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RESEARCH ARTICLE

Higher Order Occurrence of Virulent Isolates of *Pseudomonas aeruginosa* in Hospital Environments Initiate One Health Concerns Irrespective of the Biological Association

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ABSTRACT

One health links the health of humans with that of animals and the environment. *Pseudomonas aeruginosa*, a common nosocomial and opportunistic pathogen of humans and animals causing cystic fibrosis, dermatitis, soft tissue infections, gastrointestinal infections and mastitis etc., has been prevalent in many hospital environments throughout the world. In present the study, out of six hundred samples taken either directly or from close proximity of humans, bovines and equines keeping in view the one health concept of *P. aeruginosa*. All the samples were checked through conventional methods, API KIT, Biofilm detection, Colony PCR, sequencing and *in vivo* experiments for taxonomic, molecular and pathogenic characterization of *P. aeruginosa* isolates. Present study indicates the prevalence of virulent strains of *P. aeruginosa* in higher order of frequency of isolation i.e. 13.5, 9 and 7.5% in three different hospital environments including Allied Hospital (AH), District Head Quarter Hospital (DHQH) and Civil Veterinary Hospital (CVH) respectively. *P. aeruginosa* could be recovered from humans, bovines and equines throat swabs with or without symptoms (14.7%), wound pus (13.3%), surgical instruments (8.6%) and garden soil (3.3%). However, the most commonly prevalent and virulent isolates were confirmed as *P. aeruginosa* strain BTP1 (KU534099) and *P. aeruginosa* strain WPP1 (KU534100) and consequent phylogenetic analyses clustered them with the most narrowly related *Pseudomonas* species belonging to the famous *P. aeruginosa* PAO1 groups. Such a higher order prevalence of these strains in diverse environments and their physiological, genetic and pathogenic variation is quite alarming demanding special considerations from the concerned quarters for one health promotion in Pakistan.

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INTRODUCTION

Health of human beings and animals depends upon healthier environment. Due to wide host range (human and wild/domestic/livestock animals) of *P. aeruginosa*, significant number chronic and resistant zoonotic cases were reported which ultimately cause major economic losses of a medical and livestock concerning departments (Haenni *et al.*, 2015; Jamil *et al.*, 2016; Parvin *et al.*, 2016). A Clinico-morphological study of *P. aeruginosa* on young and growing poultry (broilers and layers were reported and found mortality 0.5-50% (Dinev *et al*., 2013) which is a serious disease threat to poultry pathologists and veterinary practitioners. *P. aeruginosa* responsible for severe infections in animals such as abscesses, bovine mastitis, equine metritis, canine urinary tract infections and otitis (Al-Graibawi and Ati, 2016). Herd acute mastitis due to *P. aeruginosa* in cattle associated to improper precautionary approaches affects the wounds and burns of the veterinary practitioners as well as milkman (Zadoks *et al*., 2011). Studies were conducted on prevalence of multidrug resistant *P. aeruginosa* in companion animals (dogs, cats and Chinchillas) and found dogs and cats carrier of the *P. aeruginosa* infection possibly from environment, contaminated antibiotics and vaccinations, a significant prevalence in pet as well as laboratory rodent chinchillas 41.8% were reported which is a serious risk of *P. aeruginosa* zoonosis to public

health. Alarming zoonotic disease burden of *P. aeruginosa* needs improved surveillance and prevention measures of one health (Hirakawa *et al.,* 2010; Harada *et al.,* 2012; Hameed and Ahmed, 2014). *P. aeruginosa* is frequently found wet muddy zones across stables and constant source to reproductive, respiratory, ocular and wound infections in horses as well as in veterinarians (Allen *et al.,* 2011).

Moreover *P. aeruginosa* is one of the major cause of nosocomial infections especially in patients admitted in Intensive Care Units and responsible for cystic fibrosis in patients and additionally causes dermatitis, soft tissue infections, gastrointestinal infections that ultimately lead to high mortality and morbidity (Wahab and Rahman, 2013; Cabassi *et al*., 2015).

P. aeruginosa has many virulence enzymes, toxin, and resistant factors like alkaline protease, toxin A and elastases and rhamnolipids. *P. aeruginosa* produced biofilms are responsible for multi drug resistant behavior allowing it to grow easily in hospital environment, instruments and soil (Aruna and Mobashshera, 2012; Naz *et al*., 2015). Moreover, this pathogen in clinical environment, wet and dam places becomes a continue source of infection. Therefore, it is necessary to evaluate the hospital environments and clinical samples for the better diagnosis, prevention and treatment of *P. aeruginosa*. A cross sectional study of multidrug resistant *P. aeruginosa* was conducted in different hospitals of Karachi, Pakistan and their results showed that the frequency of multidrug resistant *P. aeruginosa* was as follows; pus samples (33.3%), wound swabs (26.6%), bronchial fluid (23.3%), urine (10%) and blood samples (6.6%) out of 100 collected samples (Khan *et al*., 2014). Another epidemiological reported 29% prevalence of multidrug resistant *P. aeruginosa* in Peshawar premises (Farhatullah *et al*., 2009). Therefore, in this study, we examined the different morphological characteristics and virulence of local strains of *P. aeruginosa*, for better diagnosis and treatment of *P. aeruginosa* based infection in district Faisalabad, Pakistan ensuring one health parameters.

MATERIALS AND METHODS

Survey and Sampling: Total 600 samples were collected from two humans and one veterinary hospital of district Faisalabad, Punjab, Pakistan viz., Allied Hospital (AH), District Head Quarter Hospital (DHQH), and Civil Veterinary Hospital (CVH) during December 2012 to November 2014. Samples were taken from throat samples of humans, bovines and equines showing or not the bronchopneumonia symptoms, wound pus, clinical instruments and garden soils of hospitals. All the samples were collected aseptically and tested freshly within two hours of collection in the laboratory.

Culture media and growth condition: All the samples were pre-enriched in Luria Bertani (LB) broth and were then transferred to the *P. aeruginosa* specific Cetrimide agar medium (Johnsen and Nielsen, 2006). Also, the swarm plates of *P. aeruginosa* local strains were prepared using swarming media (Krishnan *et al*., 2012).

Identification and pathogenicity confirmation: Initially, all the isolates were identified through cultural, morphological and biochemical characters using selective media, gram staining, oxidase test and API 20 E Kit (Biomerieux) using standard protocol as per manufacturer's guidelines (Yang and Bashir, 2008). Biofilm formation by *P. aeruginosa* was detected by microtitraion plate method as describes by Hassan *et al*. (2011). Extent of swarming was determined by measuring the diameter of swarms (Krishnan *et al*., 2012). For pyocynin detection the spent cultures of freshly grown *P. aeruginosa* cultures (16 hours in LB broth) were treated (Li *et al*., 2014).

For pathogenicity determination, *in vivo* experiments were performed into the artificially created wounds in the rabbits limb approved by Animal Ethics Committee of the University as described by Spilker *et al*. (2004). Wound scoring was examined as followed, serous exudate (1), Erythema (2), Purulent exudate (3) and separation of deep tissues (4). In another experiment rabbits were administered with food contaminate with the test organism (@10^3 cfu/mL) three times a day and development of pneumonia like symptoms were observed over time (Data not shown). Each time inoculated animals were also tested for the test organism after specific interval of time during the infection.

Taxonomic identification: Colony PCR, BLAST searches and Phylogenetic analysis: Taxonomic identification was based on the BLAST searches of PCR amplified and sequenced 16S rRNA gene sequences (Janda and Abbott, 2007). Briefly, colony PCR was performed on freshly grown single isolated colonies using universal 16SrRNA gene primers fD15-CCGAATTCGTC GACAACAGAGTTTG ATCCTGGCTCAG-3 and Reverse primer; rD1 5- CCCGGGATCCAAGCTTAAGG AGGTGATCCAGCC-3. Standard protocol for PCR was followed. PCR products were purified using purification kit (Qiagen, Germany) according to the manufacturer's procedure and sent for sequencing with both forward and reverse primers to Eurofins WMG|Operon-USA. The provided DNA sequences were analyzed using BioEDIT software and were compared with those stored in gene bankat National Center for Biological Information (NCBI) (http://www. ncbi.hlm.nih.gov/genbank/) using online Basil Local Alignment Search Tool (BLAST) to fetch most reliable closely related species (Saitou and Nei, 1987). Phylogene-tic trees were constructed using MEGA6 DNA analysis software (Tamura *et al.,* 2013). Briefly, sequences were first aligned through multiple sequence analysis (MSA) using Clustal W with partial deletion (90%) followed by dendrogram construction through maximum likelihood method with 1000 bootstrap replication. The analysis involved known fixed number of sequences. All positions with less than 90% site coverage were deleted. The gram positive human bacterial pathogens were included as out groups (Tamura *et al*., 2013).

Statistical analysis: Data were analyzed statistically by chi square test and ANOVA using SPSS software version 17.

RESULTS

***P. aeruginosa* as the major contaminant of human and animal frequented clinical environments:** Out of 600 isolates obtained from various clinical environments

including human, bovine and equine *P. aeruginosa* could be isolated from each group of samples whether it was garden soil, sterilized clinical instruments, human or animal throat and wound pus samples using specific isolation procedures described above. It was noticed (Fig. 1) that the multidrug resistant bacteria prevailed in higher order percentages i.e. 13.5% (AH), 9% (DHQH) and 7.5% (CVH) not only in terms of geographical location but also as group category based on sample origin i.e 14.7% from throat swabs, 13.3% from wound pus, 8.6% from surgical instruments and 3.3% from garden soil. The isolates showing multi drug resistant behavior (Data not shown) were retained for further confirmatory analysis through pathogenicity and molecular approaches. These results depicted that the multidrug resistant *P. aeruginosa* is frequently present in hospital environments as major contaminant related to the human and animals. *P. aeruginosa* strains showing high physiological, biochemical and pathogenic variation not related to their site of origin.

Variations in swarming, pigment and biofilm production: The young culture smears of *P. aeruginosa* isolates appear to be gram-negative rods, coccobacilli, scattered throughout the microscopic field. 18% of our isolates showed swarming growth on swarming media. Isolates showed uniform turbidity in LB broth and after 48-72 hours pigment was produced at the supernatant of test tubes. *P. aeruginosa* characteristically produce three colored pigments i.e. green (pyoverdine), blue (pyocyanin) and reddish (pyorubin) on cetrimide media. Pyoverdine pigment also produces fluorescence under UV light. We examined this type of fluorescence in 32.7% pyoverdine producing isolates. For more accurate identification API 20E kit was used coupled with oxidase test. All *P. aeruginosa* isolates showed oxidase positive results. Number generated of those isolates were 2216004 and 2202000 after employing, API 20E Kit coupled with oxidase test, which showed that the tested organism was *P. aeruginosa.*

In microtitraion plate method, while reading at 490nm (OD 490) in ELISA reader, strength of the biofilm was measured using the mathematical equation described by Saxena *et al.* (2014). In the end 55% of the isolates showed strong biofilm formation while 31.7, 11.7 and 1.7% showed intermediate, weak and absent biofilm formation respectively. To summarize the data of present study 12 characteristics isolates of *P. aeruginosa* were selected for further experimentation (owing to similar behavior). Table 1 described the characteristics of candidate isolates depending upon morphological and biochemical characters.

In vivo response of P. aeruginosa isolates: Pathogenicity of candidate *P. aeruginosa* isolates was conducted in the model laboratory animals, the rabbits. In case of uninoculated animals (control group) wound healed up after certain period of time while in case of inoculation with virulent pathogen group pustular lesions (wound score 3 or 4) appeared at the site of injection. It was noticed that strong biofilm producing isolates were more virulent on rabbits as compared to the others, again irrespective of their biological origin (humans or animals).

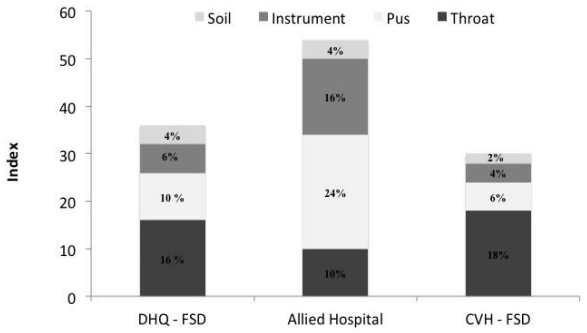

Fig. 1: Relative occurrence (%) of *P. aeruginosa* isolated from various environments (soil, clinical instruments, pus infections and throat swabs). FSD, DHQH and CVH stand for Faisalabad, District Head Quarter Hospital and Civil Veterinary Hospital, respectively.

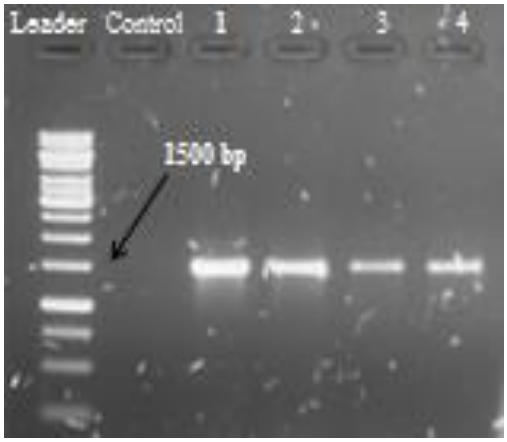

Fig. 2: PCR amplified 16srDNA fragments on 1%agarose gel. 1: Complete left; 1: Kb DNa ladder; 2: negative control; 3 & 4: *P. aeruginosa* strains BTR1 and WPP1 and 4 & 6: positive controls.

However, soil isolated *P. aeruginosa* were found to be the weak biofilm producers and consequently less pathogenic. These results indicated higher physiological, biochemical and pathogenic variation among the isolates and depending upon the behavioral similarities twelve most promising isolates were retained (three from each category of origin (irrespective of being related to humans or animals). Results also depicted that among all the sub groups (throat, wound pus, clinical instruments and garden soils) irrespective of their biological origin or sampling sites, the strains with stronger biofilms and pigments (all three pigments, as judged through conventional methods) were the most virulent as depicted by the *in vivo* experiments.

Comparative phylogenetic analysis based on partial 16S rRNA gene sequence: Isolates presented in the Table 1 were subjected to molecular characterization using 16S rRNA gene sequencing. The sequences thus obtained were submitted to the NCBI data base (accession number: KU534099 and KU534100). Sequence alignment and BLAST searches of the deduced sequences indicated the presence of only two promising strains *Pseudomonas aeruginosa* strain BTP1 (KU534099) and *Pseudomonas aeruginosa* strain WPP1 (KU534100) that clustered together with the world known nosocomial strain *P. aeruginosa* PAO1. However, when these two were analyzed together including other taxonomically related strains (Fig. 3: phylogentic analysis based on highly

conserved 422 internal nucleotides within the 16S rRNA gene) appeared to be clustering in two different sub groups within the *P. aeruginosa* clad. These sub groups represent major pathogenic strains according to the reference strains in these sub-groups. It was interesting to see that these two subgroups harbor strains from diverse origins (environment, food, humans etc). Additionally, two phylogenetically diverse clusters among the *P. aeruginosa* strains are observed one represented by PAO1 and other by DK1 strain possessing characteristic physiological differences. The results depicted that the two newly reported strains in this study belonged to sub groups that are adapted to diverse ecological conditions and possess similar pathogenic traits within the *P. aerugionsa* taxon.

DISCUSSION

Conventionally, *P. aeruginosa* are identified on the basis of their ability to produce several pigments. *P. aeruginosa* exhibit distinctive phenotypic characters in terms of pigment production having different colors, pyoverdine (green), pyocyanin (blue) and pyorubin (reddish brown) on cetrimide medium (Das and Das, 2015). In our study, it was observed that out of 60, 3.3% soil isolates lack pyoverdine pigment while they produced pyorubin and pyocyanin. Pyoverdine pigment that gives fluorescence under UV light was observed in only 31.03% of isolates. In line with our findings, Yu *et al*. (2014) observed that because of diverse ecological habitats or availability of iron, phenotypic diversity is common in *P. aeruginosa*. Some *P. aeruginosa* are high siderophores while others produce less that result in phenotypic variation in pyoverdine florescence character.

Previously, a research investigated that lung mucus membrane provides ideal condition for *P. aeruginosa* biofilm formation, resulted the colonization, biofilm formation and diagnosis as cystic fibrosis in bronchopneumonia patients (Yoon *et al*., 2002; Alvarez-Ortega and Harwood, 2007). Here we compared the strong, intermediate and weak biofilm producing *P. aeruginosa* isolates of different sampling categories with the intensity of wound in *in vivo* pathogenicity assays and found that Ts2 and Pus1 isolate which collected from bronchopneumonia patient throat and wound were strong biofilm forming and virulent *in vivo* experiment as compared to other tested strains.

Although we had identified our isolates as *P. aeruginosa* using classical methods yet employed 16SrDNA universal primers (Forward primer; fD15 CCGAATTCGTCGACAACAGAGTTTGATCCTGGCT CAG-3 and Reverse primer; rD1 5- CCCGGGATCC AAGCTTAAGGAGGTGATCCAGCC-3) based strategy for the accurate identification. The colony PCR with universal primer 16SrDNA was used to obtain a 1500bp bright band on 1% agarose gel (Fig. 2). Previously a study was conducted in which colony PCR with universal primers 16S ribosomal DNA was used to avoid the misidentification of *P. aeruginosa* and found 100% sensitivity and specificity as compare to conventional methods (Spilker *et al*., 2004).

Present study documents the significant prevalence of *P. aeruginosa* in environments of three public sector hospitals in district Faisalabad namely DHQH, AH and CVH in order of 9, 13.5 and 7.5% respectively. Frequency of isolation happened to be 4, 4 and 2% in soil samples. Similarly from instruments, patient pus and throat swabs it remained 6, 16 and 4%, 10, 24, and 6, 16, 10 and 18% respectively from DHQH, AH and CVH respectively, and it was noted that *Pseudomonas aeruginosa* strain BTP1 (KU534099) and *Pseudomonas aeruginosa* strain WPP1 (KU534100) were the most prevalent and appeared in all samples irrespective of their origin or sampling sites, the same strains remained the stronger biofilm and pigment producers (all three pigments) as judged through conventional methods and *in vivo* experiments. Our 16srDNA sequence based comparative studies through BLAST searches found that *Pseudomonas aeruginosa* strain BTP1 (KU534099) and *Pseudomonas aeruginosa* strain WPP1 (KU534100) belongs to genus *Pseudomonas*. Comparison of these sequences with already published strains showed 97, 98 and 99% sequence identity. Phylogenetic trees were constructed using neighbor-Joining method in MEGA-6 software presenting the association of the quarantined strain with the approximately 67 at present published strains (Fig. 3). Studies on different countries of world were conducted and found significant percentage of *P. aeruginosa* in clinical environment like 14% was found in Houston (Nseir *et al*., 2011), 45.2% prevalence in India (Amutha *et al.*, 2009), 1.1% incidence of *P. aeruginosa* was reported in japan during year 2008 (Kirikae *et al*., 2008). Improper cleaning and turnover of filtration system leads to biofilm formation on pipe surfaces and dam places in dairy and poultry sheds which is a continuous source of *P. aeruginosa* infection in veterinary practice (Ahmad *et al*., 2014; Widmer *et al*., 2016).

Table 1: Characteristics summary of candidate *P. aeruginosa* isolates

Isolates	Group	Source	Morphology					Biochemical characteristics	
			G –ve CB	Biofilm	Pigment			Oxidase test	API20E KIT
					1	2	3		
1	G1	TS	+	4	+	+	+	+	2216004
2		TS	+	4	+	+	+	+	2202000
3		TS	+	3	+	+	_	+	2216004
4	G2	Pus	+	4	+	+	+	+	2216004
5		Pus	+	4	_	+	_	+	2216004
6		Pus	+	3	+	+	_	+	2216004
7	G3	Soil	+	1	_	_	_	+	2216004
8		Soil	+	2	+	+	+	+	2216004
9		Soil	+	0	+	+	+	+	2202000
10	G4	Ins	+	3	_	+	_	+	2216004
11		Ins	+	4	+	+	+	+	2216004
12		Ins	+	3	+	+	+	+	2216004

1=Pyorubin, 2=Pyoverdin, 3=Pyocyanin, TS=Throat swab, Ins=Instrument, CB=Coccobacilli.

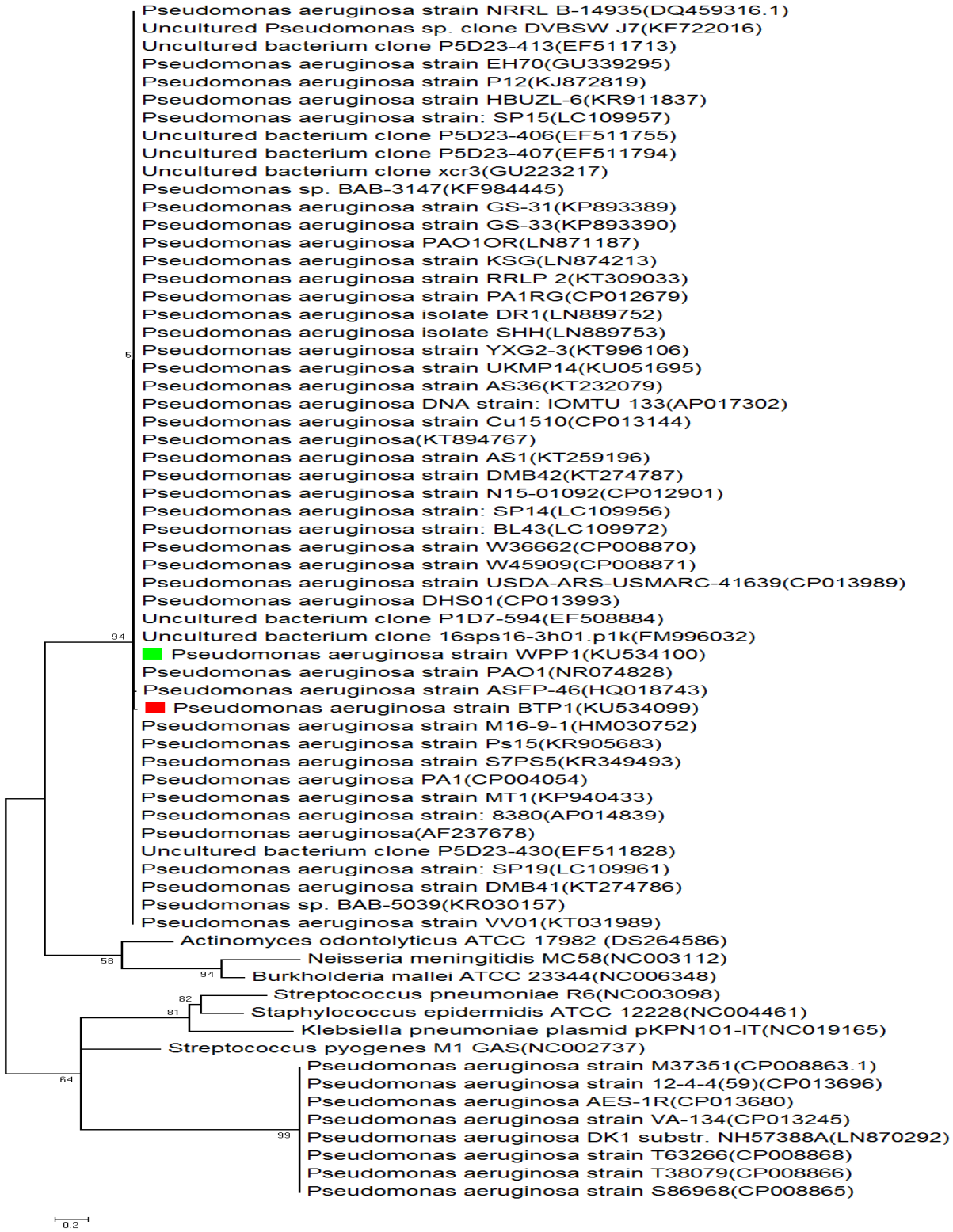

Fig. 3: Phylogenetic tree showing the inter-relationships of *Pseudomonas aeruginosa* strain BTP1 (KU534099) and *Pseudomonas aeruginosa* strain WPP1 with the most narrowly related 16S rRNA gene sequences of the genus *Pseudomonas.* DS264586, NC006348, NC003112, NC002737, NC003098, NC019165 and NC004461 were used as out group. The tree was generated using the neighbor-joining method with 1000 Bootstrap values in MEGA6. The accession number of each strain is shown in parenthesis. The analysis involved 67 nucleotide sequences.

Conclusions: Under the excessive use of antimicrobials, climate change and massive human and animal movement over long distances several bacterial pathogens are crossing the so called traditional species barriers of ecological adaptability. Here, we present the two most widely occurring and environmentally stable representative *Pseudomonas aeruginosa* strains recovered from various clinical environments i.e. garden soil, clinical instruments, wounds puss and throat swabs of humans and animal. We not only confirmed their pathogenicity but also report that irrespective of their origin of isolation (human, animal or environment) they retain their virulence highlighting their zoonotic potential and one health concerns. These observations suggest that perhaps these most prevalent

strains have somehow gained more ecological adaptability owing to miss management on part of the hospital administration and patients as well suggesting that these strains have stronger genetic adaptability characteristics, a distinctive feature of *P. aeruginosa* and have major alarming situation to health concern departments.

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Author's contribution: HH and KR conceived, designed and performed the experiments, and also compiled the manuscript; IH, FD and MSM participated in sample processing, data analysis and manuscript writing. All authors read and approved the final version of manuscript.

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