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Full Length Research Paper

Genetic diversity of common bean (*Phaseolus vulgaris* L.) landraces from South Western Kenya for resistance to *Pythium* root rot disease

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Common bean landraces represent an important source of genetic resources, which is under-exploited by Kenyan crop breeding programs. The objective of this study was to characterize 51 common bean landraces from South Western Kenya for resistance to *Pythium* root rot using 5 peroxidase gene (POX) markers. Following infection with *Pythium* spp., 11.77, 54.90 and 33.33% of the landraces were moderate-resistant, susceptible and highly susceptible, respectively. A total of 1119 alleles were amplified by the 5 primers, ranging from 3 to 8 alleles per locus. The polymorphism information content (PIC) of the POX markers varied from 0.10 to 0.47. The dendrogram generated using similarity coefficients and un-weighted pair group method with arithmetic averages (UPGMA) did not reveal any unique groupings according to their reaction to *Pythium* root rot disease. Population structure analysis separated the germplasm into 3 groups and all the groups contained landraces exhibiting both moderate resistance and susceptible to *Pythium* root rot. The lack of distinct grouping of landraces based on resistance suggests presence of significant genetic variability and different sources of resistance to *Pythium* root rot. These findings give valuable information for breeders and serve as a baseline for development of cultivars with *Pythium* root rot resistance.

Key words: genetic variability, population structure, peroxidase gene markers, *Pythium* root rot, resistance.

INTRODUCTION

Common beans (*Phaseolus vulgaris* L.), are the most important grain legume, second to maize as a food crop in Kenya. Africa contributes 17% of the world's total yield, of which 70% is from Eastern Africa. Kenya produces 400 - 1200 kg/ha, mainly from intercropping in small scale farms and an average household production of 430 kg/ha in South Western Kenya (FAOSTAT, 2013). Common beans provide a cheap source of protein and minerals (iron and zinc) to humans. In addition, consumption of bean grains provides humans with various health benefits including reduction of cholesterol level and coronary heart diseases (Mattei et al., 2011), favorable effects against cancer (Hangen and Bennink, 2002) and decreases diabetes and obesity (Ahn et al., 2013). Furthermore, common beans play a very important role in sustaining soil fertility by fixation of atmospheric nitrogen and organic

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matter to the soil. It is a dual-purpose crop producing grains as well as fodder for livestock. As a cover crop, it is efficient in suppressing weeds and prevents soil erosion.

Production of common bean in South western (SW) Kenya is constrained by various biotic stresses including insect pests especially pod-borers and weevils which may cause yield loss of up to 80%. There is also lack of cultivars with consumer quality attributes such as taste, palatability and fast cooking. However, the greatest limitation to bean production in SW Kenya are fungal diseases particularly root rots caused by *Fusarium*, *Rhizoctonia* and *Pythium* (Otsyula et al., 2003). *Pythium* root rot disease can cause yield losses of up to 100% in susceptible varieties. Farmers have abandoned these susceptible cultivars even though they produce high yields. This has led to gradual genetic erosion of once elite landraces. The use of resistant bean varieties is the most effective, economical and environmentally sustainable strategy to control *Pythium* root rot disease (Binagwa et al., 2016). However, this requires identification of resistant genotypes, and incorporation of the disease resistance into agronomically desirable varieties. There is need to characterize common bean germplasm for *Pythium* root rot resistance in order to identify markers for selection of resistant varieties. The markers can be used for discarding susceptible germplasm before tolerant varieties are screened in the glasshouse or field and will fast track the development of high yielding *Pythium* root rot-resistant cultivars for farmers.

Genetic differences that exist between common bean accessions can be associated with economically important traits and used for germplasm characterization. Molecular markers are useful tools in estimation of genetic diversity and identification of alleles of interest without interference from changes in environmental parameters. Gene-based molecular markers represent an important resource for characterization of germplasm and elucidation of gene functions. Peroxidase, a key enzyme in metabolic pathways is an example of gene-based molecular marker in plants. Peroxidases (POXs) belong to a multigene family and exhibit high sequence variability with the existence of conserved domains (Oliva et al., 2009). Conserved DNA regions of peroxidase share the same priming site and are distributed across the genomes of different genotypes in different patterns hence polymorphisms can be detected within species. POXs are glycoprotein enzymes containing heme cofactor and utilize H_2O_2 in oxidation reactions involving a range of compounds. These enzymes perform diverse roles in plants including detoxification of reactive oxygen species generated during biotic and abiotic stresses (Mittler et al., 2004; Gill and Tuteja, 2010; Zhang et al., 2014), inducing defense response against pathogens (Passardi et al., 2005), formation of lignin and suberin (Herrero et al., 2013), metabolism of auxin, healing of wounds and plant–microbial symbiosis (Passardi et al., 2005). Peroxidases also catalyzes deamination of trans-cinnamic acid in a biosynthesis pathway leading to the formation of phenolic compounds which have many vital activities in plants such as regulation of plant growth differentiation (Vicuna, 2005), inhibition of pathogens (Almagro et al., 2008), and tolerance to abiotic stresses (Gill and Tuteja, 2010; Zhang et al., 2014).

The objective of this study was to characterize common bean landraces from South Western Kenya using peroxidase gene markers for resistance to *Pythium* root rot disease. This information would be useful in understanding the genetic relationship between different landraces and selection of *Pythium* root rot-resistant parents for use in present and future breeding programs.

MATERIALS AND METHODS

Plant materials

Fifty one common bean landraces were evaluated in the greenhouse for resistance to *Pythium* root rot disease. These comprised of 25 landraces collected from farmers' fields in different agro-climatic zones of south western Kenya and 26 germplasm obtained from the National Gene Bank of Kenya (GBK), Muguga, Kenya. The germplasm from GBK were collected also from South Western Kenya region in 1983 - 1984 and preserved.

Preparation and inoculation of *Pythium* spp. pathogen

Pythium spp. was isolated from symptomatic bean plants collected from farmers' fields in southwestern Kenya. The bean plants were uprooted washed with running water and stem bases cut off, surface sterilized with 1.5% solution of sodium hypochlorite for 30 s and then rinsed three times with sterile distilled water and blot dried. The cut stem tissues were plated on Potato Dextrose Agar (PDA) media supplemented with 50 ppm streptomycin and incubated for 7 days at 24°C. The *Pythium* spp. was identified based on morphological and cultural features and confirmed using fungal identification keys as described by Watanabe (2010).

To prepare *Pythium* inocula, three day old, actively growing hyphal regions measuring 4 mm^2 were aseptically cut and grown on autoclaved millet seeds. The culture of *Pythium* spp. was then mixed with pre-sterilized loam soil in a ratio of 1:8 v/v in wooden flats measuring 48 cm × 72 cm and the inoculum allowed to establish for a period of 14 days in the dark. Two rows of bean seeds (10 seeds per landrace) were planted in the wooden flats and each treatment replicated thrice. The control experiment contained seeds sown in sterilized loam soil without *Pythium* inoculum. Replications were set using the standard randomized complete block design (RCBD). The experiment was repeated twice.

Pythium root rot disease assessment in the glasshouse

After germination, bean plants were watered after every three days to ensure optimum growth conditions. Thirty six days after planting, seedlings were uprooted and washed with tap water to remove soil from the roots and the plants were individually assessed for disease severity using the CIAT nine-point severity scale (Otsyula et al., 2003). Data on disease severity were subjected to analysis of variance (ANOVA) using the GenStat 11th Edition (VSN International Ltd., 2008) software.

Table 1. PCR markers used for amplification of peroxidase gene (POX) in common bean.

Primer	Sequence (5' - 3')		Fragment size (bp)
	Forward	Reverse	
PM55	TTGTAGATTCTCGCTCGGAA	CTTGGCATAATTGTTATTTGGT	150 - 800
POX1	CTCGACCTACAAGGAC	ATGTAGGCGCTGGTGA	100 - 800
POX8b	CACCATCAAGAGCGTCATAAC	TTGCTAGAGCGAGCTGG	100 - 200
POX11	CCTTCTTCTTGCCATCTTGC	CATATCGCTCCACGACCTTT	150 - 750
POX12b	CTCTCTCCTGGGGGTTCTATGC	GCGAGCGTGGTGATGTC	100 - 750

Genomic DNA extraction

Plant genomic DNA was isolated using cetyltrimethyl ammonium bromide (CTAB) method as described by Gyang et al., (2017). Approximately 200 mg of young leaf samples were weighed and crushed in pre-warmed extraction buffer consisting of 700 µl CTAB buffer plus 150 µl 20% sodium dodecyl sulfate (SDS) to form a homogenous paste. The homogenate was transferred into 1.5 ml centrifuge tubes and incubated in a water-bath at 55°C for 20 min. The tubes were gently inverted five times after every five minutes during the incubation period to ensure uniform distribution of the homogenized tissues in the buffer. After incubation, the samples were spun in a micro-centrifuge for 10 min at 13,800 rpm. The debris was discarded and the supernatant transferred to a new 1.5 ml centrifuge tube preceding addition of equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The contents in the centrifuge tubes were mixed 50 times by inversion to allow proper mixing before spinning for 7 min at 13,800 rpm. The top layer was transferred to new 1.5 ml centrifuge tubes followed by addition of 50 µl of 7.4 M ammonium acetate and 2 volumes of ice cold absolute ethanol. The tubes were incubated at -20°C for 1 h and then centrifuged for 10 min at 10,000 rpm to pellet the precipitated nucleic acids. The supernatant was discarded and 500 µl of a wash solution (75% ethanol and 15 mM ammonium acetate) was added to wash the pellet. The washing step was repeated twice. After every wash, the centrifuge tube was spun for 5 min at 12,000 rpm and the supernatant discarded. The DNA pellet was air dried for 10 min in the fume hood and dissolved in 70 µl TE buffer. Ribonuclease A (3 µl of 10 mg/ml) was added to the dissolved nucleic acids and incubated in a water bath at 37°C for 30 min. The dissolved DNA was stored at -20°C for subsequent molecular analysis.

Peroxidase-gene-based markers and polymerase chain reactions

Primers (Table 1) designed from peroxidase cDNA sequences of *Arabidopsis* and rice (Welinder et al., 2002; Gulsen et al., 2007; Nemli et al., 2014) were used to detect polymorphism in common bean accessions. Polymerase chain reaction (PCR) reactions were done in a total volume of 20 µl, made up of 5 µl 1× GoTaq Mix (Promega Corporation, Madison, USA), 1 µl of each of the forward and reverse POX markers (10 µM), 1 µl genomic DNA (20 ng), 12 µl nuclease-free water. Amplifications were done in an MJ Mini^{TM} Thermal Cycler machine (Bio-Rad, Singapore) as follows: initial denaturation at 94°C for 5 mins, for 30 s, followed by denaturation (30 s) at 94°C, annealing at 46 - 56°C (45 s), extension (1 min) at 72°C, then a final extension at 4°C for 7 mins. PCR reaction for each POX primer was done at least twice using DNA extracted from different plants of the same landrace and only clear bands that can be reproduced were used during analysis of data.

Gel electrophoresis

The products of the PCR reactions were ran on a 2% agarose gel for 70 mins at 65 Volts. Amplified DNA bands were photographed using a UV transilluminator and scored for further analysis. The fragment sizes were evaluated base on how they moved through the agarose gel in comparison to a 100-bp DNA ladder (Bioneer, South Africa).

Scoring of alleles and data analysis

Each band was scored as present (1) and absent (0) to generate binary matrix for the 5 POX markers. This POX data was used for analysis of genetic diversity and population structure. The polymorphic information content (PIC) was determined for each peroxidase gene locus following the equation $PIC = 1 - \sum (pi)^2$ which was described by Botstein et al., (1980) (where *pi* is the population carrying the *i*th allele). The similarity matrix generated using Nei's genetic distance (Nei and Li, 1979) was used to construct a dendrogram using the unweighted pair-group method arithmetic mean (UPGMA) by the use of MVSP 3.1 program.

Population genetic structure analysis was done with a clustering approach of a Bayesian model-base, clustering approach in the STRUCTURE version 2.3.4 program (Pritchard et al., 2000). An analysis of all 51 landraces was done using the number of clusters (*K*) ranging from 1-10, and a burn-in period of 5,000 iterations with 50,000 replications of Markov Chain Monte Carlo (MCMC). Results were not significantly affected with longer burn-in periods. The runs showing the maximum posterior probability for each *K* value was used. The *ad hoc* statistic Δ*K* was used to estimate the total sub-populations, and to determine *K* (Evanno et al., 2005). Principal component analysis (PCA) was carried out depending on the variation patterns of the POX gene, and a two dimensional representation of relationships across the 51 common bean landraces using XLSTAT program was generated. Analysis of molecular variance (AMOVA) within and among populations was done with the GenAlEx (v6.5) software (Peakall and Smouse, 2012).

RESULTS

Phenotyping of *Pythium* root rot disease

Analysis of variance showed highly significant ($p < 0.001$) differences in disease severity among the 51 landraces. Average disease severity of all the landraces ranged from 2.1 to 7.8. Based on the disease severity scores against *Pythium* root rot in the glasshouse, all the 51 common bean landraces were classified in such a way that six

(11.77%), twenty-eight (54.90%) and seventeen (33.33%) were moderate-resistant, susceptible and highly susceptible, respectively (Table 2). The landraces with the lowest *Pythium* root rot disease severity were LRC008, LRC014, LRC016, LRC018, LRC019 and GK030257. None of the 51 landraces was found to be resistant to *Pythium* root rot disease.

Polymorphism detected using POX markers

Five POX primers were used to characterize 51 common bean landraces based on the amplification of clear banding patterns. The number of bands generated among the 51 common bean landraces using these 5 primers was 1119, which ranged from 3 (POX8b and POX55m) to 8 (POX12b), with an average of 4.8 bands/primer. A sample amplification pattern of the primer POX1 is shown in Figure 1. Out of the fragments scored, 81% were polymorphic. The percentage polymorphic loci varied from 66.6% (POX 11 and P55m) to 100% (POX 8b) with an average of 80.6% bands /primer (Table 3). Primer POX8b gave 100% polymorphism, indicating the capability of POX primers to detect high levels of polymorphism among common bean (Table 3). The PIC value ranged from 0.10 (POX11) to 0.47 (POX8b).

Genetic relationships among the common bean landraces

A pair-wise comparison among the landraces were used to calculate the genetic similarity coefficient based on the proportion of shared bands. The genetic similarity among the 51 landraces ranged from 0.44 to 1.0, with an average of 0.72. The lowest value of genetic similarity (44%) was observed between S8 (highly susceptible) and S28 (susceptible) common bean landraces. Also relatively low values of genetic similarity was observed between the landraces S4 and S8 (53.8%), S13 and S8 (52%), S24 and S8, (46.2), S27 and S8 (45.8) and; S28 and S8 (45.8%). The maximum genetic similarity (100%) was observed between landraces S7 (highly susceptible) and S19 (susceptible). The genetic similarity matrices showed that the 6 landraces that were moderate resistant were not genetically similar.

Phylogenetic analysis

Based on the genetic similarity matrix, a dendrogram was constructed wherein 51 common bean landraces were separated into two major Clusters 1 and 2 (Figure 2). Cluster 1 was the largest and is divided into 2 sub-clusters (namely 1A and B) which in total contained 42 bean landraces of which 4 (S3, S27, S38 and S45) were moderate resistant. Sub-cluster 1Ais the smallest and contains only one (S27) moderate resistant landrace. Sub-cluster 1B was divided into four groups namely I – IV. Groups I and III contained 2 (S3 and S45) and 1 (S38) moderate resistant landraces, respectively, while in Groups II and IV none of the landraces were found to be moderate resistant. Cluster 2 contained 9 landraces of which two (S2 and S20) were moderate resistant. Generally, the landraces form south Western Kenya did not form specific clusters or groups

Population structure analysis

The 51 common bean landraces were analyzed for population structure using Bayesian base method without any prior classification to know the highest populations (K). The peak plateau of adhoc measure ΔK was found to be K = 3 (Figure 3), which indicated that the entire 51 landraces were distributed into three groups (POP1, POP2 and POP3) (Figure 4). The POP2 was the smallest group consisting 12 landraces (23.53%) of which 2, 14 and 6 were found to be moderately resistant, susceptible and highly susceptible, respectively. The POP3 included 17 (33.33%) landraces of which 2, 8 and 7 were found to be resistant, susceptible and highly susceptible, respectively. On the other hand, POP1 was the largest group comprising of 22 (43.14%) landraces of which 2, 6 and 14 were resistant, susceptible and highly susceptible, respectively. There were equal numbers of resistant genotypes (2) in all the three groups. The structure analysis did not differentiate resistant and susceptible landraces into separate groups.

Estimation of population genetics through AMOVA analysis

Analysis of molecular variance was calculated to estimate the partitioning of genetic variance among and within populations. Within population variance explained 100% and no variance (0%) was observed among population (Table 4). All the diversity of common bean landraces from south western Kenya resided within the populations.

DISCUSSION

The presence of genetic variability in germplasm is a pre-requisite for efficient utilization of available genetic resources for breeding programmes. Understanding genetic differences for *Pythium* root rot resistance is useful for exploiting landraces as breeding parents. Gene based molecular markers in legumes have been used to identify genotypes, study genetic diversity and determining the phylogenetic relationships (Nemli et al., 2014). This study determined the genetic variations and

Table 2. General mean score response of 51 common bean landraces from SW Kenya to *Pythium* root rot.

Entry	Genotype Code	Local name	Source of genotype	Disease severity score	Disease rating
S1	LRC 006	Esaitoti	Daraja mbili	6.2^{cdefgh}	HS
S2	LRC 008	Cinchae	Kisii Municipality	2.1^{p}	MR
S3	LRC 018	Richore	Marani	3.1^{no}	MR
S4	GK030171	NNP	Gene bank	5.4^{hijkl}	S
S5	GK030217	NNP	Gene bank	5.8^{defgh}	S
S6	GK030178	NNP	Gene bank	5.4^{hijkl}	S
S7	GK030185	NNP	Gene bank	6.5^{bcd}	HS
S8	GK036526	NNP	Gene bank	7.9^{a}	HS
S9	GK030261	NNP	Gene bank	5.2^{hijkl}	S
S10	GK030200	NNP	Gene bank	5.2^{hijkl}	S
S11	GK030204	NNP	Gene bank	4.8^{jkl}	S
S12	GK030210	NNP	Gene bank	4.3^{lm}	S
S13	GK030246	NNP	Gene bank	4.2^{lm}	S
S14	GK036524	NNP	Gene bank	5.6^{fghi}	S
S15	GK030211	NNP	Gene bank	4.8jkl	S
S16	GK030249	NNP	Gene bank	6.3^{cdef}	HS
S17	LRC 001	Ekenagwa	Kisii Municipality	6.3^{cdef}	HS
S18	LRC005	Egirini	Kisii Municipality	7.6^{a}	HS
S19	LRC010	Bunda entambe	Daraja mbili	5.4^{hijkl}	S
S20	LRC016	Manoa emwamu	Kisii Municipality	2.4^{op}	MR
S21	LRC015	Ekoko enyenge	Suneka	5.8^{defgh}	S
S22	LRC026	Nyaibu/Bunda enetu	Daraja mbili	4.7^{kl}	S
S23	LRC011	Ekebure	Daraja mbili	6.6^{bc}	HS
S24	LRC012	Enyamatobu	Kisii Municipality	7.2^{ab}	HS
S25	LRC021	Morogi	Nyacheki	5.8^{defgh}	S
S26	LRC024	Ekoko entambe	Keumbu	5.7e^{fgh}	S
S27	LRC019	Manoa endabu	Kisii Municipality	2.5^{op}	MR
S28	LRC022	Enyamwam**u**	Daraja mbili	5.3^{hijkl}	S
S29	GK030194	NNP	Gene bank	4.2^{lm}	S
S30	GK030227	NNP	Gene bank	6.2^{cdefgh}	HS
S31	GK030239	NNP	Gene bank	5.7e^{fgh}	S
S32	GK036530	NNP	Gene bank	7.8^{a}	HS
S33	LRC 023	Eosama	Nyamarambe	6.5^{bcd}	HS
S34	LRC 009	Eroyoo	Kenyenya	4.9^{ijkl}	S
S35	LRC025	Amaika inse	Kisii Municipality	4.4^{l}	S
S36	LRC020	Ritinge	Daraja mbili	4.8^{jkl}	S
S37	LRC 007	Eamini	Nyamache	5.3^{hijkl}	S
S38	LRC 014	Esaire	Daraja mbili	3.6^{mn}	MR
S39	LRC 017	Masaku	Marani	4.2^{lm}	S
S40	GK 030244	NNP	Gene bank	5.5^{ghij}	S
S41	GK 036527	NNP	Gene bank	5.4^{hijkl}	S
S42	LRC 004	Emwetemania	Masimba	4.8^{jkl}	S
S43	LRC 013	Onyoro	Daraja mbili	4.7^{kl}	S
S44	GK036523	NNP	Gene bank	6.6^{bc}	HS
S45	GK030257	NNP	Gene bank	3.3^{n}	MR
S46	GK036522	NNP	Gene bank	6.3^{cdef}	HS
S47	GK030260	NNP	Gene bank	4.2^{lm}	S
S48	GK030198	NNP	Gene bank	7.4^{a}	HS
S49	GK030259	NNP	Gene bank	6.4^{cde}	HS

Table 2. Contd.

S50	GK030167	NNP	Gene bank	6.6[bc]	HS
S51	LRC003	Enchano	Daraja mbili	7.7[a]	HS

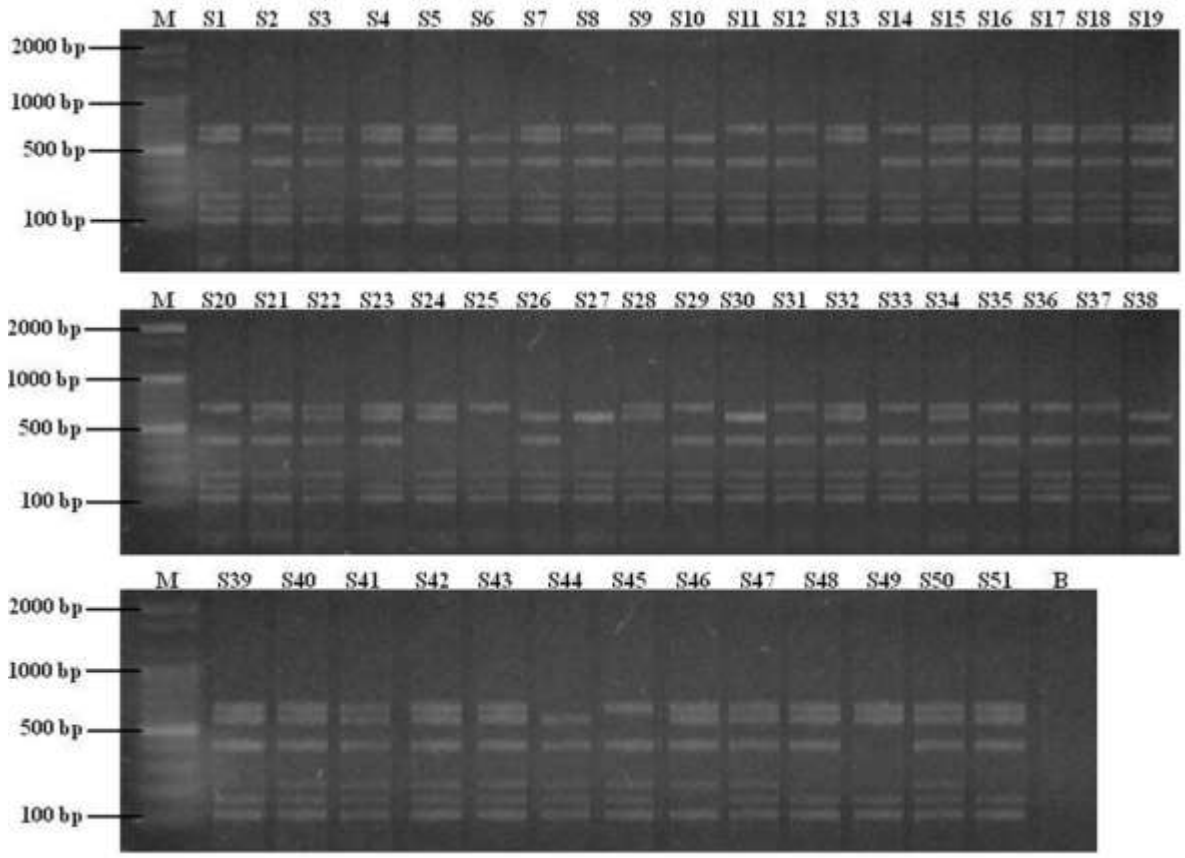

Figure 1. Electrophoretic POX marker profile of 51 common bean landraces generated by primer POX1. Lanes S1 – S51 represent the entries of the landraces (Table 2); M- Molecular marker and Lane B is sterile distilled water.

population structure in common bean landraces from South Western Kenya using peroxidase-gene markers. Plant peroxidase genes serve important roles in providing plants resistance to various biotic stresses resistance (Bela et al., 2015; Mir et al., 2015; Passardi et al., 2005), and peroxidase marker patterns might be utilized in defining relationships among plant genotypes in relation to their adaptive conditions.

In the current study, 51 common bean landraces from South Western Kenya were characterized using five POX loci for resistance to *Pythium* root rot. The POX markers were previously made using conserved motifs of rice and *Arabidopsis* peroxidase by Gulsen et al. (2007). The total amplified alleles on each locus varied from 3 - 8, with an average score of 4.8. This compares favorably with Nemli et al. (2014) who reported 1 - 8 alleles on each locus and had an average value of 4.0 when characterizing common bean genotypes from Turkey using POX markers. All the POX markers used in this study were polymorphic. However, the markers had a low average PIC value of 0.28, implying that the landraces used in the present study were closely related. The mean value of PIC observed in this study was lower than that reported by Nemli et al. (2014) and Wittayawannakul et al., (2010), who estimated variation among common bean genotypes and Garcinia species, respectively, using POX polymorphisms and got mean PIC values of 0.40 and 0.79, respectively.

The mean genetic similarity coefficients ranged from 0.44 to 1.0, with an average of 0.72, which indicates high genetic diversity among the 51 landraces. The high genetic diversity in these landraces is a valuable resource for broadening the genetic base in common bean breeding programs. The genetic differences among

Table 3. Peroxidase-gene based markers, numbers of total and polymorphic fragments and PIC values obtained from 51 common bean landraces.

POX marker	Total fragments	Polymorphic fragments	Polymorphism (%)	PIC
POX1	6	5	83.33	0.233481
POX8b	3	3	100	0.473003
POX11	6	4	66.67	0.103735
POX12b	8	7	87.5	0.339913
P55m	3	2	66.67	0.275888

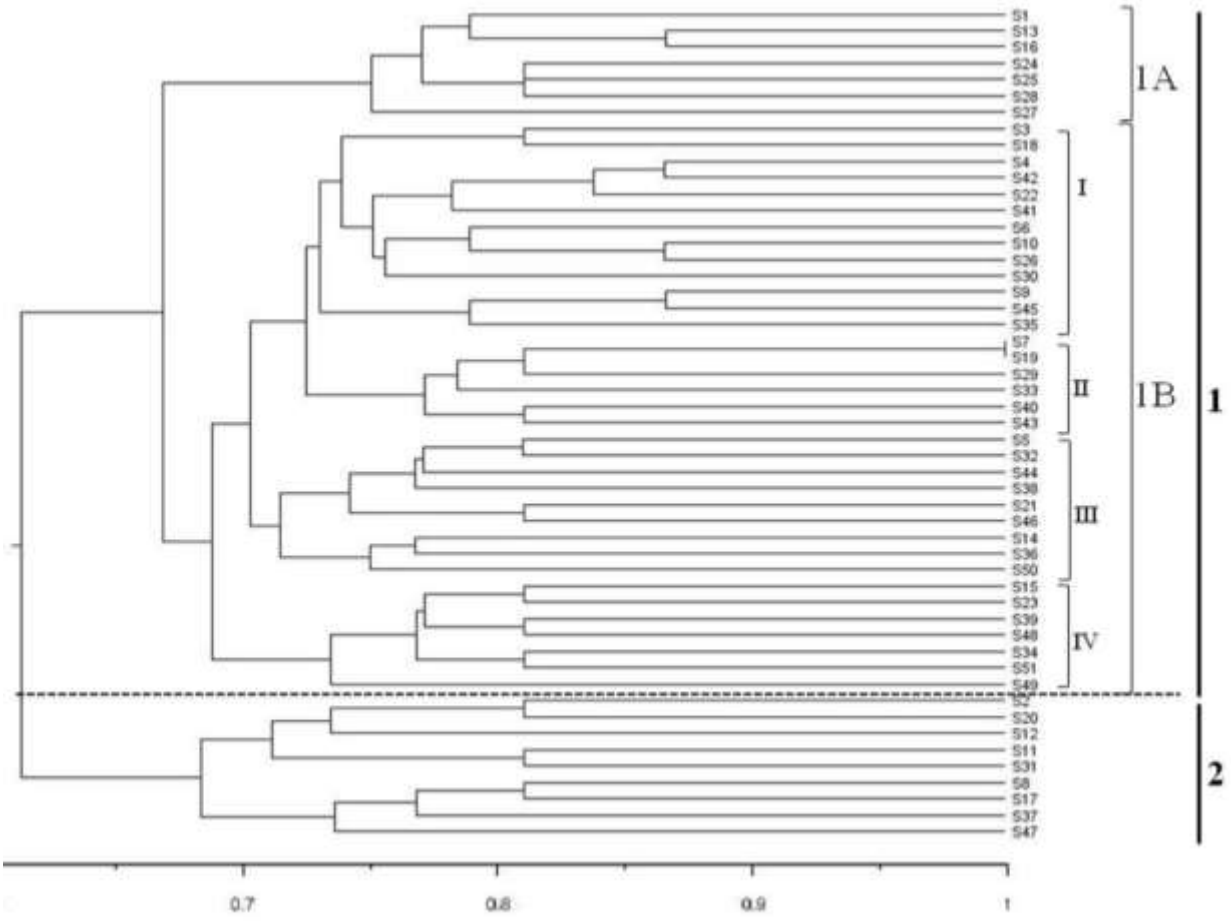

Figure 2. UPGMA dendrogram showing genetic relationship among 51 common bean landraces from SW Kenya using 5 POX markers.

moderately resistant landraces to *Pythium* root rot disease as revealed by their clustering into different clusters and groups suggest the presence of different sources and sufficient genetic variation for resistance to *Pythium* root rot. This genetic variability can be exploited for developing cultivars resistant to *Pythium* root rot. In the present study, the dendrogram constructed using UPGMA method suggested occurrence of two major clusters and illustrated no clear pattern of distribution of moderate resistant, susceptible and highly susceptible landraces. The relationship between landraces in the cluster groups could not be attributed to their resistance to *Pythium* root rot. The 51 common bean landraces were divided by STRUCTURE analysis into three groups and did not indicate any distribution pattern in terms of their reaction to *Pythium* root rot. It is therefore predicted that, combining landraces from the different clusters and groups as parents in breeding programmes would result in broadening *Pythium* root rot resistance genes in the population.

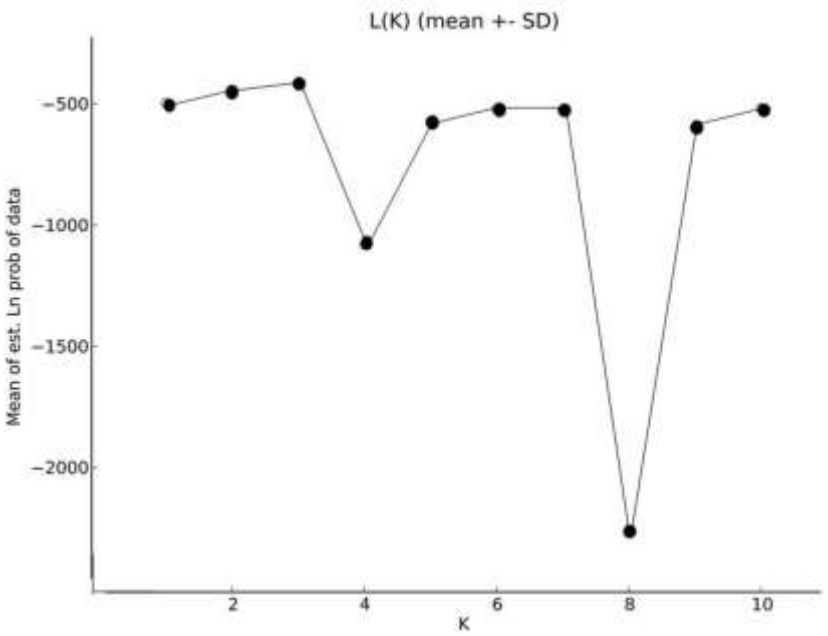

Figure 3. STRUCTURE analysis of the total genetic clusters for values of *K* (*K*=1 to 10), using delta *K* (*ΔK*) values.

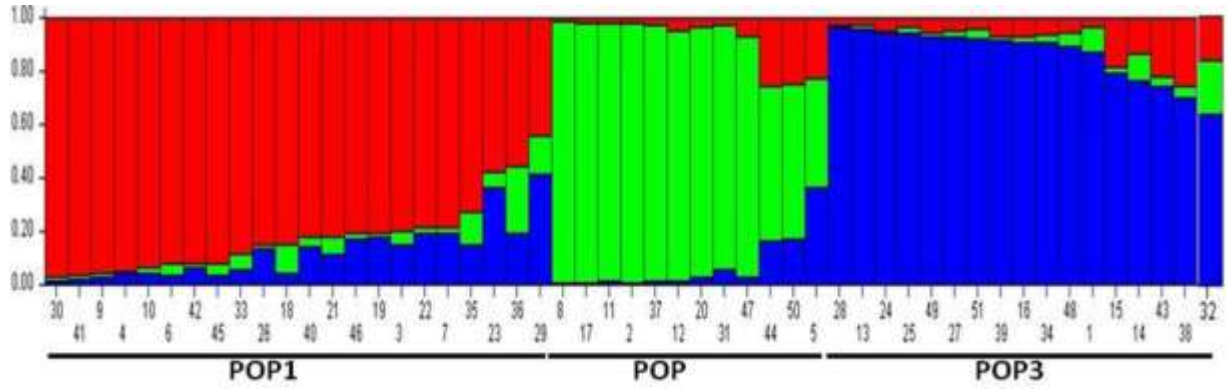

Figure 4. Population structure of 51 common bean landraces based on peroxidase-gene (POX) based primers for *K* = 3. The colors represent single sub-population and the colored segment length indicates the analyzed membership proportion of every sample to designed population. The maximum K value was determined by structure harvest to be 3, which indicates that the entire population of 51 consisted of 3 subgroups. The numbers 1 – 51 represent the entries of the landraces (Table 2) where S is excluded.

Incorporating *Pythium*-moderate resistant landraces which have other desirable agronomic and consumer quality traits such as high iron and zinc content, fast cooking ability, from the different clusters and groups as parents for breeding, would ensure the diversification of resistance to the disease while creating new hybrids. Six common bean landraces from South western Kenya with the potential moderate resistance to *Pythium* root rot were identified. This study has shown that despite the damaging effects of the disease on the crop, there is a significant amount of genetic variability among the local landraces, which could be utilized in breeding programs targeting development of *Pythium* root rot-resistant germplasm and cultivars to diversify resistance to the disease. This molecular characterization of common bean landraces from South Western Kenya will give valuable information for breeders and lead to the development of new cultivars for resistance to *Pythium* root rot. Since the POX markers used in the present study were developed to determine genetic diversity, there is need to use markers linked to the genes responsible for resistance to *Pythium* root rot in order to facilitate the effective identification of quantitative trait loci linked to this trait.

Table 4. Molecular variance of POX markers among and within 51 common bean landraces.

Source	df	SS	MS	Estimated variation	Percentage of variation%
Among populations	4	9.628	2.407	0.000	0
Within populations	46	142.529	3.098	3.098	100
Total	50	152.157		3.098	100

df = degrees of freedom, SS = sum of squares, MS = mean of squares, p-value (<0.001).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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Full Length Research Paper

Isolation and characterization of a lytic bacteriophage (VB_PAnP_PADP4) against MDR- *Pseudomonas aeruginosa* isolated from septic wound infections

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Resistance to multiple drugs by pathogens has become the sweltering problem for the treatment of bacterial infections. To overcome this problem of drug resistance, several diversified bacteriophages can be used as one of the alternatives and attractive approaches. The present study aimed to detect, isolate and partially characterize the bacteriophage used against MDR-*Pseudomonas aeruginosa* isolated from wound infections. A total number of 130 pus samples were collected and processed for the bacterial isolates from which a potential pathogen (*P. aeruginosa*) was identified and characterized using morphological, biochemical and molecular techniques (16S r-DNA technique). Among the Pseudomonas isolates, 26 multidrug-resistant strains were detected by Kirby-Bauer disc diffusion method. Bacteriophages (n=15) were collected against the MDR-*P. aeruginosa* and one of the efficient lytic phage (PADP4) was selected for this study. The transmission electron microscopic studies revealed that the selected phage PADP4 morphological features resemble the *Podoviridae* family. Phage PADP4 had the highest stability in a broad range of pH, temperatures, and high burst size (270 phages/ infected bacterial cells). Further, the potential phage, PADP4 was co-cultured with MDR-*Pseudomonas aeruginosa* and showed a significant growth reduction at 12 h of incubation and at multiplicity of infection of 1. Irrespective of the drug resistant bacteria, phage PA DP4 showed perfect lytic activity against the MDR- *P. aeruginosa*. It has been clarified that PADP4 could be a future promising agent for controlling MDR-*P. aeruginosa*.

Key words: Septic wounds, MDR-*P. aeruginosa*, bacteriophage, transmission electron microscope (TEM).

INTRODUCTION

Pseudo*monas aeruginosa* (*P. aeruginosa*) is a gram-negative bacillus, motile, a facultative anaerobe, and non-spore former; it is responsible for a wide variety of diseases in both humans and animals. Generally, *P. aeruginosa* is an opportunistic and nosocomial infectious organism that can could develop infections in severe

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burns, injured and surgical wounds and as well as in immunocompromised subjects. It is also responsible for the development of pneumonia, upper respiratory tract infections, bacteremia, endocarditis and urinary tract infections (UTI) (Kumari, 2009; Garbe et al., 2010; Kwiatek et al., 2015).

Development of natural drug resistance by bacterial pathogens is one of the greatest problems to the human health. During the clinical manifestations, *P. aeruginosa* acquired resistance to most of the antibiotics which were precisely available. During the infestation, all the MDR-pathogens formed biofilm which facilitated infections such as catheter associated urinary tract infection (UTI), and ventilator-associated pneumonia. In general, most of the bacterial pathogens were encapsulated with polysaccharide matrix, which is difficult to treat with conventional therapeutics. However, these signs contribute to a significant increase in mortality and morbidity rate particularly during sepsis. Therefore, there is an urgent need for the development of alternative therapies, in general and MDR-Pseudomonas, in particular. Mehta et al. (2007) and Yakha et al. (2015) reported that treatment with broad-spectrum antibiotics may eradicate the planktonic cells unlike biofilms; hence, an alternative strategy is required globally to protect the clinical community.

Bacteriophage therapy is a good old method in ancient times where there was no awareness of chemotherapies. Due to greatest achievement and production of antibiotics, the application of bacteriophages was overwhelmed. So far so good the MDR-bacterial strains renewed the interest and recapped the application of phages as therapeutic agents (Azizian et al., 2013). Specific bacteriophage selectively kills bacteria and this phenomenon may be an excellent alternative method to solve the global problem of multidrug resistance. However, the use of bacteriophages against several pathological abnormalities is referred to as phage therapy. Instead of whole phages, phage lysins were the primitive factors in this regard. Usually, bacteriophage therapy is used on the basis of lytic state of art against MDR-strains and has several advantages than antibiotics. The major advantages of the phage therapy include host specificity, self-replication and environmental safety of bacteriophages (Azizian et al., 2013; Chan et al., 2013).

The basic essence of bacteriophages is that they attack and destroy specific pathogens where unspecified bacteria are not affected, making them to live freely in the environment. Feasibility bacteriophage multiplies very rapidly in the host bacteria, increases the phage titer and is far more effective to control pathogens. Exclusively when the concentration of the pathogen decreases sufficiently, the phage titer also declines. This is one of the remarkable indicators of phage therapy, which has also been confirmed successful in aquaculture to protect one from experimentally induced bacterial infections. Therefore, it has also been approved by the USA research groups and they continue to work towards the development of the whole phage to be used as an alternative therapeutics (Kutter et al., 2010). This process is very much similar with optimization and purification of phage components as well as antibacterial substances opening up new opportunity against various vital infections (Piracha et al., 2014; Sulakvelidze et al., 2001).

In the present study, we have reported the detection and partial characterization of bacteriophage PADP4, isolated against MDR-*P.aeruginosa* (National Center for Biotechnology Information (NCBI); the accession number allotted to *P.aeruginosa* in this study is *P.aeruginosa* KY018605.

MATERIALS AND METHODS

Bacterial isolate

Different *P. aeruginosa* strains were isolated from pus samples of septic wounds and identified by different cultural methods (cetrimide agar media). They were further identified by using morphological and physiological test (gram staining, oxidase, IMVIC test, nitrate reduction test and catalase, carbohydrate fermentation test for glucose, maltose, lactose, galactose and sucrose). After confirmation by 16S r-DNA sequencing, *P.aeruginosa* culture was preserved for further analysis.

Screening method for MDR-pathogen

P. aeruginosa was the most predominant isolate from the septic wounds, tested for antibiotic susceptibility to identify MDR-bacteria. An MDR-bacterium must show resistance to different antibiotics with different concentrations. The following lists of antibiotics were used for the selection of MDR strains and their susceptibility patterns were recognized exclusively according to the Clinical and Laboratory Standards Institute (CLSI) guidelines; (benzylpenicillin (30 µg), ampicillin (30 µg), amoxicillin (30 µg), kanamycin (20 µg), tobramycin (20 µg), streptomycin (20 µg), cefotaxime (20 µg), vancomycin (10µg) and tetracycline (10 µg) (Towns et al., 2010; Jaafar et al., 2014). The most potent MDR-*P. aeruginosa* was selected for these studies and were submitted to NCBI for the allotment of accession number.

Molecular identification of MDR-*P. aeruginosa*

One among the *P. aeruginosa* isolates was noticed to have the highest drug resistance to a wide range of antibiotic regimen; therefore, it was selected for the molecular confirmation by 16S r-DNA sequencing. The selected and confirmed strain was inoculated with 100 mL of Luria Bertani (LB) broth and incubated at 42°C and 120 rpm for 24 h. After incubation, the culture was subjected to centrifugation at 8000 rpm /10 min. Further, the pellet was collected for DNA extraction using DNA extraction kit (Thermo fisher scientific company, USA). Meanwhile, the culture suspension was measured by UV spectrophotometer for the culture density component and the primers were used for its molecular diagnosis by PCR. Primer information is shown in Table 1. Therefore, the PCR amplicon was purified and further processed for sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A consensus sequence of 1512 bps

Table 1. Sequence information of universal primer of *Pseudomonas aeruginosa*

S/N	Universal 16S rRNA	Primer sequence (5' to 3')	Product length (bp)
1	Forward primer	GGCGACCACCTGGACTGATACTGACACTGAGG TGCGAAAGCGTGGGGAGCAGAC	785
2	Reverse primer	ACCTGGACTGATACTGACACTGAGGTGCGAA AGCGTGCGAAGCAAAC	876

16S r-DNA was generated from forward and reverse sequence data using multiple alignment software program Clustal W. The 16S r-DNA sequence was used for BLAST alignment search tool of the NCBI Genbank for the accession number (Table 1).

Bacteriophage isolation

Sewage samples were collected from nearby RIMS hospital, Kadapa, Andhra Pradesh; they were used to isolate selective bacteriophages against MDR-*P. aeruginosa* strain. The collected sewage samples centrifuged at 8000 rpm for 10 min to debris were removed and the filtrate was filtered through 0.45 µm syringe driven filters (Millipore filters, Himedia, Mumbai) mixed with LB broth containing a selected strain of MDR -pathogen. This mixture was incubated at 37°C for 15 h in a shaking incubator and later centrifuged at 10,000 rpm per 10 min. It was then filtered through a 0.45 µm membrane. So the isolated phage filtrates were screened for host-specific phages by spot test. Subsequently, the positive culture from the spot test was further used for plaque forming assay by double layer agar method, which indicates titer levels of the phages. The same experiments were repeated successively at time interval to obtain single and pure plaques or phage stocks. These lysates were filtered (50 mM Tris-CL, 99 mM NaCl, 8mM $MgSO_4$, 0.01 gelatin (pH 7.5) at 4°C with 1% chloroform (Khairnar et al., 2013; Pallavali et al., 2017).

Plaque assay

A plaques assay or agar overlay method or double layer agar method was used to quantify the titer of lytic bacteriophages as plaque forming units (PFU). 10^{-3} of bacteriophage (PADP4) dilutions (1000 fold serial dilution in SM buffer) was mixed with 18 h old culture of MDR-*P. aeruginosa* and incubated at 37°C for 30 min to allow the phages to be absorbed by the bacterial cell. To this mixer, a total of 5 mL of 45°C top LB agar was added and then poured on to the previously solidified bottom LB agar. The plates were incubated at room temperature for 18-24 h and counted to estimate the abundance of lytic bacteriophages (Santos et al., 2009).

Latent period, burst size of phage PA DP4

One step growth curve experiment was implemented to determine the latent period and burst size of the phages. The single step growth curve experiment was performed as described by Pajunen et al. (2000). 50 mL of MDR-*Pseudomonas* KY018605 culture was incubated to mid-exponential phase (reaching to O.D600 0.6); then cells were harvested and centrifuged (10000 rpm per 30 s at 4°C). The pellet was re-suspended in 0.5 mL of LB broth and mixed with 0.5 mL of the phage solution having plaque forming unit (PFU) of $1x10^9$ PFU/mL. This mixture was allowed to stand for 3 min at 37°C so that the phages were adsorbed by the host cells. The mixture was then centrifuged at 13,000 rpm for 2 min to remove the free phage particles. The pellet was re-suspended in 100 ml of LB broth and culture was incubated at 37°C with shaking at 150 rpm. Samples were examined for every 3 minutes up to 45 min and after centrifugation at 13,000 rpm for 1 minute, the phage titrations were obtained by double layer agar method (triplicates). The latent period was defined as the time interval difference between the adsorption and beginning of the initial rise in the phage count. The burst size of phage PADP4 was calculated as the ratio of the final phage titer to the initial count of infected bacterial cells during the latent period (Bolger-Munro et al., 2013; Piracha et al., 2014).

Analysis of calcium and magnesium ion effect on phage adsorption

In order to measure the effect of divalent metal ions on phage adsorption rate $CaCl_2$, $MgSO_4$ was used (Suárez et al., 2008). The 100 ml of *P. aeruginosa* overnight culture having O.D 600 0.6 was divided into four autoclaved flasks of 25 ml each. In one set of the flasks was inoculated with 500 µL phage having $1x10^9$ PFU^{-1}(control), while the second set of flasks was inoculated both with 500 µL phage and 250 µL of 10 mM $CaCl_2$, $MgSO_4$ and incubated with constant shaking at 120 rpm and 37°C. Samples were taken from both sets of flasks at different time intervals of 0, 5, 10, 15, 20, 25, 30 and 35 min. Samples were centrifuged at 13,000 rpm for 3 minutes to sediment the phage adsorbed bacteria. The supernatant was assayed for unabsorbed phages via double layer plaque assay method and counts were compared with the titer of control (Piracha et al., 2014; Suárez et al., 2008).

Thermal and pH stability of phage PADP4

Thermal stability tests were used to describe the impact of environment factors on phage growth, and their stability is required to maintain as lytic phage. The optimization studies (temperature, pH, metal ion effects) as obtained showed the level of integrity and exclusions of phage in an appropriate concentration (Suárez et al., 2008). Phage filtrates (1×10^9 PFU/ml) were taken in micro-centrifuged tubes and treated under different temperatures: 37°C (control), 40, 50, and 60°C for 30, 60 and 90 min. After incubation, double layer agar method was performed for each treated sample to evaluate the lytic ability of phages with respective temperatures compared to the control (37°C) (Kęsik-Szeloch et al., 2013; Piracha et al., 2014). For the pH stability assay, a phage suspension (1×10^9 PFU/ml) was inoculated in a series of tubes containing SM buffer at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 and incubated at 37°C for 4 h. After incubation double layer agar method was performed for each treated sample to evaluate the lytic ability of phages with respective PH compared to the control P^H 7. All assays were performed in duplicate (Suárez et al., 2008).

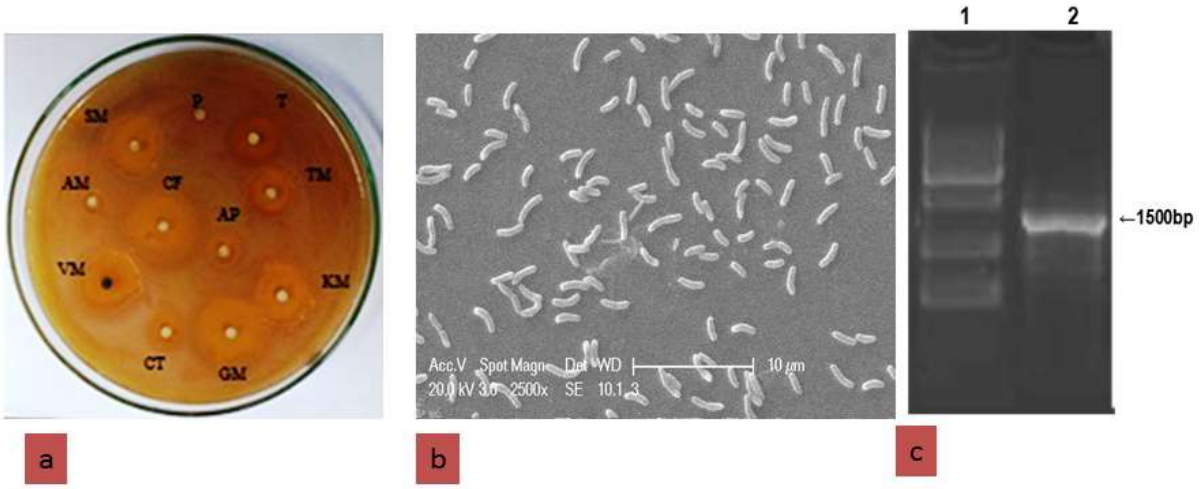

Figure 1. Antibiotic susceptibility pattern and SEM image of *Pseudomonas KY01860.* **a)** *P. aeruginosa KY018605* on Muller-Hinton agar media was sensitive to ciprofloxacin (CF), gentamycin (GM), vancomycin (VM), intermediate sensitive to kanamycin (KM), tetracycline (T), and streptomycin(SM), and resisted benzyl penicillin (P), amoxicillin (AM), ampicillin (AP), cefotaxime (CT). **b)** Scanning electron microscopy (SEM). *P. aeruginosa* grown in LB agar media for 12 h, fixed in paraformaldehyde on coverslip, dehydrated in increasing concentrations of ethanol (30-100%), and sputter coated before SEM. Bacterial cells were arranged as slight curved rods. **c)** 0.8% Agarose gel showing single 1.5 kb of 16S r DNA amplicon. Lane 1, 1 Kb DNA ladder; Lane 2, 16Sr DNA amplicon.

Host range

The selection of PADP4 bacteriophage host range was confirmed based on clinical significance such as *P. aeruginosa, S. aureus, Escherichia coli* and *Klebsiella pneumoniae* isolated from wound sepsis. The susceptibility range of bacteriophage was tested via spot assay technique along with the control (Armon and Kott, 1993).

Bacterial reduction assay

P. aeruginosa pure culture was maintained spectrophotometrically (OD_{600} at 0.6) and 1 mL of culture suspension was inoculated in 2 flasks having 150 mL of LB broth. The phage supernatant 500 µL was introduced in one flask and another flask was treated as control; both flasks were incubated in shaking incubator at 37°C and 120 rpm. The $O.D_{600}$ readings were taken after every 2 hours for 24 h using spectrophotometer. The values were compared with control and triplicates were maintained for analysis of results (Pallavali et al., 2017; Piracha et al., 2014).

Phage confirmation study by transmission electron microscope

TEM analysis was conducted to determine the characterization of bacteriophage including its morphology. The confirmed and partially characterized bacteriophage was used to treat the MDR-pathogen isolated from septic wounds. TEM analysis was carried out with FEI Tecnai G2 S-Twin, Department of Nano Technology, and University of Hyderabad, India. Further, the purified bacteriophage filtrate was filtered through 0.45 µm filters (0.45 µm PVDF syringe driven filters, Hi Media, Mumbai) with consent monitoring of centrifugation at 30,000 rpm for 60 min (Beckmann Ultracentrifuge, Proteomics laboratory, University of Hyderabad, Hyderabad). The pellet was mixed with 5 mL of SM buffer. 5 µL of phage suspension was overlaid on the formvar coated 200X200 copper grid; excess phage solution was removed with the pieces of filter paper from the edges of the grid and we waited for 3 min. 5 µL of 0.5% uranyl acetate was then applied to the grids, excess solution was immediately removed, and grids were air dried. Samples were viewed with the FEI Tecnai G2 S-Twin Transmission Electron at operated voltage of 80 KV (Kwiatek et al., 2015).

RESULTS

Isolation and identification of MDR- *Pseudomonas* KY018605

P. aeruginosa is the predominant isolate of septic wounds from 130 pus samples. Of the 26 *P. aeruginosa* isolates PA 13 was selected for further studies because it showed resistance to cefotaxime (20 µg), benzyl penicillin (30 µg), ampicillin (30 µg), tobramycin (20 µg) and amoxillin (30 µg). The drug resistance pattern, scanning electron microscopic image (SEM) and 16S r- DNA sequencing information is shown in Figure 1a, b and c.

In Figure 1a, it is seen that *P. aeruginosa* PA 13 showed resistance to these antibiotics of three different classes. Strongly, there is a fascinated multidrug resistance activity constantly identified as a specific host for isolation of bacteriophages. Scanning electron microscopy studies revealed the morphology of *P. aeruginosa* (Figure 1b); *P. aeruginosa* PA13 strain had the highest drug resistance, so it was treated and selected as the best strain for our study. Further it was characterized by 16 S r-DNA (1500bp) sequencing and substantially submitted to NCBI database, which provided its accession number as *Pseudomonas KY01860* (Figure 1c).

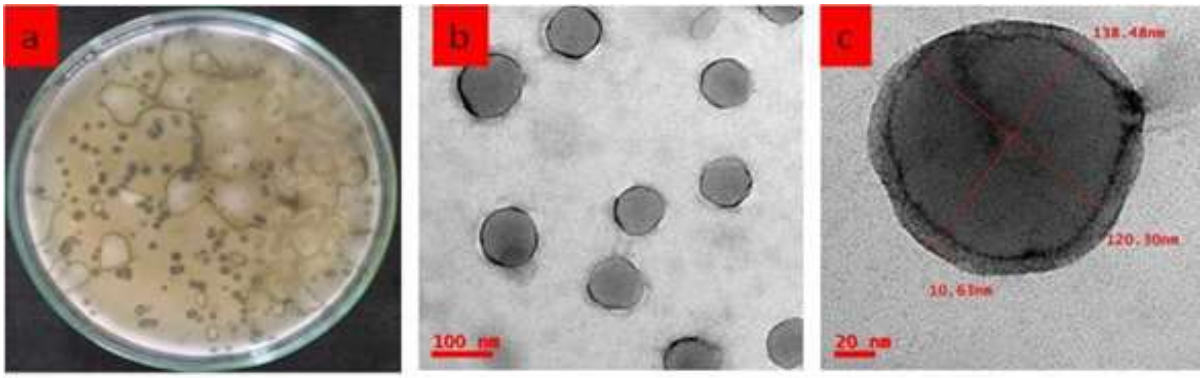

Figure 2. Phage PA DP4 bacteriolytic activities against *Pseudomonas KY018605.* **(a)** Phage V_B_PAnP_PADP4 forms plaques on the bacterial lawn by double layer agar method. **(b)** Phage PADP4 belonging to *Podoviridae* family and was characterized morphologically with Transmission Electron Microscopy (TEM) by negative staining with 2% uranyl acetate. Scale bar = 100 nm. **(c)** Enlarged image of Phage V_B_PAnP_PADP4 with scale bar = 20 nm.

Isolation of bacteriophage against *Pseudomonas KY01860*

The respective bacteriophages were detected and isolated by double layer agar method. Amongst these isolates, phage PADP4 was selected and partially characterized. Phage PADP4 showed specific lytic activity against MDR-*P. aeruginosa* (Figure 2). Transmission electron microscope is one of the most precious and widely used methods for identifying the morphological features of bacteriophages as well as their classification. Phage PA DP4 showed icosahedral symmetrical head without tail. Based on these features, it was confirmed that PADP4 belongs to *Podoviridae* family (Figure 2b, 2c). This phage isolate was named according to the newly proposed naming system vBPanP/M/S KPno, where vB = bacterial virus; Pan = REBASE abbreviation for genus/species of the host; P = Podovirus; M=Myovirus; S = Siphovirus; KPno = name and number of phage. So the name of PADP4 was given as Phage V_B_PAnP_PADP4 (Kęsik-Szeloch et al., 2013).

Biophysical characterization of Phage PA DP4

Later on, one step growth curve of PADP4 was observed at prominent latent period for 25 min, and an average burst size of PADP4 was calculated as 270 PFU/infected bacterial cell (Figure 3a). One step growth curve of bacteriophages was influenced by the host, selective medium and external factors. The bacterial reduction probability by bacteriophages was monitored from 2 to 24 h and also bacteriophage PADP4 *in vitro* assay (Figure 3b). From the figure, it shown that PADP4 has the highest lytic activity against MDR- *P. aeruginosa*, which reflects on the optical density of the infected organism and sometimes develops the biostatic condition. Hence the reduction of optical density of bacterial growth is intensified due to development of phage resistance. Various external factors were also tested for temperature, pH, effect of metal ions on the adsorption rate of bacteriophages (Figure 4). From the figure, it is seen that the most important external factor affecting bacteriophage stability is pH and temperature affects the lytic activity of PADP4. They were exclusively fluxuvated with different pH ranges that is more stable phages were obtained with a pH range of 6 to 8, but there was no appropriate activity detected at pH 2.0, 3.0, 10.0 and pH 12. But during the constant 4 h of incubation, it was observed that PADP4 showed traces of infectivity at pH 4 and 9 (Figure 4a). Thereafter thermal stability results were noticed, that temperature is one of the critical factors affecting bacteriophage activity during the phage therapy. The phage PADP4 survived at 0, 20 and 37°C, with no significant loss in plaque formation (PFU). The percentage of survival of PADP4 fluctuated randomly for every milliliter i.e. by one order of magnitude over 30, 60 min at 50 and 60°C, respectively (Figure 4b). Hence, these results suggest that extreme temperatures and pH conditions favorably affect the stability of phage PADP4.

Effects of divalent metal ions ($MgSO_4$ and $CaCl_2$) on the adsorption rate of bacteriophages were well studied and reported in Figures 5a and b respectively. From the figures, it seen that $MgSO_4$ and $CaCl_2$ ions induced the infection rate of MDR- *P. aeruginosa* PA13 by PADP4, which tremendously enhances the adsorption rate of bacteriophages reflected on PFU observed in regular time intervals of 10, 20 and 30 min. These results suggest that metal ion impact increases the rate of infectivity as well as burst size.

DISCUSSION

P. aeruginosa is one of the emerging multi drug resistant, nosocomial pathogen and causes health care complications. Development of drug resistance in *P.*

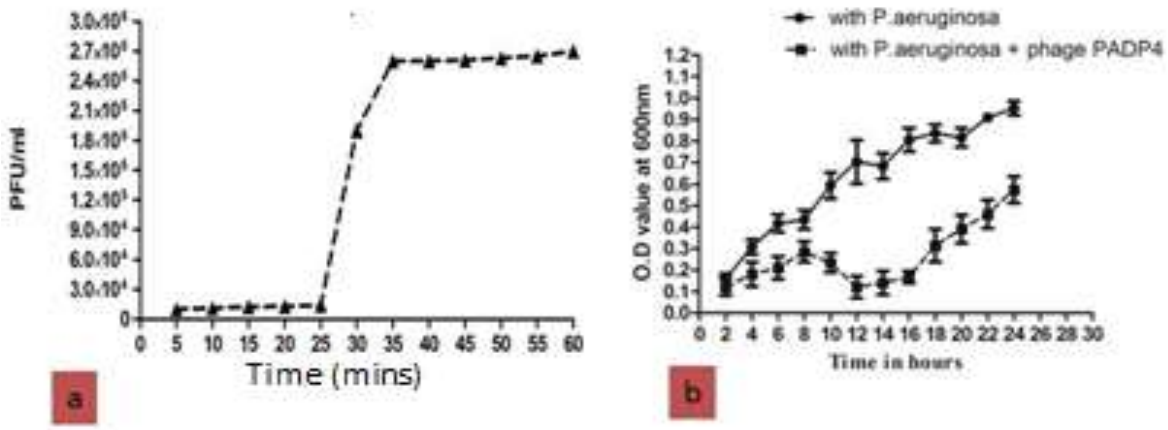

Figure 3. One step growth curve of Phage V_B_PAnP_PADP4. a) Single step growth curve experiment of phage PADP4 showed the latent period is about 25 min (the time interval between the phage adsorption and initiation of the first burst of the bacteriophage) and average burst size is 270 viral particles per single infected bacterial cell. Latent period and burst size of phage were concluded from the curve with a tri phasic arrangement. **b)** Lysis of *P. aeruginosa KY018605* strain by the phage PADP4 in LB broth medium. OD development of a control flask having uninfected *P. aeruginosa* and test flask having the *P. aeruginosa* cultures infected with its specific phage PADP4. Phage infection leads to a drastic decrease of the *P. aeruginosa* culture at 12-14 h of incubation.

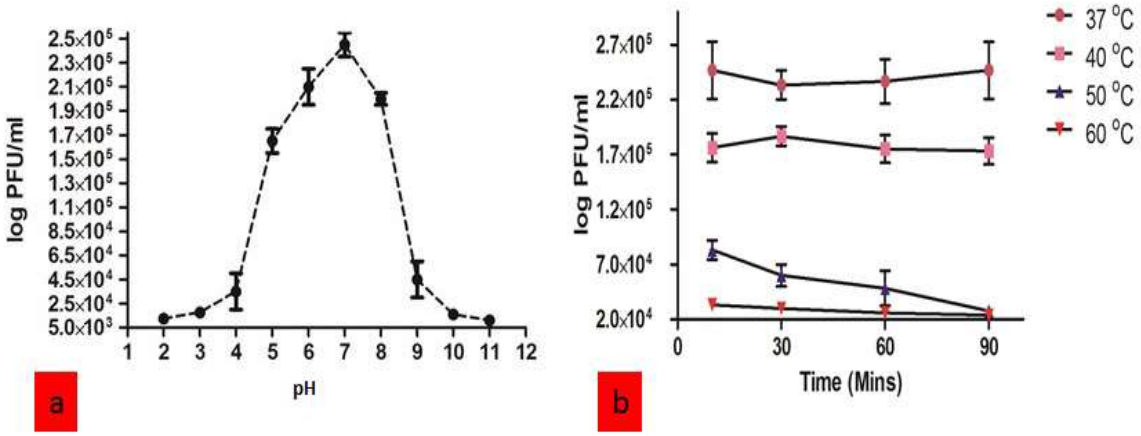

Figure 4. Effect of p^H and temperature on stability of Phage PADP4. **(a)** Influence of pH on the stability of phage infection. Phage PA DP4 suspension was incubated for 4 hat the different pH values indicated by dark circles. **(b)** Effect of temperature on the infection ability of PADP4. Results obtained with different temperatures, and times of incubations are shown. In both cases, the data showed here the mean of two independent experiments.

aeruginosa is due to the modification of genetic material, transferred drug resistance from R-plasmids, and transposons. In general, the development of drug resistance is responsible for outer membrane proteins which are able to construct biofilms as well as increase the rate of infection (Kwiatek et al., 2015). There are a number of reports available on the isolation of bacteriophaes against *P. aeruginosa* in general but the present investigation mainly focuses on the specificity of the lytic nature of the host, bacteriophage type and their partial characterization for the development of new isolate having more clinical significane. Therefore, based on these concepts, we highlighted the isolation and partial chracterization of bacteriophages such as metal requirements for the stability and infection of bacteriophages, temparture and pH and bacterial reduction assay in *invitro* method. Many reports described that receptor based integrity on host, adsorption

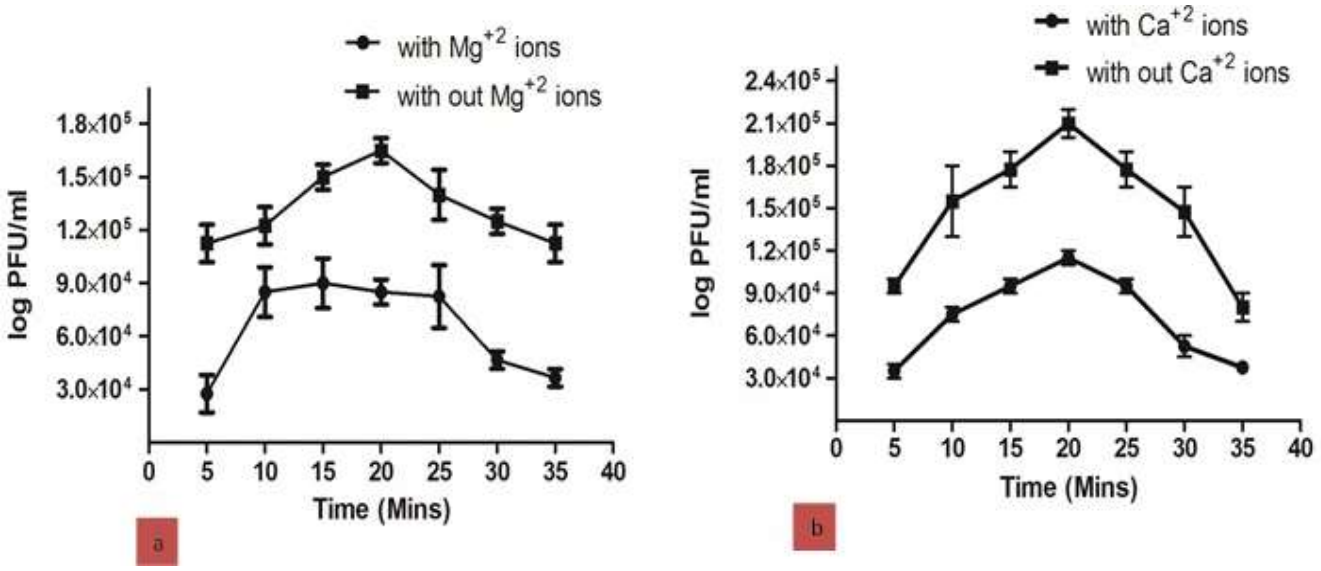

Figure 5. Effect of calcium and magnesium ions on the absorption rate of Phage PADP4. PADP4 suspensions were incubated at different time intervals (5, 10, 15, 20, 25, 30, and 35 min) in a 10 mM $MgSO_4$ and $CaCl_2$ in SM buffer. The rates of adsorption ability of PADP4 in the presence and absence of divalent metal ions were assayed by using the double layer agar method. In both cases, the data showed here the mean of three independent experiments.

fluctations leading to not only establishment of lysis but also these bacteriophages would be used as alternative drugs and theraupatics for various systemic infections (Pallavali et al., 2017; Pavlenishvili and Tsertsvadze, 1993; Rhoads et al., 2009; Sulakvelidze et al., 2001; Vandersteegen et al., 2013).

According to Bolger-Munro et al. (2013), the single step growth curve experiment illustrated that the convaluted steps in multiplication of bacteriophages target the growth curve, latency as well as burst levels. They also noticed that latent or incubation period is the right time where the bacteriophages are located inside their host and excel within 20 min; present results noticed that phage PADP4 has the 25 min as latent period.The high burst size would be the preferable feature for the phages which has theraupatic role; burst size explain the average number of phages produced from the single bacterial cell. The other physiological factors (temparature, pH, metal ion effect) play an important role in the mulptilication of phages, phage-bacterial intergration. Shafia and Thompson (1964) noticed that 0.1 M calcium ions were nessacery for the adsorption of bacteriophages (øμ-4) to Bacillus sterothermophilus NU-10 bacteria (Shafia and Thompson, 1964). Pajunen et al. (2000) noticed that latent period and burst size by one step growth curve of the phage YeO3-12 on bacteria *Y.enterocolitica* is 25 min and 100 to 140 PFU/Cell; these results is similar to our results but we noticed higher burst size (290 PFU/cell). Piracha et al. (2014) disclosed the role of calcium ions (1 mM) in the stabilization, adsorption and also infectivity rate is increased in presence of 1mM calcium ions in the solution. These metal ions plays a role in between phage and the host receptors for their accessibility to enhances the biological communication between them (Piracha et al., 2014). From this study, it was noticed that the phage PADP4 shown maximum adsorption at 10 mM concentration and at remaining concentartions the minimum range of adsorption were noticed (Data not shown).

Suárez et al. (2008) demonstrated that the study of bacteriophage QF9 is not required calcium ions and showed the same percentage of adsorption even with or without calcium ions but our results contradict with our results of phage PADP4 showed three fold higher adsorption were noticed in presece of calcium ions and magnisium ions. Fauquet et al. (2005) proven that 5 mM $cacl_2$ is required for the stability of phage PM2 and phage PM2 is stable at pH 6 to 8 (Fauquet et al., 2005). Temparature is one the cretical parameter to determine the stability and infectivity of bacteriophages (Olson et al., 2004). During the course of the analysis our finding showed that the existence of PADP4 in SM buffer at 4°C could be maintanined for two years and would exist up to 60˚C for 30 mins, but usually the maximum infectivity was observed at 37 and 40˚C with highest affinity towards their host and is companion to study of Suarez. Thorne and Holt (1974) observed phage CP-51 have optimum stability at 15°C and even survive at room temperature but at high temperature phage CP-51 lost its lytic capacity (Thorne and Holt, 1974). pH is another important factor for the stability and also for the infectivity of phages. Phage PADP4 showed stability at pH range 5 to 9 and maximum stability were noticed at pH 7. Our results are similar with those of Sharp et al. (1946) phage

T2 that showed that stability at pH range 5 to 9, but maximum stability was noticed at pH 5 to 6 (Sharp et al., 1946). Kłak et al. (2010) study revealed that Phage T4 showed the stable at pH 6–7.4 and unstable at the pH 5 and 9.2 (Kłak et al., 2010).

Conclusion

In conclusion, a highly virulent bacteriophage V_B_PAnP_PADP4 were isolated and partially characterized from sewage samples. It has therapeutic potential to treat septic wounds caused by MDR-*P.aeruginosa*. Analysis of TEM studies revealed that V_B_PAnP_PADP4 resembles *Podoviridae* family and has a strong lytic activity against *P.aeruginosa.* Thus the isolated phage will be effectively proposed to treat wound infections in near future. However, further studies about antibacterial activity of this phage on other bacterial pathogens or *invivo* host cells are required to expand the use of phages.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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