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RESEARCH ARTICLE

COMPARATIVE STUDY BETWEEN MOLECULAR AND CONVENTIONAL DETECTION METHODS OF *PANTOEA AGGLOMERANS* ISOLATED FROM IRAQI SEPTICEMIA PATIENTS

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ABSTRACT

Background: *Pantoea agglomerans* is an opportunistic human bacteria characterized by shaped aggregations or symplas-mata, yellow pigment-producing colonies that were 2 mm in size, nonhemolytic, and convex were detected on a blood agar plate. It belongs to the family *Enterobacteriaceae* and is responsible for infection diseases ranging from plant-thorn arthritis, osteoitis, osteomyelitis and traumatic wound infections to septicemia. Bacterial identification systems were performed by using manual and automated methods as phenotypic assays, and PCR method as molecular assay.

Aims and objectives: The aims of the present study were to review the impact of *Pantoea agglomerans* in septicemia cases, and study the role of 16S rRNA gene in the molecular detection of *Pantoea agglomerans* in patients with clinical diagnosis of septicemia.

Materials and Methods: In this study blood samples from 75 patients with clinical diagnosis of septicemia were used for blood culture, Gram staining, culture, VITEC-2 Compact and PCR methods. PCR was performed with primer pair targeted to the 16S rRNA gene of *Pantoea agglomerans*. The result of the PCR was compared with conventional methods of blood culture, Gram staining, culture and VITEC-2 Compact methods. The PCR positive samples were identified by presence of ~1500 bp amplicon of the 16S rRNA gene.

Results: Conventional methods of blood culture, Gram staining, culture, VITEC-2 Compact methods showed a positive results in 14 (18,6 %) of 75 patients with clinical diagnosis of septicemia. PCR detected all 14 (18,6 %) samples that were positive by conventional method. Two (2.6 %) of 61 (81,3 %) patients who were negative by conventional methods were positive by PCR method. Statistical analysis revealed that the PCR to have a sensitivity of 95.1 % in the detection of *Pantoea agglomerans* in septicemia cases.

Conclusion: Polymerase chain reaction (PCR) is a rapid, specific and sensitive method to detect *Pantoea agglomerans* in septicemia cases of human.

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INTRODUCTION

Bacteria of the family *Enterobacteriaceae* are ubiquitous pathogens, causing intestinal and systemic illness of humans and other animals. The genus *Pantoea*, a member of the *Enterobacteriaceae* family, includes a large group of Gram-negative bacteria isolated from plants, soil, and water, as well as human diseased tissues (Tian and Jing, 2014; Deletoile *et al*., 2009). In the early years, species of *Pantoea* genus are known as plant-pathogenic strains or plant associated bacteria (Brady *et al*., 2007), but the growing infection of *Pantoea* species being isolated from humans indicates that *Pantoea* is an opportunistic human (Nadarasah and Stavrinides, 2014).

The genus *Pantoea* consists of two groups of species: the core *Pantoea* species including *P. agglomerans*, *P. dispersa*, *P. ananatis* and *P. stewartii* and the "Japanese" species including *P. citrea*, *P. punctata* and *P. terrea* (Grimont and Grimont, 2005; Brady *et al*., 2008). All species of the genus *Pantoea* are diverse in their origin and geographical spread and can be isolated from feculent material, plants, and soil, where they can either be pathogens or commensals. Within the genus, *P. agglomerans* is the most commonly isolated species from clinical samples in humans, many isolates of *Pantoea* have been identified as the causal agent soft tissue or bone/joint

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infections following penetrating trauma by vegetation. As an opportunistic human pathogen, *P. agglomerans* can occur sporadically or in outbreaks (Mehar *et al*., 2013; Nadarasah and Stavrinides, 2014).

The most common infection caused by *P. agglomerans* is septic arthritis or synovitis, ostitis, osteromyelitis, cholelithiasis, occupational respiratory infections and skin allergy, peritonitis, and blood stream infections (Kim *et al*., 2008; Gora *et al*., 2009). Clinical reports predominantly involve septicemia following penetrating trauma or nosocomial infections. *P. agglomerans* infections are typically of a polymicrobial nature involving patients affected by other diseases and may represent secondary contamination of wounds (Rezzonico *et al*., 2010).

Conventional studies have highlighted the difficulties in identifying *P. agglomerans* strains based on commercial phenotypic identification systems. Identification of *Pantoea* species has long been based on phenotypic and biochemical characteristics. These physiological and biochemical tests were performed on selected isolates using API 20E and Vitek 2 systems. However, as there is a high degree of phenotypic similarity between genera of the *Enterobacteriaceae*, this has led to the misidentification of many strains. In recent years identification of strains has been based on PCR assays with species-specific primers, as is the case with *P. agglomerans*, genotypic identification was obtained by 16S rRNA gene detection and sequencing (Cheng *et al*., 2013).

MATERIALS AND METHODS

Samples collection

Samples were obtained from patients with clinical diagnosis of septicemia in Ibn Al-Baladi Hospital and Al Kadhmiya teaching hospital, Baghdad, Iraq for a period of 4 months. Seventy five cases of septicemia were investigated in this study. Patients' ages ranged from 15 to 30 years of age. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq.

Five milliliter of blood sample was obtained from 75 patients with clinical diagnosis of septicemia and directly added to the appropriate BacT/Alert broth (as a culture media), then the samples were incubated in BACT/ALERT instrument at 37°C for 7 days. Automated biochemical identification methods for positive samples were performed using VITEKk-2 compact (Biomerieux, France). The bacterial isolates from positive samples were activated using brain heart infusion broth and incubated at 37°C for 18 hrs, then the Gram stains were performed and cultured on Nutrient agar, MacConky agar, blood base agar and chocolate agar (Merck, Germany) at 37°C for 48 hours under aerobic condition as deferential and enrichment media for *P. agglomerans* from other *Enterobacteriaceae* members.

DNA extraction

Genomic DNA was extracted from the *P. agglomerans* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of an overnight *P. agglomerans* culture grown at 37°C in brain heart infusion broth (Sigma, USA) was transferred to a 1.5 ml microcentrifuge tube. The microcentrifuge tube was centrifuged at 14,000 rpm for 3 minutes to pellet the cells and the supernatant was removed. 600 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added and gently pipet until the cells are resuspended. The microcentrifuge tube was incubated in water bath at 80°C for 5 minutes to lyse the cells; then cool to room temperature. 3 µl of RNase solution (wizard genomic DNA purification kit) was added to the cell lysate and the microcentrifuge tube was inverted for 5 times to mix. The microcentrifuge tube was incubated at 37°C for 60 minutes and cool to room temperature. 200 µl of protein precipitation solution (wizard genomic DNA purification kit) was added to the RNase-treated cell lysate and vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate. The microcentrifuge tube was incubated on ice for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The microcentrifuge tube was gently mixed by inversion until the thread-like strands of DNA form a visible mass and centrifuged at 14,000 rpm for 5 minutes. The supernatant was carefully pour off and the microcentrifuge tube was drained on clean absorbent paper. 600 µl of room temperature 70% ethanol was added and then the microcentrifuge tube was gently inverted several times to wash the DNA pellet. The microcentrifuge tube was centrifuged at 14,000 rpm for 2 minutes and the ethanol was carefully aspirated. The microcentrifuge tube tube was drained on clean absorbent paper and the pellet was allowed to air-dry for 15 minutes. 100 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added to the microcentrifuge tube and the DNA was rehydrated by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the microcentrifuge tube and the DNA sample was stored at -20°C until use.

DNA quantification

The extracted DNA from the *P. agglomerans* isolates was quantified spectrophotometrically at O.D. 260/ 280 nm with ratios 1.6-1.7. The sensitivity of the 8FPL and 1492RPL primers was evaluated by PCR amplification for serial diluted concentrations (10-100 ng) of purified genomic DNA isolated from *P. agglomerans.*

Primers selection

The primers for 16S rRNA gene of *P. agglomerans* as the target gene for this study were selected according to (Cheng *et al*., 2013).

This set of primers was designed based on the conserved region in *P. agglomerans*, primers were synthesized by Alpha DNA, Kanda. The primers sequence of 16S rRNA gene and their size of product are shown in (Table 1).

Table 1. The primers sequences of 16S rRNA gene of *P. agglomerans* and their product size

Name of Primer	Sequence of Primer (5'-3')	Size of Product
8FPL	AGAGTTTGATCCTGGCTCAG	~150
1492RPL	GGTTACCTTGTTACGACT	0 bp

PCR Master Mix

The PCR reactions for detection of 16S rRNA gene of *P. agglomerans* were performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol 8FPL primer and 2.5 µl of 20 pmol 1492RPL primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil.

PCR program

PCR was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Cheng *et al*., 2013), with some modification. Briefly, the Amplification of 16S rRNA gene of *P. agglomerans* was carried out with initial denaturation at 95°C for 6 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 8FPL and 1492RPL primers for 90 seconds, and extension at 72°C for 2 minutes. The thermal cycles were terminated by a final extension for 10 minutes at 72°C. Positive control and Nuclease free water as a negative control were used too.

PCR products analysis

The analysis of PCR products of 16S rRNA gene of *P. agglomerans* were performed on 1% agarose gel. The 1 kb DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The PCR products were stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

RESULTS

Conventional methods

Of the 75 patients with clinical diagnosis of septicemia, a positive result of *P. agglomerans* in 14 (18,6 %) patients was detected by the conventional methods of blood culture, Gram staining, culture; Nutrient agar (Figure 1), MacConcky agar (Figure 2), blood base agar (Figure 3), chocolate agar (Figure 4) and VITEC-2 Compact.

Analysis of extracted DNA of *Pantoea agglomerans* isolates

After performing of the DNA extraction from *Pantoea agglomerans* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1 % agaros gel at 7 volt/ cm for 1 hour (Figure 5).

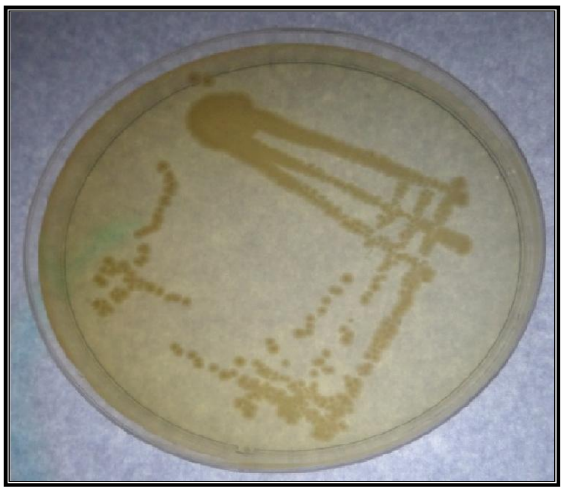

Figure 1. Yellow colonies of *Pantoea agglomerans* grow on nutrient agar media

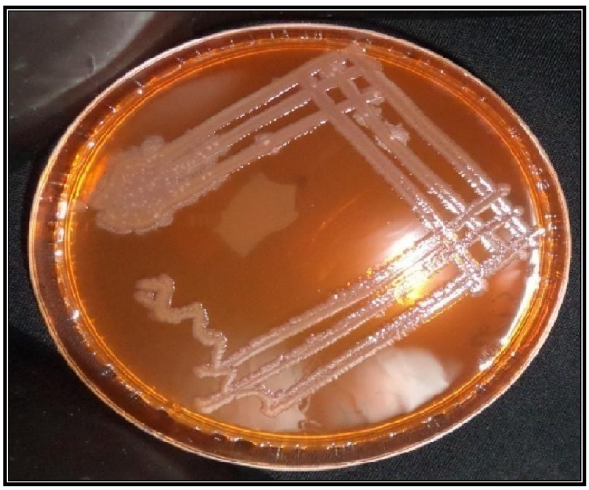

Figure 2. Creamy colonies of *Pantoea agglomerans* grow on MacConcky agar media

Figure 3. Creamy colonies of *Pantoea agglomerans* grow on blood base agar media

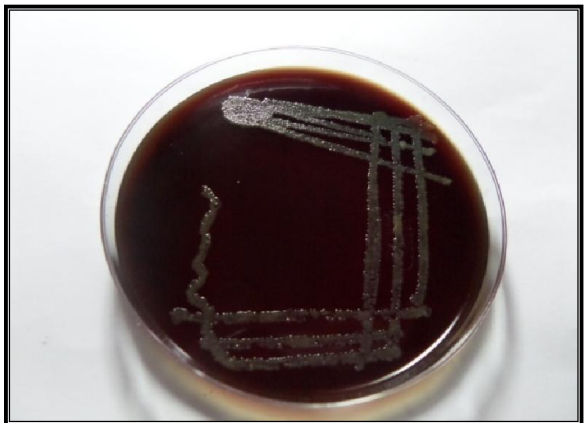

Figure 4. Creamy colonies of *Pantoea agglomerans* grow on chocolate agar media

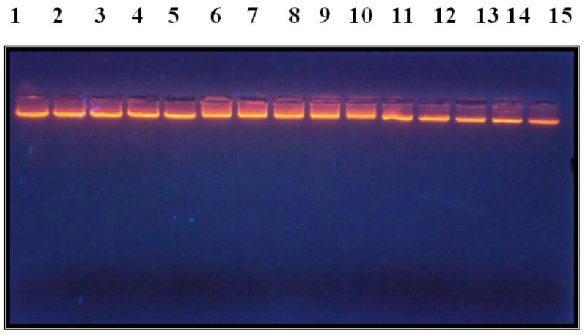

Figure 5. Gel electrophoresis of extracted DNA of *Pantoea agglomerans* isolates using 1 % agarose gel at 7 volt/ cm for 1 hour. Lane 1-15: Extracted DNA

Analysis of PCR products of *Pantoea agglomerans* 16S rRNA gene

On the basis of the 16s rRNA gene sequence, a product of ~1500 bp was amplified by PCR with 8FPL and 1492RPL primers. In the 75 patients with clinical diagnosis of septicemia, a positive result for *Pantoea agglomerans* was detected in 16 (21.2 %) patients (14 (18,6 %) patients that were positive by conventional and PCR methods; Two (2.6 %) patients who were negative by conventional methods and positive by PCR method.

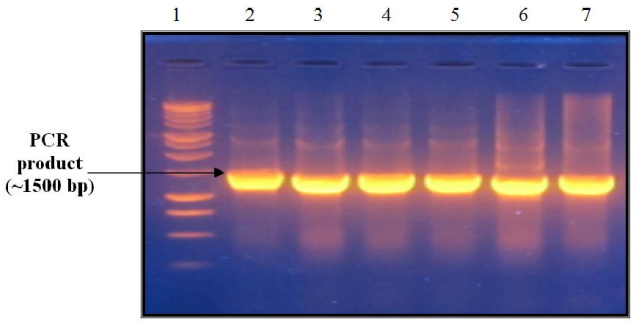

Figure 3. Gel electrophoresis of 16 rRNA PCR products of *Pantoea agglomerans* isolates using 1 % agarose gel at 7 volt/ cm for 1 hour. Lane 100: 1000 bp DNA ladder, lane 2-7: 16S rRNA PCR products of *Pantoea agglomerans* isolates

The PCR products and 1 kb DNA ladder were resolved by electrophoresis. 5 µl of the PCR product were loaded on 1% agarose gel and run at 100 volt/ cm for 45 minutes. The gel was stained with ethidium bromide solution (0.5 µg/ ml) for 15-30 minutes; finally, bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system. PCR result was considered positive for *Pantoea agglomerans* when there was presence of ~1500 bp PCR product band on agarose gel electrophoresis, no amplification was observed with negative control (Figure 6).

DISCUSSION

P. agglomerans, formerly called *Enterobacter agglomerans* and *Erwinia herbicola*, is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae* where they can be either pathogens or commensals (Derek *et al*., 2013). This study was described septicemia caused by *P. agglomerans* and identification performed by conventional methods and molecular method or PCR technique. *P. agglomerans* has been isolated from the blood stream infections; which may caused by association with the contamination of intravenous fluid, total parenteral nutrition, the anesthetic agent propofol and blood products. Biochemical tests are largely used for bacterial identification in clinical laboratories (Brady *et al*., 2008). The full VITEK-2 compact profile identified 14 (18,6 %) of 75 patients clinical diagnosis with septicemia. The advantages of conventional methods were non costly but the disadvantages of those methods were consuming time, contamination present, false positive result and require a large amount of sample (Chen *et al*., 2000).

In current study, the molecular or PCR method for detection of *Pantoea agglomerans* 16s rRNA gene (~1500 bp) using 8FPL and 1492RPL primers showed a positive result in 16 (21.2 %) of 75 patients clinical diagnosis with septicemia. The benefits of molecular methods are more sensitive, more qualitative for results, materials available, but the drawback of molecular methods is costly. These explanations made molecular methods relatively more accurate than conventional methods (Kim *et al*., 2013).

In prokaryotes, 16S rRNA gene, the small subunit of rRNA gene which contained more highly conserved regions interspersed with variable and hyper variable sequences making it easier to design universal primers (Ludwig and Schleifer, 1999). The degree of conservation may be caused by the importance of the 16S rRNA gene as a critical component of cell function. Conventionally, this gene played a limited role in the identification of microorganisms in clinical microbiology laboratories, the causes commonly resulted from the high costs, requirements for great technical skill, and the lack of user-friendly comparative sequencing analysis software and validated databases (Clarridge, 2004).

At a comparison between the conventional and molecular methods, we think that the incubation period is uncertain and inappropriate growth media, in addition to the contamination of culture in identification methods could be the reason for negative results, this may explain the negative culture results in 2 (2.7 %) of 75 patients clinical diagnosis with septicemia,

whereas the PCR method detected all 14 (18,6 %) of 75 patients who were positive by conventional method and the two (2.6 %) of 61 (81,3 %) patients who were negative by conventional methods. Statistical analysis revealed that the PCR to have a sensitivity of 95.1 % in the detection of *Pantoea agglomerans* in septicemia cases. This explains that the molecular diagnosis of *Pantoea agglomerans* by the PCR method was more sensitive and efficiency than the diagnosis of these bacteria by conventional methods**.** This data agrees with the study by Cheng *et al.* (2013) and confirms the efficacy of the PCR assay compared to conventional methods of diagnosis in the clinical setting.

Conclusion

Many evidence indicate that *P. agglomerans* are based upon inaccurate isolate identification, resulting from inadequate identification methods and both biochemical profiling tests (manual or automated) lack the resolution needed to discriminate *P. agglomerans* isolates, on the other hand, 16S rRNA gene sequence, appeared to be useful for determination of *Pantoea* spp.

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REFERENCES

Brady, C.; Cleenwerck, I.; Venter, S.; Vancanneyt, M.; Swings, J. and Coutinho, T. 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Syst. *Appl. Microbiol*., 31: 447-460.

Brady, C.; Venter, S.; Cleenwerck, I.; Vancanneyt, M.; Swings, J. and Coutinho, T. 2007. A FAFLP system for the improved identification of plant-pathogenic and plant-associated species of the genus *Pantoea. Syst Appl. Microbiol.*, 30: 413-417.

Chen, Y.; Eisner, J.; Kattar, M.; Rassoulian-Barrett, S.; LaFe, K.; Yarfitz, S.; Limaye, A. and Cookson, B. 2000. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J. Clin. Microbiol.,* 38(6): 2302-2310.

Cheng, A.; Liu, C.; Tsai, H.; Hsu, M.; Yang, C.; Huang, Y.; Liao, C. and Hsueh, P. 2013. Bacteremia caused by *Pantoea agglomerans* at a medical center in Taiwan 2000-2010. *J. Microbiol. Immunol. Infect,* 46: 187-194.

Clarridge, J. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *J. Clin. Microbiol*., 17(4): 840-862.

Deletoile, A.; Decre, D.; Courant, S.; *et al.* 2009. Phylogeny and identification of *Pantoea* species and typing of *Pantoea agglomerans* strains by multilocus gene sequencing. *J. Clin. Microbiol*., 47(2): 300-310.

Derek, D.; Morgan, W. and Stavrinides, J. 2013. Draft genome sequenc of the antibiotic-producing cystic fibrosis isolate *Pantoea agglomerans* Tx10. *Genome Announc*., 1: 5 e00904-13.

Gora, A.; Mackiewicz, B.; Krawczyk, P. *et al.* 2009. Occupational exposure to organic dust, microorganisms, endotoxin and peptidoglycan among plants processing workers in Poland. *Ann. Agric. Environ. Med.* 16:143-150.

Grimont, P. and Grimont, F. 2005. Genus *Pantoea*, p. 713-720. In Brenner, D.; Krieg, N. and Staley, J. (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. The *Proteobacteria*. Springer-Verlag, New York, NY.

Kim, H.; Lee, H.; Kim, D. *et al.* 2008. A case of peritonitis caused by *Pantoea agglomerans* in a patient undergoing continuous ambulatory peritoneal dialysis. *Korean. J. Med.,* 74: 426-9.

Kim, J.; Hyung J.; Yong, B.; Yang, W.; Kyu, J. and Kee, C. 2013. Molecular Biological Identification of Malassezia Yeasts Using Pyrosequencing. *Ann. Dermatol*., 25(1): 73-79.

Ludwig, W. and Schleifer, K. 1999. Phylogeny of bacteria beyond the 16S rRNA standard. *ASM News,* 65: 752-757.

Mehar, V.; Yadav, D.; Sanghvi, J.; Gupta, N. and Singh, K. 2013. *Pantoea dispersa*: an unusual cause of neonatal sepsis. Braz. *J. Infect. Dis.,* 17(6): 726-728.

Nadarasah, G. and Stavrinides, J. 2014. Quantitative evaluation of the host-colonizing capabilities of the enteric bacterium Pantoea using plant and insect hosts. *Microbiology*, 1(160): 602-615.

Rezzonico, F.; Vogel, G.; Duffy, B. and Tonolla, M. 2010. Application of whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification and clustering analysis of *Pantoea* species. *Appl. Environ. Microbiol*., 76: 4497-4509.

Tian, H. and Jing, C. 2014. Genome sequence of the aerobic arsenate-reducing bacterium *Pantoea* sp. strain IMH. Genome Announc 2(2): e00267-14 http://dx.doi.org/10. 1128/genome A. 00267-14.

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