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Full Length Research Paper

Phenotypic and molecular characterization of multiple-resistant gram-negative bacteria in urine of pregnant women attending antenatal clinic of Mother and Child hospital, Ondo, Nigeria

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Phenotypic and molecular characterization of multiple antibiotic resistant Gram-negative bacteria in urine samples of pregnant women in Mother and Child Hospital, Nigeria was reported. In the study, 407 apparently healthy pregnant women were recruited. Structured questionnaire was administered to the patients to obtain their socio-demographic information and the medical history. Urine samples were collected, processed and analysed using standard microbiological procedures. Detailed identification of the bacteria isolates was done using biochemical characterization using Bergey's Manual of Determinative Bacteriology and Analytical Profile Index (API) Kit. The antimicrobial susceptibility testing of the bacteria isolates was carried out using the Kirby-Bauer's disk diffusion technique. Detection of the beta lactamase resistance genes (bla $_{CTX-M}$ and *Tet* A) was done by polymerase chain reactions (PCR) with appropriate primers. The following Gram-negative bacteria were recovered comprising *Pseudomonas aeruginosa* 48 (34.0%), *Escherichia coli* 30 (21.3%), *Klebsiella* sp. 27 (19.1%), *Proteus* sp. 15 (10.6%), *Salmonella* sp. 8 (5.7%), *Providencia rettgeri,* 4 (2.8%) and *Enterobacter cloacae* 4(2.8%) and other enterobacteriaceae 5 (3.5%). Resistance of the isolates to antibiotics used varied greatly among the isolates. Resistance to antibiotics was highest with *P. aeruginosa* having 100% to augumentin, tetracylines, amoxicillin, nitrofurantoin, cotrimoxazole, ceftriazone, cefixime (97.9%) and cefuroxime (95.8%). There was diversity in the multiple antibiotic resistance (MAR) patterns among the isolates with 12 different MAR patterns observed. The selected *P. aeruginosa* profiled for resistance genes harboured *bla-CTX-M* (585bp) and *Tet* A (954bp) genes. The multiple antibiotic resistant bacteria recovered could pose great health challenge to the pregnant women and the unborn foetus.

Key words: Gram negative bacteria, antibiotics, enterobacteriaceae, resistance genes.

INTRODUCTION

Bacteriuria can be defined as the appearance or presence of bacteria causing diseases in the urethra, bladder, and pelvis of the kidney. Bacteria such as *Escherichia coli*, *Enterococcus faecalis Klebsiella* species, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Streptococcus agalactiae* are some of the causative agent of urinary tract infections (UTI). Blockage of the urinary tract, catheter usage, deficiency in oestrogen,

genetic predisposition and sexual intercourse are predominant risk factors for urinary tract infection (Tigabu et al., 2020). Asymptomatic bacteriuria (ASB) is the detection of 10^5CFU/ml of one or more 46 bacterial species, irrespective of pyuria, in a urine specimen from a pregnant women patient without any symptoms of a urinary tract infection (UTI) (Willey et al., 2020). The relatively upsurge of ASB in pregnancy, the consequences encountered by the women and their pregnancies, avoidance of treatment with undesired outcomes, screening and treatment of ASB in pregnancy with justification. There are variations in the frequency of pathogens isolated and antimicrobial resistance patterns based on different geographical regions. Asymptomatic bacteriuria occurs in 2 to 15% of pregnant women. Therefore if the ASB remains untreated, over 30% of mothers will develop acute pyelonephritis, and this has been associated with low birth weight and premature birth (Sujatha and Nawani, 2014; Smaill and Vazquez, 2019).

It is recommended that screening for bacteriuria should routinely be undertaken in the first trimester of pregnancy to identify women who are at risk in order to prevent undesirable end result as bacteriuria which is one of the risk factor in pregnant women. The importance of the microbiological analysis of urine samples collected from patients that appears healthy cannot be over emphasized.

Multiple antibiotic resistant Gram-negative bacteria are bacteria which have developed resistant to many common commercial used antibiotics. Their habitat is in the bowel and therefore causes no harm or any problems but can cause infections in the urine, skin wounds and blood when left untreated. These multidrug resistant Gram-negative bacteria infections pose a serious threat in the clinical settings with limits to the choice of antibiotics in the treatment and management of infections in patients that are hospitalized, and more especially patients in intensive care unit. Overuse and misuse of antibiotics and problems and wrong infection control practices have led to the development of multiple resistant Gram-negative bacteria infections. This study investigates into the phenotypic and molecular characterization of multiple resistant Gram-negative bacteria among pregnant women attending antenatal of Mother and Child Hospital in Ondo, Nigeria.

METHODOLOGY

Isolation of bacteria

Urine samples of four hundred and seven apparently healthy pregnant women attending antenatal clinic at Mother and Child hospital in Ondo Town were collected between July 2015 and January 2016. Samples were cultured on Centrimide and MacConkey agar (Lab M Ltd, UK) by streaked plate method, incubated at 37°Cfor 24 h for isolation of *P. aeruginosa* and other Gram-negative bacteria. Preliminary identification of isolates was by morphological, cultural characteristics and biochemical tests. Bacteria isolates were further re-confirmed using analytical profile index (API) 20E test kit (bioMérieux, Inc., France).

Antibiotic susceptibility testing

Susceptibility test of the isolates to antibiotics was carried out by the Kirby-Bauer's disc diffusion method (Bauer et al., 1966). Antibiotic discs (Abtek Biological limited, UK) which include; gentamycin (10 µg), augmentin (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), nitrofurantoin (200 µg), cotrimoxazole (25 µg), ofloxacin (5 µg), amoxicilin (25 µg), tetracycline (30 gµ), ciprofloxacin (5 µg), ofloxacin (5 µg), ceftriazone (30 µg) and cefixime (5 µg) were carefully placed on Mueller-Hinton agar plates previously seeded with 24 h old culture (0.5 Mcfarland's standard- 10^7cfu/ml). The plates were incubated at 37°C for 24 h and diameter of zone of inhibition was measured by a transparent calibrated ruler to the nearest millimetre and the results interpreted according to the guidelines of Clinical Laboratory Standard (CLSI, 2013). Multiple antibiotic resistance was defined as resistance to more than two class of antibiotics.

Molecular characterization of multiple antibiotic resistant isolates

Twelve representative multiple antibiotic resistant bacterial isolates selected on the basis of their antibiotype were profiled for detection of resistance *bla* CTX (585bp) and *Tet* A (954bp) genes by polymerase chain reaction (PCR) using appropriate primers as depicted in Table 1. The DNA of the bacteria isolates was extracted using boiling method at 100°C for 7 min in water bath, cold shocked in ice for 2 min. The PCR thermocycling conditions include initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s for *bla* CTX (585 bp) gene and 43°C for 30 s for *Tet* A (954 bp) gene, extension at 72°C for 1 min and final extension at same temperature for 5 min. The bands were then visualized with a short wave ultraviolet trans illuminator and photographed gel bioimaging system.

RESULTS

Table 2 shows the baseline characteristics of the subjects. The women recruited were between the age brackets of 15 and 49 years. Among the 407 pregnant women, 36(8.8%) was in their first trimester, 171(42.0%) in second trimester and 200(49.1%) in third trimester. Six (1.5%) of the subjects was single, while 401(98.5%) was married. Also, 37(9.1%) was students, 75(18.4%) was public or civil servants, 6(1.5%) was professionals,

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Table 1. Primers used for the detection of the resistance genes in the selected isolates.

Primer	Sequence 5^1 -3^1	Gene	Product size (bp)	References
CTX-M F CTX-M R	CGATGTGCAGTACCAGTAA TTAGTGACCAGAATAAGCGG	bla CTX-M	585	Colom et al. (2003)
TET-A	F:GCGCTNTATGCGTTGATGCA R: ATGTGTCCTGGATTCCT	TET-A	246	Adesiyan et al. (2019)

Table 2. Baseline characteristics of the pregnant women.

Characteristics	Cases n (%)
Marital status:	
Single	6 (1.5)
Married	401 (98.5)
Patients status	
Religion	
Christianity	371 (91.2).
Islamic	36 (8.8).
Occupation	
Civil/public servants	75 (18.4)
Professional/managers	6 (1.5)
Farming/artisans/trading	253 (62.2)
Students	37 (9.1)
Dependant/unemployed	16 (3.9)
Others	20 (4.9)
Pregnancy trimesters	
First trimester	36 (8.8)
Second trimester	171 (42.0)
Third trimester	200 (49.1)
Age group	
15-19	15 (3.7)
20-29	221 (54.3)
30-39	156 (38.3)
40-49	15 (3.7)

253(54.4%) was artisans or traders and 6(3.9%) was dependent or unemployed.

Five pregnant women within the age bracket 21-32 years suffering from urinary tract infection served as control and were all in their second and third trimesters. Table 3 depicted the percentage distribution of the bacteria isolates recovered viz; *P. aeruginosa* (34.0%), *E. coli* (21.3%), *Proteus* sp (10.6%), *Salmonella* sp (5.7%), *Klebsiella* sp (19.1%), *P. rettgeri* (2.8%), *E. cloacae* (2.8%) and other enterobacteriaceae 5 (3.5%). Table 4 shows the percentage occurrence of bacteria isolated from pregnant women in relation to their occupation. The percentage distribution of the bacterial isolates varies with occupation. There was no bacterial isolates recovered from pregnant women who are professionals/ managers.

Table 5 shows the antibiotic resistance profiles of the bacterial isolates. Resistance to antibiotics varies greatly among the organisms. Resistance to beta-lactam class of antibiotics was generally high among the organisms. P. aeruginosa showed highest resistance most antibiotics tested namely augmentin (100%), tetracyclines (100%),

Table 3. Percentage Distribution of Bacterial Isolates in the Urine Samples of Pregnant Women at Mother and Child Hospital Ondo.

Names of organisms	Total number of isolates	No of occurrence	Percentage
P. aeruginosa	141	48	(34.0
E. coli	141	30	(21.3)
Salmonella sp.	141	8	(5.7)
Proteus sp.	141	15	(10.6)
K. pneumoniae	141	27	(19.1)
E. cloacae	141	4	(2.8)
P. rettgeri	141	4	(2.8)
Other enterobacteriaceae	141	5	(3.5)

Table 4. Percentage distribution of bacterial isolates in relation to occupation of the pregnant women.

Occupation	*Proteus* sp.	*E. coli*	*Klebsiella* sp.	*P. aeruginosa*	*Salmonella* sp.	*P. rettgeri*	*E. cloacae*	Others
Trader	66.7	86.7	74.1	79.2	100	100	100	100
Civil /public servant	33.3	10	-	2.1	-	-	-	-
Dependant	-	3.3	-	2.1	-	-	-	-
Student	-	-	14.8	6.3	-	-	-	-
Professional/managers	-	-	-	-	-	-	-	

amoxicillin (100%), ceftriaxone (100%), cotrimoxazole (100%), nitrofurantoin (95.8%), cefuroxime (95.8%) and cefixime (97.9%). *E.coli* were only resistant to augmentin (93.3%).

Table 6 showed the multiple antibiotic resistance pattern of *P. aeruginosa* and *Klebsiella ornithinolytica* isolates. All *P. aeruginosa* and *K. ornithinolytica* isolates recovered from this study were multidrug resistance. The isolated bacteria were resistant to multiple antibiotics which ranges from four to seven classes. *K. ornithinolytica* (9.5%) were resistant to four class of antibiotics. *P. aeruginosa* (97.9%) were multi-resistant to six different class, 2.1 to 7% and 4.2 to 5% different class of antibiotics. Meanwhile, both *K. ornithinolytica* and *P. aeruginosa* exhibited 2 different multiple antibiotic resistance patterns each (Table 6).

Figures 1 and 2 showed agarose gel electrophoresis of the amplification product coding *bla* CTX (585 bp) and *Tet* A (954) genes in selected MAR *P. aeruginosa* and *K. ornithinolytica* isolates. In Figure 1, seven of the 12 representative isolates that were resistance to beta lactam antibiotics as depicted by Lanes 3, 4, 5, 7, 8, 9, and 12 harboured *blaCTX* resistance gene of molecular weight of 585 bp. Lanes 1-2 represent *K. ornithinolytica* which harboured no *blaCTX* resistance gene. Lane 11 represents the control (patient suffering from urinary tract infection) which also harboured no *blaCTX* resistance gene. Figure 2 shows the agarose gel electrophoresis of *Tet*A genes in selected 12 representatives multiple antibiotic resistant *P. aeruginosa*. Five of the 12 representative isolates that were resistant to tetracycline antibiotics as depicted by Lanes 3, 4, 5, 7 and 8, harboured *Tet* A resistance gene of molecular weight of 945 bp. Lane 11 represents control (patient suffering from urinary tract infection).

DISCUSSION

This study showed the prevalence of 36.4% bacteria amongst the sampled population of 407 pregnant women attending ante-natal clinic at Mother and Child Hospital Ondo town Nigeria. This level of prevalence is at variance to other researchers' reports. Olamijulo et al. (2016) reported a prevalence of 14.6% in a study carried out in 556 pregnant women in Lagos University Teaching Hospital, Nigeria. It is also at variance with Nguefack et al. (2019) who also reported a prevalence of 9.9% in a

Table 5. Resistance profile of the isolates cultured from urine sample of pregnant women.

Organism	Aug (30 µg)	Cpr (5 µg)	Nit (200 µg)	Gen (10 µg)	Ctx (30 µg)	Ofl (5 µg)	Cfx (30 µg)	Cxm (5 µg)	Amx (25 µg)	Tet (5 µg)	Cfr (30 µg)	Cot (25 µg)	S.D
E. coli (n=30)	28(93.3)	0	0	0	0	0	0	0	ND	ND	ND	ND	$p \geq 0.05$
Salmonella sp (n=8)	4(50.0)	0	0	0	8(100)	0	4(50.0)	8(100)	ND	ND	ND	ND	$p \leq 0.05$
E. clocae (n=4)	2(50.0)	0	0	0	0	0	0	0	ND	ND	ND	ND	$p \geq 0.05$
P. retgeri (n=4)	2(50.0)	0	0	0	2(50.0)	0	0	0	ND	ND	ND	ND	$P \leq 0.05$
Kluyvera sp (n=3)	2(66.7)	2(66.7)	0	0	0	0	0	0	ND	ND	ND	ND	$p \geq 0.05$
Klebsiella sp (n=27)	26(96.3)	1(3.7)	1(3.7)	1(3.7)	0	1(3.7)	26(96.3)	27(100)	ND	ND	ND	ND	$p \geq 0.05$
P. aeruginosa (n=48)	48 (100)	0	46 (95.8)	1 (2.1)	0	0	46 (95.8)	45 (93.8)	48 (100)	48 (100)	48 (100)	48 (100)	

Aug= Augmentin, Cpr= Ciprofloxacin, Nit= Nitrofurantoin, Gen= Gentamycin, Ctx= Ceftaxidime, Ofl= Ofloxacin, Cfx= Cefuroxime, Cxm= Cefixime, Amx= Amoxicillin, Tet= Tetracycline, Cfr= Ceftriaxone, Cotrimozaxole, ND= Not Determined, S.D= Standard Deviation.

Table 6. Multiple antibiotic resistance pattern of *P. aeruginosa* and *K. ornithinolytica*.

Name of isolates	No. of classes of antibiotics tested	Multiple resistant pattern	Frequency (n%)	Occurrence of MAR isolates (n%)
K. ornithinolytica	4	AMX, AUG,CRX,OFL	1 (50)	
K. ornithinolytica	4	AMX, AUG,CRX,OFL	1 (50)	2 (100)
P. aeruginosa	5	AUG, AMX, COT, CRX, TET,	47 (97.9)	
P. aeruginosa	7	AUG, AMX, COT, CRX, GEN, NIT, TET	1 (2.1)	48 (100)

COT –Cotrimoxazole 25 µg, CPX –Ciprofloxacin 10 µg, AMX –Amoxycilin 25 µg, OFL –Ofloxacin 5 µg, CRO –Ceftriazone 30 µg, GEN –Gentamycin 10 µg, CRO- ceftriazone 30 µg, AUG –Augmentin 30 µg, NIT- Nitrofurantoin 200 µg, TET –Tetracycline 5 µg, CRX–Cefuroxime 30 µg, CXM- Cefixime 30 µg, CAZ- ceftazidime 30 µg.

three hospitals in the developing country.

The prevalence of bacteria in increasing order in this study, *P. aeruginosa* (34.0), *E. coli* (21.3), *Klebsiella* sp (19.1), *Proteus* sp (10.6), *Salmonella* sp (5.7), other enterobacter (3.5), *Providencia rettgeri* (2.8) and *Enterobacter clocae* (2.8) is in contrast to Olamijulo et al. (2016) who reported that *Klebsiella* sp is the most common pathogen isolated in urine of pregnant women. Gram-negative bacteria are responsible for more than 85% cases of UTI and are the dominant causative agents, also they are the normal flora of the intestinal tract especially the rectum which is very close to the urethral orifice (Anyamene et al., 2002; Obiogbolu, 2004).

Bacteria were predominantly recovered among women of ages (19-32) in their second and third trimesters with none in the first trimester. This finding is quite similar to Durowaiye et al. (2011) who reported the Women in the third trimester were observed to have the highest prevalence 18.2% than those in their second and first trimester. This could be because of changes that occur in the anatomic site and physiology that is been experienced by pregnant women during the stages of pregnancy. Because of the uterus expansion there is increase in the hormonal effects which can together lead to invasion of microorganisms. These hormonal changes reduce the muscular tone of the uterus and induce the mechanical pressure from the gravid uterus and this may leads to urinary stasis which encourages the multiplication of bacteria in urine since urine is an excellent culture media for bacteria growth (Obiogbolu, 2004). Pregnant women within the age of 20-39 years old age group had the highest

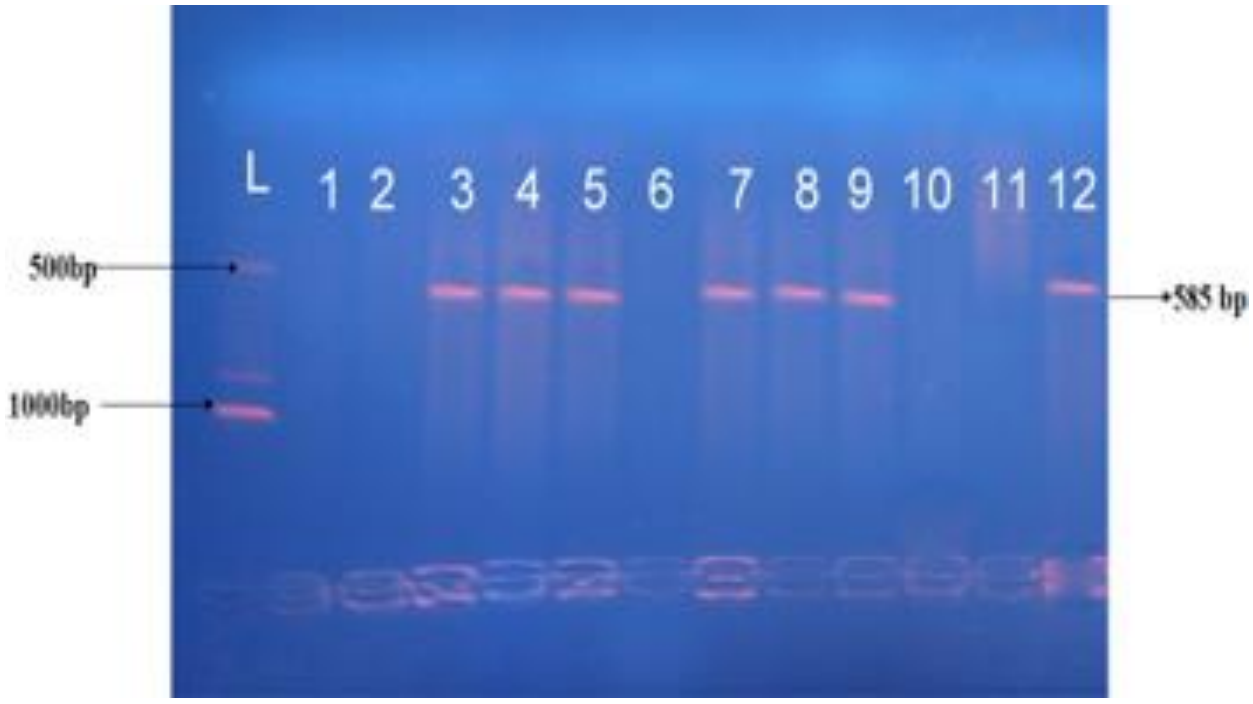

Figure 1. Agarose gel electrophoresis of the amplification product coding blaCTX (585bp) gene in selected MAR *Pseudomonas aeruginosa* isolates and *K. ornithinolytica* isolates. Ladder L: DNA marker 100 bp; Lane 1-2 *K. ornithinolytica*; Lane 3-12: *P. aeruginosa*. L1- K238;L2-K116; L3-P188; L4-P269;L5-P351;L6-P332;L7-091;L8-P355;L9-P372;L10-P081;L11-P311(control);L12-P074 L- Lader, K- *Klebsiella ornithinolytica*, P-*Pseudomonas aeruginosa*.

prevalence of bacteria. This shows that these women are at the risk of developing urinary tract infection in future if not or properly treated. The bacteria are responsible for asymptomatic bacterial infection are of faecal origin and they colonize the periurethral region.

The percentage of *Proteus* sp, *P. retgeri*, *E. coli*, *Klebsiella* sp, *P. aeruginosa*, *Salmonella* sp and *E. clocae* was predominantly high among pregnant women who were farmers/ artisans/traders. This high prevalence among these categories of women could be as a result of consumption of contaminated food, socio-economic status, attitude to personal hygiene and educational exposure.

All the bacterial isolates in this study namely; *K. ornithinolytica*, *E. coli*, *E. clocae*, *Salmonella* sp, and *P. rettgeri* were highly sensitive to nitrofurantoin, gentamycin, and ofloxacin except *P. aeruginosa*. Similarly, *E. coli* were relatively sensitive to ceftaxidime, cefixime, ciprofloxacin, gentamycin, nitrofuraintoin and cefuroxime. This finding is comparable to Olamijulo et al. (2016) who reported in their study that Gram-negative bacteria showed high sensitivity rate to gentamycin and ofloxacin. This could be because gentamycin belongs to the class aminoglycoside where antibody binds to the subunit of the bacteria ribosome, interruption of protein synthesis thereby preventing bacteria from performing vital roles needed for survival. Therefore, gentamycin can be used in the treatment of these Gram-negative bacteria in urinary tract infection.

Antimicrobial susceptibility testing in this study revealed the high resistance of *P. aeruginosa* to amoxicillin which belongs to penicillin class (beta lactam group) by the isolates recovered. Similar results were reported in ast studies by Sabharwal (2012). This could be a result of misuse and excessive usage of antibiotic and increase in the spread of beta lactamase producing isolates. Sensitivity of *P. aeruginosa* to flouroquinolones (ciprofloxacin (100%) and ofloxacin) were significant in this study thereby revealing the potency of the antibiotics against the urinary tract pathogens. However, widespread usage may lead to resistance against fluoroquinolones (Gupta et al., 2005).

P. aeruginosa in this study showed high resistance to nitrofurantoin (95.8%) which is in contrast to a report that nitrofurantoin, is a urinary antiseptic, and found to have a better susceptibility and considered safe by Peterson and Andriole (1997) and Christensen (2000).

The result from the antimicrobial susceptibility profile in this study is closely related to that obtained by Akingbade et al. (2012), who reported high resistance rate of *P. aeruginosa* to the following antibiotics: amoxicillin (92.7%), oxacillin (88.2%), cotrimoxazole (77.3%), erythromycin (72.7%), and tetracyclines (70.9%), while they also reported a relatively low resistance to gentamicin as recorded in the present study. Similarly, they reported a low resistance to ceftazidime (20%),

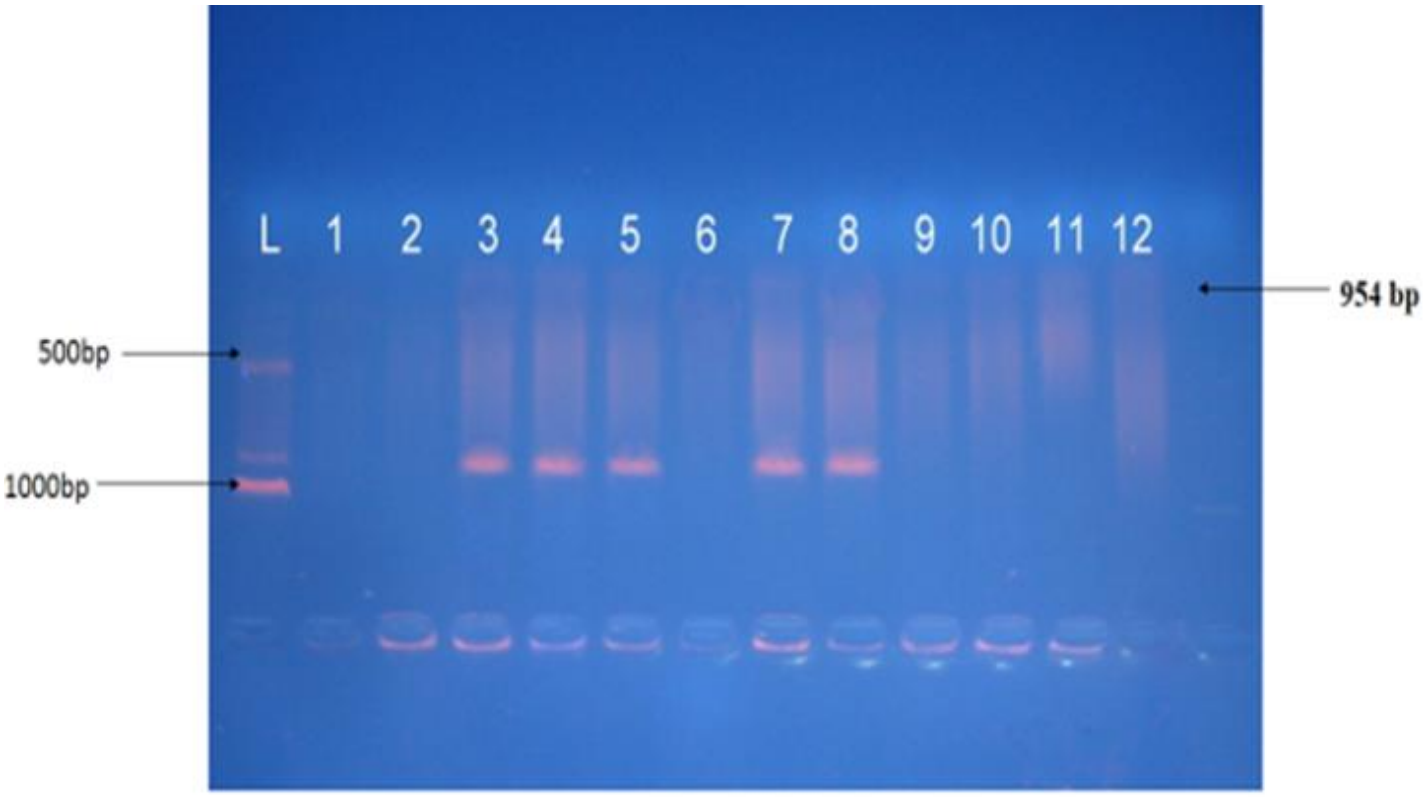

Figure 2. Agarose gel electrophoresis of the amplification product coding *Tet A* (954 bp) gene in selected MAR *P. aeruginosa* isolates. Lanes 1-12: *P. aeruginosa*. Ladder marker 100bp. Ladder, 1= 188, 2=332, 3=372, 4=269, 5=351, 6=311(control), 7=P091, 8=355, 9=081, 19=074, 11=011 and lastly 12=181. L- Lader, P-*Pseudomonas aeruginosa*.

gentamicin (26.4%) by *P. aeruginosa*, while a sharp contrast pattern was observed in the present study with high sensitivity to ceftazidime (97.9%), gentamycin (97.9%) and ciprofloxacin (100%), but high resistance to tetracycline (100%), amoxicillin (100%), cotrimoxazole (100%) and ceftriaxone (100%). Also, Akingbade et al. (2012) recorded a high resistance to ofloxacin (60.0%) which is in contrast to the present study in which a relatively high sensitivity to ofloxacin (100%) was observed.

P. aeruginosa is known to be an opportunistic pathogen which is a leading cause of morbidity and mortality rate in immunocompromised individuals including pregnant women. *P. aeruginosa* has become extremely difficult to eradication because of its remarkable capacity to resist different antibiotics. Strains of *P. aeruginosa* have been known to utilize high levels of intrinsic and acquired resistance mechanisms to attack most antibiotics. Biofilm-mediated resistance and formation of multidrug-tolerant persister cells have been a recent characterized mechanism for adaptive antibiotic resistance which is responsible for recalcitrance and relapse of infections (Pang et al., 2019). The discovery and development of alternative therapeutic strategies that present novel avenues to fight *P. aeruginosa* infections are increasingly demanded and gaining more attention. According to findings from this study, all *P. aeruginosa* recovered from the patients were multiple antibiotic resistant. This high prevalence of multiple antibiotic resistance (MAR) is more alarming and its consistent with previous researchers' findings (Pharmd et al., 2018; Shah et al., 2015). Resistivity of the isolates to various antibiotics could be because of overuse of antibiotics and self-medication resulting in drug resistance especially by *P. aeruginosa*.

The detection of beta-lactamase resistance genes in *P. aeruginosa* in the study is undoubtedly partly responsible for the high resistance rate phenotypically observed particularly against most of the beta-lactams antibiotics used. These genes code for acquired extended spectrum beta-lactamases which are involved in the resistance against beta-lactams and are located in transferable genetic elements such as plasmids or transposons of the organisms (Giedraitienė et al., 2011) and often on integrons (Kotsakis et al., 2010; Zhao et al., 2009; Nordmann et al., 2012). Ogbolu et al. (2013) reported a prevalence of 30.8, 15.4, and 23.1% for bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes, respectively from *P. aeruginosa* isolates recovered in their studies. In this study, bla_{CTX-M} was also detected in the organism. This study detected the presence of *Tet*A resistance gene among *P.*

aeruginosa.

In conclusion, the varieties of bacteria isolated in this study have great implication on the health status of pregnant women and their unborn fetuses. Antibiotics such as gentamycin, ciprofloxacin, ofloxacin and ceftazidime play a great role in the treatment of bacteria in pregnant women. Health education on personal hygiene should be emphasized by the physicians of antenatal care to all pregnant women, especially those of low socio-economic level.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Characterization of diversity and pathogenecity of *Pyricularia grisea* affecting finger millet in Kenya

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Pyricularia grisea **characterization is a prerequisite for species differentiation and understanding of the pathosystem, evolution and diversity of species. The aim of this study is to determine the morphological variation, pathogen virulence and molecular diversity of *P. grisea* isolates. Five isolates from infected heads of finger millet were collected from Bomet, Nakuru, Baringo, Busia and Machakos counties in 2019. The samples were cultured in the lab for both characterization and spore suspension preparation. Data on morphological characterization included colony diameter, color and shape of conidia. Pathogenicity test was done in the greenhouse in a randomized complete block design using KNE 741, a susceptible genotype and disease data scored. Molecular characterization involved the use of seven SSR markers. Data analyses included use of softwares such as AUDPC, Power Maker, GeneAlex and Darwin. Results showed that *P. grisea* isolates had different growth pattern with respect to color, colony diameter and conidia shape. Pathogenicity test revealed that all sites had significant different (P<0.01) virulence on the test genotype. Neck blast, scored at physiological maturity was prominent in Koibatek and Bomet strains while leaf blast was severe in Bomet and Alupe strains. Molecular analysis showed that ENA ranged from 1.30 (MGM 437) -1.99 (*Pyrm* 61-62) with an average of 1.71. PIC varied between 0.20-0.37 for primers MGM 437 and *Pyrm* 61-62, respectively. Factorial and phylogenetic analysis revealed that *P. grisea* isolates were diverse with no geographical grouping. AMOVA indicated diversity occurred within populations (87%) as opposed to among populations (13%). The high *P. grisea* variability found in the study is a clear indication of the high sexual recombination among strains collected in major growing areas of Kenya.**

Key words: Diversity, morphology, pathogenecity, *Pyricularia grisea*.

INTRODUCTION

Finger millet blast disease, caused by *Pyricularia grisea* (Teleomorph*; Magnaporthe grisea* (T.T Herbert, (M. E Barr), is the most economically important disease of finger millet (Mgonja et al*.,* 2007). It is known to cause significant losses in yield and utilization of finger millet. Worldwide losses of above 50% yield have been reported

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in finger millet and above 30% in rice production (Esele, 2002; Prajapati et al*.,* 2013). In India, an increase in 1% infection in the neck and finger results to a corresponding increase of 0.32 and 0.084% in yield losses and grain losses of 6.75 to 87.5%, respectively (Rao, 1990). In East Africa, Pagliaccia et al. (2018) reported yield losses that exceeded 80%, while in rice, *P. grisea* has been known to cause yield loss of 60-100% (Kihoro et al*.,* 2013). Blast infects finger millet at all stages from seedling stages all through panicle formation (Sreenivasaprasad et al*.,* 2004). Effect on the panicle on susceptible genotypes is drastic and may lead to total seed loss of entire finger millet crop (Gashaw et al*.,* 2014). Muimba-Kankolongo (2018) reported that favorable weather conditions (temperature of 25^{o}C and 80% humidity) precedes infection of blast diseases, which starts when a three-celled conidia lands on a leaf surface. This leads to formation of an appressorium which later forms a penetration peg, punctures the cuticle allowing entry to the epidermis. Formation of lesions then follow which later spreads to the whole plant through the epidermis forming diamond shaped grey lesions with brown or black margins. Infection from the leaves begins from the tip backwards. The disease has a wide range of hosts especially grasses and sedge species including rice (*Oryza sativa*), wheat (*Triticum aestivum)*, pearl millet and foxtail millet (*Setaria italica*). Blast affects production and utilization of these crops leading to a substantial decrease in production in Southern Asia, Eastern and Southern Africa (Takan et al*.,* 2012).

Finger millet is a small-grained cereal that is widely cultivated in arid and semi-arid areas of East and South of Africa and Southern Asia. Nutritionally, it contains 7-14% protein in its seed, iron, calcium, phosphorus, carbohydrate, zinc and gluten-free amino acids such as methionine, leusine, isoleusine, phenylalamine among others (Kumar et al*.,* 2016). *Eleusine coracana* is not only a food crop but also an important source of food security to marginal areas. Finger millet provides solutions to alleviating 'hidden hunger' affecting worldwide populations by providing essential micronutrients such as zinc (Underwood, 2000). It can also be utilized as crop with a wide range of genetic resources providing resilience to the changing climatic conditions. With all these benefits, finger millet is affected by many diseases such as root rot, smut, streak, mottling virus and blast disease. *E. coracana* is tolerant to most of these diseases however blast disease is the most devastating and destructive leading to losses in yield and poor utilization by farmers (Ramakrishnan et al*.,* 2016).

Resistance breakdown overtime due to pathogen variability interferes with the breeding objective of developing resistant genotypes (Kariaga et al*.,* 2016). Morphological and genetic diversity of *P. grisea* population is important as it offers durable resistance to the losses caused by these pathogens (Kariaga et al*.,* 2016). Characterization of *P. grisea* is important in understanding evolution, diversity and pathogenicity. Biodiversity-ecosystem functioning studies controls numerous ecosystem processes such as detection, identification and distribution of the fungi. The effect of fungal biodiversity has been used in approaches such as metagenomics, metatranscriptomics and metabolomics (Frąc et al., 2019). Pathotypes of *P. grisea* in rice, pearl millet and fox tail millet have been studied and identified; however, fewer studies have been done on the morphological and molecular characterization of blast disease in finger millet (Takan et al*.,* 2012). These studies have shown considerable variation morphologically in terms of mycelia growth, color and colony production of *P. grisea* (Getachew et al*.,* 2014). Molecular markers have been used to indicate diversity of pathogenic population. The use of SSR markers has been used to evaluate pathotype genetic diversity because of their high sexual reproduction recombination potential, co-dominance, locus specific, multi-allelic and they occur in abundance for all species. This study therefore aimed at characterization of *P. grisea* collected from different finger millet growing regions of Kenya using morphological and genetic features of the pathogen strains and their pathogenicity test to reveal the most virulent pathogen. The determination of the most virulent strain will help to come up with an effective management strategy against the pathogen.

MATERIALS AND METHODS

Isolation of *P. grisea*

Four samples each of diseased tissues (leaves and panicle) were picked from five different regions (Bomet, Baringo, Busia, Makueni and Nakuru) and ported to Egerton biotechnology laboratory for isolation of the fungus using Tuite (1969) and Aneja (2005)'s standard tissue isolation procedure with minor modifications. The margins of the infected tissues were cut in triangular shapes of 5-10 mm and surface sterilized with 0.5% sodium hypochlorite solution and dipped in sterile distilled water for 2 s to saturate the specimen and dealcoholize. The tissues were then placed in glass plates with filter paper to dry the excess water. They were then plated on growth media containing Oat meal Agar amended with 60mg/l Neuromycine sulphate to avoid bacterial contamination (Tredway et al*.,* 2003) and incubated at 25 ± 1^{o}C in artificial light on a 12 h light/dark photoperiod for 15–25 days for sporulation and growth of the fungi (Aneja, 2005). Pure colonies were obtained from each region through five subsequent sub-culturing.

Microscopic characterization of *P. grisea* isolates

A 0.5 cm section of the young sporulated fungus was picked using a micro-pin placed on a drop of water on a piece of slide to allow for classical characterization using binoculars microscope after staining with lacto phenol cotton blue and images observed using image analyzer software. Morphological observations were taken based on colony, conidia and conidiophore morphology; Colony diameters of each isolate (mm), surface texture, pigmentation, mycelial growth on different solid media, type of margin, shape, color, size (length and width) septation of the conidia (Gashaw et al. 2014)

Table 1. Primers used with annealing temperature, forward and backward primer-sequences.

Name	AT	Forward primer (5'-3')	Backward primer (5'-3')
MGM200	55	AAGCGTAAATGGCTCAATGC	GCTGATGTTGTTGCTGCTGT
MGM436	55	GACCTTTATCGGATGCGTGT	CACACAGTGGCCATCTAACG
MGM437	55	GCCCCTCAATAGATCGTCAA	ACTGCGGCATTTTAACCTGT
MGM451	55	TCTCAGTAGGCTTGGAATTGA	CTTGATTGGTGGTGGTGTTG
MGM454	55	GCAAATAACATAGGAAAACG	AGAAAGAGACAAAACACTGG
Pyrms 15-16	61	TTCTTCCATTTCTCTCGTCTTC	CGATTGTGGGGTATGTGATAG
Pyrms 61-62	61	GAGGCAACTTGGCATCTACC	TGGATTACAGAGGCGTTCG

*AT=Annealing Temperature.

Micrographs were taken to show the typical spore morphology of mycelial color, type of margin and sporulation of *P. grisea* isolates (Barnett and Hunter, 1960). Monoconidial isolates of pure culture fungi were then maintained on in agar slants at 4^{o}C. Mother cultures were also preserved in a freezer as reference cultures (Khosravi et al*.,* 2019).

Pathogen spore preparation

Spores and conidia from a 14 day cultured fungi were harvested by flooding five plates representing the five regions with sterilized distilled water and scrapping the growth by a spatula and placed on glass Petri dish (Getachew et al*.,* 2014). A spore solution of 10 µl was pippeted and placed on a haemocytometer and the number of spores counted on the chambers A, B, C, D and E. The numbers found were then used to determine the number of spores per ml by multiplying the value by 2000. The spore suspension was then adjusted to desired concentration of 1×10^5 spores/litre with the help of hemocytometer using the C1V1 formula and 0.01 % Tween 20, a surfactant which amends the properties of the carrier to ensure it dissolves. The suspension was then sieved through a double layer of muslin sleeve poured in calibrated hand sprayers.

Planting, inoculation and scoring of disease on susceptible KNE 741

Test plants were planted in a CRD design with three replicates representing the five regions and three pots for control. Eighteen small pots measuring 20 cm diameter and 40 cm height were filled with sterile soil autoclaved at 121 Pa pressure and 21^{o}C for 15 min DAP of 15.5 g added per pot. Three seeds from the most susceptible variety (KNE 741) were then sown in the pots and allowed to germinate and grow for two weeks. A spore suspension of the *P. grisea* strains from each of the five regions (Bomet, Nakuru, Koibatek, Alupe and Makueni) were used to do the pathogenicity test. Five calibrated hand sprayers were used to spray the pathogen strains to the susceptible variety. The pots were then covered with a parchment bag for 48 h to create humidity required for the growth of the pathogen. After a period of 7 days the symptoms were recorded from each variety and the most virulent pathogen determined on disease severity. Each observed symptom was assigned group I- III where; I-Highly pathogenic, II-moderately pathogenic III- mild pathogenic.

Disease severity rating (DSR) (% damage) was done on the first four leaves (flag) where five plants were randomly tagged per plot. Disease severity on tagged plants was recorded at tillering, flowering, and physiological maturity stages on KNE 741 using modified Cobb scale (Babu et al., 2013). Leaf blast scored based on percent surface area of the infected leaves was evaluated as *P. grisea* severity was used as an indication of severity (Table 1). Neck blast severity was based on the relative lesion size on the neck; a 1 to 5 progressive rating scale was used where, 1 = no lesions to pin head size of lesions on the neck region, 2 = 0.1 to 2.0 cm size of typical blast lesion on the neck region, 3 = 2.1 to 4.0 cm, 4 = 4.1 to 6.0 cm, and 5 = >6.0 cm size of typical blast lesion on the neck region.

DNA extraction of *P. grisea*

Pure cultures of *P. grisea* strains grown on Oat Meal Agar (OMA) for 5 days were used. DNA extraction was done using a modification of the Dellaporta (1994) protocol. The modifications included use of a single SDS buffer (Modified SDS Buffer Components; 20g SDS, 100ml of 1M Tris-HCl (pH 8.0), 50ml of 0.5M EDTA (pH 8.0), 20g PVP, 10g Sodium Sulphite and 82g Sodium Chloride), omission of the sodium acetate, potassium acetetate and mecarptoethanol. 70% ethanol was used instead of 80% and subsequent extraction procedure modified as follows: 50-70 mg of fungal mycelia was scrapped from the Petridish and placed in a 2ml microcentrifuge tube. 1000 µl of 2% SDS buffer was added and placed in a water bath at 65 ^{o}C for 1 h. Centrifugation was then done at 13000 rpm for 10 min. 750 µl of the supernatant was then pipetted out and placed on a sterile empty 2 ml microcentrifuge tube and an equal amount of Chloroform: Isoamyl Alcohol (CIA) 24:1 added. This was then centrifuged at 13,000 rpm for 10 min. 600 µl of aqueous was picked, placed on a fresh tube and an equal amount of cold isopropanol added to precipitate the nucleic acids. This was then incubated in a freezer (-20^{o}C) for 12 h and then centrifuged at 13,000 rpm for 10 min.

After centrifugation, the supernatant was carefully decanted leaving a pellet in the tubes. 700 µl of 70% alcohol was added and centrifuged at 13,000rpm for 10 min.

Ethanol was carefully decanted leaving the pellets. The pellets were then air-dried for 30 min, 100 µl of sterile ddH_2O added and samples incubated at 65 ^{o}C for 1 to dissolve the pellets. The samples were then stored at 4^{o}C.

DNA confirmation by gel electrophoresis

The bands of 30 isolates of *P.grisea* collected from the five sites were confirmed for presence and absence of DNA. The gel was then visualized under a UV transilluminator (Vilber Lourmat)

DNA quantification

DNA quantification was done using a 1% agarose prepared using 1X sodium borate and pre-stained with Gelred dye. The gel was placed in an agarose gel tank (Model CBS Scientific) containing sodium borate. 5 µl of DNA was mixed with 3 µl of lading dye and

Table 2. Colony diameter (mm) of *P.grisea* incubated at 25°C±1 on oat meal agar for 10 days period and spore diameter (µm, Magn x40) at 20 day growth observed under a microscope.

	Colony diameter (mm)			Spore diameter
Site	**3-day**	**5-day**	**7-day**	**(µm) Magn x40**
Alupe	$17.0^{b} \pm 0.9$	$31.3^{ab} \pm 1.6$	$75.0^{ab} \pm 2.6$	$5.34^{b} \pm 0.24$
Bomet	$27.8^{a} \pm 2.2$	$36.3^{a} \pm 2.1$	$81.0^{a} \pm 1.1$	$4.57^{b} \pm 0.46$
Koibatek	$20.7^{b} \pm 0.5$	$27.6^{b} \pm 1.4$	$76.6^{ab} \pm 2.0$	$4.95^{b} \pm 0.22$
Makueni	$17.2^{b} \pm 2.1$	$36.0^{a} \pm 2.0$	$75.0^{ab} \pm 2.6$	$6.37^{a} \pm 0.25$
Nakuru	$20.8^{b} \pm 1.4$	$29.6^{b} \pm 0.8$	$72.1^{b} \pm 1.7$	$4.92^{b} \pm 0.37$
LSD (5%)	0.5	0.54	0.66	0.79
CV%	14.6	10.05	5.14	6.30
R^2	75.7	68.5	53.3	99.10

*Means of three replicates per site ± standard error of the mean. Means followed by the same letters are not significantly different (P<0.01)

loaded on the gel wells. The gel was then run for 1 h at a voltage of 100 volts and a current of 500mA using an EC 1000 XC Power Pack and CBS Scientific MGU-502T gel tray. The gel was then visualized under a UV transilluminator (Vilber Lourmat).

DNA quantification was also done using a Nanodrop 2000c spectrophotometer (Thermo Scientific). The measurements include the concentration in ng/µl and absorbance 260:280 ratio to check for DNA purity.

SSR genotyping

Seven SSR markers were used for analyzing SSR diversity in *P. grisea* sourced from MGM database (http://ibi.zju.edu.cn/pgl/MGM/index.html) (Table 1).

PCR was performed in a 10 µl reaction volume containing 6 µl of master mix (One Taq Quickload 2x MM), 1 µl of forward and backward primers, 0.5 µl of 25Mm $MgCl_2$ (Promega), 1.5 µl of ddH_2O and 1 µl of DNA template. Amplification was done in an AB2720 thermocycler (Applied Biosystems) using the following conditions: Initial denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 30 s, annealing at 55°C for MGM coded primers for 1 min (*Pyrms* at 61°C), extension at 72°C for 2 min and final extension for 10 min at 72 °C. The PCR products were run on a 2% agarose gel. Amplicons were visualized under UV light and the fragment sizes scored based on a 100bp/1Kb molecular ladder.

Data analyses

Area Under Disease Progress Curve (AUDPC) was used to estimate the severity of leaf and head blast disease in susceptible KNE 741 finger millet variety. It was computed using Wilcoxson et al. (1975)'s formula;

$$\sum_{i=1}^{n} \left(\frac{(yi+yi+1)}{2} \times (t_{i+1} - ti) \right) \quad (1)$$

Where; yi = disease severity % on the ith scoring, ti = number of days from sowing to ith scoring, n= total numbers of scoring t (i+1) is second assessment date of two consecutive assessment and y_(i+1) is disease severity on assessment date (i+1).

The data on severity were subjected to analysis of variance for green house experiment using PROC GLM in Statistical Analysis Software (SAS institute Inc; Cary, 2002) with the following model

$$Yi = \mu + Rj + \varepsilon i \quad (2)$$

Where, Yi is the overall observation, µ is the overall mean, Ri is the ith observation due to variety and εi is the random error term (Equation 1).

Diversity data were generated by scoring for the presence (1) or absence (0) of amplification on all the gels producing a score matrix. Marker polymorphism was quantified in terms of polymorphic information content (PIC), major allele frequency, observed number of alleles and effective number of alleles using Power marker software version 3.25 (Liu and Muse, 2005). Diversity of *Pyricularia grisea* involved calculation of Nei's genetic diversity indices and Shanon's Information Index executed in PowerMarker software (Table 2). Phylogenetic analysis and grouping of isolates involved factorial and dendrogram construction using Neighbor Joining method in Darwin software, with 1000 bootstraps (Perrier, 2018; Perrier and Jacquemoud, 2015). A Principal Coordinate Analysis (PCA) was also done using Darwin software. Analysis of diversity within and among population was computed using AMOVA in GenAlEx software version 6.5 (PEP, 2012).

RESULTS

Cultural and morphological characteristics of *Pyricularia grisea*

Colony color

There was variation in colony color of all the pathogens collected from Alupe, Bomet, Koibatek, Nakuru and Makueni regions. Variation in color occurred mostly 3 to 5 days and 5-14 days at initial and later stages, respectively. At day 10, most pathogens had varied grey and black color on both the front and the back side of the petri-dish (Figure 1)

Blast isolate 1 (Alupe) was white at initial growth and finally changed to grey color at mid-stage from day 10, it was greyish-white in color. The colony had smooth margins with 5 rings observed only at the front of the Petri-dish. It had a unique appearance of red pigmentation during its growth which disappeared at later stages. Blast

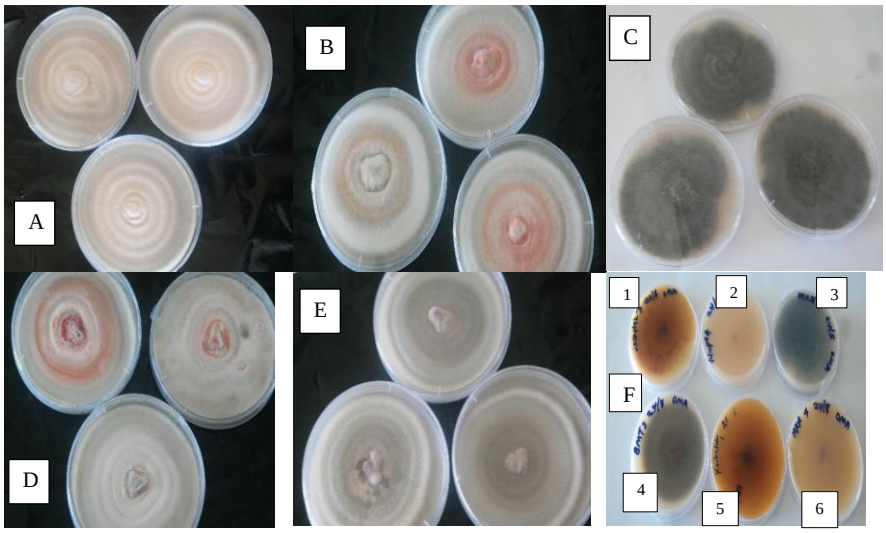

Figure 1. 10 day *Pyricularia grisea* isolates on Oat meal Agar color differentiation collected from different sites**.** Where A-Alupe isolate, B-Nakuru isolate, C-Makueni isolate, D-Koibatek isolate and E- Bomet isolate. F- Photographs taken from the back side of the petri-dish; where 1 and 5- Koibatek isolate, 2- Alupe isolate, 3- Makueni isolate, 4-Bomet isolate and 6- Nakuru isolate.

isolate 2- (Bomet) was white at initial stages of growth which turned to grey color at the final stages with a buff color observed at the back of the Petri-dish. It has smooth margins with 6 rings observed on the front and the back of the Petri-dish. Blast isolate 3- (Koibatek) had numerous variations in color. White and red pigmentation was observed during the initial stages of growth. The color later changed to greyish white at the final stages. It grows with smooth margins with 4 rings observed only at the front of the Petri-dish. Blast isolate 4- (Makueni) had grey color at the initial stage of growth on both sides of the Petri-dish which later changes to a total black color. It had no rings both at the back and the front of the Petri-dish with irregular margin as it grows. Blast isolate 5- (Nakuru) had a clear white color at the initial stages of growth with smooth margins and 5 centric rings. It finally changed to greyish white color at later stages (Figure 1).

Colony diameter and spore observation under the microscope

Colony diameter (mm) was measured on a 3rd, 5th and 7th day period and growth incubated in conditions of 25 ± 1 ^{o}C for 10 days was compared on all the five sites. On the 3rd day *P. grisea* from Bomet colony diameter had the highest mean (27.8 mm) which was significantly different (P<0.05) from those of Makueni, Alupe, Koibatek and Nakuru. On the 5th day, colony diameter from Bomet, Makueni was not significantly different (P<0.05). Pathogen strains from Koibatek and Nakuru did not differ significantly (P<0.05). Colony diameter from Alupe was not significantly different from those of all the other sites. On the 7th day, colony diameter from Bomet (81.0) had the highest growth and therefore significantly different from those of the other sites. Alupe, Koibatek, and Makueni had no significant difference (P<0.05). Nakuru had the lowest mean (72.1), however it was not significantly different (P<0.05) from those of Makueni, Koibatek and Alupe (Table 2).

Conidia characteristics

Microscopic observation showed that spore diameter of *P. grisea* was measured and the results indicated that there was significant difference (P<0.01) in spore sizes on the 20 days cultures. *P. grisea* from Makueni had the highest mean of 14.37 µm. The spores were long, pyriform with four septa at 20 days culture, more mature in terms of growth. Nakuru had two septate conidia with a rounded shape. The septae had separate margin. Alupe had a two medium sized pyriform spore with a rounded apex. Bomet had the lowest 4.57 µm with 2-4 septate conidia which had a smooth margin at the apex (Figure 2). The spores had a highest diameter of 5.34 µm when fully mature at 25 days. There was no significant difference in spore sizes from Alupe, Koibatek, Nakuru and Bomet at full maturity (Table 2). All *P. grisea* isolates from all the sites had pyriform shape with varied apex; round/sharp and round/flat, number of cells; from 2-4 celled conidia of various sizes and shapes. The middle cells were larger compared to the apex and the base cells. There was also varied growth from being large and

Figure 2. Micro-images of a twenty day culture of *P. grisea* isolates; Magn x40 collected from different growing regions of Kenya. Key: A= Blast 1- Alupe; B= Blast 2- Bomet; C= Blast 3- Koibatek; D= Blast 4- Nakuru; E= Blast 5- Makueni.

Table 3. ANOVA table for leaf and neck blast severity for KNE 741.

Source of variation	d.f	LAUDPC	NAUDPC
Site	5	1259.09***	1309.93***
Rep	1	36.75	36.75
Error		28.78	7.35
CV		7.53	4.00
R^2		97.77	99.44

***Significant at P<0.01, d.f=Degree of Freedom, AUDPC= Area Under Disease Progress Curve LAUDPC= Leaf severity values, NAUDPC=Neck severity values.

fully mature at 20th day (Isolate D) to slow development of spore characterized by small and immature spore (Isolate E) (Figure 2).

Pathogenicity test

There was variation in growth patterns, shape and color of *P. grisea* collected from the five regions. The effect of the *P. grisea* from the various sites on test plants indicated the pathogenicity levels of the pathogen. The symptoms were noted 7-20 days after inoculation as small white lesions which grew into diamond shape at the 10th day, greyish white symptoms with brown spots which later developed to be black at the 15th day and leaf necrosis by yellowing which was typical from the apex as it progressed downwards at the 20th day (Figure 5) (Wekesa et al*.,* 2019). These symptoms were a clear indication of *P. grisea* especially when compared to control which was not sprayed. *P. grisea* from Bomet and Makueni had the highest virulence (Highly pathogenic-Group I) while those from Alupe and Nakuru and finally Koibatek *P. grisea* had the least virulence (Mild pathogenic- Group III) (Table 3).

Virulence of pathogens strains from selected regions

Pathogen strains from different regions had different indication of the symptoms and infection to the susceptible variety KNE 741. From the ANOVA table, site was significant (p<0.005) for both leaf and neck blast severity (Table 4).

Mean separation from the five sites revealed that *P. grisea* from the sites were significantly different (P<0.01) in leaf and neck blast severity. Strains from Bomet, Alupe, and Makueni had higher severity values (>80) and did not differ significantly (P<0.01); while in Koibatek and Nakuru there was no significant (P<0.01) difference in leaf severity. A lower mean was recorded on the control (<20). Pathogens from Alupe, Makueni, Koibatek and Nakuru had no significant difference (P<0.01) on leaf severity. Neck severity appeared more on pathogen picked from Koibatek and Bomet and therefore they were not significantly different (P<0.01); while Alupe and Makueni had the same mean on neck blast severity

Table 4. Morphological characterization of *Pyricularia grisea* from five selected counties of Kenya.

Isolate	Location	Colony morphology	Colony color	Shape of conidia	Pathogenicity	Pathogenicity group
Blast-1	KALRO-Alupe, Busia county	Smooth margins with 5 circular rings observed on the front of the petri-dish	White and brown in color with buff color at the back	Pyriform very small	Moderate	II
Blast-2	ATC- Longisa Bomet county	Smooth margins with rings observed on both front and back side of the petri-dish	White which finally turns to grey in color	Pyriform-Large	High	I
Blast-3	ATC-Koibatek, Baringo county	Smooth margin with rings observed only on the front of the petri-dish	Brownish grey in color	Pyriform-medium	Mild	III
Blast-4	KALRO-Makueni, Machakos county	Circular in growth with irregular margins	Grey which finally turns to black color	Pyriform-Very large	High	I
Blast-5	ATC- Soil, Nakuru county	Round with smooth margins and rings at the front	White which finally turns to grey	Pyriform-Medium	Moderate	II

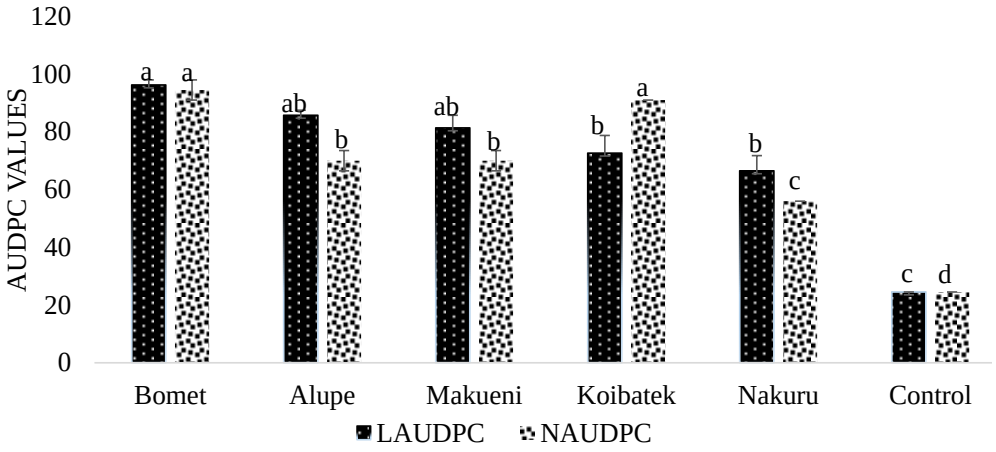

Figure 3. Comparison of leaf and neck severity of *P. grisea* on KNE 741. Means with the same letters are not significantly different (P<0.01).

(Figure 3).

Relationship between neck and leaf blast

Pearson's correlation coefficient indicated that there was a positive correlation of leaf and neck blast since a one-unit increase in leaf blast with all the factors held constant led to an increase of 0.9003 with 88.2 % of neck blast (Figures 4 and 5).

Molecular characterization of *P. grisea* from five selected major growing areas of Kenya

Confirmation of the DNA by gel electrophoresis

The bands of 30 isolates of *P.grisea* collected from the five sites were confirmed to be present after visualization under a UV transilluminator (Vilber Lourmat) (Figure 6)

Marker polymorphism and genetic diversity of *P. grisea* isolates collected in the five major growing areas of Kenya

A simple numeric scoring was used to denote the presence (1) and absence (0) of allele for each loci (Table 5). The observed allele number was two for all the selected primers indicating that only one locus was amplified by all the markers. Effective number of alleles $\{A_E=1/ (1-H_{exp})\}$ ranged from 1.3006 (MGM 437) to the highest (1.9912) (*Pyrm* 61-62). Major allele frequency ranged from 0.5333 to 0.8667. Polymorphic Information Content (PIC) varied between 0.2044 and 0.3739 for primers MGM 437 and *Pyrm* 61-62 respectively. When *P. grisea* diversity was assessed, gene diversity ranged between 0.2311 and 0.4978 with a mean of 0.4.

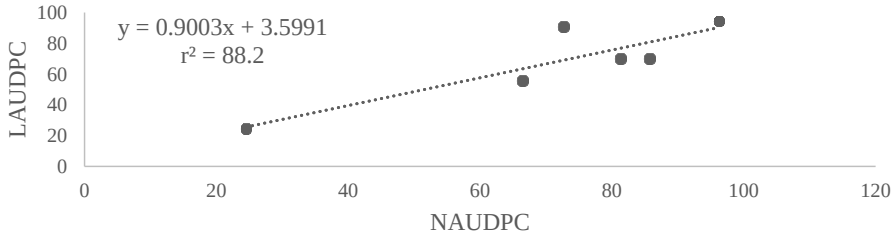

Figure 4. Relationship between neck and leaf blast of *P. grisea* sprayed on susceptible variety KNE 741.

Shannon's Information index varied between 0.3927 and 0.6909 with a mean of 0.5848 (Table 5).

Factorial and phylogenetic analysis of the strains of *P.grisea* collected in various regions

Factorial analysis revealed that the samples from all the five sites clustred randomly with no distinct pattern observed (Figure 7). Most isolates clustered as individuals showing that they were genetically distinct while a few clumped together.

The phylogenetic analysis grouped the samples into two main clusters and six sub-clusters. Cluster 1 comprised 2 sub clusters. Sub-cluster 1 comprised isolates 2, 3, 4 and 5 from Nakuru, 3 and 4 from Alupe and 3 and 6 from Koibatek which appeared as duplicates and isolate 6 from Bomet which clustered as a distinct individual. Sub cluster two had only two isolates Makueni 1 and Alupe 6 which appeared distinct. Cluster II comprised 4 sub clusters. Sub cluster I comprised isolate 4 and 5 from Bomet which appeared as duplicates and isolate 1 from Koibatek. Sub cluster II comprised isolate 1 and 2 from Bomet and 2 and 5 from Koibatek which appeared as duplicates and isolate 5 from Alupe appeared as distinct. Sub cluster III comprised distinct isolates 6 and 1 from Makueni and Nakuru respectively. Sub cluster IV had 5 duplicate isolates Makueni (2, 3, 4 and 5) and distinct isolate 1 from Alupe, 3 from Bomet and 6 from Nakuru. There was no particular pattern deduced from the grouping; however, some strains from different regions were observed to cluster in same sub clusters as duplicates with few appearing distinct (Figure 8).

Overall, phylogenetic analysis revealed that *P. grisea* isolates from the studied regions were genetically diverse within the isolate population as opposed to geographical differentiation.

Analysis of Molecular variance (AMOVA)

Results of AMOVA (Table 6) revealed there was huge diversity within the *P. grisea* isolate populations (87%) compared to among the population (13%) with a P value of 0.053.

DISCUSSION

Morphological diversity of *P. grisea* in major finger millet growing areas in Kenya

P. grisea isolates collected from Alupe, Bomet, Makueni, Koibatek and Nakuru showed high variation both morphologically and genetically. There was variation in colony color, colony diameter and structure in growth patterns both at the petridish and microscopically. Description of the *Ascomycete* fungi has been described by conidia and conidiophore morphology as the main characteristics of the fungi (Choi et al*.,* 2013). The difference in color maybe due to different growth stages of the spores which tend to vary with blast isolate and patterns of growth. Meena (2005); Getachew et al*.* (2014); and Shahriar et al*.* (2020) revealed that *Magnaporthe grisea* tends to vary due to sexual hybridization which shows variability in form and color at different asexual stage of the fungi. Colony structure on the microscope revealed significant morphological differences on the structure of conidia and conidial appendages of *P. grisea* from different regions. *P. grisea* had several flared pigmented conidiophores which are aseptate in nature. The findings are similar to those reported earlier by a study done by Klaubauf et al. (2014) who noted that the fungi have septate conidia of varying shape. This could be due to effects of different environments and ecological conditions under which the various *P. grisea* strains were collected which could have influenced the size, septation and form of the conidia. Getachew et al*.* (2013) reported similar findings and noted that environment affects the growth of the fungi size and shape. Conidia appendages aid in attachment to substrates, dispersal of spores and acclamation to new environments, which affect their variability in shape, mode of development, color and infectivity. Klaubauf et al*.* (2014) also noted different isolates differ with respect to the factors which also are a determinant of their variability in shapes, mode of development, color and

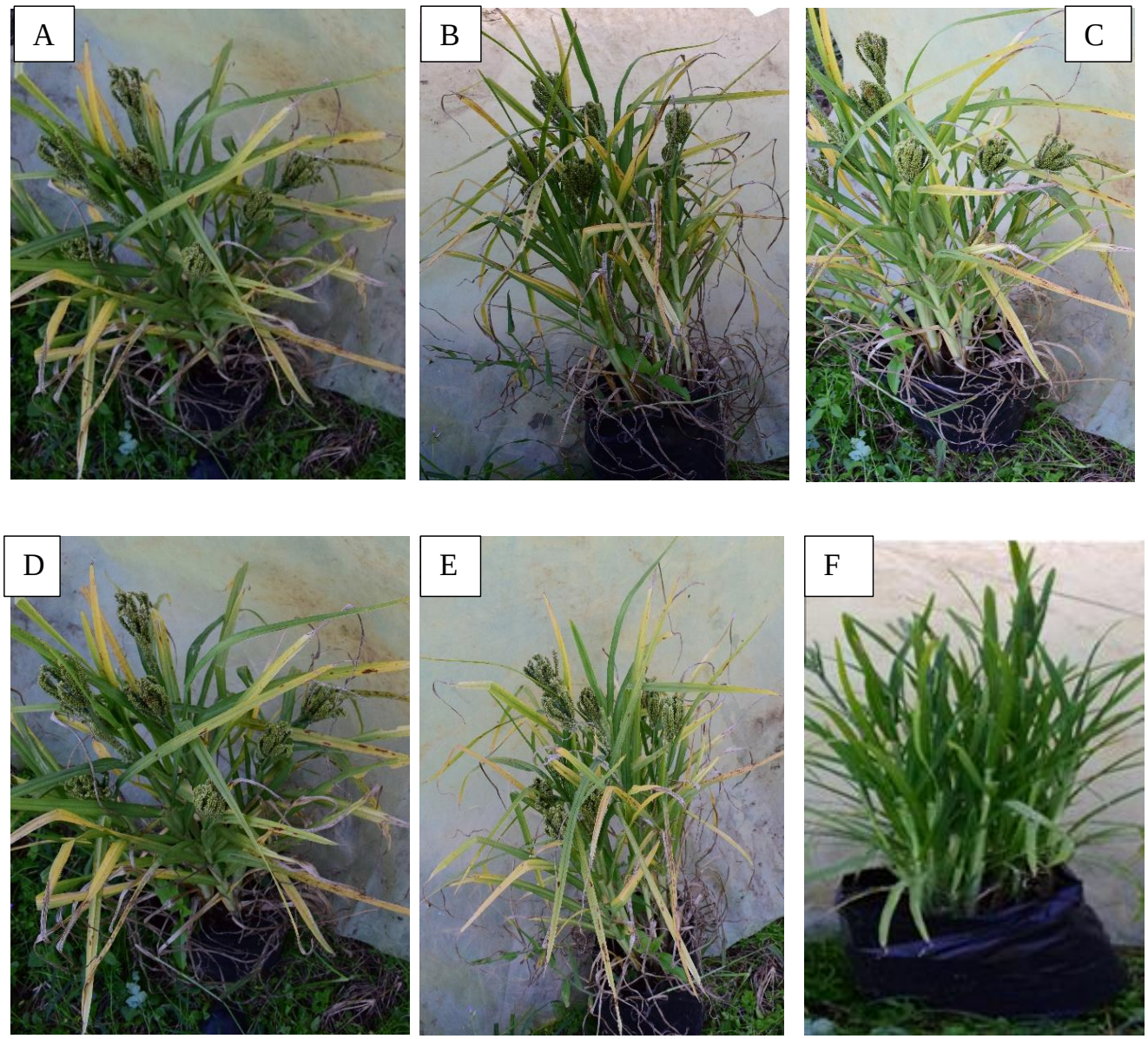

Figure 5. Effect of *P. grisea* isoltaes on susceptible KNE 741 variety. Where letters represent the pathogens sprayed on susceptible KNE-741: A= Bomet isolate, B= Makueni Isolate, C= Alupe isolate, D=Nakuru isolate, E=Koibatek isolate and F=Control (Non-sprayed)

infectivity to the host plant. The pathogen from Bomet and Makueni had higher growth on the petri-dish compared to those of Nakuru, Koibatek and Alupe. This could be due to the pathogens' aggressiveness to mature faster and produce leading to more colonization on the host plant; these types of pathogen tend to gain resistance faster and can breakdown easily hence gaining resistance. These findings are in accordance with Saleh et al. (2014) who studied the origin, diversity and, dispersion of rice blast fungi and found that faster evolution and resistance of *Magnaporthe grisea* is linked to the faster growth of the pathogen.

Pathogenic diversity of *P. grisea* in major finger millet growing areas in Kenya

Pathogenicity test which was a measure of the virulence of the pathogen collected from five different major growing areas showed that there is variation in symptoms of various *P. grisea*. This may be due to the existence of diversity that existed within the pathogens and their expression on the host. This was because an attack on the vegetative cycle of the plant can easily be translocated on the neck. Similar to the findings, Ghatak et al. (2013) observed the aggressiveness of *P. grisea*

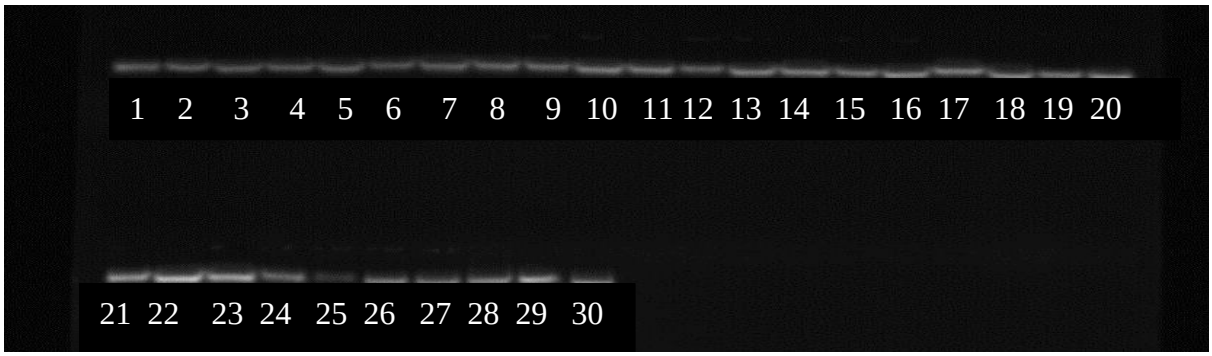

Figure 6. The presence of *P.grisea* isolates showing presence of DNA bands as viewed under a UV trans-illuminator.

Table 5. Summary statistics of genetic diversity indices of 30 samples of *P. grisea* in the five selected sites.

Marker	Sample size	Observed allele number	Effective number of alleles	Major allele freq.	PIC	Gene diversity	Shannon's information index
MGM 200	30.0000	2.0000	1.3846	0.8333	0.2392	0.2778	0.4506
MGM 436	30.0000	2.0000	1.9651	0.5667	0.3705	0.4911	0.6842
MGM 437	30.0000	2.0000	1.3006	0.8667	0.2044	0.2311	0.3927
MGM 451	30.0000	2.0000	1.6423	0.7333	0.3146	0.3911	0.5799
MGM 454	30.0000	2.0000	1.9651	0.5667	0.3705	0.4911	0.6842
PYRM 15-16	30.0000	2.0000	1.7241	0.7000	0.3318	0.4200	0.6109
PYRM 61-62	30.0000	2.0000	1.9912	0.5333	0.3739	0.4978	0.6909
Mean	30.0000	2.0000	1.7104	0.6857	0.3150	0.4000	0.5848
Std. deviation		**0**	**0.2850**	**0.1345**	**0.0682**	**0.1081**	**0.1202**

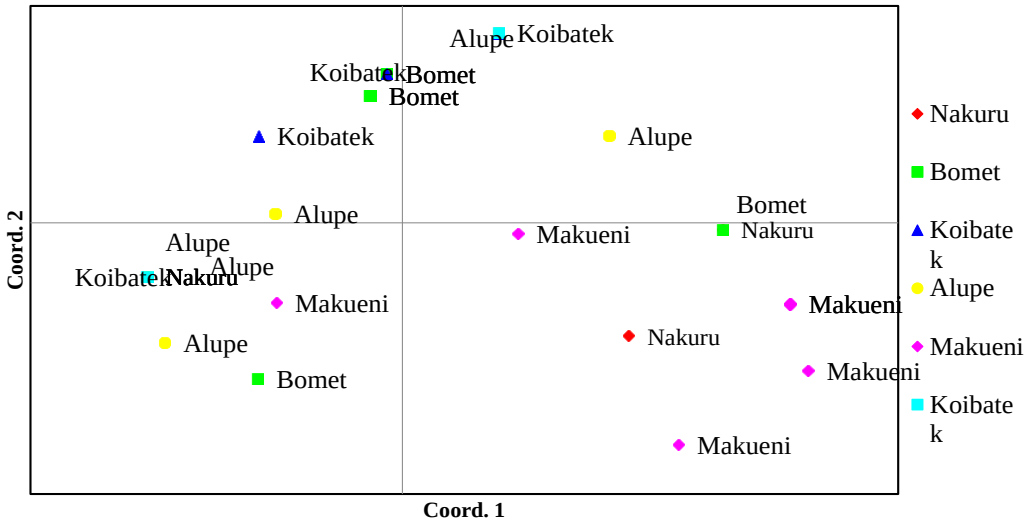

Figure 7. Factorial analyses of *Pyricularia grisea* strains collected from various growing regions.

and reported the epidemics of leaves during the early stages of the crop cycle leads to a high probability for neck infections during the reproductive stage. This is due to the numerous population shifts occurring. However, this differs with genotype resistance to the pathogen since various resistant genotypes will tend to show

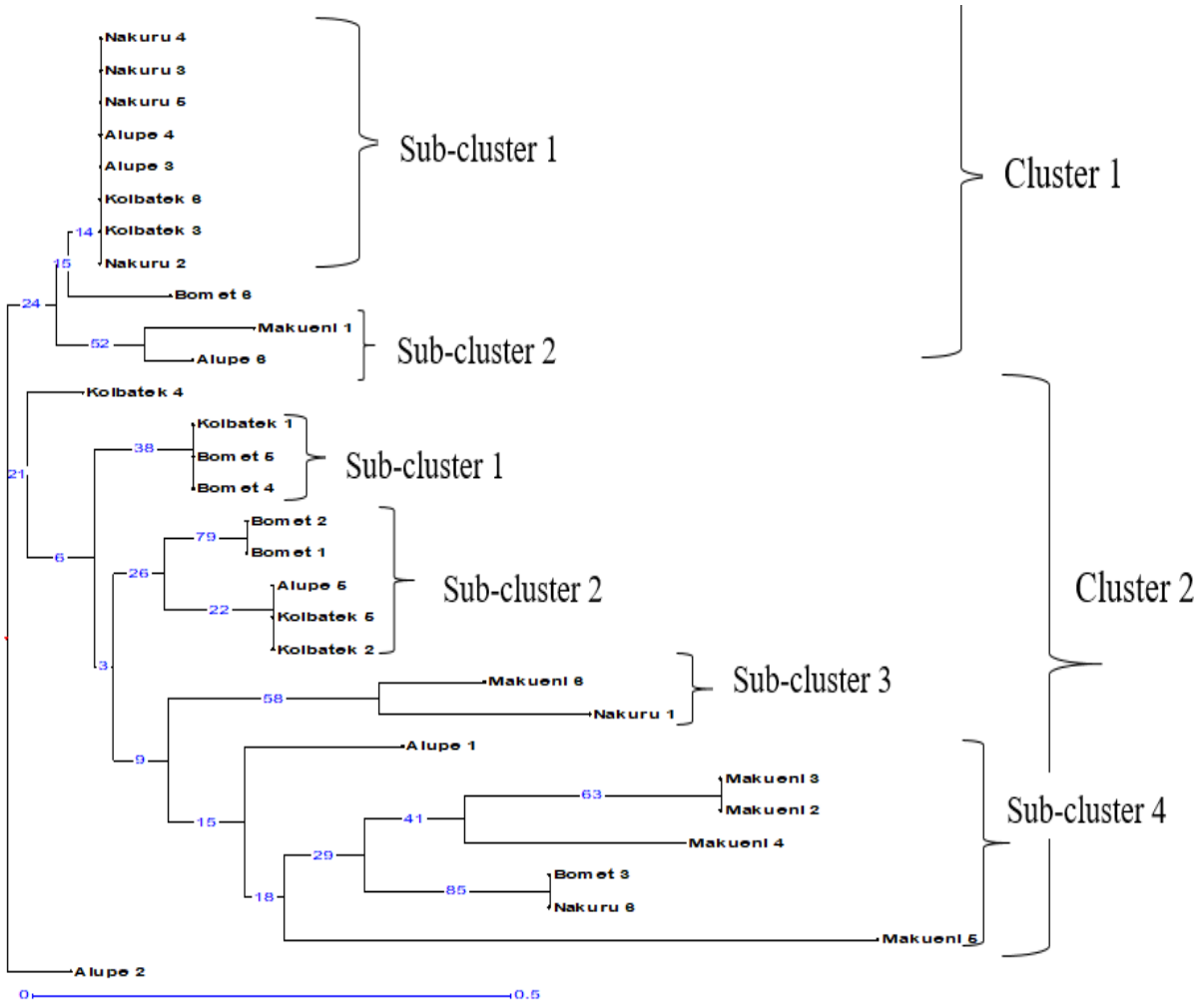

Figure 8. Dendrogram showing *Pyricularia grisea* diversity constructed using neighbor joining method.

Table 6. AMOVA table of *P. grisea* with 30 samples collected from 5 regions.

Source	df	Ms	Est.Var.	%	P.value
Among Pops	5	2.233	0.193	13	0.053
Within population	24	1.235	1.285	87	

*Est.var-Estimated variance and %-Percentage populations.

hypersensitivity therefore leading to no more spread of the disease unlike susceptible genotypes such as KNE 741 that have a high probability of expressing both types of blast disease.

Genetic diversity of *P. grisea* in major finger millet growing areas in Kenya

Genetic variability is important for proper understanding of blast mechanism and for development of strategies for the control of most fungal diseases. Few studies have focused on genetic variability of *M. grisea*; the use of RAPD by Singh and Kumar (2010) who confirmed the variability and virulence complexity of *Magnaporthe grisea*. Takan et al. (2012) used AFLP technology to show genetic variation pattern, adaptive divergence of host specific forms of *M.grisea* and the use of MGR-RFLP by Babujee and Gnanamanickam (2000) distinguished rice and finger millet blast fungi in India. Recent studies include the use of SSR markers (Jagadeesh et al., 2020; Yadav et al., 2019; Ngernmuen

et al., 2019). Similar to this study is Anjum et al. (2016) who evaluated finger millet using SSR markers to show genetic variability in *P. grisea*.

In this study, genetic diversity indicated the existence of different strains collected from different environments.

The average number of observed and effective number of alleles reported in the study (2 and 1.71) and indication of the number of frequent alleles that would achieve expected heterozygosity in the population. This finding was slightly lower than that reported by Babu et al. (2013) (6.18). This variation in the number of alleles has been reported in other studies (Kaye et al., 2003; Zheng et al., 2008; Fujita et al., 2009). This could be due to few number of markers and samples, nature of MGM markers which were more specific to rice than finger millet and possible difference in the level of genetic variation in different areas (Salem et al., 2010).

MGM markers had lower scores of PIC and genetic diversity compared to *Pyrm*. Among the MGM markers, MGM 454 scored high PIC (0.3705) and genetic diversity (0.4911) while MGM 437 had the lowest 0.2044 and 0.2311 for PIC and genetic diversity respectively. MGM 454 has a higher motif number (ct-29) compared to MGM 437 (tct-11) (http://ibi.zju.edu.cn/pgl/MGM/index.html). Higher repeat motif is linked to the coverage area of the primer which directly impacts primer amplification and detection (Wang et al., 2009). The PIC and genetic diversity values in this study were similar to those reported by Anjum et al. (2016) for MGM 454 (0.40, 0.32) and higher for MGM 437 (0.08, 0.08).

Markers *Pyrm* 15-16 had PIC values of 0.3318 and 0.3739 respectively while *Pyrm* 61-62 had 0.785 and 0.760 respectively. These values were lower than those reported by Babu et al. (2013). The higher values reported by Babu et al. (2013) could be due to the fact that the isolates of *M. grisea* used were from a combination of finger millet, foxtail millet and rice. Anjum et al. (2016) who incorporated the use of the same markers as those in the study recorded a 0.59 and 0.54 on *Pyrm* 15-16 and *Pyrm* 61-62 respectively from isolates of *P. grisea* from finger millet. The higher values reported by Babu et al. (2013) could be due to the fact that their study used rice, foxtail millet and finger millet while in this study only finger millet is used.

Genetic diversity for *Pyrm* 15-16 and *Pyrm* 61-62 was 0.42 and 0.49 respectively which was almost similar to the findings of Anjum et al. (2016) (0.59 and 0.54); while Babu et al. (2013) reported higher values of 0.803 and 0.780 for the same markers. The higher value reported could be due to genetic diversity of the pathotypes collected from different crops; while this study's own is in contrast to Anjum et al. (2016) who used *P. grisea* isolates collected from finger millet only. This variation in values with the same pathogen assessed could be due to host-pathogen specificity and adaptation of the pathogen to a particular host; this in turn could have influence on the variability of the pathogen hence higher values recorded (Tribble et al., 2013).

The results on factorial analysis showed that the samples from all the five sites clustered randomly with no distinct pattern observed. For example, Cluster 1 comprised 2 sub clusters. Sub-cluster 1 comprised isolates 2, 3, 4 and 5 from Nakuru, 3 and 4 from Alupe and 3 and 6 from Koibatek which appeared as duplicates and isolate 6 from Bomet which clustered as a distinct individual. This finding is in line with other numerous studies on finger millet who found out that there was geographical differentiation between strains (Rebib et al., 2014). This suggests the non exixtence of sexual reproduction among strains of *P. grisea* and therefore pathogen changes with time through evolution (Fry et al., 2015; Bengtsson, 2003).

Phylogenetic analysis revealed that *P. grisea* in the study regions was diverse with no geographical grouping with some strains from different regions observed to cluster in same sub clusters as duplicates with few appearing distinct. This observation was similar to findings of Longya et al. (2020) who also failed to deduce regional differentiation of rice *P. grisea* in Thailand. Similar findings have been documented by Anjum et al. (2016) and Singh and Kumar (2010).

AMOVA indicated there was huge diversity within *P. grisea* isolates (87%) and low diversity among the selected regions (13%). This implies that majority of the observed variation in the *P. grisea* was due to genetics rather than geography. The pathogen varied genetic diversity has challenges in development of management and control of the pathogen (Mia, 2013). Similar to the findings, Kumar et al. (1999) and Rebib et al. (2014) reported a huge diversity occuring within populations as opposed to among populations of *Magnaporthe grisea* of rice, which was mainly linked to the varied genetic make up of the pathogen.

CONCLUSION AND RECOMMENDATIONS

The findings of this study showed that there were differences in the finger millet blast (*Pyricularia grisea*) strains existing in the major finger millet growing area in Kenya (Alupe, Bomet, Makueni, Koibatek and Nakuru). The key variations were associated with morphology, pathogenicity and genetic diversity. The morphological test revealed that the pathogen from Bomet and Makueni as compared to those from Alupe, Nakuru and Koibatek). The pathogenicity test showed that environment plays a significant role in the physical appearance of the pathogen which is mainly associated with rainfall, temperature and humidity of the regions. Molecular diversity showed that there was large variation within the isolates as opposed to among the isolates indicating the possibility of finding same strains of the pathogen in different environments as opposed to having same strains in the same environment. Blast populations are

genetically diverse and the relationship among them can be identified by use of specific SSRs for the selected pathogen. From this study it is clear that *P. grisea* diversity is important in disease management strategies, disease dynamics and host-pathotype understanding which can lead to development of resistant finger millet hosts. Therefore there is need for more studies to be done on the *P. grisea* affecting finger millet in more areas of Kenya using different types of markers. It is also important to do sequencing on the isolates of finger millet genotypes and documented in Kenya.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Role of mass gatherings in transmission of respiratory tract infections caused by Haemophilus influenzae

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Respiratory infection is one of the infections that can be transmitted in closed places due to the ease of transmission of pathogens from infected people to healthy people through droplets from coughing and sneezing, among these bacterial causes, *Haemophilus influenza*. Therefore, this study aimed to evaluate the prevalence of this type of bacteria among pilgrims coming from different geographical locations in the world and determine the antibiotic-resistant strains. Around 1226 nasopharyngeal swabs were collected from 613 pilgrims from several different nationalities before and after completing the Hajj. These specimens were cultivated on chocolate agar medium and the pathogens were identified according to Clinical Laboratory Standards Institute (CLSI) protocol and confirmed by an automated system machine (VITEK2). There is no *H. influenzae* was detected among pilgrims before performing Hajj and the rate of transmission of *H. influenzae* was 1% after performing Hajj. The most effective antibiotics against to *H. influenza* isolates from pilgrims was Rifampicin while all the isolates were resistant to ampicillin. The continuous monitorization of the rate of potentially pathogenic bacterial during the Hajj season is required in order to develop advanced strategies to confront any outbreak of any infectious disease among the pilgrims.

Key words: Hajj season, nasopharyngeal swabs, ethnic group, Makkah.

INTRODUCTION

Hajj and Umrah season have been associated with the spread of respiratory infections between visitors and pilgrims, this is due to mass gathering resulting from the gathering of a large number of Muslims to perform Hajj rituals from all over the world in the limited geographical area and specific times (Lewnard et al., 2019). A number of health reports showed that respiratory infections were the most common health complaint among pilgrims and the first reason for hospitalization (Sarangi et al., 2000) (Farley et al., 1992; Shafi et al., 2008). Respiratory infection and meningitis are important health problems, which are often associated with crowded places due to the ease of translation of the pathogens from the infected and carrier persons to healthy persons by droplets through the air (Madani et al., 2006; Asghar et al., 2011). These causes include *Streptococcus pneumoniae, Haemophilus influenza*, and *Neisseria meningitidis*, these bacterial pathogens are densely colonized in the human nose and mouth, along with many commensals bacteria which can turn into pathogens, especially in unhealthy

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living conditions (Slack, 2015; Memish et al., 2014). Many studies, showed that the colonization of *H. influenzae* bacteria in the nasal playing a major role in the spreading of infections of the respiratory system (Whittaker et al., 2017; Ahmed et al., 2009). One of the factors that cause concern due to these health conditions during the Hajj season is the spread of bacterial strains that were not included in the vaccinations that the pilgrims receive before the Hajj and also the emergence of bacterial strains resistant to antibiotics which have become a major challenge to the health system in Saudi Arabia (Michael et al., 2019; Bozdogan et al., 2006; Stephen et al., 2007). Based on these facts, this study focused on one of the causes of respiratory tract infections, which is *H. influenzae*. *H. influenzae* bacteria belongs to Gram-negative coccobacilli and include two major categories, encapsulated and unencapsulated strains. Encapsulated strains contain six serotypes are (a, b, c, d, e, and f) (Plumb et al., 2018; Harris et al., 2017). *H. influenzae* type b, (Hib) is the major virulence factor of this organism and causes pneumonia, meningitis, epiglottitis, and bacteremia (Soeters et al., 2018; Adam et al., 2010). Un-encapsulated strains are non-typable *H. influenzae* (NTHi) due to lack of capsular serotypes and less invasive. Hib vaccine is effective in preventing Hib infection only but not effective against infection with NTHi strain (Romaneli et al., 2019). Therefore, this study aimed to evaluate the prevalence of this type of bacteria among pilgrims coming from different geographical locations in the world to determine the emergence of antibiotic-resistant strains, which would provide the basis for the adoption of appropriate preventive to limit the spread of these pathogens and control them.

MATERIALS AND METHODS

The subject of the study

The current study included, 613 pilgrims from several different nationalities around the world; 90 hajjes from Libyan, 161 Indian, 68 Syrian, 102 Nigerian, 95 Indonesian, 35 Turkish, 46 British, 11 Australian, 2 Iranian, and 3 Swedish of the total number of pilgrims who performed the Hajj in the year 1432 AH corresponding to 2010, where the sample was selected in a statistically controlled manner by specialists in biological statistics, where the method of collecting a number of random samples was adopted, regardless of nationality, or gender.

Collection of samples

Around 1226 nasopharyngeal swab were collected from of 613 Pilgrims, one swab at arrival each pilgrim to Saudi Arabia and second swab after completing the Hajj, in addition to collecting data from these pilgrims including; date of collection, nationality, sex, age, coughing, smoking, antibiotic usage, contacts numbers. All specimens were collected on a sterile transport medium (Amie's agar gel- Oxoid) and sent to the microbiology research laboratory in the faculty of medicine, where nasopharyngeal specimens insulated on chocolate agar medium under aseptic conditions inside a biological safety cabinet class 2 and the plates were incubated in a CO_2 incubator at 37°C according to clinical and laboratory standards institute (CLSI).

Isolation and Identification of *H. influenza*

H. influenzae was isolated after an overnight incubation from chocolate agar as a mucoid colony, and the identification was confirmed by characteristic appearance and microscopically by Gram stain as a Gram-negative coccobacillus followed by the confirmatory tests of suspected colonies were subculture on nutrient agar with commercial diagnostic discs, nicotinamide adenine dinucleotide (factor V) and hemin (factor X). Isolated strains were confirmed by automated system (microbial identification system) VITEK2 Compact " biomerieux".

Antibiotics susceptibility testing

Susceptibility testing included numbers of antibiotics as the following: Azithromycin, Cefotaxime, Ceftriaxone, Ciprofloxacin, Levofloxacin, Ampicillin, Rifampicin, Clindamycin, Meropenem, Augmentin, chloramphenicol, and Trimethoprim-sulfamethoxazole, and conducted according to clinical and laboratory standards institute (CLSI) (CLSI, 2012, 2018).

Statistical method used in this study

Statistical analysis was done by an experienced biostatistician, using the latest statistical.

Ethics approval

We obtained the approval of the ministry of Hajj to carry out this study in addition to obtaining written consent from the heads of pilgrim groups and from every person participating in this study before starting to collect samples.

RESULTS

A total of samples collected from 613 individuals who participated in this study who came to perform the rituals of Hajj was 1226 samples, half of the samples were collected before the Hajj and the other half after Hajj. There is no *H. influenzae* was detected among this number of pilgrims before performing Hajj. While after the Hajj, a positive rate was recorded for the transmission of bacterial carriage among the pilgrims of some nationalities participating in the Hajj, and it was as follows, among 161 Indian pilgrims was 2 (1.2%) confirmed their positivity which was statistical significance (P-value = 0.5). Among 68 Syrian pilgrims was 1 (1.5%) confirmed their positivity after completing the Hajj, and this difference was not statistically significant (P-value = 1.0). Among 35 Turkish pilgrims was 1 (2.9%) confirmed their positivity after completing the Hajj, and which was its statistical significance (P-value = 1.0). Among 11 Australian pilgrims was 1(18.2%) confirmed their positivity after completing the Hajj, and which was its statistical significance (P-value = 0.5). While there is no

Table 1. The overall prevalence of *H. influenzae* isolated from different nationalities of the Pilgrims during the Hajj season.

Nationality	No. pilgrims tested	Before Hajj		After Hajj		P-value
		No. positive pilgrims, N(%)	No. negative pilgrims, N(%)	No. positive pilgrims, N(%)	No. negative pilgrims N(%)	
Libyan	90	0 (0)	90 (100)	0 (0)	90 (100)	-
Indian	161	0 (0)	161 (100)	2 (1.2)	159 (98.8)	P= 0.5 not significant
Syrian	68	0 (0)	68 (100)	1 (1.5)	67 (98.5)	P= 1.0 not significant
Nigerian	102	0 (0)	102 (100)	0 (0)	102 (100)	-
Indonesian	95	0 (0)	95 (100)	0 (0)	95 (100)	-
Turkish	35	0 (0)	35 (100)	1 (2.9)	34 (97.1)	P= 1.0 not significant
British	46	0 (0)	46 (100)	0 (0)	46 (100)	-
Australian	11	0 (0)	11 (100)	2 (18.2)	9 (81.8)	P= 0.5 not significant
Iranian	2	0 (0)	2 (100)	0 (0)	2 (100)	-
Swedish	3	0 (0)	3 (100)	0 (0)	3 (100)	-
Total	613	0 (0)	613 (100)	6 (1)	607 (99)	P= 0.03 significant.

Table 2. Antimicrobial Susceptibility of *H. influenza* isolated from Pilgrims during the Hajj season.

Antibiotics	*Haemophilus influenza* isolates before Hajj Total (0)		*Haemophilus influenza* isolates after Hajj Total (6)	
	Susceptible No. (%)	Non-susceptible No. (%)	Susceptible No. (%)	Non-susceptible No.(%)
Azithromycine	0	0	4	2
Cefotaxime	0	0	4	2
Ceftriaxone	0	0	4	2
Ciprofloxacin	0	0	4	2
Levofloxacin	0	0	4	2
Ampicillin	0	0	0	6
Rifampicin	0	0	6	0
Clindamycin	0	0	2	4
Meropenem	0	0	4	2
Augmentin	0	0	4	2
Chloramphenicol	0	0	2	4
Trimethoprim-sulfamethoxazole	0	0	2	4

H. influenzae detected among the pilgrims of the following countries; Libyan, Nigerian, Indonesian, British, Iranian, and Swedish before and after performing Hajj (Table 1).

Antibiotics susceptibility testing of *H. influenzae* isolated during the Hajj season from pilgrims

Antibiotic sensitivity testing for six isolates of *H. influenzae* that were isolated in this study from pilgrims during the Hajj season in the city of Makkah and which were tested by disk diffusion, all of them was susceptible to rifampicin antibiotic. While all were resistant to ampicillin antibiotic on the other hand, the percentage of sensitivity was 67% to Ceftriaxone, Ciprofloxacin, Azithromycin, Meropenem, Cefotaxime, Levofloxacin, and Augmentin antibiotic (Table 2).

DISCUSSION

Hajj and Umrah are mass congregations that may put serious health hazards to visiting pilgrims and native residents via the transmission of infectious diseases especially the infections that may get spread via contact, respiratory droplet and with the influx of untreatable bacterial strains, this may lead to emerging multidrug drug-resistant strains, that may lead to outbreaks in mass congregations like Hajj and Umrah and generate resistant

strains that may get a swift global and local transmission through Hajj and Umrah gatherings where was in the recent years there was a quick drop in antibiotics response against common infections. Studies showed that exposure to the mass gathering during the performance of the Hajj rituals had a role in infection with respiratory diseases and the acquisition of this bacterial infection and its spread among the pilgrims (Shiraha et al., 2017). Studies have shown that the colonization with potentially pathogenic bacteria such as *H. influenzae* is considered a major factor in respiratory tract infection and meningitis, and it is transmitted from infected persons or carriers (Gautret et al., 2015). Some studies also showed a difference in transmission rates, which were associated with factors including social, economic, and living conditions (Rubach et al., 2011). Two important factors of concern during Hajj season the first, emergence and spread of several antimicrobial-resistant strains such as; ampicillin-resistant *H. influenzae*, and antibiotic-resistant *S. pneumoniae* (such as; penicillin, macrolide, quinolone). The other factor of concern is the possible spread strains not included in the current vaccines administered to all pilgrims before coming to Saudi Arabia (Dworkin et al., 2007). Therefore, the World Health Organization recommends monitoring epidemics in such gatherings that may spread due to this mass gathering in these seasons, and to ensure that available vaccinations for these pathogens especially the causes of respiratory tract infections (Ahmed et al., 2009). In the current study, the bacterial isolates of *H. influenzae* were recorded after performing Hajj and none of them were proven before the Hajj on all the pilgrams who underwent this study. Can be concluded from this that the pilgrams of some countries were colonizing with the causes of bacterial infection, and with exposure to crowding they became a source of infection spread as the translation rate increased to (1%) after performing Hajj. The highest percentage was recorded among Australian pilgrims (18.2%), followed by Turkish pilgrims (2.9%), followed by Syrian pilgrims (1.5%) and Indian pilgrims (1.5%), respectively while other nationalities have not been infected with *H. influenzae* during the Hajj season, either before or after performing the Hajj. In comparison with some similar studies, the results of this study were lower in the number of isolated bacteria, compared to other studies that recorded higher rates (Nik et al., 2019; Blain et al., 2014; ChunCho et al., 2019; Sarangi et al., 2000; Farley et al., 1992). This may be one of the reasons that there are no epidemics during the Hajj season, and it may be due to taking antibiotics and getting vaccinated before going to Hajj. However, also, the results of the antibiotic susceptibility tests showed that the most effective antibiotics against *H. influenzae* isolate frompilgrims who have been shown to be infected with *H. influenzae* was Rifampicin which was the sensitivity of the isolated bacterias (100%), while all the isolated strains were resistant to Ampicillin.

Conclusion

This study showed that being in crowded places increases the possibilities of transmitting infectious diseases among the participants. In the study, it was found that the level of bacterial carriage in the upper respiratory tract before performing the Hajj was very low, in other words, there are no *H. influenzae* detected among pilgrims before performing Hajj and the rate of transmission of *H. influenzae* was 1% after performing Hajj, the results of the susceptibility testing on the isolated bacterial strains showed highly sensitive to rifampicin while all the isolated strains were resistant to Ampicillin. So, the continuous monitorization of the prevalence rate of potentially pathogenic bacterial during the Hajj season is required in order to develop advanced strategies to confront any outbreak of any infectious disease among the pilgrims. These results should be subject to more detailed studies in the next Hajj seasons to evaluate the carriage rate of pathogenic bacterial strains in pilgrims and determine if any changes in the prevalence levels and the introduction of controls for this study by Saudi citizens to compare the prevalence rates of bacterial infection among pilgrims with Saudi citizens.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Full Length Research Paper

Role of heavy metals on antibiotic resistance properties in lactose fermenting isolates collected from textile industry effluents

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It is crucial to monitor the microbial ambience in heavy metal enriched industrial effluents that generally discharge into the environment. Therefore, the present study aimed to understand the load of heavy metals in multidrug-resistant lactose fermenting microorganisms isolating from textile industry effluents. Samples were collected from five different textile industrial canals. In most of the cases, the colony forming units (CFUs) per plate were uncountable at 10^{-6} dilution but countable at 10^{-10} dilution. A total of 100 lactose fermenting (LF) isolates were selected using 4 differential media and tested for the determination of minimum inhibitory concentrations (MICs) of heavy metals and antibiotic susceptibility. For nickel (Ni), the MIC was 0.3 mM for almost 98% isolates. The MIC was 0.5 mM for chromium (Cr) in almost 99% isolates. For lead (Pb), 100% isolates had a MIC of 0.4 mM. The most prevalent (36%) resistance pattern was found for sulfamethoxazole-trimethoprim (SXT) and only 10% isolates showed resistance to cefotaxime (CTX). The combined effect of heavy metals and antibiotics revealed that in most cases, the antibiotic zone of inhibition was increased. Plasmid profiling showed that among 14 selective isolates, high sized (21kb) plasmid was found in 6 isolates.

Key words: Antibiotics, effluents, heavy metals, minimum inhibitory concentration (MIC), antimicrobial resistance.

INTRODUCTION

Wastewater is widely known as crucial crossroads between diverse water bodies, for example, wastewater from hospital, household water, surface- and ground-water (Zhang et al., 2009). However, this study is mainly focusing on industrial wastewater discharged directly to the environment as effluents during the pre- and post-processing of textile products. The wastewater is actually outlined by the presence of discriminating pressures of harmful chemical compounds, heavy metals (copper, lead, chromium, nickel, cadmium, zinc etc.), and high

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content of organic materials (Kümmerer, 2003; Martinez et al., 2009; Rahube and Yost, 2010) which contribute an opportunity for the mobile elements to be incorporated into the opportunistic pathogenic microbes and naturally growing environmental bacteria (Martinez et al., 2009; Sørensen et al., 2005; Szczepanowski et al., 2009). Furthermore, once the genes responsible for antibiotic resistance are successfully integrated in gene-transmission components, they can be persistent and spread between discrete microbial species alike in the absence of antibiotic compounds (Abd Elhady et al., 2020; Allen et al., 2010; Khan, 2016; Koike et al., 2007; Martinez et al., 2009). In Bangladesh, very little information is acknowledged regarding the natural- and human-associated environmental repositories of resistance in polluted or contaminated water from textile industries. Moreover, most of the textile industries do not have the effective effluent treatment plants (ETP), although there are strict rules and regulations imposed by the government.

Urban wastewater serves as important repositories of human and animal symbiotic bacterial species in which antimicrobial resistance determinants and/or micro-organisms remain in the eventual effluents and are dispensed and/or released into the environment without any significant pre-treatments (Reinthaler et al., 2003; Tennstedt et al., 2003, 2005). In addition, by conducting a comparative study on three activated sludge-treatment plants, it was concluded that although there were no significantly noticeable increases in antibiotic resistance phenotypes detected over the period of sewage treatments, these processes may contribute to the circulation of resistant bacteria mainly to the water and soil environment (Reinthaler et al., 2003; Tennstedt et al., 2003, 2005). Additionally, Tennstedt et al. (2003) described the existence of antibiotic resistance markers in self-transmissible genetic elements of bacteria inhabiting the activated-sludge and effluent end products released from a wastewater treatment plant. In this study, the prevalence or the predominance of antibiotic and heavy metal resistance was investigated in lactose-fermenting (LF) bacterial isolates collected from wastewater containing textile effluents.

Bangladesh has a significant number of textile, dying and tannery industries, however, till now there were no systemic and extensive studies in this area except some random and small scale studies; for instance, Ashikuzzaman et al. (2015) studied gram positive bacteria with heavy metal resistance isolated from textile effluents. In addition, Md et al. (2009) studied on chromium resistance bacteria and Hossain and Anwar (2012) reported on chromium and copper resistance bacteria isolated from tannery effluents. As a result, the present study was a sure systemic and a small-scale effort to study on gram negative lactose fermenting *Enterobacteriaceae* providing emphasis on mostly phenotypic characterization.

MATERIALS AND METHODS

Study area and sample collection

The samples from textile effluents were collected in sterilized airtight 1.5-L glass bottles (Pyrex, UK) from the canals of Savar Upazila within three months of the period from June to August in 2016. The effluents discharged directly from different textile industries into the canals and the canals are connected to the nearby Bangsi and Turag rivers. Effluent samples were collected from five (n = 5) different canals and from each canal, five samples were collected with average distance of 2 m. The collected samples were transported to the laboratory and stored at 4ºC for further studies. The name of the industrious canals, dates of collection, and distances of the site of collection were given in the Table 1 and also the map of Savar Upazila was shown in Figure 1, where locations of all the textile industries were indicated including the nearby rivers. The effluents are generally discharged from different textiles through the canals. The red color indicated the different canal sites from where the samples were collected. The present study was carried out in the Common Research Laboratory as well as in the Research Laboratory for Biomedical Sciences, Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka.

Total bacterial colony (TBC) count

The textile effluent samples were serially diluted with autoclaved distilled water from 10^{-1} to 10^{-10}. Plate counting agar (PCA) media was used to count the total bacterial colony by spreading the serially diluted effluents 10^{-6} to 10^{-10}, that is, serially, total of 5 PCA plates were used for each sample of each location. Each plate (20 ml media) was inoculated with 50 µl aliquot (2.5 µl/ml) and spread with the spreader followed by incubation at 37ºC for 5 consecutive days to get the total bacterial colony (TBC). The appeared colony forming units (CFUs) were counted manually observing under light microscope (XSZ-107 BN 230 v, USA).

Selection of gram-negative LF colonies using differential media

To enrich the number of bacteria, 1 ml aliquot of the collected effluent from each sample was inoculated in 1 ml of autoclaved nutrient broth (NB) and incubated for 24 h in an orbital shaker incubator at a shaking speed of 180 rpm at 37°C. After incubation, the culture was inoculated in agar plates containing differential media using cotton swab and by streaking. Four differential media, such as MacConkey (MAC) agar, Xylose-Lysine-Deoxycholate (XLD) agar, *Shigella-Salmonella* (SS) specific agar and Thiosulphate-Citrate-Bile Salt-Sucrose (TCBS) agar were used to identify gram negative lactose fermenting *Enterobacteriaceae* colonies (Bacteriological Analytical Manual, 1998). The different visible colonies on differential agar plates were selected, picked, cultured and purified by repeated plate streaking method (Cappuccino et al., 2017). The purity of each bacterial colony morphology of the selected lactose fermenting *Enterobacteriaceae* isolates was ensured by observing their color, size and shape by comparing with those of the standard colonies of *Escherichia coli* (*E. coli*; ATCC 25922) (UniProt accession number: CP009072) under the microscope. These standard colonies that grew on the four differential media were used as positive control. The non-lactose fermenting (NLF) colonies were also isolated following the

Table 1. Information of specific locations in canals carrying textile industry effluent.

Sample	Date of collection	Location	Distance	Industrial canal
S1	June 26, 2016	L1----L2	2.0 m	Pakiza Textiles Ltd.
		L2----L3	1.5 m	
		L3----L4	2.5 m	
		L4----L5	2.0 m	
S2	August 1, 2016	L1----L2	1.0 m	Aman Spinning Mills
		L2----L3	2.0 m	Mondal Fashions Ltd.
		L3----L4	1.5 m	Green Life Knit Composite Ltd.
		L4----L5	3.0 m	Eva Garments
S3	August 7, 2016	L1----L2	2.5 m	Marma Composites
		L2----L3	2.0 m	
		L3----L4	1.5 m	Fashion Garments Ltd.
		L4----L5	2.0 m	
S4	August 21, 2016	L1----L2	1.0 m	Ultra Embroidary Ltd.
		L2----L3	3.0 m	
		L3----L4	1.5 m	Amigo Fashion Ltd.
		L4----L5	2.5 m	Shine Embroidary Ltd.
S5	August 28, 2016	L1----L2	1.0 m	Dekko Designs Ltd.
		L2----L3	3.0 m	Magpie Knit Wear
		L3----L4	1.5 m	Hameem Group AJ Super Garments Ltd.
		L4----L5	2.5 m	Sharmin Group Trouser Line Ltd.

S: Sample; L: Location; M: Meter.

same procedure where *Shigella flexneri* 2a (UniProt accession number: AE005674), and *Salmonella* Serovar paratyphi B (UniProt accession number: CP000886) standard colonies were used. All the selected isolates were cultured and grown in NB media supplemented with 0.3% yeast extract and stored at -80°C temperature after adding 15% glycerol.

Determination of minimum inhibitory concentrations (MICs) to heavy metals

The MICs of different toxic heavy metals were determined by preparing MAC agar supplementing with three metal salts, such as $K_2Cr_2O_7$ (chromium salt), $NiSO_4$ (nickel salt) and $PbNO_3$ (lead salt) to give a final concentration of 0.1, 0.3, 0.5, 1.0, and 2.0 mM for Cr^{6+}, 0.2, 0.3, 0.6, 1.25, 2.5 mM for Ni^{2+} and 0.1, 0.15, 0.3, 0.6, 1.2 mM for Pb^{2+}, respectively. Spot inoculations were performed using plate assay method by drawing square patches on the plates. The plates were marked with 40 patches. Next, each patch of the plates was inoculated with a single colony taken from previously cultured MAC agar and incubated at 37°C for 24 h (Zhou et al., 2015). As a negative control, *E. coli* (ATCC 25922) was used.

Antibiotic susceptibility test

On Mueller-Hinton (MH) agar, the isolates were screened on modified Kirby-Bauer disc diffusion method using the standard guidelines (CLSI, 2016; Talukder et al., 2002). The tested antibiotics were cefotaxime (CTX, 30 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg), tetracycline (TE, 30 µg), azithromycin (AZM, 15 µg) and ciprofloxacin (CIP, 5 µg) (Bio-Rad, USA). *E. coli* ATCC (25922) was used as a negative control.

Binary exposure experiment

Individual MH agar plates supplemented with individual heavy metal salts (nickel/chromium/lead) were prepared based on the results of MIC and antibiotic susceptibility tests. For nickel, chromium, and lead, single MH agar plates with 0.6 mM $NiSO_4$, 1.0 mM $K_2Cr_2O_7$, and 0.6 mM $PbNO_3$ were prepared, respectively. After adding individual metal salts to MH agar plates, each individual bacterial colony was then spread on the plates and allowed to grow for 10 min. Then antibiotic discs containing 30 µg cefotaxime (CTX), 25 µg sulfamethoxazole-trimethoprim (SXT), 30 µg tetracycline (TE), 15 µg azithromycin (AZM) and 5 µg ciprofloxacin (CIP) (Bio-Rad, USA) were placed on the inoculated plates following standard CLSI guidelines as described (CLSI, 2016; Talukder et al., 2002) and incubated for a period of 24 h at 37°C. Based on the zone of inhibition values measured in millimeters, the effects of metal salts on the spectrum of antimicrobial resistance were analysed (Zhou et al., 2015). A negative control [*E. coli* ATCC (25922)] was used and experimental results were repeated in triplicates (n=3) to verify their

Figure 1. Map showing the different textile industries of Savar Upazila (red color is pointing the different canal sites of different textile industries).

reproducibility.

Plasmid profiling

The plasmid DNA was extracted using the simplified alkaline lysis method developed by Kado and Liu (1981) with minor modifications, followed by electrophoresis using 0.8% agarose in Tris-Borate EDTA buffer (Talukder et al., 2002; Kado and Liu, 1981). In this experiment, reference strain *E. coli* ATCC (25922) does not carry any plasmid and was therefore used as a negative control. Plasmid bearing *E. coli*, R1 (62 MDa) and V517 (35,6,4,7,3,3,2,1 I,1.8,1.4 MDa) were taken as positive controls (Talukder et al., 2002; Henry, 1991). Molecular weights of plasmids found between the reference strains were converted to Kilo base pair (Kb) measurements. Molecular weight of the plasmids was determined by comparing them to the circular DNA ladder molecular weight markers of 80-10,000 bp (Mass Ruler DNA

Table 2. Day-wise breakup of total bacterial colony (TBC) counts at varying dilution.

Sample ID	Plate no.	Dilution factor	Total bacterial colony (TBC) count by plate observation				
			Day 1	Day 2	Day 3	Day 4	Day 5
			(CFUs/plate)				
S1L1	1	10^{-6}	42	45	48	N	N
	2	10^{-7}	36	39	43	N	N
	3	10^{-8}	24	27	33	46	N
	4	10^{-9}	12	18	21	38	N
	5	10^{-10}	7	8	15	23	N
S1L5	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	2	5	8	N	N
	4	10^{-9}	2	3	5	N	N
	5	10^{-10}	1	2	4	9	N
S2L1	1	10^{-6}	69	N	N	N	N
	2	10^{-7}	40	N	N	N	N
	3	10^{-8}	23	23	30	N	N
	4	10^{-9}	10	13	13	16	18
	5	10^{-10}	4	8	10	14	17
S2L5	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	15	21	27	38	53
	3	10^{-8}	9	16	16	18	41
	4	10^{-9}	8	13	14	21	35
	5	10^{-10}	6	18	20	23	27
S3L1	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	N	N	N	N	N
	4	10^{-9}	11	14	16	19	N
	5	10^{-10}	7	9	16	19	23
S3L5	1	10^{-6}	53	57	61	77	83
	2	10^{-7}	45	48	50	56	63
	3	10^{-8}	31	37	44	49	51
	4	10^{-9}	23	26	31	37	41
	5	10^{-10}	13	19	23	28	32
S4L1	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	N	N	N	N	N
	4	10^{-9}	51	59	63	74	N
	5	10^{-10}	27	33	48	52	N
S4L5	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	47	51	60	66	71
	3	10^{-8}	39	45	55	57	63
	4	10^{-9}	24	31	43	49	56
	5	10^{-10}	11	16	21	26	35
S5L1	1	10^{-6}	N	N	N	N	N
	2	10^{-7}	N	N	N	N	N
	3	10^{-8}	12	33	37	41	N
	4	10^{-9}	11	29	31	35	45
	5	10^{-10}	7	23	28	32	37
S5L5	1	10^{-6}	20	42	N	N	N
	2	10^{-7}	11	29	N	N	N

Table 2. Contd.

3	10^{-8}	9	14	17	19	24
4	10^{-9}	8	13	15	20	22
5	10^{-10}	2	4	5	8	15

SL: Sample location; N: Numerous; ID: Identification; CFU: Colony forming unit.

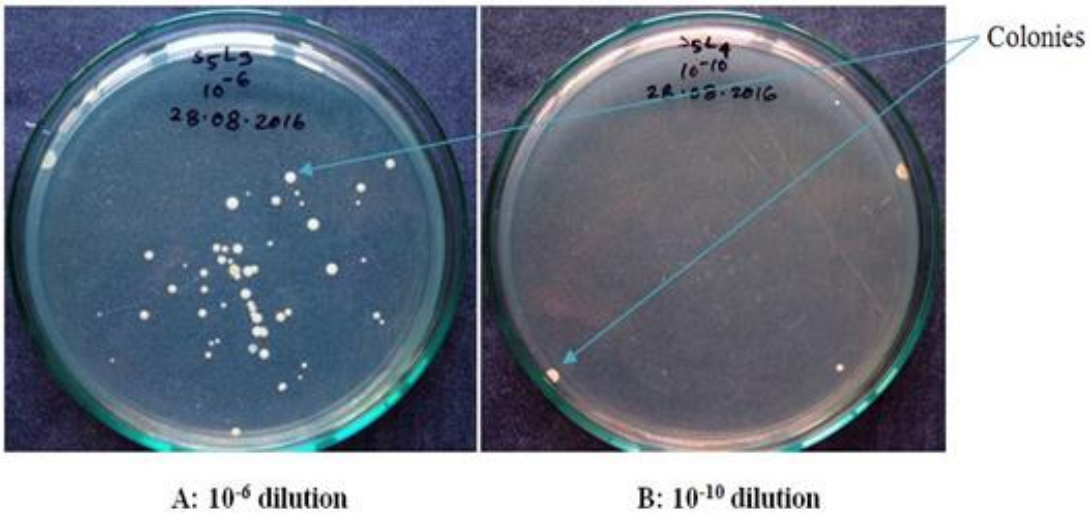

Figure 2. Representative plate observing and counting the CFUs manually in day 1.

Ladder Mix, Thermo Scientific, USA). The plasmid extraction protocol as well as the size of the plasmids were validated through the use of these negative and positive control strains.

Statistical analysis

Analysis of the data was conducted using MS-Excel, R 3.5.3 and RStudio for Windows 10 (32/64 bits). In order to analyze the data, the linear model was used to perform an ANOVA with one tail t-test and a p-value of 0.05 was considered statistically significant. The binary exposure experiment results were expressed as the means and standard deviations of three replications.

RESULTS AND DISCUSSION

Total bacterial colony (TBC) count found in textile samples

The number of colony forming units (CFUs) for each sample of each dilution for a single day and for two locations (1 and 5) was given in Table 2 and Figure 2 shows the plate with colonies which was grown in PCA media. Sample 3 was collected from the canals coming from Marma Composites and Fashion Garments Ltd. In day 1, for 10^{-6} dilution, N number CFUs/plate was observed. On the other hand, for 10^{-10} dilution, in day 1, average 10 CFUs/plate was found. On day 5, for 10^{-6} dilution, average N number CFUs/plate was observed and for 10^{-10} dilution, average N number CFUs/plate was counted also. For other samples, the total observations were given in Table 2. The results were almost consistent for the other samples as well (Table 2). The level of CFUs/plate definitely exceeded the allowable standard CFU per 2.5 µl volume of effluents. For *E. coli,* the plate count was linear and ranged from 30 to 300 CFU on a standard or regular sized petri-dish. Therefore, to ensure that a sample will yield CFU in this range requires dilution of the sample and plating of several dilutions. Generally, for this experiment, ten-fold microbial dilutions were used (Breed and Dotterrer, 1916).

Selection of gram-negative LF colonies

By microscopic and naked eye observation, 20 gram-negative LF colonies were isolated (Figure 3) using four differential media from five locations of each canal (average 4 colonies from each location). By comparing with a positive control *E. coli* ATCC (25922), well-defined, round, concave shaped colonies found in MAC (pink coloured colonies) or SS (faint pink coloured colonies), XLD (yellow coloured colonies) agar and TCBS (small

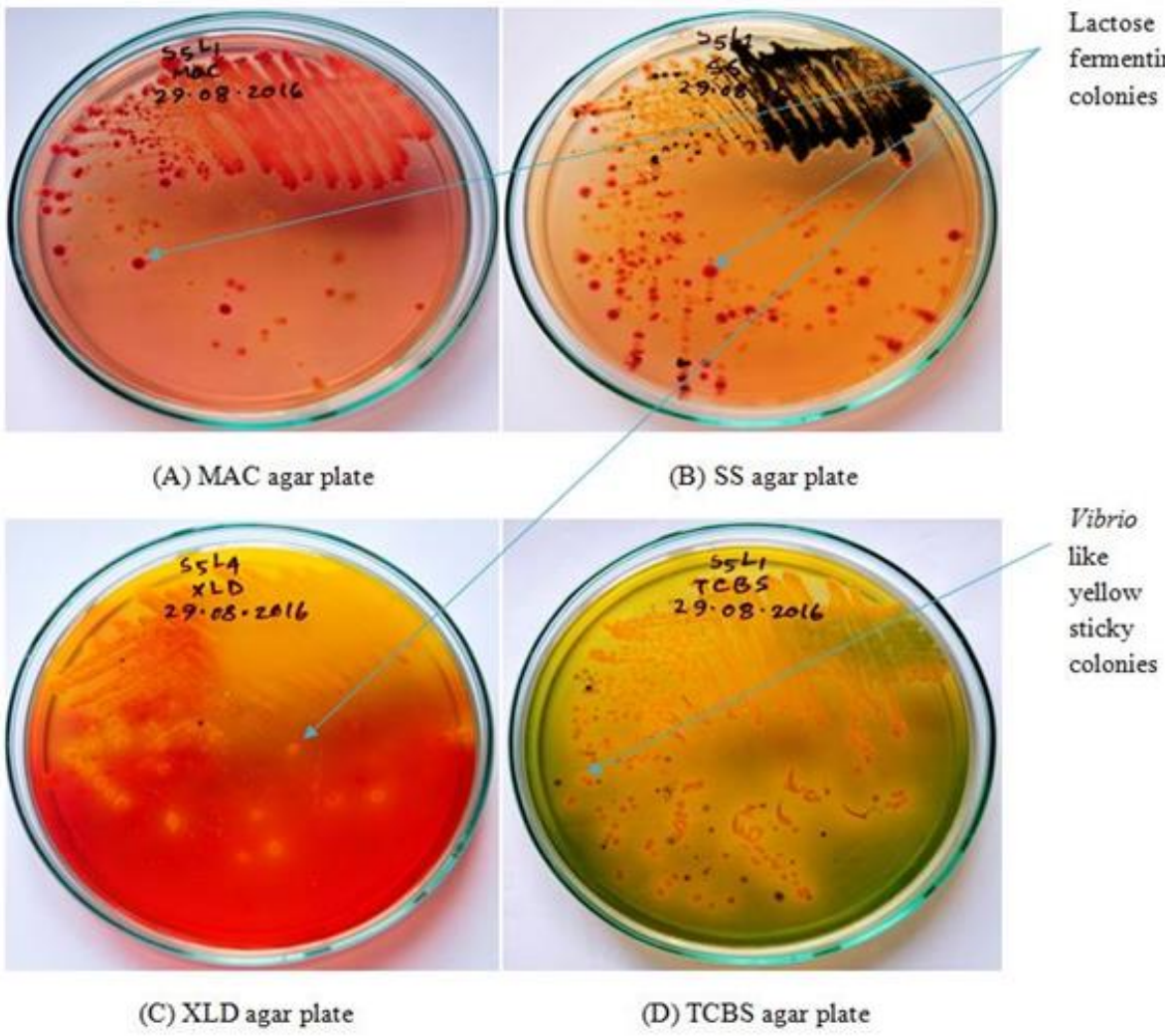

Figure 3. Representative photographs showing colony morphologies of LF and Vibrio like colonies.

clear colonies) were isolated as LF colonies.

A total of 100 LF isolates were selected from five different samples each with five different locations and stored at -4°C for further investigation. In this study, 100 NLF isolates were also isolated and stored; however, only LF isolates were characterized in this study. In general, the colonies formed in culture by different species of bacteria are quite different and are clearly distinguishable by both micro- and macroscopic observations. For observations and identification of growing available microorganisms on plates, the morphology of colonies could be of great use (Practical Handbook of Microbiology, 2015).

A normal Q-Q plot was observed in a linear model for 100 NLF and 100 LF isolates (Figure 4). In ANOVA analysis, a highly significant *p*-value (< 0.05) and a small t-scored value (0.120863) was found indicating significant similarity exists between the two sets of samples (NLF and LF isolates).

Determination of MICs to heavy metals of representative LF isolates

MICs (Figure 5) of the 100 LF bacterial isolates was tested for their resistance to 3 different metals where almost all the isolates displayed resistance to these metals tested (Ni, Cr and Pb). This is because the most common heavy metal pollutants in the environment are Cr, Mn, Ni, Cu, Zn, Cd, and Pb (Fu et al., 2017). In this experiment, 98% (n=98) isolates were grown in 0.2 mM and 0.3 mM concentrations of Ni (nickel) whereas none (n=0) of the isolates was grown in 1.25 mM and 2.5 mM concentrations of Ni. Only 3% (n=3) isolates were able to grow in 0.6 mM concentration of Ni. For Cr (chromium), 100% (n=100) isolates were able to grow in 0.1 mM and 0.3 mM concentrations. Ninety-nine percent (n=99) and 95% (n=95) isolates were able to tolerate the 0.5 mM and 1.0 mM concentrations of Cr, respectively. Only 17% (n=17) isolates had a maximum

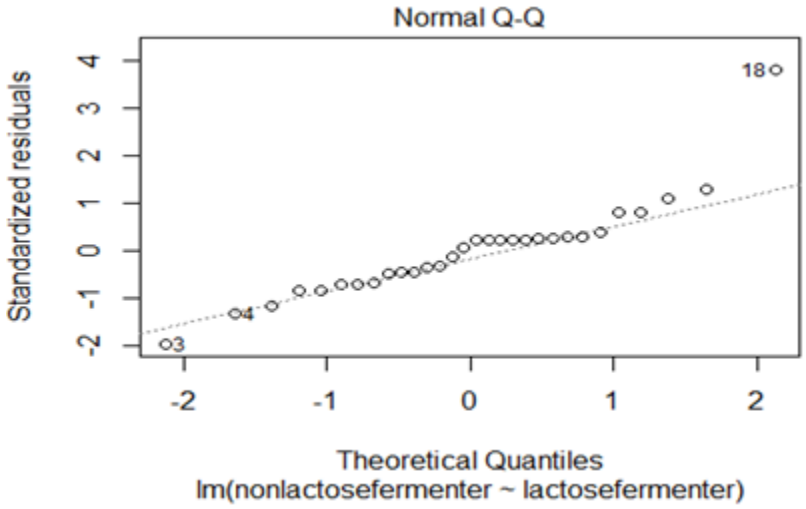

Figure 4. A linear model indicating a normal Q-Q plot for two sets of samples (NLF and LF isolates).

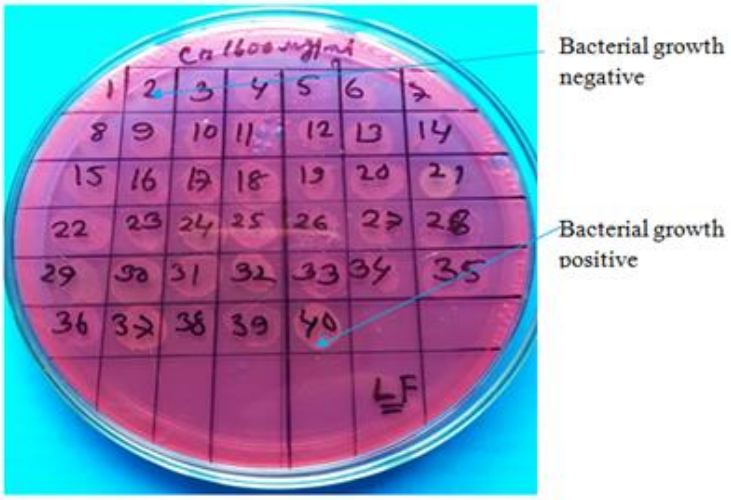

Figure 5. Representative photographs of LF colonies showing MICs of heavy metals determined by plate assay method.

tolerable concentration of 2.0 mM Cr. For Pb (lead), 100% (n=100) isolates were tolerable to 0.1, 0.2 and even 0.4 mM concentrations and 99% (n=99) isolates were also able to grow in 0.6 mM concentration. Only 20% (n=20) isolates had a maximum tolerable concentration 1.2 mM Pb. In Table 3, MICs for representative LF isolates (n=23) and *E. coli* ATCC (25922) as a negative control were given. Table 4 provides overall information about percentage (%) of the LF isolates able to tolerate different concentrations of three different heavy metals. It is also important to note that 17% (n=17) isolates were tolerable to 2.0 mM Cr concentration and 20% (n=20) isolates were tolerable to 1.2 mM Pb concentration from isolated from sample 3 (Marma Composites and Fashion Garments Ltd.). The capability of showing resistance by these bacterial isolates could be as a result of their surviving in the textile effluents containing high concentrations of all types of heavy metals.

One study (Wright et al., 2006) reported that bacteria samples including a gradient of contaminated heavy metals displayed greater tolerance to those metals as well as antibiotics compared to bacterial samples collected from a standard reference site. The experimental findings of our present study revealed the high heavy metal resistance in bacterial isolates from textile industrial effluents. This result is supported by another experiment conducted by Aleem et al. (2003)

Table 3. MICs of representative LF isolates (n=23) to heavy metals.

Lab ID	Heavy metals														
	Nickel (Ni) Conc. (mM)					Chromium (Cr) Conc. (mM)					Lead (Pb) Conc. (mM)				
	0.2	0.3	0.6	1.25	2.5	0.1	0.3	0.5	1.0	2.0	0.1	02	0.4	0.6	1.2
1	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
5	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
15	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
23	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
25	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
27	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
29	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
31	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
34	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
35	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
39	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
44	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
46	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
49	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
51	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
59	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
60	+	+	-	-	-	+	+	+	+	-	+	+	+	+	+
68	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
73	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
83	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
11	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
85	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
99	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
ATCC (25922)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

ID: Identification; Conc.: Concentration; mM: Milimolar, ATCC (25922): *E. coli* negative control strain; “+”: Growth positive; “-”: Growth negative.

Table 4. MICs (%) of representative LF isolates (n=100) to heavy metals.

Isolate	Conc. of Ni (mM)				
	0.2	**0.3**	**0.6**	**1.25**	**2.5**
Lactose fermenter	98%	98%	3%	0	0
	Conc. of Cr (mM)				
	0.1	**0.3**	**0.5**	**1.0**	**2.0**
Lactose fermenter	100%	100%	99%	95%	17%
	Conc. of Pb (mM)				
	0.1	**0.2**	**0.4**	**0.6**	**1.2**
Lactose fermenter	100%	100%	100%	99%	20%

ID: Identification; Conc.: Concentration; mM: Milimolar; Ni: Nickel; Cr: Chromium; Pb: Lead.

who showed that 77·10% of the bacterial isolates collected from the agricultural soil of Aligarh and treated with the samples of wastewater exhibited resistance to most of the toxic heavy metals up to 71·4, 65.7, 65.7, 54.2, 45.7 and 25.7% for Cr, Cd, Zn, Cu and Hg respectively. In another study, Murtaza et al. (2002) illustrated that of 80 *E. coli* isolates cultured and collected from five different regions of India, 4 regions were

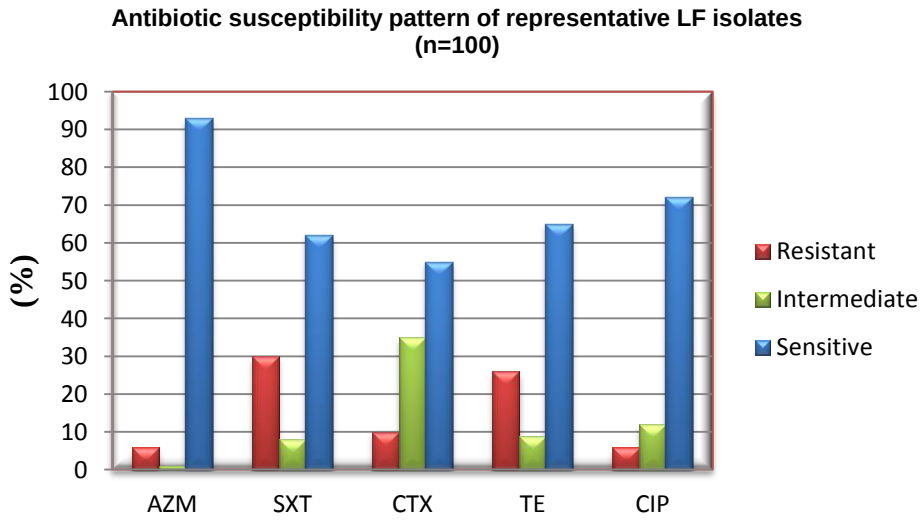

Figure 6. Distribution (%) of antibiotic susceptibility pattern of representative LF isolates (n=100) (CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline).

contaminated with effluents from household and industry while 1 was from unpolluted area where 68 isolates displayed resistance to at least any one of these metals-Cu, Cd, Pb, Co, Zn, and Fe. The bacterial resistance to heavy metals possibly indicates the intensity of environmental contamination by these toxic metals and might be directly linked to the bacterial routine exposure to those metals. On the other hand, there is a possibility of harbouring heavy metal resistant microorganisms on the uncontaminated environment or organisms may be spontaneously adapted to high toxic metal concentrations (Murtaza et al., 2002).

Determination of antimicrobial susceptibility test of representative LF isolates (n=100)

In this experiment, five antibiotics were chosen on the basis of their importance in treating human or animal *Enterobacteriaceae* infections and their use as feed additives to promote growth in animals and also on the basis of their ability to provide diversity for representation of different antimicrobial agent classes (Butler and Paterson, 2020). Among 100 LF isolates, the most prevalent (36%, n=36) resistance pattern was found for sulfamethoxazole-trimethoprim (SXT) followed by 26% (n=26) for tetracycline (TE). For azithromycin (AZM) and ciprofloxacin (CIP), the percentages for resistance pattern were equal, estimating 6% (n=6) for each which was also the lowest resistance pattern. For cefotaxime (CTX), 10% (n=10) isolates were found to be resistant to this antibiotic.

It is interesting to note that 35% (n=35) isolates were showing intermediary resistance pattern to CTX whereas only 1% (n=1) for AZM. For CIP, TE and SXT, the intermediary resistance pattern was found 12% (n=12), 9% (n=9) and 8% (n=8), respectively. Overall, 16% (n=16) isolates were found to be multidrug (more than one drug) resistant and another 12% (n=12) isolates were observed to be intermediary resistant (Figure 6). Therefore, it is possible that all these intermediary resistant isolates would turn into complete resistant gradually within a short duration of time increasing the number of multidrug-resistant isolates. Isolation of antibiotic resistant *Enterobacteriaceae* showed the contamination of industrial effluents with nearby sewage waste water. The strains of bacteria isolated from the soil of agricultural land exposing continuously by contaminated waste water exhibited high heavy metal and antibiotic resistance (Wright et al., 2006).

Bacterial isolates from the contaminated soils (Máthé et al., 2012), surface water (Koc et al., 2013) and even the shallow sediments of Antarctica (Lo Giudice et al., 2013), have also been reported to show cross-resistance to toxic heavy metal and antibiotic. The findings of the current study agreed with the findings of the previous studies. The water and soil of industrial environments are regularly contaminated with a variety of organic and inorganic pollutants (Aleem et al., 2003; Aleem and Malik, 2003; Ansari and Malik, 2007). In these environmental samples, multi-resistant bacteria are clearly correlated to the resistance or tolerance of the hazardous heavy metals. The heavy metals and various drugs including antibiotics are environmentally exposed factors resultant from pollution caused by human which employ a selective pressure for the development of resistance in bacteria (Lazăr et al., 2002). The detailed mechanisms of cross-resistance to both heavy metals and antibiotics in

Table 5. Antibiotic susceptibility pattern for representative LF isolates (n=23).

Lab ID	Isolate ID	Antibiotic Discs					Resistance to antibiotics
		CIP (5 µg)	CTX (30 ug)	AZM (15 ug)	SXT (25 µg)	TE (30 µg)	
1	S1L1 LF1	S	R	S	R	S	CTX, SXT
5	S1L2 LF1	S	R	S	R	S	CTX, SXT
15	S1L4 LF3	S	R	S	R	S	CTX, SXT
23	S2L1 LF3	R	R	I	R	I	CIP, CTX, SXT
25	S2L2 LF1	S	R	I	R	I	CTX, SXT
27	S2L2 LF3	R	R	I	R	S	CIP, CTX, SXT
29	S2L3 LF1	S	R	R	R	R	CTX, AZM, SXT, TE
31	S2L3 LF3	S	R	R	R	R	CTX, AZM, SXT, TE
34	S2L4 LF2	S	R	I	S	S	CTX
35	S2L4 LF3	S	R	I	S	S	CTX
39	S2L5 LF3	R	R	S	R	I	CIP, CTX, SXT
44	S3L1 LF4	S	I	R	S	I	AZM
46	S3L2 LF2	S	R	R	S	I	CTX, AZM
49	S3L3 LF1	S	R	S	R	R	CTX, SXT, TE
51	S3L3 LF3	S	R	I	R	R	CTX, SXT, TE
59	S3L5 LF3	S	R	R	R	R	CTX, AZM, SXT, TE
60	S3L5 LF4	S	S	I	R	R	SXT, TE
68	S4L2 LF4	S	S	I	R	S	SXT
73	S4L4 LF1	S	S	R	S	S	AZM
83	S5L1 LF3	S	I	R	I	S	AZM
11	S1L3 LF3	S	S	S	S	S	-
85	SFL2 LF1	S	S	S	S	S	-
99	S5L5 LF3	S	S	S	S	S	-
ATCC	25922	S	S	S	S	S	-

ID: Identification; LF: Lactose fermenter; CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; S: Sensitive; I: Intermediary; R: Resistance.

bacterial isolates are still unclear.

The number of resistant isolates obtained from sample 2 (6%, n=6) and sample 3 (4%, n=4) were more than that of samples 1, 4 and 5. Even 3% (n=2) and 2% (n=2) isolates were intermediary resistant which were isolated from sample 2 and 3, respectively. In Table 5, significant antibiotic susceptibility patterns for 23 LF isolates were given. From this finding it is assumable that perhaps samples 2 and 3 were more contaminated with different metals as contamination of metals directly selects metal-tolerant bacteria and at the same time co-selects antibiotic-tolerant bacteria (Wright et al., 2006).

Binary exposure experiment of representative LF isolates (n=14)

By binary exposure experiment, the effects of heavy metals on diameter of zone of inhibition of isolates to antibiotics were observed. Based on the results of MICs and antimicrobial susceptibility tests, ten isolates were finally selected for this experiment. The isolates, which were resistant to at least one antibiotic and had intermediary resistance for at least one antibiotic, were selected. Three types of MH agar plates were prepared adding Ni (0.3 mM), Cr (1.0 mM) and Pb (0.6 mM) separately. The specific metal concentrations were selected based on the maximum isolates tolerable to that specific concentration. Among 10 isolates, 9 isolates were able to tolerate these specific concentrations. Three more isolates were also taken which were susceptible to all antibiotics but still able to tolerate these specific metal concentrations. *E. coli* ATCC (25922) was included as a negative control. In Table 6, a zone of inhibition of antibiotics was given in the presence of Ni (0.3 mM), Cr (1.0 mM), Pb (0.6 mM) of representative 13 LF isolates with 1 control strain.

Table 6 showed that in most of the cases, the zone diameter increased and in some cases, the zone diameter decreased and in few cases, no change was found. Hence, from this finding, it is assumable that metal resistant isolates, having negative or no effects on antibiotic resistance, could be used for bioremediation. Since these isolates can accumulate metals into their body (Medfu Tarekegn et al., 2020) and have decreased effects or no effect on resistance, it therefore means that

Table 6. Zone of inhibition of the representative LF isolates (n=14) to the heavy metals, Ni (0.6 mM), Cr (1.0 mM), Pb (0.6 mM), respectively in the presence of antibiotics.

Lab ID	AZM (diameter in mm)		SXT (diameter in mm)		CTX (diameter in mm)		TE (diameter in mm)		CIP (diameter in mm)	
	Ab	Ab + HM	Ab	Ab + HM	Ab	Ab + HM	Ab	Ab + HM	Ab	Ab + HM
23	10±0.06	(Ni) - (Cr) 7±0.03 (Pb) 12±0.07	7±0.03	(Ni) - (Cr) 7±0.02 (Pb) 7±0.01	22±0.23	(Ni) - (Cr) 30±0.27 (Pb) 26±0.23	7±0.02	(Ni) - (Cr) 10±0.05 (Pb) 12±0.08	20±0.12	(Ni) - (Cr) 12±0.06 (Pb) 10±0.07
27	7±02	(Ni) 20±0.16 (Cr) 7±0.04 (Pb) 10±0.08	7±0.01	(Ni) 7±0.04 (Cr) 7±0.02 (Pb) 7±0.03	20±0.17	(Ni) 30±0.31 (Cr) 30±0.29 (Pb) 30±0.28	7±0.04	(Ni) 7±0.04 (Cr) 10±0.05 (Pb) 10±0.06	24±0.15	(Ni) 24±0.21 (Cr) 20±0.23 (Pb) 22±0.18
31	20±0.15	(Ni) 26±0.21 (Cr) 24±0.25 (Pb) 26±0.23	7±0.04	(Ni) 20±0.13 (Cr) 16±0.15 (Pb) 20±0.14	8±0.05	(Ni) 20±0.18 (Cr) 20±0.17 (Pb) 20±0.21	8±0.06	(Ni) 18±0.11 (Cr) 24±0.20 (Pb) 24±0.21	7±0.02	(Ni) 22±0.20 (Cr) 22±0.17 (Pb) 24±0.25
35	14±0.9	(Ni) 20±0.17 (Cr) 7±0.03 (Pb) 18±0.15	7±0.02	(Ni) 7±0.04 (Cr) 7±0.02 (Pb) 7±0.03	22±0.12	(Ni) 26±0.25 (Cr) 24±0.20 (Pb) 30±0.32	20±0.14	(Ni) 18±0.16 (Cr) 26±0.23 (Pb) 20±0.17	24±0.17	(Ni) 24±0.26 (Cr) 30±0.32 (Pb) 24±0.21
39	10±0.07	(Ni) 20±0.14 (Cr) 7±0.03 (Pb) 26±0.21	7±0.03	(Ni) 14±0.11 (Cr) 12±0.10 (Pb) 14±0.12	24±0.15	(Ni) 16±0.14 (Cr) 14±0.12 (Pb) 20±0.18	7±0.03	(Ni) 16±0.15 (Cr) 24±0.14 (Pb) 20±0.16	18±0.21	(Ni) 22±0.19 (Cr) 22±0.24 (Pb) 24±0.20
46	16±0.11	(Ni) 20±0.25 (Cr) - (Pb) -	10±0.06	(Ni) 20±0.18 (Cr) - (Pb) -	10±0.03	(Ni) 20±0.19 (Cr) - (Pb) -	20±0.12	(Ni) 14±0.15 (Cr) - (Pb) -	20±0.19	(Ni) 22±0.25 (Cr) - (Pb) -
51	14±0.09	(Ni) 20±0.16 (Cr) 20±0.18 (Pb) 28±0.26	7±0.01	(Ni) 20±0.14 (Cr) 22±0.16 (Pb) 20±0.15	22±0.14	(Ni) 26±0.21 (Cr) 20±0.18 (Pb) 22±0.20	10±0.11	(Ni) 20±0.18 (Cr) 26±0.23 (Pb) 24±0.21	10±0.06	(Ni) 20±0.23 (Cr) 22±0.26 (Pb) 22±0.19
68	14±0.05	(Ni) 20±0.19 (Cr) 7±0.04 (Pb) 16±0.12	20±0.17	(Ni) 26±0.21 (Cr) 20±0.18 (Pb) 20±0.17	22±0.16	(Ni) 32±0.30 (Cr) 28±0.25 (Pb) 30±0.27	7±0.02	(Ni) 7±0.03 (Cr) 10±0.05 (Pb) 10±0.07	26±0.23	(Ni) 24±0.18 (Cr) 24±0.20 (Pb) 22±0.18
73	16±0.12	(Ni) 24±0.20 (Cr) 7±0.03 (Pb) 10±0.05	16±0.13	(Ni) 22±0.20 (Cr) 16±0.13 (Pb) 16±0.15	14±0.09	(Ni) 20±0.18 (Cr) 16±0.14 (Pb) 18±0.15	24±0.18	(Ni) 22±0.19 (Cr) 26±0.25 (Pb) 24±0.20	22±0.18	(Ni) 22±0.20 (Cr) 16±0.13 (Pb) 16±0.16
83	20±0.17	(Ni) 20±0.18 (Cr) 22±0.16 (Pb) 24±0.22	14±0.12	(Ni) 14±0.12 (Cr) 18±0.15 (Pb) 20±0.18	12±0.07	(Ni) 18±0.21 (Cr) 16±0.17 (Pb) 22±0.23	16±0.15	(Ni) 24±0.23 (Cr) 26±0.27 (Pb) 28±0.25	22±0.16	(Ni) 20±0.21 (Cr) 20±0.22 (Pb) 22±0.25
11*	28±0.22	(Ni) 25±0.21 (Cr) 26±0.23 (Pb) 28±0.22	23±0.21	(Ni) 20±0.14 (Cr) 25±0.20 (Pb) 23±0.21	28±0.28	(Ni) 19±0.21 (Cr) 22±0.18 (Pb) 22±0.20	24±0.21	(Ni) 15±0.10 (Cr) 28±0.32 (Pb) 28±0.29	25±0.21	(Ni) 26±0.27 (Cr) 27±0.30 (Pb) 25±0.26
85*	27±0.26	(Ni) 24±0.21 (Cr) 23±0.18 (Pb) 27±0.21	22±0.25	(Ni) 18±0.15 (Cr) 25±0.23 (Pb) 22±0.18	26±0.21	(Ni) 22±0.16 (Cr) 21±0.28 (Pb) 20±0.18	23±0.20	(Ni) 15±0.14 (Cr) 29±0.20 (Pb) 27±0.25	21±0.20	(Ni) 23±0.20 (Cr) 25±0.22 (Pb) 21±0.19
99*	26±0.24	(Ni) 24±0.24 (Cr) 24±0.20 (Pb) 26±0.23	20±0.11	(Ni) 18±0.13 (Cr) 22±0.16 (Pb) 20±0.18	30±0.26	(Ni) 20±0.19 (Cr) 18±0.16 (Pb) 22±0.25	22±0.17	(Ni) 14±0.10 (Cr) 26±0.29 (Pb) 26±0.26	22±0.16	(Ni) 24±0.26 (Cr) 22±0.29 (Pb) 22±0.27
E.coli ATCC (25922)	28±0.25	(Ni) 27±0.25 (Cr) 22±0.21 (Pb) 23±0.20	28±0.15	(Ni) 27±0.21 (Cr) 23±0.25 (Pb) 24±0.24	30±0.27	(Ni) 28±0.26 (Cr) 26±0.25 (Pb) 25±0.26	30±0.24	(Ni) 29±0.28 (Cr) 25±0.20 (Pb) 26±0.27	28±0.25	(Ni) 27±0.20 (Cr) 25±0.19 (Pb) 23±0.21

ID: Identification; CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; Ab: Antibiotic; HM: Heavy metal; mm: millimeter; Ni: Nickel; Cr: Chromium;Pb: Lead;11*, 85*, 99*: LF antibiotic sensitive isolates; ATCC (25922): Negative control strain.

they are not harmful to humans and animals if these isolates discharge into the environment.

The influences of Cr on AZM and CIP and also Pb on CIP were found most significant as in both cases the zone of inhibition decreased for most isolates. For ATCC (25922), the zone of inhibition decreased for all antibiotics

in the presence of Ni, Cr and Pb. By observing the 3 antibiotic sensitive isolates and 1 negative control strain, it was quite difficult to determine the actual effects of heavy metal to antibiotics for these isolate, because the patterns seem highly variable. In the present research work, the complexity in observed results mainly relied upon the types of various heavy metals, antibiotics, and their concentrations, which might be clarified by two major aspects; firstly, the occurring reactions due to the interactions between the chemical groups present on the heavy metals and antibiotics; and secondly, the particular biological effect caused by those. More particularly, the resultant concentrations of metals or antibiotic compounds might be impaired by the chemical group involving reactions among them (Zhang et al., 2012) and, therefore, if the ultimate end products of heavy metal and antibiotic reactions are highly toxic compared to those specific starting compounds, the bacteria might show weaker resistance to those antibiotic-derived substances (Tamilselvi and Mugesh, 2008).

Nevertheless, when the toxicity of the resultant end products is lower than starting toxic substances, the bacterial resistance properties to antibiotic-derived compounds could be unaltered or increased for the effectively reduced concentrations. Similarly, the presence of Cu or Zn can improve resistance to imipenem (a β-lactam antibiotic) in *Pseudomonas aeruginosa* due to possibly causing coagulation reaction (Caille et al., 2007). Hence, it is likely that the chemical reactions can be altered by various factors such as the categories and concentrations of metals, antibiotics, as well as, the presence of toxic contaminants in the same environmental system.

Moreover, the overall effects of heavy metals on bacterial antibiotic resistance attribute might additionally lead to the prodigy of bacterial cross-resistance and the pertinent mechanisms might be much complex. In general, heavy metals had a key role on the bacterial enzymatic activities and those trace elements are vital for the growth of bacteria (Liu et al., 2012), although it could be stress causing factors impairing the protein synthesis in *Pseudomonas fluorescens* (Sharma et al., 2006). The stress factors alter the bacterial efflux pumps and the integron-comprising mobile elements which causes cross-resistance among heavy metals and antibiotics (Petrova et al., 2011). The *Vibrio* species contain cryptic plasmids and these plasmids have important role in showing resistance to both Hg and antibiotics (Wang et al., 2006; Zhang et al., 2006). Further, the heavy metals in low concentrations could promote protein expression such as metallothionein, and the metallothionein synthesis has generally been noticed to be induced by high concentrations of a few metals and further antibiotic resistance might be inhibited rather than being enhanced (Strouhal et al., 2003). Although the underlying distinct mechanisms of heavy metal induced stress on microbial antibiotic resistance are still ambiguous due to the lack of enough evidences from related studies, relevant cross-resistance studies with a number of bacteria would be supportive in explaining the environmental turnover and the associated risks of metals and antibiotics.

Plasmid profiling of representative LF isolates (n=14)

Plasmid profiling (Figure 7) showed that all the isolates had various plasmid patterns ranging from P1 to P7. Seven isolates were found to contain one plasmid and 2 isolates contained 2 plasmids. Two sensitive isolates including one resistant isolate (resistant to only TE) and ATCC (25922) were plasmid less. Only one isolate was found to contain 5 plasmids. A large sized (21kb) plasmid was found in 6 isolates. Five isolates contained 12 Kb plasmids including one sensitive isolate. Only one isolate contained 3 plasmids. Smaller sized plasmids range from 5.5 to 1.6 Kb. In Table 7, plasmid size and patterns were given for 12 representative LF isolates. In Table 8, a summary of the characters of all 14 representative LF isolates including three susceptible isolates and one negative control strain was provided.

Malik and Jaiswal (2000) reported that increasingly polluted environment is responsible for the extent of the high populations of heavy metal resistant bacteria, and they also stated the high prevalence of plasmid-containing bacterial strains in polluted sites compared to unpolluted ones. In the present study, almost all the bacterial isolates displayed a remarkable degree of resistance to the applied antibiotics and heavy metals. In addition, nowadays, it is conclusively proved that the resistance features are generally located on the extra-chromosomal DNA widely known as plasmids (Collard et al., 1994; Dhakephalkar and Chopade, 1994; Guo et al., 2006). It was reported on a previous study that plasmids are responsible for multidrug resistance and generally found ≥12 Kb in size (Jacoby and Sutton, 1991; Parvin et al., 2014). Therefore, the outcomes of our study had similar results and strongly supported with these findings. Moreover, our experimental results are also supported by the findings of previously conducted studies on plasmid directed heavy metal resistance characteristics of isolates of bacteria collected from the environments contaminated by heavy metals (Vajiheh et al., 2003; Zolgharnein et al., 2007). Furthermore, it was noticed from our study that plasmid free bacterium displayed either resistance to heavy metals or antibiotics as one TE resistant isolate did not contain any plasmid. As a result, it clearly suggests that the distinct features associated with antibiotics and heavy metal resistance detected in different isolates of bacteria related to the plasmid and/or chromosomal DNAs. Although, a broad range of plasmid DNAs are found in bacterial isolates from textile effluent contaminated environment, there was no constant correlations between antibiotic resistance property and plasmid profiles. This attribute is not unexpected because

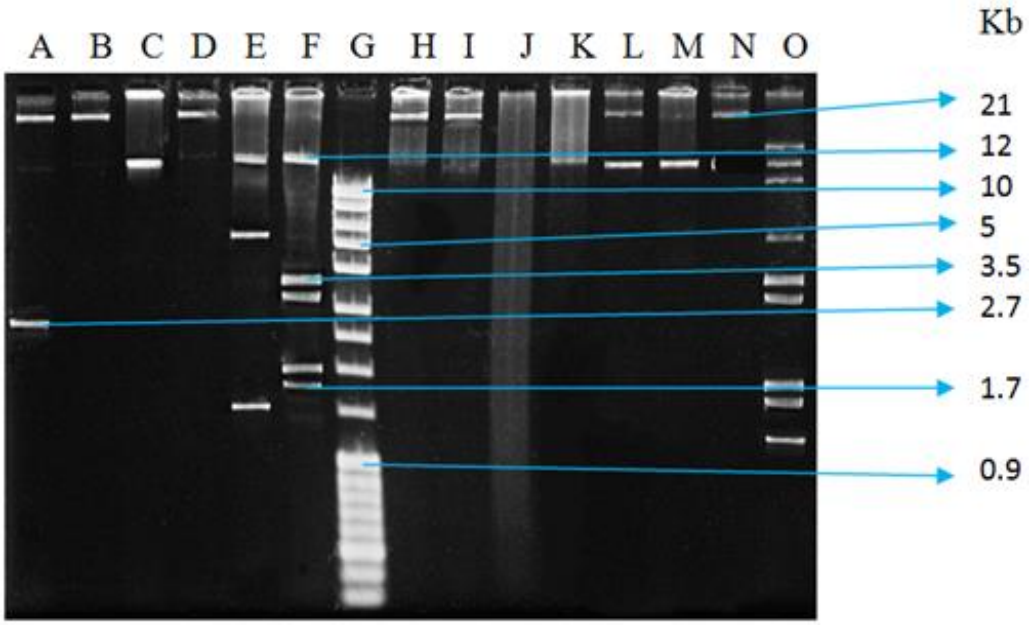

Figure 7. Plasmid profiles of representative LF isolates (n=12) (Lane A: 23; Lane B: 27, Lane C: 31, Lane D: 35, Lane E: 39, Lane F: 46; Lane G: Circular DNA Ladder; Lane H: 51, Lane I: 59, Lane J: 68; Lane K: 73, Lane L: 83, Lane M: 99 (antibiotic sensitive), Lane N: R1, Lane O: V-517).

Table 7. Plasmid patterns of representative LF isolates (n=14) showing resistance to both heavy metals and antibiotics.

Lab ID	Isolate ID	Lanes in Figure 7	Plasmid size in base pair (kb)	Plasmid pattern (P)
23	S2L1 LF3	Lane A	21, 2.7	P1
27	S2L2 LF3	Lane B	21	P2
31	S2L3 LF3	Lane C	12	P3
35	S2L4 LF3	Lane D	21	P2
39	S2L5 LF3	Lane E	12, 5.5, 1.6	P4
46	S3L2 LF2	Lane F	12, 3.5, 3.3, 2.0, 1.7	P5
51	S3L3 LF3	Lane H	21	P2
59	S3L5 LF3	Lane I	21	P2
68	S4L2 LF4	Lane J	Plasmid less	P6
73	S4L4 LF1	Lane K	12	P7
83	S5L1 LF3	Lane L	21, 12	P8
11*	S1L3 LF3		Plasmid Less	P6
85*	SFL2 LF1		Plasmid Less	P6
99*	S5L5 LF3	Lane M	12	P3
E.coli	ATCC (25922)		Plasmid less	P6
	V 517	Lane O	14,12,10,5,3.5,3,1.7,1.6,1.0	
	R 1	Lane N	21	

ID: Identification; SL: Sample location; LF: Lactose fermenter; 11*, 85*, 99*: Antibiotic sensitive LF isolates; R1: Reference strain; V517: Reference strain; P: Pattern.

the similar pattern of antibiotic resistance can be concealed by unconnected plasmid, transposon, phage and chromosomal genes (Jain et al., 2009; Vajiheh et al., 2003). Therefore, the antimicrobial resistance motifs and plasmid profiles are consistently insufficient to explain the connections between distinctive isolates of bacteria from

Table 8. Representative LF isolates (n=14) showing susceptible pattern to heavy metals and antibiotics in relation to their plasmid profiles.

Lab ID	Sample ID	Antibiotic susceptibility test			Maximum tolerable conc. to heavy metals (mM)			In the presence of maximum tolerable conc. to heavy metal, the changes of zone of inhibition of antibiotics measured in mm						Plasmid profiling (size in Kb)
								Ni (0.3 mM)		Cr (1.0 mM)		Pb (0.6 mM)		
		R	I	S	Ni	Cr	Pb	E	D	E	D	E	D	
23	S2L1 LF3	AZM,SXT,TE	CTX,CIP	-	0.6	1.0	0.6	-	-	-	-	-	-	21, 2.7
27	S2L2 LF3	AZM,SXT,TE	CTX	CIP	0.3	1.0	0.6	AZM,CTX	-	CTX,TE	AZM,SXT,CIP	AZM,CTX,TE	CIP	21
31	S2L3 LF3	SXT,CTX,CIP,TE	-	AZM	0.3	1.0	0.6	All	-	All	-	AZM,SXT,CTX,TE,CIP	-	12
35	S2L4 LF3	SXT	AZM,CTX	TE,CIP	0.3	1.0	0.6	AZM,SXT,CTX	TE	CTX,TE,CIP	AZM	AZM,CTX	-	21
39	S2L5 LF3	AZM,SXT,TE	CIP	CTX	0.3	1.0	0.6	AZM,SXT,TE,CIP	CTX	SXT,TE,CIP	AZM,CTX	AZM,SXT,TE, CIP	CTX	12, 5.5, 1.6
46	S3L2 LF2	SXT,CTX	AZM,CIP	TE	0.3	2.0	1.2	AZM,SXT,CTX, CIP	TE	-	-	-	-	12, 3.5, 3.3, 2.0, 1.7
51	S3L3 LF3	SXT,TE,CIP	AZM,CTX	-	0.3	1.0	0.6	All	-	AZM,SXT,TE,CIP	CTX	AZM,SXT,TE, CIP	-	21
59	S3L5 LF3	All	-	-	0.6	2.0	1.2	-	-	-	-	-	-	21
68	S4L2 LF4	TE	AZM,CTX	SXT,CIP	0.3	1.0	0.6	AZM,SXT,CTX	CIP	CTX,TE	AZM,CIP	AZM,CTX,TE	CIP	Plasmid less
73	S4L4 LF1	CTX	AZM	SXT,TE, CIP	0.3	1.0	0.6	AZM,SXT,CTX	TE	CTX,TE	AZM,CIP	CTX	AZM, CIP	12
83	S5L1 LF3	CTX	SXT,TE	AZM,CIP	0.3	1.0	0.6	CTX,TE	CIP	AZM,SXT,CTX,TE	CIP	AZM,SXT,CTX,TE	-	21, 12
11*	S1L3 LF3	-	-	All	0.3	1.0	0.6	-	-	-	-	-	-	Plasmid Less
85*	SFL2 LF1	-	-	All	0.3	1.0	0.6	-	-	-	-	-	-	Plasmid Less
99*	S5L5 LF3	-	-	All	0.3	1.0	0.6	CIP	AZM,SXT, CTX,TE	SXT,TE	AZM,CTX	TE	CTX	12
E.coli	ATCC (25922)	-	-	All	-	-	-	-	-	-	-	-	-	Plasmid less

ID: Identification; R: Resistance, I: Intermediate, S: Sensitive, CIP: Ciprofloxacin; CTX: Cefotaxime; AZM: Azithromycin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; Conc.: Concentration; Ni: Nickel; Cr: Chromium; Pb: Lead; E: Enhanced; D: Decreased, Kb: Kilo base pair, 11*, 85*, 99*: *A*ntibiotic sensitive LF isolates, ATCC (25922): *E. coli* negative control strain.

polluted environment and may lead to flawed epidemiologic conclusions. In summary, these significant bacterial isolates from textile industrial effluents had a greater range of resistance property against different antibiotics and heavy metals, as well as, found to contain an extra-chromosomal or plasmid DNA of 12 Kb and 21 Kb.

Conclusion

The results obtained from this study suggest that industrial effluents are enriched organic medium for supporting the potentially high growth and spreading of microorganisms, which are ultimately resistant to different antibiotics and heavy metals. Thus, environmental distributions of such bacteria are putting a deliberate threat for successfully treating the infectious diseases in some cases. In most cases, heavy metal exerts negative or no effects on antibiotic resistance of isolates. The heavy metal resistance patterns are of comparatively minor clinical concern than antimicrobial resistance; nevertheless, the obtained knowledge about heavy metal resistance may administer

some valuable evidences of antibiotic resistance, genetic study of plasmid, physiological and ecological features of bacteria inhabited in polluted water and soil environments. In summary, this study gives a general view of the state of lactose fermenting isolates growing in heavy metal enriched context to understand the aftereffects of discharging such effluents into the environment. Therefore, it is very essential to investigate the complete properties of heavy metal and antibiotic resistant bacterial isolates both phenotypically and molecularly to combat possible health hazards caused by the infection of such isolates.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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Isolation and in vitro screening of plant growth-promoting rhizobacteria from *Solanum lycocarpum* St. Hil., an endemic plant of the Brazilian tropical savannah

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***Solanum lycocarpum* St. Hil. is an endemic plant of the Brazilian tropical savannah (cerrado) that is capable of growing on acidic and nutrient-poor land, an ability which attracts attention to its rhizospheric microbiota, including plant growth-promoting rhizobacteria (PGPR). In this work, 131 bacterial strains were isolated from rhizosphere samples of *S. lycocarpum* and were tested *in vitro* for direct mechanisms of plant growth promotion (biological nitrogen fixation, phosphate solubilization and indolic compounds production) and enzyme activities. The 26 most promising isolates selected from the previous tests were used to continue the screening. Ten of these isolates showed antifungal activity against fourteen phytopathogenic fungi and twelve isolates showed antimicrobial activity against at least one of the three clinical pathogens evaluated. Seven of the 26 isolates were identified at random as belonging to the genera *Bacillus*, *Burkholderia* and *Microbacterium*, are PGPR and have potential to participate in more in-depth research aimed at the development of bio-inputs, especially the *Bacillus* strains.**

Key words: Biological control, biotechnological potential, cerrado, plant growth-promoting rhizobacteria (PGPR), phytopathogenic fungi, R*hizosphere, Solanum lycocarpum* St. Hil.

INTRODUCTION

The Brazilian tropical savannah (cerrado) biome is a mosaic of savannah, forests and grasslands with high species richness. This biome comprises approximately 2 million hectares or 24% of Brazil's land surface and is surpassed only by the Amazonian forest. The cerrado hosts approximately 6,000 vascular plant species, featuring the highest floral biodiversity among the world's savannas. The cerrado also has a high diversity of

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microorganisms. Much of the biodiversity of this microbiome is not yet known, which makes it one of the 25 most important terrestrial biodiversity hotspots on the planet (Oliveira-Filho and Ratter, 2002; Sousa et al., 2017).

Native plants are interesting for the study of bioactive compounds since they are adapted to the adverse conditions of their biome, such as aluminum-toxic acidic soil with few nutrients, water stress, exposure to UV radiation and attacks by herbivores and phytopathogens (Bailão et al., 2015). Among the plants in the cerrado, *Solanum lycocarpum* St. Hil is an important species described in the literature and is popularly known as “lobeira”. The fruit of *S. lycocarpum* is the basic food of the endangered maned wolf (*Chrysocyon brachyurus*) (Aragona and Setz, 2001). This plant also attracts interest for its medicinal properties and is used to treat hepatitis, asthma, flu and colds (Araújo et al., 2010).

Plants live in association with soil microbes (bacteria and fungi) during their growth. Free-living soil microorganisms that inhabit the rhizosphere of many plant species have diverse beneficial effects on the host plants through different mechanisms; these microorganisms are generally referred to as plant growth-promoting rhizobacteria (PGPR). In return, the plant's roots release exudates containing nutrients and compounds that can be used by PGPR for their development (Kloepper and Schroth, 1978). PGPR and their interactions with plants have been documented and used as important tools to sustainably manage agriculture. PGPR boost plant growth through various mechanisms, including phosphate solubilization (PS), biological nitrogen fixation (BNF) and production of indole-3-acetic acid (IAA) (Glick, 2012). In addition to their environmental benefits, biofertilizers formulated with PGPR have increasing economic potential. Global projections for the biological market point to an annual growth of 12.7% by 2022, estimated at US $ 11.35 billion. In Brazil, the estimates from the Brazilian Association of Control Companies (ABCBio) appear promising, with an expected annual growth ranging between 15% and 20% for the coming years (Calvo et al., 2014). PGPR may also act in the biological control of phytopathogens by producing metabolites. For example, fungi belonging to the genera *Phytophthora* and *Fusarium*, regarded as the most calamitous crop pathogens in the world, are inhibited by PGPR (Ramadan et al., 2016).

PGPR are interesting subjects for the study of new enzymes and antibiotics due to the survival strategies they develop to compete with other microorganisms in the rhizosphere, such as the production of bioactive secondary metabolites (Raaijmakers and Mazzola, 2012) and lytic enzymes. In enzymatic production processes, microorganisms have advantages over other sources due to their low cost and susceptibility to genetic manipulation (Castro et al., 2014). There is strong interest in microbial enzymes such as proteases, pectinases, cellulases, lipases, esterases and amylases in industry sectors. These sectors include food processing, detergent and textile manufacturing, medical therapy and molecular biology, agricultural and pharmaceutical research (Carrim et al., 2006; Ferreira-Filho et al., 2012; Quecine et al., 2014).

Studies on plant-microbe interactions are necessary to preserve the plant richness and bioprospecting of microorganisms associated with plants in the cerrado. Although this topic is currently underexplored, it can generate beneficial bioproducts for humanity. Therefore, this study aimed to isolate, characterize and select cultivable rhizobacteria associated with *Solanum lycocarpum* St. Hil. with potential applications in agriculture, medicine and industry.

MATERIALS AND METHODS

Bacterial isolation

Three rhizospheric soil samples of approximately 50 g each were collected in March 2018 from three *S. lycocarpum* St. Hil. plants (n = 9) located in a fragment of cerrado vegetation (S 21° 58’/WO 47° 52’) on the campus of the Federal University of São Carlos, São Paulo state, Brazil. For soil sample collection, 5 cm of topsoil was removed and 20 to 25 cm of the soil layer where roots were concentrated was collected. Collected samples were stored in sterilized plastic bags and transported to the Laboratory of Microbiology and Biomolecules (LaMiB®/UFSCar) for bacterial isolation.

The roots were agitated to remove excess soil, the three samples from each plant were mixed and approximately 50 g of rhizospheric soil were obtained per plant. Each mix was added to 90 ml of phosphate buffered saline (PBS) and shaken for 1 h at 150 rpm. The soil-PBS solution obtained was serially diluted and tryptic soy agar (TSA) (Kasvi, Sao Jose dos Pinhais, Brazil) medium supplemented with benomyl (50 µg/ml) to inhibit fungal growth was used for isolation with 10^{-4} to 10^{-5} serial dilutions. The plates were incubated at 28°C for 72 h and colonies were collected after 24 h and 72 h to obtain a bacteria pool with greater diversity of metabolism and growth rates and these colonies were purified by repeated plating at least three times on TSA (Schmidt and Belser, 1983). A total of 131 bacterial strains were isolated and inoculated into liquid tryptic soy broth (TSB) (Kasvi, Sao Jose dos Pinhais, Brazil) medium supplemented with glycerol (15% final concentration) and stored at −80°C for future experiments. The strains received codes beginning with the letters "A" or "T" which meant, respectively, picked up after 24 or 72 h of isolation; a number from 1 to 3, related to *S. lycocarpum* specimen from which it was isolated; the letters "LRZ", indicating that it was isolated from the rhizosphere; finally, sequential numbers for identification.

Growth capacity of isolates in nitrogen-free culture medium

The ability to fix atmospheric nitrogen by the bacteria was evaluated by growing the strains on the semisolid nitrogen-free medium (NFb), prepared according to Dobereiner et al. (1995) and Cattelan et al. (1999). Glass tubes were filled with 3 ml of the NFb medium and after solidification the microorganisms were inoculated in the tube. The test was performed by triplicate. After 96 h of incubation at 28°C in the dark, the formation of a white growth film near the surface of the tubes indicated a positive result. *Burkholderia ambifaria* strain RZ2MS16, was used as a positive

control (Batista et al., 2018; Fukuda et al., 2021). The strains that showed positive results in this stage were re-inoculated to confirm the result.

Indolic compound production

Indolic compound production was evaluated using the colorimetric method (Bric et al., 1991) with modifications to make it quantitative. The cultures were resuspended in sterile distilled water at an optical density of 0.1 at 550 nm (OD550 = 1.0 ± 0.05). Then, 100 µl of each solution was inoculated in separate tubes containing 5 mL of liquid 10% TSB (w/v) with 50 µg/ml L-tryptophan. The tubes were incubated for 72 h at 28 ± 2°C and were then centrifuged at 12,000 rpm for 5 min. Subsequently, the IAA concentration in each culture's supernatant was estimated using Salkowski reagent. The measurements were estimated with a standard curve, which was calculated based on known doses of the hormone (Sigma) at the following concentrations: 1, 5, 25, 50, 75, 100, 125, 150, 175 and 200 µg/ml. The reddish-pink color of the samples indicated the production of indolic compounds. The test was performed by triplicate and the measurements were averaged.

Phosphate solubilization

Qualitative and quantitative assays for phosphate solubilization were performed and measured in insoluble phosphate-containing solid medium (Berraquero et al., 1976). The presence of a clear halo around a colony indicated phosphate solubilization. The solubilization index (SI) values of the isolates that showed this capacity were then calculated according to the ratio of the diameter of the solubilization halo to the diameter of the colony halo and they were classified as having low solubilization potential (SI less than 2), medium solubilization potential (SI between 2 and 3) or high solubilization potential (SI greater than 3). Experiments were conducted in a completely randomized design with two replications per isolate in the quantitative test and four replicates per isolate in the qualitative test, in which the SI was calculated by averaging the values from these replications.

Enzymatic activity

Amylolytic activity was measured in solid agar (Hankin and Anagnostakis, 1975). Isolates were inoculated in 5% TSA medium containing 1% soluble starch. After bacterial growth, 5 ml of a 1% iodine solution was added to each plate, allowing visualization of clear halos around colonies.

A specific medium was used to evaluate lipolytic activity (Sierra, 1957). Previously sterilized Tween 80 or Tween 20 was added to the sterilized culture medium to a final concentration of 1% (w/v) to evaluate bacterial esterase and lipase activities, respectively. The presence of a halo was considered indicative of enzymatic activity.

Pectinolytic activity was determined after growing bacterial isolates on M9 medium, substituting glucose for 1% (w/v) pectin and adding 0.5% yeast extract (Miller, 1972). After bacterial growth, 10 mL of Lugol's solution was added, followed by a wash with water. The presence of a halo around a colony indicated pectinolytic activity. For pectate lyase activity, final pH of the M9 medium was adjusted to pH 8.0 with 5 M NaOH and for polygalacturonase was adjusted to pH 5.0 using 1 M HCl.

We used a culture medium containing skim milk to determine proteolytic activity (Zarnowski et al., 2007). The formation of a halo around a colony was considered indicative of proteolytic activity. The cellulolytic activity of the isolates was also evaluated (Teather and Wood, 1982). The isolates were grown on M9 medium containing 0.5% yeast extract and 1% carboxymethylcellulose (CMC). After bacterial growth, 10 ml of Congo red dye (1%) was added and the plates were washed with NaCl (5 M). The presence of a colorless halo around a colony indicated enzymatic activity.

Selection of promising PGPR

To narrow the screening and select the most promising PGPR from a pool, following assays to determine the direct mechanisms and enzymatic activity, isolates were selected that: presented positive results by more than one direct mechanism, showed SI higher than 1.1, produced indolic compounds and showed more than one type of enzymatic activity. Following this selection, 26 isolates were selected for evaluation in the following tests.

Assessment of antifungal activity

Antifungal activity was evaluated in two parts, visual agar plate and plate pairing assays (Chang et al., 1999). The potato dextrose agar (PDA) plates were divided into quadrants and the 26 selected isolates were inoculated punctually, one isolate per quadrant, 3 cm from the center of the plate. A fungal phytopathogen was point inoculated at the center of each plate. The control plate was only point inoculated with the fungal phytopathogen. The plates were sealed with parafilm and incubated at 28 ± 2°C until the mycelium reached the edge of the control plate (visual agar plate assay). The test was conducted by duplicate.

The ten isolates that showed antifungal activity in the visual assay were streaked on PDA medium and the fungal phytopathogen was inoculated punctually at 1 cm from the opposite end from which the bacterial inoculum was streaked (plate pairing assay). The control plate was point inoculated with the fungal phytopathogen. The plates were sealed with parafilm and incubated at 28 ± 2°C until the mycelium reached the edge of the control plate. Antifungal activity was calculated in terms of the inhibition index by measuring the zone of fungal growth inhibition divided by mycelium diameter of the positive control. The test was performed by triplicate.

The phytopathogenic fungi used in this study were chosen for their economic importance as phytopathogens of ornamentals, seeds, fruits and vegetables. The following species from the microbiological collection of the Laboratory of Microbiology and Biomolecules (LaMiB®/UFSCar) were used in antagonism tests: *Alternaria alternata, Ceratocystis paradoxa, Colletotrichum* sp., three strains of *Fusarium oxysporum* (ATTCC 2163, an isolate from bean and an isolate from cotton), *F. proliferatum, F. solani*, *F. verticillioides, Moniliophthora perniciosa, Phytophthora sojae, Rhizopus microsporus, Sclerotinia sclerotiorium* and *Sphaceloma* sp. (CNPUV 102).

Antagonistic activity against clinical pathogens

The overlay technique used in inhibition assay was adapted from Pugsley and Oudega (1987). Each isolate was inoculated in tryptic soy broth (TSB) and incubated for 72 h at 28°C. Then the inoculum was spread with sterile swabs on TSA plate and after incubation for 72 h at 28°C, an agar block was cut out in the form of a 1 cm diameter cylindrical disk. The clinical pathogens *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25923 and *Staphylococcus aureus* ATCC 25922 were used in this test after being reactivated and spread with swabs on the surfaces of plates containing TSA medium. After the formation of a layer of pathogenic microbial cells, the previously prepared agar blocks of twenty-six isolates selected for their performance in the direct plant-growth promotion tests (BNF, PS and IAA production) and enzymatic activity assays were added to plates, one agar block per quadrant. After incubation at

37°C for 48 h, the absence or presence of an antibiosis halo around the blocks was recorded. The tests were performed by triplicate.

Bacterial molecular identification

After all the evaluations, seven of the twenty-six best isolates were selected randomly and were identified by partial sequencing of 16S rDNA. The DNA extraction was performed using Bacteria DNA Preparation Kit (Cellco Biotec do Brasil Ltda., Sao Paulo, Brazil) according to the manufacturer's specifications. The concentration and quality of the extracted nucleic acids were analyzed on an agarose gel (1.0%) (3 volts/cm) with the GelRed nucleic acid gel stain (1.0 mg/ml) together with a 1-kb DNA Ladder RTU weight marker (Kasvi, Sao Jose dos Pinhais, Brazil). The 16S rDNA was amplified using the primers V3F (5'- CCA GAC TCC TAC GGG AGG CAG - 3', forward sequence) and V6R (5'-ACA TTT CAC AAC ACG AGC TGA CGA - 3', reverse sequence). Polymerase chain reaction (PCR) was performed in a 15 µl volume containing 3.75 mM $MgCl_2$, 0.2 µM dNTPs, 0.2 µM of each primer, 2.5 U Taq DNA polymerase (Jena Bioscience, Jena, Germany) and 1 µl template DNA. The reaction conditions for the thermocycler (Eppendorf AG, Hamburg, Germany) were as follows: initial denaturation for 10 min at 95°C; 35 cycles at 95°C for 20 s, annealing for 30 s at 52°C and primer extension for 30 s at 72°C; followed by final extension for 10 min at 72°C. The reaction products were analyzed on an agarose gel (1.0%) (3 volts/cm) with the GelRed nucleic acid gel stain (1.0 mg/mL) together with a 1-kb DNA Ladder RTU weight marker (Kasvi, Sao Jose dos Pinhais, Brazil). Then the agarose gel was analyzed on Gel Doc™ EZ System/Image Lab™ software (Bio-Rad Laboratories, CA, USA).

The 16S rDNA PCR products (approximately 1400 bp in size) were purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) and sequenced at the Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara campus, SP, Brazil. Sequencing was performed using the primer V6R. Bacterial identification was performed by comparison with sequences deposited in GenBank (National Center for Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov) with more than 97% homology, using the BLASTn tool for this purpose.

Statistical analyses

Data obtained from each semiquantitative assay using a completely randomized design with three (IAA production and the antifungal activity index) or four (phosphorus solubilization index) replicates were subjected to one-way ANOVA. The means were compared and clustered using the Scott-Knott test ($p < 0.05$). Analyses were conducted using R Statistical Software (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Bacterial isolation and selection, evaluation of the direct mechanisms of plant growth promotion and enzymatic activity assays

One hundred thirty-one bacterial isolates were successfully isolated from the rhizosphere of *S. lycocarpum* St. Hil. soil samples. The results of the 26 most promising isolates for biological nitrogen fixation, phosphate solubilization, IAA production and enzyme activity tests are shown in Table 1. Among the 131 isolates evaluated, 32.1% were able to form a typical subsuperficial whitish 'veil-like' pellicle in nitrogen-free semisolid medium after incubation and were considered to be positive. All the isolates were able to produce IAA, which varied in concentration from 0.07 to 134.1 µg/ml. The calcium phosphate solubilizing capacity trials showed that 45.8% of the isolates exhibited a positive reaction. The T1LRZ1, T1LRZ6, T3LRZ4, A1LRZ4, A1LRZ7 (*Microbacterium* sp.), A1LRZ14, A1LRZ22 (*Bacillus* sp.), A1LRZ32, A2LRZ8, A3LRZ2, A3LRZ5 and A3LRZ11 (*Burkholderia* sp.) isolates showed high solubilization potential. The T3LRZ29, A1LRZ1, A1LRZ3, A1LRZ11, A2LRZ2 (*Bacillus* sp.) and A2LRZ7 isolates showed medium solubilization potential. The remaining isolates had low potential or were considered negative in the test. Eighty-eight percent of the isolates were able to produce at least 1 of the 7 evaluated enzymes. Regarding pectinolytic activity, 22.3% of the evaluated isolates showed enzymatic activity of polygalacturonase and 40.2% of the evaluated isolates showed pectate lyase activity. Amylolytic activity was observed in 42.9% of the evaluated isolates, esterase activity in 47.3%, lipolytic activity in 27.7%, proteolytic activity in 60.7% and cellulolytic activity in 42.9%. Some isolates exhibited 4 or more enzymatic activities: T1LRZ14, A1LRZ19, A2LRZ7, T3LRZ7 (*Bacillus* sp.), T3LRZ9, T2LRZ11, A1LRZ22 (*Bacillus* sp.), A2LRZ30 (*Bacillus* sp.), A3LRZ16, A3LRZ15, A1LRZ28, A3LRZ19, A1LRZ30 and A2LRZ35 (*Bacillus* sp.).

Antagonistic activity against phytopathogenic fungi and clinical pathogens

Twenty-six bacterial isolates were evaluated in visual agar plate assays against phytopathogenic fungi. A total of 50% of the isolates showed antagonist activity against *Sclerotinia sclerotiorium*, 58% against *Moniliophthora perniciosa*, 38% against *Fusarium solani*, 40% against *Fusarium oxysporum* (ATTCC 2163), 52% against *Sphaceloma* sp., 61% against *Ceratocystis paradoxa*, 23% against *Alternaria alternata*, 46% against *Fusarium proliferatum*, 50% against *Colletotrichum* sp., 31% against *Fusarium verticillioides*, 33% against *Fusarium oxysporum* (bean), 47% against *Fusarium oxysporum* (cotton), 32% against *Phytophthora sojae* and 46% against *Rhizopus microsporus*. Ten isolates were selected for plate pairing assays and the mycelium growth inhibition percentages are shown in Table 2. Among the 26 bacteria evaluated, 7.7% exhibited antagonism to *Candida albicans*, 46% to *Escherichia coli* and 11.5% to *Staphylococcus aureus* (Table 3).

Molecular identification of bacterial isolates

The seven first sequenced isolates were chosen randomly

Table 1. Summary results of the *in vitro* screen for plant growth promotion and enzyme activity of the best rhizobacterial isolates.

Rhizobacteria isolates	Plant growth promotion			Enzymatic activity						
	BNF[a]	SI[b*]	IAA (ug/ml)[c*]	Protease	Lipase	Esterase	Pectate lyase	Poly-galacturonase	Cellulase	Amylase
T1LRZ14	-	1.167^{j}	13.2^{g}	-	+ +	+ +	+ +	+ +	-	+
A1LRZ4	+	3.143e	108.7^{a}	-	+	+	-	-	-	-
A1LRZ15	+	1.250^{j}	33.0^{d}	-	-	-	-	-	+ +	-
A1LRZ19	-	1.167^{j}	23.0^{e}	-	+ +	-	+ +	+ +	+	+ +
A2LRZ2	+	2.714^{f}	24.0^{e}	+ +	-	+ +	-	-	-	-
A2LRZ7	-	2.222^{g}	16.0^{f}	-	+ +	+	+ +	+ +	-	+ +
T3LRZ7	+	1.125^{j}	4.3^{j}	+ +	+	+	-	+	+ +	+ +
T3LRZ9	+	2.667^{f}	2.5^{j}	+ +	-	+	+ +	+	+ +	+ +
A3LRZ2	+	3.285^{d}	3.5^{j}	-	-	+ +	-	-	-	-
A3LRZ5	+	4.833^{a}	2.5^{j}	-	+ +	+ +	-	-	-	-
A3LRZ11	+	4.000^{c}	5.3^{j}	+	+ +	+ +	-	-	-	-
T2LRZ11	+	1.423^{h}	9.0^{h}	-	+	-	+ +	+ +	+ +	+ +
A1LRZ22	+	3.143^{e}	3.6^{j}	+ +	+	+	+	-	+ +	+ +
A1LRZ25	+	1.115^{j}	84.1^{b}	+ +	-	-	-	-	-	-
A1LRZ27	+	1.167^{j}	4.4^{j}	-	-	-	-	-	-	-
A2LRZ29	-	1.115^{j}	10.5^{h}	-	+ +	+ +	-	-	-	-
A2LRZ30	-	2.222^{g}	4.2^{j}	+ +	-	+	+	-	+ +	+
A3LRZ16	-	1.167^{j}	3.6^{j}	+ +	-	+	+ +	-	+ +	+
A3LRZ15	+	1.117^{j}	5.1^{j}	-	+ +	+ +	+	+	-	+ +
A1LRZ28	+	1.333^{i}	6.1^{i}	+ +	+ +	+	+ +	-	+ +	+ +
A3LRZ17	+	1.500^{h}	8.0^{j}	+ +	-	+ +	-	-	-	-
A1LRZ30	+	1.250^{j}	11.2^{h}	+	-	+ +	-	+	+ +	+ +
A1LRZ31	+	1.167^{j}	38.6^{c}	-	-	-	-	-	-	-
A1LRZ32	+	4.384^{b}	4.^{3j}	+ +	+ +	+ +	-	-	-	-
A3LRZ19	-	1.333^{i}	7.7^{i}	+	+ +	+ +	+	+	-	+ +
A2LRZ35	-	1.167^{j}	3.1^{j}	+ +	+	-	+ +	-	-	+ +

[a]Biological Nitrogen Fixation; [b]Phosphate Solubilization index; [c]Indole-3-acetic acid production. [*]Within a column, values with the same superscript letter are not different at the 5% significance level (Scott-Knott test). The character "+" indicates that the isolate showed enzymatic activity. The character "+ +" indicates high enzymatic activity. The character "-" signifies that the isolate did not show enzymatic activity.

from the twenty-six best isolates and have presented genera described in the literature as plant growth promoters (Kumar et al. 2017; Kashyap et al. 2019). The identified strains belonged to the genera *Bacillus* (strains A1LRZ22, A2LRZ2, A2LRZ30, A2LRZ35 and

Table 2. Antimicrobial capacity of rhizobacterial isolates against phytopathogenic fungi *in vitro.*

Phytopathogens	Antifungal activity index for each isolate (%)*									
	T1LRZ14	A1LRZ4	A1LRZ15	A2LRZ7	T3LRZ7	T3LRZ9	A3LRZ5	A3LRZ11	A1LRZ30	A1LRZ32
Alternaria alternata	34.4^{g}	43.8^{d}	43.8^{d}	46.9^{b}	36.3^{f}	6.3^{m}	47.5^{b}	43.8^{d}	49.4^{a}	45.6^{c}
Ceratocystis paradoxa	0.0^{n}	40.0^{e}	25.0^{i}	25.0^{i}	46.7^{b}	48.3^{a}	46.7^{b}	33.3^{g}	46.7^{b}	43.3^{d}
Colletotrichum sp.	43.3^{d}	33.3^{g}	36.7^{f}	45.0^{c}	40.0^{e}	45.0^{c}	41.7^{e}	48.3^{a}	46.7^{b}	48.3^{a}
Fusarium oxysporum (ATTCC 2163)	22.1^{j}	35.5^{f}	21.1^{j}	27.0^{i}	32.9^{g}	49.3^{a}	46.7^{b}	46.7^{b}	46.7^{b}	41.4^{e}
Fusarium oxysporum (bean)	42.3^{d}	30.3^{h}	34.4^{g}	34.4^{g}	40.0^{e}	33.3^{g}	43.3^{d}	49.4^{a}	50.0^{a}	48.3^{a}
Fusarium oxysporum (cottom)	16.7^{k}	46.7^{b}	50.0^{a}	45.0^{c}	16.7^{k}	33.3^{g}	48.3^{a}	41.7^{e}	48.3^{a}	38.3^{f}
Fusarium proliferatum	25.0^{i}	43.3^{d}	33.3^{g}	36.7^{f}	45.0^{c}	50.0^{a}	41.7^{e}	46.7^{b}	48.3^{a}	50.0^{a}
Fusarium solani	36.4^{f}	46.7^{b}	49.5^{a}	43.2^{d}	49.0^{a}	49.5^{a}	44.7^{c}	49.0^{a}	47.6^{b}	47.6^{b}
Fusarium verticillioides	40.8^{e}	36.7^{f}	41.7^{e}	41.7^{e}	50.0^{a}	43.3^{d}	38.3^{f}	31.7^{h}	48.3^{a}	41.7^{e}
Moniliophtora perniciosa	1.3^{n}	32.9^{g}	45.4^{c}	1.3^{n}	34.9^{f}	42.8^{d}	42.8^{d}	40.8^{e}	46.7^{b}	42.8^{d}
Phytophthora sojae	21.1^{j}	47.4^{b}	47.4^{b}	11.2^{l}	29.6^{h}	29.6^{h}	49.3^{a}	29.6^{h}	25.7^{i}	46.7^{b}
Rhizopus microsporus	33.3^{g}	43.3^{d}	40.8^{e}	25.0^{i}	21.1^{j}	11.2^{l}	41.7^{e}	36.7^{f}	29.6^{h}	49.3^{a}
Sclerotinia sclerotiorium	0.0^{n}	40.0^{e}	25.0^{i}	25.0^{i}	46.7^{b}	48.3^{a}	46.7^{b}	33.3^{g}	46.7^{b}	43.3^{d}
Sphaceloma sp. (CNPUV 102)	33.3^{g}	45.0^{c}	30.0^{h}	50.0^{a}	46.7^{b}	45.0^{c}	41.7^{e}	41.7^{e}	45.0^{c}	50.0^{a}

*Values with the same superscript letter, in the same row, are not different at the 5% significance level (Scott-Knott test).

T3LRZ7), *Burkholderia* (A3LRZ11) and *Microbacterium* (A1LRZ27). The nucleotide sequences were deposited in GenBank with the accession numbers MT415822, MT415823, MT415824, MT415825, MT415826, MT415827 and MT415828.

DISCUSSION

The Brazilian tropical savannah (cerrado) has a high diversity of microorganisms. However, studies focusing on the isolation and characterization of rhizobacteria from native plant specimens from the Brazilian savannah biome are rare. To our knowledge, the present work is the first report on the characterization, identification and isolation of rhizospheric bacteria from *S. lycocarpum* in Brazil. The cerrado soil is acidic, had high concentrations of aluminum, low level of exchangeable cations and low availability of nutrients, mainly carbon, nitrogen and phosphorus, which come from the slow mineralization of organic matter. Physical-chemical parameters of the soil are common in agriculture, but it is necessary to understand the soil as a dynamic living system and its biological indicators, such as the resident microbiota, are directly related to the balance of this system. PGPR have a fundamental role in soil fertility, health and quality, in addition to being involved in the biogeochemical cycles of nitrogen, phosphorus, carbon and other chemical elements (Procopio and Barreto, 2021).

The flow of a nitrogen in the cerrado soil involves FBN, process responsible for approximately 65% of the total N introduced into soil. In agriculture, the need to maintain soil fertility leads to anthropogenic application of nitrogen fertilizers, which leads to a disturbance of the biogeochemical cycle. The efficient associations between diazotrophic microorganisms and several economically and environmentally important plant species can supply most of the nutritional needs related to nitrogen, thus reducing the use of nitrogen fertilizers. In this study, 32.1% of the isolates were able to grow in NFb and this result corroborates with previous studies (Grobelak et al., 2015; Habibi et al., 2019; Fukuda et al., 2021), emphasizing the BNF potential of rhizobacteria associated with plants.

Phosphorus is the second most required element by plants and is fundamental for structural development and metabolism, it is found in greater quantities in the shallower layers of the

Table 3. Antimicrobial capacity of rhizobacteria isolates against clinical pathogens *in vitro*.

Rhizobacteria isolates	Clinical pathogens			Rhizobacteria isolates	Clinical pathogens		
	Staphylococcus aureus	***Candida albicans***	***Escherichia coli***		***Staphylococcus aureus***	***Candida albicans***	***Escherichia coli***
T1LRZ14	-	-	-	A1LRZ25	-	-	-
A1LRZ4	+	+	+ +	A1LRZ27	-	-	-
A1LRZ15	-	-	-	A2LRZ29	-	-	-
A1LRZ19	-	-	+	A2LRZ30	-	-	+
A2LRZ2	-	-	-	A3LRZ16	-	-	+
A2LRZ7	-	-	-	A3LRZ15	-	-	-
T3LRZ7	-	-	+ +	A1LRZ28	-	-	-
T3LRZ9	-	-	+	A3LRZ17	-	-	-
A3LRZ2	-	-	-	A1LRZ30	+	-	+ +
A3LRZ5	-	-	+ +	A1LRZ31	-	-	-
A3LRZ11	-	-	+ +	A1LRZ32	+	+ +	+ +
T2LRZ11	-	-	-	A3LRZ19	-	-	+
A1LRZ22	-	-	-	A2LRZ35	-	-	+

The character "+" signifies that the isolate showed antimicrobial activity. The character "+ +" indicates high enzymatic activity. The character "-" indicates that the isolate did not show antimicrobial activity.

soil. *Bacillus* sp., *Burkholderia* sp. and *Microbacterium* sp. are described in the literature as having the ability to solubilize inorganic phosphate (Midekssa et al., 2015). In the present study, 45.8% of the evaluated isolates exhibited this ability, including isolates belonging to the aforementioned genera. Twelve isolates (9.2%) featured high solubilization potential (SI greater than 3.0). Phosphate-solubilizing bacteria (PSB) are important not only for the growth of plants but also for reducing the quantity and increasing the quality (in terms of crop yield) of manufactured fertilizers (Quecine et al., 2014). The beneficial *in vivo* effects of PSB have been evidenced in studies conducted with *Triticum aestivum* L. (wheat). The addition of an inoculum containing strains of *Bacillus* sp., *Burkholderia* sp. and *Microbacterium* sp. to a conventional phosphate fertilizer induced better plant development than the use of the fertilizer alone (Chauhan et al., 2013).

In the present work, rhizobacteria isolates produced indolic compounds, which plants can use as growth regulators. A previous study recorded a significant increase in the number of stem branches after inoculation with indolic compound-producing PGPR in *Brassica napus* (Asghar e al., 2004). The ranges of the beneficial and toxic quantities of IAA common to all plants are not yet known and the effects of these amounts may vary. Still, these isolates deserve attention since they have shown other mechanisms for promoting plant growth that can be combined with the indolic compounds production (Taiz and Zeiger, 2013).

Over 70% of diseases that affect crops of high economic importance are caused by fungi (Agrios, 2005). *Fusarium* spp., for example, causes diseases in soy (*Glycine max*), tobacco (*Nicotiana tabacum*) and beans (*Phaseolus vulgaris*), reducing both the quality and quantity of crops, resulting in losses (Matarese et al., 2012). A study reported strains of *Burkholderia* sp. isolated from sugarcane inhibiting growth of *Fusarium verticillioides* (Mendes et al., 2007). *Bacillus* sp. has also shown an antagonistic capability against the phytopathogen *Alternaria alternate* (Abdalla et al., 2014). Isolates belonging to the genera *Bacillus* and *Burkholderia* demonstrated excellent results for mycelial inhibition of phytopathogenic fungi and in antibiosis tests against clinical pathogens in this study.

Rhizospheric and endophytic bacteria isolated from medicinal plants of the cerrado represent a large reservoir of secondary metabolites; several species producing bioactive compounds can be found within this niche (Piza et al., 2016). By analyzing the results of this study, we highlight isolates (A1LRZ32 and A1LRZ4) that exhibited antibiosis against the three evaluated pathogens. Several studies have reported that bacteria of different genera, including *Bacillus* and *Burkholderia*, exhibit enzymatic activities of amylases, cellulases, esterases, lipases, pectinases and proteases (Khan et al., 2017; Liu and Kokare, 2017). Enzymes from cerrado soil microorganisms have already been characterized structurally and functionally and even successfully applied to the manufacture of products (Silva et al., 2010; Istvan et al., 2018).

Our study presented several isolates with 5 or more enzymatic activities, confirming the usefulness of rhizobacteria isolated from a cerrado plant as a source for bioprospecting enzymes of industrial interest. This finding also reinforces the need to protect this biome, which has great economic notoriety in addition to its ecological importance. Some PGPR strains are already

being used commercially in the composition of bioproducts for nutrition and control of plant diseases, such as Nemix (*Bacillus* spp.) (AgriLife/Chr Hansen, Brazil) and Life® (PGPR consortia) (Biomax, India). Several species of *Bacillus* sp. have great advantages over other bacterial genera due to their rapid growth and their ability to form resistant spores against variations in pH, temperature, agrochemicals, fertilizers and storage time, allowing its use in the formulation of stable bioproducts and in biological control programs (Fantinel et al., 2018).

In conclusion, bacteria belonging to the genera *Bacillus*, *Burkholderia* and *Microbacterium* isolated from the rhizosphere of *S. lycocarpum* St. Hil. have *in vitro* potential for promoting plant growth, both directly and indirectly (antagonisms). The rhizobacteria associated with *Solanum lycocarpum* have enzymatic and antibiosis activities against pathogens, showing their importance and usefulness as sources of bioprospecting of enzymes and secondary metabolites, although further evaluation is necessary. This also shows the importance of the cerrado as a reservoir of biodiversity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of the efficacy of *Trichoderma* and *Pseudomonas* species against bacterial wilt *Ralstonia* isolates of tomato (*Lycopersicum* species)

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Ralstonia solanacearum **causes bacterial wilt of tomato and limits the crop production, and antagonistic microorganisms use to suppress the disease, of which** ***Trichoderma*** **and** ***Pseudomonas*** **species are the most effective agents to control bacterial wilt. In the present study, attempt was made to isolate these two microorganisms to evaluate their effectiveness to control** ***R. solanacearum*****, the causal agent of bacterial wilt disease of tomato under greenhouse conditions. Thus** ***R. solanacearum*****,** ***Pseudomonas*** **and** ***Trichoderma*** **spp. were isolated from wilted and healthy tomato plants grown from farmer's field in Ziway and Meki, Oromia Ethiopia. The virulence of the pathogen and the antagonistic effect of the bacteria and fungi were evaluated against** ***R. solanacearum in vitro*** **and** ***in vivo*** **condition. Based on the** ***in vitro*** **results the best two isolates were selected to show their antagonistic effect under greenhouse condition in single and combined designs. The result showed the pathogenicity test of the isolates were evaluated under greenhouse condition, and isolate AAURS1 showed highest virulence (75%) followed by isolate APPRCRS2 with pathogenicity of 50%. With regard to antagonism test, isolates AAURB20 and AAUTR23 showed the highest inhibition against** ***R. solanacearum*** **with inhibition zone of 16 and 15 mm, respectively. Among the treatments co-inoculation was more effective and reduced disease incidence by 13.33% and increased the bio-control efficacy by 72.22% when compared with individual treatment and negative control. The isolates significantly increased the plant height and dry weight by 72.33 cm and 12.18 g, respectively. Thus, the combined use of the biocontrol agents significantly reduced the incidence of tomato bacterial wilt disease. However, their performance should be evaluated using other yield parameters under field conditions to produce healthy tomato seedling to minimize the use of chemicals and reduce environmental pollution.**

Key words: Biocontrol, *Pseudomonas*, *Ralstonia solanacearum*, *Trichoderma*.

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is the second most important vegetable crop in the world next to potato (Gebisa et al., 2017). The center of origin of *Solanum lycopersicum* has been localized in the narrow band between the Andes mountain ranges and the Pacific coast of western South and extends from southern Ecuador to northern Chile, including the Galapagos Islands (Peralta et al., 2008). Tomatoes production accounts for about 4.8 million hectares of harvested landarea globally with an estimated production of 162

million tones. China leads world tomato production with about 50 million tones followed by India with 17.5 million tonnes (FAOSTAT, 2014). In Africa, the total tomato production for 2012 was 17.938 million tons with Egypt leading the continent with 8.625 million tones (FAOSTAT, 2014). It is an economically important vegetable in Ethiopia. According to the CSA (2019), the country produced 27,774.538 tons of tomato in 5235.19 hectares of land in 2018.

Ralstonia solanacearum is ranked as the second most important bacterial pathogen among the top ten economically important soil borne pathogens that cause severe yield losses on different solanaceous crops in different parts of the globe (Mansfied et al., 2012). Different studies showed the bacterial wilt pathogen inflict 50 to 100% loss on potato in Kenya (Muthoni et al., 2012), 88% on tomato in Uganda (Katafiire et al., 2005), 70% on potato in India (APS, 2005). It is one of the most destructive and widespread disease of tomato in Ethiopia and its prevalence is as high as 55% in major tomato producing areas of the country (Biratu et al., 2013). Different methods, mainly pesticides are employed to control bacterial wilt of tomatoes.

Chemical controls with Actigard (e.g., acibenzolar-S-methyl) and phosphorous acid effective to control bacterial wilt under at greenhouse and to a lesser extent field conditions (Pradhanang et al., 2005). The use of excessive agrochemicals is negatively perceived by consumers and supermarket chains due to residual chemicals in horticultural products. In addition use of chemical pesticides contaminate groundwater, enter food-chains, and pose hazards to animal health and to the user spraying the chemicals. Consequently, several members of the European Union (EU) (Sweden, Denmark, and Netherlands) decided in the mid-late 1980s to decrease the use of chemicals in agriculture by 50% and ban some of them through time within a 10-year period (Butt et al., 2001).

However, effective and long term control is possible by using a combination of diverse methods including the use of resistant/tolerance varieties, cultural practices, biological and chemical control as parts of an integrated pest management strategy to control bacterial wilt caused by *R. solanacearum* (Persley, 1986). The use of biological control agents alone and/or together with other control methods as part of integrated pest management (IPM) practices is widely employed to overcome these problems (Barbari, 2016).

Soil bacteria and fungi which flourish in the rhizosphere of plants and, stimulate plant growth are collectively known as plant growth promoting microorganisms (PGPM). The most abundant and useful microorganisms in the rhizosphere are *Pseudomonas*, *Bacillus*, *Burkholderia*, *Agrobacterium*, *Streptomyces*, *Trichoderma, Penicillium*, and *Gliocladium.* These microorganisms are used with the aim of improving crop yield by augmenting nutrient availability, enhancing plant growth and protection of plants from diseases and pests (Vessey, 2003). They are capable of secreting hydrolytic enzymes and causing mycoparasitism on pathogens and narrow spectrum antagonistic activity compared to synthetic pesticides, and thus used singly or in combination with one another and chemicals in integrated pest management (IPM) to suppress plant-pathogens (Handima and Kalaivani, 2014).

Trichoderma and *Pseudomonas* species are the most frequently isolated fungi and bacteria in all the root ecosystems, respectively. *Trichoderma* spp. effective in controlling phytopathogens due to their ability to grow toward the hyphae of other fungi, coil around them and degrade the cell walls of the pathogen. Morsy et al. (2009) showed that, the dual application of *Trichoderma viride* and *Bacillus subtilis* decreased the percentage of pathogen infection and increased survival rate than single inoculation in tomato. The biocontrol potential of two *Trichoderma* spp. on *sclerotia* rot disease of tomato plants in Chile and Iceland was evaluated and the result showed that, *Trichoderma harzianum* and *T. viride* reduced the disease by 74.50 and 68.75%, respectively (Kator et al., 2015).

Several studies also showed that the application of these antagonists have a dramatic effect on bacterial wilt disease caused by *R. solanacearum* on tomato. Narasimha et al. (2013) showed that *Trichoderma asperellum* (T4 and T8) isolates delayed wilt development by *R. solanacearum*, effectively decreased the disease incidence (51%), improved plant growth promotion and increased fruit yield under field conditions.

Another study also showed that *Trichoderma* spp. AA2 and *Pseudomonas fluorescens* PFS were most potent inhibiting the growth of *Ralstonia* species and the field study indicated *Trichoderma* spp. and *P, fluorescens* alone were able to prevent 92 and 96% of the infection and combination of both were more effective, preventing 97% of infection compared to chemical control methods that prevented 94% of infection (Yendyo and Pandey, 2017). This shows the promising potential of native isolates of *Trichoderma* spp. and *P, fluorescens* as biocontrol agents against *Ralstonia* spp.

In order to identify successful microorganisms as biocontrol agents, continuous screening of new isolates is needed for effective formulation against specific

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pathogens. Therefore, this study was initiated with the objective of evaluating the efficacy of *Trichoderma* and *Pseudomonas* spp. individually and in combination against bacterial wilt pathogen, *R. solanacearum* of tomato under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Sample collection

Soil samples from the rhizosphere of healthy and bacterial wilt infected tomato plants were collected from different fields from Ziway and Meki along the Rift Valley, which is one of the most important vegetable producing areas in the country. Diseased plant samples were selected based on visible characteristic symptom of bacterial wilts (Yendyo and Pandey, 2017).

Isolation of *R. solanacearum* from wilted tomato plants

Isolation of the wilt pathogen was undertaken according to Kelman (1954). Diseased tomato stem samples were washed with tap water, and surface sterilized with 70% ethanol for 2 min and rinsed repeatedly in sterile water for 5 min. The samples were then suspended in the 5 ml sterile distilled water for 10 min to make them turbid due to oozing of bacterial cells from cut ends of diseased tissue. The bacterial suspensions were prepared to appropriate dilutions from which, 1 ml of the bacterial suspension was spread onto the surface of solidified Triphenyltetrazolium chloride agar (TZC) medium and incubated at 28±2°C for 48 h.

Identification of virulent/avirulent isolates of *R. solanacearum*

The virulent and a virulent isolates of the pathogen were differentiated by Kelman method (1954) on TZC agar medium and compared with isolates obtained from Ambo Plant Protection Research Center. The virulent isolates were detected based on their pink or light red colored colonies with characteristic red center and whitish margin, whereas the avirulent isolates were differentiated on their colonies characterized by smaller, off-white and non-fluidal or dry texture on TZC medium after 24 h of incubation.

Pathogenicity test

Virulence of the isolates was carried out by inoculating them on the tomato seedlings according to Margaret et al. (2011). Tomato seeds were planted directly in 20 × 18 cm plastic pots containing sand and soil in the ratio of 2:1 (3 kg of soil and 1.5 kg of sand) soil and sand was obtained from AAU. Bacterial isolates were grown on nutrient broth medium for two days at 30°C, suspended in sterile distilled water and adjusted to OD 600 nm = 0.1 (approximately inoculum size of 10^8 CFU/ml) (Ran et al., 2005). Inoculation was made at the four true leaf stages by injecting into the stem with a needle. Plants inoculated with sterile water served as control and pots were regularly watered. Tomato plants were observed for development of typical wilt symptoms, and the severity of bacterial wilt was recorded based on the severity scale as follows: (% of shoot wilted, using a scale of 0-5 where 0=No symptoms, 1=one leaf wilted (1%-25%), 2=2 or 3 leaves wilted (26%-49%), 3=half plant wilted (50%-74%), 4=all leaves wilted (75%-100%), 5=Plant dead) (Tans-Kersten et al., 2001).

Biochemical characterization of isolates

The selected virulent isolates were also inoculated on nutrient agar plates and incubated at 28°C for 24 h for biochemical characteristics including Gram reaction, catalase test, oxidase test, motility and indole production test.

Isolation of antagonists from tomato rhizosphere soil

Isolation of the bacterial and fungal antagonists was carried out using soil dilution method according to Johnsen and Nielsen (1999). Ten grams of rhizosphere soil sample collected from healthy tomato plants was prepared to appropriate dilutions (10^{-1} to 10^{-5}) and 10^{-3} to 10^{-5} plated on to KB (King's B medium) for rhizobacteria and PDA for *Trichoderma* spp. (fungal antagonists). The Petri plates were incubated at 25°C for 7 days for fungal antagonists and at 28°C for two days for rhizobacteria.

In vitro antagonism test against the pathogen

The antagonism tests were carried out on the fungal and bacterial isolates against the bacterial wilt pathogen *in vitro* used disk diffusion method (Nguyen and Ranamukhaarachchi, 2010). The bacterial wilt pathogen was grown on nutrient broth for 48 h from which, 100 µml was swapped onto Petri plates with nutrient agar. And the bacterial antagonist grow on nutrient broth for 48 h and *Trichoderm*a were grown in Potato Dextrose Broth (PDB) (20 g/l dextrose, 4 g/l potato extract and 15 g/l agar) for 7 days and sterilized Paper disc (5 mm) was immersed in each test antagonist solution and was spotted at the center of the pathogen-inoculated-plate. Paper disc immersed in sterile distilled water and spotted at the center of the plates with the pathogen was used as control. Plates were incubated at 28°C for 48 h to measure inhibition zone of diameter.

Morphological characterization of antagonist fungi

Morphological characterizations of the fungal antagonists were performed by growing them on PDA at 25°C for 7 days. They were characterized by observing their cultural characteristics (colony color on the front and reverse side of the plate, growth rate, conidiophore branching, conidial shape and compared with the culture collection from Addis Ababa University (AAU).

Biochemical characterization of antagonist bacteria

The selected bacterial antagonistic isolates were characterized by the biochemical tests including Gram differentiation and Gram reaction, growth at 41°C, catalase test, oxidase test, pigment production, gelatin liquefaction, hydrogen cyanide production, ammonia production, phosphate solubilization, and carbohydrate fermentation test by using standard methods (Bhargavi and Tallapragada, 2016).

Compatibility test

In vitro compatibility test between the selected bacterial and fungus isolate was conducted using dual culture method in order to determine whether they can be used in combination. Thus, an overnight culture of the bacterium grown in King's B broth was streaked on one side of a Petri-dish containing NA of 2% sucrose.

Table 1. Variations in pathogenicity of *R. solanacearum* isolates on the host tomato variety (Gelilema).

Isolate	Infection (%)	Scale	Pathogenicity
AAURS1	75	4	Highly pathogenic
APPRCRS2	50	3	Moderately pathogenic
AAURS3	25	1	Weekly pathogenic
AAURS4	25	1	Weekly pathogenic

The other side of the Petri-dish was inoculated with 1 cm disc of 7 days old *Trichoderma* spp. The plates were then incubated at 25°C to test the presence of inhibition diameter between the two isolates.

Antagonistic test of the isolates against the test pathogen on tomato under greenhouse condition

Tomato seeds from local Gelelima and Galilea varieties that were obtained from Melkasa Agricultural Research Center were sown in seedling bed. After 25 days, the seedlings were transplanted in pots filled with potting mixture (soil: sand at 2:1 w/w/) at the rate of three seedlings per pot. Inoculum of the pathogen and the selected biocontrol agents; *Pseudomonas* and *Trichoderma* were prepared at 10^8 cfu ml^{-1} and conidial suspension of (10^8 spores ml^{-1}), respectively as described by Sivan et al. (1984). 50 ml of the mixed inoculum of the pathogen and antagonists were inoculated into the pots at the same time using soil drench method (Algam et al., 2010). Each treatment was replicated thrice in completed randomized design (CRD). The treatments were:

T1: *R. solanacearum* + *Trichoderma* (AAURS+AAURB20)
T2: *R. solanacearum* + *Pseudomonas* (AAURS+AAUTR23)
T3: *R. solanacearum* + *Trichoderma* spp. + *pseudomonas* (AAURS+AAURB20+AAUTR23)
T4: Inoculated control with *R. solanacearum* (diseased control) (AAURS); and
T5: Un-inoculated control (healthy control) (DW)

According to Song et al. (2004), wilt incidence was calculated using the following formula:

$$Wilt\ incidence\ (\%) = \frac{scale\ X\ number\ plants\ infected}{highest\ scale\ X\ total\ number\ of\ plants} X100$$

$$BE\ (\%) = \frac{DIC - Disease\ incidence\ of\ antagonist\ treated\ group}{DIC} X100$$

GPE (%) = {(Plant parameter of antagonist treated group - Plant parameter of control) / Plant parameter of control} ×100

where BE = Biocntrol efficacy, DIC = Disease incidence of control, GPE=growth promotion efficacy.

Plant growth was measured in terms of shoot height and shoot dry weight 2 months after sowing. For dry weight measurement, plants were dried in an oven at 70°C for 3 days to constant weights.

Data analysis

All statistical analysis was performed with SAS software. Measured variables were submitted to one-way ANOVA. Duncan's multiple range test was used for mean values discrimination at 5% level.

RESULTS AND DISCUSSION

Cultural and biochemical tests for identification of *R. solanacearum*

A total of fifteen bacterial isolates were collected from infected tomato plants with bacteria wilt, of which four isolates that showed the typical cultural characteristics of virulent *R. solanacearum* were selected for *in vivo* pathogenicity studies (Table 1). These isolates exhibited pink or light red colonies or red center with whitish margin. All of them were rod shaped, Gram negative, non-spore forming, motile, and catalase and oxidase positive and indole negative bacteria (data not shown). These results conformed to the characteristics of virulent strains of *R. solanacearum* on TZC medium after 24 h of incubation reported elsewhere (Kelman, 1954; Narasimha et al., 2013).

Pathogenicity tests

The result showed that bacterial wilt of tomato occurred within 15 to 21 days after inoculation. All isolates were pathogenic on tomato plants and produced typical symptoms of wilt. Isolate AAURS1 exhibited the highest disease incidence (75% wilting) followed by 50% of wilting with APPRCRS2, whereas isolates AAURS3 and AAURS4 induced weak infection on the host (Table 1). Other reports also showed 50 to 71% wilting on different tomato varieties (Abo-Elyousr and Asran, 2009). El-Ariqi et al. (2005) also reported that different isolates of *R. solanacearum* caused 52 to 97% of wilting.

Selim et al. (2011) have also reported that different isolates of *R. solanacearum* showed different wilt incidence ranging from 40 to 96%.

Isolation and screening of plant growth promoting antagonist

A total of twenty rhizobacterial and six fungal isolates were collected and preliminarily screened for their antagonistic property on the test pathogen. They were evaluated against two isolates of *R. solanacearum* using paper disc diffusion method under *in vitro* conditions.

Table 2. Antagonistic activity of antagonists against *R. solanacearum* under in vitro condition grown on NA medium and incubated at 28°C for 2 days.

Isolate	Group	Inhibition zone in mm (mean±SD)	
		AAURS1	APPARCRS2
AAURB1	Rhizobacteria	9.0±0.00^{cdef}	7.5±0.70^{d}
AAURB2	"	6.5±0.71^{fg}	7.0±1.41^{de}
AAURB3	"	9±0.00^{cdef}	10.0±0.00^{bcd}
AAURB4	"	7.5±2.12^{defg}	7.5±0.70^{d}
AAURB5	"	0	
AAURB6	"	9.5±1.41^{cdef}	8.00±1.41^{d}
AAURB7	"	8.5±0.71^{cdef}	10.5±0.70^{bcd}
AAURB8	"	10±0.00^{cdef}	10±0.00^{bcd}
AAURB9	"	7±1.41^{efg}	8.5±2.12^{cd}
AAURB10	"	0	
AAURB11	"	9.5±0.62^{cdef}	7.5±2.12^{d}
AAURB12	"	8±0.00^{cdef}	9.0±0.00^{bcd}
AAURB13	"	7.5±0.70^{defg}	9.0±1.41^{bcd}
AAURB14	"	4.5±0.71^{g}	7±0.00^{de}
AAURB15	"	0	2.5±0.71^{ef}
AAURB16	"	9.5±0.66^{cdef}	10±0.00^{bcd}
AAURB17	"	8±1.41^{cdef}	8±2.83^{d}
AAURB18	"	9.0±0.04^{dce}	7.5±0.71^{d}
AAURB19	"	11±0.30^{bc}	13±1.41^{abc}
AAURB20	"	15.±0.71^{a}	16±0.70^{a}
AAUTR21	Fungi	9.5±0.71^{cdef}	10±0.00^{bcd}
AAUTR22	"	9±0.00^{cdef}	10±1.41^{bcd}
AAUTR23	"	14±1.41^{ab}	13.5±0.70^{ab}
AAUTR24	"	10±1.41^{cde}	8±1.41^{d}
AAUTR25	"	10.5±0.70^{cd}	9.5±0.70^{bcd}
AAUTR26	"	10.5±0.70^{cd}	9.5±0.14^{bcd}

Data are presented as mean value ±standard division of three replicates. Values with different letters within each column indicate significant difference at $p < 0.05$

The data showed that the bacterial isolate, AAURB20 had the highest mean inhibition diameter of 15 and 16 mm followed by the fungus, AAUTR23, isolate with inhibition diameters of 14 mm against the two test pathogens AAURS1 and APPRCRS2, respectively (Table 2). This implies that the antagonists have potential to be used in the greenhouse for *in vivo* bio protection of tomato plant. The *in vitro* antagonistic activity of *P. fluorescens* was also reported by Alyie et al. (2008) where *P. fluorescens* isolates (PF20) had the greatest inhibition zone *in vitro* against *R. solanacearum* with the inhibition diameter of 14.15 mm and other two isolates (PR-3-I-x, PR-4-I-x) showed 3.2 and 3.5 mm, respectively. This suggests that the mode of action or the type of antibacterial metabolite production may vary among the isolates tested (Williams and Asher, 1996). The inhibitory activity of *P. fluorescens* against the pathogen in the study is in line with that of Henok et al. (2007), Aliye et al. (2008) and Yendyo and Pandey (2017) where they reported that isolates of *P. fluorescens* had significantly inhibited under the bacterial growth of *R. solanacearum* under *in vitro* conditions.

The *in vitro* antagonistic activity of *T. asperellum* was reported by Narasimha et al. (2013) that inhibit the growth of *R. solanacearum* with inhibition zone ranging from 11 to 27 mm diameter.

Morphological and biochemical characterization *P. fluorescens*

Based on the antagonistic potential characteristics, twelve isolates of *P. fluorescens* were studied in detail for colony, colour, growth type, cell shape, and fluorescens of the isolates. All the isolates showed similar results with regard to round yellow colony texture on King's B

Table 3. Carbohydrate fermentation test results of different indigenous bio-control agents.

Isolate	Fructose	Glucose	Lactose	Maltose
AAURB 1	+	+	±	+
AAURB 3	+	+	+	+
AAURB6	+	+	+	+
AAURB 7	+	+	+	+
AAURB 8	+	+	+	+
AAURB 11	±	+	±	±
AAURB 12	±	+	+	+
AAURB16	±	+	±	±
AAURB 17	+	+	+	+
AAURB 18	±	+	±	±
AAURB19	+	+	+	+
AAURB 20	+	+	+	+

+=Positive,-=negative± intermediate reaction.

medium with production of fluorescent pigment gelatin liquefaction positive, catalase, oxidase, Gram stain negative, positive KOH and lack of growth at 41°C. This, together with rod shape cell morphology and fast growth further confirmed the isolates to be *P. fluorescens* as reported by earlier workers (Meera and Balabaskar, 2012).

Carbohydrate fermentation test for bacterial isolates

The isolates utilized the tested carbohydrates and produced yellow color on the medium, which was an indication of the utilization of each carbohydrate. All isolates were capable of utilizing glucose followed by maltose, fructose and lactose (Table 3). The utilization of different carbohydrate sources by the isolates was similar with *P. fluorescens* reported by Henok et al. (2007).

Morphological characterization of fungi

The fungal isolates were characterized by fast growth with dark green mycelia colony on PDA. Microscopic study revealed that it produced globes to ellipsoidal conidial shape, which was much branched.

Plant growth promoting rhizobacteria (PGPR) characterization of rhizobacteria

Among isolates that were screened for their plant growth promoting activities viz., HCN production, ammonia production, and phosphate solubilization. Isolate AAURB20 and AAURB19 exhibited strong HCN production followed by isolates AAURB8 and AAURB16 (Table 4). Among test isolates, AAURB 7 and AAURB 20 displayed three PGP characters; whereas most of the isolates exhibited only one of the PGP characters (Table 5). The strains of *P. fluorescence* isolated from rice fields are found to produce HCN against *S. oryzae* (Meera and Balabaskar, 2012).

Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia. In this study, isolate AAURB7 and AAURB20 produced ammonia. Another study showed that 95% of the isolates from the rhizosphere of rice crops produced ammonia (Joseph et al., 2007).

Phosphorous is a major essential macronutrient for biological growth and development. With regard to solubilization of inorganic phosphate four isolates (33%) (AAURB7, AAURB16, AAURB19, and AAURB20) were able to solubilize phosphate in the plate-based assay, by showing a clear halo zone around the colony. Several species of *Pseudomonas* such as *P. fluorescens*, *P. aeruginosa* and *Bacillus* species have been reported as good phosphate solubilizers in agricultural soils (Jha et al., 2013).

Compatibility test

The compatibility test between the selected isolate, AAURB20 and selected fungal isolate AAUTR23 indicated that, the colonies of the fungus and the bacterium met on the 7th day without showing inhibitory activity with one another. This observation was the basis for testing a combination of the two antagonists as “mixed culture” in the greenhouse trial. Similarly, under *in vitro* compatibility between *T. viride* and *P. fluorescens* was reported by Ephrem et al. (2011) with no inhibition between them.

Table 4. Morphological characterization of fungi.

Isolate characters	AAUTR21	AAUTR22	AAUTr23	AAUTR24	AAUTR25	AAUTR26
Colony growth rate (cm)	8-9 cm in 6 days	8-9 cm in 6 days	8-9 cm in 3 days	8-9 cm in 5 days	8-9 cm in 4 days	8-9 cm in 4 days
Colony colour	Green	Green	Dark green	Dark green	Dark green	Dark green
Reverse colony colour	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
Conidiospore	Branched	Branched	Branched	Branched	Branched	Branched
Conidial shape	Globes to ellipsoidal	Globes to ellipsoidal	Globes to ellipsoidal	Ellipsoidal	Globes to ellipsoidal	Globes to ellipsoidal

Table 5. Characterization of rhizobacteria for their PGPR characters.

Isolate	HCN production	NH_3 production	Inhibition zone(mm)	Phosphate solubilization	Multiple PGP characters
AAURB 1	+	+	9	-	2
AAURB 3	+	+	9	-	2
AAURB6	+	-	9.5	-	1
AAURB 7	+	+++	8.5	+	3
AAURB 8	++	-	10		1
AAURB 11	+	-	9.5	-	1
AAURB 12	+	-	8	-	1
AAURB16	++	+	9.5	+	3
AAURB 17	-	+	8	-	1
AAURB 18	-	+	9	-	1
AAURB19	+++	++	11	+	3
AAURB 20	+++	+++	15	++	3

+: Low production; ++: medium production; +++: strong production; -: no production

Effects of isolates on disease incidence and biocontrol efficacy

The biocontrol efficacy and antagonistic effect of the treatments on disease incidence was highly significant ($p \leq 0.05$) when compared with the control treatments. The highest disease incidence of 80 and 60% was recorded from the control (Pathogen infection only) on Galilea and Gelelima varieties, respectively. All treatments reduced disease incidence ranging from 13 to 35% and biocontrol efficacy of 48 to 72% (Table 6). Similar results were also reported by Selim et al. (2011) plants treated with PGPR isolates significantly disease reduced ranging from 15 to 57% compared to infected control, as well as greater amount of biomass compared to the control.

The combined treatments exhibited the lowest value (13.33%) of disease incidence as well as the highest value (72.22%) of biocontrol efficacy against *R. solanacearum*, on Gelelima variety. While isolate AAURB20 exhibited the highest (31.11%) disease incidence and lowest value (61.11%) of biocontrol efficacy on Galilea variety, and 35 and 48% on Gelelima variety, respectively (Table 6). The results could be attributed to the synergistic effect between the combinations of the two microorganisms in this treatment. These results were in harmony with those reported by Yendyo and Pandey (2017) that *Trichoderma* spp. and *P. fluorescence* seem to be more effective than treatment using each individual biocontrol agent that has been achieved 97% of

Table 6. Effect of AAURB20, AAUTR23, and their combination (AAURB20+AAUTR23) on disease incidence.

Treatment	Disease incidence (%)		Biocontrol efficacy (%)	
	Galilea variety	Gelelima variety	Galilea variety	Gelelima variety
AAURs1+AAURB20	31.11^{c}	35.56^{c}	61.11^{b}	48.1^{c}
AAURs1+AAUTR23	26.67^{cd}	22.22^{d}	66.67^{ab}	63.0^{b}
AAURs1+AAURB20+AAUTR23	22.22^{d}	13.33^{de}	72.22^{a}	70.37^{a}
AAURs1(control)	80^{a}	60.00^{b}	-	-

Data are presented as mean value of three replicates. Values with different letters within each column indicate significant difference at $p < 0.05$.

Table 7. Effect of plant growth promotion of antagonists on tomato.

Treatment (pathogens+bioagents+variety)	Plant height(cm)		Plant dry weight(g)	
	Mean	GPE (%)	Mean	GPE (%)
AAURs1+AAURB20+V1	54±2.65^{bcd}	26.5	9.46±0.73^{abc}	52.21
AAURs1+AAUTR23+V1	54±2.65^{bcd}	40.35	9.54±0.65^{abc}	51
AAURs1+AAURB20+AAUTR23+V1	67±3.81^{a}	55.4	11.25±1.23^{ab}	47.6
AAURs1+V1	43±3.61^{c}	-	6.27±1.20^{c}	-
Distil water+V1	55.67±3.21^{abcd}	-	10.22±3.25^{ab}	-
AAURs+AAURB20+V2	58±3.46^{ab}	30.36	10.79±1.24^{ab}	42.4
AAURs+AAUTR23+V2	64.67±4.16^{abc}	45.27	12.18±1.82^{ab}	66.2
AAURS1+AAURB20+AAUTR23+V2	72.33±3.23^{a}	61.66	12.73±0.48^{a}	81.5
AAURs1+V2	44.67±2.31^{cd}	-	7.81±1.42^{bcd}	-
Distil water+V2	57.33±4.13^{bcd}	-	11.65±2.61^{ab}	-

Data are presented as mean value ± standard division of three replicates, and each replicate contains three plants. Values with different letters within each column indicate significant difference at $p < 0.05$. GPE= Plant promotion efficacy; V1=Galilea variety; V2=Gelelima variety.

biocontrol efficacy.

The dual application of *T. viride* and *B. subtilis* decreased the percentage of pathogen infection and increased survival rate than single inoculation in tomato (Morsy et al., 2009). Another study showed that the number of wilted chickpea infected with *Fusarium oxysporium* plants was reduced by 67.93% due to inoculation/suppression by *T. harzianum* (Subhani et al., 2013). The highest percentage of disease incidence was found on galilee variety, which may be due to variety resistance. These results were in harmony with those reported by Chatterjee et al. (1997) which stated that differences of wilt incidence and severity were due to diversity of host plants, the virulence of the pathogen, and other environmental factors.

Plant growth promotion efficacy of antagonists in greenhouse condition

Results of this experiment showed that antagonists (bioagents) stimulated plant growth promotion under greenhouse conditions and indicated that tomato plants treated with rhizobacteria and *Trichoderma* strains significantly grew better than control biomass increase of tomato plants treated with rhizobacteria and *Trichoderma* strains are shown in Table 7.

Significant differences ($P \leq 0.05$) among treatments regarding plant height and biomass were observed. Plants treated with combined isolates of AAURB20+AAUTR23 showed the highest values of plant height, and dry weight (72.33 cm and 12.73 g), respectively, when compared with the control (AAURS1) and plants treated by individual isolates AAURB20 and AAUTR23 in variety two (Table 7). Likewise plants treated with isolates AAURB20+AAUTR23 showed high GPE (%) (62% and 81.5%) for height and dry weight, respectively in variety two (Table 7).

Significant differences ($P \leq 0.05$) among treatments regarding plant height and biomass were also noted on variety one (Table 7). Plants treated with combined isolates of AAURB20+AAUTR23 presented the highest values of plant height and dry weight (67 cm and 11.25 g), respectively, when compared with the control

(AAURS1) and plants treated by individual isolates AAURB20 and AAUTR23 in variety one (Table 7). Generally, combined treatments showed the best performance compared to individual treatments. Significant differences were observed in the vegetative growth parameters due to the inoculation of isolated bio-inoculants. This result was in harmony with that of Nguyen and Ranamukhaarachchi (2010) on tomato. The use of beneficial microorganisms as biocontrol agents led to enhance plant growth parameters (70.4 cm plant height and 19.5 g of dry weight). Such enhancement may be due to induce plant resistance (De Meyer et al., 1998), production of extracellular enzymes and antifungal or antibiotics, which reduce the negative effect of biotic stress on plant and produce growth promoting substances (Szczech and Shoda, 2004). Similar results also reported by Selim et al. (2011) plants treated with PGPR isolates significantly reduced disease compared to infected control, as well as caused greater amount of biomass compared to the control.

Conclusion

A total of twenty rhizobacterial and six fungal isolates were preliminarily screened for their antagonistic property on the test pathogen using paper disc diffusion method under *in vitro* conditions. Isolate, AAURB20 gave the highest mean inhibition diameter of 15 and 16 mm followed by the fungus, *AAUTR23,* isolate with inhibition diameters of 14 mm against the two test pathogens AAURS1and APPRCRS2, respectively.

Based on the *in vitro* inhibition test and PGB properties, one isolate of *Pseudomonas* spp. and one isolates of *Trichoderma* had shown better inhibitory effects on the test pathogen strains selected for green house study. Combined treatments of *Trichoderma* and *Pseudomonas* spp. (AAURB20+AAUTR23) showed best performance compared to individual treatments, although individual isolates reduce bacterial wilt incidence and increase biomass of tomato significantly compared to the pathogen inoculated control. Therefore, the use of this bioagent would be important to manage bacterial wilt at greenhouse conditions. However, further study will be required to use the bio-agent in the field condition for bio-control development program.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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