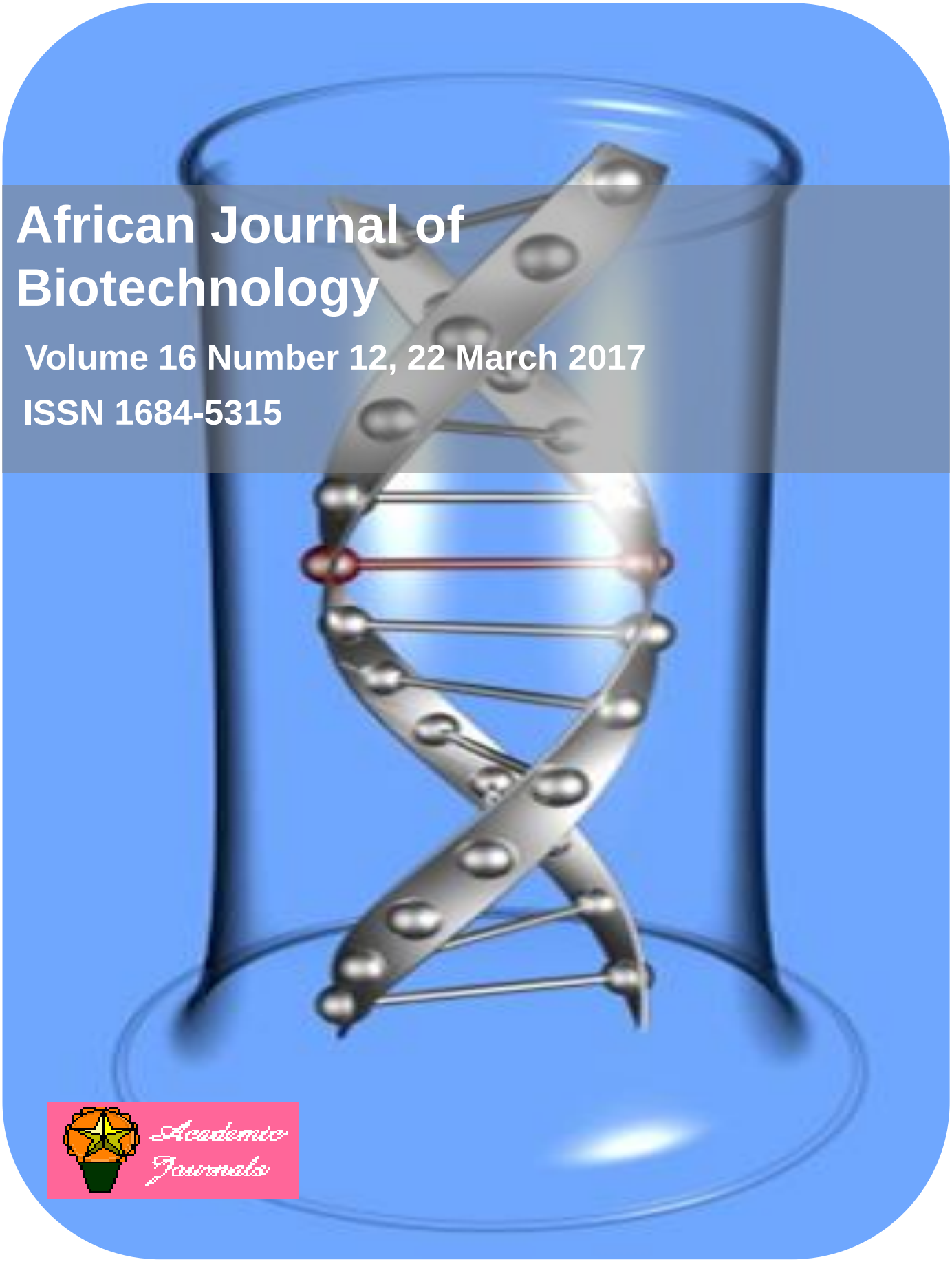

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Full Length Research Paper

Microsatellite, inter simple sequence repeat and biochemical analyses of *Rosa* genotypes from Saudi Arabia

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***Rosa damascena* Mill. as a main economic crop in the world is planted for beauty and essential oil production in Ta'if region. For the management and improvement of this important crop, genetic variability was evaluated amongst six *Rosa* genotypes grown in different plantations using microsatellite (simple sequence repeats, SSR), inter simple sequence repeat (ISSR) and biochemical markers. The six SSR primers showed low level of variation, whereas all ISSR primers generated high levels of polymorphism ranging from 66.7 to 100%. The biochemical markers revealed slight polymorphism between the three *Rosa* species under study. The dendrogram resulted from the combined data of SSR and ISSR splits the 6 genotypes into two main clusters. The first comprised the four *R. damascena* accessions, and the second grouped *R. damascene,* Trigintipetala'and *R. hybrid* together. ISSR markers can be recommended for the genetic variability analysis in *Rosa* genome.**

Key words: *Rosa damascena,* microsatellite, inter simple sequence repeat, dendrogram, genetic relationship.

INTRODUCTION

In Saudi Arabia, *Rosa damascena* is mainly grown for the production of oil that could be considered one of the most expensive oils in the world (Farooq et al., 2013). Despite the history of *R. damascena,* some doubt has been cast upon the source and origin of this species. A lot of roses have been selected and recombined to produce *R. damascena* in the Middle East regions including Ta'if (Widrlechner, 1981). Hence, there is consistent need for evaluating the genetic differences among *Rosa* species and cultivars to provide a continuous development of new germplasm for rose breeding programs and rose oil industry in Ta'if.

Molecular markers could aid breeding by providing dependable tools to investigate the variability among parents and their progenies (Rusanov et al., 2005). Although, *Rosa* plants have high levels of polyphenols

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Table 1. Geographical locations of *Rosa* genotypes in Ta'if region, Saudi Arabia

S/N	Species	Geographic origin	Latitude North	Longitude East	Altitude (m)
1	*R. damascena*	Ta'if city	21°16′N	40°24′E	1672
2	*R. damascena*	Al Hada	21°22′N	40°17′E	2034
3	*R. damascena*	Ash Shafa	21°04'N	40°18′E	2225
4	*R. damascena*	Misan	21°15'N	40°24′E	2500
5	*R. d.* ‘Trigintipetala‘	Ta'if city	21°16′N	40°24′E	1672
6	*R. hybrida*	Ta'if city	21°16′N	40°24′E	1672

Table 2. SSR primers used in the analysis of *Rosa* species showing clear and valid products.

Primer code	Sequence (5’-3’)	Annealing Temperature (˚C)	Bands number	Polymorphic bands	Polymorphism (%)
SSR 1-F	CAGATTCGCCGTAGCCCTTAC	58	1	1	100
SSR 1-R	ATCCGAACCCCGACCTGAC				
SSR 2-F	ATCATGTGCAGTCTCCTGGT	54	1	0	0.00
SSR 2-R	AATTGTGGGCTGGAAATATG				
SSR 3-F	GTGGATTTTCAGAGATACGC	52	1	0	0.00
SSR 3-R	TCACAGACAGGACCACCTAT				
SSR 4-F	GCCATCACTAACGCCACTAAA	54	1	1	100
SSR 4-R	GCGTCGTTCGCTTTGTTT				
SSR 5-F	ACAGGCCTCTGTTCACCATC	54	1	0	0.00
SSR 5-R	CACACATGCACAACTCAGAGAA				
SSR 6-F	CGGTGGAGAGGATGATGTG	54	1	0	0.00
SSR 6-R	GCAACAAGAACCAGCACAGA				
Total			6	2	33.3

and polysaccharides that make extraction of protein and DNA difficult (Kaul et al., 2009), significant efforts have been done to use these markers for identification, gene expression and biodiversity in *Rosa* lines, cultivars and hybrids, such as proteins (Dafny-Yelin et al., 2005), isozymes (Grossi et al., 1997; Jayasree et al., 1998), RAPDs (Kiani et al., 2008; Mirzaei and Rahmani, 2011), inter simple sequence repeat (ISSR) (Jabbarzadeh et al., 2010) and simple sequence repeats (SSR) (Stafne et al., 2005; Babaei et al., 2007; Farooq et al., 2013; Nadeem et al., 2014).

Among molecular markers, microsatellite or simple sequence repeats and ISSR as rapid techniques are useful in several areas of plant genetic studies. ISSR-PCR produces variable patterns of loci that are reproducible, abundant and polymorphic (Bornet and Branchard, 2004). Furthermore, SSR markers as codominant factors are able to reveal many alleles that can discriminate among closely related hybrids and cultivars (Nadeem et al., 2014). However, SSRs may be expensive to produce and laborious in particular species. So SSR primers developed from one species could be used for other studies on related species and genera (Stafne et al., 2005).

To increase our understanding of the genetic relationships between *Rosa* species, we used SSR, ISSR and biochemical markers in genetic diversity analysis of four *R. damascena* accessions and two related species; *R. damascena* ‘Trigintipetala‘ and *R. hybrida* grown in Ta'if region, Saudi Arabia.

MATERIALS AND METHODS

Plant materials

Roses were obtained from different sites of Ta'if region in Saudi Arabia (Table 1). In this study, six genotypes (four accessions of *R. damascene* and two related species; *R. damascena* ‘Trigintipetala’, *R. hybrida*) were investigated.

DNA extraction

Young leaves (0.5 g) of genotypes were taken for DNA extraction. Rose DNA extraction was done based on the cetyl trymethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1987).

SSR and ISSR primers

In this study, SSR and ISSR primers were selected from previous studies of Zhang et al. (2006) and Jabbarzadeh et al. (2010) (Tables 2 and 3). Fourteen primers were selected that produced good banding patterns for analysis.

Table 3. ISSR primers used in the analysis of *Rosa* species showing clear and valid products.

Primer code	Sequence (5'-3')	Annealing Temperature (˚C)	Bands number	Polymorphic bands	Polymorphism (%)
ISSR 1	CTCTCTCTCTCTCTCTTG	49	6	5	83.3
ISSR 2	GTGGTGGTGGC	36	3	2	66.7
ISSR 3	CAGCAGCAGCAGCAG	49	5	5	100
ISSR 4	CAACAACAACAACAA	36	4	4	100
ISSR 5	AGAGAGAGAGAGAGAGT	49	6	5	83.3
ISSR 6	GATAGATAGATAGATA	36	5	5	100
ISSR 7	CCAAGAGAGAGAGAGAGAGT	53	3	3	100
ISSR 8	GAGAGAGAGAGAGAGAACC	53	5	5	100
Total			37	34	91.9

PCR conditions

Amplifications were done with a 13 µL total reaction/sample that included 10 µL Taq Master Mix, 1 µL each, forward and reverse primers, and 1 µL DNA. Thermal cycling was done on a Techne TC-3000 (Barloworld Scientific, Ltd. Staffordshire, UK) with the following program: 105°C heated lid, initial denaturation of 94°C for 5 min, and 35 cycles of 94°C for 1 min, 58°C (changed according to primer) for 1 min, 72°C for 1 min, finishing with a final extension of 72°C for 5 min and a final hold at 4°C. Products were confirmed on an agarose gel and stained by ethidium bromide then observed under UV light and photographed by gel documentation system.

Isozyme analysis

The isozymes; α- and β-esteras (EST), acid phosphatase (ACP), alcohol dehydrogenase (ADH) and peroxidase (PRX) were separated in 10% native-polyacrylamide gel electrophoresis as described by Stegemann et al. (1987). For gels staining, protocols of Scandalios (1964) were used for α- and β-EST, Wendel and Weeden (1989) for ACP, Weeden and Wendel (1990) for ADH and Heldt (1997) for PRX. Gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid and photographed.

Protein analysis

To extract proteins, 1 g of leaves of each genotype was mixed with 1 M Tris-HCl buffer, pH 8.8 and homogenized using a mortar and pestle. After centrifugation, the supernatants were transferred to new tubes and kept in deep-freeze until use. Electrophoresis was carried out by the modified discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (DISC SDS-PAGE) method (Laemmli, 1970). Gels were stained overnight in coomassie brilliant blue-R250 solution then destained and photographed.

Data analysis

The bands of SSR, ISSR and protein were evaluated by comparing with 100 bp DNA ladder and blue wide range prestained protein ladder (Cleaver Scientific Ltd, UK), respectively, using gel analyzer program (version 3). To determine the genetic relationship among *Rosa* genotypes, bands of SSR and ISSR patterns were treated as a unit character and coded 1 or 0 for their presence or absence, respectively. Clustering was performed using UPGMA procedure and represented in a phenogram by using SAHN and TREE modules, respectively. The NTSYS-pc 2.2 (Numerical Taxonomy and Multivariate Analysis System, Exeter Software) program was utilized in all previous analysis (Rohlf, 1998).

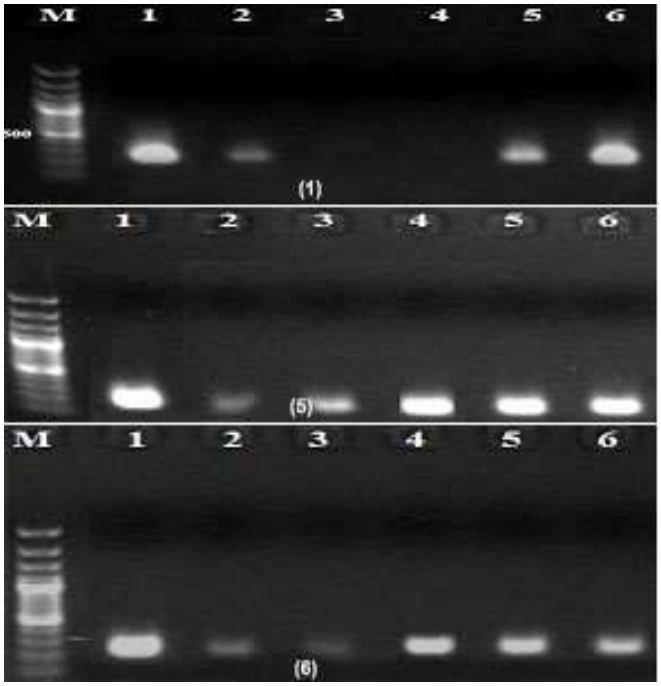

Figure 1. SSR patterns of *Rosa* genotypes amplified with primers 1, 5 and 6. M, marker.

RESULTS AND DISCUSSION

For management and successful improvement of the rose crop, genetic variation within different accessions, cultivars and species of *Rosa* is needed (Kaul et al., 2009). Of the nine specific SSR primers tested in *Rosa*, six produced clear and valid products (Table 2). The six primers produced six bands with molecular sizes that ranged from 150 to 422 bp as shown in Figure 1. Additionally, this study has shown that the annealing

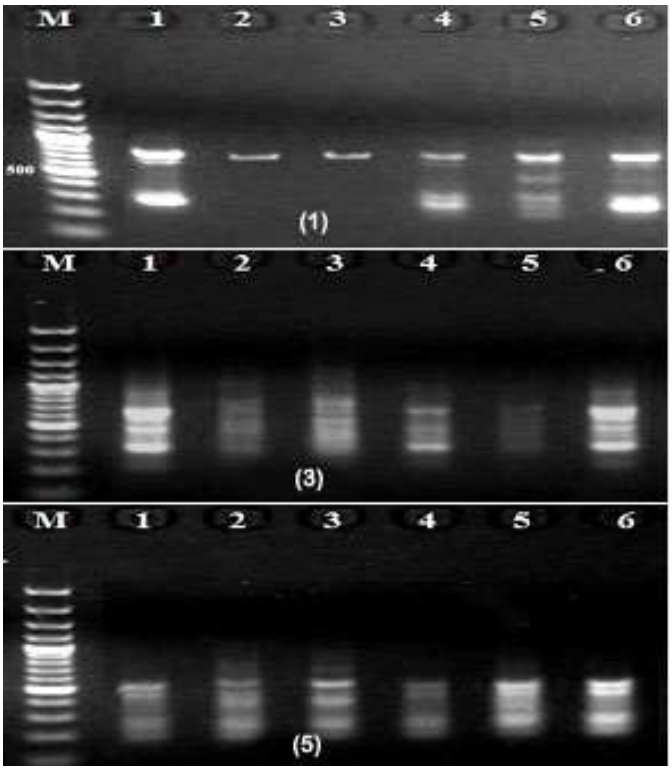

Figure 2. ISSR patterns of *Rosa* genotypes amplified with primers 1, 3 and 5. M, marker.

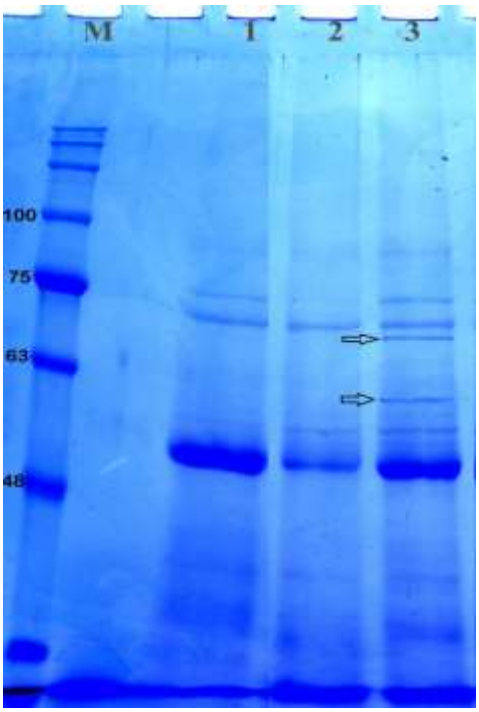

Figure 3. SDS-PAGE pattern of *R. damascena* (1), *R. d.* 'Trigintipetala' (2) and *R. hybrida* (3). M, marker. Arrows indicate unique bands.

temperatures for the 6 SSR primers varied from 52-58°C (Table 2). Notably, only 2 out of 6 primers showed low level of variation among rose genotypes tested. These findings contrast that of Zhang et al. (2006) who observed that SSRs revealed a significant level of variation and could differentiate easily among rose cultivars studied. On the other hand, they are in accordance with previous studies depending on SSR markers that did not exhibit any variability among cultivars of *R. damascena* in Turkey and Bulgaria (Baydar et al., 2004; Rusanov et al., 2005). Although, SSR markers give rapid data from small amount of plant sample, they are costly to produce and can be very exhausted in particular species (Stafne et al., 2005).

For further evaluation of the genetic relatedness among *Rosa* genotypes, eight ISSR primers ranging between 11 and 20 bases were used. The annealing temperatures for the eight ISSR primers varied from 36-53°C (Table 3). A total of 37 bands were amplified from the genotypes, of which 34 were polymorphic. The bands sizes ranged from 235-1100 bp (Figure 2). The ISSR patterns revealed 11 unique bands; 2 in *R. damascene*, 3 in *R. damascena* 'Trigintipetala', six in *R. hybrid*. All primers generated high levels of polymorphism ranging from 66.7 to 100%. These results were in conformity with those of the Pakistani and Iranian scientists that scored high levels of diversity among rose genotypes collected from different regions from Pakistan and Iran (Mirali et al., 2012).

The data of SSR and ISSR were combined to estimate the genetic similarity values and generate a dendrogram showing the relationship among rose genotypes under study. The similarity matrix showed that the highest value (0.966) was between two accessions of *R. damascena* (collected from Ta'if city and Misan), whereas the lowest value (0.333) was found between one accession of *R. damascena and R. hybrida* (data not shown). The resultant dendrogram grouped the six genotypes into two main clusters. The first comprised the four *R. damascena* accessions, and the second grouped *R. damascena* 'Trigintipetala'and *R. hybrid* together (Figure 3). Occurrence of genetic variations among accessions of *R. damascena* might be due to the geographical differences that might cause an evolution through genetic drift, mutation and recombinations. The climatic conditions can also affect and lead to variability within and between *Rosa* species (Farooq et al., 2013).

Two biochemical approaches; isozyme and SDS-PAGE, were used to discriminate among *R. damascena*, *R. damascena* 'Trigintipetala'and *R. hybrida.* Seven enzyme systems were used as mentioned in Table 4. The three species did not generate any bands for the two MDH and malic isozymes. One to three bands per isozyme were produced for the remaining five systems. *R. damascena* 'Trigintipetala 'was distinguished by three

Table 4. Biochemical markers detected in *R. damascena, R. d.* 'Trigintipetala 'and *R. hybrida*

System	Monomorphic band	Polymorphic band		Total bands	Polymorphism (%)
		Unique	Shared		
SDS-PAGE	7	2	2	11	36.3
ACP	1	0	0	1	0
ADH	1	0	0	1	0
α-EST	0	2	1	3	100
β-EST	1	2	0	3	66.7
MDH	0	0	0	0	0
MALIC	0	0	0	0	0
PRX	0	1	0	1	100

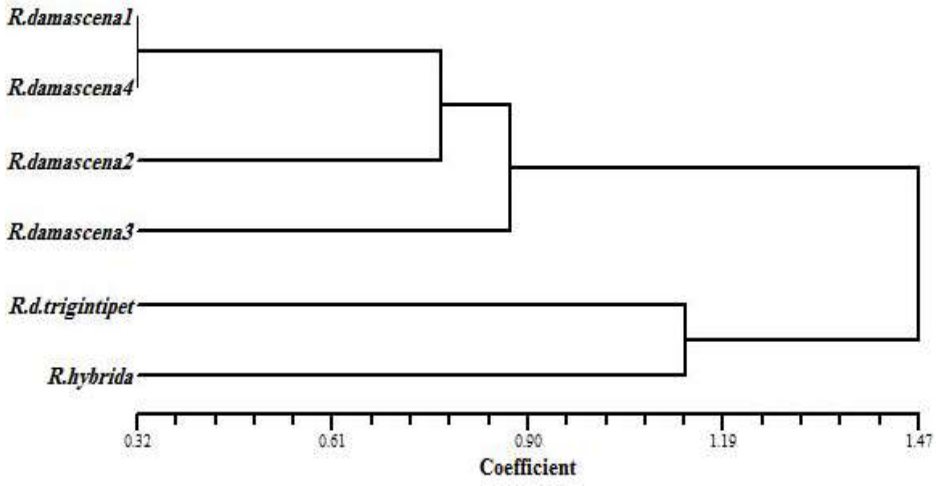

Figure 4. UPGMA phenogram showing genetic relationship among *Rosa* genotypes.

unique bands for estesrases, whereas *R. damascena* and *R. hybrida* were severally characterized by only one unique band for PRX and β-EST systems, respectively. On the other hand, the produced SDS-PAGE of leaf protein profile of *R. damascena, R. damascena* 'Trigintipetala'and *R. hybrid* is shown in Figure 4. A total number of 11 bands were recorded. Molecular weight (Mw) of the protein subunits ranged from 34.7 to 91.5 kDa. The profile revealed seven monomorphic bands in all species and four polymorphic bands including two unique bands, with polymorphism percentage 36.3% (Table 4). The two unique bands at 70.0 and 60.2 kDa characterized *R. hybrida* only (Figure 4). The limitation of information obtained from biochemical markers may be due to the presence of high contents of polyphenols and polysaccharides that make the extraction of protein difficult (Kaul et al., 2009).

Conclusion

Evaluation of genetic diversity by various molecular systems provided different levels of information that could be important in the management of *Rosa* germplasm. ISSR markers were more efficient based on better reproducibility and polymorphism percentage. SSR and biochemical markers revealed slight polymorphism within and between *Rosa* species collected from different plantations. This study needs further confirmation using a large number of accessions and species that can clearly reveal and explain the variability at the species level. However, our results may help in the initiation of intraspecific and interspecific cross-breeding programs for improvement of roses in Ta'if.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Babaei A, Tabaei-Aghdaei SR, Khosh-Khui M, Omidbaigi R, Naghavi MR, Esselink GD, Smulders MJM (2007). Microsatellite analysis of Damask rose (*Rosa damascena* Mill.) accessions from various regions in Iran reveals multiple genotypes. BMC Plant Biol. 7(1):12.

Baydar N, Baydar H, Debener T (2004). Analysis of genetic relationships among *Rosa damascena* plants grown in Turkey by using AFLP and microsatellite markers. J. Biotechnol. 111:263-267.

Bornet B, Branchard M (2004). Use of ISSR fingerprints to detect microsatellites and genetic diversity in several related *Brassica* taxa and *Arabidopsis thaliana*. Hereditas 140:245-248.

Dafny-Yelin M, Guterman I, Menda N, Ovadis M, Shalit M, Pichersky E, Zamir D, Lewinsohn E, Adam Z, Weiss D, Vainstein A (2005). Flower proteome: changes in protein spectrum during the advanced stages of rose petal development. Planta 222:37-46.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11-15.

Farooq A, Kiani M, Khan MA, Riaz A, Khan AA, Anderson N, Byrne DH (2013). Microsatellite Analysis of *Rosa damascena* from Pakistan and Iran. Hortic. Environ. Biotechnol. 54(2):141-147.

Grossi C, Raymond O, Jay M (1997). isozyme polymorphism of *Rosa* spp. and cultivar identification. Euphytica 98:11-19.

Heldt WH (1997). A leaf cell consists of several metabolic compartments. Plant Biochemistry and Molecular Biology. Oxford University Press, Oxford.

Jabbarzadeh Z, Khosh-khui M, Salehi H, Saberivand A (2010). Inter simple sequence repeat (ISSR) markers as reproducible and specific tools for genetic diversity analysis of rose species. Afr. J. Biotechnol. 9(37):6091-6095.

Jayasree N, Devi BP, Vijaya N (1998). Somatic embryogenesis and isozymes in rose species *Rosa hybrida* l. cv. king's ransom. Indian J. Genet. 58(4):449-454.

Kaul K, Dhyani D, Sharma RK (2009). Evaluation of DNA extraction methods for RAPD, SSR and AFLP analyses of wild rose species. Floricult. Ornam. Biotechnol. 3(1):25-30

Kiani M, Zamani Z, Khalighi A, Fatahi R, Byrne DH (2008). Wide genetic diversity of *Rosa damascena* Mill. Germplasm in Iran as revealed by RAPD analysis. Sci. Hortic. 115:386-392.

Laemmli U (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Mirali N, Aziz R, Nabulsi I (2012). Genetic characterization of *Rosa damascene* growing in different regions of Syria and its relationship to the quality of the essential oils. Int. J. Med. Arom. Plants 2(1):41-52.

Mirzaei L, Rahmani F (2011). Genetic relationships among *Rosa* species based on random amplified polymorphic DNA (RAPD) markers. Afr. J. Biotechnol. 10(55):11373-11377.

Nadeem M, Wang X, Akond M, Awan FS, Khan AI, Riaz A, Younis A (2014). Hybrid identification, morphological evaluation and genetic diversity analysis of *Rosa* × *hybrida* by SSR markers. Aust. J. Crop Sci. 8(2):183-190.

Rohlf FJ (1998). NTSYSpc: Numerical Taxonomy and Multivariate Analysis System, version 2.02. Exeter Software, New York.

Rusanov K, Kovacheva N, Vosman B, Zhang L, Rajapakse S, Atanassov A, Atanassov I (2005). Microsatellite analysis of *Rosa damasena* Mill. Accessions reveals genetic similarity between genotypes used for rose oil production and old damask rose varieties. Theor. Appl. Genet. 111:804-809.

Scandalios JC (1964). Tissue-specific allozyme variations in maize. J. Hered. 55:281-285.

Stafne ET, Clark JR, Weber CA, Graham J, Lewers S (2005). Simple sequence repeat (SSR) markers for genetic mapping of raspberry and blackberry. J. Am. Soc. Hortic. Sci. 130(5):722-728.

Stegemann H, Afifiy AMR, Hussein KRF (1985). Cultivar Identification of dates (*Phoenix dectylifera*) by protein patterns. 2nd International Symposium of Biochemical Approaches to Identification of Cultivars. Braunschweig, West Germany. 44 p.

Weeden NF, Wendel JF (1990). Genetics of plant isozymes. In. Soltis, D.E. and P.S. Soltis, (Ed.). Isozymes in plant biology. Chapman and Hall Press, London. pp. 46-72.

Wendel JF, Weeden NF (1989). Visualization and interpretation of plant allozymes. In: Soltis, D.E. and P.S. Soltis, (Ed.). Allozymes in plant biology, Advances in plant sciences, series 4. Dioscorides Press, Portland, OR. pp. 5-45.

Widrlechner MP (1981). History and utilization of *Rosa damascena*. Econ. Bot. 35:42-58.

Zhang LH, Byrne DH, Ballard RE, Rajapakse S (2006). Microsatellite marker development in rose and its application in tetraploid mapping. J. Am. Soc. Hortic. Sci. 131(3):380-387.

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Full Length Research Paper

PCR validation of predicted virulence factors in a collection of *Xanthomonas campestris (*pv. *musacearum* and *vascolurum*) strains

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Bacterial plant pathogens belonging to the *Xanthomonas* genus are adapted to their host plants and are not known to colonize other environments. *Xanthomonas* colonize host parts such as leaves, stems and roots before entering vascular tissues and engaging in an invasive pathogenic phase. These bacterial strains have evolved strategies to adapt to life in this environment. The host-pathogen interactions of *Xanthomonas vasicola* (Xv) need to be well understood to properly map the target genes in the host and pathogen so as to understand the mechanism of resistance. Genotypic characterization, based on the analysis of restriction fragment length polymorphism of virulence factor fragment products was performed on members of the *X. vasicola pv. musacearum* (Xcm) and *X. vasicola* pv. *vasculorum* (Xvv) from varying geographical locations. The study showed that Xcm and Xvv are different from each other based on amplification of virulence factors within fragments of their DNA. Bacterial strains of similar species can have unique Type four pili (Tfp) and Tfp pilus assembly protein PilF a fimbrial biogenesis protein was amplified in all *Xanthomonas* strains except NCPPB1131 only. Type III effector protein RipT was confirmed to be present in all strains of Xcm and Xvv but not NCPPB1131 and NCPPB1132. All the Xcm and Xvv strains under test yielded bands of type III effector HopAF1 except Xvv206, NCPPB1131 and NCPPB1132. YopJ type III secretion system effector protein hybridizes in DNA of all Xcm strains tested but not in NCPPB1131 or NCPPB1132. This study confirmed the predicted presence or absence of virulence factors especially effectors across bacterial strains and within strains of the same species and other clusters conserved in gram negative bacteria.

Key words: Banana, effectors, pathogen-host, *Xanthomonas* wilt, *Xanthomonas campestris*, *Xanthomonas vasicola*.

INTRODUCTION

Banana production in Eastern Africa is on the decline due to diseases particularly Banana Xanthomonas Wilt (BXW) (Kubiriba et al., 2012; Tushemereirwe et al., 2004). This correlates to 80% yield loss and thus 32% loss in

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household income. Currently the most effective control methods are cultural based and the deployment of resistance would be most ideal and less expensive on time and resources. Efforts are being made to develop transgenic BXW resistant banana varieties using Hypersensitive Response Assisting Protein (Hrap) or Plant Ferredoxin like Protein (Pflp) gene originated from sweet pepper (*Capsicum annuum*). The interaction of Xcm and its banana host needs to be well understood to properly map the target genes in host and also understand the mechanism of resistance to support future control programs (Tripathi et al., 2010).

Xanthomonas is a large genus of Gram-negative bacteria that cause disease in hundreds of plant hosts, including many economically important crops. For bacteria to adapt specifically to their hosts, they sense favorable environmental stimuli and then they move toward favorable conditions. Bacteria have evolved receptors and sensors in their cell walls to detect chemical and environmental signals such as the presence of chemo-attractants and chemo-repellents (Mhedbi-Hajri et al., 2011). Pathogenic species and pathovars within species show a high degree of host plant specificity and exhibit tissue specificity, invading either the vascular system or the mesophyll tissue of the host. Plant-pathogenic *Xanthomonas pathovars* require a type three secretion system (TTSS) to secrete and translocate effector proteins in order to cause disease. The repertoire of effectors can vary between species and strains within species and is believed to be a key determinant in the host range of a given pathogen (Baltrus et al., 2011). The draft genome analysis of the two bacteria species (Xcm4381 and Xvv702) revealed several genetic differences between the two strains that might be important for host specificity, virulence and epiphytic fitness, including differences in the repertoires of secreted and translocated effector proteins, Tfp and enzymes for lipopolysaccharide biosynthesis (Studholme et al., 2010). They both encode homologues of the candidate T3SS effectors including AvrBs2, AvrGf1, HopW1; HopAF1; PilvD; RipT; YopT-like cysteine protease and a variety of homologues of XopF, XopK, XopL, XopN, XopP, XopQ, XopR, XopX, XopZ, XopA, XopB, XopG, XopH, XopI, XopY, XopAA, XopAD, XopAE and XopAK, which are conserved in a subset of *Xanthomonas* genomes. However Xcm4381 encodes two predicted YopJ-like C55 cysteine proteases that are absent from Xvv702, whereas Xvv702 encodes a protein XopAF (also known as AvrXv3) which is absent from Xcm4381, but shares 35% identity with the HopAF1-like genes. Such differences in effector repertoires are said to be significant for host adaptation.

Lipopolysaccharide (LPS), which are produced by Gram-negative bacteria, are powerful activator of inherent immune responses. The lipopolysaccharide locus in Xvv702 showed no significant sequence similarity to that of Xcm4381. The respective Type IV pilus (Tfp) cluster showed little sequence similarity between proteins, respectively encoded on the Xvv702 and the Xcm4381 TFP clusters. An 8-kb gene cluster in Xcm4381 is reported to encode TFP components FimT, PilV, PilW, PilX, PilY1 and PilE. A different gene cluster in Xvv 702 encodes homologues of TFP components FimT, PilE, PilY1, PilW and PilV. These differences might be adaptive for attachment to and motility on different plant surfaces.

There is need to clearly identify and understand the infection process applied by Xcm and Xvv so as to propose pathogen protein targets for suppressing the motility, mode of host plant surface identification, vascular rapid multiplication and evasion of host plant resistance mechanism. This study therefore aimed at experimentally validating presence or absence of predicted virulence factors in different strains of Xcm, Xsp.and Xvv.

MATERIALS AND METHODS

Bacterial culture conditions

Bacterial cultures of strains in Table 1 were obtained from National Collection of Plant Pathogenic Bacteria (NCPPB) at the Food and Environmental Research Agency (FERA, York, UK). The samples were received on filter paper in glass vials, recovered through streaking on solid Kings Broth (KB). The KB was prepared using 10 g Peptone meat, 10 g N-Z casein; 1.5 g $MgSO_4$-$7H_2O$; 1.5g K_2HPO4; 12.6 g glycerol to a liter of dH_2O and stabilized at pH 7.2. Cultures were further purified by re-streaking onto new KB in a fixed incubator for 12 h. A single loop colony from each strain was transferred into 10 ml liquid KB and grown at 28°C in a shaking incubator at 150 rpm overnight. All of the isolates used in this study were then put in glycerol stock and kept at -20°C for further use during the experiment and -80°C for long term storage.

Total genomic DNA extraction from cultures

Genomic DNA from each of the strains was extracted using modified protocols by Mahuku (2004). Overnight cultures were centrifuged at 4000 rpm 4°C for 10 min to obtain a cell pellet, and supernatant was poured off and the pellet dried. Cells were suspended and dissolved in 2 ml of TE buffer (25 mM Tris Hcl pH 8.0, 10 mM EDTA). 300 µl cells were lysed by adding 12 µl lysozyme 20 mg/µl. 1.5 µl RNase 10 mg/µl were then added and mixed by inverting 10 times and incubated at 25°C for 10 min. 17 µl 10% SDS were then added and mixed by inverting 10 times then incubated on ice for 5 min. Proteins were pelleted by adding 170 µl 8 M ammonium acetate (not pH), mixed by inverting 10 times, vortexed 20 s and spanned 4°C at 4000 rpm for 30 min. The supernatant was carefully pippeted off into clean tubes avoiding the pellet and membrane formed on the surface. DNA was pelleted by adding 0.75% Vol Isopropanol, mixed by inverting until a ball of DNA was visible; then centrifuged at 4°C, maximum speed for 10 min. The supernatant was pippeted off and air dried for 5 min. The pellet was washed with 100 µl 70% ethanol; centrifuged for 1 min maximum speed, the ethanol pipetted off, spinned short burst and pipette off excess ethanol; air dry pellet 5 mins. The pellet was then dissolved in 200 µl TE, incubated in 65°C water bath for 30 min to 1 h. The quality and quantity of DNA was then quantified using a NanoDrop® ND-1000 (NanoDrop Technologies USA,

Table 1. Bacterial strains used in the study collected from National Collection of Plant Pathogenic Bacteria (NCPPB).

Code	Species	Host	Origin country	Sampling date	Donor reference
Xcm4383	*Xanthomonas vasicola pv. musacearum*	*Musa*-Banana	Uganda	2007	071/wsk/05
Xcm4387	*X. vasicola pv. musacearum*	*Musa*-Banana	Democratic Republic of Congo	2007	IMI392223
Xcm4389	*X. vasicola pv. musacearum*	*Musa*-Banana	Rwanda	2007	IMI393640
Xcm4433	*X. vasicola pv. musacearum*	*Musa*-Banana	Burundi	2008	20709953
Xcm4434	*X. vasicola pv. musacearum*	*Musa*-Banana	Kenya	2008	20702295
Xcm2251	*X. vasicola pv. musacearum*	*Musa sp.*	Ethiopia	1969	B3645
Xcm2005	*X. vasicola pv. musacearum*	*Ensette ventricosum*	Ethiopia	1967	B3100
Xcm4392	*X. vasicola pv. musacearum*	*Musa-Banana*	Tanzania	2007	394051
Xvv206	*X. vasicola pv. vasculorum*	*Zea mays*	South Africa	1948	
Xvv1326	*X. vasicola pv. vasculorum*	*Saccharum offinarum*	Zimbabwe	1962	

www.nanodrop.com). Agarose gel electrophoresis of each sample DNA was run on 0.8% agarose gel for 45 min at 100 V.

Primer design and validation of putative virulence factors

The primers for 26 virulence factors were designed using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012) to amplify the full length gene. The Primer-Search program was used to confirm the specificity of the primers (cut off of 20% mismatch) against the sequenced Xcm4381 and Xvv702. Each 25 µl of PCR mixture contained 1.5 mM $MgCl_2$, 0.2 µM forward and reverse primers, PCR buffer (Invitrogen, Carlsbad, CA), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.5 units Taq polymerase (Invitrogen, Carlsbad, CA), and 10 ng DNA. After an initial denaturing step, PCR was conducted for 28 cycles of 30 s at 94°C, 30 s at 55°C, and 70 s at 72°C. The primers were tested on DNA of Xcm4381 and Xvv702 and those validated used to amplify bacterial strains in Table 1.

Cluster analysis

The data was converted as follows: Absence of a band (-) changed to (0) and presence of a band (+) changed to (1) before performing a hierarchical cluster analysis. The distance was measured using the Binary Squared Euclidean distance and separated by the between groups method. A dendogram was then produced by the SPSS 16 Edition program.

RESULTS

Primers used in the study are detailed in Table 2 and amplified with DNA from test bacterial strains whose response is listed in Tables 3 and 4. Virulence factors were categorized into lipopolysaccharides (LPS), type iv pilus proteins (Tfp) and type three secretion system (TTSS) proteins for analysis across 8 strains of Xcm, 2 strains of NCPPB and 3 Xvv strains. Only primers that amplified with DNA of the selected strains are given against a 1000 bp ladder. The amplification of DNA was scored as present or absent for the different strains in the study, converted to binry data (0 for -) and (1 for +). The converted data was subjected to cluster analysis using a two-step approach in SPSS 16.0 (SPSS, 2008). The binary data was then clustered using the square Euclidean distance method.

Lipopolysaccharides variation in selected Xcm and Xvv strains

Xcm strains had lipopolysaccharides

ABC transporter permease/ATP-binding protein (permease); S-adenosyl-L-methionine (SAM) dependent methyltransferase, truncated O-antigen biosynthesis protein and hypothetical protein ZP_06489485 amplified but had no product with NCPPB1131, NCPPB1132, Xvv206, Xvv1326 and Xvv1381 strains (Figure 1).

Predicted membrane protein (YP_007652590) was present in all Xcm strains (LPS-E) and also in NCPPB1132, Xvv 1326 and 1381 but not observed in Xsp 1131 and Xvv206 (LPS-). Nicotinamide adenine dinucleotide (NAD) dependent epimerase was observed in Xcm4387, Xcm4389, Xcm4433, Xcm 4434, Xcm2251, Xcm2005, Xcm4392, NCPPB1131, NCPPB1132 and unexpectedly in Xvv206, Xvv1326 and Xvv1381, interestingly we did not observe it in Xcm 4383 (LPS-F). The protein, indolepyruvate ferredoxin oxidoreductase (LPS-G) was amplified in all the Xcm strains except Xcm4392 and although expected in Xvv strains, it was not amplified (Figure 2).Xcm4383, Xcm2005, Xcm4392, NCPPB1131, NCPPB1132 and all the Xvv strains did not amplify the lipopolysaccharide biosynthesis protein (Figure 3). Ferredoxin oxidoreductase (Xvv702 based) hybridised only in Xcm4387, Xcm4433, Xcm4434 and Xcm2251 strains (LPS-K); also in Xcm2005, Xvv206 and Xvv1326. Another protein short chain dehydrogenase/reductase was amplified in Xcm4387, Xcm2005 and Xcm4392 just like was expected in

Table 2. Primer sequences for genes used in the study and their expected products.

Code	Genes	Expected product size (Kb)		Forward primer	Reverse primer
		Xcm	Xvv		
LPS-A	ABC-transporter-permease	783	NP	ATGCAGAGCCATGCAAATCTG	TCATAGGACGTCCGCAAAACC
LPS-B	SAM-dependent methyltransferases-WsaE	1656	NP	ATGGGCGTTGATCTCCAAGC	TCAATTGGCCAATAAGCGGC
LPS-C	Truncated O-antigen biosynthesis protein	1701	NP	ATGGCGGCATATGCTTCTGG	TCAAGCATCTGCCTGCGC
LPS-D	Hypothetical protein-ZP_06489485	1269	NP	ATGCTTGATCGTTTGCTACGGC	CTACACCCGGTCAACCTGG
LPS-E	Predicted membrane protein	399	NP	TCATCGATGCCCCCGAAAAGTG	GTGATCGATCAGAAGTTCTTC
LPS-F	NAD dependent epimerase	945	NP	TCAGCGCTTGAGCACGATGTGGC	ATGGCACAGAAGAATGACAAG
LPS-G	Indolepyruvate ferredoxin oxidoreductase	1302	1302	CTACTCCGTGACCCGGCG	ATGGCGGCGGGGCAGTCC
LPS-H	Indolepyruvate ferredoxin oxidoreductase	1302	1302	ATGGCGGCGGGGCAGTC	CTACTCCGTGACCCGGCG
LPS-K	Lipopolysaccharide biosynthesis protein	2838	2838	TCATCCGGCTTGAATCAATCCG	ATGGCGCACCGCGTGACTG
LPS-M	Short chain dehydrogenase	NP	729	ATGCAACGCGTTCTTATTATCG	TCAGAGCTTGATCCTACGAAAAACG
LPS-N	Putative transmembrane GtrA	NP	405	ATGATTAGTCGACAGTTCATCG	TCATTGTGGTGTGGCGATCTTG
LPS-P	GDP-mannose 4,6-dehydratase	NP	969	TCAGATTGAAAGTCGACGCATATC	ATGAGTAAGAATGCCCTGATC
Tfp-A	Tfp pilus assembly protein PilE	426	NP	TCAAAAACATTGTGACGCGCCATC	ATGTACAGAATTGCTCGCTCG
Tfp-B	Tfp pilus assembly protein PilE	NP	381	TTGATCGAGTTGATGATCGTCG	CTACCAACAGCCCGGAGTG
Tfp-C	Tfp pilus assembly protein FimT	NP	474	ATGATCACGATCGTCGTGC	TCATTGGCAGGAGCTCTTCTG
Tfp-D	Tfp_fimbrial biogenesis protein	534	NP	ATGCGATACGCTCCTCTGG	TCACGAAGGGCAGGGATG
Tfp-F	Tfp pilus assembly protein PilV	411	NP	TCATAGACGGGTCTTGATAG	ATGTCTGGTATCGGTTTGATCG
Tfp-G	Tfp pilus assembly protein PilW	975	NP	TCATGCATTGCGATTCCGAAG	ATGCGCGGCGTGACGCTG
Tfp-H	Tfp pilus assembly protein PilX	480	NP	TTACTGCTTGATGTAGTTAGTCTG	GTCTCGCTCATTGTTGTGCTG
Tfp-K	Tfp pilus assembly protein PilY1	2151	NP	TTAGTTCCTGATGATCTCGCG	TTGGCCGACGTGGCCATG
Rip-T	Type III effector protein Ript	720	NP	GTGTTCAATGGTGATGAGATAG	CTAAGGCACGAGACGAGC
HopA	Type III effector HopAF1	861	860	ATGCTTGTCATGATGCCTGTTACC	CTATTCCGCAGCGACGAG
HopW	Type III effector HopW1	1503	1503	TCAGGAGGTAATCCCCTTTTTGG	TTGCAAAAACAAGCGGGCCTC
	Type III secretion system effector protein XopAF	NP	657	CTATTTAACAAGATCTGTTACAAATC	ATGACTGATGGTTTAGATCTTTGCG
Yop1	Virulence factor yopJ-like 1	1077	NP	TTGCAGGACTTCATTGACGC	TTAGCTCCCATACCCGGAGTCG
Yop2	Virulence factor yopJ-like 2	1068	NP	ATGGACATTGAAAATCTCCCC	TCAGGATTCTAAGGCGTGACG

NP, No amplification product expected.

Xvv206, Xvv1326 and Xvv1381; the other Xcm strains including NCPPB1131 and NCPPB1132 did not yield the protein (Figure 3).

Strain Xcm2005 unexpectedly amplified putative transmembrane GtrA and GDP-mannose 4,6-dehydratase protein just like Xvv206, Xvv1326 and Xvv1381, the rest of the test strains did not yield any strands of the protein (Figure 4).

Type IV pili of Xcm, Xvv and Xsp strains

Here we show that bacterial strains of similar species can have unique Tfp. Primers specific to Xcm4381, amplified a unique product for Xcm2251 of size 1500 kb, whereas Xcm4383, Xcm4387, Xcm4389, Xcm4433, Xcm4434, amplified similar product except NCPPB1131, NCPPB1132 (Tfp-A). Tfp pilus assembly protein

Table 3. PCR-amplification of virulence genes in genomic DNA of 8 selected Xcm strains.

Code	Bacterial strains	Xcm (kb)	Xvv (kb)	Xcm 4383	Xcm 4387	Xcm 4389	Xcm 4433	Xcm 4434	Xcm 2251	Xcm 2005	Xcm 4392
	Proteins		**Host**	**Musa**	**Musa**	**Musa**	**Musa**	**Musa**	**Musa**	**Ensete**	**Musa**
LPS-M	Short chain dehydrogenase	NP	729	-	+	-	-	-	-	+	+
LPS-N	Putative transmembrane GtrA	NP	405	-	-	-	-	-	-	+	-
LPS-P	GDP-mannose 4,6-dehydratase	NP	969	-	-	-	-	-	-	+	-
Tfp-B	Tfp pilus assembly protein PilE	NP	381	-	-	-	-	-	-	+	-
Tfp-C	Tfp pilus assembly protein FimT	NP	474	-	-	-	-	-	-	-	-
XopAF	Type III secretion system effector protein	NP	657	-	-	-	-	-	-	-	-
LPS-A	ABC-transporter-permease	783	NP	+	+	+	+	+	+	+	+
LPS-B	SAM-dependent methyltransferases-WsaE	1656	NP	+	+	+	+	+	+	+	+
LPS-C	Truncated O-antigen biosynthesis protein	1701	NP	+	+	+	+	+	+	+	+
LPS-D	Hypothetical protein-ZP_06489485	1269	NP	-	+	+	+	+	+	+	+
LPS-K	Lipopolysaccharide biosynthesis protein	2838	2838	-	+	+	+	+	+	-	-
LPS-G	Indolepyruvate ferredoxin oxidoreductase	1302	1302	+	+	+	+	+	+	+	-
Yop1	Virulence factor yopJ-like 1	1077	NP	+	+	+	+	+	+	+	+
Yop2	Virulence factor yopJ-like 2	1068	NP	+	+	+	+	+	+	+	+
LPS-H	Indolepyruvate ferredoxin oxidoreductase	1302	1302	+	+	+	+	+	+	+	-
Tfp-A	Tfp pilus assembly protein PilE	426	NP	+	+	+	+	+	+	+	+
Tfp-F	Tfp pilus assembly protein PilV	411	NP	+	+	+	+	+	+	+	+
Tfp-G	Tfp pilus assembly protein PilW	975	NP	+	+	+	+	+	+	+	+
Tfp-H	Tfp pilus assembly protein PilX	480	NP	-	+	+	+	+	+	+	+
Tfp-K	Tfp pilus assembly protein PilY1	2151	NP	-	+	-	+	-	+	+	+
Tfp-D	Tfp_fimbrial biogenesis protein	534	NP	+	+	+	+	+	+	+	+
LPS-E	Predicted membrane protein	399	NP	+	+	+	+	+	+	+	+
LPS-F	NAD dependent epimerase	945	NP	-	+	+	+	+	+	+	+
Rip-T	Type III effector protein Type III effector Ript	720	NP	+	+	+	+	+	+	+	+
HopA	Type III effector HopAF1	861	860	+	+	+	+	+	+	+	+
HopW	Type III effector HopW1	1503	1503	-	+	+	+	+	+	+	+

NP, No amplification product expected; +, amplification product based on expected amplicon size; -, no amplification.

PilE specific to Xvv702 was amplified in Xcm2005, Xvv1326 and Xvv1381 DNA but tested negative in DNA of the other *Xanthomonas* test strains (Tfp-B). Tfp pilus assembly protein FimT was amplified in DNA of Xvv1326 and Xvv1381 but not Xvv206 nor any of the other strains (Tfp-C) (Figure 5).

Tfp pilus assembly protein PilF a fimbrial biogenesis protein was amplified in all *Xanthomonas* strains except NCPPB1131 only (Figure 6).

Tfp pilus assembly protein, PilV(Tfp-F) and PilW (Tfp-G) were not amplified in NCPPB1131/1132, Xvv1326 and Xvv1381 but amplified for the rest of the *Xanthomonas* strains under test (Figure 7).

DNA of Xcm4383, NCPPB1131 and NCPPB1132

Table 4. PCR-amplification of virulence genes in genomic DNA of 3 Xvv and 2 NCPPB strains.

Code	Bacterial strains	Xcm (kb)	Xvv (kb)	NCPPB 1131	NCPPB 1132	Xvv 206	Xvv 1326	Xvv 1381
	Proteins		**Host**	**Musa**	**Musa**	**Maize**	**S/cane**	**S/cane**
LPS-M	Short chain dehydrogenase	NP	729	-	-	+	+	+
LPS-N	Putative transmembrane GtrA	NP	405	-	-	+	+	+
LPS-P	GDP-mannose 4,6-dehydratase	NP	969	-	-	+	+	+
Tfp-B	Tfp pilus assembly protein PilE	NP	381	-	-	-	+	+
Tfp-C	Tfp pilus assembly protein FimT	NP	474	-	-	-	+	+
XopAF	Type III secretion system effector protein	NP	657	-	-	-	+	+
LPS-A	ABC-transporter-permease	783	NP	-	-	-	-	-
LPS-B	SAM-dependent methyltransferases-WsaE	1656	NP	-	-	-	-	-
LPS-C	Truncated O-antigen biosynthesis protein	1701	NP	-	-	-	-	-
LPS-D	Hypothetical protein-ZP_06489485	1269	NP	-	-	-	-	-
LPS-K	Lipopolysaccharide biosynthesis protein	2838	2838	-	-	-	-	-
LPS-G	Indolepyruvate ferredoxin oxidoreductase	1302	1302	-	-	-	-	-
Yop1	Virulence factor yopJ-like 1	1077	NP	-	-	-	-	-
Yop2	Virulence factor yopJ-like 2	1068	NP	-	-	-	-	-
LPS-H	Indolepyruvate ferredoxin oxidoreductase	1302	1302	-	-	+	+	-
Tfp-A	Tfp pilus assembly protein PilE	426	NP	-	-	+	+	-
Tfp-F	Tfp pilus assembly protein PilV	411	NP	-	-	+	-	-
Tfp-G	Tfp pilus assembly protein PilW	975	NP	-	-	+	-	-
Tfp-H	Tfp pilus assembly protein PilX	480	NP	-	-	+	+	+
Tfp-K	Tfp pilus assembly protein PilY1	2151	NP	-	-	+	-	-
Tfp-D	Tfp_fimbrial biogenesis protein	534	NP	-	+	+	+	+
LPS-E	Predicted membrane protein	399	NP	-	+	-	+	+
LPS-F	NAD dependent epimerase	945	NP	+	+	*	*	*
Rip-T	Type III effector protein Type III effector Ript	720	NP	-	-	+	+	+
HopA	Type III effector HopAF1	861	860	-	-	-	+	+
HopW	Type III effector HopW1	1503	1503	-	-	+	+	+

NP, No amplification product expected; +, amplification product based on expected amplicon size; -, no amplification: *amplification product of different size.

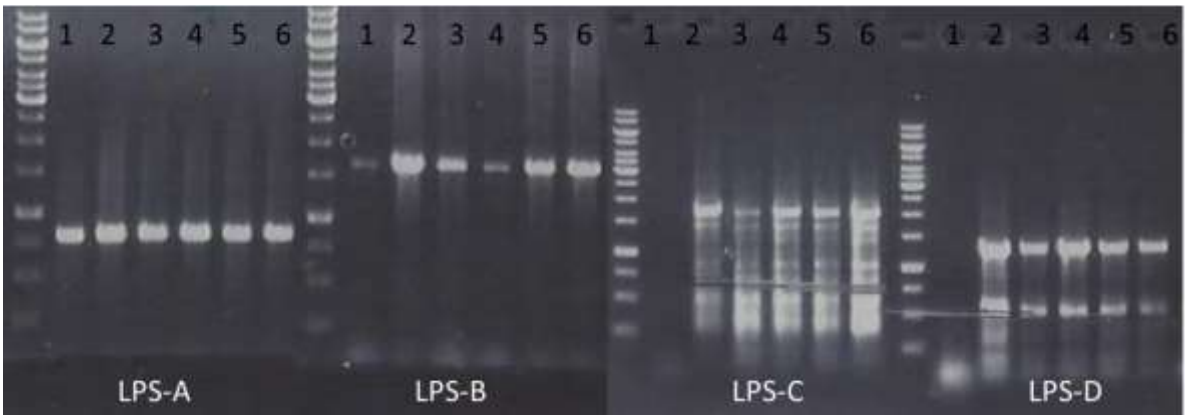

Figure 1. Hybridisation of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (Lane 4), Xcm2251 (lane 6) against 1000 bp ladder for primers of ABC transporter permease (LPS-A); SAM-dependent methyltransferases (LPS-B); truncated O-antigen biosynthesis protein (LPS-C) and hypothetical protein ZP_06489485(LPS-D).

did not amplify with Tfp pilus assembly protein PilX primers although the other strains had products

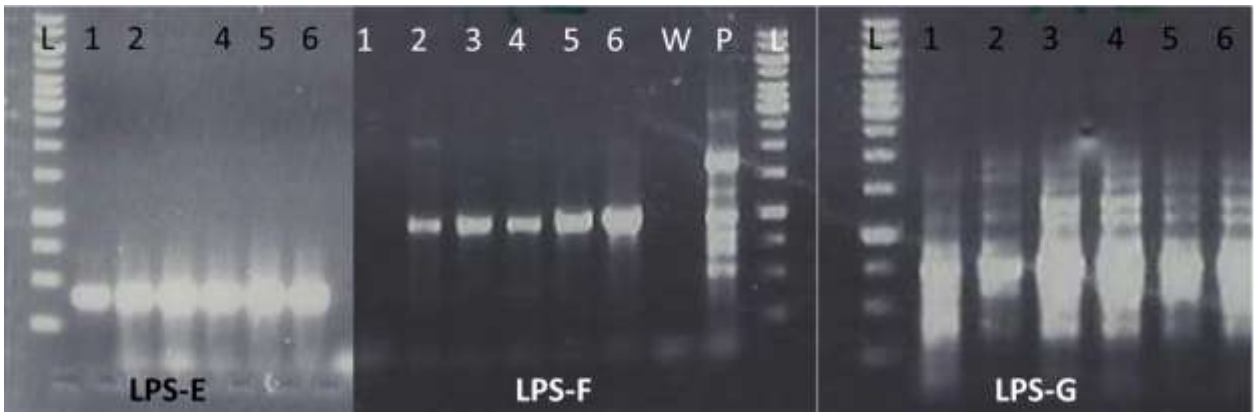

Figure 2. Hybridisation of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (lane 4), Xcm2251 (lane 6), W, Water; P, Arabidopsis DNA against 1000 bp ladder (L) for primers of predicted membrane protein (LPS-E); NAD, dependent epimerase (LPS-F) and ferredoxin oxidoreductase (LPS-G). Gel image for Xvv strains not presented.

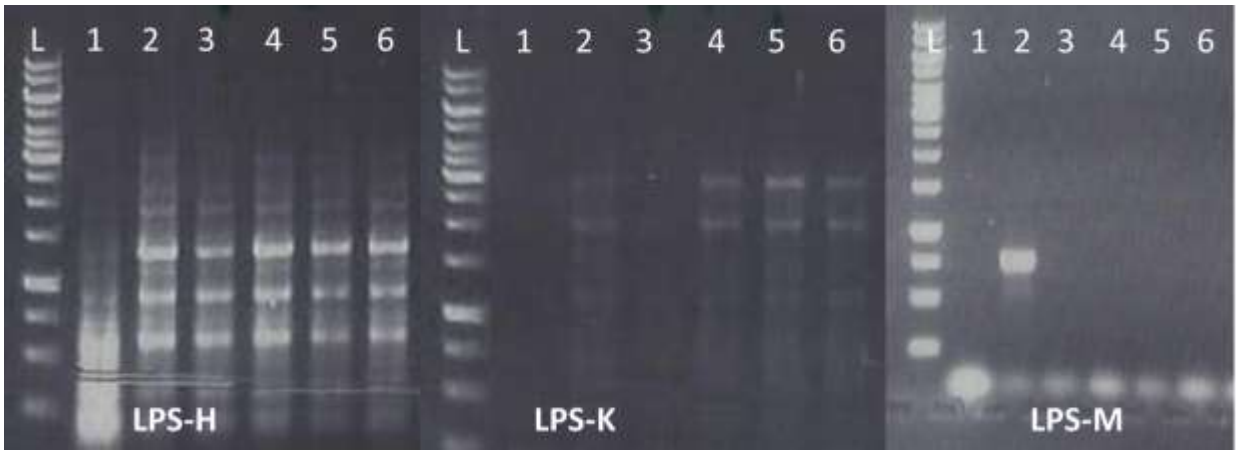

Figure 3. Amplification of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (lane 5), Xcm2251 (lane 6) against 1000 bp ladder (L) for primers of lipopolysaccharide biosynthesis protein (LPS-H); Ferredoxin oxidoreductase (LPS-F) and short chain dehydrogenase (LPS-G).

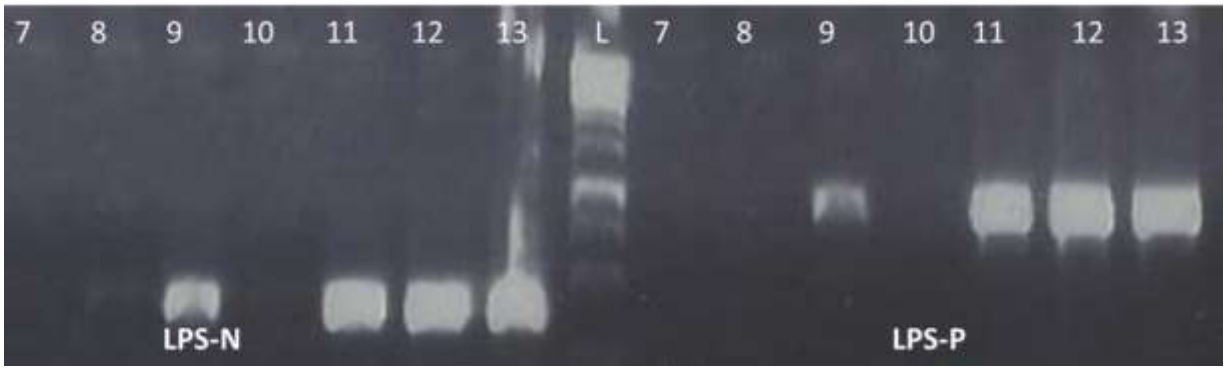

Figure 4. Hybridization of NCPPB1131(lane 7), NCPPB 1132(lane 8), Xcm2005(Lane 9), Xcm4392(Lane10), Xvv206(Lane 11), Xvv1326(Lane 12) and Xvv1381 (Lane 13) against 1000 bp ladder (L) for primers of putative transmembrane GtrA-like cell surface polysaccharide biosynthesis protein (LPS-N) and GDP-mannose 4,6-dehydratase (LPS-P).

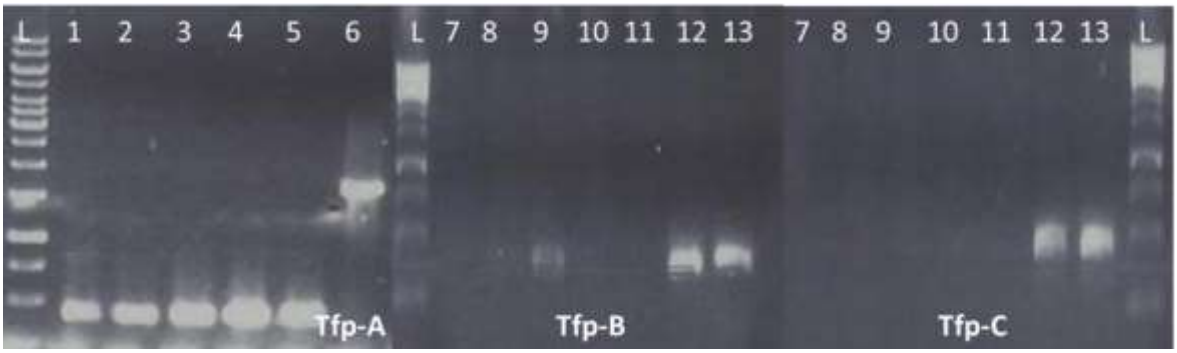

Figure 5. Hybridisation of Xcm4383 (lane 1), Xcm4387 (lane 2); Xcm4389 (lane 3); Xcm4433 (lane 4), Xcm4434 (lane 4), Xcm2251 (lane 6), NCPPB1131 (lane 7), NCPPB 1132(lane 8), Xcm2005 (lane 9), Xcm4392 (lane 10), Xvv206 (lane 11), Xvv1326 (lane 12) and Xvv1381 (lane 13) against 1000 bp ladder (L) for primers of Tfp pilus assembly protein PilE(Xcm) (Tfp-A); Tfp pilus assembly protein PilE(Xvv) (Tfp-B) and Tfp pilus assembly protein (Tfp-C).

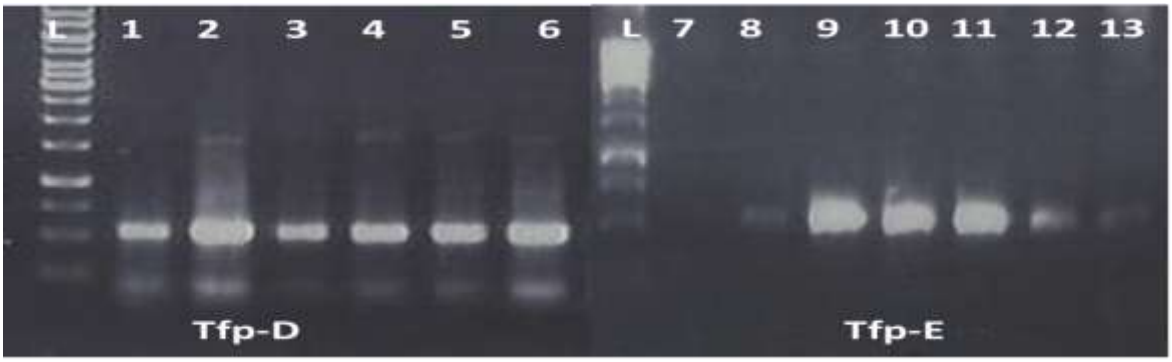

Figure 6. Hybridization of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (lane 4), Xcm2251 (lane 6), NCPPB1131(lane 7), NCPPB 1132 (lane 8), Xcm2005 (lane9), Xcm4392 (lane 10), Xvv206 (lane 11), Xvv1326 (lane 12) and Xvv1381 (lane 13) against 1000 bp ladder (L) for primers of Tfp fimbrial biogenesis protein (Xcm) (Tfp-D) and Tfp fimbrial biogenesis protein (Xvv) (Tfp-E).

as expected (Figure 8).

PilY1 a Tfp pilus assembly protein (tip-associated adhesion) was amplified in DNA of Xcm4387, Xcm4433, Xcm2251, Xcm2005, Xcm4392, and Xvv206, whereas no product was observed for strains Xcm4383, Xcm4389, Xcm4434, NCPPB1131, NCPPB1132, Xvv1326, and Xvv1381 (Figure 9).

Type three secretion system (TTSS) effectors

In this study we confirm that indeed there can be differences in the effectors across bacterial strains and within strains of the same species and other clusters conserved in gram negative bacteria. Type III effector protein RipT was confirmed to be present in all strains of Xcm and Xvv but not NCPPB1131 and NCPPB1132 (RipT). All the Xcm and Xvv strains under test yielded bands of type III effector HopAF1 except Xvv206, NCPPB1131 and NCPPB1132 (Figure 10).

Another type III effector HopW1 was amplified in all Xcm and Xvv strains tested except Xcm4383, NCPPB1131 and NCPPB1132 (Figure 11).

YopJ type III secretion system effector protein amplifies in DNA of all Xcm strains tested but not in NCPPB1131, NCPPB1132 and the Xvv strains tested (Figure 12). Xvv702, Xvv1326 and Xvv1381 strains yielded the only products for type III effector protein XopAF, with no amplification in any of the other *Xanthomonas* strains under test (results not presented).

Clustering of virulence factors

Cluster analysis of the presence or absence of virulence factors in bacterial strains separated the factors into two major clusters (Figure 13). The putative transmembrane GtrA, GDP-mannose 4,6 dehydratase, Tfp Pilus protein

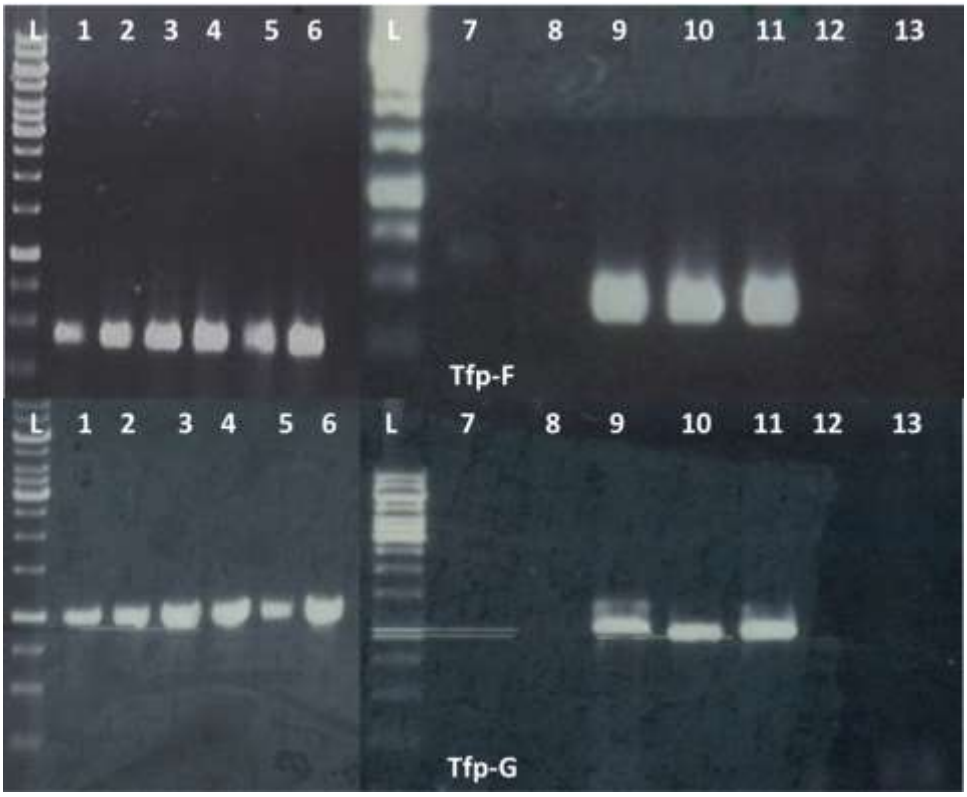

Figure 7. Hybridization of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (lane 4), Xcm2251 (lane 6), NCPPB1131(lane 7), NCPPB 1132(lane 8), Xcm2005 (lane 9), Xcm4392 (lane 10), Xvv206 (lane 11), Xvv1326 (lane 12) and Xvv1381 (lane 13) against 1000 bp ladder (L) for primers of Tfp pilus assembly protein PilV (Tfp-F) and Tfp pilus assembly protein PilW (Tfp-G).

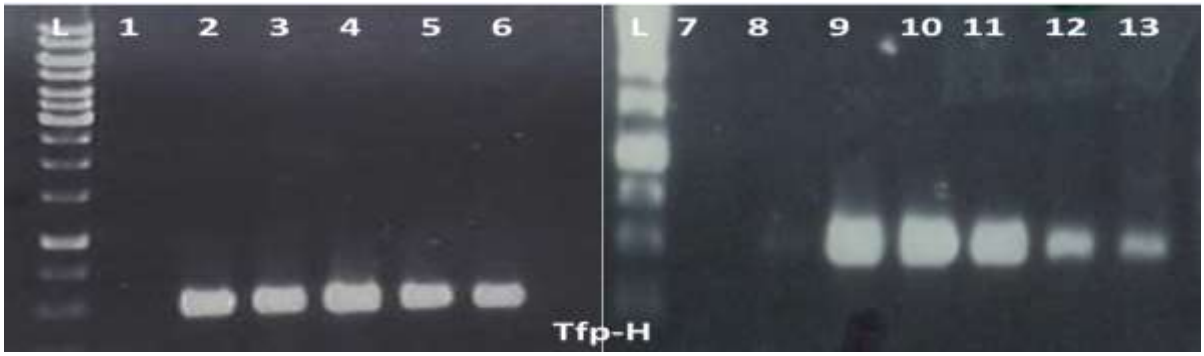

Figure 8. Amplification of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (lane4), Xcm2251 (lane 6), NCPPB1131(lane 7), NCPPB 1132 (lane 8), Xcm2005 (lane 9), Xcm4392 (lane 10), Xvv206 (lane 11), Xvv1326 (lane 12) and Xvv1381 (lane 13) against 1000 bp ladder (L) for primers of Tfp pilus assembly protein PilX (Tfp-H).

PilE, Tfp pillus assembly protein FimT, TTSS effector protein XopA and short chain dehydrogenase are in a unique cluster from the others assessed. These were mainly amplified in the Xvv strain and not in Xcm strains except for Xcm2005.

The cluster analysis further indicated that Xcm, Xvv strains and NCPPB did not cluster together (Figure 14). This is similar to observations made by Wasukira et al. (2012). Among the Xcm cluster strains Xcm4383 from Uganda clusters on its own within the major group,

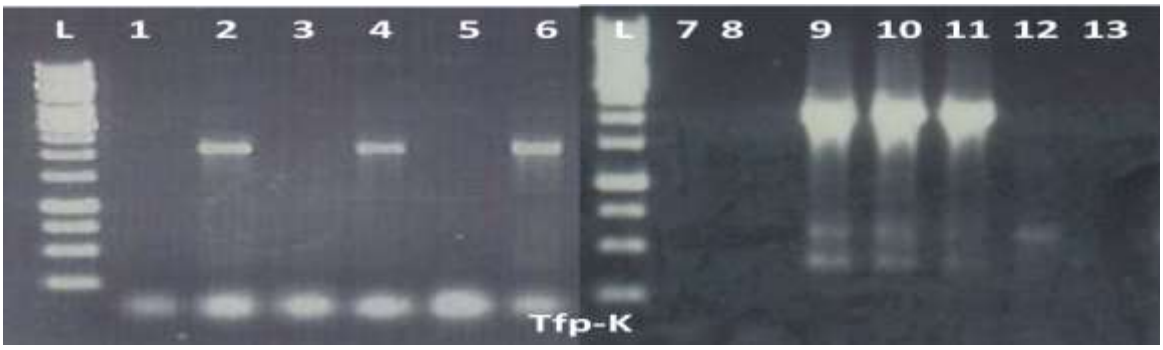

Figure 9. Amplification of Xcm4383 (lane 1), Xcm438 7(lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (lane 4), Xcm2251 (lane 6), NCPPB1131 (lane 7), NCPPB 1132 (lane 8), Xcm2005 (lane 9), Xcm4392 (lane 10), Xvv206 (lane 11), Xvv1326 (lane 12) and Xvv1381 (ane 13) against 1000 bp ladder (L) for primers of Tfp pilus assembly protein PilY1 (Tfp-K).

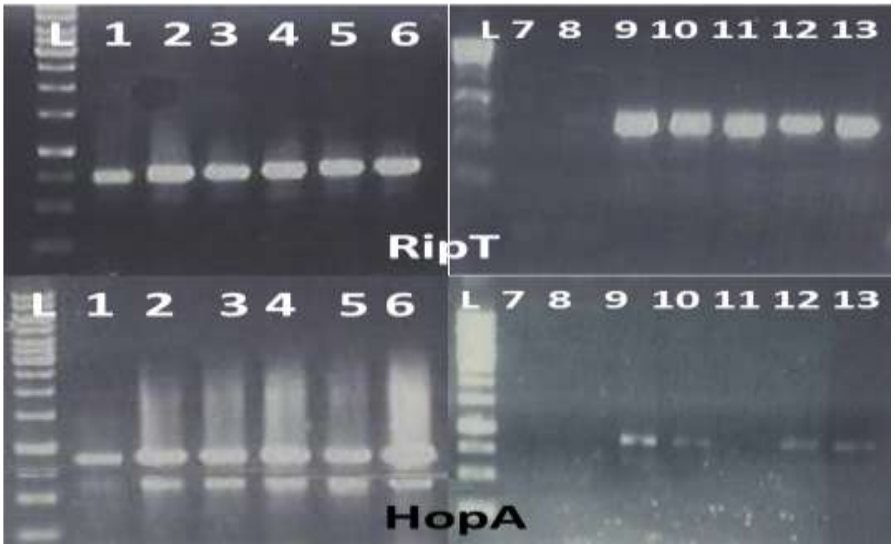

Figure 10. Amplification of Xcm4383 (lane 1), Xcm4387(lane 2), Xcm4389(Lane 3), Xcm4433(Lane 4), Xcm4434 (Lane 4), Xcm2251(Lane 6), NCPPB1131(lane 7), NCPPB 1132 (lane 8), Xcm2005 (lane 9), Xcm43924392 (lane 10), Xvv206(lane 11), Xvv1326 (lane 12) and Xvv1381 (lane 13) against 1000 bp ladder (L) for primers of type III effector protein ript (RipT) and type III effector HopAF1 (HopA).

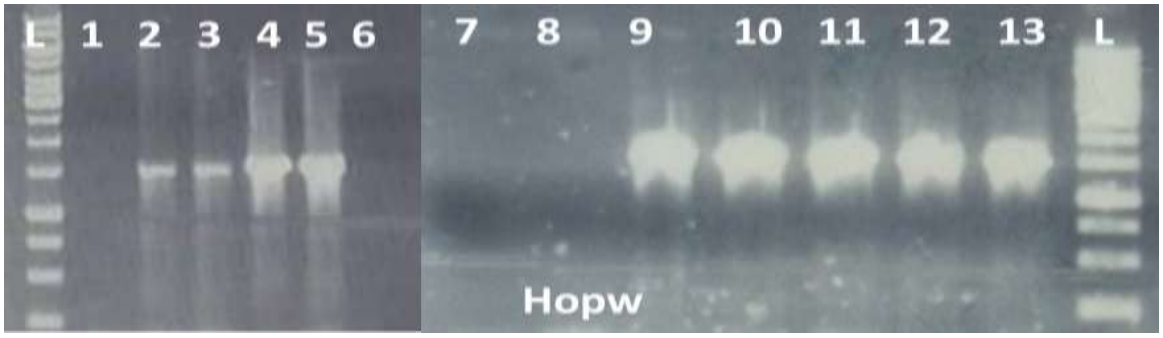

Figure 11. Amplification of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389 (lane 3), Xcm4433 (lane 4), Xcm4434 (lane 4), Xcm2251 (lane 6), NCPPB1131 (lane 7), NCPPB 1132 (lane 8), Xcm2005 (lane 9), Xcm4392 (lane 10), Xvv206 (lane11), Xvv1326 (lane12) and Xvv1381 (lane 13) against 1000 bp ladder (L) for primers of type III effector protein hopw1 (Hopw).

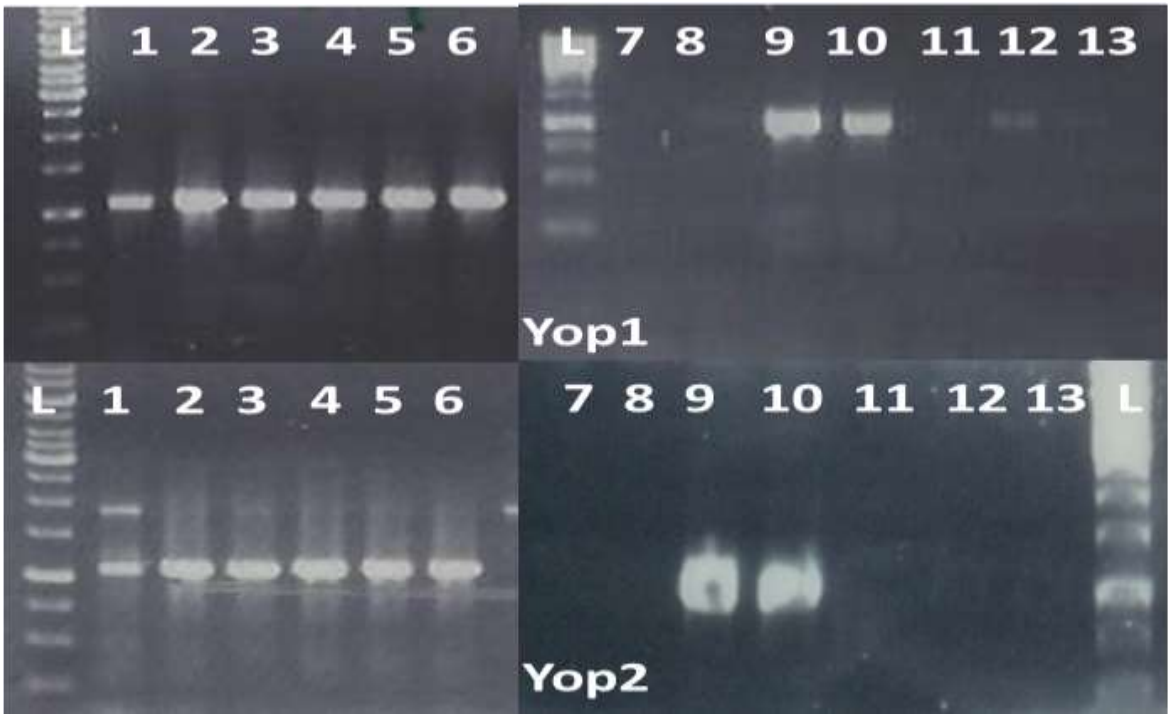

Figure 12. Amplification of Xcm4383 (lane 1), Xcm4387 (lane 2), Xcm4389(lane 3), Xcm4433 (lane 4), Xcm4434 (lane 4), Xcm2251 (lane 6), NCPPB1131 (lane7), NCPPB 1132 (lane 8), Xcm2005 (lane 9), Xcm4392Xcm4392 (lane 10), Xvv206 (lane 11), Xvv1326 (lane 12) and Xvv1381 (lane 13) against 1000 bp ladder (L) for primers of type III secretion system effector protein YopJ-like 1 (Yop1) and type III secretion system effector protein YopJ-like 2 (Yop2).

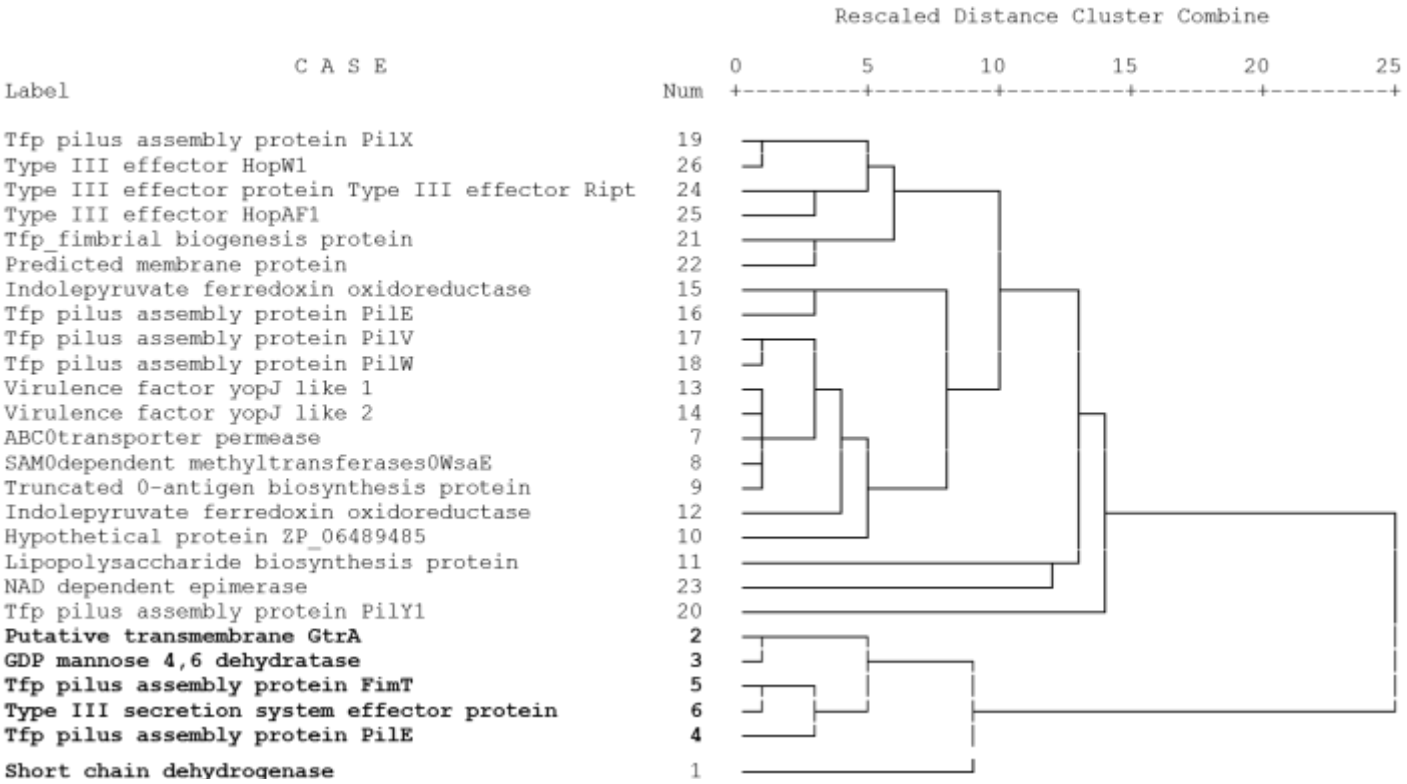

Figure 13. Dendogram showing linkage of virulence factors from amplification of 26 virulence factors against 13 *X. campestris* strains.

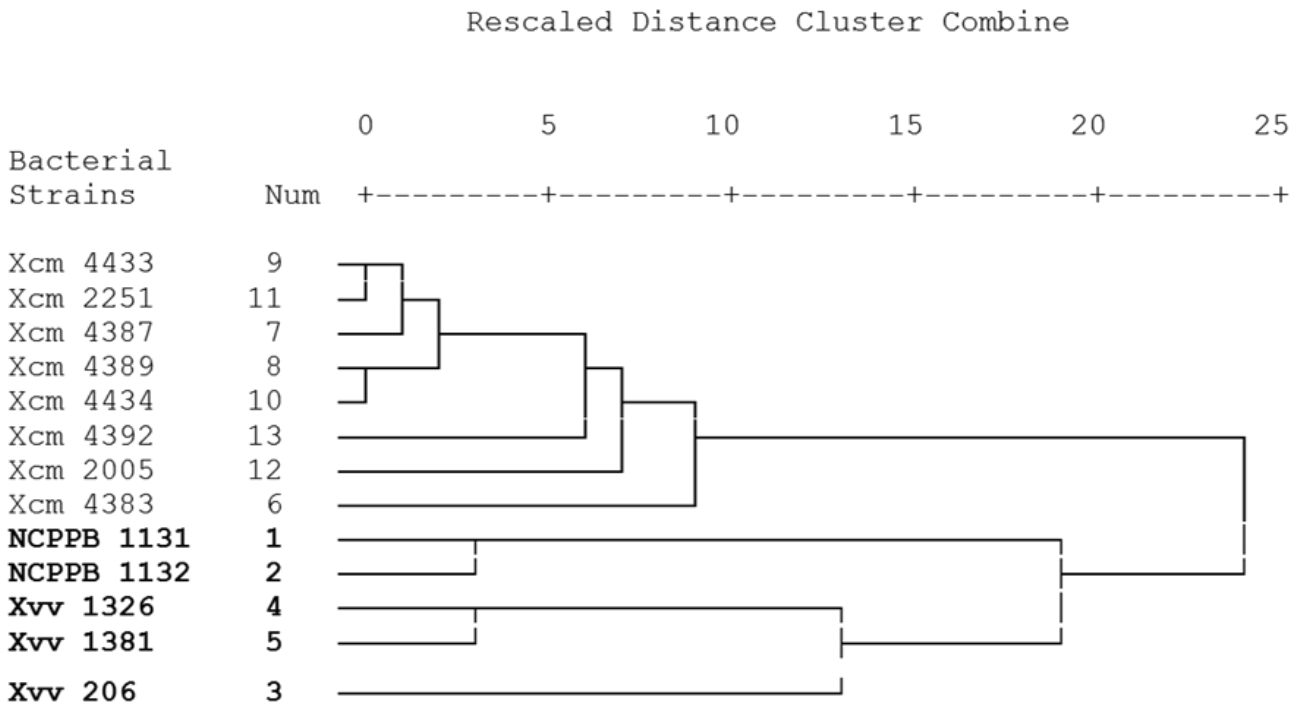

Figure 14. Dendogram showing clustering of *Xanthomonas* strains into 2 major clusters based on presence or absence (amplification) of 25 virulence factors against 13 *Xanthomonas* strains.

another unique clustering is Xcm2005 and Xcm4392 that are further from the other Xcm strains. However the NCPPB1131 and NCPPB1132 were generally grouped with the Xvv strains. NCPPB1131 and NCPPB1132 form a separate sub cluster while Xvv1326 and Xvv1381 sub cluster together and in this major cluster Xvv206 from maize is further apart from the others.

DISCUSSION

Type four pili in Xcm and Xvv

In this study we have shown that Xcm and Xvv strains under test differ in their Tfp composition as was earlier predicted by Studholme et al. (2010). We experimentally confirmed that the structural subunit PilE is present in all the strains of *Xanthomonas* under test however it was not detected in NCPPB1131 and NCPPB1132. These strains do not possess the TTSS and are not related to Xcm nor Xvv (Studholme et al., 2011). PilE were able to amplify a product in Xcm2005 (Enset), Xvv1326, Xvv1381 (sugar cane) and no product was observed for Xvv206(maize) thus indicating a possible loss in the gene locus. The ability of pathogenic bacteria to infect plants involves more than their ability to form the Hrp-TTSS and transfer virulence effectors. Qian et al. (2005) reported that infection is initiated with bacterial attachments to and colonization of host tissues via surface structures and appendages. Attachment of plant pathogenic bacteria to host specific surfaces/cells is necessary for colonization of host tissue and is mediated by surface-exposed adhesins, which generally behave as lectins, recognizing oligosaccharide residues of glycoprotein or glycolipid receptors on the host cell (Pizarro-Cerda and Cossart, 2006; Kline et al., 2009). Pili are implicated in crucial host-pathogen interactions, colonization, tropism determination, biofilm formation, and invasion and signaling events. Tfp is said to contribute to the optimal establishment, colonization, and spread of vascular bacteria pathogens via the plant xylem vessels. In *Xanthomonas*, type IV pili may be associated with the establishment of an aggregated bacterial population necessary to counteract the turbulent environment of the xylem, facilitating its adherence to the vessels in conjunction with other components, such as exopolysaccharides (Sluys et al., 2002). Tens of genes are involved in TFP synthesis and regulation, with the majority of them being generally named pil/fim genes. Moreira et al. (2004) reports of the presence and divergence of copies of pilE-fimT cluster in ancestors of xanthomonadaceae is consistent with the findings of this study where fimT was amplified in only the Xvv1326 and Xvv1381 strain from sugarcane. PilV is reported to be essential for pilus mediated cell adherence. The involvement of minor pilins (that is, PilE, PilV, PilW, PilX, FimT) in pilus assembly may play roles in priming of pilus extension or prevention of pilus retraction; in control of

pilus length; or in pilus-specific functions including adherence, transformation competence or motility (Dunger et al., 2014; Giltner et al., 2012). The putative type IV pilus protein PilY1 is likely important for attachment to surfaces. Moreira et al. (2004) reported a cluster pilE-pilY1-pilX-pilW-pilV-fimT which was common to both *Xylella fastidiosa* and *X. axonopodis pv. citri*. In this study it was experimentally confirmed that strains of the Xcm and Xvv may vary in clusters of Tfp. The roles of various Tfp between Xcm and Xvv should be further investigated for their interaction with the host and non-host(s).

Presence lipopolysaccharides in Xcm and Xvv strains

LPS are referred to as endotoxins and are known to play a wide range of roles during bacterial infection (Munford and Varley, 2006; Todar, 2014; Volk, 1966). LPSs share a common structure for all Gram-negative bacteria composed of a membrane-anchored phosphorylated and acylated 1–6-linked glucosamine (GlcN) disaccharide, named lipid A, to which a carbohydrate moiety of varying size is attached (Casabuono et al., 2011). The latter may be divided into a lipid A, proximal core oligosaccharide and a distal O-antigen, whose presence or absence determines the smooth or rough appearance of the bacterial colony. Studholme et al. (2010) had predicted that lipopolysaccharide locus in Xvv702 and Xcm4381 were not significantly similar which has been experimentally confirmed by this study. According to Wasukira et al. (2014), further genome sequencing did not reveal significant variation in this locus among isolates of Xvv. However, a predicted membrane protein was detected in strains of Xvv1326 and Xvv1381 but not Xvv206 which was isolated from maize unlike the former isolated from sugar cane. This was confirmed by Wasukira et al. (2014) who reported that the LPS structure of Xvv702 and Xvv206 were similar. LPSs apparently play diverse roles in bacterial pathogenesis of plants. As major components of the outer membrane, they are involved in the protection of bacterial cells, contributing to reduce the membrane permeability and thus allowing growth of bacteria in the unfavorable conditions of the plant environment. LPSs can be recognized by plants to elicit or potentiate plant defense-related responses (Casabuono et al., 2011; Desaki et al., 2006; Nam, 2001; White et al., 1996). It has also been inferred by Ssekiwoko et al. (2006) and Tripathi and Tripathi (2009) that *X. vasicola pv. musacearum* spread is limited to the xylem vessels and the wilting symptoms are as a consequence of large amounts of exopolysaccharides blocking water flow in the plants. One of the most widely studied effects of LPSs on plant cells is their ability to prevent the hypersensitive response (HR) induced in plants by avirulent bacteria (Mohammed, 2015; Newman et al., 2000). Newman et al. (2000) were able to show that LPS pre-treatment prevents the hypersensitive reaction caused by strains of *Xanthomonas campestris* pv. *vesicatoria* carrying the avirulence gene *avrBs1* (a gene-for-gene interaction) and by *X. campestris* pv. *campestris* (a non-host interaction). *X. axonopodis pv. citri* 306 shares 93% nucleotide sequence similarity with Xcm4381 (Studholme et al., 2010) and Casabuono et al. (2011) suggested that the O-antigen region of *X. axonopodis pv. citri* 306 LPS could be involved in the innate immunity of citrus. Horizontal gene transfer events (HGT) are frequently observed in genomic regions that encode functions involved in biosynthesis of the outer membrane located lipopolysaccharide (LPS). Also different strains of the same pathogen can have substantially different LPS biosynthetic gene clusters as has been shown for NAD dependent epimerase and lipopolysaccharide biosynthesis protein in this study. This can be attributed to the advantage in evading the host immune system since LPS is highly antigenic. Although LPS has been suggested as a potentiator of plant defense responses, interstrain variation at LPS biosynthetic gene clusters has not been reported for any plant pathogenic bacterium. Aritua et al. (2008), study found that although banana and sugarcane strains were of similar phylogenic group, their host ranges were different, since only the *X. campestris pv. musacearum* pathovars could cause disease in banana (Studholme et al., 2010; Wasukira et al., 2012). This is consistent with the observation that lipopolysaccharide (LPS) O antigen is pathovar/strain-specific and may be involved in host-range selection and pathogenicity by acting as a barrier against plant toxins (da Silva et al., 2002).

Presence of Type III secretion system effectors (TTSS)

TTSS is defined as a multi-subunit protein apparatus that is used to secrete or inject effector proteins which contribute to interactions with eukaryotic cells (Costa et al., 2015; Peeters et al., 2006). *Xanthomonas* species genomes possess 2 pathogenesis associated gene clusters that include hypersensitive response and pathogenicity (hrp) and the gum genes that encode synthases for extracellular polysaccharides xanthan. These pathogens show high level of host plant specificity inclusive of tissue specificity. They invade either the xylem elements of the vascular system or the intercellular spaces of the mesophyll. Differences in the complement of effectors between different *Xanthomonas* strains of the same pathovar may determine host specificity at the cultivar level. Effector recognition may also underpin the host range restriction of pathovars within a species and of the species themselves to particular plants (Buttner and He, 2009; Coburn et al., 2007). In this study we were able

to confirm that the genes RipT, HopAF1 and YopJ-like1 and YopJ-like2 were amplified within all Xcm strains under test. It was also observed that Xvv206, Xvv1326 and Xvv1381 did not yield any product for YopJ-like proteins. Homologs of the Yersinia virulence effector YopJ are found in both plant and animal bacterial pathogens. These YopJ family members act as cysteine proteases which is required for inhibition of the mitogen-activated protein kinase (MAPK) and nuclear factor kB (NF-kB) signaling for induction of localized cell death in plants (Orth et al., 2006). Although effector protein hopW1 and XopAF (AvrXv3) was amplified in Xvv1326 and Xvv1381, it was not detected in Xvv206. Jalan et al. (2013) in characterizing pathotypes of *X. citri* subsp. *Citri* showed that XopAF was present in Xcaw12879 but not XccA306 thus contributing to virulence in Xcaw. These observations are variable with predictions of Studholme et al. (2011) where NCPPB1131 was shown to be closely related to *X. saccharum* and also *X. albilineans.* However the strains lacked the Hrp TTSS of Xcm, and yet NCPPB1131 and NCPPB1132 possess a gum gene cluster similar to Xcm. This confirms the prediction by Studholme et al. (2010) who had indicated that genome of Xvv702 (sugar cane) encodes different protein compared to Xcm4381 (musa). Kvitko et al. (2009) proposed that unraveling functional redundancy among effectors can ease the study of individual effectors and elucidation of functional overlaps should help us understand how the various effectors in a repertoire may function as a system in hosts.

These results provide direction for functional studies on host specificity of plant pathogenic bacteria by using variable strains to study the role of single or combination of virulence factors/genes in the interaction with plants. Ali et al. (2013) recommend that comparative genomic analysis of intra-species by multiple strains is a modern trend for studying bacterial pathogens. These results will provide targets for insights into the molecular nature of virulence and host specificity; advance understanding of the dynamics responsible for banana *Xanthomonas* wilt epidemic development.

In conclusion the presence and or absence of virulence determinants is required to understand the mechanisms used by bacterial pathogens to establish infection. The construction of gene disruption mutants and their individual *in vivo* phenotype analysis is a common approach for the functional characterization of targeted genes. We used PCR-based screening to detect presence or absence of predicted virulence factors which is a fast and simple technique. This can be a first step towards characterization of predefined target genes from strains of *X. campestris pv. musacearum* and *X. vasicola pv. vasculorum*. This study has indicated as predicted that some of the predicted virulence factors are conserved within species whereas others can vary within species. The two specie strains also showed difference in the Tfp whose function is to aid in motility and attenuation to the host and thus tissue specificity. A management strategy for the disease can be based on genes which target the Tfp cluster. Lipopolysaccharides which act as PAMP are conserved within the Xcm and also vary with that found in Xvv a factor that explains the effect on host and non-host of each bacterium. Among the TTSS that were screened, XopAF was never amplified in Xcm strains, RipT, HopAF1 and YopJ-like1/2 were amplified in all Xcm. The correct identification of novel secreted effectors using protein sequence is a first step towards a more complete characterization of the complex pathogen-host interaction. This study has provided an indication of target virulence factors that can be used in further understanding the Xcm/Xvv interaction with the banana host especially targets for functional analysis of the virulence factors.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Ali A, Soares SC, Barbosa E, Santos AR, Barh D, Bakhtiar SM, David W (2013). Bacteriology & Parasitology Microbial Comparative Genomics : An Overview of Tools and Insights Into The Genus Corynebacterium. J. Bacteriol. Parasitol. 4(2):1.

Aritua V, Parkinson N, Thwaites R, Heeney JV, Jones DR, Tushemereirwe W, Smith J (2008). Characterization of the Xanthomonas sp . causing wilt of enset and banana and its proposed reclassification as a strain of X . vasicola. Plant Pathol. 57:170-177.

Baltrus DA, Nishimura MT, Romanchuk A, Chang JH, Mukhtar MS (2011). Dynamic Evolution of Pathogenicity Revealed by Sequencing and Comparative Genomics of 19 *Pseudomonas syringae* Isolates. PLoS Pathog. 7(7):e1002132.

Buttner D, He SY (2009). Type III Protein Secretion in Plant Pathogenic Bacteria. Plant Physiol. 150:1656-1664.

Casabuono A, Petrocelli S, Ottado J, Orellano EG, Couto AS (2011). Structural Analysis and Involvement in Plant Innate Immunity of *Xanthomonas axonopodis* pv. *citri* Lipopolysaccharide. J. Biol. Chem. 286:25628-25643.

Coburn B, Sekirov I, Finlay BB (2007). Type III secretion systems and disease. Clin. Microbiol. Rev. 20(4):535-549.

Costa TRD, Felisberto-rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G (2015). Secretion systems in Gram-negative insights. Nat. Pub. Group 13(6):343-359.

da Silva ACR, Ferro JA, Reinach FC, Farah CS, Furlan LR, Quaggio RB, Monteiro-Vitorello CB, Van Sluys MA, Almeida NF, Alves LMC, do Amaral AM, Bertolini MC, Camargo LEA, Camarotte G, Cannavan F, Cardozo J, Chambergo F, Ciapina LP, Cicarelli RMB, Coutinho LL, Cursino-Santos JR, El-Dorry H, Faria JB, Ferreira AJS, Ferreira RCC, Ferro MIT, Formighieri EF, Franco MC, Greggio CC, Gruber A,

Katsuyama AM, Kishi LT, Leite RP, Lemos EGM, Lemos MVF, Locali EC, Machado MA, Madeira AMBN, Martinez-Rossi NM, Martins EC, Meidanis J, Menck CFM, Miyaki CY, Moon DH, Moreira LM, Novo MTM, Okura VK, Oliveira MC, Oliveira VR, Pereira HA, Rossi A, Sena JAD, Silva C, de Souza RF, Spinola LAF, Takita M A, Tamura RE, Teixeira EC, Tezza RID, Trindade dos Santos M, Truffi D, Tsai SM, White FF, Setubal JC, Kitajima JP (2002). Comparison of the genomes of two Xanthomonas pathogens with differing host specificities. Nature 417(6887):459-463.

Desaki Y, Miya A, Venkatesh B, Tsuyumu S, Yamane H, Kaku H, Minami E, Shibuya N (2006). Bacterial Lipopolysaccharides Induce Defense Responses Associated with Programmed Cell Death in Rice Cells. Pla. Cell Physiol. 47(11):1530-1540.

Dunger G, Guzzo CR, Andrade MO, Jones JB, Farah CS (2014). *Xanthomonas citri* subsp. *citri* Type IV Pilus Is Required for Twitching Motility, Biofilm Development, and Adherence. Mol. Plant Microbe Interact. 27(10):1132-1147.

Giltner CL, Nguyen Y, Burrows LL (2012). Type IV pilin proteins: Versatile molecular modules. Microbiol. Mol. Biol. Rev. 76(4):740-772.

Jalan N, Kumar D, Andrade MO Yu F, Jones JB, Graham JH., White FF, Setubal JC, Wang N (2013). Comparative genomic and transcriptome analyses of pathotypes of *Xanthomonas citri* subsp . *citri* provide insights into mechanisms of bacterial virulence and host range. BMC Genomics 14(1):551.

Koressaar T, Remm M (2007). Enhancements and modifications of primer design program Primer3. Bioinformatics 23(10):1289-1291.

Kubiriba J, Karamura EB, Tushemereirwe WK, Tinzaara W (2012). Community mobilization : A key to effective control of banana xanthomonas wilt. J. Dev. Agric. Econ. 4(5):125-131.

Kvitko BH, Park DH, Velásquez AC, Wei CF, Russell AB, Martin GB, Schneider DJ, Collmer A (2009). Deletions in the repertoire of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion effector genes reveal functional overlap among effectors. PLoS Pathog. 5(4):e1000388.

Mahuku GS (2004). A simple extraction method suitable for PCR based analysis of plant, fungal, and bacterial DNA. Plant Mol. Biol. Rep. 22(1):71-81.

Mhedbi-Hajri N, Darrasse A, Pigné S, Durand K, Fouteau S, Barbe V, Manceau C, Lemaire C, Jacques MA (2011). Sensing and adhesion are adaptive functions in the plant pathogenic xanthomonads. BMC Evol. Biol. 11(1):67.

Mohammed A (2015). Importance and Characterization of Coffee Berry Disease (*Colletotrichum kahawae*) in Borena and Guji Zones, Southern Ethiopia. J. Plant Pathol. Microbiol. 6(9):1-6.

Munford RS, Varley AW (2006). Shield as Signal: Lipopolysaccharides and the Evolution of Immunity to Gram-Negative Bacteria. PLoS Pathog. 2(6):e67.

Nam J (2001). New Aspects of Gene-for-Gene Interactions for Disease Resistance in Plant. J. Plant Pathol. Microbiol. 17(2):83-87.

Newman MA, von Roepenack E, Daniels M, Dow M (2000). Lipopolysaccharides and plant responses to phytopathogenic bacteria. Mol. Plant Pathol. 1(1):25-31.

Orth K, Xu Z, Mudgett MB, Bao ZQ, Palmer LE, Bliska JB, Mangel WF, Peeters N, Lechner E, Vailleau F, Baud C, Gentzbittel L, Sartorel E, Genschik P, Boucher C (2006). Ralstonia solanacearum requires F-box-like domain-containing type III effectors to promote disease on several host plants. PNAS 103:14620-14625.

Orth K, Xu Z, Mudgett M B, Bao Z Q, Palmer E L, Bliska J B, Mangel F W, Staskawicz B, Dixon E J. (2000). Homologs Disruption of Signaling by Yersinia Effector YopJ, a Ubiquitin-Like Protein Protease. Science, 290;1594-1597.

Pizarro-Cerda J, Cossart P (2006). Bacterial Adhesion and Entry into Host Cells. Cell 124:715-727.

Qian W, Jia Y, Ren S, He Y, Feng J, Lu L, Sun Q, Ying G, Tang D, Jie T, Hua W, Wei H, Pei W, Lifeng J, Bo L, Zeng S, Gu W, Yi L, Gang R, Li T, Yingchuan Y, Zhijian F, Gang C, Baoshan F, Rongxiang Q, Boqin C, Zhu Z, Guo PT, Ji L, He C (2005). Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv . *campestris*. Genome Res. 15(6):757-767.

Sluys MA, Camargo LEA, Menck CFM, Silva ACR, Ferro JA, Oliveira MC, Setubal JC, Kitajima JP, Simpson AJ (2002). Comparative Genomic Analysis of Plant Associated Bacteria. Ann. Rev. Phytopathol. 40:169-189.

Ssekiwoko F, Tushemereirwe WK, Batte M, Ragama P, Komakech A (2006). Reaction of Banana Germplasm to innoculation with *Xanthomonas campestris* pv. *musacearum*. Afr. Crop Sci. J. 14(2):151-155.

Studholme DJ, Kemen E, Maclean D, Schornack S, Aritua V, Thwaites R, Grant M, Smith J, Jones JDG (2010). Genome-wide sequencing data reveals virulence factors implicated in banana Xanthomonas wilt. FEMS Microbiol. Lett. 310:182-192.

Studholme DJ, Wasukira A, Paszkiewicz K, Aritua V (2011). Draft Genome Sequences of *Xanthomonas sacchari* and Two Banana-Associated Xanthomonads Reveal Insights into the Xanthomonas Group 1 Clade. Genes 2(4):1050-1065.

Todar K (2014). Colonization and Invasion by Bacterial Pathogens. Text Book of Bacteriology. pp. 3-5.

Tripathi L, Mwaka H, Tripathi JN, Tushemereirwe WK (2010). Expression of sweet pepper Hrap gene in banana enhances resistance to *Xanthomonas campestris* pv. *musacearum*. Mol. Plant Pathol. 11(6):721-731.

Tripathi L, Tripathi JN (2009). Relative susceptibility of banana cultivars to *Xanthomonas campestris* pv. *musacearum*. Afr. J. Biotechnol. 8(20):5343-5350.

Tushemereirwe WK, Kangire A, Kubiriba J, Maureen N, Gold SC (2004). Diseases threatening banana biodiversity in Uganda. Afr. Crop. Sci. J. 12(1):19-26.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012). Primer3-new capabilities and interfaces. Nucleic Acids Res. 40(15):1-12.

Volk W (1966). Cell wall lipopolysaccharides from xanthomonas species. J. Bacteriol. 91(1):39-42.

Wasukira A, Coulter M, Al-Sowayeh N, Thwaites R, Paszkiewicz K, Kubiriba J, Smith J, Grant M, Studholme DJ (2014). Genome Sequencing of *Xanthomonas vasicola* pv. *vasculorum* Reveals Variation in Plasmids and Genes Encoding Lipopolysaccharide Synthesis, Type-IV Pilus and Type-III Secretion Effectors. Pathogens 3(1):211-237.

Wasukira A, Tayebwa J, Thwaites R, Paszkiewicz K, Aritua V, Kubiriba J, Smith J, Grant M, Studholme DJ (2012). Genome-wide sequencing reveals two major sub-lineages in the genetically monomorphic pathogen *Xanthomonas campestris* pv. *musacearum*. Genes 3(3):361-377.

White FF, Chittoor JM, Leach JE, Young SA, Zhu W (1996). Molecular analysis of the interaction between *Xanthomonas oryzae* pv . *oryzae* and rice. In Rice genetics III. Proceedings of the Third International Rice Genetics Symposium, 16-20 Oct 1995. Manila (Philippines): IRRI. pp. 255-266.

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A study on metallo-β-lactamases producing *Pseudomonas aeruginosa* in water samples from various parts of Malaysia

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Pseudomonas aeruginosa **is a Gram negative aerobic rod shaped bacterium and is an opportunistic pathogen that usually causes nosocomial infection in immunocompromised patient with various infections and affects normal healthy human as well.** ***P. aeruginosa*** **is also an omnipresent pathogen that can be inhabited in soil, water, vegetable, human and animal. Metallo-β-lactamases (MBL) producing** ***P. aeruginosa*** **are known to be resistant to almost the entire anti-pseudomonas agent via mechanism of low outer membrane permeability, β-lactamases synthesis and the efflux systems. This study was conducted to detect the potential of metallo-β-lactamases producing** ***P. aeruginosa*** **presence in water samples from various parts of Malaysia. In this study, 52 water samples were collected from various parts of Malaysia. These** ***P. aeruginosa*** **isolates were processed to these phenotypic methods, Hodge test which is used to detect the carbapenemase production, Imipenem- EDTA combined disc test (CDT). Imipenem-EDTA double disc synergy test (DDST) was used to determine metallo-beta-lactamases producing** ***P. aeruginosa*****. Among 52 various sources of water samples, 13 water samples had positive** ***P. aeruginosa*** **isolates. 6 out of 13 positive isolates have shown positive results in Hodge test, CDT and DDST. All metallo-β-lactamases producing isolates are multi-drug resistant. Among water samples from various parts of Malaysia, MBL producing** ***P. aeruginosa*** **are highly found in Selangor followed by KL, and this** ***P. aeruginosa*** **are detected from drain followed by river. Although some advantages and disadvantages exist among these phenotyping methods, still CDT and DDST are optimal to detect MBL.**

Key words: Metallo-β-lactamases, multi-drug resistance, phenotyping method, *Pseudomonas aeruginosa.*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that usually causes nosocomial infection in immuno-compromised patient with severe infections, such as cystic fibrosis, cancer, burn, urinary tract infection. They

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can also affect normal healthy human, which might cause dermatitis conjunctivitis, otitis externa and gastrointestinal infection (Hallin et al., 2012; Adesoji et al., 2015). *P. aeruginosa* is a highly adaptable bacterium, can survive in a wide range of environment and change its properties in response to changes in the environment (Lambert, 2002). The outbreaks of *P. aeruginosa* that are associated with different water sources such as drains, lakes and wastewater had been reported by Quick et al. (2014).

Antibiotic-resistant bacterial can be found in water sources from hospital, industrial or domestic wastes into watercourses (Chartier et al., 2014). It is due to contribution of the un-metabolized antibiotics in the water, which is excreted from humans. It may be a potential risk causing problem to human health (Email et al., 2010). Hospital wastewaters contain antimicrobial resistant bacterial with a level of at least a factor of 2 to 10 times higher than in domestic wastewater. Antimicrobial resistant bacterial gene transfer is great at high cell densities and under high antibiotic concentrations (Chartier et al., 2014). The effluents of the industries also act as one of influence on the pollution of the water bodies; these effluents can change the physical, chemical and biological nature of the water body (Hussain and Rao, 2013).

The characteristic features of the climate in Malaysia are uniform temperature, high humidity and ample rainfall. Malaysia mainly has 2 seasons, which are southwest monsoon season and northeast monsoon season (General Climate of Malaysia, 2016). November, December and January are the months with maximum rainfall, in the east coast while June and July are the driest months in most districts. Over the rest of the Peninsula with the exception of the southwest coastal area, the monthly rainfall pattern shows two periods of maximum rainfall separated by two periods of minimum rainfall (Chen et al., 2013; General Climate of Malaysia, 2016). Altering climatic conditions can change the volume and quality of water availability in both time and space, influencing the water usage practices. For an example, intensity of rainfall, or the period of time without rain will affect the quality of water in rivers and lakes through alteration in the timing and volume and temperature. Besides that, floods also have a probability of spreading diseases by over following the open sewage or inadequate sewage infrastructure (Corcoran et al., 2010).

Phenotyping methods are based on the ability of metal chelators, such as EDTA and thiol-based compounds, inhibit the activity of MBL (Manoharan et al., 2010). Double Disk Synergy Test (DDST) and Combined Disc Test (CDT) are most commonly used to detect the MBL producing *P. aeruginosa* then Hodge test. Modified Hodge test detects only carbapenemase activity, which prevents the use of EDTA and therefore, does not confirm the metal dependence of the carbapenemase (Kali et al., 2013). Modified Hodge test, DDST and the CDT are easy, reliable, simple to perform and cheaper (Qu et al., 2009). The CDT is the most sensitive techniques for detecting MBL compared to DDST (Biradar and Roopa, 2015). DDST is used to distinguish MBL producing gram negative bacilli from MBL non producing gram negative bacilli. For those MBL producing *P. aeruginosa* isolates positive by DDST, are also positive by CDT for MBL production (Bhalerao et al., 2010).

Most of the studies were concentrated on the isolation of β- lactamase gene from clinical samples (Kumar et al., 2012; Upadhyay and Joshi, 2015). Therefore, the aim of present study is to detect the potentiality of MBL producing *P. aeruginosa* in water samples from various parts of Malaysia. The objective of this study is to determine the presence of *P. aeruginosa* from water source, to evaluate the MBL producing *P. aeruginosa* by using modified Hodge test, double disk synergy test (DDST) and combined disk test (CD).

MATERIALS AND METHODS

Bacteria isolation

Fifty-two water samples of different water source were collected from various parts of Malaysia. Each water sample was then diluted by using serial dilution method, 10^{-1} to 10^{-5}. The diluted sample was inoculated on the Nutrient agar (NA), Cetrimide Nalidixic Acid agar (CA), Blood agar (BA) and MacConkey agar (MAC). The inoculated agar was incubated at 37˚C for 16 to 24 h Ce´ line (Slekovec et al., 2012; Pellegrini et al., 2009).

Characterization and identification

The colonies on the Nutrient agar, Cetrimide Nalidixic Acid agar, Blood agar and MacConkey agar were observed for morphological characteristics and were processed for various identification tests (Nasreen et al., 2015) such as, gram staining to distinguish gram positive bacteria from gram negative bacteria; oxidase test to differentiate pseudomonas from enterobacteriaceae, and biochemical tests (Indole, Methyl red, Voges-Proskauer, citrate, urease and triple sugar iron) to identify the species of bacteria (James and Natalie, 2007).

Antibiotic susceptibility testing

Antibiotic susceptibility test was carried out on *P. aeruginosa* isolates by using the disc diffusion and Kirby-Bauer methods, in accordance to the Clinical and Laboratory Standards Institute (CLSI, 2011) guidelines (Bashir et al., 2011).The identical colonies in bacterial culture from nutrient agar were inoculated into nutrient broth and were incubated at 37˚C for 3 to 4 h. The inoculum density was adjusted to a MacFarland 0.5 standard and it was inoculated on the Mueller-Hinton agar plate using a lawn culture technique. The inoculated plate was allowed to dry (Nasreen et al., 2015).

The following antibiotics were placed on the inoculated agar by disc diffusion method, Ciprofloxacin (5 µg), Gentamicin (10 µg), Amikacin (30 µg), Co-trimoxazole (25 µg), Tetracyline (30 µg), and Cefuroxime (30 µg). The plates were incubated at 37°C for 16 to 18 h. The zone of inhibition around the disk were measured and categorized into susceptible, intermediate and resistant.

Hodge test

The Hodge test was performed by preparing a 0.5 McFarland dilution of *E.coli* ATCC 25922 in 5 ml of nutrient broth. The diluted *E. coli* indicator organism was streaked on the Mueller Hinton agar using lawn culture technique and allowed to dry for 3 to 5 min. The test organism in a straight line from edge of disk to the edge of the plate and placed a carbapenem disc. The carbapenem disc is Imipenem (10 µg) or Meropenem (10 µg) placed at the center of the plate. The plates were incubated at 37°C for 16 to 24 h (Chaudhari et al., 2011; Georgios et al., 2014).

Imipenem-EDTA double- disc synergy test (DDST)

DDST was performed in accordance to the CLSI recommendations for the disk diffusion method. 0.5 McFarland of test organism was streaked on the Mueller Hinton agar by using sterile cotton swab to get a lawn culture. Placed imipenem (10 ug) disc in 20 mm center to center from a blank disc contained 10 µl of 0.5 M EDTA (750 ug) or EDTA disc (750 ug). The plate was incubated at 37°C for 16 to 18 h (Bhalerao et al., 2010; Chaudhari et al., 2011; Kali et al., 2013).

Imipenem-EDTA combined disc test (CDT)

0.5 McFarland of test organism was streaked on Mueller Hinton agar by using sterile cotton swab to get a lawn culture and was allowed to dry for 3 to 5 min. Placed 2 imipenem (10 ug) disc on the agar at distance of 25 mm. 10 µl of EDTA solution was added to one of them to obtain desire concentration of 750 µg. Then the plates were incubated at 37°C for 16 to 18 h (Arunagiri et al., 2012; Kali et al., 2013).

RESULTS

Bacteria isolation and identification

In this study, among 52 water samples, 20 water samples from Selangor, 15 water samples from Kuala Lumpur, 9 water samples from Penang, 5 water samples from Melaka and 3 water samples from Kedah with different sources were collected. Out of 20 water samples from Selangor, 16 water samples were gram negative bacilli GNB which is demonstrated by gram staining. 4 water samples contained gram positive bacteria GPB. In 16 GNB, 6 showed positive in selective agars, which produced yellow greenish color fluorescent pigment in CA (Figure 1), beta hemolysis and greenish in color in BA (Figure 2), non-lactose fermented in MAC, which indicates *Pseudomonas* spp. (Figure 3). The remaining 10 water samples with GNB had no growth on CA, which indicates these GNB are not *Pseudomonas* species. In oxidase test, 8 out of 16 GNB showed positive result and the remaining 8 GNB showed negative results. The water samples with GNB were processed to biochemical test to identify the species of bacteria. Out of 16 GNB, 6 indicated *P. aeruginosa*, which showed negative results in Indole, Methyl red and Voges-prokauer. Positive results in Citrate and urease, motile in Motility test and Triple Sugar Iron test showed no gas and hydrogen sulphide H_2S production, the remaining 10 indicated

Figure 1. Shown yellow greenish colour fluorescent pigment in CA agar.

Figure 2. Beta haemolysis and greenish colour in BA.

Figure 3. Non-fermented in MAC agar.

Table 1. Isolation rates of *Pseudomonas aeruginosa* from water sample in different state of Malaysia.

State	No. of sample examined (n=52)	No. of positive isolates (%)
Selangor	20	6 (46.1%)
Kuala Lumpur	15	5 (38.4%)
Penang	9	1 (7.6%)
Kedah	3	0
Melaka	5	1 (7.6%)

other than *P. aeruginosa* (Table 1).

In 15 water samples from Kuala Lumpur, 12 contained GNB 3 contained GPB. Among 12 water samples with GNB, 7 had *Pseudomonas* spp. grown in CA, BA, and MAC. The remaining 5 water samples with GNB had no growth on CA, which indicates these gram negative bacilli are not *Pseudomonas* spp. In oxidase test, 6 out of 12 GNB showed positive result, the remaining 6 GNB showed negative result In biochemical tests, out of 12 GNB, 7 indicated *P. aeruginosa* and the remaining 5 indicated other than *P. aeruginosa*.

Out of 9 water samples from Pinang, 6 had GNB, 3 had GPB. Out of 6 water samples with GNB, One had *Pseudomonas* spp. grown on CA, BA, and MAC. The remaining 5 water samples with GNB were not grown on CA, which indicates these GNB are not *Pseudomonas* spp. In oxidase test, 2 out of 6 GNB showed positive result, the remaining 4 GNB showed negative result. In biochemical tests, out of 6 GNB, 1 indicates *P. aeruginosa* whereas the remaining 5 indicates other than *P. aeruginosa.*

Among 3 water samples from Kedah, 3 were GNB, but none were grown on CA, which indicates these GNB are not *Pseudomonas* spp. In oxidase test, 1 out of 3 GNB showed positive result, another 2 GNB showed negative result. In biochemical tests, all GNB indicates other than *P. aeruginosa.*

Lastly, among 5 water samples from Melaka, all of 5 contained GNB. In these 5 GNB, 1of *Pseudomonas* spp. was grown in CA (Figure 1) BA (Figure 2) and MAC (Figure 3). The remaining 4 GNB were not grown on CA, which indicates these GNB are not *Pseudomonas* spp. In oxidase test, 2 out of 5 GNB showed positive result, the remaining 3 GNB showed negative results. In biochemical tests, of 5 GNB, 1 was indicated *P. aeruginosa*, the other 4 showed other than *P. aeruginosa.*

Thus, among 52 water samples, 13 water samples were found positive for *P. aeruginosa* isolates. The rates of *P. aeruginosa isolated* from 13 water samples in different states of Malaysia showed 6 out of 20 water samples from Selangor were positive *P. aeruginosa* isolates, which was 46.1%. 5 out of 15 water samples from Kuala Lumpur were positive *P. aeruginosa* isolates which was 38.4 %, while 1 out of 9 water samples from Penang was positive *P. aeruginosa* isolates, which was 7.6%. 1 out of 5 water samples from Melaka was positive *P. aeruginosa*, which was 7.6% and lastly Kedah had not showed any positive *P. aeruginosa* isolates among 3 water samples.

Antibiotic susceptibility testing

The antibiotic susceptibilities of the *P. aeruginosa* isolated in different states of Malaysia were as shown from Tables 2 to 5 and Figures 8 and 9. Out of 6 *P. aeruginosa* isolated from Selangor, number 7 *P. aeruginosa* isolate was highly susceptible to Gentamicin (GEN), Amikacin (AK) and Ciprofloxacin (CIP), Intermediate to Co-trimoxazole (COT), resistant to Tetracycline (TE) and Cefuroxime (CXM). Number 11 and 14 *P. aeruginosa* isolates were highly susceptible to Gentamicin, Amikacin and Ciprofloxacin, but resistance to Co-trimoxazole, Tetracycline and Cefuroxime. Whereas number 16, 18 and 19 *P. aeruginosa* isolates were resistant the entire antimicrobial disc test (Table 2b; Figure 4c and d). Of the 5 *P. aeruginosa* isolated from Kuala Lumpur number 4 and 7 *P. aeruginosa* isolates were highly susceptible to Gentamicin, Amikacin and Ciprofloxacin, resistant to Co-trimoxazole, Tetracycline and Cefuroxime (Figure 4a and b). Number 13 were intermediate to Gentamicin, susceptible to Amikacin and Ciprofloxacin. Number 5 and 14 *P. aeruginosa* isolates were resistant to all of the antimicrobial tests (Table 2a). The only one *P. aeruginosa* isolated from Penang was susceptible to Gentamicin, Amikacin and Ciprofloxacin, resistant to Co-trimoxazole, Tetracycline and Cefuroxime (Table 2b). *P. aeruginosa* isolated from Melaka was resistant to all of the antimicrobial drugs used. (Table 3) In addition, It has found that most of the *P. aeruginosa* isolates (n=13) were resistant to Co- trimoxazole, Tetracycline and Cefuroxime compared to Gentamicin, Amikacin and Ciprofloxacin (Table 4). The multidrug resistance *P. aeruginosa* isolates were indicated by resistant more than three antimicrobial drug resistant. Figures 7 and 8 showed antimicrobial susceptibility and resistance of *P. aeruginosa* isolates.

Hodge test

All 13 MDR *P. aeruginosa* isolates were tested for carbapenemase production. Among these 13 MDR *P. aeruginosa* 3 out of 6 from Selangor were positive of

Table 2a. Antibiogram of *pseudomonas aeruginosa* isolated in Selangor (n=6).

No. of bacterial strain	Gentamicin (10 µg)			Amikacin (30 µg)			Ciprofloxacin (5 µg)		
	Susceptibility (mm) >15	Intermediate (mm) 13-14	Resistance (mm) <12	Susceptibility (mm) >17	Intermediate (mm) 15-16	Resistance (mm) <14	Susceptibility (mm) >21	Intermediate (mm) 16-20	Resistance (mm) <15
7	24	-	-	28	-	-	26	-	-
11	26	-	-	28	-	-	39	-	-
14	25	-	-	27	-	-	37	-	-
16	-	-	10	-	-	11	-	-	14
18	-	-	8	-	-	10	-	-	14
19	-	-	10	-	-	12	-	-	12
No. of bacterial strain	**Co-trimoxazole (25 µg)**			**Tetracycline (30 µg)**			**Cefuroxime (10 µg)**		
	Susceptibility (mm) >18	Intermediate (mm) 16-17	Resistance (mm) <15	Susceptibility (mm) >25	Intermediate (mm) 23-24	Resistance (mm) <22	Susceptibility (mm) >18	Intermediate (mm) 15-17	Resistance (mm) <14
7	-	16	-	-	-	18	-	-	7
11	-	-	11	-	-	14	-	-	7
14	-	-	10	-	-	12	-	-	7
16	-	-	10	-	-	12	-	-	7
18	-	-	9	-	-	10	-	-	7
19	-	-	11	-	-	14	-	-	8

Table 2b. Antibiogram of *P. aeruginosa* isolated in Kuala Lumpur (n=5).

No. of bacterial strain	Gentamicin (10 µg)			Amikacin (30 µg)			Ciprofloxacin (5 µg)		
	Susceptibility (mm) >15	Intermediate (mm) 13-14	Resistance (mm) <12	Susceptibility (mm) >17	Intermediate (mm) 15-16	Resistance (mm) <14	Susceptibility (mm) >21	Intermediate (mm) 16-20	Resistance (mm) <15
4	27	-	-	30	-	-	41	-	-
5	-	-	12	-	-	11	-	-	14
7	25	-	-	29	-	-	38	-	-
13	-	14	-	23	-	-	31	-	-
14	-	-	11	-	-	13	-	-	14

carbapenemase production, 2 out of 5 from Kuala Lumpur were positive of carbapenemase production and only one *P. aeruginosa* isolates from Penang and Melaka were positive. Thus, among 13 MDR *P. aeruginosa* isolates, 7 were carbapenemase positive and 6 were no

Table 2b. Contd.

No. of bacterial strain	Co-trimoxazole (25 µg)			Tetracycline (30 µg)			Cefuroxime (10 µg)		
	Susceptibility (mm) >18	Intermediate (mm) 16-17	Resistance (mm) <15	Susceptibility (mm) >25	Intermediate (mm) 23-24	Resistance (mm) <22	Susceptibility (mm) >18	Intermediate (mm) 15-17	Resistance (mm) <14
4	-	-	12	-	-	16	-	-	7
5	-	-	10	-	-	12	-	-	7
7	-	-	11	-	-	15	-	-	7
13	-	-	8	-	-	11	-	-	7
14	-	-	13	-	-	15	-	-	7

Table 3. Antibiogram of *P. aeruginosa* isolated in Penang (n=1).

No. of bacterial strain	Gentamicin (10 µg)			Amikacin (30 µg)			Ciprofloxacin (5 µg)		
	Susceptibility (mm) >15	Intermediate (mm) 13-14	Resistance (mm) <12	Susceptibility (mm) >17	Intermediate (mm) 15-16	Resistance (mm) <14	Susceptibility (mm) >21	Intermediate (mm) 16-20	Resistance (mm) <15
9	26	-	-	29	-	-	38	-	-
No. of bacterial strain	**Co-trimoxazole (25 µg)**			**Tetracycline (30 µg)**			**Cefuroxime (10 µg)**		
	Susceptibility (mm) >18	**Intermediate (mm) 16-17**	**Resistance (mm) <15**	**Susceptibility (mm) >25**	**Intermediate (mm) 23-24**	**Resistance (mm) <22**	**Susceptibility (mm) >18**	**Intermediate (mm) 15-17**	**Resistance (mm) <14**
9	-	-	10	-	-	14	-	-	7

Table 4. Antibiogram of *P. aeruginosa* isolated in Melaka (n=1).

No. of bacterial strain	Gentamicin (10 µg)			Amikacin (30 µg)			Ciprofloxacin (5 µg)		
	Susceptibility (mm) >15	Intermediate (mm) 13-14	Resistance (mm) <12	Susceptibility (mm) >17	Intermediate (mm) 15-16	Resistance (mm) <14	Susceptibility (mm) >21	Intermediate (mm) 16-20	Resistance (mm) <15
4	-	-	12	-	-	13	-	-	13
No. of bacterial strain	**Co-trimoxazole (25 µg)**			**Tetracycline (30 µg)**			**Cefuroxime (10 µg)**		
	Susceptibility (mm) >18	**Intermediate (mm) 16-17**	**Resistance (mm) <15**	**Susceptibility (mm) >25**	**Intermediate (mm) 23-24**	**Resistance (mm) <22**	**Susceptibility (mm) >18**	**Intermediate (mm) 15-17**	**Resistance (mm) <14**
4	-	-	7	-	-	13	-	-	7

Table 5. Antibiogram of *P. aeruginosa* (n=13).

State	No. of positive isolates	Gentamicin (10 µg)			Amikacin (30 µg)			Ciprofloxacin (5 µg)		
		Susceptibility n (%)	Intermediate n (%)	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)	Resistance n (%)
Selangor	6	3	0	3	3	0	3	3	0	3
Kuala Lumpur	5	2	1	2	3	0	2	3	0	2
Penang	1	1	0	0	1	0	0	1	0	0
Melaka	1	0	0	1	0	0	1	0	0	1
Total	13	6(46.1%)	1 (7.6%)	6 (61.5%)	7 (53.8%)	0 (0%)	6 (46.1%)	6(46.1%)	0(0%)	7 (53.8%)

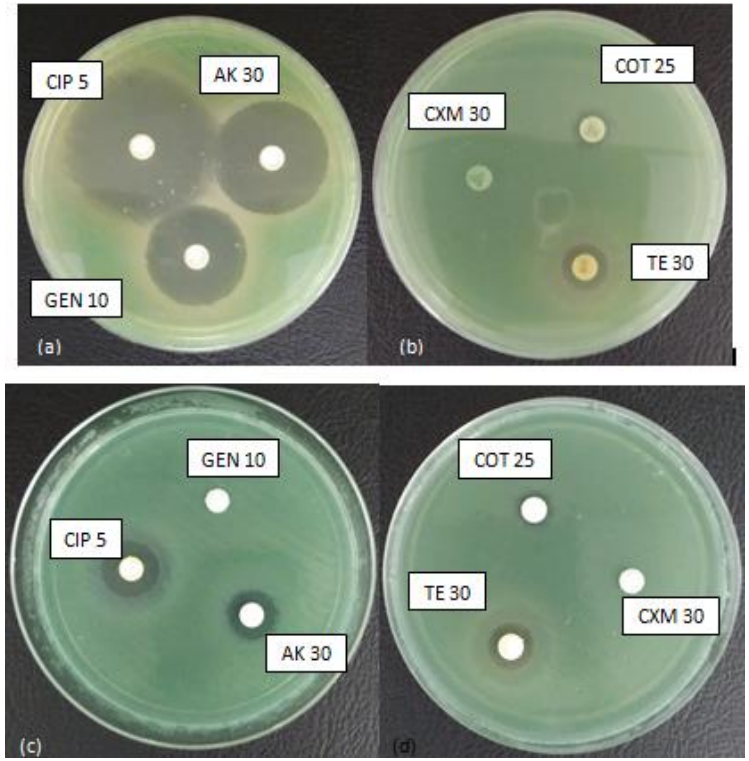

Figure 4. a and b: Antibiogram of number 7 *P. aeruginosa* strain in Kuala Lumpur, which highly susceptible to CIP, AK and GEN while resistance to COT, CXM and TE. c and d: Antibiogram of number 18 *P. aeruginosa* strain in Selangor, which resistance all to CIP, AK, GEN, COT, CXM and TE.

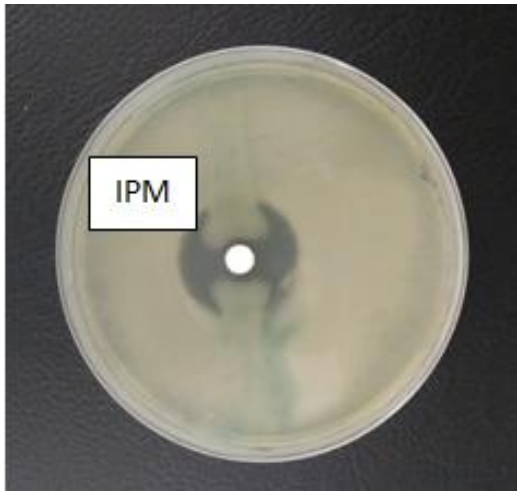

Figure 5. Interpretation of the Hodge test, positive for carbapenemase production.

Table 6, Carbapenemase production detection by Hodges test.

State	No. of positive isolates	Co-trimoxazole (25 µg)			Tetracycline (30 µg)			Cefuroxime (30 µg)		
		Susceptibility n (%)	Intermediate n (%)	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)	Resistance n (%)	Susceptibility n (%)	Intermediate n (%)	Resistance n (%)
Selangor	**6**	0	1	5	0	0	6	0	0	6
Kuala Lumpur	**5**	0	0	5	0	0	5	0	0	5
Penang	**1**	0	0	1	0	0	1	0	0	1
Melaka	**1**	0	0	1	0	0	1	0	0	1
Total	**13**	0 (0%)	1 (7.6%)	9 (92.3%)	0 (0%)	0(0%)	13 (100%)	0 (0%)	0(0%)	13 (100%)

carbapenease production (Table 5 and Figure 5).

Imipenem-EDTA combined disc test (CDT)

Out of the 13 MDR *P. aeruginosa* isolates, Selangor and Kuala Lumpur gave MBL positive, which were 5 out of 6, 4 out of 5 and only one from Melaka respectively. CDT had detected 10 MBL positive and only 3 MBL negative among the 13 MDR *P. aeruginosa* isolates. The MBL positive observed increase in EDTA with imipenem inhibition zone more than 7 mm in the imipenem alone (Table 6 and Figure 6).

Imipenem-EDTA double- disc synergy test (DDST)

Among 13 MDR *P. aeruginosa* isolates, 4 out of 6 from Selangor gave positive result, which showed the presence of synergistic zone of inhibition between two discs and indicated MBL production. 2 out of 5 from Kuala Lumpur showed MBL production. The only *P. aeruginosa* isolates from Melaka showed MBL production. However the only *P. aeruginosa* isolate from Penang showed negative result with no MBL production. Among 13 MDR *P. aeruginosa* isolates, 7 showed MBL

Figure 6. Interpretation of the Imipenem-EDTA CDT, imipenem with EDTA disc produced ≥ 7 mm of inibition zone than impenem, which indicate MBL positive.

Table 7. Beta-lactamase detection by Imipenem-EDTA combined disc test.

State	No. of positive isolates	CDT	
		Positive	Negative
Selangor	6	5	1
Kuala Lumpur	5	4	1
Penang	1	0	1
Melaka	1	1	0
Total	13	10	3
Total (%)		76.9%	23.0%

production and 6 showed non-MBL production, which gave the same results after repeated tests (Table 7 and Figure 7).

Comparison of Hodge test, CDT and DDST for detection of MBL producing P. aeruginosa

Among 13 MDR *P. aeruginosa* isolates, Hodge test detected 7 carbapenemase production, which gave 53.8% positive results, while 6 showed non-carbapenenmase production in Hodge test, gave 46.1% negative results (Table 8). The numbers of positive results in Hodge test were same with DDST, which were 7 MBL productions, 53.8% and 6 non-MBL production 46.1%. Whereas CDT detected 10 MBL positive 76.9% and only 3 MBL negative 23%. Based on the positive and negative results among these phenotyping method, CDT showed the highest sensitivity among Hodge test and DDST, yet Hodge test and DDST gave more specific results

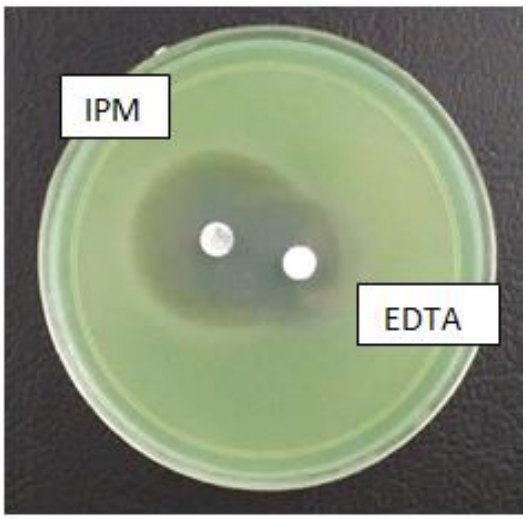

Figure 7. Interpretation of Imipenem-EDTA DDST, growth inhibition zone between two disks, which indicate positive for MBL.

Table 8. MBL-producing gram negative bacilli indicated by imipenem-EDTA double-disc synergy test.

State	No. of positive isolation	DDST	
		Positive	Negative
Selangor	6	4	2
Kuala Lumpur	5	2	3
Penang	1	0	1
Melaka	1	1	0
Total	13	7	6
Total (%)		53.8%	46.1%

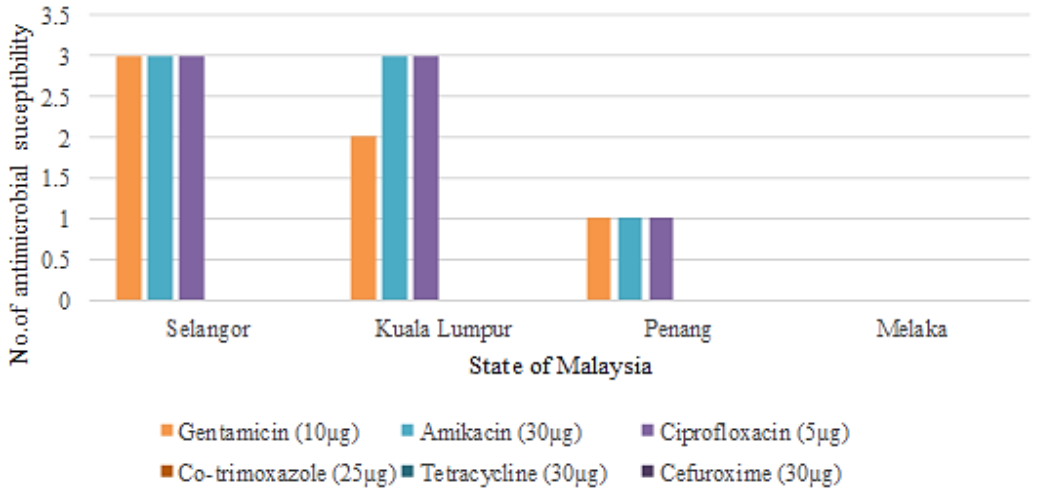

Figure 8. Antimicrobial susceptibility pattern of *P. aeruginosa* in different states of Malaysia.

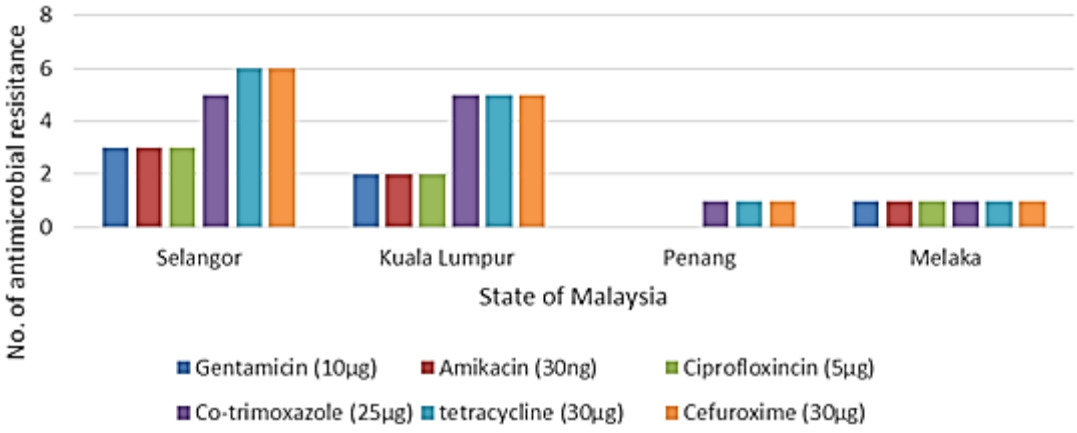

Figure 9. Antimicrobial resistance pattern of *P. aeruginosa* in different states of Malaysia.

compared to CDT. Both Hodge test and DDST gave similar results.

DISCUSSION

Urban areas use clean water and produce large amounts of wastewater. Water is an important requirement in many industrial processes as well as hospital Corcoran et al. (2010). Antibiotics were used as a treatment for patient in hospitals. These antibiotics and their metabolites were excreted with urine and faeces and ended up in the wastewater stream (Chartier et al., 2014; Email et al., (2010). Hospital wastewaters were a source

of bacteria with acquired resistance against antibiotics which has higher level compared with domestic wastewater Chartier et al. (2014). Industrial wastewater has the potential to be a highly toxic source of pollution if wastewater from industry drains directly into rivers and lakes without appropriate treatment (Corcoran et al., 2010). *P. aeruginosa* is a highly adaptable bacterium, they are able to grow well in water systems and have intrinsic antimicrobial resistance due to low outer membrane permeability as well as an extensive efflux pump system (Igbinosa et al., 2012). Therefore it increases the risk of health and environment.

Ministry of Health (2013) and Association of Private Hospitals of Malaysia (2016) showed that among the states in Malaysia, Selangor has the highest number of hospitals (18 hospitals including government and private hospital), followed by Kuala Lumpur (14 hospitals), while 12 hospitals in Penang, 9 hospitals in Kedah and 6 hospitals in Melaka. According to Makky et al. (2012), their study revealed that antibiotic resistance *P. aeruginosa* was isolated from hospital drains. Many studies on *P. aeruginosa* strains from clinical samples in hospitals were carried out (Butt et al., 2005; Aghamiri et al., 2014; Akya et al., 2015; Devi et al., 2015). In the present study, 52 water samples were collected from different states of Malaysia. 13 water samples were found positive for *P. aeruginosa* isolates. Among the *P. aeruginosa* isolates from various water sources in the different states of Malaysia, both Selangor and Kuala Lumpur (KL) have the highest rates of *P. aeruginosa* isolation compared to other states like Penang, Melaka and Kedah respectively. These 13 *P. aeruginosa* isolates were processed for antimicrobial susceptibility test. Out of these 13 *P. aeruginosa* isolation, 6 of *P. aeruginosa* isolation from Selangor indicated multi-drug resistance; 2 out of 5 of *P. aeruginosa* isolation from KL were MDR; *P. aeruginosa* isolates from Penang and Kedah did not show any MDR in antimicrobial susceptibility test; *P. aeruginosa* isolates from Melaka showed one MDR. Makky et al. (2012) mentioned that the development of resistant through mutation can also play an important role in development of β-lactam resistance.

In our study, Ciprofloxacin (5 µg), Gentamicin (10 µg), Amikacin (30 µg), Co- trimazole (25 µg), Tetracycline (30 µg), and Cefuroxime (30 µg) were used in antimicrobial susceptibility test. The 13 *P. aeruginosa* isolates were highly resistant to Tetracycline and Cefuroxime (100%) followed by Co-trimazole (92.3%), while moderate resistance with Ciprofloxacin 53.8%, Gentamicin and Amikacin (both 46.1%) respectively. According to Adesoji et al. (2015) *P. aeruginosa* isolates were more resistant to older generation antibiotics such as Sulfamethoxazole, Tetracycline, and Streptomycin than new generation antibiotics like Gentamicin, Kanamycin and Nalidixic acid. A recent study showed that *P. aeruginosa* isolates from clinical samples were found to be resistant to multiple-drug. The resistance level was 100% in Iran, 20.69% in Nepal, 19.6% in Malaysia (Nasreen et al., 2015). Akinpelu et al. (2014) stated that *Pseudomonas* strains from river were resistant to Gentamicin, followed by Co-trimazole, Tetracycline and Ciprofloxacin respectively. *P. aeruginosa* isolates from water sample in recent study (Nasreen et al., 2015) showed 93.7% isolates resistant to both Tetracycline and Gentamycin, 71.8% resistant to Co-trimoxazole and the *P. aeruginosa* isolates were found completely sensitive to Ciprofloxacin (100%). There are similarities between both studies, *P. aeruginosa* isolates are resistant to both Gentamicin and Co-trimoxazole but low resistant to Ciprofloxacin. In contrast, other study (Bhalerao et al., 2010) indicated *P. aeruginosa* isolates from clinical samples showed highly resistant to Amikacin (97.06%), followed by Ciprofloxacin (96.43%), Co-trimazole (95.24%), Gentamicin (85.7%) and Cefuroxime (75%). Based on these studies, it revealed *P. aeruginosa* isolates from water samples and clinical samples as well are highly resistant to Co-trimoxazole compared to Gentamicin and Tetracycline. Whereas, *P. aeruginosa* isolates from water samples are more susceptible to Ciprofloxacin than *P. aeruginosa* isolates from clinical samples, which showed high resistance (Bhalerao et al., 2010).

In this study, Hodge test detected 7 carbapenemase production, which gave 53.8% positive results, the number of positive results in Hodge test were same with DDST, which is 7 MBL production, 53.8%. While CDT detected 10 MBL positive 76.9% and only 3 MBL negative 23%. Based on the results among these phenotyping methods, CDT showed most sensitivity to Hodge test and DDST, yet Hodges test and DDST gave more specific results compared to CDT. Both Hodge test and DDST had similar results.

The metallo-β-lactamase producing *P. aeruginosa* were highly found in the Selangor followed by Kuala Lumpur. And the metallo-β-lactamase producing *P. aeruginosa* were detected from drain water followed by river. Selangor have more hospitals and industries than other states, therefore it may also increase the water contamination. Every water source should be tested for contamination with *P. aeruginosa*, especially metallo-β-lactamase-producing *P. aeruginosa* isolates, to prevent the infection caused by this organism. Water source should be properly filtered and treated to improve the water quality, and must be disinfected to prevent the emergence of these *P. aeruginosa*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Adesoji AT, Ogunjobi AA, Olatoye IO (2015). Molecular characterization of selected multidrug resistant Pseudomonas from water distribution

systems in southwestern Nigeria. Ann. Clin. Microbiol. Antimicrob. 14(39):1.
Aghamiri S, Amirmozafari N, Mehrabadi JF, Fouladtan B, Kafil HS (2014). Antibiotic Resistance Pattern and Evaluation of Metallo-Beta Lactamase Genes Including bla-IMP and bla-VIM Types in Pseudomonas aeruginosa Isolated from Patients in Tehran Hospitals. ISRN Microbiology. Volume 2014, Article ID 941507:6.
Akinpelu AT, Akinloye OM, Olukemi BC, Adegoke AE, Olayinka S (2014). Antibiotic resistant pattern of isolated bacteria from Obere River in Orile-Igbon, Oyo State, Nigeria. Afr. J. Microbiol. Res. 8(12):1318-1321.
Akya A, Salimi A, Nomanpour B, Ahmadi K (2015). Prevalence and Clonal Dissemination of Metallo-Beta-Lactamase-Producing Pseudomonas aeruginosa in Kermanshah. Jundishapur J. Microbiol. 8(7):e20980.
Arunagiri K, Sekar B, Sangeetha G, John J (2012). Detection and Characterization of Metallo-β-lactamases in Pseudomonas aeruginosa by Phenotypic and Molecular Methods from Clinical Samples in a Tertiary Care Hospital. West Indian Med. J. 61(8):778.
Association of Private Hospitals of Malaysia (2016). Hospitals/Medical Centers. Available at: http://www.hospitals-malaysia.org/portal/index.asp?menuid=42.
Bashir D, Thokar MA, Fomda BA, Bashir G, Zahoor D, Ahmad S, Toboli AS (2011). Detection of metallo-beta-lactamase (MBL) producing Pseudomonas aeruginosa at a tertiary care hospital in Kashmir. Afr. J. Microbiol. Res. 5(2):164-172.
Bhalerao DS, Roushani S, Kinikar AG, Akhter (2010). Study of Metallo-beta lactamase producing Pseudomonas aeruginosa in Pravara Rural Hospital. Pravara Med Rev 5(3):16-19.
Biradar S, Roopa C (2015). Prevalence of Metallo-Beta-Lactamase Producing Pseudomonas aeruginosa and its antibiogram in a tertiary care centre. Int. J. Curr. Microbiol. Appl. Sci 4(9):952-956.
Butt T, Usman M, Ahmad RM, Saif I (2005). Emergence of Metallo-beta- Lactamase producing Pseudomonas aeruginosa in Pakistan. J. Pak. Med. Assoc. 55(7):302.
Chartier Y, Emmanuel J, Pieper U, Prüss A, Rushbrook P, Stringer R, Townend W, Wilburn S, Zghondi R (2014). Safe management of wastes from health-care activities. 2nd ed. Switzerland: World Health Organization.
Chaudhari MS, Javadekar TB, Ninama G, Pandya N, Damor J (2011). A study of metallo-beta-lactamase producing pseudomonas aeruginosa in clinical samples of S.S.G hospital. Natl. J. Med. Res. 1(2):60-63.
Chen CT, Tsay JD, Yen MC, Matsumoto J (2013). The Winter Rainfall of Malaysia. J. Clim. 26(3):936-58.
Corcoran E, Nelleman C, Bake E, Bos R, Osborn D,Savelli H (2010). Sick Water? The central role of wastewater management in sustainable development. UNEP/Earthprint.
Devi PV, Reddy PS, John MS (2015). Prevalence of Metallo-Lactamases Producing Pseudomonas aeruginosa among the Clinical isolates: A study from tertiary care hospital. Int. J. Curr. Microbiol. Appl. Sci. 4(4):955-996.
Email VD, Tamhankar AJ, Khandal RK, Sen S, Aggarwal M, Marothi Y, Iyer RV, Tonderski KS, Lundborg CS (2010). Antibiotics and antibiotic- resistant bacteria in waters associated with a hospital in Ujjain, India. BMC Public Health 10(1):414.
Georgios M, Egki T, Effrosyni S (2014). Phenotypic and Molecular Methods for the Detection of Antibiotic Resistance Mechanisms in Gram Negative Nosocomial Pathogens. Trends Infect. Dis. InTech, DOI: 10.5772/57582.
Hallin M, Deplano A, Roisin S, Boyart V, Ryck RD, Nonhoff C, Byl B, Glupczynski. Y, Denisa O (2012). Pseudo-Outbreak of Extremely Drug-Resistant Pseudomonas aeruginosa Urinary Tract Infections Due to Contamination of an Automated Urine Analyzer. J. Clin. Microbiol. 50(3):580-582.
Hussain M, Prasad Rao TVD (2013). Effect of Industrial Effluents on Surface Water Quality - A Case Study of Patancheru, Andhra Pradesh, India. Curr. World Environ. 8(3):445-454.
Igbinosa EO, Odjadjare EE, Igbinosa IH, Orhue PO, Omoigberale MNO, Amhanre NI (2012). Antibiotic Synergy Interaction against Multidrug-Resistant Pseudomonas aeruginosa Isolated from an Abattoir Effluent Environment. Sci. World J. 2012:308034.
James Cappuccino, Natalie Sherman (2007).Microbiology: A Laboratory Manual, Benjamin Cummings; 8 edt.
Kali A, Srirangaraj S, Kumar S, Divya HA, Kalyani A, Umadevi S (2013). Detection of metallo-beta-lactamase producing Pseudomonas aeruginosa in intensive care units. Aust. Med. J. 6(12):686-693.
Kumar SH, De AS, Baveja SM, Gore MA (2012). Prevalence and Risk Factors of Metallo β-lactamase Producing Pseudomonas aeruginosa and Acinetobacter species in Burns and Surgical Wards in a Tertiary Care Hospital. J. Lab. Physicians 4(1):39-42.
Lambert PA (2002). Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. J. R. Soc. Med. 95:22-26.
Makky EA, Ibrahim MM, El-Gamal MS (2012). Survey of Hospital Drains Antibiotic Resistant Bacteria for Hygiene and Health Care. International Conference on Chemical, Biological and Medical Sciences (ICCBMS'2012) August 25-26, 2012 Kuala Lumpur (Malaysia), Pp. 1-4.
Manoharan A, Chatterjee S, Mathai D (2010). Detection and characterization of metallo beta lactamases producing Pseudomonas aeruginosa. Ind. J. Med. Microbiol. 28:241-244.
Ministry Of Health Malaysia (2013). List of Government Hospitals. Available at: http://www.moh.gov.my/english.php/database_stores/store_view/3.
Nasreen M, Sarker A, Malek MA, Ansaruzzaman Md, Rahman M (2015). Prevalence and Resistance Pattern of Pseudomonas aeruginosa Isolated from Surface Water. Adv. Microbiol. 5(1):74.
Pellegrini C, Mercuri. PS, Celenza G, Galleni M, Segatore B, Sacchetti E, Volpe R, Amicosante G, Perilli M (2009). Identification of blaIMP-22 in Pseudomonas spp. in urban wastewater and nosocomial environments: biochemical characterization of a new IMP metallo-enzyme variant and its genetic location. J. Antimicrob. Chemother. 63(5):901-908.
Qu TT, Zhang JL, Wang J, Tao J, Yu YS, Chen YG, Zhou JY, Li LJ (2009). Evaluation of Phenotypic Tests for Detection of Metallo-β-Lactamase- Producing Pseudomonas aeruginosa Strains in China. J. Clin. Micro. 47(4): 1136- 1142.
Quick J, Cumley N, Wearn CM, Niebel M, Constantinidou C, Thomas CM, Pallen MJ, Moiemen NS, Bamford A, Oppenheim B, Loman NJ (2014). Seeking the source of Pseudomonas aeruginosa infections in a recently opened hospital: an observational study using whole-genome sequencing. BMJ Open 4(11):e6278.
Slekovec C, Plantin P, Cholley P, Thouverez M, Talon D, Bertrand X, Hocquet D (2012). Tracking down Antibiotic-Resistant Pseudomonas aeruginosa Isolates in a Wastewater Network. PLoS One 7(12):e49300.
Upadhyay S, Joshi SR (2015). TEM mediated extended spectrum cephalosporin resistance in clinical & environmental isolates of Gram negative bacilli: A report from northeast India. Ind. J. Med. Res. 142:614-617.

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Full Length Research Paper

Ethanolic *Allium sativum* extract down-regulates the *pelF* gene involved in *Pseudomonas aeruginosa* biofilm formation

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Exploration of efficacious plant extracts that can reduce or inhibit *Pseudomonas aeruginosa* biofilm formation is necessary. *Allium sativum* is a suitable candidate because of its relative abundance. This study was carried out to determine the effect of ethanolic *A. sativum* extract on the expression of the *P. aeruginosa* biofilm gene, *pelF*. The presence of the *pelF* gene in the isolates used for this study was confirmed via polymerase chain reaction (PCR) and agarose gel electrophoresis. *P. aeruginosa* cultures treated with 1 g/ml of the *A. sativum* extract had the least turbidity (6.7 absorbance value at A_{600}). The expression profile of the *pelF* gene in the treated cultures was determined via PCR using cDNA synthesized from RNA isolated from the treated *P. aeruginosa* cultures. The amplicons from the PCR were analyzed via agarose gel electrophoresis and a concentration dependent down-regulation of the *pelF* gene was observed. Further quantification of the *pelF* gene's expression was performed via real-time PCR using *rpoB* as the reference gene. A 4-fold down-regulation of the gene was observed at 0.5 and 1 g/ml concentrations, respectively. This study suggests that the suppression of the *pelF* gene of *P. aeruginosa* by *A. sativum* extracts plays a role in the inhibition of *P. aeruginosa* biofilm formation. This is the first study to elucidate the effect of *A. sativum* on the expression of any of the *pel* genes.

Key words: *Allium sativum,* biofilm, cDNA, down-regulation, gene expression, *pelF*, *Pseudomonas aeruginosa*, quantitative polymerase chain reaction (qPCR).

INTRODUCTION

Several cases of *Pseudomonas aeruginosa* infections have been reported which implicate the organism as a leading cause of nosocomial infections. An estimated 10% of all nosocomial infections are caused by *P.*

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aeruginosa, especially in immuno-compromised individuals (Aloush et al., 2006; Lund-Palau et al., 2016). Keratitis, pneumonia, scleritis, cystic fibrosis, urinary tract infections, wound infections resulting from invasive and surgical procedures, open and burn wounds are usually caused by *P. aeruginosa* (although, often in association with other bacteria) (Okamoto et al., 2001; Fernandez-Barat et al., 2017; Murugan et al., 2017).

The virulence of *P. aeruginosa* has been shown to depend on the expression of genes that encode its virulence factors. These virulence factors are classified based on the type of infections caused (chronic or acute infections). Virulence factors expressed during chronic infections include biofilms, alginate, elastase secretion, acyltransferase, pyoverdin and pyochelin (Lang et al., 2016). Virulence factors associated with acute *P. aeruginosa* infections include exotoxin A, pili, phospholipase C and exoenzyme S (Faraji et al., 2016; Valadbeigi et al., 2017).

P. aeruginosa infections are difficult to treat due to various mechanisms that confer on it intrinsic resistance to common antibiotics (Tanya and Daniel, 2009; Khalaji et al., 2013). The inefficiency of common antimicrobials to treat infections makes it necessary for plant candidates to be exploited in achieving prophylaxis. Hyper-virulent strains which demonstrate susceptibility to antibiotics still cause difficult-to-treat infections in patients. Murugan et al. (2017) studied patients with scleritis caused by *P. aeruginosa* VRFPA10. Their analyses of the *P. aeruginosa* VRFPA10 genome showed genomic islands which they attributed the hyper-virulence to.

Biofilm formation in *P. aeruginosa,* a quorum-sensing controlled mechanism, has been reported to increase its resistance to antibiotics 1000-fold. The *las* quorum sensing system of *P. aeruginosa* controls the transcription of the *pel* gene operon which consists of the following genes: *pelA, pelB, pelC, pelD, pelE, pelF* and *pelG* (Bacalso et al., 2011; Xu et al., 2013). The *pel* genes (pellicle biosynthetic genes) have been shown to code for proteins involved in the synthesis of the exopolysaccharide moieties during *P. aeruginosa* biofilm formation (Vasseur et al., 2005; Sakuragi and Kolter, 2007). The roles of the individual *pel* genes in the Pel biosynthetic pathway have not been fully established. The *pelF* gene however, has been one of the most extensively studied and it encodes a glycosyltransferase. It is the only gene in the operon that produces a protein that localizes in the cytoplasm (Vasseur et al., 2005; Franklin et al., 2011).

Several plant extracts have been studied for their quorum sensing (QS) inhibitory properties (Singh et al., 2012). In this study, *Allium sativum* (garlic) extract was used to treat *P. aeruginosa* isolated from wounds. Garlic has been reported to inhibit quorum sensing in *P. aeruginosa,* thereby, allowing better penetration of antimicrobials into the biofilm matrix (Cavallito and Bailey, 1944; Hurley et al., 2012; Harjai et al., 2009). Studies have also demonstrated the ability of garlic extracts to inhibit biofilm formation in planktonic bacteria (Arzanlou et al. 2007; Mohsenipour and Hassanshahian, 2015). Being a plant that has been widely used for its medicinal properties, bacteria are unlikely to develop resistance to garlic extracts. This study seeks to determine the effects of *A. sativum* on the expression of the *P. aeruginosa* biofilm gene, *pelF.* Also, this study seeks to establish the prospect for use of garlic extracts either in individual or combined treatment of wound infections by *P. aeruginosa.*

MATERIALS AND METHODS

Bacterial isolation

P. aeruginosa were isolated from 10 wound swabs collected from patients in the medical ward of Barau Dikko Teaching Hospital Kaduna (this was done with the approval of the hospital's management). Fresh garlic bulbs were purchased from the Central Market, Kaduna, Nigeria. This study was carried out in the Microbiology Laboratory of Kaduna State University, Nigeria.

Cetrimide agar (Merck, Germany) was prepared according to the manufacturer's instructions. Briefly, 45 g of the agar powder was weighed and dissolved 1 L of distilled water by heating at 50°C in a water bath. Then, 3 ml of glycerol (Merck, Germany) was added and the mixture was autoclaved at 121°C for 15 min. Sterilized media was aseptically poured into sterile petri dishes, covered and allowed to solidify. The wound swabs were aseptically streaked on the solidified media and incubated for 24 h at 37°C.

Exactly two drops of the oxidase reagent (Dalynn, Canada) were used to moisten a piece of filter paper. Greenish colonies from the 24 h culture were smeared on the moistened filter paper using an applicator stick. Colour change was observed within 30 s (Cheesbrough, 2000).

Preparation of the ethanolic extract of *A. sativum*

Fresh whole bulbs of *A. sativum* (garlic) were washed with distilled water and the outer cover was removed using a sharp knife disinfected with 75% ethanol. Exactly, 500 g of the washed garlic was cut into pieces and was ground using a disinfected blender; it was then transferred to a sterile beaker containing 100 ml of 70% ethanol. The beaker was covered and left to stand for 24 h at room temperature. The mixture was then filtered using Whatman No 1 filter paper. The filtrate was poured into a crucible and placed in a water bath to concentrate at 50°C. The filtrate was exposed to the air to allow the ethanol to evaporate leaving only the pelleted *A. sativum* (*A. sativum*) extract.

To prepare various concentrations of the *A. sativum* extracts, the pellets were crushed into fine powder using a mortar and pestle. Then, 10, 5, 3.3, 2.5 and 2 g of the powder were each dissolved in 10 mL of distilled water to obtain the following concentrations of the extract (g/mL): 10, 5, 3.3, 2.5 and 2.

Preparation of broth cultures of *P. aeruginosa*

Nutrient broth (Merck, Malaysia) was prepared according to the manufacturer's instructions. Briefly, 1.2 g of the powder was dissolved in 150 ml of distilled water by swirling. The dissolved broth was autoclaved at 121°C for 15 min.

To prepare the broth culture of the test organism, 10 ml of the nutrient brought was poured into a test tube. A single colony of the

P. aeruginosa isolates was transferred to the nutrient broth using a sterile wire loop and mixed. It was incubated at 37°C with shaking at 200 rpm until the culture turbidity matches 0.5 McFarland standard (corresponding to approximately 1.5 X 10^8 CFU/ml) (Ndip et al., 2005).

Detection of the *pelF* and *rpoB* genes

To proceed with this study, it was important to detect the presence of the *pelF* gene in the representative *P. aeruginosa* isolate so as to justify the proceeding gene expression study. The presence of the *rpoB* gene, which was used as the reference gene (housekeeping gene) in quantifying the fold change in gene expression was also detected.

Specific pair of primers for the *P. aeruginosa pelF* and *rpoB* genes were designed using the online primer design tool, NCBI primer BLAST. The specificity of the designed primers to the *pelF*and *rpoB* gene sequences was confirmed using the online tool IDT OligoAnalyzer 3.1.

Probing for, and the amplification of the *pelF* and *rpoB* genes were carried out in the *P. aeruginosa* isolate employed in this study using the polymerase chain reaction technique (PCR) as described by Gemiarto et al. (2015). This was performed to ascertain the presence of the genes in the test organism. Genomic DNA was isolated from the *P. aeruginosa* culture using phenol-chloroform extraction method. Purity of the extracted DNA was ascertained spectrophotometrically using the A_{260}/A_{280} ratio.

Exactly, 2 µl of the extracted *P. aeruginosa* genomic DNA was added to 12.5 µl of 2X GoTaq Green PCR Master Mix (Promega, USA), 0.4 µl each of the appropriate forward and reverse primers designed using the NCBI primer BLAST (Basic Local Alignment Search Tool) and nuclease-free water to a final volume of 25 µl and mixed gently.

The PCR was carried out in a thermocycler (Applied Biosystems, USA). An initial denaturation at 95°C for 10 min was carried out followed by 30 cycles of denaturation at 94°C for 30 s, annealing temperature of 55°C for 45 s and elongation at 72°C for 45 s; then a final elongation at 72°C for 5 min was done. Analysis of the PCR products was carried out via agarose gel electrophoresis (using 1% agarose in TAE buffer and ethidium bromide dye).

After the PCR amplification, the suspected DNA amplicons (*pelF* and *rpoB*) were separated using agarose gel electrophoresis. This was followed by the gel extraction of the obtained DNA bands suspected to be those of *pelF* and *rpoB* gene from the agarose gel and subjecting same to sequencing. The degree of alignment of the sequenced DNA to those of the *pelF* and *rpoB* genes determined in the NCBI gene bank was obtained using the NCBI BLAST.

Treatment with various concentrations of *A. sativum* extract

For each concentration of the extract, 8 ml of freshly prepared nutrient broth was poured into a test tube and 1 ml of the *P. aeruginosa* broth cultured was added to it. Then, 1 ml each of the various concentrations (10, 5, 3.3, 2.5 and 2 g/ml) of the *A. sativum* extract was added to the respective test tubes. For each concentration, the treatment was carried out in triplicates. The treated broth cultures including an untreated control, were incubated in 100 ml conical flasks (to provide a greater surface area for attachment and biofilm formation), at 37°C for 24 h.

The overnight cultures of the treated cells and the untreated control were sub-cultured on cetrimide agar to compare the cell densities. This is to check for viability of the cells after the treatment.

Turbidity measurement of treated cultures

The turbidity of the treated *P. aeruginosa* cultures was determined using a spectrometer (Labomed, USA). Briefly, sterile swab sticks were used to gently dislodge cells that were attached to the bottom of the conical flask. The culture was mixed by swirling for 1 min. Then, about 2 ml of each of the treated cultures (and the untreated control) was transferred into a quartz cuvette. The measurements were taken at A_{600}.

Analysis of *pelF* gene expression following treatment with *A. sativum* extract

Total RNA from the *P. aeruginosa* cultures treated with the *A. sativum* extract was isolated according to the manufacturer's instructions, using the SV Total RNA Isolation System (Promega, USA). Purity of the extracted RNA was ascertained spectrophotometrically using the A_{260}/A_{280} ratio. The isolated RNA was reverse-transcribed to synthesize cDNA, according to the manufacturer's instructions, using the AccuPower RT-PCR premix (Bioneer, USA).

The synthesized cDNA were analyzed on 1% agarose gel to determine if the *pelF* gene was expressed. This step is necessary before carrying out qPCR to quantify the gene expression.

Real-time quantitative PCR for differential *pelF* gene expression

Quantitative PCR (qPCR) was carried out to ascertain the effect of the ethanolic *A. sativum* extract on the *pelF* gene. The *rpoB* gene was used as the housekeeping gene and the cDNA synthesized in the section above was used as the template. The qPCR was performed using the 2X GoTaq qPCR mastermix (Promega, USA) and the reaction was set up according to the manufacturer's protocol. The ABI StepOne (Applied Biosystems, USA) real-time thermal cycler was used and the cycling conditions used are as follows: heat activation at 95°C for 2 min then 40 cycles of denaturation (95°C for 15 s), annealing (57°C for 30 s) and extension (60°C for 60 s). A final denaturation from 60 to 95°C for 30 s was carried out. Quantification of the fold change in gene expression was calculated using the formula employed in the Pfaffl (2001) method.

$$R = \frac{(E_{target})^{\Delta C_{Ttarget}\,(control\text{-}treated)}}{(E_{ref})^{\Delta C_{Tref}\,(control\text{-}treated)}}$$

Where, R = ratio; E = efficiency of the primers; ΔC_T = difference between C_T (cycle threshold) of control and treated samples; Ref = reference gene (*rpoB*); target = the amplified gene (*pelF* gene).

The cDNA that was reverse-transcribed from the untreated *P. aeruginosa* was used to generate five-fold serial dilutions for the derivation of a standard curve. Differential expression of the *pelF* gene was derived by comparing C_T (cycle threshold) values obtained from it with those of the housekeeping gene used (*rpoB*).

Changes in gene expression are only considered to be significant when the fold change is ≥2 (where the gene is said to be up - regulated) or ≤-2 (where the gene is said to be down-regulated).

RESULTS AND DISCUSSION

Isolation and identification of *P. aeruginosa*

Of the 10 wound swab samples collected, 5 produced green colonies when cultured on cetrimide agar (a selective medium for *P. aeruginosa*), and they were

Table 1. List of primers used.

Gene and NCBI Accession number	Primer sequence	Length (bp)	T_m (°C)	PCR Product size (bp)
pelF (NC_010079.1)	*pelF*-F 5'-GTGGCTTCAGTGCTTGTAGGTA-3'	22	60.29	452
	pelF-R 5'-TGCTTGATTGAGTTGTTGCCG-3'	21	60.00	
rpoB (NC_002516.2)	*rpoB*-F 5'- TGTGGGTGATCTCGGAAAGC-3'	20	60.4	1382
	rpoB-R 5'- CCTGGAATACGTCGGCTACC-3'	20	59.97	

*T_m = Melting temperature; *bp = base pair.

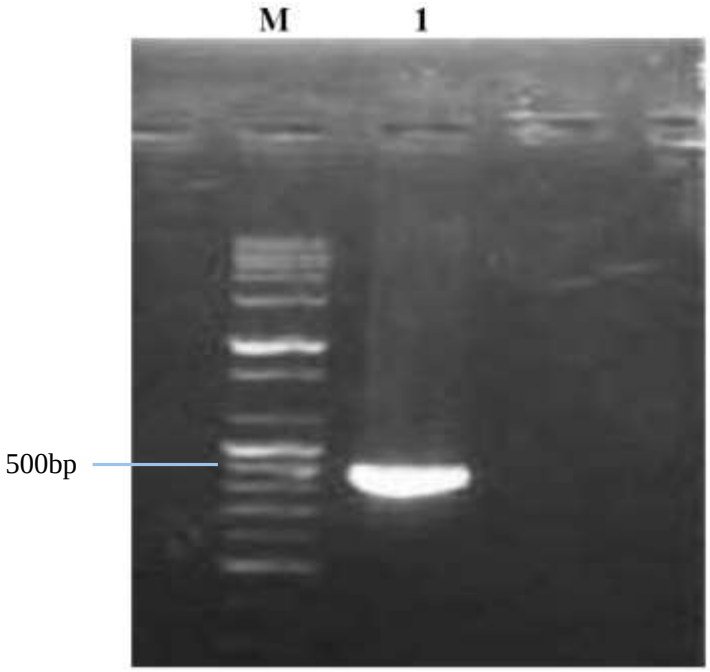

Figure 1a. Agarose gel of the PCR amplicon obtained from the *P. aeruginosa* DNA. Lane M contains a 100 bp DNA ladder; lane 1 contains the PCR amplicon suspected to be *pelF.*

confirmed to be *P. aeruginosa* by the production of an intense purple color during the oxidase test.

Detection of the *pelF* and *rpoB* genes in the isolates

Following the isolation of DNA from the isolate, pairs of specific primers (Table 1) were used to amplify the *pelF* and the *rpoB* genes and the amplicons were analyzed via agarose gel electrophoresis using 1% agarose in TAE buffer and ethidium bromide dye. Amplicon sizes corresponding to the expected product size of the primers used (452 and 1382 bp respectively) were obtained. The results obtained are as shown in Figure 1a and b.

Pel, produced by *P. aeruginosa* has been reported to also serve the function of preserving the capacity of the organism to continue to produce biofilms even when other exopolysaccharide genes have undergone mutation (Colvin et al., 2012). This validates the choice of the *pel* genes as a suitable candidate for down-regulating biofilm production in *P. aeruginosa.* In all the *P. aeruginosa* isolated from the wound swabs collected for this study, *pelF* was detected. This suggests that this QS-regulated gene is ubiquitous and highly conserved (Bacalso et al., 2011). The *rpoB,* which is responsible for the synthesis of the beta subunit of the RNA polymerase is an important reference gene for qPCR because it is expressed at a consistent level as long as the cell is alive.

Treatment of *P. aeruginosa* broth cultures with various concentrations of *A. sativum* extract

The various broth cultures of the test organism for this study were treated with 1, 0.5, 0.33, 0.25, 0.20 and 0 g/ml concentrations of the ethanolic *A. sativum* extract.

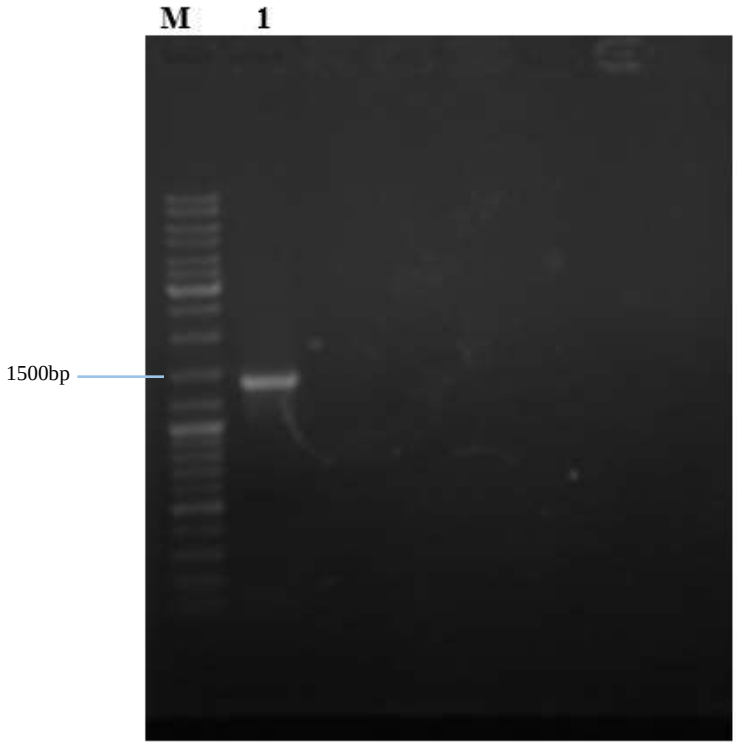

Figure 1b. Agarose gel of the PCR amplicon obtained from the *P. aeruginosa* DNA. Lane M contains a 1kb DNA ladder; lane 1 contains the PCR amplicon suspected to be *rpoB.*

Absorbance readings of the treated cultures at A_{600}, following overnight incubation showed that: with increasing concentration of the extract, a decrease in the turbidity of the treated cultures was observed. This was recorded as an increase in the amount of light that passes through the culture; the less the turbidity, the more light passes through it. The culture treated with 1 g/ml of the extract had the least turbidity (6.7 absorbance value) and that treated with 0.20 g/ml of the extract had the highest turbidity (2.0 absorbance value) (as shown in Figure 2).

Figure 2 shows that the concentration of the *A. sativum* applied is inversely proportional to the intensity of the turbidity of the culture and the highest turbidity (least light absorption) was observed in the untreated control (absorbance value: 1.6) and highest when treated with the 1 g/ml extract concentration (6.7 absorbance value). The intensity of the culture turbidity observed is dependent on amount of cells that form the biofilm at the base of the culture flask used. As the concentration of the extract was increased, the amount of cells layered at the base of the flask decreased. This clearly shows that the decreasing turbidity was concentration dependent. This also does not agree with earlier findings which reported that garlic extract increases biofilm formation by *S. mutans* (Lee et al., 2011).

In this study, the broth treated with the highest concentration (1 g/ml) of *A. sativum* extract has the highest absorbance value (6.7) and the broth treated with the lowest concentration had the lowest absorbance value (2.0). The rapid drop in the turbidity observed between concentrations 0.5 and 0.33 g/ml may imply that the minimum effective dose of the active component of the extract lies within that concentration range.

Determination of the expression profile of the *pelF* gene in the treated cultures

RNA (which is the first product of gene expression before its translation into proteins) was isolated from the *P. aeruginosa* cultures that were treated with the various concentrations of the ethanolic *A. sativum* extract. The isolated RNA, after being reverse transcribed into cDNA and amplified using a conventional PCR thermocycler; was analyzed via agarose gel electrophoresis using 1% agarose in TAE buffer and ethidium bromide dye. A down-regulation of the *pelF* gene was observed and estimated as the increasing faintness of the amplicon bands obtained on the agarose gel with increasing concentration of the extract used in the treatment of the broth cultures (as shown in Figure 3).

Figure 3 clearly shows a consistent decrease in the expression of the quorum sensing controlled gene, *pelF,*

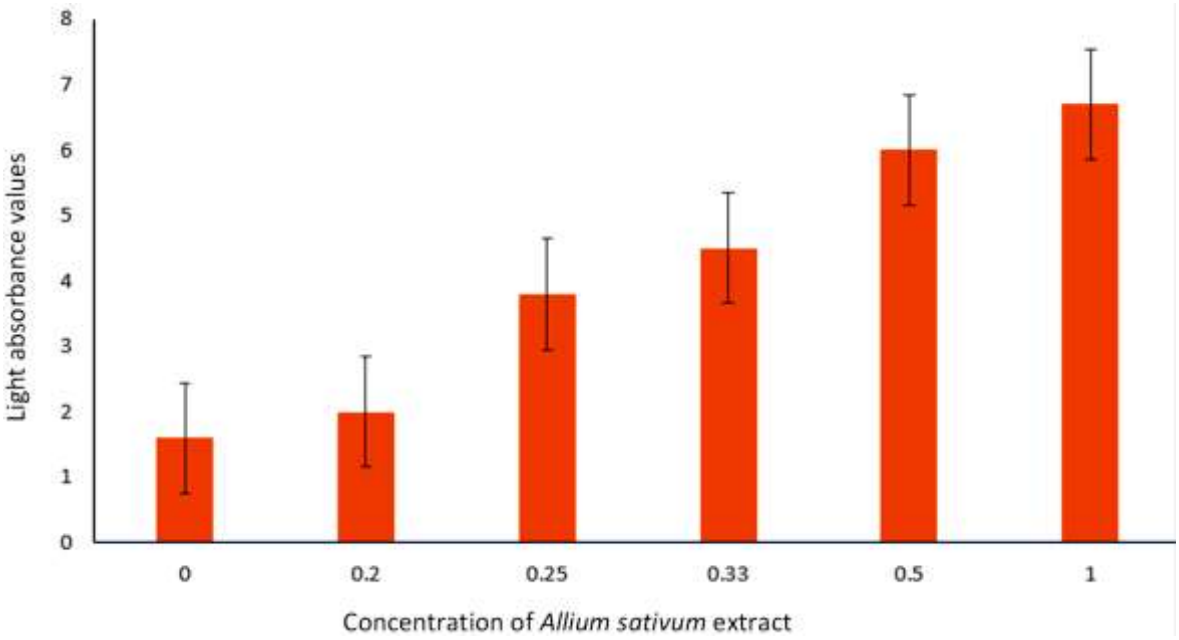

Figure 2. Absorbance of the treated *Pseudomonas aeruginosa* broth cultures at A_{600}.

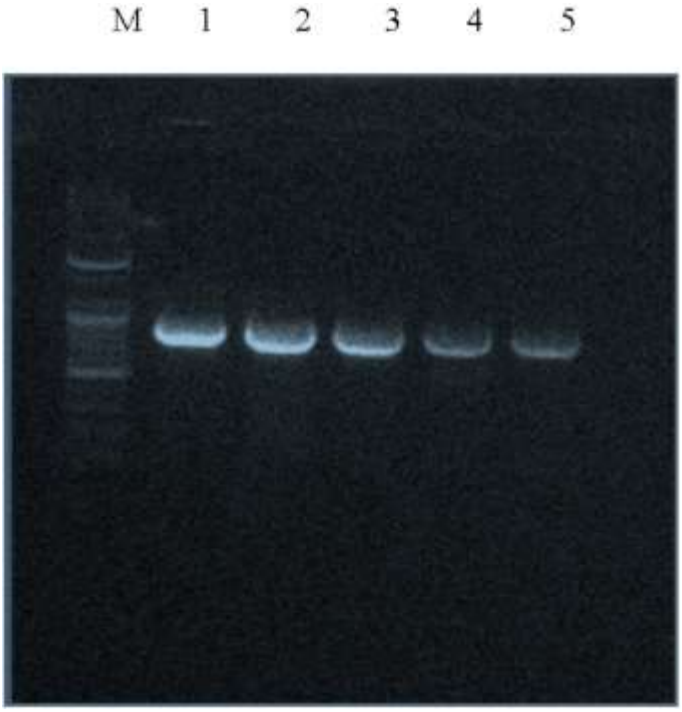

Figure 3. Expression level of *pelF* following treatment with *A. sativum* extract. Lane M, 1 kb ladder; lanes 1, 2, 3, 4 and 5; *pelF* from *P. aeruginosa* treated with 0.2, 0.25, 0.33, 0.5 and 1 g/ml of the extract.

with increasing concentration of the extract. A study suggested that the thiosulfinates contained in garlic (*A. sativum*) is responsible for quorum sensing inhibition in *P. aeruginosa* (Vadekeetil et al., 2014). There are various

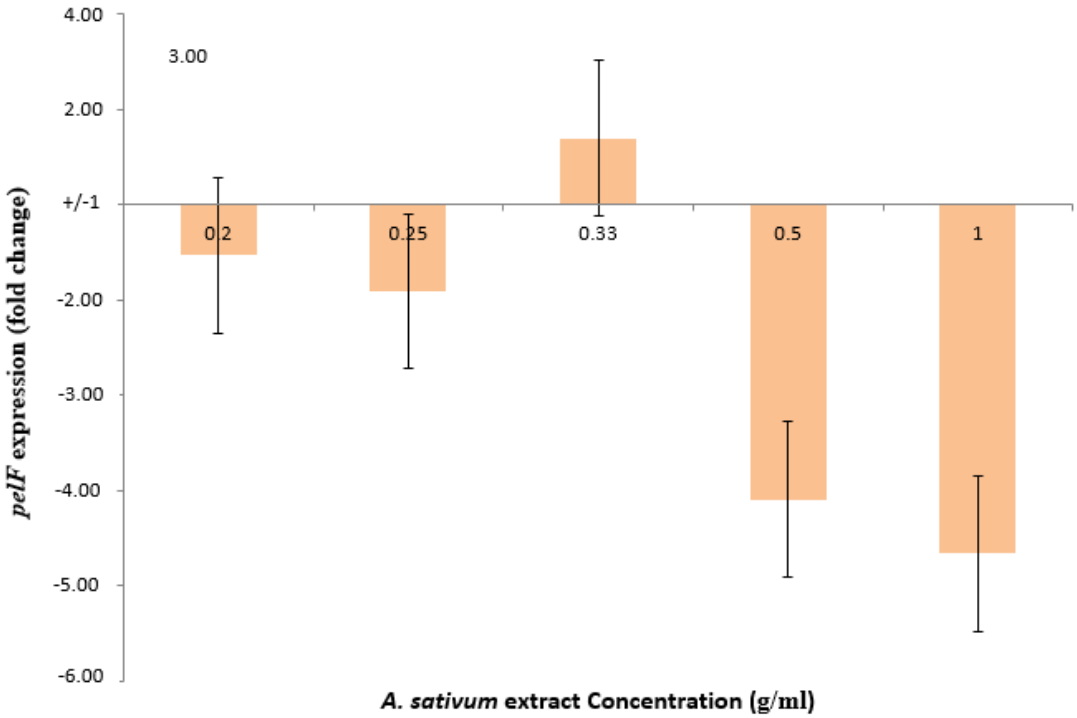

Figure 4. The real-time PCR differential expression profile of *pelF* after treatment with various concentrations of *A. sativum* extract. All fold changes have been normalised to *rpoB* as the reference gene. Data are means of fold changes with standard deviations from two independent experiments amplified in triplicates.

processes through which quorum sensing is inhibited (Gemiarto et al., 2015) and *A. sativum*-induced QS inhibition may be associated with the degradation of the signalling molecule (Vadekeetil et al., 2014).

To confirm that this increasing faintness in band quality is as a result of the down-regulation of the *pelF* gene and not due to cell death, the treated cultures were cultured on cetrimide agar plate and the growth obtained was compared against an untreated control. The growth obtained suggested that the test organism was still viable after treatment with the aforementioned concentrations of the *A. sativum* extract (data, not published).

Real-time quantitative PCR for differential *pelF* gene expression

Figure 4 shows that at extract concentrations of 0.2-0.33 g/ml, there was no significant up-regulation or down-regulation of the *pelF* gene. This could be due to the fact that these concentrations were not high enough to cause any fold-changes in the gene expression. At higher concentrations (0.5 and 1 g/ml), the *A. sativum* extract resulted in over 4-fold down-regulation of the *pelF* gene. Since the exact mechanism through which the expression of the *pel* genes are regulated is yet unknown (Sakuragi and Kolter, 2007), the role of the *A. sativum* extract in the down-regulation of the pelF gene cannot be inferred with precision.

In an inducible gene operon, the expression of genes located downstream in the operon is usually regulated by the products synthesized by genes located closer to the promoter. Hence, the garlic extracts used in this study are very likely to have interacted with products synthesized by genes located from *pelA* to *pelE*, thereby lowering the induction of *pelf*, the result of which may be observed as the decreased transcription of the gene and the observed down-regulation. One clear fact is that this down-regulation of the *pelF* gene must have occurred before transcription of the gene; this conclusion is made because qPCR cannot measure post-transcriptional control of gene expression.

On a general note, the quorum sensing inhibitory properties of *A. sativum* have been extensively elucidated. The organosulphur compound, allicin, found in garlic has been shown to act as a QS inhibitor (Bodini et al., 2009; Smyth et al., 2010). Hence, to confirm that the decrease in culture turbidity shown in Figure 2 and the expression of *pelF* shown in Figure 3 was not as a result of cell death but as a decline in biofilm formation, the treated cultures were sub-cultured and colonies were observed. The colony count observed confirms that the *P. aeruginosa* were not killed by the concentrations of the extract applied. This is in agreement with results from

studies in which *P. aeruginosa* was treated with natural organo-sulfur compounds as found in garlic (Arzanlou et al., 2007; Cady et al., 2012; Ratthawongjirakul and Thongkerd, 2016). They also observed no reduction in planktonic growth after treatment but they observed a decline in biofilm formation. A reduction in biofilm formation makes *P. aeruginosa* more susceptible to antibiotics (Bjarnsholt et al., 2005; Indu et al., 2006). Hence, this finding could be exploited in using *A. sativum* extracts in combination with antibiotics or other plant extracts for more effective antibiotic therapy. The prospects for achieving this is clearly reflected in the inhibition of *P. aeruginosa* biofilm formation when a blend of essential oils from *Cinnamomum cassia*, *A. sativum* and *Mentha piperita* were used to treat planktonic *P. aeruginosa* PA01 strains (Lang et al., 2016).

This study could be extended to explore the gene expression of other *pel genes* in response to treatment with *A. sativum* extracts. Components of the extract employed here could be used individually to assess their effect on the expression of the *P. aeruginosa pel* genes. This study shows that increasing concentrations of *A. sativum* resulted in an increasing down-regulation of an important *P. aeruginosa* biofilm gene, *pelF.* This down-regulation was not due to extract-induced death of planktonic cells. This study supports the prospects for the use of *A. sativum* extracts as an antipathogenic remedy in combined therapy with antibiotics against resistant bacteria.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

REFERENCES

Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y (2006). Multidrug-resistant Pseudomonas aeruginosa: risk factors and clinical impact. Antimicrob. Agents Chemother. 50(1):43-48.

Arzanlou M, Arab R, Alaei R (2007). Therapeutic efficacy of garlic (*Allium sativa*) against burn wound infection by *Pseudomonas aeruginosa.* Res. J. Biol. Sci. 2(6):634-638.

Bacalso M, Xu T, Yeung K, Zhen D (2011). Biofilm formation of *Pseudomonas aeruginosa* PA14 required las1 and stimulated by the Pseudomonas quinolone signal although salicyclic acid inhibition is independent of the pas pathways. J. Exp. Microbiol. 15:84-89.

Bjarnsholt T, Jensen PO, Rasmussen TB, Christophersen L, Calum H, Hentzer M, Hougen H, Rygaard J, Moser C, Eberl L, Hoiby N, Givskov M (2005). Garlic blocks quorum sensing and promotes rapid clearing of pulmonary Pseudomonas aeruginosa infections. Microbiology 151:3873-3880.

Bodini SF, Manfredini S, Epp M, Valentini S, Santori F (2009). Quorum sensing inhibition activity of garlic extract and p-coumaric acid. Lett. Appl. Microbiol. 49:551-555.

Cady NC, McKean KA, Behnke J, Kubec R, Mosier AP, Kasper SH, Burz DS, Musah RA (2012). Inhibition of biofilm formation, quorum sensing and infection in *Pseudomonas aeruginosa* by natural products-inspired organosulfur compounds. PLoS One 7(6):1-12.

Cavallito CJ, Bailey JH (1944). Allicin, the antibacterial principle of *Allium sativum* isolation, physical properties and antibacterial action. J. Am. Chem. Soc. 66(11):1950-1951.

Cheesbrough M (2000). District Laboratory Practice in Tropical Countries (Part 2). Cambridge Shire, Cambridge University Press.

Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, Howell PL, Wozniak DJ, Parsek MR (2012). The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environ. Microbiol. 14(8):1913-1928.

Faraji F, Mahzounieh M, Ebrahimi A, Fallah F, Teymournejad O, Lajevardi B (2016). Molecular detection of virulence genes in *Pseudomonas aeruginosa* isolated from children with Cystic Fibrosis and burn wounds in Iran. Microb. Pathog. 99:1-4.

Fernandez-Barat L, Ferrer M, De Rosa F, Gabarrus A, Esperatti M, Terraneo S, Rinaudo M, Li Bassi G, Torres A (2017). Intensive care unit-acquired pneumonia due to *Pseudomonas aeruginosa* with and without multidrug resistance. J. Infect. 74:142-152.

Franklin MJ, Nivens DE, Weadge JT, Howell PL (2011). Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. Front. Microbiol. 2(167):49-64.

Gemiarto AT, Ninyio NN, Lee SW, Logis J, Fatima A, Chan EWC, Lim CSY (2015). Isoprenyl caffeate, a major compound in manuka propolis, is a quorum-sensing inhibitor in *Chromobacterium violaceum*. Antonie van Leeuwenhoek 108(2):491-504.

Harjai K, Kumar R, Singh S (2010). Garlic blocks quorum sensing and attenuates the virulence of *Pseudomonas aeruginosa.* FEMS Immunol. Med. Microbiol. 58(2):161-168.

Hurley MN, Camara M, Smyth AR (2012). Novel approaches to the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. Eur. Respir. J. 40:1014-1023.

Indu MN, Hatha AAM, Abirosh C, Harsha U, Vivekanandan G (2006) Antimicrobial activity of some of the south-indian spices against serotypes of *Escherichia coli*, *Salmonella, Listeria monocytogenes* and *Aeromonas hydrophila*. Braz. J. Microbiol. 37:153-158.

Khalaji Y, Doosti A, Ghorbani-Dalini S (2013). Molecular evaluation of antibiotic resistance prevalence in *Pseudomonas aeruginosa* isolated from cockroach in south West Iran. Int. J. Med. Med. Sci. 5(9):420-424.

Lang M, Rodrigues S, Boulho R, Duteil E, Bazire A, Bedoux G (2016). An Essential Oil Blend Prevents *Pseudomonas aeruginosa* PA01 from Forming Biofilms. J. Bacteriol. Parasitol. 7(2):1-8.

Lee HJ, Park HS, Kim KH, Kwon TY, Hong SH (2011). Effect of garlic on bacterial biofilm formation on orthodontic wire. Angle Orthod. 81(5):895-900.

Lund-Palau H, Turnbull AR, Bush A, Bardin E, Cameron L, Soren O, Wierre-Gore N, Alton EW, Bundy JG, Connett G, Faust SN (2016). *Pseudomonas aeruginosa* infection in cystic fibrosis: pathophysiological mechanisms and therapeutic approaches. Expert Rev. Respir. Med. 10(6):685-97.

Mohsenipour Z, Hassanshahian M (2015). The Effects of *Allium sativum* Extracts on Biofilm Formation and Activities of Six Pathogenic Bacteria. Jundishapur J. Microbiol. 8(8):1-7.

Murugan N, Malathi J, Umashankar V, Madhavan HNR (2017). Virulence genome analysis of *Pseudomonas aeruginosa* VRFPA10 recovered from patient with scleritis. Genom. Data 12:1-3.

Ndip RN, Dilonga HM, Ndip LM, Akoachere JFK, Akenji TN (2005). *Pseudomonas aeruginosa* isolates recovered from clinical and environmental samples in Buea, Cameroon: current status on biotyping and antibiogram. Trop. Med. Int. Health 10(1):74-81.

Okamoto K, Gotoh N, Nishino T (2001). Penem Antibiotics: *Pseudomonas aeruginosa* reveals high intrinsic resistance to penem resistance mechanisms and their interplay. Antimicrob. Agents Chemother. 45(7):1964-1971.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29(9):e45.

Ratthawongjirakul P, Thongkerd V (2016) Fresh garlic extract inhibits *Staphylococcus aureus* biofilm formation under chemopreventive and chemotherapeutic conditions. Songklanakarin J. Sci. Technol. 38(4):381-389.

Sakuragi Y, Kolter R (2007). Quorum-sensing regulation of the biofilm matrix genes (pel) of *Pseudomonas aeruginosa*. J. Bacteriol. 189(14):5383-5386.

Singh BN, Singh HB, Singh A, Singh BR, Mishra A, Nautiyal CS (2012). *Lagerstroemia speciosa* fruit extract modulates quorum sensing – controlled virulence factor production and biofilm formation in

Pseudomonas aeruginosa. Microbiology158:529-538.
Smyth AR, Cifelli PM, Ortori CA, Righetti K, Lewis S, Erskine P, Holland ED, Givskov M, Williams P, Cámara M, Barrett DA (2010). Garlic as an Inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis-a pilot randomized controlled trial. Pediatr. Pulmonol. 45:356-362.
Tanya S, Daniel Y (2009). *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance. J. Med. Microbiol. 58:1133-1148.
Vadekeetil A, Chhibber S, Harjai K (2014). Analysis of quorum sensing inhibition and phytochemicals in different extracts of garlic. Int. J. Pharm. Bio. Sci. 5(3):873-884.
Valadbeigi H, Sadeghifard N, Salehi MB (2017). Assessment of biofilm formation in *Pseudomonas aeruginosa* by antisense *mazE*-PNA. Microb. Pathog. 104:28-31.
Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A (2005). The pel genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. Microbiology 151:985-997.
Xu Z, Fang X, Wood TK, Huang ZJ (2013). A systems-level approach for investigating *Pseudomonas aeruginosa* biofilm formation. PloS One 8(2):e57050.

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Full Length Research Paper

In vitro evaluation of lactic acid bacteria isolated from traditional fermented *Shamita* and *Kocho* for their desirable characteristics as probiotics

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The study was conducted to evaluate *in vitro* probiotic properties of lactic acid bacteria (LAB) isolated from fermented Shamita and Kocho. Sixteen samples, 8 each of Shamita and Kocho, were collected from Arat-Kilo and Merkato sites in Addis Ababa, respectively. The average pH values of Shamita and Kocho samples were 3.52 and 3.44, respectively. A total of 140 LAB were isolated, of which 101 isolates (72%) were found to inhibit one or more of the sensitive test organisms *Shigella boydii* and *Salmonella typhimurium* but none of them had antimicrobial activity against *Staphylococcus aureus*. The inhibition diameters on agar medium ranged from 8.5 to 17.5 mm. The 101 isolates having antagonistic effects against the test organisms were evaluated for their bile tolerance. Thirty six isolates (36%) tolerated 0.3% bile salts for 48 h with 55 to 93% survival. The 36 bile tolerant isolates were evaluated for their acid tolerance and 25 (69%), 30 (83%) and 34 (94%) tolerated pH 2, pH 2.5 and pH 3 for 3 h, respectively. Further extension of the incubation period for 6 h reduced the number of isolates to 21 (58%) and 33 (92%) at pH 2 and pH 3. Thirty of the bile tolerant isolates (83%) showed 80 to 94% survival at pH 2.5 for 6 h. These isolates were selected as LAB candidates with probiotic potential. Based on their phenotypic characteristics, the 30 isolates were identified as *Lactobacillus* (17 isolates), *Leuconostoc* (6 isolates) and *Pediococcus* (4 isolates) and *Lactococcus* (3 isolates). Antibiotic resistance patterns of the 30 isolates showed 100% resistance against oxacillin but lower resistance to levofloxacin; 57% of the isolates were resistant to penicillin. However, all the isolates were sensitive to erythromycin and gentamicin. Multiple drug resistance patterns were observed in two isolates one each from Shamita *Lactobacillus* (S9) and Kocho *Lactobacillus* (K64) having multiple resistances to penicillin, levofloxacin and oxacillin. Nine isolates (30%) were selected as probiotic candidates for further test on different fermented foods.

Key words: Lactic acid bacteria; *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus* spp., acid tolerance, antimicrobial activity, bile tolerance.

INTRODUCTION

Fermentation is an ancient widely practiced technology and fermented foods are an essential part of diets in all parts of the world. Traditional fermented foods are indigenous to a particular area and have been developed

by the local people using age-old techniques and locally available raw materials (Rose, 1977). Traditional fermentation processes are increasingly attracting the attention of scientists and policy makers as a vital part of food security strategies and for their commercial value (Van de Sande, 1997).

Fermentation is a process in which raw substrates are converted into fermented food products by the action of microorganisms or their enzymes to desirable compounds that result in new aroma, flavor, taste and texture. Fermentation helps to increase the sensory quality, palatability and acceptability of the products (Campbell-Platt, 1987). Some of these processes involve ethanol production by yeasts or organic acids by lactic acid bacteria (LAB). Natural or spontaneous fermentation is considered as a means of improving the nutritional quality and safety of foods. It also helps to extend the shelf-life of foods by preventing the outgrowth of spoilage microorganisms and foodborne pathogens (Motarjemi, 2002).

LAB are well-known for their capacity to produce a variety of inhibitory substances including metabolic end products such as organic acids like lactic acid or acetic acid, carbon dioxide, hydrogen peroxide, diacetyl, reuterin, reutericyclin, antifungal peptides and bacteriocins. These substances are very important in food preservation (Holzapfel et al., 1995; El-Ziney et al., 2000). The application of LAB strains and/ or their antimicrobial products to inhibit unwanted bacteria in food was introduced to the concept of preservation. The majority of foodborne contaminants either pathogenic or non-pathogenic are sensitive to the organic acids accumulating that result in a low pH value of the growth environment and other antimicrobial substances produced by LAB. Therefore, the interest in the application of LAB and their metabolites in the prevention of food spoilage and the extension of the shelf-life of foods has increased during the last decade (Stiles, 1996). LAB also plays a role to maintain and promote human health. Some species of LAB reportedly have a beneficial role in health and well-being of the host which is defined as the probiotic effect (Guarner and Schaafsma, 1998).

Traditional preparation of fermented foods generally depends on naturally occurring LAB. However, the use of defined starter cultures is becoming popular in modern fermentation technology. The use of LAB strains as defined starters is desirable to improve more the safety and quality of fermented foods. Given the generally poor sanitary condition of traditional fermented foods, the use of selected LAB with high antimicrobial activity against the most frequent foodborne pathogenic bacteria could be an affordable way to improve the safety of fermented foods (Omar et al., 2006).

In Ethiopia, a wide range of traditional fermented foods and beverages are produced from different raw materials such as cereals, Enset (false banana), honey, milk, etc. (Kebede et al., 2002; Mogessie, 2006). Some of the most popular Ethiopian traditional fermented foods and beverages are Injera, Dabo, Ambasha, Kocho, Bulla, Ergo, Siljo, Tella, Tej, Arekie, Borde, Shamita and Kineto (Mogessie, 2006). Most of the customs and rituals involving the Ethiopian traditional fermented foods and beverages are still popular in urban areas, village communities and rural households. Shamita is a traditional Ethiopian plant fermented beverage of roasted barley which contains low alcohol with a thick consistency. It is consumed as a meal replacement commonly by those people who cannot afford a reasonable meal (Ketema et al., 1999). Kocho is a traditional Ethiopian fermented product which is prepared from the starchy pulp separated from the fibers of Enset plant (*Enset ventricosum*) corm and pseudo-stem and left to ferment spontaneously at ambient temperature in an earthen pit (Berhanu, 1987).

In a recent review the potential of probiotics in African fermented foods was suggested to have an impact on both nutrition and health (Franz et al., 2014). There are several studies that show the importance of LAB to improve food safety and quality of fermented food/beverage products in Ethiopia (Ketema et al., 1999; Asnake and Mogessie, 2010; Anteneh et al., 2011). There has been at least one report for search of isolates with desirable characters as probiotics from the traditional Ethiopian fermented product Shamita (Ketema et al., 1999). More studies are needed on probiotic properties of LAB of indigenous fermented foods that may help to obtain products with effective probiotic cultures for future application. This study was aimed at *in vitro* evaluation of desirable characteristics as probiotic properties of LAB isolated from Shamita and Kocho.

MATERIALS AND METHODS

Description of Kocho preparation processes

According to Berhanu (1987), the scrapings from pseudo-stems and pulverized corms of four to eight mature Enset plants (*Enset ventricosum*) are decorticated and pounded pulp (from which the fiber is removed) mixed and kneaded into a mash. The mash is rolled into a ball, then covered with fresh Enset leaves and kept in an earthen pit lined with fresh leaves of Enset. This is covered with discarded plant parts and left to ferment for 2-3 days. The fermented dough well mixed, kneaded and covered with fresh

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Enset leaves and placed in a pit lined with fresh Enset leaves, and top-pressed with heavy materials (like large stone) to ensure the airtight condition in the pit which leads to anaerobic condition. This is left to ferment spontaneously and produce the final product after a few weeks to several months or years depending on ambient temperature and the need of the family. The final product is usually squeezed, mashed on chopping board with knife to shorten the fiber. Kocho is an intermediate product as a kind of traditional fermented dough for baking Kocho bread.

Description of Shamita preparation processes

According to Ketema et al. (1999), Shamita is prepared by adding lightly roasted barley malt flour, salt, ground linseed and small amounts of spices and mixing the contents with water in air-dried and clean clay jar. The microorganisms responsible for the fermentation come mostly from back-slopping using a small amount of Shamita from a previous fermentation as well as from ingredients and equipment. Thereafter, the clay jar is sealed and left to ferment overnight.

Sample collection

Shamita and Kocho samples were selected based on the prediction of the presence of LAB in these fermented products. Based on this, a total of 16 samples, 8 each from Shamita (liquid suspension) and Kocho (solid mass) were collected between January and August 2012 from Arat-Kilo and Merkato sites in Addis Ababa, respectively. The samples were collected using sterilized 250 ml bottles and kept in a refrigerator until analysis.

pH measurement of samples of Shamita and Kocho

The pH of each sample was determined using a digital pH-meter (NIG 333, Naina Solaris Ltd, New Delhi, India), after mixing 10 ml of Shamita and 10 g of Kocho separately with 90 ml distilled water in a laboratory blender as suggested by Erkmen and Bozkurt (2004).

Isolation of lactic acid bacteria

For isolation of LAB, each of the 25 ml of Shamita and 25g of Kocho was separately mixed with 225 ml of buffered peptone water (0.1%, w/v) and homogenized using a laboratory blender. A volume of 0.1 ml of appropriate dilutions of Shamita and Kocho samples was each spread in duplicates on sterile MRS (de Man Rogosa Sharpe, Oxoid, London, England) agar plates. The plates were incubated at 32°C for 48 h in an anaerobic jar (Gas Pack Anaerobic System, BBL, New Delhi, India) (Vanden-Berg et al., 1993).

Purification and identification of lactic acid bacteria

Purification and identification of LAB were done using criteria as described by Kivanc et al. (2011), Harrigan and McCance (1990) and Wood and Holzapfel (1995).

Designation of the isolates

The isolates were designated with S for Shamita and K for Kocho, followed with different numbers.

Cell morphology

Cellular morphology of isolates was determined according to Tittsler and Sandholzer (1936).

KOH-test (test on lipopolysaccharide)

Gram reaction of the isolates was detected using 3% KOH method outlined by Gregersen (1978).

Catalase-test

Presence of catalase was determined by transferring colonies from 48 h old culture on MRS agar plate to a clean microscopic glass slide using a sterile wire loop and followed by adding two drops of 3% solution of hydrogen peroxide (H_2O_2) (Kovacs, 1956).

Cytochrome oxidase-test

This test was conducted following the method outlined by Kovacs (1956).

Acid and gas production from glucose

A colony of each LAB isolate grown for 48 h was inoculated into 5 ml MRS broth tubes containing 5% glucose and 0.01% phenol red and adjusted to pH 7.4. The tubes were incubated for 48 h at 32°C. The presence of free air space just above the broth in the inverted Durham tube was recorded as heterofermentative and the absence as homofermentative LAB. The change in color of the medium from red to yellow was considered as an indicator of acid production (Mueller, 1990).

Growth of the isolates at different temperatures

Growth of the isolates at 15°C, 30°C and 45°C was measured using the method described by Barbu (2008).

Growth of the isolates at different salt concentrations

Growth of the isolates at increasing salt concentrations (2, 4, and 6.5%) was tested following the protocol given by Ahmed and Kanwal (2004).

Test microorganisms

The test organisms (*Staphylococcus aureus* ATCC25923, *Salmonella typhimurium* ATCC13311 and *Shigella boydii* ATCC9289) were obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia, and used to evaluate the antimicrobial activity of LAB isolated from Shamita and Kocho.

Determination of antimicrobial activity of LAB isolates using agar well diffusion method

The antimicrobial activities of LAB isolates against selected foodborne pathogens were determined using the agar well diffusion

Table 1. pH values of Shamita and Kocho and number of LAB isolated from the samples.

Sample type	pH					No. of isolates	%
	Minimum	Maximum	Mean*	S.D	%CV		
Shamita	3.28	3.95	3.52	0.21	5.9	71	51
Kocho	3.22	3.81	3.44	0.20	5.8	69	49

*= Average of 8 samples each from Shamita and Kocho, being reading in duplicates of each samples, S.D = standard deviation, CV= coefficient of variation.

method described by Saranya and Hemashenpagam (2011). Hundred (100) µl of the cell free supernatants were used.

Bile and acid tolerance test

LAB isolates with antagonistic activity towards the test organisms were examined for bile tolerance following the procedure described in Dunne et al. (2001). Bile tolerant isolates were used for acid tolerance test following the procedure described by Hyronimus et al. (2000).

Antibiotic resistance test of LAB isolated from Shamita and Kocho

Clinical and Laboratory Standards Institute (CLSI, 2012) methods were applied. The antibiotic discs used in this study were penicillin (6 µg), erythromycin (15 µg), gentamicin (10 µg), levofloxacin (5 µg) and oxacillin (1 µg).

Statistical data analysis

The average pH samples of Shamita and Kocho from the duplicates of independent experiments were statistically analyzed using SPSS version 20. Variation in pH between Shamita and Kocho was compared using independent samples *t*-test at 0.05 p-values and the coefficient of variation (%, CV) value was calculated to determine if significant variation occurred in pH within the samples.

RESULTS AND DISCUSSION

In this study, a total of 16 samples, 8 each of Shamita and Kocho were collected and analyzed aseptically for presence of LAB. The average pH values of Shamita and Kocho samples were 3.52 and 3.44, respectively (Table 1). The variation in pH values within the samples of Shamita and Kocho was not significant (CV<10%) (Table 1). Similarly, there was no significant difference in pH values between Shamita and Kocho samples (p>0.05).

A total of 160 different colonies were selected and purified, of which 140 colonies were confirmed as LAB isolates. Out of these, 71 isolates (51%) and 69 isolates (49%) were from Shamita and Kocho samples, respectively (Table 1). The isolates were Gram positive, catalase negative, rod or cocci shaped and appeared in single, pairs, chains or tetrad cellular arrangement.

The pH value of Kocho in this study was much lower than the ones reported by Berhanu (1987) where the pH value of Kocho samples was around 4.2. Ayele and Berhanu (1998) reported that the pH of commercial fermented Kocho was 4.3, a higher pH than found in the present study. Mogessie and Tetemke (1995) also reported that the pH of ready to consume Shamita was around 4.2. Similarly, Ketema et al. (1999) reported that the average pH value of Shamita samples collected from local brewers in Addis Ababa was around 4.22 which was higher than the result of the present study. The difference in the pH value of the present study and other related studies could be due to the duration of fermentation or type of microorganisms involved during the fermentation as the samples were collected from local producers in open markets.

Grouping of the lactic acid bacteria isolates to different genera

Grouping of the isolates were done according to the criteria described by Harrigan and McCance (1990) and Wood and Holzapfel (1995). Some of the criteria used for grouping of LAB are shown in (Table 2).

Based on their morphological, biochemical and physiological characteristics, the 30 selected isolates were grouped into 4 different genera belonging to *Lactobacillus* (17 isolates, 57%), *Leuconostoc* (6 isolates, 20%), *Pediococcus* (4 isolates, 13%) and *Lactococcus* (3 isolates, 10%) as shown in Table 3. *Lactobacilli* were the most frequently isolated genus from Shamita and Kocho samples followed by *Leuconostoc* isolates from Kocho.

Morphological, biochemical and physiological characterization of LAB isolates

Of the total 140 LAB isolates, 30 isolates were selected for further identification based on their probiotic potential, such as antimicrobial activity, acid and bile tolerance. The 30 isolates were tested for their motility, oxidase activity, acid and gas production from glucose, growth at various

Table 2. Criteria used for classification of lactic acid bacteria isolates to different genera.

Cell shape	Glucose fermentation		Growth at T (°C)		Growth in medium with NaCl (%)	LAB genera
	Homo[1]	Hetero[2]	10	45	6.5	
Rod in pairs or chains	+	+	±	±	±	*Lactobacillus*
Cocci pairs or chains	+	-	+	-	-	*Lactococcus*
Cocci pairs or chains	-	+	+	-	±	*Leuconostoc*
Cocci in pairs or tetrads	+	-	±	±	±	*Pediococcus*

1= Homofermentative, 2 = heterofermentative, +=positive result, - =negative result.

temperatures and NaCl concentrations. All the 30 isolates were found to be non-motile in stab cultures, oxidase negative but positive for acid production from glucose.

Out of the 30 isolates, 20 isolates (67%) were homofermentative, and the other 10 isolates (33%) were considered as heterofermentative based on their glucose fermentation profile (Table 3). Among the homo-fermentative isolates, 8 isolates (40%) were obtained from Shamita and 12 isolates (60%) from Kocho. All the heterofermentative isolates were from Kocho. All the isolates were able to grow at 10°C and 15°C but unable to grow at 45°C. Likewise, 7 isolates (23%), 1 isolate (3%) and 4 isolates (13%) were not able to grow at 37°C, 4% and 6.5% of NaCl concentrations, respectively. Whereas, 23 isolates (77%), 29 isolates (97%) and 26 isolates (87%) were able to grow at 37°C, 4% and 6.5% of NaCl concentrations, respectively (Table 3).

This was in line with Kebede (2007) who indicated that all the strains isolated from Borde (another traditional Ethiopian cereal beverage) showed growth at 10°C, 15°C and 37°C incubation but reduction in growth rate was observed at 45°C. Similarly, Evelyne and Laksmi (2011) reported that all LAB isolated from Indonesian fermented Sayur Asin were able to grow at 10°C and 6.5% NaCl, but unable to grow at 45°C.

Similarly, Abdulkadir et al. (2011) reported that lactobacilli were dominant, from Ergo (traditional fermented milk) samples. Eyassu et al. (2012) also reported that *Lactobacillus* species isolated from Ititu (fermented camel milk) was the dominant genus which comprised about 58% of the total LAB isolates followed by *Lactococcus* species which accounted for 25%. Additionally, Girum et al. (2005) reported that LAB isolated from ready to consume Borde and Shamita were tentatively grouped into *Lactobacillus* (60 isolates), *Leuconostoc* (15 isolates), *Pediococcus* (18 isolates) and *Streptococcus* (25 isolates).

All the lactobacilli isolated from Shamita were homofermentative. However, in a microbiological study of Shamita fermentation by Ketema et al. (1999), found that the most dominant lactic flora comprised of both heterofermentative and homofermentative *Lactobacillus* spp. and that homofermentative LAB predominated after 24 h of Shamita fermentation. In a related work, Asnake and Mogessie (2010) reported that about 94% of the LAB isolated from Awaze, Qotchqotcha and Tef dough (all fermented Ethiopian foods) was homofermentative while 6% of the isolates were heterofermentative.

Antimicrobial activity of LAB isolates against selected foodborne pathogens

All the 140 isolates were subjected to antimicrobial activity test against 3 test organisms of which 101 isolates (72%) were found to inhibit one or more of the sensitive test organisms *Shigella boydii* ATCC9289 and *S. typhimurium* ATCC13311 (Table 4). No isolate showed antimicrobial activity against *S. aureus* ATCC25923. Out of these isolates (101), 76 isolates (75%) showed inhibitory effects against both *S. boydii* and *S. typhimurium* compared to 9 isolates (9%) and 16 isolates (16%) against *S. boydii* and *S. typhimurium* alone, respectively (Table 4).

The isolates from different sources showed variations in their inhibitory activity against the test organisms *S. boydii* and *S. typhimurium*. Eighty percent (80%) of the isolates from Kocho showed inhibitory activity against both test organisms, whereas only 30% from Shamita were active against the two test organisms. Among the test organisms, *S. typhimurium* was found to be the most sensitive. It was inhibited by 92 isolates (91%), whereas 85 isolates (84%) inhibited *S. boydii*.

In a similar work, Esayas et al. (2008) reported that out of 112 LAB isolated from Ergo only 12 isolates belonging to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* showed antimicrobial activity against some pathogenic bacteria, including *Salmonella typhi*, *Shigella flexineri, S. aureus* and *Escherchia coli* with inhibition zone ranged from 7 to 12 mm in diameters. However, Girum et al. (2005) observed that all the LAB isolates (118 isolates) originated from Borde and Shamita belonging to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* were found to inhibit the growth of the test strains, such as *S. aureus, Shigella flexneri, Salmonella* spp. and *E. coli* O157:H7 with inhibition zone ranged from 15 to 17 mm in diameters.

Table 3. Morphological, physiological and biochemical characteristics of LAB isolates.

LAB isolates code	Color and Shape of colony	Cell morphology and arrangement	Glucose fermentation	Gas production from glucose	Growth at T (°C)				Growth in medium with NaCl (%)		Identified as LAB genera
					10	15	37	45	4	6.5	
S6, S21, S26	W/R	Rods in pairs or chains	HoF	-	+	+	-	-	+	+	*Lactobacillus*
S9	W/R	Cocci in pairs or chains	HoF	-	+	+	-	-	+	-	*Lactococcus*
S38	C/R	Cocci pairs or chains	HoF	-	+	+	-	-	+	-	*Lactococcus*
S44, S47	C/R	Rods pairs or chains	HoF	-	+	+	-	-	+	+	*Lactobacillus*
S45	C/R	Cocci in pairs or tetrads	HoF	-	+	+	+	-	+	+	*Pediococcus*
K7	C/F	Cocci in pairs or tetrads	HoF	-	+	+	+	-	+	+	*Pediococcus*
K12	C/F	Rods in pairs or chains	HeF	+	+	+	+	-	+	+	*Lactobacillus*
K14	C/F	Cocci in pairs or chains	HoF	-	+	+	+	-	+	-	*Lactococcus*
K16	C/R	Cocci in pairs or chains	HeF	+	+	+	+	-	+	+	*Leuconostoc*
K19	C/F	Rods in pairs or chains	HoF	-	+	+	+	-	+	+	*Lactobacillus*
K24	C/R	Rods in pairs or chains	HeF	+	+	+	+	-	+	+	*Lactobacillus*
K25	C/R	Rods in pairs or chains	HoF	-	+	+	+	-	+	+	*Lactobacillus*
K26	C/R	Cocci in pairs or chains	HeF	+	+	+	+	-	+	+	*Leuconostoc*
K30	C/F	Cocci in pairs or tetrads	HoF	-	+	+	+	-	+	+	*Pediococcus*
K32, K33	C/R	Rods in pairs or chains	HoF	-	+	+	+	-	+	+	*Lactobacillus*
K38, K64	C/F	Rods in pairs or chains	HoF	-	+	+	+	-	+	+	*Lactobacillus*
K59	C/F	Cocci in pairs or tetrads	HoF	-	+	+	+	-	+	+	*Pediococcus*
K61	C/F	Rods in pairs or chains	HoF	-	+	+	+	-	-	-	*Lactobacillus*
K65	W/F	Rods in pairs or chains	HoF	-	+	+	+	-	+	+	*Lactobacillus*
K70	C/R	Cocci in pairs or chains	HeF	+	+	+	+	-	+	+	*Leuconostoc*
K73, K76	C/F	Rods in pairs or chains	HeF	+	+	+	+	-	+	+	*Lactobacillus*
K75	C/F	Cocci in pairs or chains	HeF	+	+	+	+	-	+	+	*Leuconostoc*
K79	W/F	Cocci in pairs or chains	HeF	+	+	+	+	-	+	+	*Leuconostoc*
K80	W/R	Cocci in pairs or chains	HeF	+	+	+	+	-	+	+	*Leuconostoc*

W/R=white raised, W/F=white flat, C/R=creamy raised, C/F=creamy flat, HoF=homofermentative, HeF=heterofermentative, +=positive result, - =negative result, T° =temperature in degree centigrade.

Bile tolerance patterns of LAB isolates at 0.3% bile salt concentration

Among the isolates subjected to bile tolerance test from Shamita, 29 isolates (66%) and 10 isolates (23%) survived for 24 and 48 h, respectively. Whereas, 50 isolates (88%) and 26 isolates (46%) from Kocho tolerated incubation for 24 and 48 h, respectively (Table 5). A total of 101 LAB isolates having antagonistic effects were evaluated for their tolerance to bile salt. Out of the 101 isolates, 79 isolates (78%) and 36 isolates (36%) tolerated incubation in MRS broth supplemented with 0.3% bile salt for 24 and 48 h, respectively (Table 5).

Survival rate of the isolates ranged from 69 to 95% for 24 h incubation period in MRS broth supplemented with 0.3% bile salt (Table 6). *Lactobacillus* (S6) isolate was the most tolerant

Table 4. Antagonistic activity of LAB isolated from Shamita and Kocho against test organisms by agar well diffusion method.

Source	No. of LAB isolates with inhibitory effects against test organisms		
	S. boydii* and *S. typhimurium	***S. boydii***	***S. typhimurium***
Shamita	21 (30%)	9 (9%)	14 (14%)
Kocho	55 (80%)	0 (0%)	2 (2%)
Total	76 (75%)	9 (9%)	16 (16%)

Table 5. Bile tolerance patterns of LAB isolated from Shamita and Kocho at 0.3% bile salt.

Source	No. of tested isolates	No. of survived isolates at 0.3% bile	
		24 h	48 h
Shamita	44	29 (66%)	10 (23%)
Kocho	57	50 (88%)	26 (46%)
Total	101	79 (78%)	36 (36%)

with 95% survival rate at 24 h which was isolated from Shamita, followed by *Lactobacillus* (S47) from Shamita and *Lactobacillus* (K24) from Kocho with 94% survival rate at 24 h (Table 6). On the other hand, survival rate of the isolates ranged from 55 to 93% for 48 h incubation period. *Lactobacillus* (K24) isolated from Kocho was the most effective with 93% survival rate among the isolates for 48 h incubation, followed by *Leuconostoc* (K80) with 90% survival rate and *Lactobacillus* (K12) with 89% survival rate both from Kocho (Table 6).

In a related study, Asnake and Mogessie (2010) showed that 58% of the 257 tested LAB isolates belonging to the genus *Lactobacillus, Lactococcus*, *Pediococcus* and *Leuconostoc* survived bile concentration greater than 0.3% for 5 days incubation period. A study conducted by Evelyne and Laksmi (2011) indicated that, all the 25 isolates of LAB belonging to the genus *Lactobacillus* obtained from Indonesian fermented Sayur Asin survived in an environment containing 0.3% and 0.5% bile salt for 4 h. Similarly, Jacobsen et al. (1999) studied 47 *Lactobacillus* spp. isolated from Ghanian fermented maize and dairy products for their probiotic activities by *in vitro* techniques and reported that 46 isolates (98%) showed tolerance to 0.3% bile salt in MRS broth for 4 h of incubation. In addition, Anteneh et al. (2011) indicated that 9 (33%) out of the 27 LAB strains isolated from Ethiopian locally fermented products tolerated 0.3% bile for 48 h incubation.

An acid tolerance pattern of LAB isolates at different pH values

Thirty-six LAB isolates that showed bile tolerance were evaluated for their acid tolerance patterns. Testing of the acid tolerance of the isolates showed that, out of 36 isolates, 25 isolates (69%), 30 isolates (83%) and 34 isolates (94%) tolerated pH 2, pH 2.5 and pH 3 for 3 h, respectively. Further extension of the incubation period for 6 hours reduced the surviving isolates to 21(58%) and 33 (92%) at pH 2 and pH 3, respectively. But, the number of surviving isolates at pH 2.5 after incubation for 6 h was similar to the result obtained for incubation at the same pH for 3 h (Table 7).

In a similar study, Anteneh et al. (2011) reported that out of 27 LAB isolates tested for acid tolerance, 9 isolates (33%) showed a survival rate of ≥50% at pH 2.5 for 3 and also for 6 h. Asnake and Mogessie (2010) reported that out of 257 LAB isolates belonging to the genera *Lactobacillus, Lactococcus*, *Pediococcus* and *Leuconostoc*, only 10 isolates (4%) were tolerant to pH 2 for 3 h. However, all the LAB isolates did not survive at pH 2 for 6 h. With respect to pH 2.5, 41 isolates (16%) and 14 isolates (5%) were found to be tolerant for 3 and 6 h incubation time, respectively. The authors also pointed out that 198 isolates (77%) could survive at pH 3 for the first 3 h, but further incubation for 6 h decreased the number of surviving isolates to 172 (67%). According to Ketema et al. (2009), about 44% of the 99 LAB isolates had 100% survival rate for 3 h in MRS broth at pH 3 but only 7 isolates (7%) for 3 h, 2 isolates (2%) for 6 h had 100% survival rate at pH 2.5, respectively. The authors also added that about 4 isolates (4%) showed 60-75% survival rate at pH 3 for 3 h, whereas about 40 (41%) showed 90 to 100% survival rate for 6 h at pH 3.

Generally, survival rate of the isolates ranged from 77 to 97% at different pH values for 3 and 6 h incubation period (Table 8). *Lactobacillus* (K24) was the most

Table 6. Survival rate of representatives of LAB isolated from Shamita and Kocho grown in MRS broth with 0.3% bile salt.

No.	Isolates (code)	Mean count in log cfu/ml (% survival rate) of the isolates grown in MRS broth with 0.3% bile salt		
		Initial	24 h	48 h
1	*Lactobacillus* (S6)	6.52	6.16 (95)	5.41 (83)
2	*Lactococcus* (S9)	6.45	4.84 (75)	4.69 (73)
3	*Lactobacillus* (S21)	6.21	5.67 (91)	5.09 (82)
4	*Lactobacillus* (S26)	6.61	4.92 (74)	3.61 (55)
5	*Lactococcus* (S38)	6.09	4.22 (69)	3.95 (65)
6	*Lactobacillus* (S44)	6.56	5.22 (80)	4.59 (70)
7	*Pediococcus* (S45)	6.14	5.36 (87)	4.86 (79)
8	*Lactobacillus* (S47)	6.41	6.02 (94)	4.71 (77)
9	*Pediococcus* (K7)	6.35	5.63 (89)	5.57 (88)
10	*Lactobacillus* (K12)	6.42	5.91 (92)	5.72 (89)
11	*Lactococcus* (K14)	6.56	5.89 (90)	5.67 (86)
12	*Leuconostoc* (K16)	6.37	5.44 (85)	5.19 (81)
13	*Lactobacillus* (K19)	6.49	5.69 (88)	5.31 (82)
14	*Lactobacillus* (K24)	6.65	6.24 (94)	6.19 (93)
15	*Lactobacillus* (K25)	6.14	5.37 (87)	4.78 (78)
16	*Leuconostoc* (K26)	6.22	5.52 (89)	4.91 (79)
17	*Pediococcus* (K30)	6.05	5.36 (89)	5.13 (85)
18	*Lactobacillus* (K32)	6.12	5.31 (87)	4.79 (78)
19	*Lactobacillus* (K33)	6.43	5.45 (85)	5.39 (84)
20	*Lactobacillus* (K38)	6.07	5.53 (91)	5.23 (86)
21	*Pediococcus* (K59)	6.33	5.48 (87)	5.21 (82)
22	*Lactobacillus* (K61)	6.31	5.42 (86)	5.19 (82)
23	*Lactobacillus* (K64)	6.59	6.04 (92)	5.09 (77)
24	*Lactobacillus* (K65)	6.15	5.24 (85)	4.14 (67)
25	*Leuconostoc* (K70)	6.52	5.54 (85)	5.18 (79)
26	*Lactobacillus* (K73)	6.42	5.43 (85)	5.13 (80)
27	*Leuconostoc* (K75)	6.44	5.53 (86)	5.36 (83)
28	*Lactobacillus* (K76)	6.28	5.58 (89)	5.29 (83)
29	*Leuconostoc* (K79)	6.71	6.13 (91)	5.87 (87)
30	*Leuconostoc* (K80)	6.18	5.76 (93)	5.54 (90)

Table 7. Acid tolerance patterns of LAB isolated from Shamita and Kocho at different pH values after 3 and 6 h exposure.

Source	No. of tested isolates	No. of survived isolates (%)					
		3h			6h		
		pH 2	pH 2.5	pH 3	pH 2	pH 2.5	pH 3
Shamita	10	5 (50%)	8 (80%)	9 (90%)	2 (20%)	8 (80%)	9 (90%)
Kocho	26	20 (77%)	22 (85%)	25 (96%)	19 (73%)	22 (85%)	24 (92%)
Total	36	25 (69%)	30 (83%)	34 (94%)	21 (58%)	30 (83%)	33 (92%)

tolerant at pH 2 for 3 h incubation period with 89% survival rate, followed by *Lactobacilli* isolates (K12, K25, K33), *Lactococci* isolates (K14, S9) and *Leuconostoc* (K70) with 88% survival rate. Similarly, *Lactobacillus* (K24) was the most tolerant at pH 2 for 6 h incubation period with 88% survival rate, followed by *Lactobacillus*

Table 8. Survival rate of representatives of LAB isolated from Shamita and Kocho at different pH values after 3 and 6 h exposure.

No.	Isolates (code)	Mean count in log cfu/ml (% survival rate) of LAB isolates						
		Initial	3 h			6 h		
			pH 2	pH 2.5	pH 3	pH 2	pH 2.5	pH 3
1	*Lactobacillus* (S6)	6.42	5.32 (83)	5.61 (87)	5.76 (90)	5.11(79)	5.59 (87)	5.73 (89)
2	*Lactococcus* (S9)	6.15	5.43 (88)	5.51 (90)	5.79 (94)	4.78 (78)	5.49 (89)	5.72 (93)
3	*Lactobacillus* (S21)	6.49	4.78 (74)	5.86 (90)	5.87 (90)	0 (0)	5.71 (88)	5.73 (88)
4	*Lactobacillus* (S26)	6.58	5.27 (80)	5.81 (88)	5.89 (90)	0 (0)	5.69 (86)	5.79 (88)
5	*Lactococcus* (S38)	6.32	0 (0)	5.74 (91)	6.12 (97)	0 (0)	5.45 (86)	5.95 (94)
6	*Lactobacillus* (S44)	6.46	5.63 (87)	6.16 (95)	6.29 (97)	0 (0)	6.08 (94)	6.19 (96)
7	*Pediococcus* (S45)	6.34	0 (0)	5.72 (90)	5.74 (91)	0 (0)	5.46 (86)	5.71 (90)
8	*Lactobacillus* (S47)	6.41	0 (0)	5.62 (88)	5.85 (91)	0 (0)	5.57 (87)	5.68 (89)
9	*Pediococcus* (K7)	6.31	5.38 (85)	5.46 (87)	5.56 (88)	5.28 (84)	5.37 (85)	5.48 (87)
10	*Lactobacillus* (K12)	6.37	5.61 (88)	5.68 (89)	5.76 (90)	5.53 (87)	5.62 (88)	5.71 (90)
11	*Lactococcus* (K14)	6.41	5.64 (88)	5.72 (89)	5.77 (90)	5.49 (86)	5.55 (87)	5.59 (87)
12	*Leuconostoc* (K16)	6.25	5.41 (87)	5.47 (88)	5.83 (93)	5.23 (84)	5.37 (86)	5.68 (91)
13	*Lactobacillus* (K19)	6.59	5.72 (87)	5.81 (88)	5.86 (89)	5.56 (84)	5.64 (86)	5.77 (88)
14	*Lactobacillus* (K24)	6.47	5.75 (89)	5.91 (91)	5.98 (92)	5.68 (88)	5.79 (89)	5.92 (91)
15	*Lactobacillus* (K25)	6.16	5.41(88)	5.46 (89)	5.54 (90)	5.26 (85)	5.44 (88)	5.49 (89)
16	*Leuconostoc* (K26)	6.11	5.38 (88)	5.49 (90)	5.56 (91)	5.29 (87)	5.42 (89)	5.51 (90)
17	*Pediococcus* (K30)	6.53	5.69 (87)	5.74 (88)	5.84 (89)	5.42 (83)	5.59 (86)	5.67 (87)
18	*Lactobacillus* (K32)	6.17	5.32 (86)	5.43 (88)	5.48 (89)	5.18 (84)	5.31 (86)	5.41 (88)
19	*Lactobacillus* (K33)	6.13	5.37 (88)	5.43 (89)	5.49 (90)	5.27 (86)	5.34 (87)	5.42 (88)
20	*Lactobacillus* (K38)	6.68	5.65 (85)	5.78 (87)	5.88 (88)	5.34 (80)	5.61 (84)	5.76 (86)
21	*Pediococcus* (K59)	6.31	5.23 (83)	5.39 (85)	5.47 (87)	4.97 (79)	5.08 (81)	5.35 (85)
22	*Lactobacillus* (K61)	6.38	5.06 (79)	5.32 (83)	5.45 (85)	4.91 (77)	5.11 (80)	5.37 (84)
23	*Lactobacillus* (K64)	6.39	5.12 (80)	5.23 (82)	5.46 (85)	4.98 (78)	5.15 (81)	5.36 (84)
24	*Lactobacillus* (K65)	6.21	0 (0)	5.33 (86)	5.54 (89)	0 (0)	5.16 (83)	5.35 (86)
25	*Leuconostoc* (K70)	6.27	5.51 (88)	5.56 (89)	5.87 (94)	5.37 (86)	5.49 (88)	5.63 (90)
26	*Lactobacillus* (K73)	6.29	5.24 (83)	5.37 (85)	5.48 (87)	5.13 (82)	5.26 (84)	5.38 (86)
27	*Leuconostoc* (K75)	6.24	5.02 (80)	5.23 (84)	5.39 (86)	4.86 (78)	5.07 (81)	5.17 (83)
28	*Lactobacillus* (K76)	6.26	0 (0)	5.42 (87)	5.54 (88)	0 (0)	5.31 (85)	5.41 (86)
29	*Leuconostoc* (K79)	6.28	5.34 (85)	5.44 (87)	5.52 (88)	5.15 (82)	5.37 (86)	5.46 (87)
30	*Leuconostoc* (K80)	6.18	4.93 (80)	5.31 (86)	5.78 (94)	0 (0)	(85)	5.43 (88)

(K12) and *Leuconostoc* (K26) with 87% survival rate (Table 8).

Among the isolates exposed to pH 2.5 for 3 h, *Lactobacillus* (S44) was the most tolerant with 95% survival rate, followed by *Lactobacillus* (K24) and *Lactococcus* (S38) with 91% survival rate. *Lactobacillus* (S44) was also the most effective at pH 2.5 for 6 h with 94% survival rate, followed by *Lactobacillus* (K24), *Leuconostoc* (K26) and *Lactococcus* (S9) with 89% survival rate. Similarly, *Lactobacillus* (S44) was found to be the most tolerant to pH 3 for 6 h with 96% survival rate, followed by *Lactococcus* (S38) with 94% survival rate (Table 9). The results revealed that 30 isolates had 80 to 94% survival rate at pH 2.5 for 6 h and these isolates were selected as LAB candidates with probiotic potential (Table 8).

As reported by Hyronimus et al. (2000), all LAB isolated from cattle feces did not survive at pH 2.5 for 3 h. Charteris et al. (1998) also reported a complete loss of viability of lactobacilli isolated from traditional Greek cheese at pH 2.5 for 3 h. Thirabunyanon et al. (2009) reported that 3 of 5 lactobacilli isolated from Thailand fermented dairy milk survived at pH 2.5 for 3 h. When compared to the weak tolerance to low pH seen in some LAB investigated in earlier works (Charteris et al., 1998; Hyronimus et al., 2000), isolates of this study had a higher survival rate at pH 3 and pH 2.5 as well as at pH 2. Therefore, it is possible to consider the isolates have the potential to be good as candidates for probiotics as they have shown better survival rate in *in vitro* selection

Table 9. Antibiotic resistance patterns of LAB isolated from Shamita and Kocho.

Source	Isolates	No. of tested isolates	No. of resistant isolates (%)		
			Pen (6 µg)	Lev (5 µg)	Oxa (1 µg)
Shamita	Lactobacilli	5	5	0	5
	Lactococci	2	2	1	2
	Pediococci	1	1	0	1
Kocho	Lactobacilli	12	7	2	12
	Lactococci	1	0	0	1
	Leuconostoc	6	1	0	6
	Pediococci	3	1	0	3
Total		30	17 (57%)	3 (10%)	30 (100%)

Pen=Penicillin, Lev=levofloxacin, Oxa=oxacillin.

criteria during the experiment. This could be also an indication of their possible survival in the acidic condition of the stomach of the human host before their transit to the small intestine.

Antibiotic resistance or susceptibility patterns of LAB isolated from Shamita and Kocho

A total of 30 LAB isolates were tested for antibiotic resistance or susceptibility patterns. 100% resistance by isolates of Shamita and Kocho was observed against oxacillin. But, a relatively lower proportion of resistance for levofloxacin by isolates from Shamita and Kocho was observed. In addition, 17 isolates (57%) were resistant to penicillin (Table 9). None of them showed resistance to erythromycin and gentamicin (data not shown). Among the *Lactobacillus* isolates, 5 isolates (29%) from Shamita and 7 isolates (41%) from Kocho were resistant to penicillin. Likewise, 2 isolates (12%) of lactobacilli from Kocho were resistant to levofloxacin. *Lactococcus*, *Leuconostoc* and *Pediococcus* isolates showed varying degrees of resistance against penicillin and levofloxacin (Table 9).

Antibiotic resistance to penicillin by the LAB isolates was higher than of the work of Asnake and Mogessie (2010) who reported that only 43% of the isolates showed resistance to penicillin. On the contrary, Ketema et al. (2010) and Abdulkadir et al. (2011) reported that all their LAB isolates were sensitive to penicillin. Although susceptibility towards the inhibitors of cell wall synthesis such as penicillin and ampicillin has been observed in many species of LAB (Danielsen and Wind, 2003; Delgado et al., 2005; Zdolec et al., 2011), more than half of the isolates tested in the current study were found to be resistant to penicillin.

Multiple drug resistance patterns were observed in two LAB isolates one each from Shamita *Lactococcus* (S9) and Kocho *Lactobacillus* (K64) with multiple resistances to penicillin, levofloxacin and oxacillin (data not shown).

Probiotic potential patterns of the selected LAB isolates

A total of 30 LAB isolates were screened as candidates with probiotic potential based on *in vitro* evaluation of antimicrobial activity, bile and acid tolerance results. Screening was also done after ranking the results of antimicrobial activity, acid-bile tolerance and antibiotic resistance profile to identify isolates with the best probiotic potential among the 30 selected isolates. A ranking of the results was done from the overall sum calculated after standardizing the raw data to 5 point scale as indicated in (Table 10).

Out of 30 isolates, 9 isolates (30%) were selected as best probiotic candidate of LAB isolates based on their standardized probiotic potential results (Table 10). The 9 selected isolates were *Lactobacillus* (K12), *Lactobacillus* (K19), *Lactobacillus* (K38), *Leuconostoc* (K79), *Lactobacillus* (S44), *Lactobacillus* (K25), *Leuconostoc* (K26), *Lactobacillus* (K32) and *Lactobacillus* (K64). Among these isolates, *Lactobacillus* (K12) isolate showed the best overall probiotic property, followed by *Lactobacillus* (K19), *Lactobacillus* (K38) and *Leuconostoc* (K79) isolates (Table 10).

Seven *Lactobacillus* and 2 *Leuconostoc* isolates were selected as the best LAB genera with probiotic potential. The results also show that LAB isolated from Kocho have the best overall probiotic potential compared to isolates from Shamita (Table 10).

Conclusion

The morphological, physiological and biochemical

Table 10. Probiotic potential patterns of LAB isolated from Shamita and Kocho.

Isolates (code)	Mean diameter of inhibition zone in (mm)		%survival rate at 0.3% bile for 48 h	%survival rate at pH 2.5 for 6 h	No. of antibiotic resisted by the isolates	Rank
	S. boydii	*S. typhimurium*				
Lactobacillus(K12)	12.5	12	89	88	2	1
Lactobacillus (K19)	13	13.5	82	86	2	2
Lactobacillus (K38)	14.5	13.5	86	84	1	
Leuconostoc (K79)	17.5	13	87	86	1	
Lactobacillus (S44)	12.5	11.5	70	94	2	
Lactobacillus (K25)	12	10	78	88	2	
Leuconostoc (K26)	10.5	9.5	79	89	2	3
Lactobacillus (K32)	12.5	11	78	86	2	
Lactobacillus (K64)	13.5	14.5	77	81	3	
Lactobacillus (S21)	9	9.5	82	88	2	
Lactobacillus (S47)	12.5	10.5	77	87	2	
Pediococcus (K7)	11.5	12.5	88	85	1	4
Lactococcus (K14)	12	11.5	86	87	1	
Lactobacillus (K24)	-	11.5	93	89	2	
Lactobacillus (K76)	17.5	14	83	85	1	
Leuconostoc (K80)	16	12.5	90	85	1	
Lactococcus (S9)	10	-	73	89	3	
Leuconostoc (K16)	12.5	11.5	81	86	1	
Pediococcus (K30)	12.5	11	85	86	1	5
Lactobacillus (K33)	12.5	11	84	87	1	
Pediococcus (K59)	13	11	82	81	2	
Leuconostoc (K70)	10	13	79	88	1	
Leuconostoc (K75)	14.5	14	83	81	1	
Lactobacillus (S26)	8.5	12.5	55	86	2	
Lactococcus (S38)	9.5	11.5	65	86	2	
Pediococcus (S45)	12	-	79	86	2	6
Lactobacillus (K61)	10.5	10.5	82	80	2	
Lactobacillus (K65)	8.5	13	67	83	2	
Lactobacillus (S6)	10	-	83	87	2	7
Lactobacillus (K73)	12.5	10.5	80	84	1	

- = no inhibition zone.

characteristics of isolates belonging to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Lactococcus* were present in Shamita and Kocho. *Lactobacillus* isolates were the most frequently isolated groups from Shamita and Kocho samples followed by *Leuconostoc* isolates from Kocho.

A total of nine isolates, of which seven *Lactobacillus* isolates; six from Kocho (K12, K19, K25, K32, K38, K64) and one from Shamita (S44), and two *Leuconostoc* isolates (K26, K79) from Kocho were found to have potentially good probiotic characteristics. It is suggested that these isolates can be good candidates for the dairy industry as probiotic cultures in the future. More tests are required to show their potential as health promoting LAB.

Conflict of Interests

The authors have not declared any conflict of interest.

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REFERENCES

Abdulkadir B, Tsige K, Ketema B (2011). Antimicrobial susceptibility pattern of lactic acid bacteria isolated from *ergo*, a traditional Ethiopian fermented milk, Jimma, southwest Ethiopia. Ethiop. J. Educ. Sci. 7 9-17.

Ahmed T, Kanwal R (2004). Biochemical characteristics of lactic acid producing bacteria and preparation of camel milk cheese by using starter culture. Pak. Vet. J. 24:87-91.

Anteneh T, Tetemke M, Mogessie A (2011). Evaluation of the *in vitro* and *in vivo* probiotic qualities of lactic acid bacteria recovered from locally fermented products. Int. J. Probiotics Prebiotics 6:101-108.

Asnake D, Mogessie A (2010). Evaluation of the probiotic properties and antibiotic resistance of lactic acid bacteria isolated from *awaze, qotchqotcha* and *tef* dough, traditional Ethiopian fermented foods. Int. J. Food Saf. 12:187-191.

Ayele N, Berhanu AG (1998). Effect of heat treatment on the antimicrobial properties of *tef* dough, *injera*, *kocho* and *aradisame* and the fate of selected pathogens. World J. Microbiol. Biotechnol.14:63-69.

Barbu V (2008). Phenotypical characterization of several lactic acid bacteria strains isolated from wheat's epiphyte microbiota. Roum. Biotechnol. Lett.13:4074-4085.

Berhanu AG (1987). Kocho fermentation. J. Appl. Bacteriol. 62:473-477.

Campbell-Platt G (1987). *Fermented Foods of the World*: *A Dictionary and Guide*. Butterworths, London, pp. 193-195.

Charteris WP, Kelly PM, Morelli L, Collins JK (1998). Development and application of in-vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 84:759-768.

Clinical and Laboratory Standards Institute (2012). Performance Standards for Antimicrobial Disk Susceptibility Tests; Eleventh Edition. Approved standard M02-A11. CLSI, Wayne, P.A., USA.

Danielsen M, Wind A (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int. J. Food Microbiol. 82:1-11.

Delgado S, Florez AB, Mayo B (2005). Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr. Microbiol. 50:202-207.

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgelard G, Daly C, Killey BC, O'Sullivan G, Shananhan F, Collins JK (2001). *In-vitro* selection criteria for probiotic bacteria of human origin: correlation with *in-vivo* findings. Am. J. Clin. Nutr. 73:386S-292S.

El-Ziney MG, Debevere J, Jakobsen M (2000). Reuterin. In: *Natural Food Antimicrobial Systems*, pp. 567-587, (Naidu, A. S., ed). CRC Press, London.

Erkmen O, Bozkurt H (2004). Quality characteristics of retailed *sucuk* (Turkish dry-fermented sausage). Food Technol. 42:63-69.

Esayas A, Fekadu B, Santhanam A (2008). Isolation and characterization of inhibitory substance producing lactic acid bacteria from *Ergo*, Ethiopian traditional fermented milk. Livest. Res. Rural Dev. 20:1-5.

Evelyne M, Laksmi L (2011). Isolation and screening lactic acid bacteria from sayur asin as starter for probiotic beverages. The 3rd International Conference of Indonesian Society for Lactic Acid Bacteria, January 21-22, Bulaksumur, Yogyakarta, Indonesia, pp. 1-7.

Eyassu S, Araya A, Kurtu MY, Zelalem Y (2012).Isolation and characterization of lactic acid bacteria from *ititu*: Ethiopian traditional fermented camel milk. J. Camelid Sci. 5:82-98.

Franz CMA, Huch M, Mathara JM, Abriouel H, Benomar N, Reid G, Galvez A, Holzapfel WH (2014). African fermented foods and probiotics. Int. J. Food Microbiol. 190:84-96.

Girum T, Eden E, Mogessie A (2005). Assessment of the antimicrobial activity of lactic acid bacteria isolated from *borde* and *shamita*, traditional Ethiopian fermented beverages, on some foodborne pathogens and effect of growth medium on the inhibitory activity. Int. J. Food Saf. 5:13-20.

Gregersen T (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. J. Appl. Microbiol. Biotechnol. 5:123-127.

Guarner F, Schaafsma GJ (1998). Probiotics. Int. J. Food Microbiol. 39:237-238.

Harrigan WF, McCance ME (1990). Laboratory Methods in Food and Dairy Microbiology, 8th ed. Academic Press Inc., London, pp. 286-303.

Holzapfel WH, Geisen R, Schillinger U (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24:343-362.

Hyronimus B, Le Marrec C, Sassi AH, Deschamps A (2000).Acid and bile tolerance of spore forming lactic acid bacteria. Int. J. Food Microbiol. 61:193-197.

Jacobsen CN, Nielsen VR, Hayford AE, Moller PL, Michaelsen KF, Parregaard A, Sandstro B, Tvede M, Jakobsen, M (1999). Screening of probiotic activities of forty-seven strains of *lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Appl. Environ. Microbiol. 65:4949-4956.

Kebede A (2007). Isolation, characterization and identification of lactic acid bacteria involved in traditional fermentation of *borde*, an Ethiopian cereal beverage. Afr. J. Biotechnol .6:1469-1478.

Kebede A, Fekadu B, Langsrud T, Narvhus JA (2002). Indigenous processing methods and raw materials of borde, an Ethiopian fermented beverage. J. food Technol. Afr. 7:59-64.

Ketema B, Tetemke M, Mogessie A (1999). Microbiology of the fermentation of *shamita*, a traditional Ethiopian fermented beverage. *SINET:* Ethiop. J. Sci. 22:113-126.

Ketema B, Tetemke M, Mogessie A (2009). *In vitro* evaluation of probiotic potential of lactic acid bacteria isolated from *wakalim*, a traditional Ethiopian fermented beef sausage. Ethiop. J. Health Sci. 19:21-27.

Ketema B, Tetemke M, Mogessie A (2010). Antibiotic susceptibility patterns of LAB isolated from *wakalim*, a traditional Ethiopian fermented beef sausage. J. Food Saf. 30:213-223.

Kivanc M, Yilmaz M, Cakir E (2011). Isolation and identification of lactic acid bacteria from *boza*, and their microbial activity against several reporter strains. Turk. J. Biol. 35:313-324.

Kovacs N (1956). Identification *Pseudomonas pyocyana* by the oxidase

reaction. Nat. 178:703.
Mogessie A (2006). A review on the microbiology of indigenous fermented foods and beverages of Ethiopia. Ethiop. J. Biol. Sci. 5:189-245.
Mogessie A, Tetemke M (1995). Some microbiological and nutritional properties of *borde* and *shamita*, traditional Ethiopian fermented beverages. Ethiop. J. Health Dev. 9:105-110.
Motarjemi Y (2002). Impact of small scale fermentation technology on food safety in developing countries. Int. J. Food Microbiol. 75:213-229.
Mueller T (1990). Comparison of methods for differentiation between homofermentative and heterofermentative lactic acid bacteria. Zentralbl.Microbiol. 145:363-366.
Omar NB, Abriouel H, Lucas R, Martinez-Canamero M, Guyot JP, Galvez A (2006). Isolation of bacteriocinogenic *Lactobacillus plantarum*strains from ben saalga, a traditional fermented gruel from Burkina Faso. Int. J. Food Microbiol. 112:44-50.
Rose AH (1977). Alcoholic beverages. In: *Economic Microbiology*, pp.413-418, (Rose, A.H., ed). Academic Press, London.
Saranya S, Hemashenpagam N (2011).Antagonistic activity and antibiotic sensitivity of lactic acid bacteria from fermented dairy products. Adv. Appl. Sci. Res. 2:528-534.
Stiles ME (1996).Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70: 331-345.
Thirabunyanon M, Boonprasom P, Niamsup P (2009). Probiotic potential of lactic acid bacteria isolated from fermented dairy milk on antiproliferation of colon cancer cells. Biotechnol. Lett. 31:571-576.
Tittsler RP, Sandholzer LA (1936). The use of semi-solid agar for the detection of bacterial motility. J. Bacteriol. 31:575-580.
Van de Sande T (1997). Socio-economic pitfalls of enhancing indigenous capabilities in household fermentation. Food Contr. 8:303-310.
Vanden-Berg DJC, Smith A, Pot B, Ledeboer AM, Kerstens K, Verbakel JMA, Verrips CT (1993). Isolation, screening and identification of lactic acid bacteria from traditional food fermentation processes and culture collections. J. Food Biotechnol. 7:189-205.
Wood BJB, Holzapfel WH (1995). The Genera of Lactic Acid Bacteria. Chapman and Hall, London, pp.1-392.
Zdolec N, Filipovic I, FlecK ZC, Maric A, Jankuloski D, Kozacinski L, Njari B (2011). Antimicrobial susceptibility of lactic acid bacteria isolated from fermented sausages and raw cheese. Vet. Arch. 81:133-141.

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Full Length Research Paper

Differentiation of *Urochloa brizantha* cultivars by inter-simple sequence repeat (ISSR) markers in seed samples

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Approximately 80-90% of cultivated grassland areas in Brazil are covered by *Urochloa brizantha* [syn. *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf.]. Some genotypes of *Urochloa* have being widely used with a wrong nomenclature, like species and cultivars. In this way, the *Urochloa* cultivar identification is primordial for breeding programs and seed production. Considering the importance of genetic purity in commercialized seed lots, the present work aimed to evaluate the use of inter-simple sequence repeat (ISSR) markers in six cultivars of *U. brizantha* (Xaraés; Piatã; Basilisk; MG4; MG5 and Marandu) to discriminate and determine the contamination in seed batches. Results showed that it is possible to discriminate all cultivar with only two primers in pure samples. Basilisk was confirmed as a *U. brizantha* cultivar. ISSR markers showed a low polymorphism level. It was not possible to separate samples intentionally contaminated even at 5%.

Key words: *Brachiaria*, molecular markers, varietal purity.

INTRODUCTION

The *Urochloa* genus (syn. *Brachiaria*) belongs to the Poaceae family and was introduced in Brazil from Africa, having as its main centre of origin and diversification, the east of the continent (Vilela, 2005). In Brazil, sixteen species were introduced from Africa and the most important were *Urochloa brizantha*, *Urochloa decumbens*, *Urochloa ruziziensis* and *Urochloa humidicola* (Karia et al., 2006.), which represent the majority of commercial seeds. In addition to its use as pasture, forage species have been used to cover the soil in crop-livestock integration (Crusciol et al., 2009). Currently, it is estimated that more than 50% of the area cultivated with pastures in the Center-west region of the country are cropped with this grass (Macedo, 2006).

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There are seven major worldwide collections of *Urochloa*, all *ex situ*, which have a total of 987 accesses of 33 described species (Keller-Grein et al., 1996). However, problems with incorrect identifications are still frequent among *Urochloa* species, as well as in the accessions of germplasm collections (Assis et al., 2003). Presently, Embrapa Gado de Corte is responsible for the largest and most important resources of Forage Germplasm Bank of the *Urochloa* used for the plant breeding efforts (Valle et al., 2008).

Currently, nine cultivars of *U. brizantha* are registered, but just Marandu; Xaraés; BRS Piatã; BRS Paiaguás; Basilisk; MG4; MG5 Vitória and MG-13 Braúna are commercially available (Brasil, 2016), and there are still doubts about the identification of some of these cultivars. An important cultivar, originally introduced as *Urochloa decumbens*, is Basilisk, collected originally in Uganda, sent to Australia and subsequently introduced from International Research Institute (IRI) to São Paulo in the early 1960s and was the first *Urochloa* cultivar (Karia et al., 2006). According to Renvoize et al. (1996), Basilisk was wrongly identified as a *U. decumbens* but it is actually an *U. brizantha*, data is supported by Ambiel et al. (2008, 2010).

The Marandu cultivar was introduced in 1967 in São Paulo, and occupies the largest grassland area in Brazil (Macedo, 2006). More recently, the cultivars Xaraés, BRS Piatã and BRS Paiaguás were released by Embrapa Gado de Corte (CNPGC) (Brasil, 2016).

The cultivars, MG-4, MG-5 'Vitória' and MG-13 'Braúna' were released by Matsuda company after an accession selection from CIAT genebank (International Center for Tropical Agriculture) (Matsuda, 2016). Despite some data pointing that Xaraés and MG-5 are not the same material (Ambiel et al., 2008, 2010), for the National Cultivar Register, they are under the same number (Brasil, 2016).

Assis et al. (2003) succeeded in using morphological markers to differentiate six species of *Urochloa* (*U. brizantha*, *U. humidicola*, *U. decumbens*, *Urochloa jubata*, *U. ruziziensis* and *Urochloa dictyoneura*) using vegetative and reproductive characteristics and pubescence. However, there is still no standard for varietal discrimination of *U. brizantha* cultivars, which can result in commercial seed lots with high varietal mix due to technical difficulties to identify the degree of contamination (Zanine et al., 2007).

For the seed production and selling of *U. brizantha*, the minimum seed purity percentage is 60% and contamination with other cultivars, must be below 0.15% (Brazil, 2008). Therefore, techniques for discrimination and for varietal purity are important for quality control in many points of the production chain, because of the easy identification for synonyms cases and homonyms between cultivars and to assist in individual selection that can be used to compose germplasm banks or in breeding plans (De Paula et al., 2012). Besides the aspect related to seed quality control, another reason for the significant increase is the interest in the cultivar characterization and identification for protection of commercial cultivars in an increasingly competitive markets.

Among the available molecular methods, those based on the polymerase chain reaction (PCR) has advantages over the other methods because they use reduced quantities of DNA and electrophoretic profiles are obtained faster (Ferreira and Grattapaglia, 1998; Ramos et al., 2006). The development of variants of this technique enabled the emergence of various types of molecular markers, including inter-simple sequence repeat (ISSR). This type of marker has been widely used in studies for varietal identification, and also in the seed technology, being a simple and efficient technique to generate high levels of polymorphism (Reddy et al., 2002). ISSR molecular markers were developed by Gupta et al. (1994) and Zietkiewicz et al. (1994). The amplified products of the PCR reaction produced in the ISSR reaction correspond to sequences of different sizes that are located between identical microsatellite repeat regions and oriented in opposite directions. In addition to presenting high levels of polymorphism, they are robust due to the fact that they have greater anchoring surface and also higher temperatures reassociation, thus having high reproducible products (Lin et al., 2010; Haq et al., 2011).

The advantages of using the ISSR technique is that it requires small DNA amounts per reaction, the speed to obtain relevant genetic information for studies of population diversity is low, and also they require little infrastructure equipment for laboratories, when compared with other markers (Satya et al., 2012) and seed can be used as a DNA source, even if the extracted amounts are low, but the quality is satisfactory. ISSR have been widely used to detect the intraspecific polymorphisms in plants (Pharmawati et al., 2004). ISSR can be used to analyze multiple loci in a single reaction (Marotti et al., 2007) and to produce fragments with high reproducibility as compared to other non-specific PCR-based markers such as random amplified polymorphic dna (RAPD) (Wolfe and Liston, 1998). Saini et al. (2004) obtained in rice, the highest percentage of polymorphic ISSR markers as compared to amplified fragments length polymorphism (AFLP) markers, but smaller than simple sequence repeats (SSR). Azevedo et al. (2011) investigated the genetic variability within genotypes of *U. ruziziensis* by ISSR. These markers are shown to be effective for such evaluation, suggesting that the populations of this *Urochloa* species retain a wide genetic variability. In the case of *U. brizantha*, despite the commercial importance of this species as a forage plant, there are still relatively few studies on the genetic diversity within existing accesses in germplasm collections in Brazil. Also, unlike *U. ruziziensis*, most commercial cultivars, *U. brizantha* have an apomictic reproduction which justifies the lowest variability among the few available accesses.

Some economically important crops have been

Table 1. List of *U. brizantha* cultivars used in this study.

Cultivar	Register Date	Maintainer	Protected for
*Basilisk	10/05/1999	MATSUDA	Public domain
Marandu	10/05/1999	EMBRAPA	Public domain
Xaraés	11/09/2001	EMBRAPA/GERMISUL/MATSUDA	Public Domain
BRS Piatã	23/06/2003	EMBRAPA	EMBRAPA
MG-4	10/05/1999	MATSUDA	Public domain
MG-5 Vitória	22/03/2000	EMBRAPA/GERMISUL/MATSUDA	Public domain

*Wrongly identified as *U. decumbens* cv. *Basilisk* (Renvoize et al., 1996).

evaluated with these markers in accesses and wild species, to obtain data for breeding assisted programs as in Silva et al. (2011) that used ISSR markers to identify inter-and intraspecific variability in accessions of *Manihot* (*Manihot esculenta*, *Manihot caerulescen*, *Manihot dichotoma* and *Manihot flabellifoli*). Also, several studies used ISSR markers as a tool for the delimitation of plant species (Dogan et al., 2007; Wood and Nakazato, 2009; Anand et al., 2010).

The DNA molecular markers have been used to discriminate cultivars and evaluate their genetic purity in seed samples. Due to the advances in molecular marker techniques, and the greater demand of the seed market, these tools have become a viable alternative for routine analysis. The ability to access the genetic variability directly from DNA has increased the process of intellectual protection of genetic materials, in association with other discriminatory analysis (Almeida et al., 2009, 2011). While in Brazil, the cultivar registration do not require molecular characterization, in other countries, with consolidated certification systems of plant material, molecular techniques have allowed developing a varietal plant molecular fingerprint, which facilitates the control of the quality at every stage in the process (Bianchi et al., 2004; Wickert et al., 2007; De Paula et al., 2012).

As exemplified above, the use of ISSR has specific characteristics to be explored in genetic analyses and varietal discrimination. Thus, the present study aimed to investigate the potential of using ISSR to discriminate the six cultivars of *U. brizantha* available and to check if this type of molecular marker could potentially determine the degree of varietal contamination among seed lots of this species.

MATERIALS AND METHODS

Genetic seeds of cultivars, *U. brizantha* Marandu, BRS Piatã, Xaraés, Basilisk, MG4, MG5 (Table 1) were provided by EMBRAPA - Campo Grande (MS) and Matsuda Seeds. MG-13 Braúna was not analysed because it was released after the start of this work.

DNA extraction and PCR

After the removal of the glumes, DNA was extracted from seeds of each cultivar as in Ambiel et al. (2008, 2010). The pellet obtained was then washed twice with 100 µl of 70% ethanol and after drying, it was resuspended in 100 μl^{-1} 1× TE buffer (2.5 mM Tris-HCl and 0.25 mM EDTA). The DNA quantification was performed in a spectrophotometer (Eppendorf BioPhotometer Plus), measuring the absorbance at a wavelength of 260 and 280 nm. The integrity of the purified DNA was assessed by electrophoresis in a 0.8% agarose gel using ethidium bromide as stain.

Initial selection was performed with 28 ISSR primers (Table 2), whereas only ten of these were useful for determining the cultivars electrophoretic pattern. PCR reactions were performed in a final volume of 25 µl containing: 25 ng of DNA, 2.5 µl (10×) *Taq* buffer (Invitrogen), 2 µl $MgCl_2$ 50 mM, 0.25 µL dNTPs (1.25 mM), 0.5 µM of each primer (Fermentas) and 2.5 U of *Taq* polymerase. The amplifications were performed in a thermocycler (PCT - 100, MJ Research) programmed for one cycle at 94°C for 1.5 min for initial denaturation, followed by 40 cycles at 94°C for 40 s, 45°C for 45 s, and 72°C for 1.5 min, the final cycle at 94°C for 45 s, 44°C for 45 s and 72°C for 5 min for final extension or elongation.

Separation of the amplified fragments was performed in a 1.2% agarose gel with 0.5 × SB buffer (10 mmol L^{-1} NaOH, adjusted to pH 8.5 with boric acid) (Brody and Kern, 2004). Gels were stained with ethidium bromide and visualized using Electrophoresis Analysis System (Biosystems). Each amplification reaction was repeated at least twice and only clearly distinct and reproducible bands were scored. The analysis of the bands was performed with the Quantum program - Capt (Vilber -Lourmat) to determine the electrophoretic pattern of the six *U. brizantha* cultivars.

Discrimination and varietal purity analysis

To test the potential of ISSR markers to discriminate the *U. brizanta* cultivars, the pattern of amplified products were tabulated as presence (1) or absence (0) for each polymorphic fragment. One genetic similarity matrix was generated using the Jaccard coefficient and a dendrogram constructed by clustering method unweighted pair-group method using arithmetic averages (UPGMA) using the software NTSYS 2.1 (Rohlf, 2004).

In order to simulate different levels of contamination varietal, a simulation of DNA contamination was performed on Marandu extracted DNA with DNA from the other cultivars in 1, 2.5 and 5% concentration. This experiment was performed in triplicate, with extraction of DNA from three samples of each studied cultivar.

After that, a PCR was performed with polymorphic primers that discriminated the Marandu cultivar from the other cultivars (P4, P7, P9, P10, P15, P23 and P24). The PCR products, of intentionally contaminated DNA samples, were separated on 1.5% agarose gel to verify the possible presence of the band belonging to the contaminant cultivar. The analyses were performed using the program Quantum - Cap, as mentioned above.

Table 2. ISSR primer nucleotide sequence used in this study.

Primer	Sequence (5 '- 3')	Primer	Sequence (5 '- 3')
P1	(CT)8 RG	P18	(GT)6 YR
P2	(AT)9 T	P19	(GT)6 AY
P3	(AG)8 G	P20	CAA(GA)4
P4	(GA)8 T	P21	(GT)6 YG
P6	(CT)8 A	P22	(GAG)4 RC
P7	(AC) 8 CT	P23	(AG)6 YC
P8	(TG)8 GG	P24	(GA)6 RG
P9	(AG)8 GYT	P25	(CA)7 YG
P10	(GA)9 YC	P26	(CTC)4 RC
P11	(GA)8 YG	P27	(GT)6 RG
P12	(CT)8 RA	P28	(GTG)4 RC
P13	(CT)8 RC	P29	(CA)7 YC
P15	(CA)6 RY	P30	(CAC)4 CCRC
P16A	(CA)6 RG	P31	GGGC(GA)6

R= (G or A); Y= (C or T).

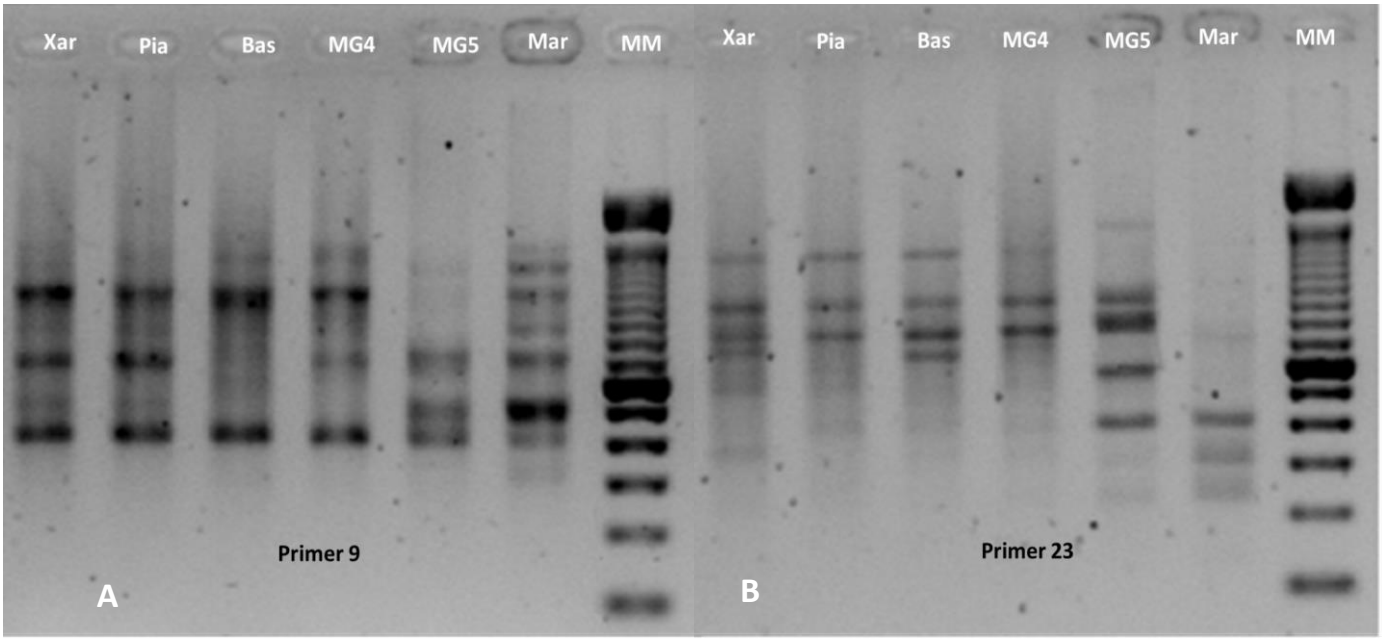

Figure 1. Example of polymorphisms of *U. brizantha* genotypes: *Xaraés* (Xar), BRS *Piatã* (Pia), *Basilisk* (Bas), MG4, MG5 and *Marandu* (Mar) and molecular markers (MM) using primers P9 (A) and P23 (B).

RESULTS AND DISCUSSION

Discrimination of cultivars

In this study, 28 ISSR primers were tested in six *U. brizantha* cultivars (Xaraés, Basilisk, Piatã, MG4, MG5 and Marandu), and 10 primers produced polymorphic bands (an example of two primers, P9 and P23 is shown in Figure 1A and B). Using these primers, a total of 65 bands were generated ranging from 120 to 2274 bp, and 10 of these were polymorphic (Table 3). From the ISSR amplified bands, a dendrogram was constructed. The grouping analysis was done using the UPGMA algorithm, by which it was possible to identify the similarity of *U. brizantha* cultivars (Figure 2). Despite the high similarity coefficient between the genotypes, some cultivars showed genetic differences. The Xaraés and MG5 cultivars have the same origin (CIAT 26110) and record

Table 3. Primer fragment length, number of bands (total and polymorphic) used in ISSR analysis of *U. brizantha* cultivars.

Primer	Fragment length (bp)	Number of bands	
		Total	Polymorphic
P4	813-1889	4	2
P7	380 -1256	7	1
P9	430 – 2274	7	1
P10	163 -1663	8	1
P15	423 – 2129	5	4
P16A	113-433	3	2
P20	140-1664	14	7
P23	120-240	4	2
P24	140-555	4	1
P26	309-1538	9	7

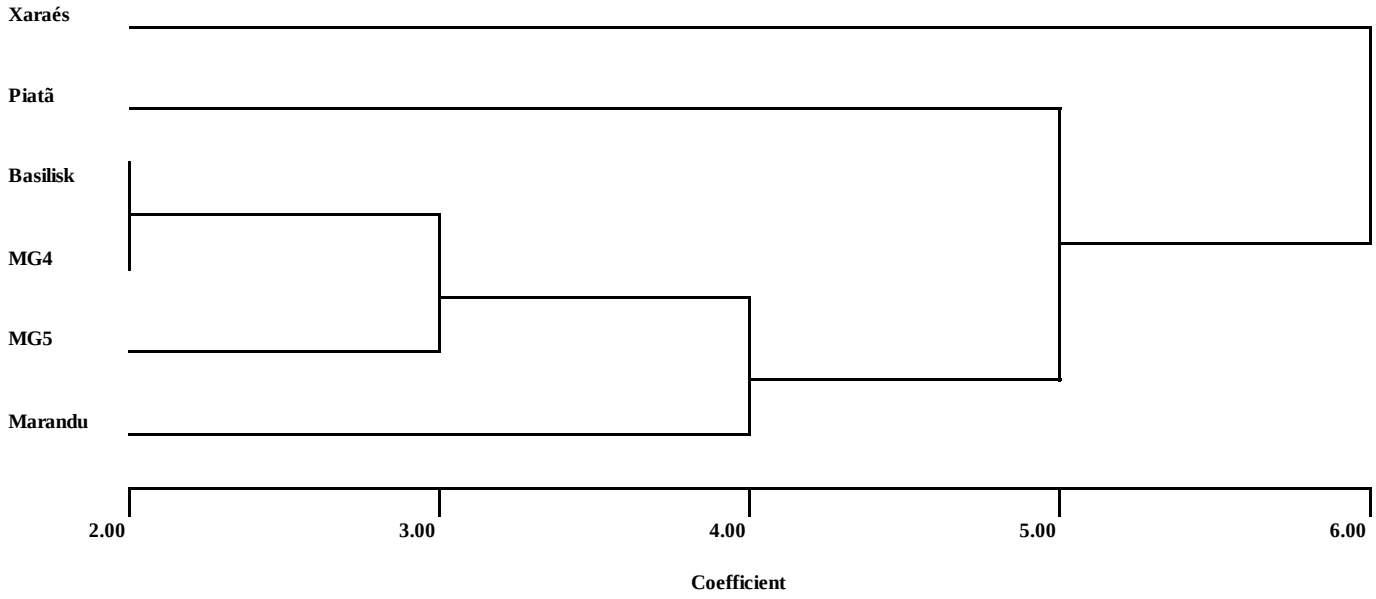

Figure 2. Grouping of six cultivars of *U. brizantha* by UPGMA method using polymorphic ISSR.

(04509) in the National Register of Cultivars (RNC)– being maintained by Embrapa and Matsuda. However, this data show that these cultivars have some genetic divergence (Figure 2), which was also verified by Ambiel et al. (2008, 2010) using the RAPD markers.

Also, it was observed that the cultivar Basilisk, erroneously classified as a *U. decumbens* genotype (Renvoize et al., 1996) was grouped with *U. brizantha* cultivars, which proved to be very similar to cultivar MG4, based on the polymorphisms obtained with the selected ISSR primers. These results confirm what was observed by others using RAPD markers (Ambiel et al., 2008; Almeida et al., 2011). Renvoize et al. (1996) suggested that the cultivar 'Basilisk', widely used and commonly identified as *U. decumbens* is actually a cultivar of *U. brizantha*. So, this error of taxonomic identification was also confirmed in this study using ISSR markers. It is important to mention that it was possible to identify and distinguish all cultivars of *Urochloa* studied in this work using the 10 selected ISSR primers.

Ten primers were used to discriminate the cultivars, but a single primer that was sufficient *per se* to identify all cultivars was not found. P4, P10 and P24 primers shown to be polymorphic among Marandu and MG4, P7 primer differentiates Marandu and Xaraés, the P9 and P23 primers discriminates Marandu from MG5 (Figure 1A and B), the P15 primer differentiated Marandu from BRS Piatã while the primer for P23 was polymorphic for Marandu and Basilisk (Figure 1B). Thus, even with the low variability among commercial cultivars *U. brizantha*, this

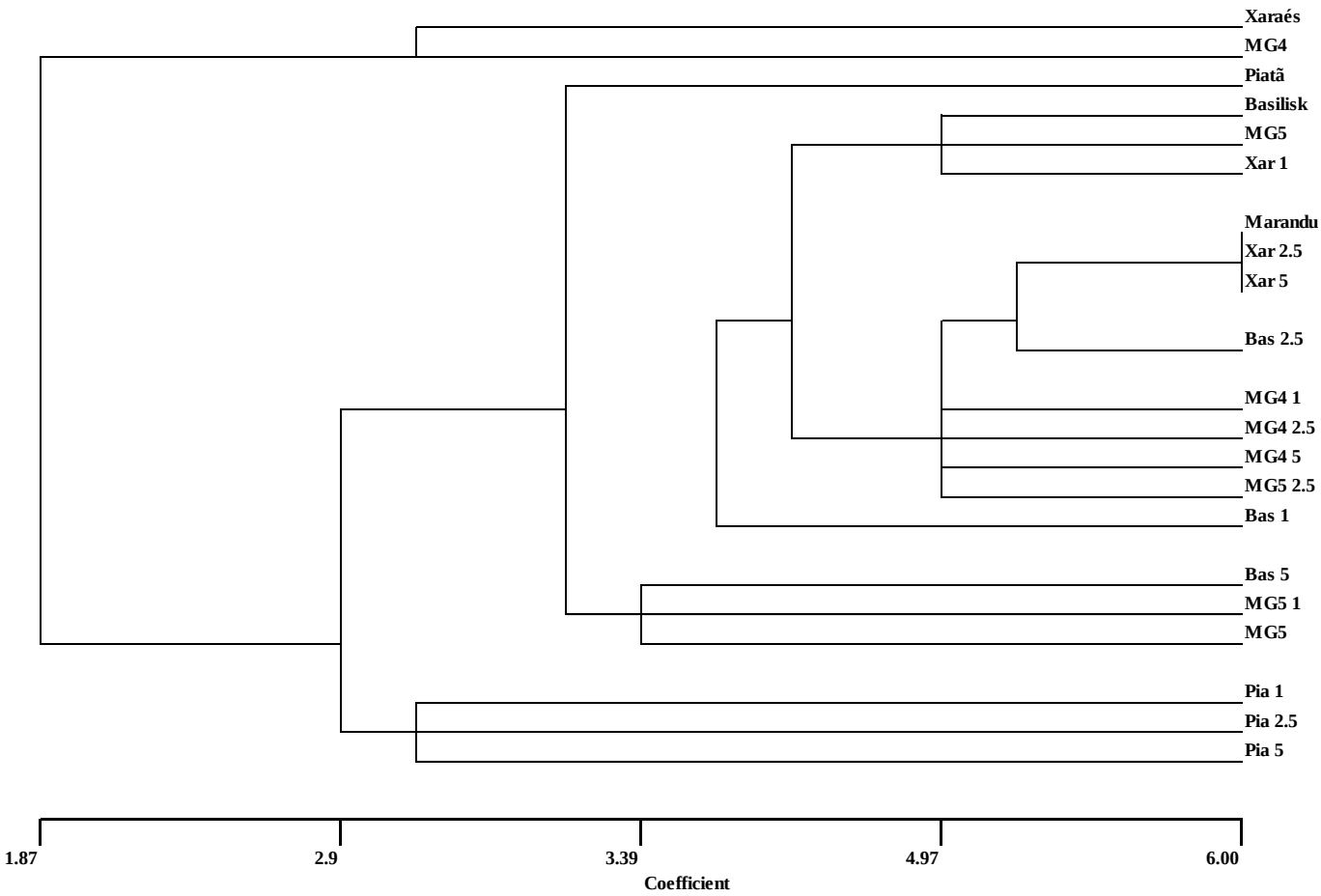

Figure 3. Grouping of *U. brizantha* genotypes: Xaraés (Xar), BRS Piatã (Pia), Basilisk (Bas), MG4 and MG5 contaminated with Marandu DNA at 1, 2 and 5%.

work showed that the use of ISSR primers allowed differentiation between these genotypes.

Silva et al. (2011) used ISSR molecular markers for the genus *Manihot,* which allowed the observation of highly reproducibility markers, revealing large intra- and interspecific divergence among accessions of *Manihot* species. Also, in studies with orchids, phylogenetic analysis showed that for genetic diversity, ISSR can respond optimally to evolutionary issues in complex species consistently and with high reliability values (Rodrigues et al., 2015).

Vigna et al. (2011) developed 15 microsatellite markers to study the genetic variability within *U. brizantha* in a germplasm collection and observed that this collection does not display a considerable variability. Thus, this work showed that the use of ISSR markers can complement the information obtained with other markers.

Genetic purity of seed lots

The second objective was to investigate the ISSR potential as markers to determine the degree of contamination of seed batches of *U. brizantha*. For this analysis simulation, a contamination of 1, 2.5 and 5% of DNA from each genotype with *U. brizantha* cultivar Marandu was made.

Several authors have studied levels of varietal purity in commercial species. Jorgensen et al. (2007) analysed the purity in batches of *Brassica napus* by ISSR, and their results indicated that there was the possibility of separating the varieties with these markers and to estimate the purity in most cases. These authors observed that in harvested seed analysed, three cultivars were contaminated with other varieties over the allowed limit (0.03% for food and <1% for feed).

The ISSR markers used in this study to explore cross-contamination with DNA, between cultivars were not efficient for determining the required levels of contamination (Figure 3) where the samples mixed with Marandu DNA did not group together; just the contaminated samples of Piatã grouped in just one cluster, but apart from the pure sample, the others are mixed with another samples or with the pure material.

According to Brasil (2008), for the purity analysis of *U. brizantha* (Hochst. Ex A. Rich) Stapf., the maximum allowed level of other cultivar at every 180 g of seeds is 30 seeds (Brasil, 2008 and 2011), for example between 0.11 and 0.13%, therefore less than 1% (considering the average number of seeds in a *U. brizantha* seed gram ranging between 123 and 145, approximately) (Brasil, 2009). In this work, even with levels higher than allowed, 5% (Figure 3), ISSR primers were able to differentiate the contamination but not identify it. Therefore, this PCR showed no efficiency in determining the contaminants from the cultivars in any tested level of contamination.

Conclusions

ISSR molecular markers proved to be useful for varietal identification of *U. brizantha* cultivars and may determine genetic differences between commercially used cultivars. The ISSR markers were not efficient to determine contamination levels at 1, 2.5 and 5% DNA between genotypes of *U. brizantha*. It was possible to reconfirm the cultivar identification of Basilisk as a cultivar of *U. brizantha.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Almeida CMA, Lima SN, Lima GSA, Brito JZ, Donato VMTS, Silva IMV (2009). Caracterização molecular de cultivares de cana-de-açúcar utilizando marcadores ISSR. Ciênc. Agrotec. 33:1771-1776.

Almeida MCC, Chiari L, Jank L, Valle CB (2011). Diversidade genética molecular entre cultivares e híbridos de *Brachiaria* spp. e *Panicum maximum*. Ciênc. Rural 41:1998-2003.

Ambiel AC, Machado Neto NB, Guaberto LM, Vanderlei TM (2008). Agrupamento de acessos e cultivares de três espécies de Brachiaria por RAPD. Acta Scient. Agron. 30:457-464.

Ambiel AC, Machado Neto NB, Guaberto LM, Vanderlei TM (2010). *Brachiaria* germplasm dissimilarity shown by the RAPD markers. Crop Breed. Appl. Biotechnol. 10:55-64.

Anand KK, Srivastava RK, Chaudhary LB, Singh AK (2010). Delimitation of species of the *Astragalus rhizanthus* complex (Fabaceae) using molecular markers RAPD, ISSR and DAMD. Taiwania 55:197-207.

Assis GML, Euclydes RF, Cruz CM, Valle CB (2003). Discrimination species of *Urochloa* based on different groups of morphological characters. Rev. Bras. Zootec. 32:576-584.

Azevedo ALS, Costa PP, Machado MA, De Paula CMP, Sobrinho FS (2011). High degree of genetic diversity among genotypes of the forage grass *Brachiaria ruziziensis* (*Poaceae*) detected with ISSR markers. Genet. Mol. Res. 10:3530-3538.

Bianchi VJ, Fachinello JC, Schuch MW, Sansavini S (2004). Molecular characterization of cultivars of peach and nectarine with microsatellites. Rev. Bras. Frutic. 26:490-493.

Brasil (2008). Ministério da Agricultura e Reforma Agrária. Instrução Normativa nº 30, de 21 de maio de 2008.

Brasil (2009). Ministério da Agricultura e Reforma Agrária. Regras para análise de sementes. Brasília, DF, CLAV, 2009. 365p.

Brasil (2011). Ministério da Agricultura e Reforma Agrária. Instrução Normativa nº 30, de 9 de Junho de 2011.

Brasil (2016). Ministério da Agricultura e Pecuária. Registro Nacional de Cultivares. Available at: <http://extranet.agricultura.gov.br/php/snpc/cultivarweb/cultivares_registradas.php >

Brody JR, Kern SE (2004). Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. Biotechniques 36(2):214-217.

Crusciol CAC, Soratto RP, Borghi E, Mateus GP (2009). Integração lavoura-pecuária: benefícios das gramíneas perenes nos sistemas de produção. Inf. Agron. 125:1-15.

De Paula LA, Bianchi VJ, Fachinello JC (2012). Molecular characterization and genetic variability among port peach grafts based on codominant markers. Pesq. Agropecu. Bras. 47:193-199.

Dogan B, Duran A, Hakki EE (2007). Phylogenetic analysis of *Jurinea* (Asteraceae) species from Turkey based on ISSR amplification. Ann. Bot. Fenn. 44:353-358.

Ferreira AME, Grattapaglia D (2012). Introduction to using RAPD and RFLP markers in genetic analysis. 3.ed. Brasilia: EMBRAPA-CENARGEN, Brasil. P.220

Gupta M, Chyi YS, Romero-Severson J, Owen JL (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor. Appl. Genet. 89:998-1006.

Haq QMR, Singh U, Arif M, Zaidi N (2011). Genetic variability within *Fusarium solani* as revealed by PCR-fingerprinting based on ISSR markers. Indian Phytopathol. 61:305-310.

Jorgensen T, Hauser TP, Jorgensen RB (2007). Adventitious presence of other varieties in oilseed rape (*Brassica napus*) from seed banks and certified seed. Seed Sci. Res. 17:115-126.

Karia CT, Duarte JB, Araújo ACG (2006). Cultivar development of the genus *Brachiaria* (Trin.) Griseb in Brazil. Planaltina: Embrapa Cerrado. 57. (Embrapa Cerrado Documents, 163).

Keller-Grein G, Maass BL, Hanson T (1996) Natural variation in Brachiaria and existing germplasm collections. In: Miles JW, Maass BL and Valle CB (Eds.). *Brachiaria*: biology, agronomy, and improvement. 45 Cali, CIAT. pp. 16-42.

Lin XC, Lou YF, Liu J, Peng JS, Liao GL, Fang W (2010). Crossbreeding of *Phyllostachys* species (Poaceae) and identification of their hybrids Liao using ISSR markers. Genet. Mol. Res. 9:1398-1404.

Macedo MCM (2006). Respostas morfofisiológicas de *Brachiaria spp*. ao alagamento do solo e a síndrome da morte do capim-marandu. Embrapa Amazônia Oriental.

Marotti I, Bonetti A, Minelli M, Catizone P, Dinelli G (2007). Characterization of some italian common bean (*Phaseolus vulgaris* L.) landraces by RAPD, semi-random and molecular markers ISSR. Genet. Resour. Crop Evol. 54:175-188.

Matsuda (2016). Espécies forrageiras. Available at http://www.matsuda.com.br/

Pharmawati M, Yan G, Mcfarlane IJ (2004). Application of RAPD and ISSR markers to analyze molecular relationships in *Grevillea* (*Proteaceae*). Aust. Syst Bot. 17:49-61.

Ramos NP, Brunelli KR, Camargo LDA, Marcos Filho J (2006). Sensibilidade dos Microssatélites para determinar a pureza varietal em sementes de milho. Rev. Bras. Semen. 28:99-105.

Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:9-17.

Renvoize SA, Clayton WD, Kabuye CHS (1996). Morphology, taxonomy, and natural distribution of *Brachiaria* (Trin.) Griseb. In. Miles JW et al. (Ed.). *Brachiaria*: biology, agronomy, and improvement. Cali: CIAT, 1996. pp. 1-17.

Rodrigues JF, van den Berg C, Abreu AG, Novello M, Veasey EA, Oliveira GC, Koehler S. (2015). Species delimitation of *Cattleya coccinea* and *C. mantiqueirae* (Orchidaceae): insights from phylogenetic and population genetics analyses. Plant Syst. Evol. 301(5):1345-1359.

Rohlf FJ (2004). NTSYS-Pc: numerical taxonomy and multivariate analysis system, version 2.1, user's guide New York: Exeter Software.

Saini N, Jain N, Jain S, Jain RK (2004). Assessment of genetic diversity

within and among Basmati and Basmati rice varieties non-using AFLP, ISSR and SSR markers. Euphytica 140:133-146.
Satya P, Karan M, Sarkar D, Sinha MK (2012). Genome synteny and evolution of AABB allotetraploids in *Hibiscus* section Furcaria revealed by interspecific hybridization, ISSR and SSR markers. Plant Syst. Evol. 298:1257-1270.
Silva KVP, Alves AAC, Martins MIS, Melo CAF, Carvalho R (2011). Variabilidade genética entre acessos do gênero *Manihot* por meio de marcadores moleculares ISSR. Pesq. Agropecu. Bras. 46:1082-1088.
Valle CB, Simioni C (2008). Melhoramento genético da Braquiária. In. Resende, RMS; Valle CB; Jank, L. *Melhoramento de forrageiras tropicais*. Campo Grande: Embrapa Gado de Corte.
Vigna BBZ, Jugmann L, Francisco PM, Zucchi MI, Valle CB, Souza AP (2011). Genetic diversity and population structure of the *Brachiaria brizantha* germplasm. Trop. Plant Biol. 4:157-169.
Vilela H (2005). Pastagem: seleção de plantas forrageiras, implantação e adubação. Aprender Fácil. Viçosa.
Wickert E, Lemos EDM, Pereira FM, Mayer M (2012). Marcadores fAFLP na caracterização de três genótipos de umezeiro selecionados como porta-enxertos para pessegueiro. Pesq. Agropecu. Bras. 42:1741-1746.
Wolfe AD, Liston A (1998). Contributions of PCR-based methods to plant systematics and evolutionary biology. In. Molecular systematics of plants II. Springer US. pp. 43-86.
Wood TE, Nakazato T (2009). Investigating species boundaries in the *Giliopsis* group of *Ipomopsis* (*Polemoniaceae*): Strong discordance among molecular and morphological markers. Am. J. Bot. 96:853-861.
Zanine AM, Dias PF, Souta M, Ferreira DJ, Santos EM, Pinto FB (2007). Uso de funções discriminantes para comparação de cultivares dos gêneros *Cynodon* e *Digitaria* quanto à produção de matéria seca e teores de macronutrientes. Arch. Latinoam. Prod. Anim. 15(4):159-164.
Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain-reaction amplification. Genomics 20:176-183.

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