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Note

Characterization of first hemin-requiring *Pseudomonas aeruginosa* small-colony variants from the blood of an octogenarian male-patient with double pneumonitis☆

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ABSTRACT

A hemin-requiring *Pseudomonas aeruginosa* small-colony variant (SCV) was isolated from the blood of an octogenarian male-patient with double pneumonitis. The isolate was capable of growing on both sheep blood and chocolate agars but not on MacConkey agars without blood ingredient. Furthermore, the isolate revealed to grow only around the X-factor impregnated discs when examined using the X and V disc strips. However, not only RapID-NH system but also the VITEK2 system failed to identify the isolate. The isolate was finally identified as *P. aeruginosa* by the sequence of the 16S rRNA genes and the MALDI-TOF MS analysis. Interestingly, the isolate represented positive reaction for δ-aminolaevulinic acid (ALA)-test despite the requirement of hemin. Detailed analysis indicated that the isolate produced protoporphyrin IX from ALA. Therefore, the reason for the hemin dependence was deduced the dysfunction of *hemH*-encoded ferrochelatase behaving at the end of biosynthetic pathway of heme. However, the genetic analysis of *hemH* gene demonstrated no variations of both the DNA and the amino-acid sequences. To the best of our knowledge, this is the first clinical isolation of a hemin-dependent *P. aeruginosa* SCV from blood.

Small colony variants (SCVs) constitute a slow-growing subpopulation of bacteria representing atypical colony morphologies and unusual biochemical characteristics. The SCVs have been described for many genera and species, including *Pseudomonas aeruginosa* [1,2], *Escherichia coli* [3,4], and *Enterococcus faecalis* [5]. Clinical *P. aeruginosa* SCVs are more resistant to antibiotics, more hyperpiliated, and better able to form biofilms than their wild-types, and these SCV phenotypes have been related to persistent and recurrent infections [6–9].

Large scale investigation [2] indicated that *P. aeruginosa* SCVs were isolated from lung and other airway specimens of 33 out of 86 *P. aeruginosa*-positive cystic fibrosis (CF) patients, among which no auxotrophic isolate was included. An interesting finding was also reported that the methionine-dependent mutants of *P. aeruginosa* SCVs were isolated from seven out of 16 patients [10]. No other auxotrophic *P. aeruginosa* SCVs including hemin-dependence have ever been reported.

Abbreviations: SCV, small-colony variant; ALA, δ-aminolevulinic acid; BHI, Brain-Heart-Infusion; CFU, colony-forming-unit; CF, cystic fibrosis.

☆ All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. As stated above, all authors meet the above ICMJE authorship criteria.

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To the best of our knowledge, this was the first clinical isolation of a hemin-dependent *P. aeruginosa* SCV from blood of the patient with no cystic fibrosis. We investigated the detailed characteristic of this isolate and mechanism of hemin-dependence.

An octogenarian male-patient, who had suffered from bronchiectasis and had treated with erythromycin for decades, was hospitalized in Kansai Medical University Medical Center, Moriguchi, Osaka, Japan in February 2015, with the complaint of developing a feeling of malaise accompanied by excessive production of sputum. He was soon diagnosed as double pneumonitis. On the day of his hospitalization, two sets of peripheral blood culture were collected for bacteriological examination by means of the BacT/Alert 3D system (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan). At the 72 hrs of incubation, two FA bottles (SYSMEX bioMérieux) in the system showed the positive signal for bacterial growth, demonstrating Gram-negative, short or elongated rod-shaped morphology, by means of Gram stain. The isolate shaped tiny colonies (less than 1 mm in size) after 24 h incubation, and formed small colonies (from 2 to 3 mm in diameter) after 48 hrs incubation on both sheep blood and chocolate agar (Nissui Pharmaceutical Co., Ltd.) plates, but not on MacConkey agar plates.

VITEK2 system (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan) yielded the profile number 1300100000, indicating *Acinetobacter lwoffii* with an identification probability of 96.00%, regardless of the addition of hemin. RapID-NH system (AMCO Co., Ltd, Tokyo, Japan) indicated *Aggregatibacter actinomycetemcomitans* with 99.99% probability. However, in the molecular identification, the coding 16S rRNA of SCV isolate was directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and Applied Biosystems ABI3130 (Applied Biosystems). The sequence was retrieved from three databases of base sequence (NCBI, lebibi and EZTaxon). Comparative sequence of the SCV analysis showed 100% 16S rRNA sequence similarity to that of the type strain of *P. aeruginosa* ATCC 15692 (NCBI), 97.1% similarity to that of the type strain of *P. aeruginosa* HE978271 (lebibi) and 100% similarity to that of the type strain of *P. aeruginosa* JCM5962 (EZtaxon), respectively. Furthermore, the SCV isolate showed the same pattern as *P. aeruginosa* ATCC 27853, when analyzed using MALDI-TOF-MS Microflex and Bruker Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany), with the score value of 2.281. Therefore, we identified the SCV isolate as *P. aeruginosa.*

Moreover, when examined the hemin and NAD auxotrophy using X-, and V-factor-containing Taxo™ differentiation discs X and V (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) on Mueller-Hinton agar plates (Nippon Becton Dickinson Co., Ltd.), the isolate grew only around the X-factor impregnated discs as shown in Fig. 1. The microorganism was subsequently identified as a hemin-requiring *P. aeruginosa* with the phenotype of SCV. After administration of meropenem for 13 days, the patient's complaint on admission was eased and he was discharged on the 13th hospital day.

A hemin-dependent *P. aeruginosa* isolate grown on the sheep blood agar was subjected to the experiments described below.

Brain-Heart-Infusion (BHI) broth (Eiken Chemical Co., Tokyo, Japan) was used to examine the effects of hemin (Wako Pure Chemical Industries, Ltd. Osaka, Japan) on the growth of the SCV isolate. Media were prepared by adding hemin at concentrations of 0, 5, 10, and 20 μg/ml, respectively. The SCV isolate inoculated into broth media were incubated at 35 °C. The number of viable cells was calculated as colony-forming unit (CFU) per milliliter. At 0, 24, 48, and 72 hrs, the samples (100 μl) were removed and 10-fold serial dilutions were performed. Each diluent (100 μl) was spread in duplicate onto sheep blood agars for colony counts. The inoculated plates were incubated at 35 °C for 48 hrs. The growth curves of the SCV isolate obtained under various concentrations of hemin were shown in Fig. 2. The BHI broth media supplemented with 10 and 20 μg/ml of hemin were demonstrated to support sufficient growth in comparison with the both media with 5 μg/ml and 0 μg/ml of hemin. The growth curves of the SCV isolate under supplementation with 10 and 20 μg/ml of hemin were shown to be almost the same with each other. Little growth appeared at concentration of 5 μg/ml hemin. We previously reported that hemin-dependent *E. coli* could be identified with the MicroScan WalkAway system by adding hemin to the suspension at proper concentrations [4]. However, in this case, the VITEK2 system failed to identify the hemin-dependent *P. aeruginosa* SCVs, notwithstanding the addition of hemin at concentrations of 10 μg/ml. This could probably be ascribed to the shortage of reaction times in VITEK2 system, because the isolate represented to form mucoid colonies and exhibited slow growth phenotypes.

In the subsequent study, stability of the isolate with SCV phenotype was assessed to determine the occurrence frequency of revertants without hemin dependency. The SCV isolate was cultured in BHI broth including hemin at concentrations of 10 μg/ml at 35 °C for 48 hrs. The broth culture of the SCV isolate around the log to stationary phase of growth was washed twice with sterile physiological saline solution. The number of cells in the broth culture was counted by the dilution plating method to determine CFU. An aliquot (100 μl) of the bacterial suspension was spread onto fifty MacConkey agar plates. The occurrence rate of revertants was calculated by the number of revertants appearing among the number of SCVs.

During the course of this experiment, only 1 colony of the revertant was obtained when the aliquot of the broth without dilution was spread on the plate. The frequency of emergence of revertants from SCVs was outstandingly low (frequency $<2.0 \times 10^{-9}$). We subsequently examined the genetic clonality between the SCV isolate and the revertant strain by means of the POT method [11,12], and confirmed the clonal identity, both

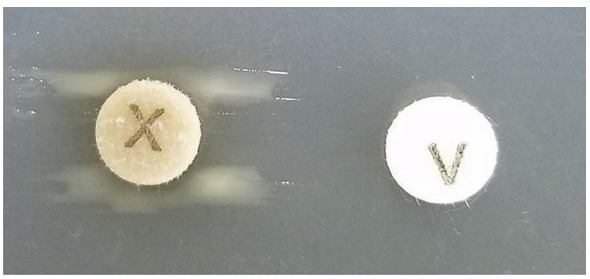

Fig. 1. Auxotrophy examination of the isolate using the X and V disc strips. Growth of the isolate apparently surrounded the X-factor-impregnated strip on the Mueller–Hinton agar, indicating that the isolate was hemin dependent.

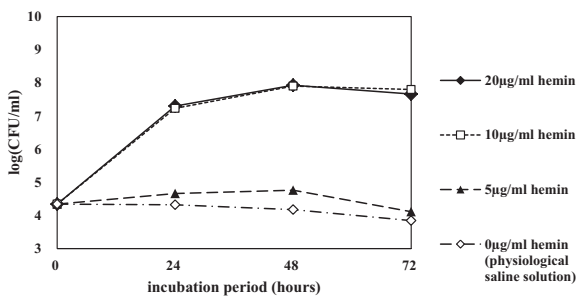

Fig. 2. Growth curves of the SCV isolate in BHI broth under the supplementation with various concentrations of hemin. Supplementation with 10 and 20 μg/ml of hemin sufficiently support the growth.

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