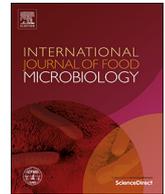




ELSEVIER

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Aversion center blackening of muskmelon fruit caused by *Pseudomonas oryziphantans*, an opportunistic pathogen of humans and warm-blooded animalsOkhee Choi^{a,1}, Yeyeong Lee^{b,1}, Byeongsam Kang^c, Juyoung Bae^c, Seunghoe Kim^b, Jinwoo Kim^{a,b,c,*}^a Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea^b Department of Plant Medicine, Gyeongsang National University, Jinju 52828, Republic of Korea^c Division of Applied Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea

ARTICLE INFO

Keywords:

Center blackening

Muskmelon

Pseudomonas oryziphantans

ABSTRACT

This is the first report of bacterial center blackening in muskmelon fruit caused by *Pseudomonas oryziphantans*, which is known as an opportunistic pathogen of humans and warm-blooded animals. The aim of this study was to investigate the microbiological characteristics of this infection. Bacterial center blackening, which can cause aversion in consumers, was observed in muskmelon fruit in South Korea in the fall of 2017. Symptoms included severe black pigmentation in the pulp surrounding the seeds inside muskmelon fruit. Dark brown pigmentation and gram-negative, non-spore-forming, rod-shaped pseudomonads were consistently recovered from the black pigmented pulp tissue of muskmelons. The symptoms after artificial inoculation were the same as those of the natural infection, while the control fruit exhibited no symptoms of infection. Using pathogenicity tests, analytical profile index (API) tests, whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and 16S rRNA gene and *gyrB* region sequencing, the dominant species was identified as *P. oryziphantans*. The recent outbreak indicates that *P. oryziphantans* poses a potential threat to the global production and transportation of muskmelon as well as food safety.

1. Introduction

Muskmelon or cantaloupe (*Cucumis melo* L.; family: *Cucurbitaceae*) is one of the most popular fruits worldwide. It is largely consumed for its sweetness, flavor, texture, and phytonutrients (Lester, 2008). It is also popular as a health food because it is rich in ascorbic acid, carotene, folic acid, potassium, and bioactive compounds, which are beneficial to human health (Lester and Hodges, 2008).

In 2017, bacterial center blackening, which can cause aversion in consumers, was observed in commercial muskmelon fruit in Jinju, South Korea. Symptoms included severe black pigmentation in the pulp around the seeds inside muskmelon fruit. No symptoms were observed in the bark of muskmelon fruit; however, the severe center blackening was undesirable to consumers. Severe stages of the disease are less of a risk to consumers, because infection can be identified and avoided; however, consumers should be careful when eating melons exhibiting low levels of black pigmentation, indicating the early stage of disease

development.

This study was conducted to inform melon growers and consumers of current outbreaks of aversion center blackening in muskmelon fruit. We performed identification tests of the microbial agent causing blackening of muskmelon fruit center using pathogenicity tests, analytical profile index (API) tests, whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and 16S rRNA gene and *gyrB* (DNA gyrase B subunit) region sequencing. Here, we present the first report of bacterial center blackening caused by *Pseudomonas oryziphantans* in muskmelon fruit. *P. oryziphantans*, an opportunistic pathogen of humans and warm-blooded animals, is known to cause septicemia, peritonitis, endophthalmitis, skin and soft tissue infections, and bacteremia (Freney et al., 1988; Panagopoulos et al., 2016; Tena and Fernández, 2015; Woo et al., 2014). The recent occurrence of the disease indicates that the species poses a potential threat to the global production and transportation of melon as well as food safety.

* Corresponding author at: Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea.

E-mail address: jinwoo@gnu.ac.kr (J. Kim).¹ Okhee Choi and Yeyeong Lee contributed equally to this work.<https://doi.org/10.1016/j.ijfoodmicro.2018.11.003>

Received 16 June 2018; Received in revised form 26 October 2018; Accepted 4 November 2018

Available online 06 November 2018

0168-1605/ © 2018 Elsevier B.V. All rights reserved.

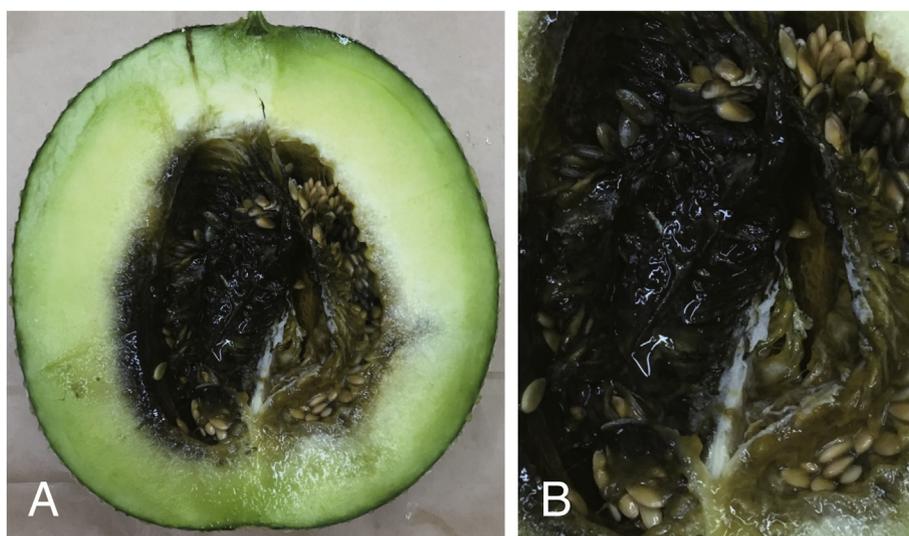


Fig. 1. (A) Symptoms of center blackening in muskmelon fruit sampled from a commercial market. (B) Enlarged image.

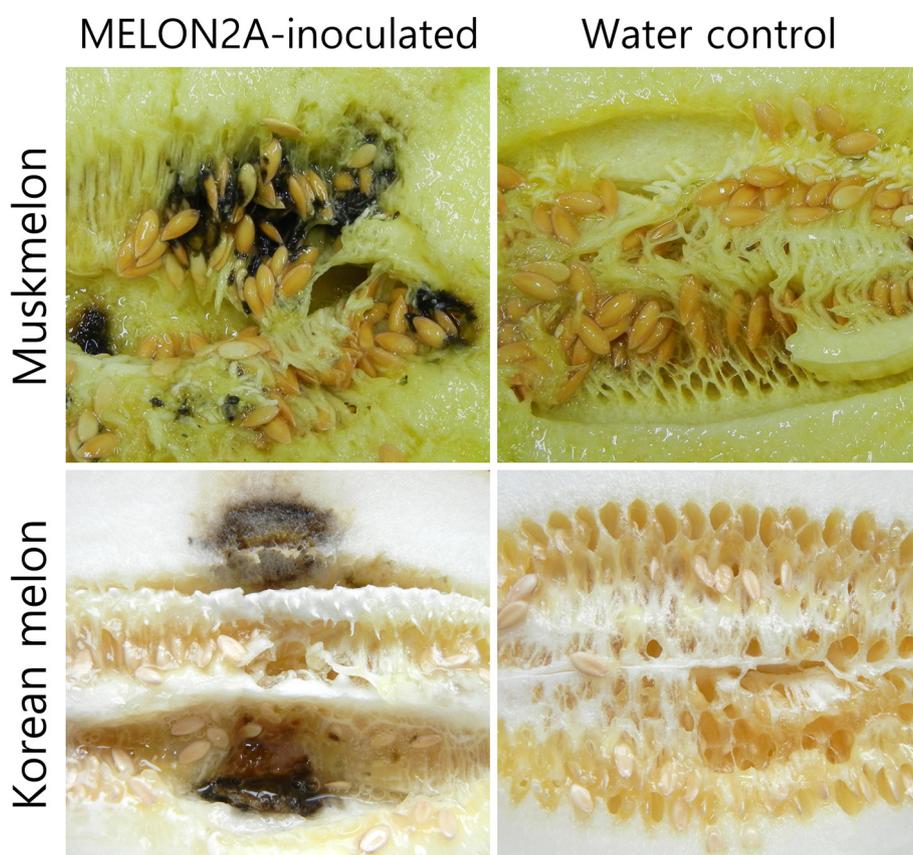


Fig. 2. Symptoms induced artificially 7 days after inoculation: muskmelon and Korean melon fruit inoculated with the bacterial isolates exhibited center blackening 7 days after inoculation; whereas those treated with sterilized distilled water were disease-free.

2. Materials and methods

Bacterial isolation was performed as described previously (Choi et al., 2016) with some modifications. Briefly, pulp tissues (3–5 mm) showing black pigmentation were transferred into 1.5-mL Eppendorf tubes containing 1 mL of sterilized distilled water and crushed with a pipette tip. The crushed tissues were left for 10 min and vortexed vigorously, and 100 μ L of each liquid suspension were serially diluted, and spread onto King's B (KB) and 0.5 \times tryptic soy agar (TSA) media. The plates were then incubated at 28 $^{\circ}$ C for 3–5 days. Colonies producing

dark-brown diffusible pigments grew predominantly on both media. Since there was no difference between media, KB agar medium was used for subsequent culture. The resulting dominant colonies were re-streaked onto new KB agar medium.

The inoculation techniques for the pathogenicity tests were performed as described previously with some modifications (Kim et al., 2012). Commercial muskmelon (cv. Santafe) and Korean melon (cv. Keum Je) fruits were purchased from a market. Fruits were surface-disinfected with 70% ethanol and dried. Prior to the inoculation, the isolates were streaked onto KB agar and incubated at 28 $^{\circ}$ C overnight;

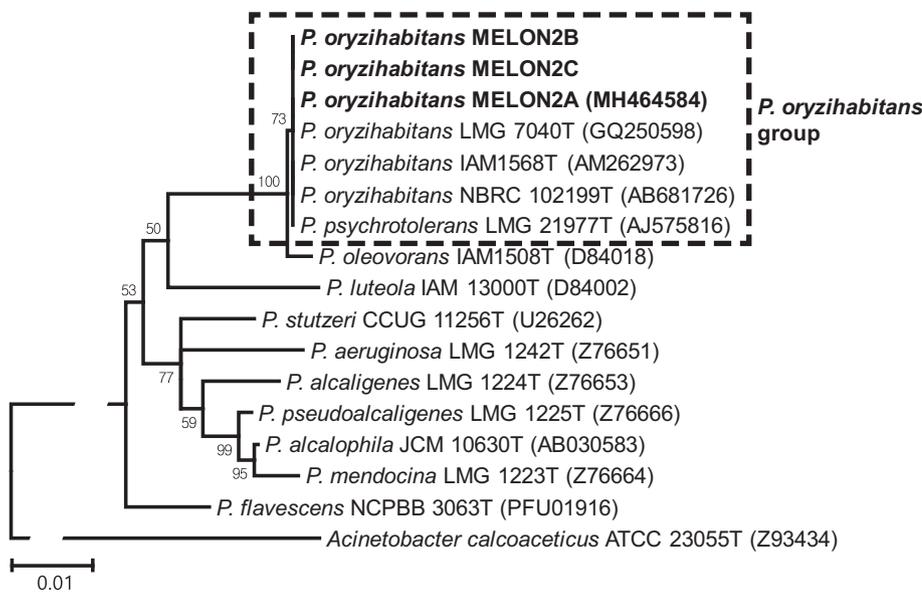


Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships among *Pseudomonas* spp. using the neighbor-joining method. The bacterial isolates from muskmelon fruit is shown in bold font. The numbers above the branches represent the bootstrap values. The bar indicates a change of 0.01 per sequence position.

colonies were harvested and suspended in 1 mL sterile distilled water. Each suspension was adjusted to a final dilution of 10^8 CFU/mL, and the inoculation dose was confirmed by serial titration. Three fruits were inoculated per isolate by injection with 1 mL bacterial suspension. Three additional fruits were injected with sterilized distilled water as a negative control. The inoculated fruits were placed in a plastic box containing moistened paper towels at room temperature for 1 week.

The physiological and biochemical characteristics of the three bacterial isolates were determined using the API 20NE system (Biomérieux, Marcy l'Etoile, France), according to the supplier's instructions. Whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and a search using the MALDI Biotyper software (ver. 3.0; Bruker Daltonics, Germany) were performed according to the manufacturer's instructions. Oxidase and tobacco hypersensitive reaction tests were performed as described previously with some modifications (Lelliott et al., 1966).

To confirm the identity of the causal bacteria, partial 16S rRNA and *gyrB* genes were amplified with primers 27mF (5'-AGAGTTTGATCMTGGCTCAG-3'), 1492mR (5'-GGYTACCTTGTTACGACTT-3'), and *gyr* AF3 (5'-CCGGCGGTAAGTTCGATGATAACTC-3'), *gyr* AR-B3 (5'-TGATCGCGGTACGGCCTTCACGGGC-3'), respectively (Lane, 1991; Sawada et al., 2002). Total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's directions. PCR was performed on T100 Thermal Cycler (Bio-Rad, California, USA) using PCR Premix (Bioneer, Daejeon, Korea), 1 μ L genomic DNA, and 1 mM each primer; under the following conditions: 98 °C for 2 min; 30 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 70 °C for 1 min; and final extension at 72 °C for 4 min. The amplified products were separated by electrophoresis on 0.8% (w/v) agarose gels. PCR amplification yielded only a single visible DNA product, and the product band was cleaved from the ethidium bromide stained gel and purified using the Expin Gel SV, 200 prep kit (GeneAll Biotechnology, Seoul, Korea), following the manufacturer's directions. The purified PCR products were sequenced by Macrogen Services (Daejeon, Korea) in both directions using the aforementioned primers (Lane, 1991). Additional primers 800R (5'-TACCAGGGTATCTAATCC-3') and 518F (5'-CCAGCAGCCGCGGTAA TACG-3') were used to generate sequences with complete coverage in both directions.

The DNA sequences were analyzed using the BLAST program, and the DNA sequences of the 16S rRNA gene were compared using the NCBI/GenBank database (<http://www.ncbi.nlm.nih.gov/blast/>). A phylogenetic tree was generated using the neighbor-joining method and

Tajima-Nei distance modeling in MEGA 4.1 (Tamura et al., 2007). The sequences of related bacterial strains were downloaded from the GenBank database.

3. Results and discussion

Unappealing center blackening was observed in commercial muskmelon fruit in South Korea. Symptoms included severe center blackening of the pulp around the seeds inside muskmelon fruit, while no symptoms were observed in the bark of the fruit (Fig. 1). Gram-negative, non-spore-forming, rod-shaped pseudomonads (Kodama et al., 1985) were consistently recovered from the black-pigmented pulp tissues of fruit. Three bacterial isolates (labeled MELON2A–C; isolated from the same fruit) were selected for further pathogenicity testing and identification. The resulting yellow colonies on the KB agar media were opaque, circular, and raised with entire margins, and the colonies produced dark brown pigments.

In the pathogenicity tests, muskmelon and Korean melon fruit inoculated with the bacterial isolates exhibited center blackening 7 days after inoculation, whereas those treated with sterilized distilled water were disease-free (Fig. 2). *P. oryzihabitans* was re-isolated from the lesions of the inoculated fruit, fulfilling Koch's postulates. The colony features of the bacteria re-isolated from the inoculated fruit were the same as those of the original isolates. The identity of the re-isolated bacteria was confirmed using the 16S rRNA gene sequencing.

The API 20NE tests revealed that D-glucose, L-arabinose, D-mannose, D-mannitol, potassium gluconate, malate, and trisodium citrate were utilized by all three bacterial isolates, while adipate, caprate, maltose, N-acetyl-glucosamine, and phenyl-acetate were not. All three bacterial isolates were positive for catalase and did not reduce nitrates into nitrites, but they were negative for the production of arginine dihydrolase, urease, β -glucosidase, β -galactosidase, protease, and indole. In addition, the oxidase, potato rot, and tobacco hypersensitive reaction tests were negative. MALDI-TOF MS yielded valid scores 2.073–2.234 (score value ≥ 2.0 ; identification accurate to the species level) for all three bacterial isolates.

Sequencing results revealed that three isolates (MELON2A–C) had identical 16S rRNA gene and *gyrB* region sequences. BLAST analysis revealed that the nucleotide sequences of the 16S rRNA gene sequence (1408 bp; GenBank Accession No. MH464584) from the bacterial isolate MELON2A causing center blackening shared 99% similarity to *P. oryzihabitans* (NR117269; Mulet et al., 2010). The *gyrB* region (674 bp, MH464585) of MELON2A shared 100% sequence identity with *P.*

oryzihabitans strain LMG 7040T (FN554210) (Mulet et al., 2010). The phylogenetic analysis revealed that the isolates from muskmelon fruit formed a clade with the reference strain of *P. oryzihabitans* group (Fig. 3).

Based on the symptoms, microbiological characteristics, pathogenicity assays, API tests, MALDI-TOF MS, and 16S rRNA gene and *gyrB* region sequencing, the pathogen was identified as *P. oryzihabitans*. To our knowledge, this is the first report of *P. oryzihabitans* causing center blackening in muskmelon fruit.

P. oryzihabitans, a potential nosocomial pathogen, causes infections in immunocompromised individuals (Dussart-Baptista et al., 2007; Freney et al., 1988; Giacometti et al., 1998; Kodama et al., 1985). Infection symptoms of *P. oryzihabitans* are rather vague and similar to those of other diseases or illnesses, such that the cause of these symptoms can be difficult to identify. Most patients experience chills and increased body temperature (Lejtkowicz et al., 2003). *P. oryzihabitans* is found frequently in various environments, including rice paddies, rice and onion seeds, eggshells, water, and bath sponges (Calle-Bellido et al., 2012; Cottyn et al., 2009; Dussart et al., 2003; Marín et al., 2000; Musgrove et al., 2004). These reports indicate that environmental sources could be a reason for the development of an infectious disease in the body (Dussart et al., 2003; Dussart-Baptista et al., 2007). It remains unknown how the causative bacteria of this disease enter the fruit, but the recent occurrence of the disease indicates that *P. oryzihabitans* poses a potential threat to the global production and transportation of muskmelon as well as to food safety.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education of the Republic of Korea [2015R1A6A1A03031413].

References

- Calle-Bellido, J., Rivera-Vargas, L.I., Alameda, M., Cabrera, I., 2012. Bacteria occurring in onion (*Allium cepa* L.) foliage in Puerto Rico. *J. Agric. Univ. P. R.* 96, 199–219.
- Choi, O., Kang, B., Cho, S.K., Park, J., Lee, Y., Kim, W.L., Kim, J., 2016. First report of *Pseudomonas syringae* pv. *syringae* causing bacterial leaf blight on *Miscanthus × giganteus*. *J. Plant Dis. Prot.* 123, 137–140.
- Cottyn, B., Debode, J., Regalado, E., Mew, T.W., Swings, J., 2009. Phenotypic and genetic diversity of rice seed-associated bacteria and their role in pathogenicity and biological control. *J. Appl. Microbiol.* 107, 885–897.
- Dussart, L., Dupont, J.P., Zimmerlin, I., Lacroix, M., Saiter, J.M., Junter, G.A., Jouenne, T., 2003. Occurrence of sessile *Pseudomonas oryzihabitans* from a karstified chalk aquifer. *Water Res.* 37, 1593–1600.
- Dussart-Baptista, L., Bodilis, J., Barray, S., Frébourg, N., Fournier, M., Dupont, J.P., Jouenne, T., 2007. Recurrent recovery of *Pseudomonas oryzihabitans* strains in a karstified chalk aquifer. *Water Res.* 41, 111–117.
- Freney, J., Hansen, W., Etienne, J., Vandenesch, F., Fleurette, J., 1988. Postoperative infant septicemia caused by *Pseudomonas luteola* (CDC group Ve-1) and *Pseudomonas oryzihabitans* (CDC group Ve-2). *J. Clin. Microbiol.* 26, 1241–1243.
- Giacometti, A., Cirioni, O., Quarta, M., Schimizzi, A.M., Del Prete, M.S., Scalise, G., 1998. Unusual clinical presentation of infection due to *Flavimonas oryzihabitans*. *Eur. J. Clin. Microbiol. Infect. Dis.* 17, 645–648.
- Kim, J., Choi, O., Kim, T.S., 2012. An outbreak of onion center rot caused by *Pantoea ananatis* in Korea. *Plant Dis.* 96, 1576.
- Kodama, K., Kimura, N., Komagata, K., 1985. Two new species of *Pseudomonas*: *P. oryzihabitans* isolated from rice paddy and clinical specimens and *P. luteola* isolated from clinical specimens. *Int. J. Syst. Evol. Microbiol.* 35, 467–474.
- Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stacke-brandt, E., Goodfellow, M. (Eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons, New York (155–147).
- Lejtkowicz, F., Belavsky, L., Kudinsky, R., Gery, R., 2003. Bacteraemia and sinusitis due to *Flavimonas oryzihabitans* infection. *Scand. J. Infect. Dis.* 35, 411–414.
- Lelliott, R.A., Billing, E., Hayward, A.C., 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. *J. Appl. Bacteriol.* 29, 470–489.
- Lester, G.E., 2008. Antioxidant, sugar, mineral, and phytonutrient concentrations across edible fruit tissues of orange-fleshed honeydew melon (*Cucumis melo* L.). *J. Agric. Food Chem.* 56, 3694–3698.
- Lester, G.E., Hodges, D.M., 2008. Antioxidants associated with fruit senescence and human health: novel orange fleshed non-netted honey dew melon genotype comparisons following different seasonal productions and cold storage durations. *Postharvest Biol. Technol.* 48, 347–354.
- Marín, M., García de Viedma, D., Martín-Rabadán, P., Rodríguez-Créixems, M., Bouza, E., 2000. Infection of hickman catheter by *Pseudomonas* (formerly *Flavimonas*) *oryzihabitans* traced to a synthetic bath sponge. *J. Clin. Microbiol.* 38, 4577–4579.
- Mulet, M., Lalucat, J., Garcia-Valdes, E., 2010. DNA sequence-based analysis of the *Pseudomonas* species. *Environ. Microbiol.* 12, 1513–1530.
- Musgrove, M.T., Jones, D.R., Northcutt, J.K., Cox, N.A., Harrison, M.A., 2004. Identification of Enterobacteriaceae from washed and unwashed commercial shell eggs. *J. Food Prot.* 67, 2613–2616.
- Panagopoulos, G.N., Megaloikononimos, P.D., Lontos, M., Giannitsioti, E., Drogari-Apiranthitou, M., Mavrogenis, A.F., Kontogeorgakos, V., 2016. *Pseudomonas oryzihabitans* infected total hip arthroplasty. *J. Bone Jt. Infect.* 1, 54–58.
- Sawada, H., Kanaya, S., Tsuda, M., Suzuki, F., Azegami, K., Saitou, N., 2002. A phylogenomic study of the OCTase genes in *Pseudomonas syringae* pathovars: the horizontal transfer of the *argK-tox* cluster and the evolutionary history of OCTase genes on their genomes. *J. Mol. Evol.* 54, 437–457.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- Tena, D., Fernández, C., 2015. *Pseudomonas oryzihabitans*: an unusual cause of skin and soft tissue infection. *Infect. Dis.* 47, 820–824.
- Woo, K.S., Choi, J.L., Kim, B.R., Kim, J.E., Kim, K.H., Kim, J.M., Han, J.Y., 2014. Outbreak of *Pseudomonas oryzihabitans* pseudobacteremia related to contaminated equipment in an emergency room of a tertiary hospital in Korea. *Infect. Chemother.* Mar. 46, 42–44.