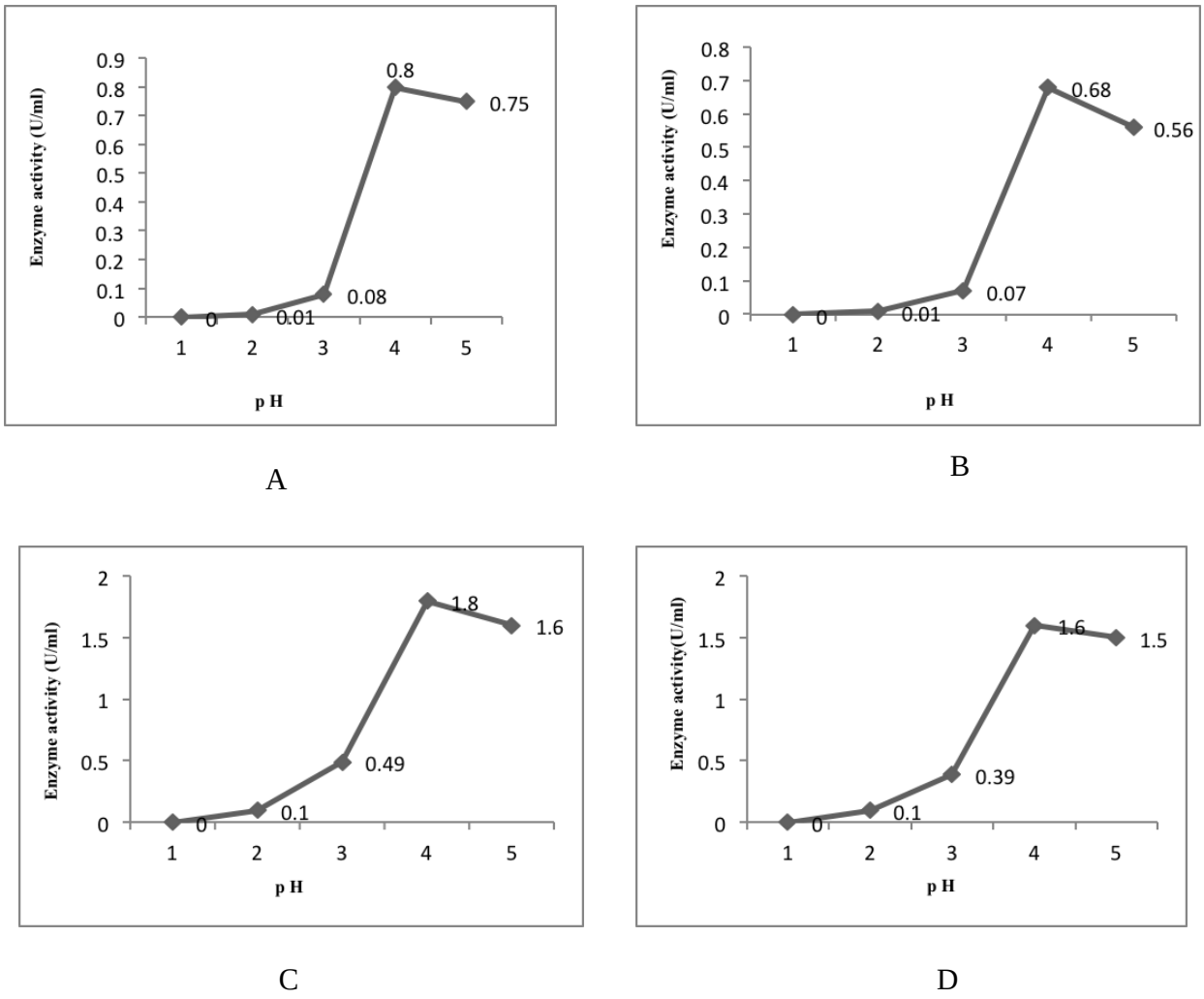

Figure 3. (A) Effect of pH on cellulase (EXG) activity (U/ml) in *T. harzianum;* (B) cellulase (EG) activity (U/ml) in *T. harzianum* (C). Effect of pH on cellulase (EXG) activity (U/ml) in *T. reesei* (D) changing cellulase (EG) activity (U/ml) in *T. reesei.*

The maximum enzyme activities were obtained with yeast extract (1.0%) which brought about an improvement in all the two cellulase components, including EXG and EG was found to be 1.96 and 1.76 U/ml in *T. reesei* and 0.88 and 1.29 U/ml in *T. harzianum* shown in Figure 6, respectively. Peptone was second important nitrogen source used by *Trichoderma* sp. in cellulase production. It was reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source.

DISCUSSION

Cellulases are commercially famous enzymes known for their vast roles in biomass consumption and others. Keeping this view, a comparative study was done on two important species of *T. harzianum* and *T. reesei* and the effect of different parameters which have direct or indirect bearing on the crude cellulase activity were assessed. It was observed that the cellulase activity was less at 20, more at 30°C and maximum enzyme production was found at 35°C at 112 h in both species under study. This observation was similar to the finding of Mekala et al. (2008) which showed that cellulase production was maximum at 33°C incubation and decreased with high temperature in case of *T. harzianum*. Rajshekharan et al. (2011) also stated that optimum temperature for the fermentation of media components in *T. reesei* was found to be ideal at 32°C. Many workers have also reported different temperatures for maximum cellulase production either in flask or in fermentor studies using *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Murao et al., 1988; Lu et al.; 2003).

Cellulase activity was found to be increased at acidic pH 4 and decrease at pH 5 due to the fact that cellulases are acidic proteins and affected by neutral pH values

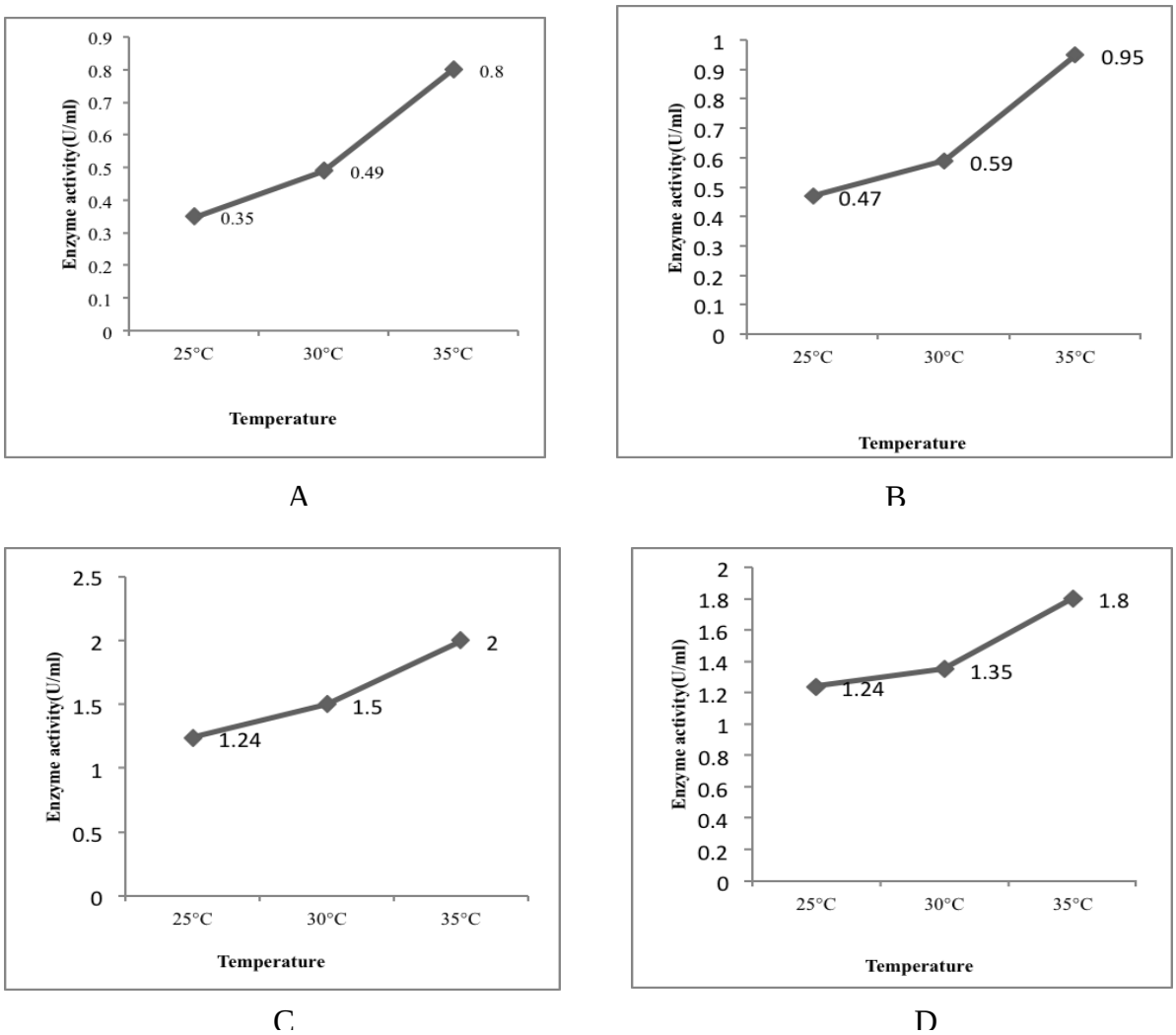

Figure 4. (A) Effect of temperature on cellulase (EXG) activity (U/ml) in *T. harzianum;* (B) cellulase (EG) activity (U/ml) in *T. harzianum;* (C) Effect of temperature on cellulase (EXG) activity (U/ml) in *T.reesei;* (D) changing cellulase (EG) activity (U/ml) in *T.reesei.*

(Juhasz et al., 2004). The optimal pH for fungal cellulases varies from species to species, though in most cases, the optimum pH ranges from 3.0 to 9.0 (Ishfaq et al., 2011). *T. harzianum* strain Ce 17A which showed maximum exoglucanase production at a pH range of 3 - 5 and then decreased above pH 5 (Mekala et al., 2008).

The optimal pH for fungal cellulases varies from species to species though in most cases the optimum pH ranges from 3.0 to 6.0 (Niranjane et al., 2007).

Cellulase production is also dependent on the concentration of carbon source in the production media. Addition of different carbon sources had both positive and negative effects on cellulase production (Rai et al., 2012). The maximum enzyme production was obtained using the carbon source 1% (w/v) sucrose and with 1% (w/v) of cellulose, this can be due to immediate need of energy. Mandels and Reese (1957) also reported that maximum yields of cellulase were obtained on 1% different carbon substrate using *T. viride.* For other sources of carbon (glucose and maltose) cellulase activity was found to be less as compared to the sucrose and cellulose. Malik et al. (1986) have also reported that negligible cellulases were produced with glucose as carbon source from *T. harzianum*.

Various inorganic nitrogen sources have been optimized by different workers for cellulase production (Sherief et al., 2010; Solomon et al., 1997; Lee et al., 2010). In the present study, yeast followed by peptone was found to have more cellulase activity in both species which was in accordance with the results obtained by Gautam et al. (2010). Though the addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and enzyme production, as was reported before, they were not an effective replacement for inorganic

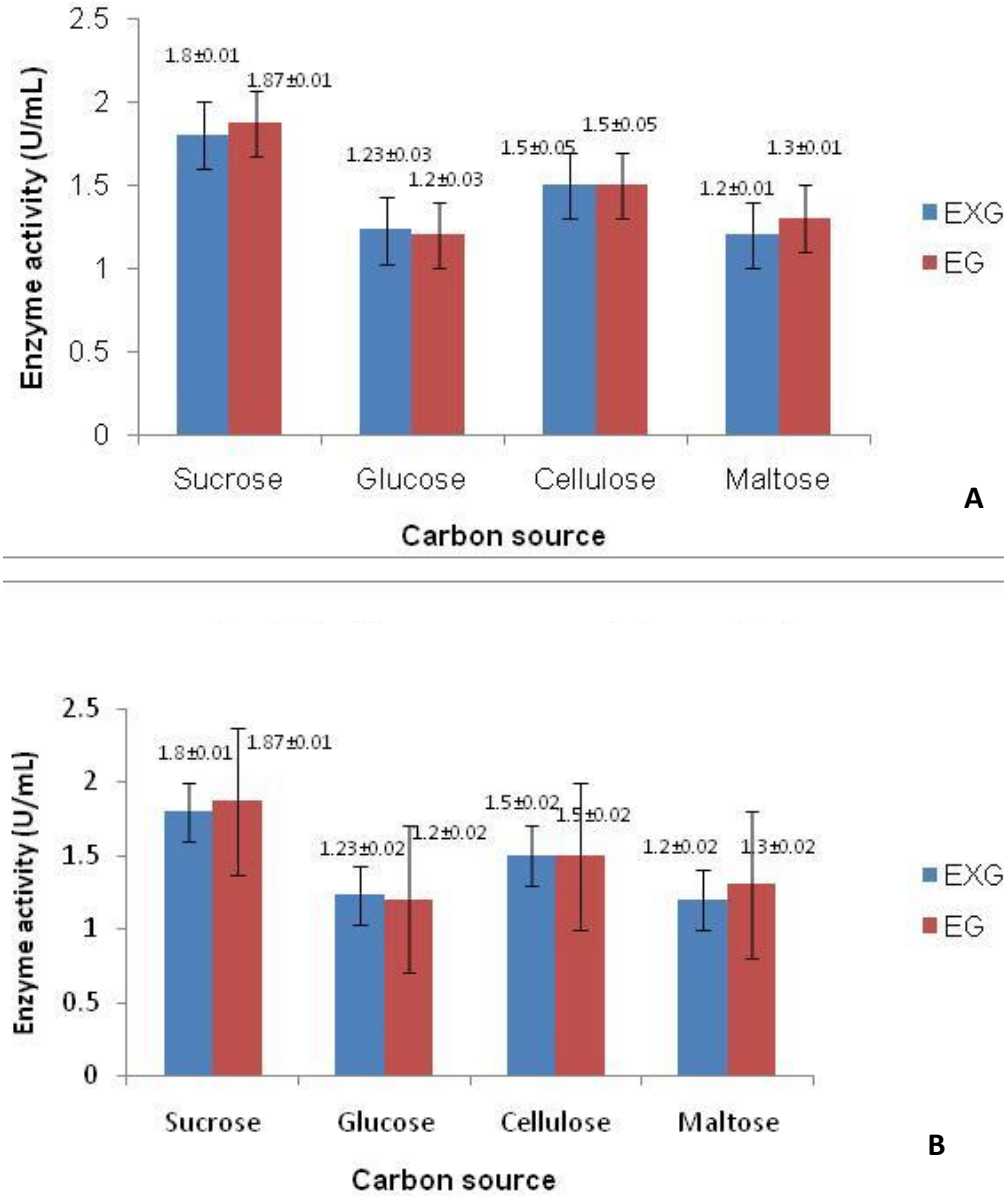

Figure 5. (A) Effect of different carbon sources on cellulase (EXG and, EG) activities in *T. harzianum.* (B) Effect of carbon sources on cellulase (EXG and EG) activities in *T. reesei.* The lines in the bar graph represent the standard error (SE).

nitrogen sources because of their higher cost.

Conclusion

In this investigation, celluloltyic potential of the two potential strains of *Trichoderma* based on crude cellulase activity was tested. *T. reesei* was already being commercially exploited due to its higher cellulase activity. *T. harzianum* though depicting lower cellulytic potential than *T. reesei,* also have possibility for its usage in commercial production of enzyme and a have wider scope to be further exploited for the commercial production of cellulases by increasing the cellulase yield by further characterizing it based on stability and applicability of the enzymes from these two species.

Conflict of Interests

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

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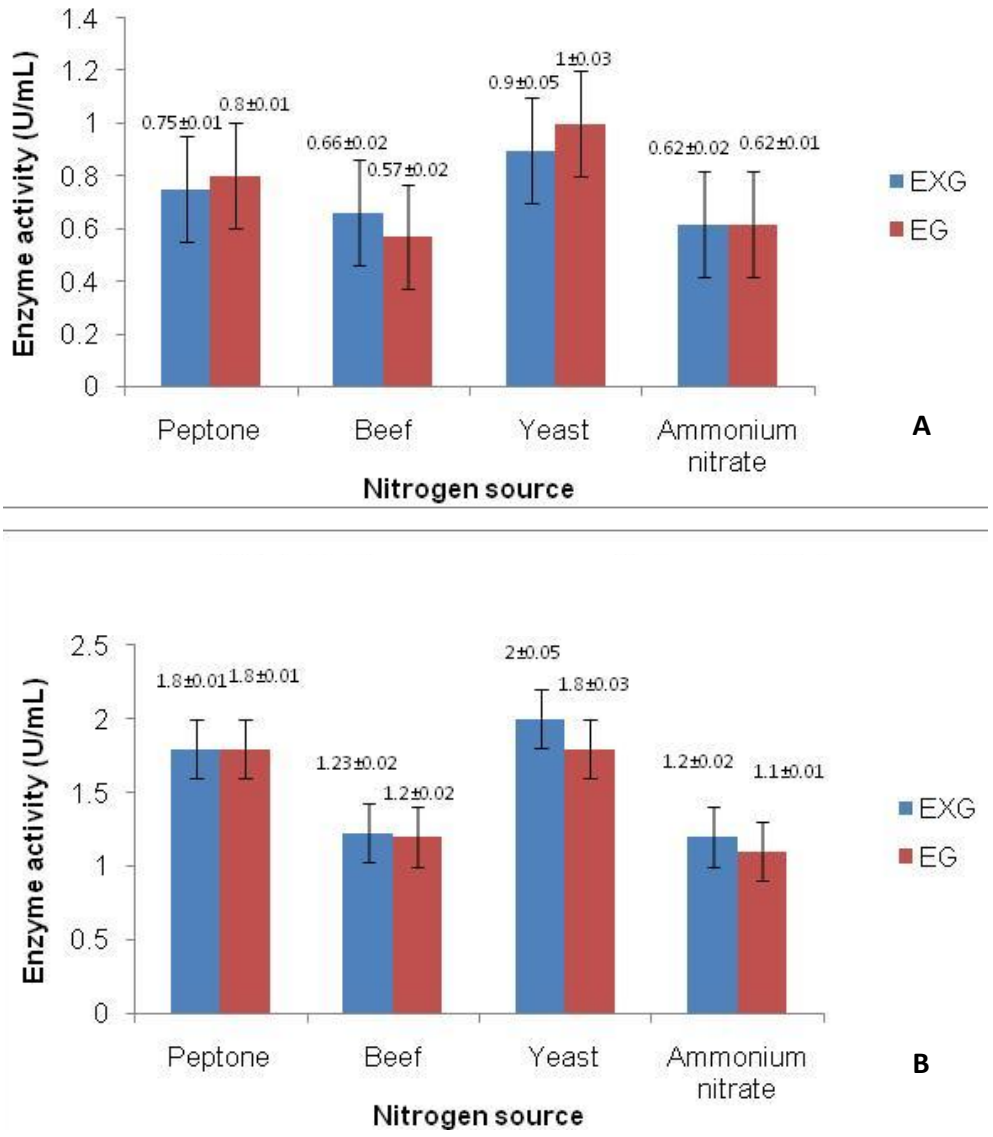

Figure 6. (A) Effect of different nitrogen sources on Cellulase (EXG and EG) activities in *T. harzianum.* (B) Effect of nitrogen sources on Cellulase (EXG and, EG) activities in *T. reesei.* The lines in the bar graph represent the standard error.

as antagonist soil borne pathogens'' and we thank the Head, Division of Plant Pathology, Indian Agricultural Research Institute (IARI) where the facilities for the experimentation was provided.

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Full Length Research Paper

Pectate lyase production at high and low pH by *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*

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Unripe avocado fruit in Mexico are affected by *Colletotrichum* spp. Although the literature indicates that unripe fruit are resistant to the fungus because of presence of compounds in the fruit, there is evidence on the intervention of pectate lyase (PL) as a virulent factor in *Colletotrichum gloeosporioides*. The objective of this work was to detect PL from the fungus in response to avocado cell wall under two pH conditions, to simulate infection in unripe (low pH) and ripe (high pH) fruit. *Colletotrichum* isolates were grown in a second liquid media at either pH 4 or 7. Enzyme activity was measured in the clear supernatant and PL was detected by immunoblot with an antibody against *C. gloeosporioides* PL, which detects a single PL. Results show that, *in vitro*, the fungus responds with higher enzyme activity when in contact with avocado cell wall at pH 7, than at pH 4. However, two of the isolates examined produced PL at pH 4. This behavior under acid conditions may be related to their pathogenicity in unripe fruit. To our knowledge, this is the first documentation of PL secretion by *C. gloeosporioides* at low pH and may help explain its ability to attack young avocado fruit.

Key words: Anthracnose, metabolites, pectinases, *Persea americana.*

INTRODUCTION

Anthracnose is one of the main phytopathological problems encountered in the commercial production of avocados in Mexico. Anthracnose appears both in the field and also post-harvest. This disease is known to be caused by *Colletotrichum gloeosporioides* (Teliz and Mora, 2010), *Colletotrichum acutatum* (Avila-Quezada et al., 2007) and also by *Colletotrichum boninense* (Silva-Rojas and Avila-Quezada, 2011). Recently, *Neofusicoccum parvum* has also been reported to cause similar symptoms (Molina-Gayosso et al., 2012).

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It is known that the infection process first involves the germination of conidia on the fruit skin. These develop appressoria from which penetration hyphae emerge to breach the cuticle. It seems that the fungus then becomes latent due to adverse physiological conditions in the young host fruit – for example, epicatechin levels are high in unripe fruit but the level falls with ripening allowing the fungus to grow and invade the tissues (Prusky and Plumbley, 1992).

Anthracnose development is the result of the interaction between the fruit and the fungus. For example, the enzyme endopolygalacturonase produced by *C. gloeosporioides* is able to macerate avocado tissues. Some researchers have found that the fungus also produces pectate lyase (PL) during colonization (Barash and Khazzan, 1970). PL is involved as a virulence factor for *C. gloeosporioides*. The infection process has been shown to occur only in ripe avocado fruit (Yakoby et al., 2000). Many researchers have suggested ways to detect PL using antibodies (Wattad et al., 1997). Western blot analysis of the proteins secreted by *C. gloeosporioides* into pectolytic enzyme-inducing media (Prusky et al., 1989) reveals the presence of PL bands.

It is well known that cell-wall-degrading enzymes often play a role in the pathogenesis of a fungus on its host (Annis and Goodwin, 1997). A significant amount of work has been published on the role of pectolytic enzymes but, in avocado, this has focused on ripe fruit. This would seem to suggest that anthracnose is restricted to the postharvest period and it assumes that the fungus produces PL only where the pH is above 6.0 (Prusky et al., 2001; Wattad et al., 1994).

Nevertheless, PL synthesis has been documented only in ripe fruit. The absence of studies focusing on enzyme production by *C. gloeosporioides* in unripe fruit is likely because, in most countries, the disease is unknown in unripe fruit.

Since, in Mexico, anthracnose causes significant economic loss in both ripe and unripe avocado fruit, the objective of this study was to simulate PL production under acid conditions *in vitro*. To increase our understanding on the infection process in unripe fruit, a medium was used which had been amended with avocado cell walls as inductor.

The new information created here sheds new light on interactions between the fungus and unripe avocado fruit (cv Hass) in Mexico.

MATERIALS AND METHODS

The investigation involved *Colletotrichum* strains collected from avocado orchards in Michoacan, Mexico and identified in previous studies (Silva-Rojas and Avila-Quezada, 2011). A total of seven isolates were re-plated to confirm their identities and viabilities.

Five strains of *C. gloeosporioides*: GAQ31 (NCBI EF424484), GAQ35, GAQ42, DTO09 and HSR02 and two of *C. acutatum* GAQ34 (NCBI EF175780) and GAQ01 (NCBI EF221831) were selected from our collection for the study.

Spore suspensions of $1x10^6$ conidia/mL taken from monoconidial cultures were used to verify the pathogenicity of these *Colletotrichum* spp. and to induce PL synthesis under *in vitro* conditions. Seven healthy avocados (*Persea americana* cv Hass) were inoculated with 10 µL of the spore suspension and placed in a humid chamber until symptoms developed, according to Yakoby et al. (2000).

PL production by *Colletotrichum*

A volume of 1 mL of each isolate containing $1x10^6$ conidia was added to an Erlenmeyer flask containing 40 mL of liquid modified Murashige-Skoog (M3S) medium containing $MgSO_4$.7H2O, KH_2PO_4, peptone bios D, bacto yeast extract, sucrose and chloramphenicol.

Each strain had three repetitions and the experiment was performed three times. Samples were shaken at 150 rpm, at room temperature (20°C), for five days as suggested by Wattad et al. (1995). After this, the fungus was harvested, washed with sterile water and transferred to a second liquid medium.

This second medium contained avocado cell walls that promote PL synthesis by the fungus following the methodology proposed by Wattad et al. (1994). Environmental variations among samples were minimized as the experiment was conducted under controlled conditions.

Hydrogen potential (pH) can be used to indicate fruit ripeness in avocado, with unripe fruit being characterized by a low pH and ripe fruit by a more neutral pH. Therefore, in some tests, the second media was adjusted to pH 4 to simulate infection in a young avocado fruit while in others, the acidity was adjusted to pH 7 to simulate infection in a ripe fruit. Because the fungus can modify the pH of the medium, phthalate buffer was added to minimize pH change by the fungus. After five days of continuous shaking in the second media, a clear supernatant was recovered and concentrated to a final volume of 5 mL by an evaporator OA-SYSTM (USA) under an atmosphere of reagent grade N_2. Once concentrated, samples were dialyzed into Thermo Scientific MWCO 10K membranes to remove impurities and salts as described by Miyara et al. (2008).

The activity of proteins secreted into the pectolytic enzyme-inducing media was measured at 232 nm using polygalacturonic acid (PGA) as substrate (Miyara et al., 2008). The reaction mixture contained 50 mM Tris HCl buffer pH 8.5, 0.2% PGA as a substrate and 0.5 mM $CaCl_2$. A total of 950 µL of the reaction mixture and 50 µL of each protein extract were placed in a quartz cuvette. Samples were incubated for 10 min at 37°C and read on a spectrophotometer JENWAY 6405 UV / Vis (USA) at 232 nm. The entire procedure was performed three times for each sample.

For each of the seven isolates, activity was calculated from a linear regression line ($r^2 = 0.999$). Where Y axis was represented by the optical density (values 0, 0.1, 0.2, 0.3, 0.4 to 1.0) at 232 nm for several known concentrations of protein and the X axis was represented by our PL samples from 1 to 20 µg/mL.

Electrophoresis

Total soluble protein separation was carried out using 12.5% polyacrylamide gel electrophoresis under denaturing and reducing conditions (SDS-PAGE) according to Laemmli (1970) and Wattad et al. (1994) at 140 V for 1 h in a transfer chamber (TRANS-BLOT SDS BIO-RAD). The transfer of proteins from gel to nitrocellulose (NC) membrane (Trans-Blot ®) was at 15 V for 15 min.

Immunodetection by Western blot

Immunodetection of PL was carried out with an antibody obtained

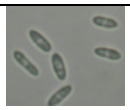
Isolate	Colonies on Petri plate	Conidia	Inoculated avocado fruits
GAQ34			
GAQ01			
GAQ31			
GAQ35			
GAQ42			
DTO09			
HSR02			

Figure 1. Colonies, conidia and pathogenicity tests of *C. gloeosporioides* and *C. acutatum* on avocado fruit cv Hass. Mycelium was grey color with salmon conidia masses. Conidia was 7.6 - 18.9 µm in length and 3.2 - 4.6 µm wide. All inoculated avocado fruits with five *C. gleosporioides* isolates represented by GAQ31, GAQ35, GAQ42, DTO09, HSR02, and two *C. acutatum* isolates represented by GAQ34 and GAQ01, showed anthracnose symptoms.

from balb mice. The antibody against *C. gloeosporioides* PL detects a single PL secreted by this fungus at a molecular mass of 39 kD (the antibody was donated by the Institute of Postharvest Science and Fresh Produce of the Volcani Center in Bet Dagan, Israel) using a commercial kit for Western blot (Western Blot Chromogenic WesternBreeze Inmmunodetection® Kit, Invitrogen, USA).

RESULTS AND DISCUSSION

C. gloeosporioides has been reported as the main causal agent of anthracnose and also of the disease known as pepper spots (Willingham et al., 2000).

Previous work has documented the main characteristics of *C. gloeosporioides* and of *C. acutatum* as grey/olive-green mycelia, some of which form pink/salmon conidial masses; unicellular, hyaline, cylindrical and curved conidia (Bailey and Jeger, 1992). These characteristics were all observed in this study.

Typical symptoms of anthracnose were found on the avocado fruit six days after inoculation (Figure 1). The isolates GAQ34 (*C. acutatum)*, GAQ35 and GAQ42 (*C. gloeosporioides*) caused most damage to the fruit and also showed greater growth on PDA and more abundant conidial masses.

In this study, we presented *C. acutatum* and *C. gloeosporioides* with avocado cell walls to simulate the infection process. We found that PL activities revealed the presence of five bands at pH 7 of 39 kD corresponding to the PL bands in *C. gloeosporioides* as reported by Yakoby et al. (2000). A recent published manuscript by Alkan et al. (2013) where the whole genome of *Colletotrichum* was reported also indicated the presence of a single protein for PL similar to that previously reported. Some of these PL sequences have been reported and deposited in the GenBank (NCBI) under the accession

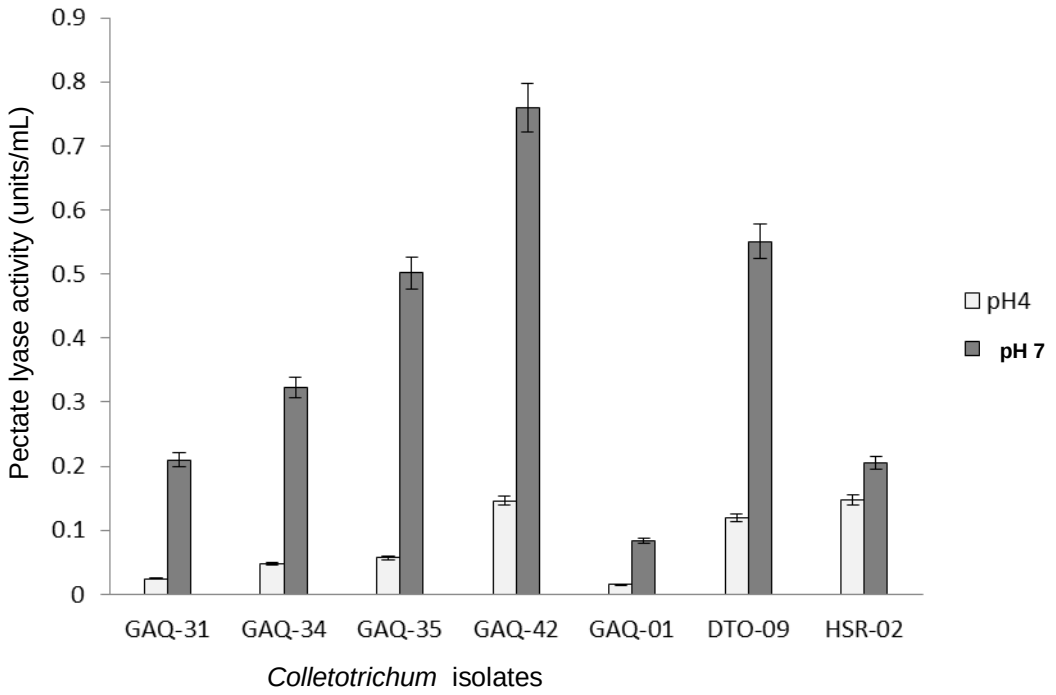


Figure 2. Enzymatic activity of the fungus at pH 7 (solid bars) and at pH 4 (open bars) on M3S liquid media amended with avocado cell wall material. All experiments were conducted three times. *C. acutatum* is represented by GAQ34 and GAQ01 and *C. gloeosporioides* by GAQ31, GAQ35, GAQ42, DTO09 and HSR02. Letters in bars represent significant differences among isolates (P<0.01) based on an analysis of variance.

numbers EQB56455, EQB58288 and EQB56345.

PL activities were significantly different (P<0.01) among the isolates tested. Isolates GAQ34 (*C. acutatum*), GAQ35 (*C. gloeosporioides*) and GAQ42 (*C. gloeosporioides*) had the highest activities at pH 7. These isolates also showed larger diameters of decay lesions and greater abundance of salmon-colored conidial masses. Moreover, when compared with the other isolates, they had the highest activities at pH 7 and the lowest at pH 4, also showing greater activity differences between pH 7 (1.4 U/mL) and pH 4 (0.1 to 0.2 U/mL) (Figure 2).

These activity differences may be explained by a difference in pathogenicity. For example, higher enzyme activity at pH 7 may be related to a pathogen's preferential ability to attack ripe avocado fruit. PL activity at this pH by these isolates was 1.4 U/min. However, strains showing greater PL activity at low pH could be more damaging to younger fruit as has been observed in Michoacan.

PL activity at pH 4 was 0.34 U/min for *C. acutatum* and 0.35 U/min for *C. gloeosporioides* as calculated by linear regression (r^2=0.99). All PL activities recorded were significantly different (P<0.01) among the seven isolates and two pHs.

Both species produced PL which was detected by immunoblot when exposed to avocado cell wall material. Treatments at pH 7 showed higher PL production than those at pH 4 (Figure 3A to D). Enzyme production by *C. gloeosporioides* (DTO09 and HSR02) could be related to its pathogenicity to young fruit, since they revealed a band of 39 kD at pH 4 detected by the antibody against PL. These results differ from those reported in studies carried out in other countries such as in Israel (Wattad et al., 1994; Miyara et al., 2008) and others (Herron et al., 2000) who state that PL is produced by the fungus only under alkaline conditions in the range from pH 6 to 11.2. Our results also conflict with others that state that anthracnose is a disease restricted to postharvest fruit, and report that the threshold for PL synthesis is from pH 5.1 upwards (Yakoby et al., 2000).

In addition, the isolates DTO09 and HSR02 also showed enzymatic activity at pH 4 which could explain why the fungus can be pathogenic in unripe fruit.

An important contribution of this work is that PL was produced by *C. gloeosporioides* at pH 4. In Mexico, anthracnose has been found in very young avocado fruit (Avila-Quezada et al., 2002) (Figure 4), therefore, these findings would seem to help explain the pathogenicity process by the enzyme activity recorded at pH 4 in the *in vitro* simulation of young fruit. Another observation is that the various strains had different specificities, for example with isolate GAQ42 producing PL at pH 7 but not at pH 4. It can be predicted that this strain would not affect young fruit.

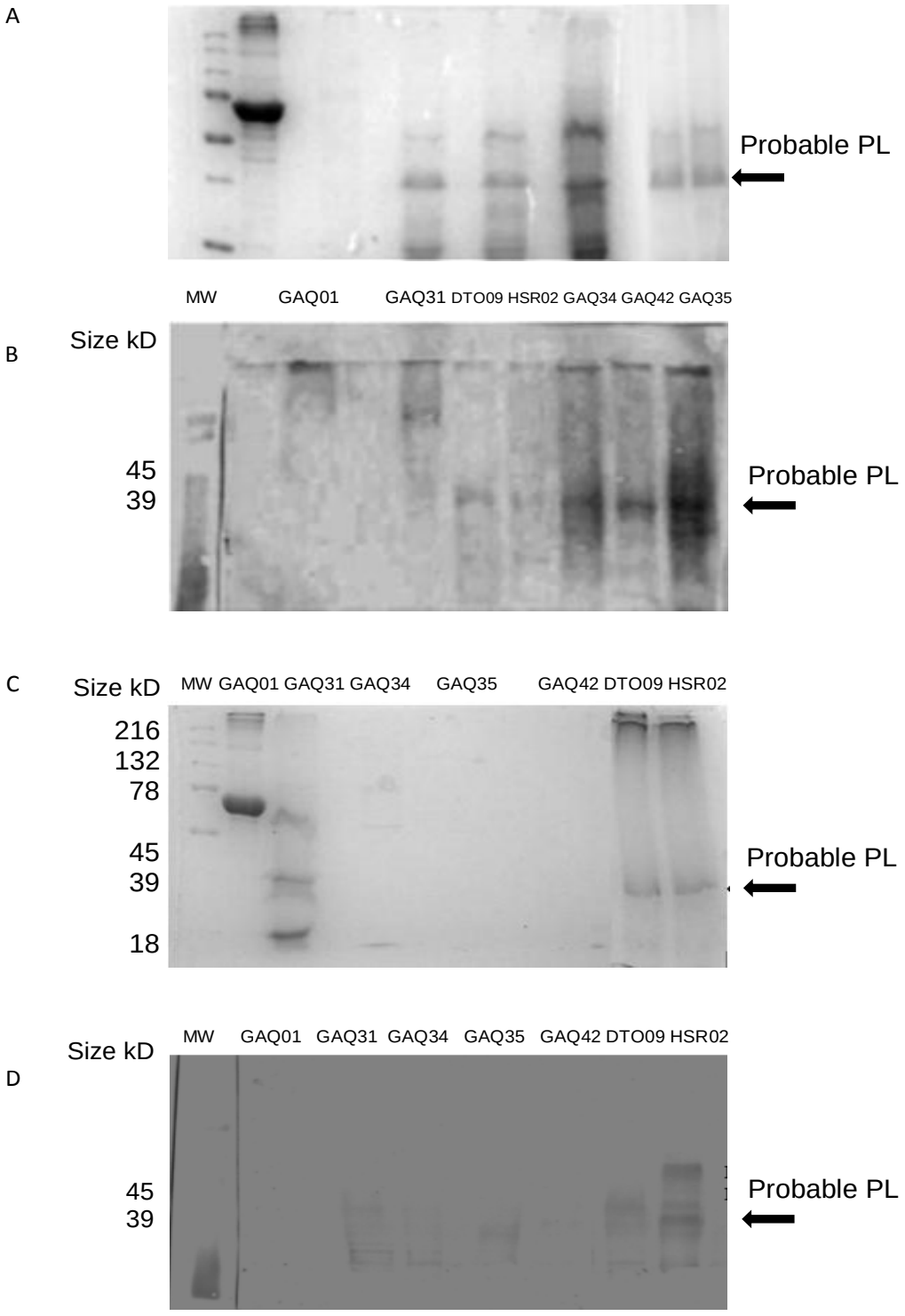


Figure 3. Pectate lyase (PL) secretion from seven *Colletotrichum* isolates which were grown for 5 days in pectolytic enzyme-inducing media. Clear supernatant (40 mL) was concetrated by evaporator at 40°C and samples (10 µL) were subjected to Western blot (immunoblot) analysis by running on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 h at 140 V constant voltage and transferred to a nitrocellulose membrane, which was analyzed with PL antibodies as described by Wattad et al. (1995). Western blot analysis of *C. gloeosporioides* and *C. acutatum* pectate lyase (PL) were carried out at pH 7 and 4. All experiments were conducted three times. The arrow indicates the precipitate at 39 kDa. *C. acutatum* is represented by GAQ34 and GAQ01 and *C. gloeosporioides* by GAQ31, GAQ35, GAQ42, DTO09 and HSR02. A) pH 7 in gel. B) pH 7 in membrane. C) pH 4 in gel. D) pH 4 in membrane. MW = molecular weight.

Figure 4. Very young fruit of avocado cv. Hass with anthracnose symptoms (Michoacan, Mexico).

On the other hand, isolates DTO09 and HSR02 which have similar enzymatic activities at pH 4, and can produce PL at pH 4 and at pH 7, may be able to infect both ripe and unripe fruit.

It is reported that *C. gloeosporioides* and *C. acutatum* produce phytotoxic secondary metabolites necessary for pathogenicity. Thus, *C. gloeosporioides* PL is produced in culture media above pH 5.8 because the pelB gene is expressed at pH values above 5.1 as claimed by Yakoby et al. (2000). Drori et al. (2003) found that pelB is expressed from pH 4.9 upwards, reaching maximum expression at pH 6. Prusky et al. (1989) suggest that the gene for protein secretion is translated under low pH conditions and that it then remains in the mycelium until the pH rises sufficiently to permit secretion. Some authors noted that PG mRNA production and protein secretion polygalacturonases (PG) occurs under low pH conditions (5 to 5.8) (Yakoby et al., 2000).

In earlier studies, Prusky et al. (1989) found that the PL of *C. gloeosporioides* macerates avocado wedges twice as rapidly as the endopolygalacturonase produced by the fungus. In this study, the authors found that some Mexican *Colletotrichum* isolates secreted PL at pH 4.0 and were more aggressive than the isolates from Israel (Guestky et al., 2005).

It is known that the PL of *C. gloeosporioides* is a polypeptide secreted by the pathogen in the presence of the host and causes maceration of avocado pericarp. In the Michoacan State microclimate, the fungus is able to macerate young avocado fruit pericarp. This observation would seem to find its explanation in our results which provide compelling evidence of enzymatic activity and PL under low pH conditions and detected by immunoblot testing.

Previous studies have shown that pectinases with lyase activity produced by *C. acutatum* have different patterns of pH regulation than those produced by *C. gloeosporioides*. Apparently in *C. acutatum*, these enzymes are expressed and secreted at very low pH values-at pH 3 according to Patiño-Torres (2010). However, one explanation for the lack of expression of PL from *C. acutatum* could be that the antibody was designed against *C. gloeosporioides* PL and it is possible that it is unable to detect PL from other *Colletotrichum* species.

Nevertheless, our hypotheses were properly tested because PL was expressed under low pH conditions such as will be the case when *C. gloeosporioides* challenges an unripe avocado fruit. Future work should include the use of immunoblot analyses and should also employ antibodies against *C. acutatum* PL tested under low pH conditions.

Conclusions

C. acutatum (represented by GAQ34 and GAQ01) and *C. gloeosporioides* (isolates GAQ31, GAQ35, GAQ42, DTO09 and HSR02) were pathogenic to avocado fruit cv. Hass and showed enzymatic activity at pH 4 and 7.

Only one isolate of *C. acutatum* (GAQ34) and four of *C. gloeosporioides* (GAQ35, GAQ42, DTO09, HSR02) produced pectate lyase (PL) detected by immunoblot assay at pH 7. This indicates that these isolates may be pathogenic in ripe (higher pH) avocado fruit.

C. gloeosporioides represented by the isolates HSR02 and DTO09 synthesized PL at pH 4 detected by immunoblot assay, indicating that these isolates are likely to be pathogenic in younger (lower pH) avocado fruit.

Conflict of Interests

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

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Full Length Research Paper

Development of a loop-mediated isothermal amplification assay for rapid detection of *Streptococcus suis* serotype 2

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The development of a loop mediated isothermal amplification (LAMP) assay for the rapid and sensitive detection of *Streptococcus suis*, an economically important swine pathogen is reported. Primers were designed targeting conserved region of the capsular polysaccharide (*cps2J*) gene of *S. suis* serotype 2 and 1/2. The LAMP assay produced reliable amplification in 60 min at isothermal conditions of 60°C. Genomic DNA extracted from *S. suis* serotype 2 and other related bacterial species causing illness in swine were used to optimize and validate the sensitivity and specificity of the assay. Based on a serially diluted genomic DNA of *S. suis*, the LAMP assay was found to be 100-fold more sensitive than the currently used conventional polymerase chain reaction for *S. suis*. These results indicate that LAMP can be an alternate method for the rapid and sensitive detection of *S. suis*, especially in resource-poor countries.

Key words: *cps2J* gene, loop-mediated isothermal amplification, *Streptococcus suis*.

INTRODUCTION

Streptococcus suis is an important bacterial pathogen that causes a wide range of diseases primarily in swine but also in humans and other animal species including cats, dogs, horses, birds, deer and ruminants (Devriese et al., 1990; Hommez et al., 1988; Wertheim et al., 2009). Infection in swine is associated with sudden death, dysentery, septicemia, endocarditis, arthritis, pneumonia, meningitis and abortion causing annual losses of more than 300 million dollars in the US alone (Staats et al., 1997). The organism is known to inhabit the upper respiratory tract of apparently healthy pigs, which can serve as a source of infection to other pigs (Marois et al., 2007). In humans, *S. suis* causes severe meningitis followed by hearing loss in 50-70% of infected patients (Dupas et al., 1992; Lun et al., 2007). Human cases of *S. suis* infections in North America are limited to persons working

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Table 1. Primers used to amplify the *cps2J* gene of *Streptococcus suis.*

Assay	Primer name*	Sequence (5' - 3')	Reference
LAMP	F3	ATGAATTTTTAACAGCCGATTC	This study
	B3	CGGAAATTATACATTTAGTAACTGG	This study
	FIP	GTCATAACAAACCATTGCCAATTCACCACGGAAACTTTTGGAAC	This study
	BIP	AATCGTTTATACCACGTAGAATGCAACCATCAGAATGATGCCA	This study
	BLP	ATGGAGAACAATATCGAAACAGCTC	This study
Conventional PCR	F	GCAGCGTATTCTGTCAAACG	[20]
	R	CCATGGACAGATAAAGATGG	[20]

* F3 = Forward outer; B3 = reverse outer; FIP = forward inner; BIP = reverse inner; BLP = reverse loop; F = forward; R = reverse.

with animals while it is the most frequently diag-nosed cause of streptococcal meningitis in Southeast Asia (Huy et al., 2012; Mai et al., 2008; Suankratay et al., 2004).

Based on the polysaccharide capsular antigens, thirty-five serotypes of *S. suis* (types 1 to 34 and type 1/2) have been described (Staats et al., 1997). On the basis of sequence analysis of the 16S rRNA and *cpn*60 genes, serotypes 32 and 34 have now been reclassified as *Streptococcus orisratii* (Hill et al., 2005). A wide variation in virulence has been reported among the *S. suis* serotypes but serotype 2 is considered to be the most virulent worldwide and is most frequently isolated from outbreaks in swine as well as in humans (Staats et al., 1997).

While it is not difficult to cultivate and identify *S. suis* under laboratory conditions, it takes about 2-3 days to correctly identify the organism. Also, bacterial culture has low sensitivity and is often complicated by the presence of multiple microbes and prior use of antimicrobials. Polymerase chain reaction (PCR)-based assays have been developed and used for the detection of *S. suis* (Maroise et al., 2004). While conventional PCR is a sensitive molecular diagnostic tool, it is time consuming and labor intensive as it requires extensive prior sample preparation to remove PCR inhibitors and post PCR analysis such as gel electrophoresis. Conventional PCR assays also need expensive thermocyclers that may not be accessible to resource poor countries (Fredricks and Relman, 1998).

To overcome the limitations of the conventional PCR assays and to make the technology accessible to resource limited laboratories, loop mediated isothermal amplification (LAMP) assays have been developed and used for molecular detection of pathogens (Hara et al., 2007; Hong et al., 2004; Notomi et al., 2000). LAMP is a simple, rapid, and cost-effective method for pathogen detection as compared to conventional PCR. The LAMP method uses four primers targeting six regions on the target DNA. Additionally, two more primers (loop primers) can be used to accelerate the reaction. The incorporation of colorimetric indicators such as calcein or hydroxyl-napthol blue makes it possible to visually detect amplification results (Goto et al., 2007, 2008; Tomita et al., 2008). Since amplification in LAMP is done at a constant temperature, no specialized thermocycler instrumentation is needed, making it easier to use in resource limited laboratories.

In the current study, the development of a serotype specific LAMP assay for the rapid and sensitive detection of *S. suis type 2* is reported.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were: *S. suis* serotype 1 DSM 9683, serotype 2 ATCC 43765, *Mycoplasma hyopneumoniae 232, Haemophilus parasuis* strain 29775. *S. suis* serotype 7 and 9, *Pasteurella multocida*, *Actinobacillus pleuropneumonia, Actinobacillus suis*, *Staphylococcus aureus*, *Klebseilla pneumoniae*, *Salmonella* spp., *Escherichia coli* were controlled reference strains obtained from the archives of the Minnesota Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN.

DNA extraction

Total genomic DNA was extracted from overnight grown colonies of various bacteria using a commercial kit (PrepMan® Ultra, Applied Biosystems). In brief, colonies were picked with a moist sterile swab and dislodged into 200 µl of phosphate buffered saline (PBS) in a micro-centrifuge tube. The bacterial cells were pelleted by centrifugation, re-suspended in 200 µl of buffer and placed in a boiling water bath for 15 min. The DNA was separated from cell debris by centrifugation at 8,000 g for 2 min and the resulting supernatant was collected and used in subsequent analysis.

Development of LAMP assay

Primers targeting the conserved region of capsular (*cps2J*) gene (GenBank accession number. JN980172) of *S. suis* serotype 2, were designed using a PrimerExplorer V4 software (http://primerexplorer.jp/e/index.html). The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to ensure primer specificity. The sequences of the primers are shown in Table 1. The LAMP reaction was carried out in a heating block and the reaction consisted of 12.5 µl of 2× reaction mix containing 40 mM Tris–HCl, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $[NH]_4SO_4$, 0.2% Tween 20, 1.6 M betaine, 50 µM of calcein, 2.8 mM each deoxynucleotide triphosphates, 0.8 µM of each internal

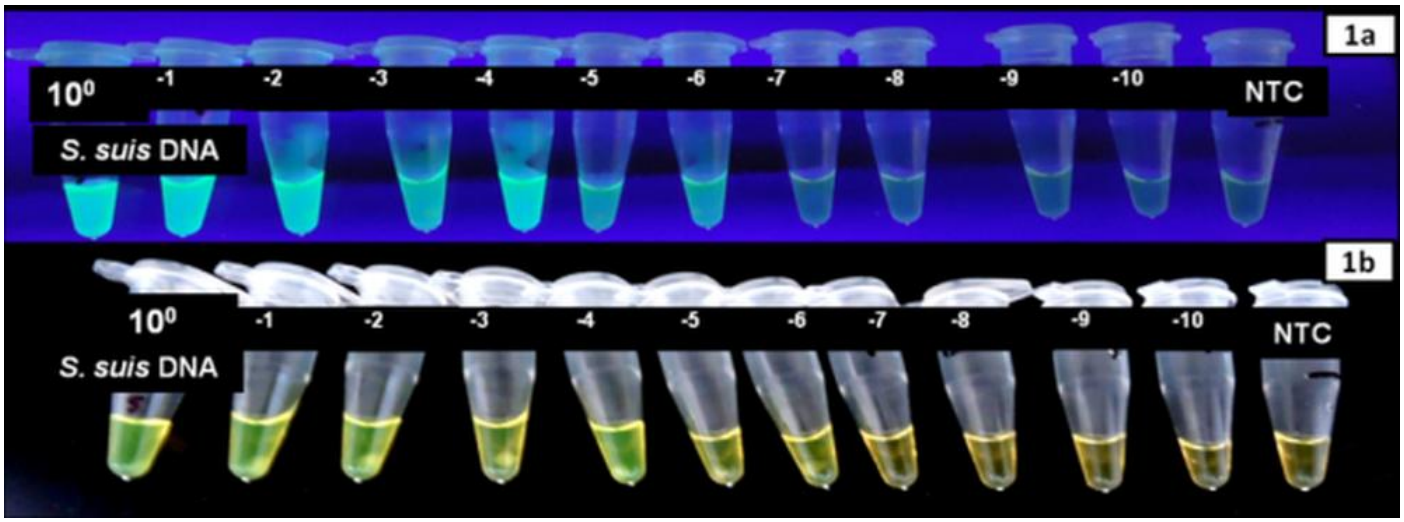

Figure 1. Visual detection of loop-mediated isothermal amplification products from 10-fold serial dilutions of *S. suis* DNA template corresponding to 10^{0} - 10^{-10} dilutions. a). Observation for color change with naked eye. Positive reactions are indicated by bright yellow color in tubes 1 to 7 (10^{0} - 10^{-6}). b). Upon UV irradiation, tubes 1 to 7 (DNA dilutions of 10^{0} - 10^{-6}) showed strong fluorescent green color, while tubes 8 and 9 had a weak fluorescent green color. No change of color was observed in tubes 10 and 11 (10^{-9} and 10^{-10}) including lane 12 that served as a no template negative control.

primers (BIP and FIP), 0.4 µM of each outer primers (B3 and F3) 0.4 µM of loop (LB), 1 µl of the Bst DNA polymerase (8 U), 2 µl of target DNA and appropriate amount of PCR grade water to make a final volume of 25 µl. Optimum time and temperature for LAMP reaction was determined by performing the reaction at different temperatures ranging from 56 to 64°C (in increments of 2°C) for time periods ranging from 15 to 60 min (in increments of 15 min). The reaction was stopped by increasing the temperature to 80°C for 2 min.

Validation of the LAMP assay

The sensitivity of the LAMP assay was determined using 10-fold serial dilutions of the genomic DNA from *S. suis* serotype 2 (corresponding to 428 to 0.0428 fg/µl of DNA). Triplicates of each dilution were tested in three times in three different days. Upon completion of the amplification reaction, the tubes were inspected for color change with unaided eye and for fluorescence upon ultraviolet irradiation. In addition, the LAMP products were separated on a 2% agarose gel and visualized under UV light upon ethidium bromide staining. The specificity of the LAMP assay was tested by using DNA extracts from several related bacterial species causing illnesses in swine as mentioned under the section on bacterial strains.

Conventional PCR

For comparative purposes, the newly developed LAMP assay and the *S. suis* serotype-specific conventional PCR were run in parallel. PCR was performed using commercial PCR Master Mix kit (Qiagen). The reaction mixture contained 10 µM of each primer, 12.5 µL of master mix (Qiagen) and 2 µL of template DNA in a total volume of 25 µL. The cycling program consisted of an initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, annealing at 65°C for 1 min and extension at 72°C for 3 min with a final extension at 72°C for 10 min. PCR products were separated on a 1.2 % agarose gel. From positive reactions, an amplicon of *ca.* 688 bp was obtained (Okuwumabua et al., 2003).

RESULTS

The LAMP assay successfully amplified serotype specific capsular polysaccharide (*cps2J*) gene from *S. suis* after 60 min at isothermal conditions of 60°C (Figure 1a). Initially, the LAMP amplification products were inspected for color change with unaided eye and were later visualized under ultraviolet light for florescence. Negative controls without added template retained light yellow color of the pre-incubation reaction mix while positive reactions showed green fluorescence (Figure 1a and 1b). When LAMP products were subjected to 2% agarose gel electrophoresis, typical ladder-like amplification products were detected in positive reactions (Figure 2a).

To determine the sensitivity of the LAMP assay, serial 10-fold dilutions of a *S. suis* DNA extract (original DNA concentration 428 ng/µl) were tested. Clear amplification that was detectable by florescence and by typical LAMP products on agarose gel was observed in dilutions as high as 10^{-6} with estimated DNA concentration of 0.428 pg/µl (Figures 1b and 2a). Although as low as 4.28 fg/µl of template DNA showed amplification products on agarose gel, they were not as clear as those with higher template concentrations. Therefore, a reliable detection limit of the LAMP assay was determined to be 0.428 pg/µl of template DNA. The conventional PCR that was run in parallel with LAMP had a detection limit of 42.8 pg/µl of template DNA (Figure 2b), which were 100 fold less than the newly developed LAMP assay. To ascertain the specificity of the

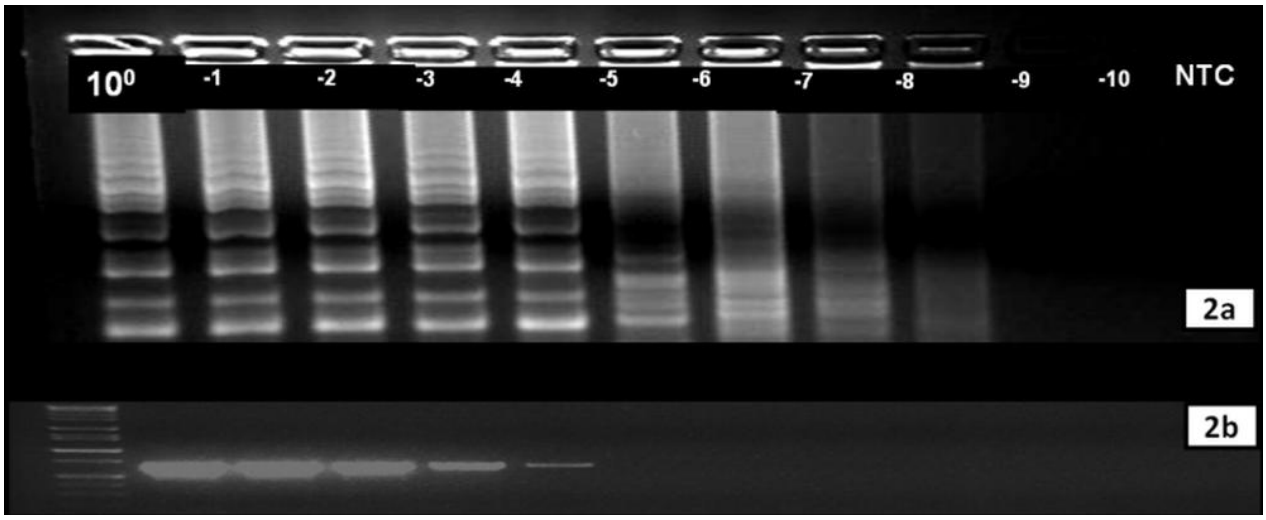

Figure 2. Agarose gel electrophoresis of amplification products. a). Typical ladder-like pattern, a characteristic feature of loop-mediated isothermal amplification (LAMP), can be seen. Lanes 1 - 9 depict LAMP products from 10-fold serial dilutions of *S. suis* DNA template corresponding to 10^{0} - 10^{-8} dilutions. No product was detected in lane 10 and 11 (10^{9} and 10^{10}). Lane 12 was a no template negative control. b). Conventional polymerase chain reaction products showing single bands in lanes 1-5 (dilutions 10^{0} - 10^{-4}).

assay, genomic DNA from pure cultures of other bacterial species known to colonize swine respiratory tract were tested and found negative. The newly developed LAMP did not cross react with *S. suis* serotypes 1, 7 and 9.

DISCUSSION

S. suis is one of the most economically important swine pathogen causing heavy losses to the swine industry (King et al., 2001). Recent reports indicate that *S. suis* is an emerging zoonotic pathogen that poses serious occupational health hazard to persons working with pigs (Tara et al., 2008). Serotype 2 is known to be the most virulent and most frequently isolated serotype from *S. suis* outbreaks (Staats et al., 1997). Multiplex PCR assays have been developed for the detection of several serotypes of *S. suis* (Okuwumabua et al., 2003).

In the present study, we successfully developed and validated a LAMP assay for sensitive and specific detection of *S. suis* serotype 2. Primers were designed based on the sequences of *S. suis cps2J* gene known to be specific to *S. suis* serotype 2 and the LAMP assay successfully amplified this gene and yielded reliable amplification products in 60 min (Figure 2a).

One of the characteristic features of LAMP is its ability to amplify nucleic acids under isothermal conditions (Notomi et al., 2000) allowing the use of simple and inexpensive equipment. As compared to the currently employed conventional PCR used to detect *S. suis*, the newly developed LAMP assay was 100-fold more sensitive. The use of two to three primer pairs designed to target six to eight sequences makes LAMP a highly sensitive and specific assay as shown in the current study as well as in previous reports (Das et al., 2012; Venkatesan et al., 2012).

The robustness of LAMP is also ascribed to the formation of large amounts of pyrophosphate ion that enables visual monitoring of amplification products under natural light or UV irradiation (Mori et al., 2001). In the newly developed *S. suis* LAMP assay, calcein was used as a fluorescence detection reagent; strong green fluorescence was visually detected from positive reactions within 60 min. The degree of fluorescence corresponded to typical ladder-like LAMP amplification products upon agarose gel electrophoresis (Figures 1b and 2a).

Although, not all *S. suis* serotypes were tested, the LAMP assay tested negative for *S. suis* serotypes 1, 7 and 9 suggesting that the new LAMP is specific for serotype 2 of *S. suis*. This is not surprising because specific primers showing 100% sequence identity with the *cps2J* gene sequence of *S. suis* serotype 2 were used. In contrast to the 33 *S. suis* serotypes, the *cps2J* gene in *S. suis* serotype 2 and serotype 1/2 has been reported to have high sequence homology (Zhang et al., 2013). Since we did not test serotype 1/2, we cannot tell if our LAMP assay could discriminate between these two strains. Also, no cross reaction was observed with other bacterial species known to colonize swine respiratory tract. These results indicate that LAMP can be used as a rapid and diagnostic tool for the detection of *S. suis*. This study is based on using well characterized bacterial isolates. Further studies are necessary to determine if this assay will be applicable to samples collected from the field.

Conflict of Interests

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

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Full Length Research Paper

High viral load is necessary to detect human immunodeficiency virus by polymerase chain reaction in blood-soiled needles

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The estimated risk of contamination through percutaneous injuries involving HIV-contaminated sharp objects is 0.32%. It is well known that a high viral load is one of the conditions that increase the risk of HIV-1 transmission in occupational accidents. In order to estimate the level of viral load that could be necessary for HIV-1 transmission to health care workers undergoing occupational accidents with contaminated needles, blood-soiled needles were obtained from HIV-positive patients just after drawing blood to determine HIV-1 viral load by NASBA. Detection of HIV-1 genome was performed by reverse-transcription polymerase chain reaction (RT-PCR) after RNA extraction directly wash-outs collected from needles immediately after blood drawing. Samples collected from needles used on 102 HIV-1-positive patients were RT-PCR tested but only 7 were HIV-1-positive. Each patient sample was compared to its respective viral load. All 7 RT-PCR-positive samples were from patients with viral loads higher than 160,000 RNA copies/mm^3. Since the needle samples tested here could be considered to be of very high risk of HIV-1 transmission following an occupational accident, RT-PCR detection of HIV-1 genome was only possible in patients with high viral load. These results indicate that, among all risk factors, high HIV-1 viral load probably is the most important risk factor for HIV transmission to health care workers during occupational exposure.

Key words: HIV, viral load, polymerase chain reaction (PCR), RNA, occupational accidents.

INTRODUCTION

Since HIV/AIDS was first identified 30 years ago, more than 60 million people have been infected and approximately 30 million people have died (Jiang et al., 2014). The HIV/AIDS has become one of the greatest

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Table 1. Characteristics of 102 patients enrolled in the study.

Variable	Patient (%)
Median Age (years)	37.5 (10-66)
Male	65 (63.7)
Female	37 (36.3)
CD4+ cells/mm3 (median)	327.5
CD4 < 200 cells/mm3	34 (33.3)
Viral load (median, log10)	4.46
Undetectable viral load	29 (28.4)
≤ 150,000 RNA copies/mL	58 (56.8)
≥150,000 RNA copies/mL	15 (14.7)

problems of public health worldwide (Kopfer and McGovern, 1993) and occupational accidents with blood or body fluids may pose a high risk to health care workers (HCWs). Both blood-borne hepatitis viruses (hepatitis B [HBV] and hepatitis C [HCV] viruses) and the human immunodeficiency virus (HIV) represent the vast majority of cases of occupational infection (Garcia and Facchini, 2009). Prüss-Üstün et al. (2005) reported that the total number of infections attributable to injuries with sharp instruments, due to occupational exposure to percutaneous injuries, was 16,000 HCV, 66,000 HBV and 1,000 HIV infections among HCWs. After percutaneous occupational exposure of infected blood, the highest risk for HIV infection is 0.3% and after mucosal exposure is 0.09% (Ippolito, 1993). However, among all risk factors, the ones associated with the highest risk are exposure to a large quantity of blood from the source patient, as indicated by a device (e.g., a needle) visibly contaminated with the patient's blood; a procedure involving a needle placed directly in a large vein or artery; and a deep injury (Carpenter, 1997). However, the exact amount of HIV-1 remaining in these needles has not been measured.

The detection of HIV-1 RNA copies and their use as a viral load is just a surrogate measure of the viral titer, assessing only the amount of cell-free virus in the peripheral blood, but it has been assumed that a low viral load (e.g., < 1,500 RNA copies/mL) or one that is below the limit of detection is probably associated with a lower risk of HIV transmission, but it does not rule out the possibility of viral infection (CDC, 2005). Measurements of HIV-1 viral loads may play an important role in assessing the infectivity of the source patient in the context of an occupational exposure.

Based on this assumption and the high sensitivity of reverse-transcription polymerase chain reaction (RT-PCR), we investigated the ability of RT-PCR to detect HIV-1 genome in the whole blood retained in needles used to collect blood for HIV-1 viral load measurements, since this approach mimics the occupational exposures associated with the highest risk for HIV-1 infection. We also compared the RT-PCR results with the viral load of these patients, aiming at the definition of which viral load level posed the highest risk to HCWs after an occupational exposure.

MATERIALS AND METHODS

We analyzed blood-soiled needles used on HIV-1 positive patients, just after blood drawing to determine the HIV-1 viral load. Viral load was measured by the NASBA assay according to manufacturer's protocol, and reported as the number of HIV-1 RNA copies/mL. Patients were enrolled at the Blood Collection Center of the Hospital das Clínicas da Faculdade de Medicina de RibeirãoPreto, São Paulo State, Brazil, after signing a written consent form.

We collected blood samples, one by one, directly from the veins of the patients using sterile, previously unused needles (25×8 mm; Becton Dickinson, USA) after blood drawing, washed with 140 µl phosphate-buffered saline (PBS), and then properly discarded. PBS washings were transported to the virology laboratory under refrigeration where the RNA was extracted from the samples. RNA extraction was done with the QIAamp RNA viral kit (QIAGEN, Inc., Valencia, California, USA) according to the manufacturer's instructtions. The first cDNA strand was synthesized in a 20 µL final volume containing 0.1mMdNTP (Pharmacia, Piscataway, NJ), 7.5 U of Superscript™ (Invitrogen, Carlsbad, CA), 4 µL of 5X reaction buffer (Invitrogen, Carlsbad, CA), 25 pmol of random primers (pdN6; Pharmacia, Piscataway, NJ) and 11 µL (in average, 2.4 µg) of total RNA. The mixture was then incubated at 42°C for 1 h, and then for 1 min at 70°C to denature the reverse transcriptase. Amplification of HIV-1 was performed with two sets of primers in a two-step nested RT-PCR strategy using the primers described by Rezende et al. (2002). In our laboratory, this RT-PCR protocol had an analytical sensitivity of 500 RNA copies/mL (data not shown).

The study was conducted from December 2000 to May 2001, and it was approved by the Ethics Committee of the Hospital das Clínicas da Faculdade de Medicina de RibeirãoPreto. Clinical data of the patients were blindly obtained by chart review and correlated with the PCR results.

RESULTS

Samples of 102 patients were studied, 63.7% male and median age of 37.5 years. Most recent examination showed median CD4 count of 327.5 cells/mm^3, undetectable viral load in 29 patients and median viral load of 4.46 log10 in the rest of them (Table 1). Most patients had antecedent opportunistic infection (66%), CD4 nadir below 200 cells/mm3 (73%) and two of them had been diagnosed recently. Zidovudine, lamivudine and indinavir were the antiretroviral drugs most commonly used.

Out of 102 PBS washings, RT-PCR detected only 7 samples positive for HIV-1, and in these samples, the viral load ranged from 160,000 to 870,000 HIV-1 RNA copies/mL (Table 2). RT-PCR negative results included 29 patients with undetectable viral load (below 50 RNA copies/mL), 58 patients with viral load below 150,000 copies/mL, and 8 patients with viral load higher than 150,000 copies/mL (Table 1). All patients with viral load higher than 150,000 copies/mL were classified in the CDC category C3.

DISCUSSION

Our results show that most high viral load is considered a

Table 2. Viral load measurements on HIV-positive samples by RT-PCR.

Patient	Viral load (copies/mL)
1	160,000
2	210,000
3	220,000
4	300,000
5	530,000
6	620,000
7	870,000

risk factor for HIV infection on occupational exposures thus, we have investigated the presence of HIV-1 remaining on needle-retained blood samples of 102 HIV-positive patients through the ability of PCR to detect HIV-1 genome. This approach mimics an occupational exposure to a hollow-bore needle that had been used for accessing a large blood vessel, and where there would still be blood present on needles. Garcia and Facchini (2009) found that percutaneous exposures occur more often than the mucocutaneous exposures, and also needles were usually more frequently involved in percutaneous exposures. While preventing exposures to blood and body fluids is the primary means of preventing occupationally HIV infection, appropriate post-exposure management is an important element of workplace safety (CDC, 2005), and knowing the viral load at the moment of the occupational accident might give us an idea of the risk of HIV-1 transmission involved in the accident.

To reach our goal, we investigated which levels of HIV-1 viral load, according to NASBA values, would be detected by a RT-PCR protocol used in our laboratory that readily detects RNA copies as low as 500 copies of HIV-1 RNA/mL. NASBA values were used for comparison due to the fact that it is the technique elected by the Brazilian Government to measure the HIV-1 viral loads of patients followed at the AIDS clinics. The rational for this study is that both molecular assays are readily available in our hospital, and RT-PCR could be used to assess the risk for a possible HIV-1 contamination during occupational exposures. Our results show that RT-PCR results showed a good correlation with the viral load obtained on the same sample, since RT-PCR was positive only in patients with high viral loads, ranging from 160,000 to 870,000 HIV-1 RNA copies/mL. The fact that more than half of the samples with high viral load were RT-PCR negative is probably due to the amount of blood that was present on the needles.

The size of the inoculum is also an important factor in the assessment of a possible risk of infection by a microorganism. The HIV-1 inoculum associated with occupational exposure is yet to be determined. Based on the facts that the lowest HIV-1 viral load detected by RT-PCR was 160,000 HIV-1 RNA copies/mL and that in a needle stick injury, the volume of the inoculum ranges from 10 to 100 µL (Kopfer and McGovern, 1993) the data acquired in this study show that the viral titer in the patients' needles ranged from 1,600 to 16,000 virus/mL. This finding is important because it suggests that viral load as low as 1,600 virus/mL may be implicated in HIV-1 transmission after occupational exposures.

Heimer et al. (1992) demonstrated that PCR is a suitable technique to detect HIV-1 proviral DNA in needles used in injecting drug users since they were able to detect the HIV-1 proviral DNA, especially when there was visible blood in the returned needle. In this study, instead of detecting the proviral DNA, we tried to detect the HIV-1 RNA genome on the blood that was still present on the needles used for collecting blood from HIV-1 patients. Thus, the data presented here differ from those previously published and provides a rough estimate of what happens in a real occupational accident. Even though our study may be criticized due to the fact that it did not use the RT-PCR to produce a quantitative data on the HIV-1 levels contaminating the needles used to draw blood from our patients, the correlation with NASBA measurements agrees with other studies showing that the higher the source of patient's viral load, the higher the risk of acquiring HIV-1 after a percutaneous accident. Prüss-Üstün et al. (2005) reported that 4.4% of HIV infections in HCWs globally were due to sharps injuries. Precise worldwide data are not available on the annual number of needle sticks and other percutaneous injuries among HCWs. However, estimates indicate that 600,000 to 800,000 of such injuries occur annually in the United States (NIOSH, 1999), but only a few cases of HIV-1 infection following occupational accidents have been described, probably due to the amount of virus present in the needles involved in the accident. According to the CDC (2001), 23,951 cases of AIDS among HCWs were reported, representing 5% of the 469,850 adults or adolescents with AIDS. In 57 HCWs with documented occupationally acquired HIV infection, most (86%) were exposed to blood and most (88%) had percutaneous injuries, but the amount of virus in the device involved in the accident has never been investigated as it was done on this study. In this study, HIV-1-specific RT-PCR was positive only in patients with high viral loads, suggesting that this low RT-PCR positivity could be related to a low amount of virus retained in the needles and this could explain why there are few cases of HIV-1 infection following the accidents, since the immune response could more easily manage to control the infection resulting from a small amount of viruses. The small amount of viruses would also be more readily eliminated with specific anti-retroviral treatment. Furthermore, our data presents a cut-off (160,000 HIV-1 RNA copies/mL) that can be used in future studies set out to determine the risk of HIV-1 infection following occupational exposures, and from the these data, it can also be inferred that easy access to Highly Active Antiretroviral Therapy (HAART) and stimulating patient's good adherence to treatment will result in lower viral load and will make it more difficult for HIV-1 transmission to occur following an occupational exposure.

Conflict of Interests

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

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African Journal of Microbiology Research

Full Length Research Paper

The identification of *Bordetella bronchiseptica* from canine isolates of sequencing 16S ribosomal DNA

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The amplification and sequencing of 16S rDNA are useful tools to identify important bacteria that cause diseases in the respiratory tracks of a variety of domestic animals. *Bordetella bronchiseptica*, one of such bacterium, has demonstrated some changes in its metabolic core, generating a greater need for identification through amplification and sequencing of 16S rDNA. We applied this methodology to eleven nasal and pharyngeal isolates from dogs to detect *B. bronchiseptica*. Eight strains were completed from which seven showed 98-99% homology; and three strains were only partially sequenced. All eleven strains sequences were deposited in the GenBank (NCBI). What we found was that the sequencing of *B. bronchiseptica* 16S rDNA is an excellent tool that certifies conventional methods of identification and avoids environmental challenges.

Key words: *Bordetella bronchiseptica*, 16S rDNA, canine bacteria.

INTRODUCTION

Bordetella bronchiseptica is a commensal bacterium that has been isolated from the respiratory tracts of various domestic species, such as pigs, dogs, cats, rabbits, sheep, goats, horses, rats, hamsters and guinea pigs (Dawson et al., 2000; Abdoulaye et al., 2006; Rougier, 2006). It is associated with the clinical diseases atrophic Rhinitis in pigs (Pedersen, 1975; Ross et al., 1997) and Canine Infectious Tracheobronchitis or kennel cough (Keil and Fenwick, 1998; Mochizuki et al., 2008). This last disease is an illness that affects dogs of all age groups whether house pets or in kennels. Crossed transferences among species, such as dogs to cats (Foley et al., 2002), or rabbits to humans (Gueirard et al., 1995), have been observed. Since *B. bronchiseptica* has also been isolated from humans who suffer from compromised immune responses (Woolfrey and Moody, 1991; Ner et al., 2003;

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Table 1. Strains of *B. bronchiseptica* used in this study.

Strain	Source	Homology* (%)	GenBank access no.
151837083	Pet dog	99	JQ953661
151829626	Pet dog	99	JQ953654
Maxim	Pet dog	98	JQ953662
1518596000	Pet dog	98	JQ953656
MP1	Pet dog	99	JQ953657
231003011	Stray dog	99	JQ953652
331003011	Stray dog	98	JQ953655
361003011	Stray dog	98	JQ953663
151703011	Stray dog	98	JQ953653
78B1	Stray dog	85	JX129161
4F1	Stray dog	99	JQ953651
4617	ATCC†	ND‡	ND‡
10580	ATCC	98	JQ953658

*With respect to sequences already deposited in the GenBank. †American Type Culture Collection. ‡Not determined. The nucleotide sequences were aligned with those available in the GenBank (National Center for Biotechnology Information) data base (www/ncbi.nlm.nih.gov) using the Basic BLAST (Basic Local Alignment Search Tool) software. The sequences were deposited in the GenBank, and accession numbers were issued.

Wernli et al., 2011), it is important to analyze strains that are present in dogs to determine whether they are a source of contagion for people.

B. bronchiseptica is commonly found through conventional methods, that is, colony and microscopic morphology and biochemical profiles (Rath et al., 2008). However, culture conditions may affect the phenotypic and biochemical characteristics, leading to false-negative results.

The use of the polymerase chain reaction (PCR) for the identification of *B. bronchiseptica* has become more widely used. Thus, various primers such as those amplifying insertion sequences and gene coding for alcaligenin *alcA,* (Register and De Jong, 2006; Register and Sanden, 2006) and flagellin gene *flaA* (Hozbor et al., 1999) have been tested; however, the lack of specificity limits their use.

The amplification, sequencing and comparison of the gene coding for 16S ribosomal RNA (16S rDNA) have become useful in bacterial taxonomy. Essential for survival, ribosomal genes are highly conserved in bacteria, and help guarantee success as a means of identification. This genetic material is not as sensitive to environmental conditions as morphology and metabolic signatures.

In a previous work, there were partial sequencing (aprox 500 pb) for 16S rDNA *B. bronchiseptica*, canine gene isolates deposited at GenBank by our group and O'Connor SP (GeneBank Access number NR_024949). We reported a series of eight full sequences and three partial sequences (750-800 pb) after applying the 16S rDNA gene sequencing technology on *B. bronchiseptica* isolates from pet and stray dogs. The aim of this work was to provide several 16S rDNA sequences for *B bronchiseptica.* After identification through conventional bacteriological procedures, the amplification and sequencing methodology should provide an accurate identification of *B. bronchiseptica*.

METHODOLOGY

The isolates used in this work are listed in Table 1. From the eleven pharyngeal and/or nasal canine isolates, five were collected from pet and six from stray dogs captured by the Centers for Canine Control (CCC) in the Mexico City metropolitan area. The two reference strains from the American Type Culture Collection (ATCC) were 4617 and 10580.

The isolates were subjected to a panel of biochemical tests that included oxidase, catalase and gelatinase activity, as well as some metabolites such as indol (Cowan and Steel, 1974). Urease activity as well as carbohydrate fermentative metabolites were determined by means of a miniaturized BBL Crystal enteric/nonfermentative ID kit.

Isolates were grown on Brain Heart Infusion (BHI) agar for 48 h at 37°C. A bacterial suspension adjusted to $1.5x10^9$ CFU/ml of PBS was centrifuged at 1000 g for one minute. The DNA was extracted using a DNAeasy Blood and Tissue kit, according to the manufacturer's instructions.

The 16S rDNA region was PCR amplified with the universal primer pair RD1 (AAGGAGGTGATCCAGCC) and FD1 (AGAGTTTGATCCTGGCTCAG), according to Louws et al. (1999). 100 ng of total DNA from each isolate was used for the reaction. The reaction buffer contained 20 mM Tris HCl (pH 8), 2.5 mM $MgCl_2$, 2.5 units of Taq DNA polymerase 0.5 µmol of each primer and 0.2 mM dNTPs.

The mixture was placed in a 2720 thermocycler with an initial denaturation step for 3 min at 94°C. Then, the mixture underwent 30 additional cycles, each consisting of a denaturation for 1 min at 94°C; an annealing for 30 s at 55°C; and an extension for 2 min at 72°C. The reaction ended with a one-step extension for 7 min at 72°C, and it was stored at 4°C. PCR products (20 µl each) were

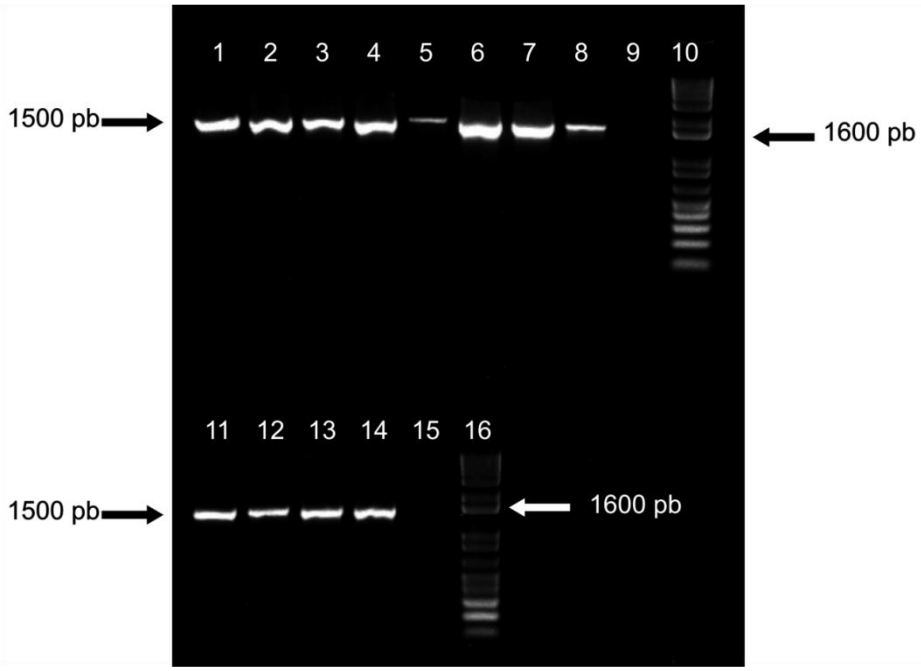

Figure 1. *Bordetella bronchiseptica* strains 16S rDNA PCR products. Lanes: 1– 231003011, 2– 151703011, 3– 331003011, 4– 361003011, 5- 10580 ATCC, 6- 78B1, 7- VIH6, 8- 151837083, 10- Biomarker 1Kb, 11- 151829626, 12- Maxim, 13- 1518596000, 14- MP1, 15- negative and 16- Biomarker 1 Kb. Lane 7 shows the PCR product from a DNA isolate that is not included in this paper.

analyzed by 1% agarose gel electrophoresis, visualized and documented.

For sequencing, the PCR products were purified with Wizard kits, according to the manufacturer's instructions. The amplicons were sequenced using a 3100 Genetic Analyzer. Nucleotide sequences were compared with those available in the GenBank (National Center for Biotechnology Information) database (www/ncbi.nlm.nih.gov) using Basic BLAST (Basic Local Alignment Search Tool) software.

Comparison of 16S rDNA regions V4-V5 (Sun et al., 2013), were performed using the ClustalW software (Larkin et al., 2007).

RESULTS AND DISCUSSION

All isolates, including the ATCC reference strains, were identified morphologically as *B. bronchiseptica,* this is, all showed Gram-negative coccobacillary microscopic morphology. Most of the strains, certainly the ATCC reference strains, complied with the whole metabolic pattern and the corresponding enzyme activities (Cowan and Steel, 1974). They expressed oxidase and catalase activities and degraded citrate. But, they did not ferment any of the ten carbohydrates tested (arabinose, mannose, sucrose, melibiose, rhamnose, sorbitol, mannitol, adonitol, galactose and inositol), neither did they produce indol. The strains showed urease activity, although in some isolates the activity was uncertain even after 48 h of incubation. Since this enzymatic activity is a key property that distinguishes *B. bronchiseptica* from other species within its genus (Dénes et al ., 2006), the expression was corroborated with the Christensen's tube method (MacFaddin, 2000). These measurements indeed confirmed very low quantities of urease. It is important to point out that urease activity may be regulated by culture medium composition, for example, by some $MgSO_4$ salts (Dénes et al ., 2006). Urease activity is one aspect of the metabolism of *B. bronchiseptica,* which occasionally generates misleading results when using only a biochemical and metabolic criteria, raising the need of molecular identification or confirmation as described in this paper.

The 16S rDNA is a gene present in all bacteria with 1500 bp coding for a portion of the 30S ribosomal subunit. The analysis of 16S rDNA has been widely used to establish the phylogenetic relationships among bacterial groups (Garrity et al., 2005; Trüper and Schleifer, 2006). The eleven isolates of *B. bronchiseptica* and the two ATCC reference strains produced an amplicon of 1500 bp with the primer pair FD1 and RD1 described by Louws et al. (1999) (Figure 1). From the eleven isolates, eight complete sequences were obtained and submitted to GenBank for identification. The remaining three sequences were obtained with one of the primers (FD1), with average sizes of 750 to 800 bp and were also submitted. The sequences were deposited in the GenBank under the following accession numbers: JQ953661-63, JQ953651-57, JQ9536559 and JX129161 (Table 1). All but one of the whole sequences showed 98-

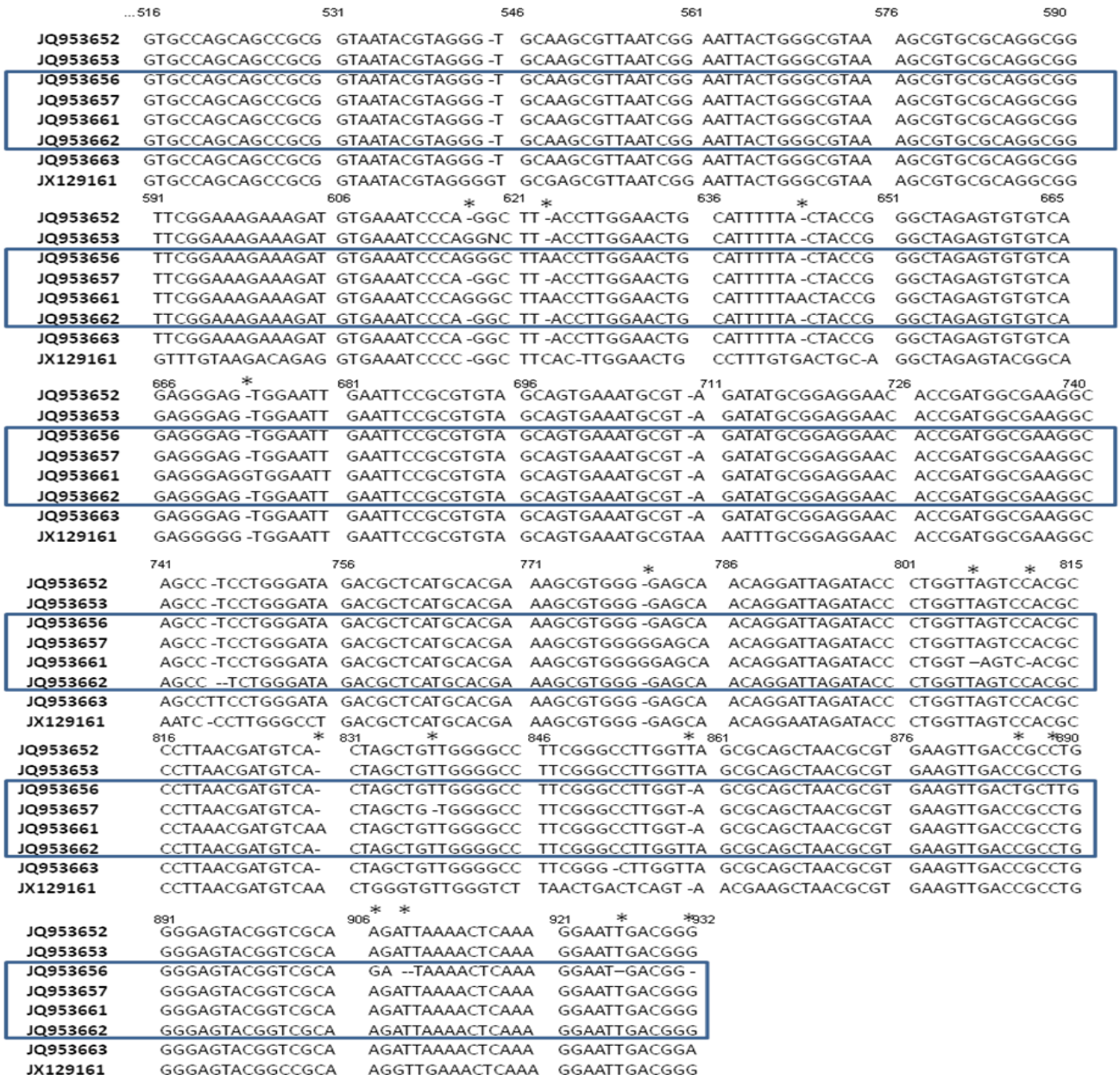

Figure 2. *Bordetella bronchiseptica* V4-V5 16S rDNA alignments. Regions V4-V5 (nucleotide 516 and so on) from 16S rDNA whole sequences from *B. bronchiseptica* were aligned by ClustalW. Sequences belonging to *B. bronchiseptica* 16S rDNA from pet dogs are embedded in square boxes. Differences are marked by *.

99% homology with *B. bronchiseptica*. The strain 78B1 showed 84% homology. The S16 rRNA has been used as a taxonomic tool because it interacts with its linking proteins and keeps the ribosome functioning. Horizontal transfer is considered to be highly unlikely for these genes; however some studies challenged that assumption (Kitahara et al., 2012). A thorough study by Sun et al. (2013) compared the intragenomic variation of the 16S rDNA with a substantial database made of sequences from Archea and bacteria. They found that indeed 16S rDNA is a conserved gene, although its mosaic design allows for determining whether the higher variability is within the gene sequence. The fragments named V4-V5 (515-533 and 907-927, respectively) are suggested to be appropriate to analyze sequences from suspected strains from the same species (contain the least intragenomic heterogeneity). In Figure 2, we compared the regions V4-V5 from the eight full sequences and observed that seven out of eight contained variations below the higher intragenomic heterogeneity (under 87%). This is in agreement with an adequate variability among strains found by Sun in his 2013 sequences comparison study. The strain 78B1

was slightly under such value (86%).

The comparison of 16S rDNA sequence between pet and stray dogs seems to indicate little differences. When comparing regions V4 and V5 (Sun et al., 2013) from stray and pet dogs, we observed in three out of four cases a deletion at nucleotide 859. In two of the pet-dog sequences, we also observed insertions of adenine at nucleotide 623 and guanine at nucleotides 617 and 780. Several deletions were also found at nucleotide sites: 806, 811, 838, 908, 909, 926 and 932. Insertions of adenine were noted at nucleotide sites: 644, 673 and 830, and three sets of transitions T/C (nucleotide 886 and 888) or G/A (nucleotide 906).

The strain 78B1 was classified as *B. bronchiseptica,* despite having only 84% homology with strains previously registered in GenBank. Homology of V4-V5 region was 86%, which is only 1% below the suggested strain variation. At first the GenBank itself suggested a possible "chimera" sequence, that is, the product of a contaminated culture. In response, the electropherogram of the strain was revised, and it was verified that there was no contamination. After the sequence was resubmitted, the strain was determined to belong to *B. bronchiseptica*. Exceptions to the established criterion of sequence homology equal to or greater than 97% for bacteria have been reported previously (Janda and Abbott, 2007; Petti, 2007). Such is the case of *Clostridium tetani* and *Clostridium innocuum*, which shows a 20% difference in the 16S rDNA gene sequence (Clarridge, 2004).

The biochemical allocation followed by a molecular analysis of 16S rDNA provides specific evidence that *B. bronchiseptica* belongs to the species. The information gathered from this work will enrich the data belonging to *B. bronchiseptica*. Rarely found in humans, *B. bronchiseptica* was isolated from an immune-compromised person who was suspected of contracting the bacteria through contact with a pet (Wernill et al., 2011). We were not able to compare pet 16S rDNA sequences to any *B. bronchiseptica* isolated from humans. There is only one partial sequence that is not long enough to contain the V4-V5 regions.

In the present study, we established the identity of *B. bronchiseptica* strains from dogs isolates first through canonical microbiological methods, which narrows the identification to species followed by16S rDNA sequencing to guarantee the strains' identity. Such methods combined in that specific order would diminish confusion with other closely related species with strains. Furthermore these sequences widen data bank information for the *B. bronchiseptica* 16S rDNA. A rich database for 16S rDNA is necessary for gene sequence-based identification schemes, that is, MicroSeq 500 (Woo et al., 2001); which offers the best alternatives in identifying strains lacking typical biochemical profiles.

Conflict of Interests

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

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Full Length Research Paper

The immunomodulatory effect of *Lactobacillus plantarum* strains on mononuclear cells isolated from human peripheral blood

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The immunomodulatory effect of nine strains of *Lactobacillus plantarum* by stimulation of human peripheral blood mononuclear cells (hPBMCs) from healthy donors was investigated. The production of pro-inflammatory, anti-inflammatory and regulatory cytokines by mononuclear cells following exposure of live bacterial cells was measured using multiplex analysis Luminex. The stimulation and production of cytokine by hPBMCs was evaluated to be strain specific and dependent on the density of cells used for stimulation. A significant increase of IL-6 and IL-1β secretion was demonstrated following stimulation of hPMBCs by high densities of lactobacilli ($P < 0.05$). By contrast, low density of lactobacilli strains increased the production of TNF-α ($P < 0.05$) in seven out of nine strains tested. The results have also shown the importance of testing probiotics with using multiplex methods that can help to explain the effectiveness/ineffectiveness of probiotics in the treatment and prevention of gastrointestinal diseases and allergies.

Key words: *Lactobacillus plantarum*, immunomodulation, cytokine.

INTRODUCTION

Immunomodulation effect is one of the mechanisms that demonstrate the beneficial effects of potentially probiotic bacteria on human health (van Hemert et al., 2010; Biswas et al., 2013). Several reports have shown that the variation in immunomodulatory effect of probiotic or strains of lactic acid bacteria (LAB) is a specific property of each strain (Vissers et al., 2010; Perez-Cano et al., 2010). Generally, microorganisms have an influence on

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Table 1. Species, origin and tested densities of lactobacilli.

Strain	Origin	Cell density C1 (log CFU/ml)	Cell density C2 (log CFU/ml)
L. plantarum CCDM 178	Original culture	5.11	9.28
L. plantarum CCDM 181	Silage	4.79	8.90
L. plantarum CCDM 182	Silage	4.67	8.85
L. plantarum CCDM 183	Dairy product	4.97	9.15
L. plantarum CCDM 194	Original culture	5.60	9.11
L. plantarum CCDM 381	Cow milk	4.94	9.11
L. plantarum CCDM 388	Goat cheese	4.86	9.04
L. paraplantarum CCDM 378	Beer contaminant	4.72	8.89
L. plantarum subsp. *argentoratensis* CCDM 806	Fermented cassava tubers	5.48	9.04

differentiation process of T helper cell (Th) towards Th1 or Th2 (Fang et al., 2000). Th1-type cells produce pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-2 activate macrophages and promote cell-mediated, and protective against intracellular infections (Ezendam and Henk van Loveren, 2006). *In vitro* studies have shown an increased production of Th1 cytokines in human monocytes and peripheral blood mononuclear cells followed by stimulation with some lactobacilli strains (Chiba et al., 2009; van Hemert et al., 2010). Th1 immune response is also related to the development of autoimmune diseases (arthritis, multiple sclerosis), and inflammatory bowel disease (IBD). Th2-type cells produce a variety of anti-inflammatory cytokines including interleukin IL-4, IL-5, IL-6 and IL-13 and promote humoral immune responses against extracellular pathogens (Doknor et al., 2012). Some lactobacilli are able to induce IL-10 production by immune system cells and strain change the balance of Th1/Th2 toward Th2 (Maragkoudakis et al., 2006). The most common microorganisms used for immunomodulation of immune cells are from genera *Lactobacillus* and *Bifidobacterium* (Fang et al., 2000). Positive effects conferred to lactobacilli include inhibition of pathogenic microorganisms, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium difficile*, *Salmonella typhimurium* and *Escherichia coli* (Bernardeau et al., 2008). Several studies demonstrated a health benefit of lactobacilli strains such as diarrhoea prevention, reduction of lactose intolerance, ability to displace enteropathogens from Caco-2 cells, decreased risk of bacterial vaginosis, reduced symptoms associated with irritable bowel syndrome and increased immune response (Lee et al., 2011; Zago et al., 2011; Borchers et al., 2009; Antonio et al., 2005).

The aim of this work was to compare the *in vitro* production of proinflammatory, anti-inflammatory and regulatory cytokines by mononuclear cells from healthy donors following exposure of different densities of live bacterial cells of *Lactobacillus plantarum* strains.

MATERIALS AND METHODS

Microorganisms

Nine strains of *L. plantarum* isolated from various sources, as shown in Table 1, were obtained from Culture Collection of Dairy Microorganisms Laktoflora® (Prague, Czech Republic). Sterile 10 ml aliquot of De Man-Rogosa-Sharpe (MRS) broth (MERCK, Germany) was inoculated with 1 % (v/v) of each of strains and cultivated at 37°C for 24 h. Subsequently, bacterial suspensions were centrifuged (3000 g, 15 min) and were washed three times in saline solution. Washed cells were resuspended in 6 ml X-vivo medium (Cambrex, USA). For enumeration of lactobacilli counts, 1 ml of each sample, was 10-fold serially diluted and plated onto MRS agar (MERCK, Germany), and cultivated anaerobically for 72 h at 37°C. The final working concentration in X-vivo medium was adjusted to 10^8 - 10^9 (C1) and 10^4-10^5 cells/ml (C2).

Isolation of mononuclear cells

Human blood was obtained from healthy adult donors from Blood Transfusion Centre of General Faculty Hospital (Prague). Human peripheral blood mononuclear cells (hPBMCs) were prepared using the method of Doknor et al. (2012) and Maragkoudakis et al. (2006) with some modifications. hPBMCs were isolated from blood by Ficoll-Hypaque gradient (Sigma-Aldrich, Switzerland). Cells at interphase ring layer (buffy coat) were carefully separated following centrifugation (600 g, 30 min), resuspended to 50 ml X-vivo medium (Cambrex, USA), washed twice and centrifuged at 400 g, 10 min and subsequently at 200 g, 10 min. Purified hPBMCs were resuspended in 5 ml X-vivo medium, counted into Bürker cell under microscope and adjusted at a final concentration of 10^7 cells/ml.

Stimulation of hPBMCs and cytokine determination

Mononuclear cells were stimulated with two different densities of lactobacilli, as shown in Table 1, or with either the pokeweed mitogen (PWM; Sigma-Aldrich, Switzerland). Negative control contained unstimulated hPBMCs and X-vivo medium. The microplate with samples was incubated for 3 days at 37°C. The supernatants were collected and stored at -20°C for analysis of cytokine profile. Determination of cytokine produced by stimulation of hPBMCs by lactobacilli was realised using Fluorokine MAP Human Base Kit A (R&D Systems, USA) for IFN-γ, IL-1β, IL-6, IL-10, IL-17 and TNF-α by multiplex analysis using Luminex 100 Analyzer (Luminex Corp., USA).

Table 2. Comparison of INF-γ, IL-17, IL-1β, IL-6, IL-10 and TNF-α levels (pg/ml) produced by hPBMCs following 3-days stimulation of two different densities of *L. plantarum* strains.

Strain	IFN-γ		IL-10		IL-17*		IL-1β*		IL-6*		TNF-α*	
	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2
178	2.5 ± 0.1	1.5 ± 0.1	24.2 ± 1.6	3.0 ± 0.5	6.8 ± 0.5	1.5 ± 0.5	162.4 ± 6.1	2400.0 ± 34.0	703.0 ± 42.6	4515.1 ± 85.0	719.2 ± 31.7	445.8 ± 13.6
181	0.7 ± 0.0	0.7 ± 0.1	5.8 ± 0.8	2.1 ±0.1	2.8 ± 0.1	0.5 ± 0.0	134.5 ± 18.0	1287.5 ± 59.3	744.1 ± 43	2331.8 ± 74.8	802.3 ± 22.1	472.9 ± 11.2
182	0.8 ± 0.1	0.3 ± 0.1	7.9 ± 0.3	0.7 ± 0.1	2.9 ± 0.3	1.2 ± 0.2	155.0 ± 12.9	1819.9 ± 62.3	994.5 ± 37.2	1014.3 ± 46.9	847.1 ± 13.5	42.4 ± 3.7
183	0.9 ± 0.0	0.1 ± 0.5	6.8 ± 0.4	0.7 ± 0.1	2.8 ± 0.3	1.1 ± 0.0	170.3 ± 17.7	1484.7 ± 53.8	984.1 ± 47.9	750.4 ± 15.4	756.9 ± 25.7	65.2 ± 3.4
194	0.7 ± 0.3	0.3 ± 0.1	1.4 ± 0.1	1.1 ± 0.3	1.1 ± 0.1	0.7 ± 0.0	26.7 ± 4.1	502.1 ± 7.8	49.2 ± 5.8	595.1 ± 7.0	160.7 ± 10.1	365.1 ± 10.9
381	3.4 ± 0.2	2.3 ± 0.4	8.4 ± 0.4	15.4 ± 1.3	2.0 ± 0.3	0.6 ± 0.2	161.2 ± 15.2	2238.6 ± 38.3	648.9 ± 18.7	2242.2 ± 36.7	650.5 ± 19.5	382.5 ± 16.1
388	0.5 ± 0.2	1.6 ± 0.4	5.8 ± 0.5	2.1 ± 0.5	2.6 ± 0.0	0.1± 0.0	104.0 ± 12.4	2266.0 ± 13.0	430.3 ± 32.2	2470.1 ± 16.1	581.3 ± 17.7	270.4 ± 14.3
378	1.8 ± 0.3	2.7 ± 0.1	1.3 ± 0.1	41.4 ±5.7	2.0 ± 0.2	2.3 ± 0.3	58.8 ± 2.8	682.3 ± 7.2	315.8 ± 35.3	2219.5 ± 43.2	303.9 ± 29.4	652.2 ± 34.0
806	0.6 ± 0.1	4.2 ± 0.2	7.7 ± 0.9	1.3 ± 0.2	0.9 ± 0.0	1.4 ± 0.3	241.0 ± 13.0	617.6 ± 8.7	192.9 ± 19.0	367.8 ± 11.8	764.3 ± 26.7	161.0 ± 19.3
NC	0.0 ± 0.0		0.1 ± 0.0		0.0 ± 0.0		0.8 ± 0.1		3.9 ± 0.3		0.9 ± 0.2	
P	0.6843		0.9770		0.0251*		0.0006*		0.0179*		0.0452*	

IL: interleukin; INF: interferon; TNF: tumor necrosis factor; NC- negative control; Value mean ± SD, n = 2; Two concentrations of lactobacilli (C1 and C2), as shown in Table 1, and 10^7 hPBMC/ ml were used. Levels of produced cytokines were compared by the ANOVA test. *Significant differences (P < 0.05) compared with cytokine levels produced by hPBMCs following 3-days stimulation of lactobacilli concentration, C1 and C2.

Statistical analysis

Results were evaluated by software Luminex IS 2.3 (Luminex Corp., USA). Differences in cytokine levels produces by hPBMCs were analysed using the ANOVA test with a level of significance of P < 0.05.

RESULTS AND DISCUSSION

Two different concentrations (C1 and C2) of nine *L. plantarum* strains were examined for pro-inflammatory, anti-inflammatory and regulatory cytokine production by stimulated PBMCs from healthy donors. Cytokine profile was determined using multiplex analysis in contrast to most studies where ELISA method was used (Doknor et al., 2012; Salva et al., 2010; Maragkoudakis et al., 2006). The main advantages of this method are low volume of sample, determination of wide spectrum of cytokine and missing binding of antigen to plate in comparison to the ELISA method. Pro-inflammatory cytokines TNF-α, IL-1β and IL-6 are the first cytokines produced in response to bacterial cells (Perdigon et al., 2002). Lactobacilli are also strong inducers of pro-inflammatory cytokines as was described in many studies (Vissers et al., 2010; Maragkoudakis et al., 2006). In our study, a significant increase of IL-6 and IL-1β secretion was determined followed by stimulation of hPMBCs by high densities of lactobacilli (P < 0.05) when compared with low densities of lactobacilli. The highest levels of IL-1β (2400.0 pg/ml) and IL-6 (4515.1 pg/ml) was detected after stimulation by *L. plantarum* CCDM 178. IL-1β influences immune response by activating lymphocytes (Biswas et al., 2013). Together with IL-6 induces the synthesis of hepatocytes in acute phase proteins. Interleukin 6 (IL-6) also stimulates T and B lymphocytes and immunoglobulin production. By contrast low density of lactobacilli strains increased the production of TNF-α (P < 0.05) in seven out of nine strains tested unlike IL-6 and IL-1β (Table 2). *L. plantarum* strains tested in study from Vissers et al. (2010) induced higher INF-γ, IL-12, TNF-α production compared with *L. acidophilus* strains and thus influenced the differentiation process of T helper cell (Th) toward Th1. In another study, Shida et al. (2006) observed levels of IL-12, TNF-α, IL-10 and INF-γ after stimulation by different densities of *Lactobacillus casei* Shirota. The results show that a lower dose (1 and 10 µg/ml) of *L. casei* Shirota induced a more effectively production of IL-12 and INF-γ than a high dose (100 µg/ml) which induced a higher production of TNF-α and IL-10. The anti-inflammatory IL-10 was able to inhibit the production of TNF-α, IL-1β and IL-6 and thus increasing

Th2 immune response (Maragkoudakis et al., 2006). No significant differences were observed between the production of IL-10 and INF-γ after hPMBCs stimulation by both low and high lactobacilli densities. Lee et al. (2011) tested the immune responses following stimulation by newly isolated lactobacilli from Korean infant faeces and found that isolated strains of *Lactobacillus gasseri*, *Lactobacillus fermentum* and *Lactobacillus plantarum* induced increased production of IFN-γ. In study of Pochard at el. (2002), lactobacilli (*L. plantarum*, *L. casei*, *L. rhamnosus*) and *Lactococcus lactis* reduced the production of IL-4 and IL-5 which play together with IL-9 and IL-13 an important role in a development and maintenance of allergic response. The next investigated pro-inflammatory cytokine in this study was interleukin 17 (IL-17) which is produced by the activated T lymphocytes and stimulates the expression of IL-6. High levels of IL-17 are associated with inflammatory bowel disease and other autoimmune disorders (Doknor et al., 2012). Decreased production of IL-17 was detected after stimulation by high densities of lactobacilli ($P < 0.05$).

In conclusion, results have shown that the stimulation of production of cytokines by hPBMCs is strain specific because each strain of *L. plantarum* influenced the stimulation of production of cytokine in a different manner. Production of cytokines by hPMBCs was also affected by used densities of lactobacilli. Levels of IL-17 and TNF-α increased after stimulation of hPMBCs by high densities of most *L. plantarum* strains. A significant increase of IL-6 and IL-1β secretion was determined following stimulation of hPMBCs by high densities of lactobacilli ($P < 0.05$). Testing probiotics and lactic bacteria with using multiplex methods can help to explain the effectiveness/ineffectiveness of probiotics in the treatment and prevention of gastrointestinal diseases and allergies. Therefore, the immunomodulatory effect of probiotics and lactic bacteria on mononuclear cells from healthy donors with allergies and autoimmune diseases and its dependence on dose of bacteria and time of stimulation were observed in other studies.

Conflict of Interests

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

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Full Length Research Paper

Phenotypic and genotypic identification of extended spectrum β-lactamases (ESBLs) among clinical isolates of *Escherichia coli*

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Extended spectrum β-lactamases (ESBLs) are emerging, diverse group of plasmid - associated antibiotic resistant enzymes that are presenting a public health concern. ESBLs were detected in *Escherichia coli* by performing phenotypic tests on 18 out of 35 *E. coli* isolates recovered from urine samples of patients with urinary tract infections in three Eygpt hospitals and identified as positive ESBLs according to CLSI screening criteria. Results of phenotypic confirmatory tests revealed that, broth microdilution test, combination disc test and double disc synergy test confirmed 14(78%), 16(89%) and 16(89 %), respectively of *E. coli* isolates as positive ESBLs. Genotypic screening using PCR was performed by testing for bla_{CTX-M}, bla_{TEM} and bla_{SHV} enzymes. Sixteen phenotypic ESBL isolates were positive for β-lactamase genes. Ten isolates produced both bla_{CTX-M} and bla_{TEM}, and 6 isolates produced bla_{CTX-M} only, while bla_{SHV} was not detected in any isolate. The sensitivity and specificity of combination disc and broth microdilution tests compared to PCR were 100%, while double disc synergy test showed sensitivity and specificity of 87.5 and 100% respectively. ESBL isolates were found to have multi-drug resistance pattern. No correlation could be made between type of ESBL and antimicrobial susceptibility profile of the isolate.

Key words: Extended spectrum β-lactamase, *bla* (TEM), *bla* (CTX-M), urinary tract infection.

INTRODUCTION

The prevalance of Extended spectrum beta-lactamase producing strains of Enterobacteriaceae are increasing globally. Recently, reports from different countries showed increasing number of β-lactamases of various types; OXA, CTX-M, TEM and SHV-derived (Tschudin-Sutter et al., 2012). The situation in developing countries is more serious due to inappropriate use of antibiotics, lack of routine susceptibility testing of clinical isolates, thus leading to poor treatment outcome and dissemination of different resistance genes in the community (Ama´ bile-Cuevas, 2010). Occurrence of resistance mechanisms may also have an epidemiological impact since these bacteriae are not only the cause of outbreaks but have become endemic in many hospitals throughout

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the world (Taneja and Sharma, 2008).

The guidelines of phenotypic detection for ESBLs are based on the principle that most ESBLs hydrolyze the third-generation of cephalosporins although they are inhibited by clavulanate (Paterson and Bonomo, 2005). The Clinical Laboratory Standards Institute guidelines (CLSI) for ESBL detection in *E. coli*, *Klebsiella* spp. and *Proteus mirabilis* recommend initial screening with either 8 mg/L of cefpodoxime, 1 mg/L each of cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by confirmatory tests such as, the double disc synergy test, the combination disc method, and specific E-test ESBL strips with both cefotaxime and ceftazidime in combination with clavulanate at a concentration of 4 µg/ml (CLSI, 2009a).

The genotypic tests for the detection of ESBLs primarily consist of polymerase chain reaction (PCR) amplification of the bla_{TEM} and bla_{SHV} genes with oligonucleotide primers. PCR may be followed by sequencing for characterization of the enzyme. Alternative approaches to sequencing include restriction fragment length polymorphism (RFLP), restriction site insertion PCR, ligase chain reaction, PCR with single-strand conformational polymorphism, and real-time PCR (Arlet et al., 1995; M'Zali et al., 1996; Kim and Lee, 2000; Chanawong et al., 2001).

Urinary tract infections (UTI) are prevalent worldwide (Gonzalez and Schaeffer, 1999). They may be caused by members of Enterobacteriaceae with *E. coli* presenting 70-95% of the causes of UTI, followed by *Klebsiella* spp (Behzadi et al., 2010). On the other hand, *Pseudomonas aeruginosa* play a major role in complicated and catheter - associated UTI (Tielen et al., 2013). Antibiotic resistance in uropathogens is increasing and is the cause for failure of treatment of complicated and uncomplicated UTI. Treatment of UTI caused by ESBL producing bacteria is challenging since ESBLs mediate resistance to extended spectrum third-generation cephalosporins and monobactams used for treatment (Ena et al., 2006). The recommended antibiotic treatment for such infections is carbapenems (Paterson and Bonomo, 2005). However, the use of piperacillin/tazobactam combination, colistin and fosfomycin was also reported to have a successful outcome comparable to that of carbapenems (Tumbarello et al*.,* 2006; Kanj and Kanafani, 2011).

This study is aimed to investigate ESBLs in *E. coli* as clinical isolates by both phenotypic and genotypic methods with evaluating their antimicrobial susceptibility profiles.

MATERIALS AND METHODS

Bacterial strains

A total of 35 *E. coli* isolates were recovered from 130 bacterial isolates obtained from the urine sample of patients clinically diagnosed as urinary tract infection and were collected at the Clinical Microbiology Laboratories of Shebeen El kom, Theodor Bilharz, and Kasr Al Ainy hospitals in Egypt. Urine samples showed bacterial colony count $\geq 10^3$ CFU/ml upon culture. All isolates were obtained after getting consent from patients. The study was carried out according to the guidelines of the Ethics committee of Cairo University. Isolates were identified by standard microbiological and biochemical tests including; culture on MacConkey agar (Oxoid, UK), Eosin methyllene blue agar (Difco USA), Chrom agar for *E. coli* (Oxoid, UK), indole, methyl red, Voges-Proskauer and citrate test (Oxoid, UK). They were then confirmed using API 20-E kit (Biomerieux, France). For long time preservation, all isolates were maintained in 25% glycerol in Luria-Bertani broth (LB broth) at -70°C.*E. coli* strain ATCC 25922 was used as a quality control strain for antibiotic susceptibility test. *Klebsiella pneumoniae* strain ATCC 700603 (bla_{SHV-18}) was used as a quality control strain for confirmatory tests of ESBLs.

ESBLs screening test

All isolates were screened for the presence of ESBLs by selecting the isolates showing reduced susceptibilities to cefotaxime and/or ceftazidime (MIC ≥ 2 µg/ml) (Tofteland et al., 2007). Cefotaxime sodium and ceftazidimepentahydrate (EL Nasr pharmaceuticls Co, Egypt) were used to prepare stock solution (using water for injection as a diluent) at a concentration of 200 µg/ml. The test was performed using agar dilution susceptibility testing according to the protocol of CLSI guidelines (CLSI, 2009a).

ESBL phenotypic tests

Double disc diffusion synergy test (DDS)

Test was performed according to a modified version of the Jarlier double-disc synergy method (Tofteland et al., 2007). Cefotaxime, ceftazidime, cefepime and aztreonamdiscs (Oxoid,UK) were placed around an amoxicillin/clavulanic acid disc at a distance of 25 mm (center to center)**.** A keyhole phenomenon was regarded as positive for ESBL production.

Combination Disc test (CD)

Test was carried out using discs of both ceftazidime 30 µg, ceftazidime-clavulanic acid 30/10 µg and cefotaxime 30 µg, cefotaxime-clavulanic acid 30/10 µg**.** Using a freshly prepared stock solution of clavulanic acid at concentration of 1000 µg/ml. Ten µl of clavulanic acid stock solution (as potassium clavulanate, Sigma Aldrich) was added to ceftazidime (30 µg) and cefotaxime (30 µg) discs within one hour before they were applied to the plates, allowing about 30 min for the clavulanic acid to be absorbed and the discs to be dried enough for application. Discs were used immediately after preparation or discarded. Results were interpreted according to the standards established by the CLSI (2003). An increase in the zone diameter by ≥ 5-mm for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone indicated the presence of an ESBL positive isolate.

Broth microdilution method (BMD)

Test was performed according to CLSI guidelines (CLSI, 2009b). The used antibiotics were cefotaxime and ceftazidime alone and in combination with clavulanic acid. Antibiotic/clavulanic acid was prepared by adding 4 µl of clavulanic acid stock solution (1000 µg/mL) to each antibiotic dilution. A decrease in the minimal inhibitory concentration (MIC) for either cefotaxime or ceftazidime by ≥ 3 two-fold dilutions when tested in combination with clavulanic acid indicated the presence of an ESBL isolate. The MIC was the lowest concentration of drug at which the microorganism tested did not demonstrate visible growth.

Table 1. PCR mixture (25 µl reaction).

Component	Volume (µl)
10× Dream Taq buffer (Fermentas, EU)	2.5
dNTP Mix, 10 mM (Fermentas, EU)	0.5
Forward primer (10 pM)	1.5
Reverse primer (10 pM)	1.5
Dream Taq polymerase (5 u/ µl) (Fermentas, EU)	0.2
Bacterial DNA	2
Nuclease free water	Up to 25

Table 2. Primers used in PCR.

Amplicon	Primers sequence (5'-3')	Product size (bp)
bla TEM	ATG AGT ATT CAA CAT TTC CG CCA ATG CTT AAT CAG TGA GG	858
*bla*SHV	ATG CGT TAT ATT CGC CTG TG AGC GTT GCC AGT GCT CGA TC	859
*bla*CTX-M	SCS ATG TGC AGY ACC AGT AA ACC AGA AYV AGC GGB GC	581

Genotypic identification of ESBLs

Rapid extraction of total genomic DNA was carried by suspending from 1-5 bacterial colonies in 100 µl of nuclease free water. Suspension was heated at 100°C for 10 min. After centrifugation at 9 000 Xg for 30 s, 2 µl of supernatant were used as template in a 25 µl PCR reaction (Nunes et al., 1999).

PCR for *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$

PCR reactions were carried out in thermalcycler (Techne FTGRAD2D, UK) using amplification protocol consisting of 30 cycles with an initial denaturation at 94°C for 5 min and final extension at 72°C for 4 min. Each cycle consisted of denaturation at 94°C for 1 min, annealing for 1 min at 53°C for *bla*$_{SHV}$, 47.9 °C for *bla*$_{TEM}$or at 52°C for *bla*$_{CTX-M}$ and final extension at 72°C for 1 min. *Klebsiella pneumoniae* strain ATCC 700603 (*bla*$_{SHV-18}$) was used as a positive control for *bla*$_{SHV}$ in PCR. PCR reaction mixture is listed in Table 1. Primers used for detection of *bla*$_{CTX-M}$,*bla*$_{TEM}$ *and bla*$_{SHV}$with the amplicon size are listed in Table 2 (Tofteland et al., 2007).

Antimicrobial susceptibility pattern of positive ESBLs isolates

The susceptibility pattern of isolates was determined by disc diffusion susceptibility test according to CLSI guidelines (CLSI, 2009a) using the following antibiotic discs (Oxoid, UK): amoxicillin (AML 10 µg), amoxicillin/clavulanic acid (AMC 20 µg/10 µg), piperacillin (PRL 100 µg), cephalexin (CL 30 µg), cefuroxime (CXM 30 µg), cefoxitin (FOX 30 µg), ceftriaxone (CRO 30 µg), cefoperazone (CFP 30 µg), cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg), aztereonam (ATM 30 µg), imipenem (IPM 10 µg), meropenem (MEM 10 µg), amikacin (AK 30 µg), Doxycycline (DO 30 µg), ciprofloxacin (CF 5 µg), gentamicin (G 10 µg), Cefoperazone/sulbactam (SCF 10 µg/5 µg) and sulphamethoxazole/trimethoprim 19:1(SXT 25 µg).The susceptibility pattern was determined using the CLSI interpretation chart.

Statistical analysis

Sensitivity and specificity of phenotypic confirmatory tests compared to PCR were calculated using VassarStats website (http://www.Vassarstats.net).

RESULTS

In this study, 35 *E. coli* isolates were obtained from 130 bacterial isolates recovered from urine samples of patients with urinary tract infections. All isolates were screened for ESBLs according to CLSI (2009) screening criteria. Eighteen isolates were identified as ESBL by screening test. In order to confirm the presence of ESBLs, three phenotypic confirmatory tests namely: DDS, BMD and CD were performed. DDS, BMD and CD detected 14 (78%), 16 (89%) and 16 (89%) isolates respectively as ESBL- producing *E. coli*. Figures 1 and 2 show representative photos of DDS and CD test.

Genotypic characterization of the isolates showed that all the 16 *E. coli* isolates carried genes coding for ESBLs. All isolates had *bla*$_{CTX-M}$ gene but not the *bla*$_{SHV}$ gene, while 62.5% of the isolates carried *bla*$_{TEM}$ gene(Figures 3-5).

The results of BMD and CD in the current study indicated 100% sensitivity and specificity (95% CI=75% to 100%) compared to PCR. On the other hand, DDS showed sensitivity and specificity of 87.5% (95% CI=60% to 97%) and 100% (95% CI=19.7% to 100%) respectively.

All ESBL isolates were resistant to amoxicillin, cephalexin, cefuroxime, cefotaxime, cefoperazone, ceftriaxone,

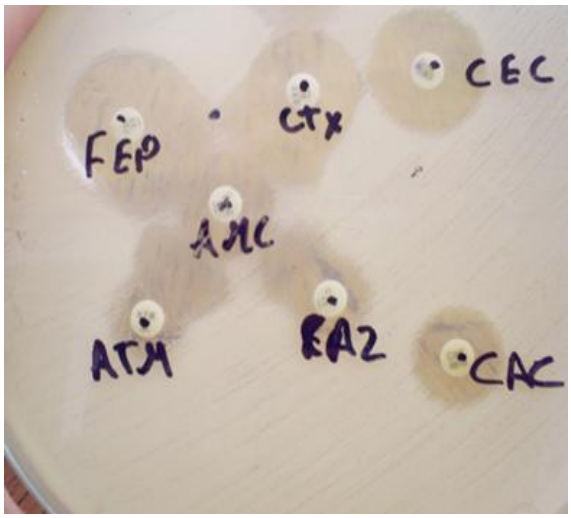

Figure 1. Representative photo of Double Disc Synergy test (DDS). Synergy between cefotaxime (CTX), ceftazidime (CAZ), Aztreonam (ATM) and cefepime (FEP) with amoxicillin/clavulanic acid (AMC) in the center showing keyhole phenomenon

Figure 2. Representative photo of the combination disc test (CD) showing difference in diameters of inhibition zones of cefotaxime (CTX)/ cefotaxime- clavulanic acid (CTX/C) and ceftazidime (CAZ)/ceftazidime-clavulanic acid(CAZ/C).

aztreonam (except 1 isolate was intermediate) and piperacillin. Percentage resistance to ciprofloxacin, cefepime, gentamycin and doxycycline was 62, 81, 56 and 68%, respectively. On the other hand, the isolates showed high susceptibility to imipenem, meropenem, cefoperazone/ sulbactam, and amikacin. Results of the phenotypic and genotypic characterization of the 16 *E. coli* isolates are summarized in (Table 3).

DISCUSSION

In this study, the incidence of ESBL production in *E. coli* isolates causing UTI was investigated. The prevalence of *E. coli* in urine culture was 27%. Among 18 isolates identified as ESBLs by screening test, 16 (89%) were confirmed by 2 phenotypic methods. BMD and CD methods were able to detect more ESBL-producing *E. coli* than DDS. This was in agreement with the previous finding of Tenover et al. (2003) who showed that the BMD method succeeded in detecting some ESBL-producing *E. coli* isolates which other methods failed in detecting. In this study, BMD and CD showed sensitivity and specificity of 100%. This observation was also supported by Taneja and Sharma, (2008) who reported that phenotypic confirmatory tests are highly sensitive and specific compared to genotypic methods in all

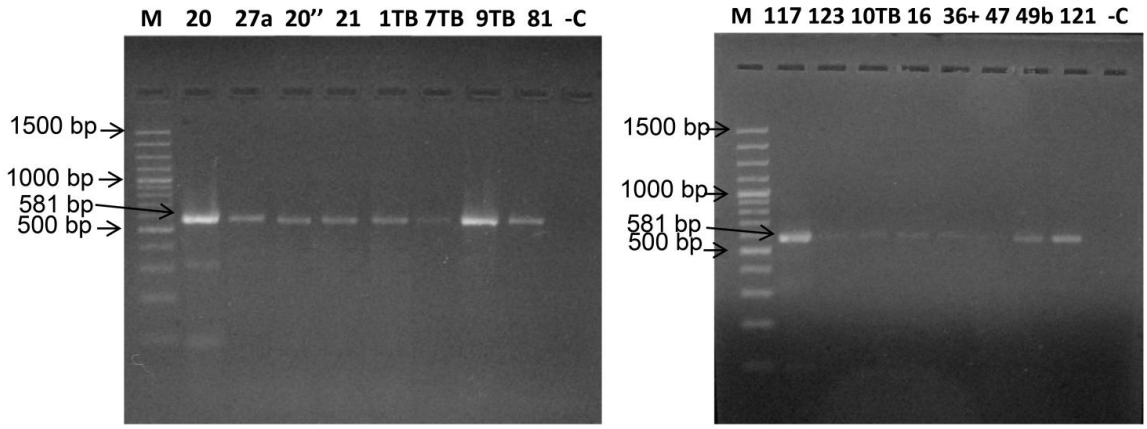

Figure 3. Photograph of agarose gel showing the PCR products of *bla* CTX-M gene of 16 *Escherichia coli* isolates. Lane (M): 100 bp DNA ladder, last lane (-c): negative control.

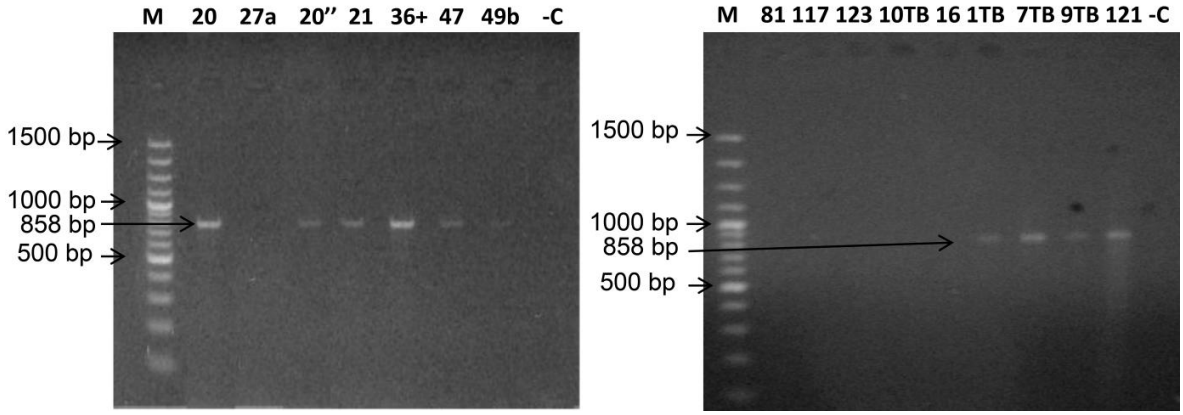

Figure 4. Photograph of agarose gel showing the PCR products of *bla* TEM gene of 16 *Escherichia coli* isolates. lane (M): 100 bp DNA ladder, last lane (-c): negative control.

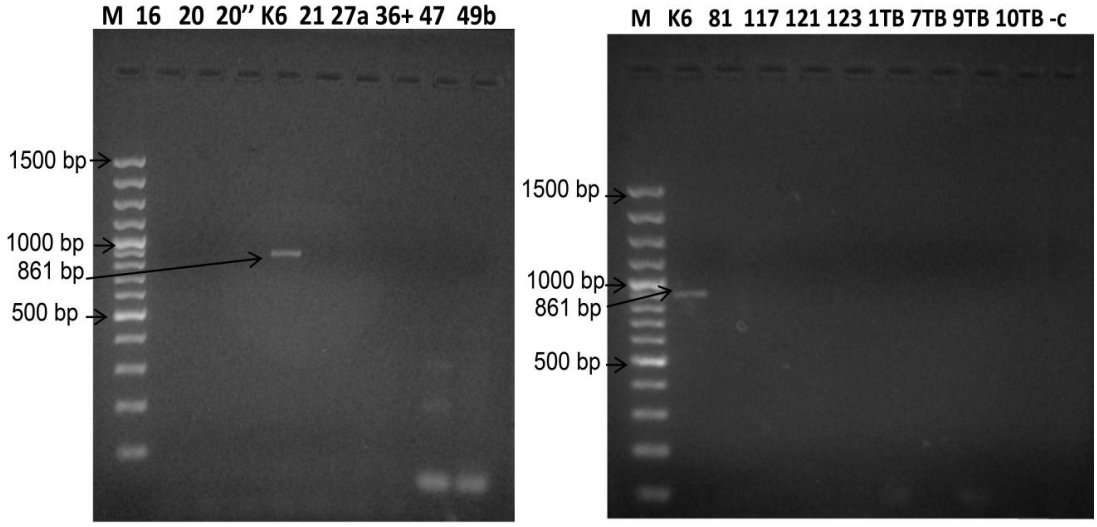

Figure 5. Photograph of agarose gel showing the PCR products of *bla*SHV gene of 16 *Escherichia coli* isolates. lane (M): 100 bp DNA ladder,lane (K6): positive control, last lane (-c): negative control.

Table 3. Phenotypic and genotypic characterization of the 16 *Escherichia coli* isolates.

Isolates number	Antimicrobial susceptibility pattern																				PCR		
	AMC	IPM	CTX	CAZ	FOX	ATM	FEP	CRO	AML	CXM	CL	AK	DO	CF	PRL	G	SXT	SCF	CFP	MEM	CTX-M	TEM	SHV
16	I	S	R	I	S	R	I	R	R	R	R	S	I	R	R	R	R	S	R	S	(+)	(-)	(-)
20	R	S	R	I	S	R	R	R	R	R	R	S	S	R	R	R	I	I	R	S	(+)	(+)	(-)
20"	I	S	R	R	S	R	R	R	R	R	R	S	S	R	R	I	S	I	R	S	(+)	(+)	(-)
21	I	S	R	I	S	R	I	R	R	R	R	S	R	S	R	S	R	S	R	S	(+)	(+)	(-)
27a	R	S	R	I	S	R	R	R	R	R	R	S	R	S	R	R	R	I	R	S	(+)	(-)	(-)
36+	I	S	R	R	S	R	R	R	R	R	R	I	R	R	R	R	R	I	R	S	(+)	(+)	(-)
47	R	S	R	R	S	R	R	R	R	R	R	S	S	R	R	R	R	I	R	S	(+)	(+)	(-)
49b	I	S	R	R	S	R	R	R	R	R	R	S	R	S	R	S	R	I	R	S	(+)	(+)	(-)
81	I	S	R	R	S	R	R	R	R	R	R	I	R	S	R	R	S	S	R	S	(+)	(-)	(-)
117	R	S	R	R	S	R	R	R	R	R	R	S	S	R	R	S	S	S	R	S	(+)	(-)	(-)
121	I	S	R	I	S	R	R	R	R	R	R	S	R	S	R	S	R	I	R	S	(+)	(+)	(-)
123	R	S	R	I	S	R	R	R	R	R	R	I	R	S	R	S	S	S	R	S	(+)	(-)	(-)
1TB	R	S	R	R	I	R	R	R	R	R	R	I	R	R	R	R	R	S	R	S	(+)	(+)	(-)
7TB	R	S	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R	I	R	S	(+)	(+)	(-)
9TB	I	S	R	R	S	I	I	R	R	R	R	S	R	R	R	S	R	I	R	S	(+)	(+)	(-)
10TB	R	S	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R	S	R	S	(+)	(-)	(-)

R (resistant), I (intermediate), S (susceptible), (CTX) cefotaxime, (CAZ) ceftazidime. Amikacin (AK), Amoxicillin (AML), Amoxicillin/clavulanic acid (AMC), Aztreonam (ATM), Cefepime (FEP), Cefoperazone (CFP), Cefoperazone/sulbactam 2:1 (SCF), Cefotaxime (CTX), Cefoxitin (FOX),Ceftazidime (CAZ), Ceftriaxone (CRO), Cefuroxime sodium (CXM), Cephalexin (CL), Ciprofloxacin (CF), Doxycycline (DO), Gentamicin (G), Imipenem (IPM), Meropenem (MEM), Piperacillin (PRL), Sulphamethoxazole/trimethoprim 19:1 (SXT), + (positive), - (negative).

instance except in presence of AmpC-type β –lactamases in addition to ESBLs. In this case, AmpC-type β-lactamases resist inhibition by clavulanate and mask the synergistic effect of clavulanate and cephalosporins against ESBLs (Paterson and Bonoma, 2005). The rate of ESBL-producing *E. coli* as indicated by both phenotypic and genotypic tests was 46%. This rate is not representative of the whole country, and was lower than that observed in a study of UTI in China, where the rate of ESBL-producing *E. coli* was 60.9% during 2011-2012 (Lai et al., 2014). However a rate of 91% was observed for ESBL-producing *E. coli* isolated from urine culture in 11 Spanish hospitals from February 2002 to May 2003 (Rodríguez-Baño et al., 2008).

Genotypic characterization of the isolates revealed that, the prevalence of CTX-M, TEM and SHV genes were 100, 62.5 and 0%, respectively. This finding was in accordance with Rossolini et al. (2008) who reported that, CTX-M enzymes are the most common type of ESBL found in micro-organisms in most areas of the world. The most widely distributed CTX-M enzyme is CTX-M-15 which was detected in Europe, some countries in Asia, Africa, North America, South America and Australia (Pitout, 2010). In addition, genes encoding CTX-Mβ-lactamases have been associated with ISEcp1, ISCR1 insertion elements which are located on conjugative plasmids and which play an important role in the expression and continuous spread of these β-lactamases (Poirel et al., 2003). In contrast to infections caused by TEM- and SHV- derived enzymes, which are limited to nosocomial type, *E. coli*- producing CTX- M enzyme is likely to cause both community- onset and nosocomial infections (Laupland et al., 2008).

The high resistance towards oxyimino-cephalosporins and aztereonam in addition to the lower resistance pattern towards cephalosporins in combination with β-lactamase inhibitors like cefoperazone/sulbactam are consistent with the characteristics of bacteria producing ESBLs (Pitout, 2010). It is worth mentioning that, no ESBL isolate was

resistant to imipenem or meropenem. In this study, all isolates showed multi-drug resistance pattern. In that context, Paterson (2000) reported that, resistance due to ESBLs may not indicate only resistance towards extended spectrum cephalosporins and monobactam antibiotics but also confers resistance towards floroquinolones and aminoglycosides. This cross resistance may be due to localization of the genes coding for ESBLs on plasmids which may have other genes coding resistance towards floroquinolones, aminoglycosides, trimethoprim, sulphonamides, tetracyclines and/or chloramphenicol.

Correlating the antimicrobial susceptibility pattern of the isolates with their genotypic characters revealed that there is no precise association between the type of ESBL and susceptibility to different antibiotics, since the presence of both CTX-M and TEM enzymes did not confer increased resistance to β - lactam antibiotics. These results could be justified by the findings of Spanu et al. (2002) who reported that, susceptibility is multifactorial, depending on ESBL, substrate specificity, production of additional β-lactamases and changes of the outer membrane permeability.

Conclusion

Out of 35 *E. coli* isolates from urine samples of patients with urinary tract infections, 16 were identified as ESBLs by both phenotypic and genotypic methods. Identified ESBLs were either of CTX-M and TEM types or CTX-M alone. All isolates were multi drug resistant. No difference in antibiotic susceptibility pattern was observed between isolates carrying either CTX-M alone or a combination of CTX-M and TEM genes.

Conflict of Interests

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

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Full Length Research Paper

Influence of methanol fruit and leaf extracts of *Myristica fragrans* (Myristicaceae) on the activity of some antibiotics

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The emergence of bacterial resistance to antibiotics is a serious draw back in the management of infections. In this study, the antibacterial activity of the methanol fruit and leaf extracts of *Myrisctica fragrans* Houtt. (Myristicaceae) against typed strains of *Staphylococcu saureus*, *Bacillus suibtilis*, *Enterococcus faecalis*, *Proteus vulgaris*, *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* were determined using a modified Kirby-Bauer agar well diffusion method. Their influence on the minimum inhibitory concentration of ciprofloxacin, tetracycline, erythromycin and amoxicillin against the test organisms were also determined using themicro-dilution method. The extracts exhibited antibacterial activity against all the test organisms. In the presence of sub-inhibitory concentrations of the leaf and fruit extracts (1.0 and 2.0 mg/mL respectively), the antibacterial activity of amoxicillin against all the organisms was nullified. The antibacterial activity of ciprofloxacin against all the organisms was also cancelled by the fruit extract while the leaf extract acted similarly against all the test organisms except *E. faecalis* and *S. typhi*both of which saw four-fold reduction in susceptibility. The antibacterial activity of erythromycin against all the test organisms were nullified by both extracts except *S. aureus* and *B. subtilis* where the leaf extract caused an 8-fold reduction in activity. In the presence of the leaf extract, tetracycline lost activity against *S. aureus*, *P. aeruginosa* and *P. vulgaris*, its activity was reduced 16-fold against *B. subtilis* and *E. faecalis* and 32-fold against *E. coli*. The fruit extract caused a complete loss of activity of tetracycline against *S. aureus*, *P. aeruginosa*, *B. subtilis* and *E. faecalis*; there was 16-fold, 8-fold and 32-fold reduction in activity against *P. vulgaris*, *S. typhi* and *E. coli* respectively. The extracts of *M. fragrans* enhanced the resistance of these organisms to all the antibiotics used.

Key words: Antibiotic resistance, antibacterial, resistance enhancing, minimum inhibitory concentration, *Myristica fragrans*.

INTRODUCTION

The discovery of penicillin by Alexander Fleming paved the way for major research into the discovery and

synthesis of various kinds of antibiotics (Bennett and Chung, 2001). However, infectious diseases that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapy (Levy and Marshall, 2004).

Many mechanisms have been established for microbial resistance to antimicrobial agents and include drug inactivation or modification, alteration of target site, alteration of metabolic pathway, reduced drug accumulation in the organisms and alteration of membrane permeability (Nelson, 2002).

Some of the factors that promote bacterial resistance to antimicrobial agents include mis-diagnosis, drug counterfeiting, misuse and abuse of antibiotics and disposal of antibiotics in food production and animal rearing, indiscriminate prescribing, noncompliance and under dosing (Spratt, 1994).

Efforts are being made to curb the problem of microbial resistance to antibiotics including improving infection control, developing new antibiotics, using antibiotics more appropriately and modulating microbial resistance to already existing antibiotics (Adu et al., 2009; Gbedema et al., 2010).

The problem of bacterial resistance to antibiotics, has limited the use of cheap and routine antibiotics, and this has necessitated the need for a continued search for new antimicrobials (Sibanda and Okoh, 2007). The search for new antibiotics is usually difficult considering the number and nature of mechanisms and factors associated with resistance (Levy and Marshall, 2004, Stewart and Costerton, 2001).

Nutmeg is the dried kernel of the seeds of *Myristica fragrans* Houtt. which belongs to the family Myristicaceae. It is a popular food additive used in many formulations. *M. fragrans* is also used in the perfumery and pharmaceutical industries, in toothpaste and as a major ingredient in some cough syrups. In traditional medicine, nutmeg and its oil are used for disorders related to the nervous and digestive systems such as infantile diarrhoea. Nutmegs, maces and their oils are largely used for flavouring and as carminative (Evans, 2002). The aim of the study is to determine the antibacterial activity of methanol fruit and leaf extracts of *M. fragrans* and their influence on activity of some antibiotics including amoxicillin, ciprofloxacin, tetracycline and erythromycin.

MATERIALS AND METHODS

Collection and preparation of plant materials

The fresh leaves of *M. fragrans* were obtained from the Physique Garden at the Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The fruits were obtained from the Kumasi central market. They were authenticated by Dr. G. H. Sam, Department of Herbal Medicine where a voucher specimen of the leaves (KNUST/HM1/2013/L-014) has been deposited. The leaves were washed, dried at 28 to 30°C for 5 days and milled into coarse powder using a laboratory mill machine (Type 8, Christy & Norris, UK). The fruits were also similarly processed. All the chemicals and culture media were purchased from Sigma-Aldrich, St Louis, MO, USA unless otherwise stated.

The powdered leaves and fruits materials (200 g each) were soxhlet extracted using methanol and concentrated under reduced pressure using a rotary vapor (Buchi, Germany). The concentrates were lyophilized. A brown semi-solid extract was obtained for the leaves giving a yield of 12.25% w/w; whiles fruits had yield of 7.76% w/w (related to the dried material).

Test organisms

Enterococcus faecalis ATCC 29212, *Salmonellatyphi* ATCC 19430, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* 25922, *Bacillus subtilis* NCTC 10073 and *Proteus vulgaris* NCTC 4175.

Preliminary phytochemical screening

The methanol leaf and fruit extracts were screened for secondary metabolites using methods described by Sofowora (1993) and Harborne (1998).

Determination of antimicrobial activity

The antimicrobial activity of the extracts was determined using both the Kirby-Bauer agar disc diffusion method and the broth dilution method using micro-dilution method (Eloff, 1998).

Petri dishes containing 20 mL of Muller-Hinton agar were poured and allowed to set. Overnight cultures of the test organisms grown at 37°C in Muller-Hinton broth were diluted to 0.5 McFarland standards with saline. Ten microliters of the bacterial culture was spread over the surface of the agar and allowed to dry for 10 min. Filter paper discs (6 mm in diameter) soaked in the various concentrations (20.0, 10.0, 5.0 and 2.5% w/w) of the extracts were placed on the inoculated agar. Discs containing tetracycline (10µg/disc) were placed as positive control.

These were incubated at 37°C for 24 h. The antibacterial activity against each test organism was quantified by determining mean zone of growth inhibition. The procedure was done in triplicate and the mean zones of inhibition recorded.

The minimum inhibitory concentrations (MIC) of the extracts and the test antibiotics (ciprofloxacin, tetracycline, erythromycin and amoxicillin) against the various organisms were determined using the micro-dilution method (Eloff, 1998). The 96-well plates were prepared by dispensing 150 µL of inoculated broth and 50 µL of plant extract or antibiotics constituted in broth or 50µL broth (MHB) in the case of negative control in each well. The plates were incubated at 37°C for 24 h. Presence of bacterial growth was determined by the addition of 20 µL (0.2 mg/mL) of 3-(4,5-

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Table 1. Antibacterial activity of methanol leaf and fruit extracts of *M. fragrans* against test organisms. Mean zones of inhibition are mean of zone of growth inhibition plus or minus standard deviation of triplicate experiments.

Concentration (%w/w)/organisms	Mean zones of inhibition (mm) ±SEM							
	40.0		20.0		10.0		5.0	
	LE	FE	LE	FE	LE	FE	LE	FE
S. aureus	13.4±0.3	16.5±0.1	12.5±0.0	14.0±1.0	11.0±0.2	13.0±0.0	9.8±0.2	11.5±0.1
P. vulgaris	13.6±0.1	22.0±1.0	12.2±0.2	20.5±0.1	10.8±0.3	17±2.0	9.1±0.2	15.0±1.0
P. areuginosa	15.0±0.0	12.5±0.1	13.8±0.2	10.5±0.2	12.0±0.3	10.0±1.0	9.7±0.4	8.5±0.1
B. subtilis	12.5±0.1	15.5±0.0	11.4±0.3	13.5±0.2	10.8±0.2	12.0±1.0	9.3±0.3	9.5±0.1
S. typhi	13±0.3	16.0±1.0	11.5±0.2	13.5±0.1	9.5±0.1	10.2±0.2	0.0	0.0
E. faecalis	13.8±0.4	16.4±0.2	11.6±0.1	13.8±0.1	10.4±0.2	11.3±0.2	8.4±0.1	9.4±0.3
E. coli	12.3±0.2	13.5±0.2	10.2±.2	11.5±0.0	8.5±0.1	9.5±0.1	8.0±0.1	8.5±0.1

FE = Fruit extract, LE = leaf extract; SEM: standard error mean.

Table 2. Minimum inhibitory concentration (mg/mL) of methanol leaf and fruit extracts of *M. fragrans* against test organisms.

Extract	Minimum inhibitory concentration (mg/mL) of extract against the test organisms						
	S. aureus	*P. aeruginosa*	*B. subtilis*	*P. vulgaris*	*S. typhi*	*E. faecalis*	*E. coli*
Leaf extract	4.0	8.0	2.0	4.0	2.0	2.0	4.0
Fruit extract	16.0	16.0	8.0	16.0	8.0	16.0	16.0

dimethylthiazole-2- yl)-2,5-diphenyltetrazolium bromide (MTT).

The minimum inhibitory concentrations (MIC) of the antibiotics against the various organisms were re-determined as above in the presence of sub-inhibitory concentrations of the extracts (1.0 and 2 mg/ml for the methanol leaf and fruit extracts, respectively).

RESULTS

Phytochemical tests

The preliminary phytochemical analysis indicated the presence of alkaloids, glycosides (including saponin glycosides) and both condensed and true tannins in both extracts.

DISCUSSION

The antimicrobial activity of *M. fragrans* has already been established by other workers (Singh et al., 2005). In this study, the methanol extracts of both the leaves and fruits of *M. fragrans* were found to have antibacterial activity against all the test organisms used and the activity increased with increasing concentration. Though, the leaf extract exhibited a more potent activity against all the test organisms than the fruit extract, as indicated by lower MIC values (Table 2), the fruits extract consistently showed larger zones of growth inhibition against all the test organisms except *P. aeruginosa* where the reverse was true (Table 1). This may be attributed to factors associated with the diffusion of the active constituents into the agar. The minimum inhibitory concentrations of the leaf extracts ranged from 2.0 mg/mL against *B. subtilis, S. typhi* and *E. faecalis* to 8.0 mg/mL against *P. aeruginosa;* while that of the fruit extract ranged from 8.0 mg/mL for *B. subtilis* and *S. typhi* to 16.0 mg/mL for all the other test organisms.

Resistance of microorganisms to antibiotics has usually been blamed on factors associated with the organisms though other factors may be responsible. Thus, under certain circumstances, it may be more appropriate to describe the situation as reduced activity of the antibiotic rather than increased resistance of the organism. This is because it may be possible that the lack of activity of the antimicrobial agent against the organism may be due to factors outside the organism.

In this study the reference antibiotics (amoxicillin, ciprofloxacin, erythromycin and tetracycline) showed activity against all the test organisms with MIC ranging from 1 to 128 µg/ml (Table 3). In the presence of sub-inhibitory concentrations of the methanol extract of *M. fragrans* leaves (1.0mg/ml) and fruits (2.0mg/ml) the MIC of the antibiotics ranged from 8 to 512 µg/ml (Table 4). The extracts thus caused drastic reduction in the antibacterial activities of all the antibiotics.

Amoxicillin lost its activity completely against all test organisms at all the concentrations used in the presence of both extracts (Table 4). Ciprofloxacin also lost its activity completely against all the test organisms used except *P. vulgaris* and *E. faecalis* in the presence of the

Table 3. Minimum inhibitory concentration of selected reference antibiotics against the test organisms.

Antibiotic	Minimum inhibitory concentration (µg/mL) of antibiotic against the test organisms						
	S. aureus	*P. areuginosa*	*B. subtilis*	*P. vulgaris*	*S. typhi*	*E. faecalis*	*E. coli*
Amoxicillin	8.0	-	16.0	32.0	32.0	64.0	64.0
Ciprofloxacin	1.0	1.0	2.0	2.0	1.0	2.0	1.0
Erythromycin	64.0	128.0	64.0	64.0	128.0	128.0	128.0
Tetracycline	16	32.0	8.0	8.0	16.0	8.0	4.0

- = No activity.

Table 4. Minimum inhibitory concentration (MIC) of reference antibiotics in the presence of sub-inhibitory concentrations of *M. fragrans* extracts.

Antibiotic	MIC (µg/mL) of antibiotic against the test organisms in the presence of *M. fragrans* extract													
	S. aureus		*P. areuginosa*		*B. subtilis*		*P. vulgaris*		*S. typhi*		*E. faecalis*		*E. coli*	
	LE	FE	LE	FE	LE	FE	LE	FE	LE	FE	LE	FE	LE	FE
Amoxicillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	-	-	8.0	-	-	-	8.0	-	-	-
Erythromycin	512.0	-	-	-	512.0	-	-	-	-	-	-	-	-	-
Tetracycline	-	-	-	-	128.0	-	-	128.0	64.0	128.0	128.0	-	128.0	128.0

-= No activity, FE = fruit extract, LE = leaf extract.

leaf extract both of which had a four-fold reduction in activity. Similarly, erythromycin lost activity against all the test organisms except *S. aureus* and *B. subtilis*, both of which also, in the presence of the leafextract, had an eight-fold reduction in activity.

Tetracycline was more resilient to the action of the extracts. Both extracts nullified its activity against *S. aureus* and *P. aeruginosa*. The methanol leaf extract nullified its activity against *P. vulgaris* while the fruits extract nullified its activity against *B. subtilis* and *E. faecalis*. There were four-fold and eight-fold reduction in activity against *S. typhi* by the leaf and fruit extracts, respectively. There was a sixteen-fold reduction in activity against *E. faecalis* and *B. subtilis* in the presence of the leaf extract and against *P. vulgaris* in the presence of the fruit extracts. *E.coli* showed a thirty-two-fold reduction in activity in the presence of both the leaf and fruit extracts.

The reduction in activities of the antibiotics seen in this study can be due to several factors that may be associated with the interaction between the antibiotics and the organism, the antibiotic and the phytoconstituents of the extracts or the organism and the phytoconstituents.

In the first instance, the phytoconstituents may act to enhance the various resistance mechanisms the organisms employ to evade the antibiotic action. The phytoconstituents may be protein activators, or co-enzymes, binding to and activating enzymes that are involved in the resistance mechanisms of the organisms (Lambert, 2002). The mechanisms may be genetic and naturally associated with the organisms or may be introduced as a result of the environment in which the organism finds itself. Thus, an organism very sensitive to a particular antimicrobial agent in one environment may be resistant to the same antimicrobial agent in another environment. In the second instance, the phytoconstituents may interact with the antibiotics by binding to the active moieties or react chemically with them resulting in loss of activity (Adu et al., 2009). In the third situation, the phytoconstituents may interact with the surface structures of the organism reducing the permeability of the cell to the antibiotics. It is known that certain substances protect organisms from the lethal effects of certain agents (Keweloh et al., 1989) and it may be possible that some phytoconstituents of *M. fragrans* exhibited this effect.

The effects of all these are that the extracts may prevent the antibiotics from reaching the target sites by inhibiting the penetration of the antibiotic into the organism or produce conformations that make the antibiotics unable to fit its receptor. It may also bind to or modify the functional groups that are responsible for the activity (Barza et al., 1976).

Minimum inhibitory concentration (MIC) is an important laboratory diagnostic tool used to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents (Andrews, 2001). MIC is generally regarded as a basic laboratory measurement of the activity of an antimicrobial agent against an organism (Budak and Ubeyli, 2011).

The antibiotics used in this study act by different mechanisms and the mechanism of resistance employed by the test organisms also differ for each antibiotic and also for each organism. This presupposes that the resistance enhancement exhibited by the extract may not involve the known resistance mechanisms of the organisms to the antibiotics neither does it involve the mechanism at the site of action of these antibiotics. Probably, the extracts created a new environment or condition that does not allow the antibiotic to penetrate the organism or does not allow the antibiotics access to their sites of action.

The results of the study indicate that concurrent administration of certain substances with antibiotics may result in treatment failures due to interaction of these substances with the antibiotics outside the infecting organisms, a situation that can be best described as resistance enhancing. It further suggests that certain factors including concomitant administration of antibiotics and other substances including certain foods and food additives can alter bacteria susceptibility to antibiotic therapy.

Since *M. fragrans* is used as food additive, it can be said that certain foods containing fruit extracts of *M. fragrans* cannot be taken when a patient is on antibiotic therapy. The finding indicates that various materials used as foods or food additives should be investigated for possible bacterial resistance enhancing effects as found in this study. The study further indicates that some attention should be paid to the choice of foods for patients on antibiotic therapy less we risk treatment failures, the ultimate effects of bacterial resistance to antibiotics.

Conclusion

The study confirms that the methanol leaf and fruit extracts of *M. fragrans* have antibacterial activity. In the presence of sub-inhibitory concentrations of the extracts, there was profound loss of antibacterial activity of amoxicillin, erythromycin, tetracycline and ciprofloxacin against all the test organisms.

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Conflict of Interests

The authors declare no conflict of interests.

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Full Length Research Paper

Characterization of different isolates of *Zucchini yellow mosaic virus* from cucurbits in Saudi Arabia

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During the 2012 growing season, thirty three samples were collected from squash, pumpkin and muskmelon plants showing virus-like symptoms in Riyadh and Al-Madena regions of Saudi Arabia. Eleven of these samples were found positive for *Zucchini yellow mosaic virus* (ZYMV) by double antibody sandwich ELISA (DAS-ELISA). In the host range study for the five selected ZYMV isolates, 11 out of the 22 mechanically inoculated test plants were infected and showed variable symptoms. The amplification of viral DNA product through reverse transcription-polymerase chain reaction amplification (RT-PCR) using a primer pair specific for ZYMV, yielded fragments of approximately 1185 bp. Southern blot hybridization confirmed the results obtained through RT-PCR, using a specific DNA probe homologous to ZYMV. Nucleotide sequences for the coat protein gene from all five Saudi isolates of ZYMV indicated a similarity of 97.1-100.0% between them. Comparative analysis of the nucleotide sequences of coat protein gene from the Saudi isolates and other ZYMV isolates obtained from NCBI, showed a relatively high nucleotide sequence similarity that ranged between 92.0-98.8%. The highest similarity was found with Syria, Jordan, Iran, Hungarian, Austria, Slovenia and Germany isolates (97.1 to 98.8%). The nucleotide sequence data obtained for the five ZYMV isolates was deposited in the GenBank under the accession numbers JQ899263, JQ899264, JQ899265, JQ899266 and JQ899267.

Key words: ZYMV, DAS-ELISA, RT-PCR, Hybridization, Sequence.

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV) is a species belonging to the genus *Potyvirus* in the family *Potyviridae* and characterized by a monopartite, positive-sense, single-stranded RNA genome encapsidated in flexuous, filamentous particles. It was first reported in Italy in 1981 (Lisa et al., 1981) and is responsible for major economic losses in cucurbit crops in many parts of the world causing severe mosaic, necrosis and malformation. Since

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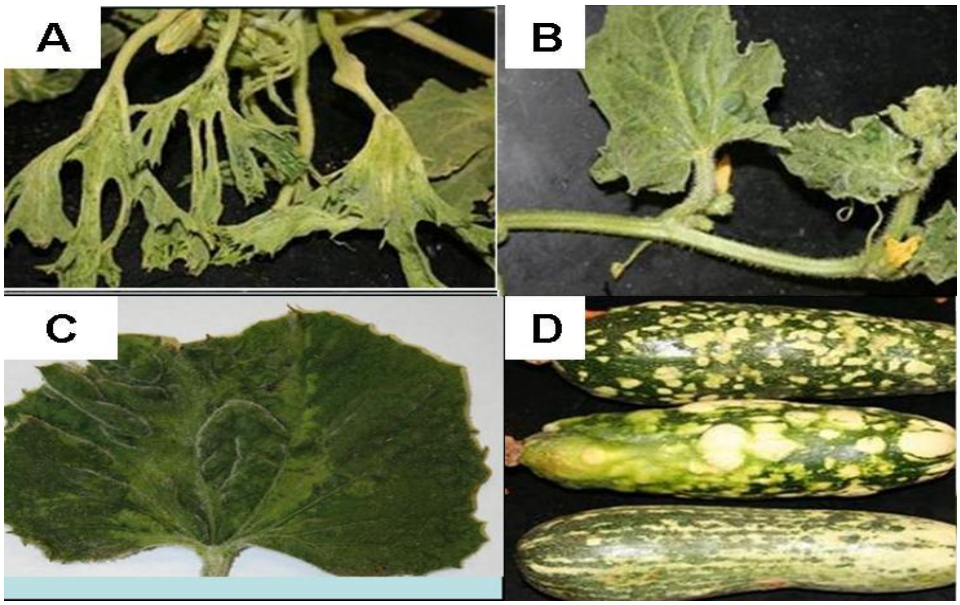

Figure 1. ZYMV symptoms on naturally infected cucurbit plants. Leaves of (A) Squash showing mosaic and malformation, (B) muskmelon showing yellow-green mottle and blistering, (C) pumpkin showing mosaic blisters and vein clearing and (D) fruits showing, warty raised yellow areas.

then it has been reported in many countries throughout the world (Lisa and Lecoq, 1984; Provvidenti et al., 1984; Davis, 1986; Dolores and Valdez,1988; Al-Shahwan, 1990; Yardimci and Korkmaz, 2004; Glasa and Pittnerova, 2006; Safaeizadeh, 2008; Coutts et al., 2011b; Vucurovic et al., 2012). Yield losses of up to 100% due to infection by this virus have been reported (Al-Shahwan et al., 1995; Ghorbani, 1988). ZYMV was reported in Saudi Arabia for the first time in the central region of the country in 1990 (Alshahwan, 1990). Because it causes abundant yield losses, studies to determine its biological, serological, and molecular characteristics were conducted to better understand the scope of diversity in its population structure (Mahgoub et al., 1998; Tóbiás and Palkovics, 2003; Bananej et al., 2008; Safaeizadeh, 2008; Yakoubi et al., 2008; Chikh Ali et al., 2009). The virus is transmitted from infected plants to healthy ones by several aphid species in a non-persistent manner (Gal-On et al., 1995). ZYMV has caused devastating epidemics in a number of commercial cucurbits worldwide, and several distinct biological strains of ZYMV have been described (Provvidenti et al., 1984; Desbiez and Lecoq, 1997). Strains of ZYMV isolated from distinct geographic origins exhibit biological diversity, especially in their host range, symptomatology and aphid transmission (Desbiez et al., 1996, 2002). Lack of studies concerning variability of ZYMV in Saudi Arabia encouraged initiation of the present investigation. The objective of this study was, therefore, to characterize five ZYMV isolates infecting three cucurbit species in Saudi Arabia using biological, serological and molecular methods, as well as sequence data analysis.

MATERIALS AND METHODS

Source of viral isolates and host range test

Eleven squash samples (*Cucurbita pepo* L.), were collected from Riyadh (Al-Hair and Wadi Eldwaser), ten muskmelon (*Cucumis melo* L.) samples were collected from Al-Kharj, and seven pumpkin (*Cucurbita maxima* L.) and five squash samples were collected from Al-Madena governorate showing malformed, yellow-green mottled and blistered leaves. Vein banding was also observed on leaves showing vein clearing, and warty raised yellow areas appeared on infected fruits (Figure 1). Samples were tested by double antibody sandwich ELISA (DAS-ELISA) as described by Clark and Adams (1977), to detect *Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* (WMV), *Alfalfa mosaic virus* (AMV) and ZYMV. Based on ELISA results, five ZYMV isolates which were singly detected in their hosts were selected to represent the plant species from which they were detected and the locations from which they were collected. Inoculums of these five ZYMV isolates were prepared from freshly collected leaf samples of squash from Riyadh (Al-Hair; ZYMV-SA-1, Wadi Eldwaser, (ZYMV-SA-4), Al-Madena (ZYMV-SA-2), muskmelon from Riyadh (Al-Kharj; ZYMV-SA-3), and pumpkin from Al-Madena (ZYMV-SA-5). Inoculums were prepared in 0.01 M potassium phosphate buffer containing 0.1% sodium sulphite (Na_2SO_3), pH 7.2 using an extraction ratio of 1:4 (w/v), and were applied on leaves of selected host plants that were previously dusted with 600-mesh carborundum. Plants utilized for the host range test are indicated in Table 1. After inoculation, the plants were maintained in a greenhouse at 25-30°C. For biological purification, single local lesion assay was performed according to Khan and Monroe (1963) using *Chenopodium amaranticolor* Coste & Reyn as a local lesion host. A single local lesion out of the several observed was used for back inoculation on squash which was used as propagative host for the ZYMV isolates. Viral symptoms on plants were recorded two weeks after inoculation, and then at regular intervals during the next 4 weeks. All plants showing no symptoms were assayed for virus infections by back inoculation to *C. pepo, C. melo, Chenopdium*

Table 1. Reaction of different plant species to inoculation with the five Saudi isolates of ZYMV and to the DAS-ELISA assay.

Host range	Host reaction					ELISA assay
	ZYMV-SA-1	ZYMV-SA-2	ZYMV-SA-3	ZYMV-SA-4	ZYMV-SA-5	
Cucurbita pepo, Vegetable Marrow	YM	M, Ma	M, Vb	M,	M	+
Cucumis melo, Russian	M	M, b	M, Vb	Mm, b	M	+
Cucumis sativus L. cv.Beit Alpha	Mm	Mm	M, Vb	Mm	M	+
Citrullus lanatus (Thunb.) Matsum & Nakai), cv.Sugar Baby	Mo	Mo	M	Mo	M	+
Cucurbita maxima, Duch. E Lam cv. Connecticut Field	Mm	M	M, Vb	M, Vb	M, Vb	+
Luffa acutangula L.	M	M	M	M	M	+
Cucurbita okeechobeensis (Small) L. H. Bailey	Mm	M	M	Mm	M	+
Chenopodium quinoa Willd	CL	CL	CL	CL	CL	+
Chenopodium amaranticolor Coste & Reyn	CL	CL	CL	CL	CL	+
Phaseolus vulgaris L. cv. Black Turtel 2	CL	CL	-	CL	-	+
Pisum sativum L. cv. *Alsk*	SL	SL	-	SL	-	+

CL = Chlorotic local lesion, M = mosaic, Vb = vein banding, b = blisting, Ma = malformation, Mm = mild mottle, YM = yellow mosaic, Mo = mottle, SL = symptomless, - = no reaction, + = positive reaction.

quinoa Willd, *C. amaranticolor*, and by DAS-ELISA (Clark and Adams, 1977).

ELISA detection

Detection of cucurbit viruses in the leaf samples collected from different locations was carried out using the DAS-ELISA method. ELISA kits were purchased from Agdia (Agdia Inc., 30380, Country Road, Elkhart, Indiana, 46514, USA), and used to test for the presence of ZYMV and other cucurbit viruses in the collected plant samples. The plates were read at 405 nm using a microtiter plate reader (Bio-Tek ELx808). Samples were considered positive when the absorbance values at 405 nm (A_{405}) exceeded the mean of the negative controls (healthy) by at least a factor of two (Al-Shahwan et al., 1995; Sammons et al., 1989). All samples were assayed in three repeats. The presence of ZYMV isolates was also confirmed by reverse transcription-polymerase chain reaction amplification (RT-PCR) as detailed below.

Extraction of viral RNA and RT-PCR

To confirm the presence of ZYMV isolates by RT-PCR, total RNA was extracted from eleven samples [six squash samples collected from Riyadh region (Al-Hair and Wadi Eldwaser), Al-Madena, three muskmelon samples from Riyadh region (Al-Kharj) and two pumpkin samples collected from Al-Madena region] which tested positive for ZYMV by DAS-ELISA using the SV–Total RNA Isolation System according to the manufacturer's protocol (Promega).

A pair of ZYMV specific forward (ZYU: 5'-gct cca tac ata gct gag aca gc-3`) and reverse primer (ZYD1186: 5`- tag gct tgc aaa cgg agt cta atc -3`) which amplify part of the cytoplasmic inclusion gene, the coat protein gene and the 3' untranslated region (UTR) of the ZYMV genome, as described by Choi et al. (2002) and Yoon (1999) were used. RT-PCR was performed using the One Step RT-PCR Kit (Qiagen). RT-PCR reactions were amplified using the following cycling parameters, hold at 50°C for 30 min (RT step), hold at 95°C for 15 min (hot start to PCR), then subjected to one cycle of amplification: 94°C for 1 min, 35 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, followed by 72°C for 5 min. Amplified DNA fragments of the expected size (1185 bp) were electrophoresed in 1% TAE buffer (40 mM Tris, 40 mM acetate, and 1 mM EDTA). DNA was stained with ethidium bromide added to the gel at a concentration of 0.5 µg/ ml. DNA was visualized on a UV transilluminator and photographed using DNA documentation gel analysis. A 1 kb DNA marker (Promega) was used to determine the size of RT-PCR amplified cDNA products (Sambrook et al., 1989).

Preparation of the cDNA probe of ZYMV and nucleic acid hybridization

The purified DNA fragment (1185 bp) which was amplified from ZYMV using the ZYU and ZYD1186 primer pair was used as template to synthesize a cDNA Dig labeled probe. The probe was labeled by PCR using the Genius™ System (Boehringer Mannheim Corp.) according to the manufacturer's instructions. The capillary transfer of DNA from the gel to nitrocellulose membrane support was done as previously described (Southern, 1975). The DNA was fixed on the membranes by ultraviolet cross-linking for 30 s. Hybridization and immunological detection were carried out using the Genius II DNA Labeling and Detection Kit (Boehringer Mannheim, IN) according to the manufacturer's instructions.

DNA sequencing and data analysis

Bands of the expected size from five selected Saudi isolates of ZYMV (ZYMV-SA-1, ZYMV-SA-2, ZYMV-SA-3, ZYMV-SA-4 and ZYMV-SA-5) were excised and recovered with a Wizard PCR clean up kit (Promega). The nucleotide sequence of the isolated ZYMV gene was carried out in two directions with the specific complementary and homologous primer at King Faisal Specialist Hospital & Research Center, Riyadh, Saudi Arabia using an AB3730xl DNA Analyzer model Hitachi. Sequence analyses were performed and the homology tree analyses were done using the DNAMAN trial version 5.2.10 program. The GenBank accession numbers for the different ZYMV isolates obtained from NCBI were used in the comparison (Table 2).

RESULTS

Isolation, detection and host reaction of the causal virus

In the present study, some ZYMV isolates occurring in

Table 2. Comparison of nucleotide identities of the CP gene sequences for Saudi Arabia isolates with those of ZYMV isolates originating in different countries.

ZYMV isolates	Country	Isolate	JQ899263	JQ899264	JQ899265	JQ899266	JQ899267
JQ899263	KSA	ZYMV-SA-1	100%	100.0%	99.2%	99.2%	98.6%
JQ899264	KSA	ZYMV-SA-2	100.0%	100%	99.2%	98.6%	98.6%
JQ899265	KSA	ZYMV-SA-3	99.2%	99.2%	100%	98.6%	97.7%
JQ899266	KSA	ZYMV-SA-4	99.2%	98.6%	98.6%	100%	97.1%
JQ899267	KSA	ZYMV-SA-5	98.6%	98.6%	97.7%	97.1%	100%
EU999757	Syria	SYR-B2	98.5%	98.5%	97.7%	98.0%	98.0%
AJ420015	Austria	Austria 10	98.1%	98.1%	97.3%	97.4%	97.4%
AJ420018	Slovenia	Slovenia 1	98.0%	98.0%	97.1%	97.3%	97.3%
AJ251527	Hungarian	10	98.0%	98.0%	97.1%	97.3%	97.3%
FJ705272	Iran	Azr.Mak.W	98.7%	98.7%	97.9%	98.0%	98.0%
EU999758	Jordan	JOR-B3	98.1%	98.1%	97.3%	97.4%	97.4%
AJ420019	Germany	Berlin 1	98.8%	98.8%	98.0%	98.1%	98.1%
AY074810	China	Ningbo	95.0%	95.0%	94.1%	93.9%	93.5%
AY074809	China	Beijing	95.0%	95.0%	94.2%	94.2%	94.2%
AJ420020	Italy	Italy 1	95.2%	95.2%	94.4%	94.4%	94.4%
AY278998	Vietnam	KR-PA	93.1%	93.1%	92.3%	92.0%	92.0%
AF486823	China	Hainan	93.9%	93.9%	93.1%	93.1%	93.1%
AF127931	Taiwan	TW-TC1	93.5%	93.5%	92.6%	92.6%	92.6%
AJ316228	China	SG	92.9%	92.9%	92.1%	92.1%	91.5%
AF127934	Taiwan	TW-PT5	92.9%	92.9%	92.1%	92.3%	91.5%
AF435425	China	Hangzhou	93.8%	93.8%	92.9%	92.9%	92.2%
AJ429071	Korea	A	93.3%	93.3%	92.7%	92.5%	92.5%
D13914	USA	Florida	93.2%	93.2%	92.4%	92.4%	92.4%
DQ645729	Spain	ZYMV C16	92.9%	92.9%	92.0%	92.0%	92.0%
AF014811	Singapore	Singapore	84.8%	84.8%	84.1%	84.1%	83.5%
AJ515907	China	SXS	82.5%	82.5%	81.8%	82.1%	81.1%
AJ515908	China	MM	82.2%	82.2%	81.5%	81.7%	80.7%
AJ515911	China	WM	82.4%	82.4%	81.7%	81.9%	81.0%
AY611025	China	BJ-03	83.0%	83.0%	82.3%	82.4%	81.6%

symptomatic squash, muskmelon and pumpkin plants in different locations in Saudi Arabia were detected and characterized. Out of thirty three samples, eleven samples [six squash samples collected from Riyadh region (Al-Hair and Wadi Eldwaser), Al-Madena, three muskmelon samples from Riyadh region (Al-Kharj) and two pumpkin samples collected from Al-Madena region] were positive for the presence of ZYMV by DAS-ELISA. Five of these samples were singly infected whereas the other six showed mixed infection with one or more of the following viruses: SqMV, CMV and WMV. Ten of the tested samples were found to be infected with one or more of the previously expected viruses other than ZYMV, whereas the rest of the samples were negative to all viruses. The Five samples that were selected for further studies of ZYMV were all singly infected and designated ZYMV-SA-1, ZYMV-SA-2, ZYMV-SA-3, ZYMV-SA-4 and ZYMV-SA-5 as indicated before. These five ZYMV isolates were characterized by host reactions, RT-PCR and nucleotide sequence. Eleven out of the 22 inoculated plant species in the host range test were infected with at least one of the five tested ZYMV isolates. However nine of the eleven positive plants were infected with all five ZYMV isolates (Table 1).

It is clear that these five ZYMV isolates differ in their host range. Whereas ZYMV-SA-1, ZYMV-SA-2 and ZYMV-SA-4 isolated from squash infected *Pisum sativum* L. *cv.* Alsk, and *Phaseolus vulgaris* L. cv. Black Turtel 2 showing symptomless infection on the former and chlorotic local lesions on the later, the two other ZYMV isolates (ZYMV-SA-3 and ZYMV-SA-5) isolated from pumpkin and muskmelon did not infect any of these two plant species at all. All six cucurbit species tested in this investigation were infected with the five ZYMV isolates and showed the following symptoms on these plant species: mild mottle, mottle, mosaic, yellow mosaic, vein banding, formation of blisters and malformation. All five ZYMV isolates induced mosaic symptoms on *Luffa acutangula* L. and chlorotic local lesions on each of *C. quinoa* and *C. amaranticolor* (Table 1). The other plant

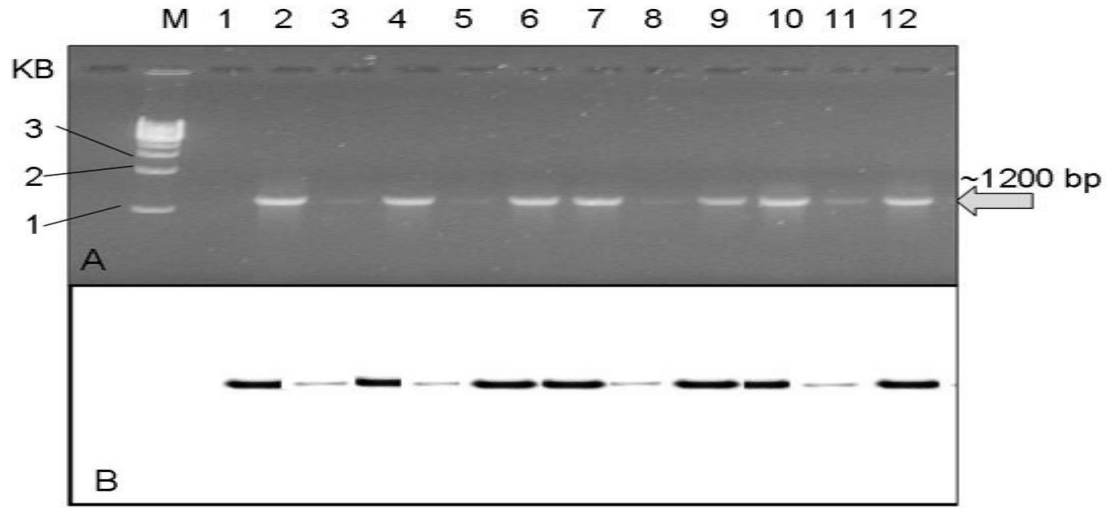

Figure 2. (A): 1% agarose gel electrophoresis of RT-PCR amplified products (1185 bp fragment) containing the complete coat protein gene using specific primers. Electrophoresis analysis of the RT-PCR products showed that single fragments of ~1.2 kb were amplified from symptomatic squash, muskmelon and pumpkin leaves collected from different locations. Lanes from left to right correspond to: squash samples collected from Riyadh region (Al-Hair: lanes 2, 3, Wadi Eldwaser: Lanes 4, 5), and Al-Madena (lanes 6, 7). Muskmelon samples from Riyadh region (Al-Kharj; lanes 8, 9, 10), and pumpkin samples collected from Al-Madena region (lanes 11, 12). Lane M: 1 kb DNA Ladder (Promega). (B) Southern blot hybridization analysis using a non-radioactive DIG–labelled cDNA probe, showing corresponding hybridization signals with the nucleic acid amplified products (1185 bp fragment). No RT-PCR and hybridization reaction was observed with uninfected squash tissues (lane 1).

species that were not infected by these isolates were *Gomphrena globosa* L.*, Datura stramonium,* L.*, Solanum lycopersicon* L.*, Nicotiana tabacum* L., cv. Samsun, Xanti-nc and White Burley*, Nicotiana rustica* L., *Nicotiana benthamiana* L.*, Capsicum annuum* L., *Beta vulgaris* L.*, Brassica oleracea* L.*, Raphanus sativs* L*,* and *Solaum nigrum* L.

Detection of ZYMV by RT-PCR and Southern blot hybridization

RT-PCR amplification of viral nucleic acid was carried out on the total RNA purified from the eleven infected samples which tested positive for ZYMV by DAS-ELISA and uninfected plant materials using an oligonucleotide primer pair specific for the complete coat protein gene of ZYMV (Figure 2A). Electrophoresis analysis of the resulting RT-PCR products showed single fragments of ~1.2 kb from RNA extracted from squash samples collected from Riyadh region (Al-Hair: lanes 2, 3, Wadi Eldwaser: lanes 4, 5), and Al-Madena (lanes 6 and 7). Muskmelon samples from Riyadh region (Al-Kharj; lanes 8, 9, 10), and pumpkin samples collected from Al-Madena region (lanes 11 and 12). No RT-PCR amplified product was observed with uninfected squash leaves (lane 1). Figure 2B illustrates Southern blot hybridization analysis using a non-radioactive DIG-labeled cDNA ZYMV probe which hybridized to the RT-PCR products amplified from symptomatic squash, muskmelon and pumpkin leaves collected from different locations (lanes 2 to 11). No hybridization reaction was observed with uninfected squash tissues (lane 1).

Sequence analysis of the ZYMV-CP gene

Following RT-PCR amplification, DNA sequences for the coat protein (CP) gene were determined for the five Saudi isolates of ZYMV based on the host plant and the location as mentioned earlier. Phylogenetic analysis using the 836 bp fragment comprising the complete CP gene fragment of the five Saudi Arabia isolates of ZYMV, along with sequences of 24 different isolates from GenBank which had been utilized in previous studies as reference sequences showed that ZYMV isolates clustered in seven groups. Saudi Arabian isolates belonged to one group to which Syria isolate also belonged. Comparing the nucleotide sequences for the five Saudi isolates indicated similarity between them that ranged between 97.1 and 100.0%. Similarity was also found between the five Saudi isolates and the remainder of the ZYMV isolates that were obtained from GenBank that ranged between 92.0 and 98.8%. The results also showed that the highest similarity was found with the following isolates: SYR-B2 isolate from Syria (97.7 to 98.5%); JOR-B3 isolate from Jordan (97.3 to 98.1%); Azr.Mak.W isolate from Iran (97.9 to 98.7%); isolate 10 from Hungary (97.1 to 98.0%); Austria 10 isolate from Austria (97.3 to 98.1%); Slovenia 1 isolate from Slovenia

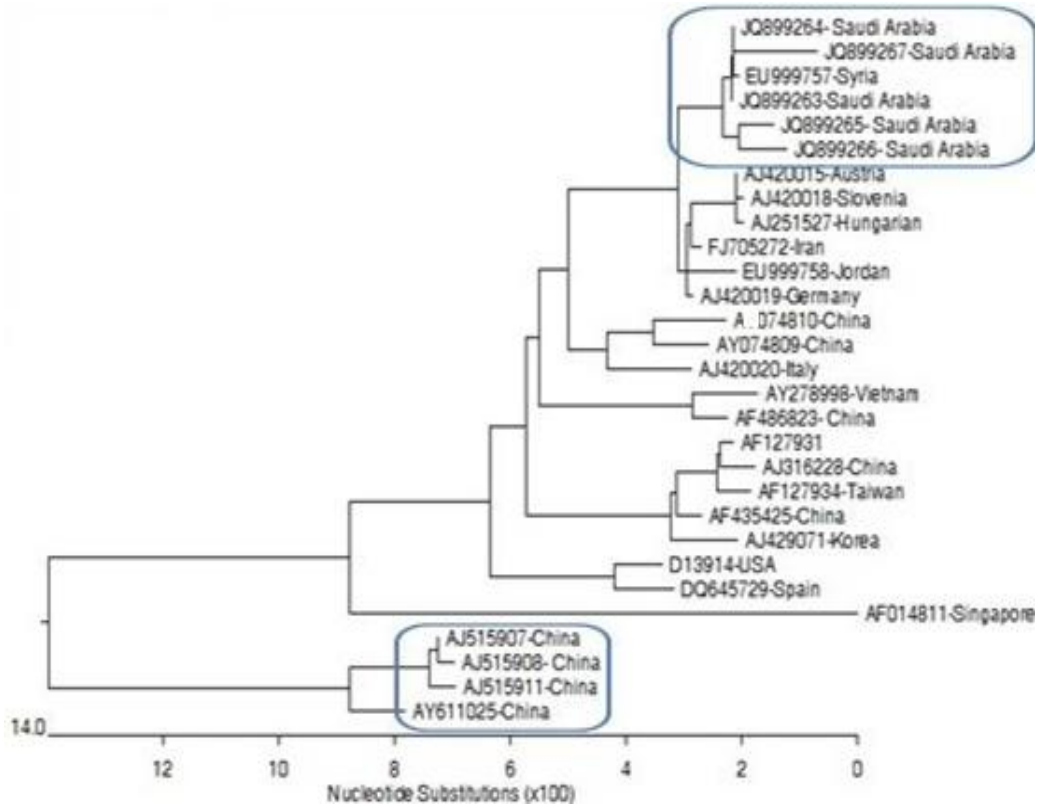

Figure 3. The homology tree based on multiple sequence alignments between five Saudi Arabia isolates of ZYMV and 24 different isolates from GenBank.

(97.1 to 98.0%); Berlin 1 isolate from Germany (98.0 to 98.8%) when compared with the five Saudi Arabia isolates. The lowest similarity was found with the SXS, WM, MM, SG, BJ-03, Hainan, Hangzhou, Ningbo and Beijing isolates from China (80.7 to 95.0%); isolate from Singapore (83.5 to 84.4%); ZYMV C16 isolate from Spain (92.0 to 92.9%); KR-PA isolate from Vietnam, KR-PA (92.0 to 93.1%), isolate A from South Korea (92.5 to 93.3%), Florida isolate from USA (92.4 to 93.2%), TW-PT5 and TW-TC1 isolates from Taiwan (91.5.6 to 93.5%%); Italy 1 isolate from Italy (94.4 to 95.2%) when compared with the five isolates of ZYMV from Saudi Arabia (Figure 3 and Table 2). The nucleotide sequences of the coat protein gene (841 bp) which were determined for all five Saudi isolates have been deposited in GenBank under the accession numbers JQ899263, JQ899264, JQ899265, JQ899266, JQ899267 (corresponding to ZYMV-SA-1, ZYMV-SA-2, ZYMV-SA-3, ZYMV-SA-4 and ZYMV-SA-5, respectively).

DISCUSSION

Out of the 22 plant species inoculated with the five Saudi isolates of ZYMV, symptoms were observed on 11 of them. All cucurbit species were infected by all five tested ZYMV isolates, however the symptoms were somewhat variable (Table 1). Among the five Saudi isolates, ZYMV-SA-2 isolated from squash in Al–Medina showed stronger symptoms on the infected hosts. These symptoms included mild mottle, mottling, mild mosaic, vein banding, mosaic, yellow mosaic and malformation. The ZYMV isolates showed variable reactions on the non-cucurbit species which included chlorotic local lesion and mosaic, whereas some of these plants did not show symptoms. Plant species exhibiting symptoms in the host range test were confirmed positive for ZYMV by ELISA. Some isolates of ZYMV have been reported to produce systemic infection without visible symptoms in *P. sativum* and tested positive by ELISA and was also reported for similar response in previous studies (Lecoq et al., 1981; Provvideenti et al., 1984; Singh et al., 2003; Safaeizadeh, 2008). Our study revealed minor differences in the host range and symptom expression of the Saudi isolates we studied. However, ZYMV-SA-2 from El-Madena was more virulent than the other four isolates. Distinct ZYMV isolates are assumed to give distinctive symptoms on infected plants. Since this is not always true in nature, incorporation of indicator plants in host range studies is generally useful, as they may help in differentiation of viral isolates. An example of this is the responses of *G. globosa, P. vulgaris* and *P. sativum* to the Saudi isolates

in this investigation. The minor differences in the host reactions observed with these isolates may be attributed to their different infection histories, which could have resulted in different host range adaptability, as suggested earlier (Chikh Ali et al., 2009).

The present study demonstrates the successful use of RT-PCR and sequencing to directly detect ZYMV in infected squash, pumpkin and muskmelon plants for the first time in various regions of Saudi Arabia. It is also worth mentioning that our DAS-ELISA and RT-PCR results were in complete agreement. More than 35 viruses have been isolated from different cucurbits so far and some of them present a serious threat to successful cucurbit production worldwide (Provvidenti, 1996), causing, in some cases, yield losses as high as 100% (Al-Shahwan et al., 1995, Abou-Jawdah et al., 2000; Coutts et al., 2011a). CMV, ZYMV, WMV and *Papaya ringspot virus* (PRSV) are transmitted from diseased plants to healthy plants by aphids in a non-persistent manner. This means that they acquire the virus from an infected plant almost immediately, but are able to infect healthy plants for only a short time, usually several hours to few days. Only a small number of aphids are needed to spread the virus throughout the field.

The nucleotide sequences were obtained for the CP gene in the five Saudi ZYMV isolates, and were used to investigate their genetic diversity and to establish their relationships with ZYMV isolates reported elsewhere in the world. Our results showed limited variability among the Saudi isolates and high similarity with other isolates from neighboring countries (Iran, Jordan and Syria), with the Iranian isolates showing the highest similarity with the Saudi isolates. These isolates and the Saudi isolates are not only similar in their nucleotide sequence, but they also share infection of the same cucurbit plants with somewhat minor differences in the symptoms induced on these plants (Table 1). The occurrence of similar ZYMV isolates in these neighboring countries may suggest spread of isolates of a common origin. High similarity was also observed between the Saudi ZYMV isolates and isolates from distant countries (Germany, Hungary, Austria, and Slovenia). The occurrence of ZYMV isolates that have nucleotide sequences similar to the Saudi isolates in these countries may suggest the common origin of distantly distributed isolates, which can be attributed to the widespread seed transmission of this viral isolate as has been reported in previous studies (Tóbiás and Palkovics, 2003; Safaeizadeh, 2008).

In conclusion, this study provided new information regarding the genetic make-up of the natural population of ZYMV isolates infecting cucurbits in Saudi Arabia. It also indicated the relative importance of ZYMV as compared to other viruses that affect cucurbit plants in the country. No substantial biological or molecular differences were observed between the characterized Saudi isolates in spite of the large area in which the study was conducted and the different plant species from which the samples were collected. These isolates did not only show high similarity among themselves but they also showed significant similarity with isolates of ZYMV in some neighboring countries, as well as in distantly located European countries too, suggesting probable transmission of this virus between these countries through transmission methods such as seeds and aphid vectors. Knowledge of the scope of variability in the population of ZYMV isolates intended in this research is not only essential for better understanding of the complexity and epidemiology of the pathogen, but also for designing of potentially effective, better adapted and durable control strategies such as determining resistance gene deployment strategies, as natural resistance genes can be rapidly overcome by adapted virus strains.

Conflict of Interests

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

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