RESEARCH ARTICLE

Detection and *in silico* Analysis of *rmpA* Gene from Soilborne Pathogenic Bacteria

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ABSTRACT

Soil serves as an ecosystem for diverse microbes that perform various roles and that range from useful organisms in biological and geological processes to dangerous transmitters of diseases. Most types of soilborne bacteria are harmful to humans and causing severe problems. *Klebsiella pneumonia* considers a serious nosocomial pathogen that causes pneumonia, UTIs, wound, and liver abscesses. One hundred fifty (150) soil samples isolated randomly during the period of November 2014- February 2015. Bacterium *Klebsiella pneumoniae* was the commonest pathogen (36.70%), followed by *Staphylococcus aureus* (22.78%), *Staphylococcus epidermidis* (12.65%), *Bacillus spp*. (10.12%), *Pseudomonas aeruginosa* (5.06%), *Enterobacter cloacae* (5.06%), *Streptococcus spp.*(2.53%), *Citrobacter spp.*(1.26%) and *Pantoea spp.*(1.26%), respectively. All *K. pneumoniae* isolates detected and confirmed by conventional PCR using *rmpA* gene size 530bp. The positive isolates were sequenced and search for homology. The multiple sequence alignment showed that our bacterial gene have differences in NAD-dependent epimerase and NAD(p)-binding domain sequence at different sites. The differences in the nucleotides at gene sequence for the isolated strain which may give new approach for developing higher sensitive *K. pneumoniae* strains and for better understanding of the *rmpA* gene structure that should provide new tools for disease management.

Keywords: *In silico*, *Klebsiella pneumonia*, Phylogeny, Soilborne.

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INTRODUCTION

Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae, and protozoa. Each of these groups has characteristics that define them and their functions in soil.[1] There are too many species of soil pathogens. A few species of bacteria in the soil can prove dangerous or even deadly to humans.[2]

A wide range of public health issues, such as infectious diseases, acute toxic effects, and allergies can arise from the exposure to the microorganisms.[3] People, especially the patients, are not only the sole source for the production of the airborne microorganisms. Other sources, however, can produce airborne microorganisms, such as different institutions where sick and injured people are treated and other outdoor conditions and surroundings.[4] In addition to their negative effects on the environment, airborne microbes are also metabolically active and well adapted for the harsh atmospheric conditions.[5] The infectious disease coming from *Klebsiella* is caused mainly by *K. pneumonia* and *K. oxytoca*. They are opportunistic bacterial pathogens associated with nosocomial infections such as urinary tract infection (UTI), pneumonia and septicemia.[6] In this study, we aimed to identify types of bacteria in soil, confirmed by molecular methods, and study the evolutionary aspect of the detected species.

MATERIALS AND METHODS

Samples

A total of 150 isolates were collected randomly from various areas in AL-Samawa City during the period from November 2014 to February 2015. The isolates were collected under sterilized conditions using sterilized cotton swabs.

Isolation and Identification of Klebsiella pneumonia

Several different media were used for the culture of *Klebsiella pneumoniae* from the isolates such as nutrient agar, NIH broth, EMB agar, Brain heart infusion broth, S.S. agar, MacConkey agar, MSA and Blood agar.[7] The media were prepared under sterilized conditions at the laboratory of Biology department/ College of Science/Al Muthanna University. The identification of gram-negative bacteria was performed by standard biochemical methods (Catalase test, Oxidase test, API 20E test) according to[7].

Molecular Detection of Klebsiella pneumonia

Bacterial genomic DNA of *Klebsiella pneumoniae* isolates were extracted by using (ZR Bacterial DNA MidiPrep™,

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Zymoresearch, USA), and done according to company instructions. For the identification and detection of *Klebsiella pneumoniae, rmpA* gene was used. The gene primer (Table 1) was designed by Aher.[8]

PCR master mix reaction was prepared by using (2xHot start PCR Mastermix), and this master mix was done according to company instructions. The mastermix including (1ng/µL) DNA template, 2xHot start PCR mastermix(12.5 µL), each of forward and reverse gene primer (0.5 µL), Nuclease-free H2O (10.5 µL) for the total of (25µL). After that, these PCR master mix reaction components placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components needed for PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, stabilizer and tracking dye). Then the tube placed in Exispin vortex centrifuge for 3 minutes. They were then transferred to Mygene PCR thermocycler. The PCR thermocycler conditions were set as 30–35 cycles of Denaturation 94ºC for 30 second; Annealing 55ºC for 30 second; Extention 72 ºC for 5 minutes.

Statistics Analysis

Statistical analysis was performed using the SPSS computing program for the analysis of the obtained results (SSPS, 2008).

RESULTS

Isolation and Identification of Bacteria

A total of 150 isolates were collected randomly from different areas in AL-Samawa City during the period of November 2014-February 2015. Our results include 100 isolates from the soil, 79 of them were positive in the growth culture while 11 were negative (with Percentage of 79%). *Klebsiella pneumoniae* was the commonest pathogen (36.70%) followed by *Staphylococcus aureus* (22.78%), *Staphylococcus epidermidis* (12.65%), *Bacillus spp.* (10.12%), *Pseudomonas aeruginosa* (5.06%), *Enterobacter cloacae* (5.06%), *Streptococcus spp.* (2.53%), *Citrobacter spp.*(1.26%) and *Pantoea spp.*(1.26%), respectively (Figure 1).

Diagnosis of Bacterial Isolates

The bacterial isolates were activated using Alternative Thioglycolate Medium (NIH Thioglycolate Broth) for sterility purposes, also to isolate and grow the largest possible number of bacterial species which cultured by streaking on different media at a temperature between 35–37Cº for 24–48 hour.[9] The results showed mucoid colonies with diffusing red pigment on MacConkey agar and were non-hemolytic on blood agar. Bacterial swab stained by gram stain and examined under the light microscope showed that *K. pneumoniae* are small gram-negative rods.

Molecular Detection of K. pneumonia

Three positive isolates were subjected to conventional PCR for the confirmation of *K. pneumoniae* using *rmpA* gene as a diagnostic gene. It has been shown that all of them were positive for *rmpA* gene (Figure 2).

Bioinformatics Analysis

For sequence analysis, *rmpA gene* was deposited at the NCBI database under the GeneBank accession number KX668629. The sequenced PCR products were submitted for the sequence of similarity using BLASTnt of NCBI. The results showed that our gene sequence was showed a high degree of homology with *K. pneumoniae* strain B-941 and *K. pneumoniae* strain Mich. 61204 (94% of identity), and 93% for other studied species (Table 2). The ORF region that encode for *rmpA* protein was detected using ORF finder program. The result showed that the actual part of *rmpA* gene is 327bp in size encodes for *rmpA* protein (108 amino acids). Two structural domains in *rmpA*

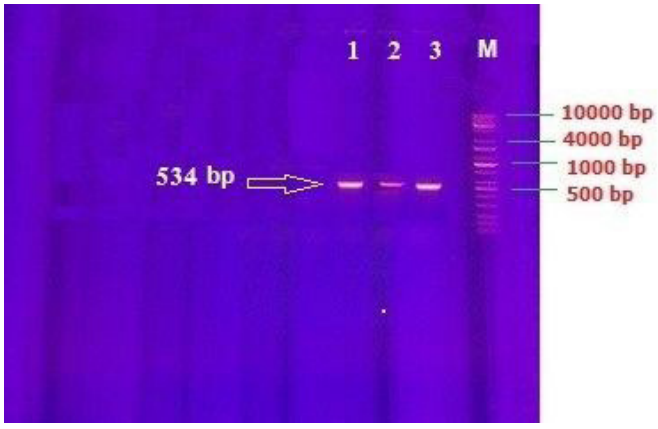

Figure 1: The Percentage of soil isolated bacterial species.

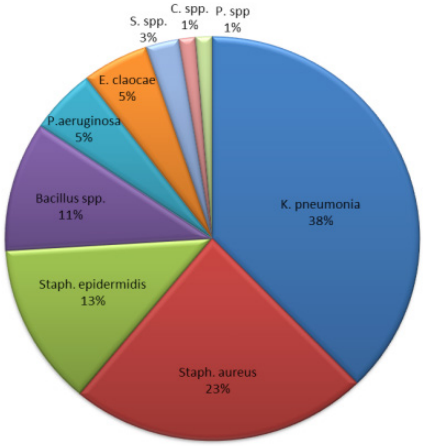

Fig. 2: Agarose gel electrophoresis image that shows the PCR product analysis of *rmpA* gene (Virulence factor gene) in *Klebsiella pneumoniae*. Where M: marker (10000bp), lane (1-3) positive for virulence factor gene at (530bp) PCR product.

Table 1: The primer used in this study

gene	Primer Sequence		size	source
rmpA	F	TCTTCACGCCTTCCTTCACT	530 bp	Aher et al. (2012)
	R	GATCATCCGGTCTCCCTGTA		

Table 2: The homology between *K. pneumoniae rmpA gene* and other studied strains

Species strain	*Sequence ID*	*Identity*
Klebsiella pneumoniae	KX668629	-
Klebsiella pneumonia strain B-941/14	KP760052.1	94%
Klebsiella pneumonia strain Mich. 61 O4 lipopolysaccharide biosynthesis gene	KU310493.1	94%
Klebsiella pneumonia strain F-35	KP760055.1	93%
Klebsiella pneumonia strain I-6208	KP760056.1	93%
Klebsiella pneumonia strain B-690/14	KP760057.1	93%
Klebsiella pneumonia strain B-958/14	KP760053.1	93%

protein, NAD-dependent epimerase, and NAD (p)- binding domain (Figure 3), were analyzed further by multiple sequence alignment (Figure 3).

The MSA showed a highly conserved area in all studied strains. However, there are differences in the type of nucleotides in some places. Changes were observed in our sequence at the positions (C:18,C:24,C:42,G:63,C:151,T:163), respectively, where nucleotides C, G, and T only located in our strain while other sequences were highly conserved with C, T, A, and G.

DISCUSSION

Clinical and Epidemiological Features

Klebsiella pneumonia was the commonest pathogen (36.70%) followed by *Staphylococcus aureus* (22.78%), *Staphylococcus epidermidis* (12.65%), *Bacillus spp.* (10.12%), *Pseudomonas aeruginosa* (5.06%), *Enterobacter cloacae* (5.06%), *Streptococcus spp.* (2.53%), *Citrobacter spp.*(1.26%), and *Pantoea spp.*(1.26%), respectively. A previous study by Jones *et al.,*[10] showed that *Klebsiella pneumonia* was the third most commonly isolated gram-negative microorganism behind *Pseudomonas aeruginosa and Escherichia coli.* Also, *Klebsiella spp.* are reported to be responsible for (7–10%) of bloodstream infections in Europe, North America, and South America according to data collected by the SENTRY Antimicrobial Surveillance Program.[11] According to a study by Rudan *et al.*,[12] reported that *Klebsiella pneumonia* and *Staphylococcus aureus* were the most responsible cause of severe cases of pneumonia among children aged under 5 years, and since the individuals spend most of their time outdoors[13], So we focused on the pathogen *Klebsiella pneumonia* in this study for this reason since it comprises the majority of soil pathogens.

Diagnosis of Bacterial Isolates

The bacterial isolates were collected under sterilized conditions using sterilized cotton swabs. Samples had been activated using Alternative Thioglycolate Medium (NIH Thioglycolate Broth) for sterility purpose and also in order to isolate and grow the most significant possible number of bacterial species[9], which cultured by streaking on different media at a temperature between (35–37C°) for (24–48 hour). In the primary culture, It was found that culturing on media without using the anti-fungal (Nystatin) and doing subculture on media containing Nystatin was better to get bacterial growth, as reported in a study by Haidaris and Brownlow showing that soil fungi are the most common environmental contaminants that grow rapidly as fuzzy colonies on a bacterial plate. They found out two prominent sources of contamination, which are the introduction of microbes from the environment and the mix-up of two or more closely related strains of bacteria[14]. The bacteria capable of transmission through aerosols and can survive in high RH and in low temperatures.[15,16] A previous study by[17] demonstrated that the survival of *K. pneumonia* was, to some extent, dependent upon relative humidity 60%.

Molecular Analysis

In our study, we used conventional PCR, which is a commonly used technique to determine the presence of virulence gene *rmpA* (encoding for uridine diphosphate galactourinate

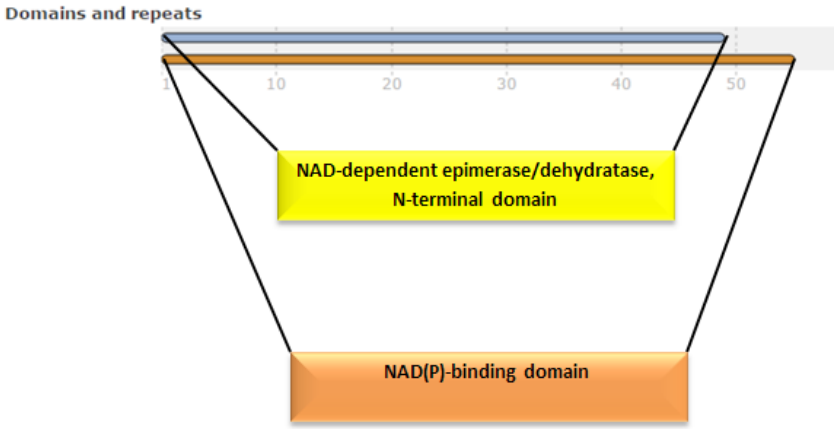

Figure 3: The main domains in open reading frame encoding for *rmpA* gene in *Klebsiella pneumonia*

4-epimerase) that associated with the virulence in *K. pneumoniae*. A study by Regué[18] proved that the mutant strains of *K. pneumoniae* (without *rmpA* gene) were non-virulent in laboratory animals; this fact proves the important role of *rmpA* gene in pathogenicity of *K. pneumoniae*.

All *K. pneumoniae* isolates tested by conventional PCR in this study were positive for *rmpA* gene. The positive isolates of *rmpA* gene were sequenced for homology using BLASTnt. The sequenced PCR product results showed that *rmpA* gene sequence was showed a high degree of homology with *K. pneumoniae* strain B-941 and *K. pneumoniae* strain Mich. 61 O4 (94% of identity), and 93% for other studied species. The *K. pneumoniae* strain B-941/14 was isolated in Russia from endotracheal aspirate of *K. pneumoniae sub.pneumoniae wabG* gene (620bp) which encodes for glucosyltransferase[19] which showed a high similarity with our gene 94% of identity, Also the *K. pneumoniae* strain Mich. 61 O4 lipopolysaccharide biosynthesis gene was isolated by Fange[20] that encodes for phosphogluconate dehydrogenase in *K. pneumoniae*.

According to Cortes,[21] capsule associated gene (*rmpA*) promotes infection by resistance to phagocytosis. This gene was commonly found in *K. pneumoniae* isolates, it seem to be at the basis of pathogenicity of *K. pneumoniae*.[21] Previous research conducted by Ho[22] on mutant ugd based on both anti K1 serum test by double immunodiffusion assay and string test, all mutants obtained lost the K1 serotype and mucoviscosity while remaining O1 serotype positive, suggesting that these mutants produce little or no CPS. Moreover, the deletion of *rmpA* in the proposed *lps* locus led to the loss of O1 serotype, confirming the proposed boundary between cps and *lps* loci. Since the deletion mutants make little or no CPS, they are expected to lose pathogenicity also.[23]

The multiple sequence alignment (MSA) showed that there are some differences between our sequence and other studied strains. In our sequence, the differences showed changes at regions: (C:18), (C:24), (C:42), (G:63),(C:151), (T:163). These differences in our gene sequence may indicates special characterizations for this strain. Also in fungi the *rmpA* gene is predicted to encode a putative UDP glucose-4-epimerase gene (*rmpA*), required for the biosynthesis of Galf as well as for Galp metabolism in *Aspergillus niger*. The mutation in the *rmpA* gene of mutant (A to G) caused the change of a codon from AAC to GAC which consequently resulted in the change of Asn to Asp at position 191 in the *rmpA* protein.[24] A study by Seifert 25 on RHD1gene in *Arabidopsis thaliana* concluded that the flux of galactose from UDP-D-Gal into different downstream products is compartmentalized at the level of cytosolic *rmpA* iso-forms. This suggests that substrate channeling plays a role in the regulation of plant cell wall biosynthesis, and the single *rmpA* isoforms from *Homo sapiens* and *Escherichia coli* and the amino-terminal moiety of Gal10 from *Saccharomyces cerevisiae* are 53%, 49%, and 48% identical to RHD1, respectively.

A previously detection of *rmpA* gene by Aher and Kumar[8] in isolated strains of *K. pneumoniae* from goats revealed that this gene was the second most prevalent virulence gene. A NAD-dependent epimerase/dehydratase (N-terminal) domain is predicted to occupy amino acids 1 to 50 in *rmpA* sequence where the NAD (p) binding domains occupy amino acids 1 to 55 in the gene (Figure 3). The *rmpA* catalyzes the conversion of UDP-D-glucuronate to UDP-D-galacturonate, which is also present in both bacterial capsules and LPS. The effects of a *rmpA* null mutation on colonization and virulence were studied in *K. pneumoniae* 52145, which is a highly virulent strain able to colonize different surfaces. A *rmpA* deletion reduced colonization and rendered the strain completely avirulent in an experimental model of pneumonia. This suggests that the *rmpA* mutation could have important, measurable effects on colonization and virulence.[21] The ORF results of the region encodes for *rmpA* protein showed that the actual part of *rmpA* gene is (327bp) in size encodes for *rmpA* protein (108 amino acids). The proteins encoded by this operon are also involved in the degradation of urea in ammonia and carbamate in carbonic acid, causing PH decreasing and allow survival to acid environment, like metabolic acidosis in hosts with sepsis.[25]

CONCLUSION

The soil considers as a suitable environment for aerosolized pathogenic bacteria. *Klebsiella pneumoniae* was the most rate bacteria in the outdoor environment that cause serious disease. *Klebsiella pneumoniae* comprises the high incidence rate of the nosocomial pathogenic bacteria. *The rmpA* gene is responsible of *Klebsiella pneumoniae* virulence, which contributes to the biosynthesis of lipopolysaccharides. *rmpA* gene sequence in this study was different in specific regions from other studied strains.

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