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Molecular Relatedness between Isolates *Yersinia pseudotuberculosis* from a Patient and an Isolate from Mountain Spring Water

A 40-yr-old buddhist monk was admitted to the hospital with abdominal pain, fever, and confusion. He had a history of drinking untreated mountain spring water in his temple, and experienced the above symptoms for several days before admission. In past medical history, he had suffered from hepatic cirrhosis. *Yersinia pseudotuberculosis* was isolated from his blood and ascitic fluid. The mountain spring water that he had ingested was cultivated and *Y. pseudotuberculosis* was also isolated. For identification of pathogenic *Y. pseudotuberculosis*, each isolate from the three sources (blood, ascitic fluid, and drinking water) was also analysed for the *inv* gene for *Y. pseudotuberculosis* and the *virF* gene for virulent plasmid by PCR. All strains were positive for both the *virF* and the *inv* genes and also positive for autoagglutination test. For relationship study, each isolate from the three sources was also analysed with serotyping and restriction endonuclease analysis of virulence plasmid DNA (REAP) using *Bam*HI. All belonged to the serotype 4b and REAP pattern D. Thus, all these findings supported that the mountain spring water was the source of the *Y. pseudotuberculosis* infection in this case.

Key Words : *Yersinia pseudotuberculosis Infections; Bacteremia; Epidemiology, Molecular*

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INTRODUCTION

Yersinia pseudotuberculosis causes gastroenteritis, mesenteric lymphadenitis and terminal ileitis in human, particularly in children (1, 2). A septicemic form of *Y. pseudotuberculosis* infection has been reported rarely. It is usually seen in patients with underlying disorders such as diabetes, hepatic cirrhosis, or iron overload (1). In Korea, several cases of septicemic form *Y. pseudotuberculosis* infections have been reported (3-7). However, no report has been supported by a molecular method. In this case, we report the first septicemic case of *Y. pseudotuberculosis* infection supported by a molecular method in Korea.

CASE REPORT

Clinical and laboratory history

A 40-yr-old buddhist monk was admitted to the hospital with abdominal pain, fever, and confusion in June 2001. He had a history of drinking untreated mountain spring water in his temple, and experienced the above symptoms for several days before admission. In past medical history, he had suffered from hepatic cirrhosis. His body temperature was 38.8℃ and there were diffuse abdominal tenderness and rebound tenderness. The routine complete blood count with differential counts revealed anemia (hemoglobin, 10.4 g/dL), neutrophilia (9,650/μL), and thrombocytopenia (57,000/μL). Blood culture, ascitic fluid culture, and stool culture were performed. Stool culture was negative and Gram-negative rods were isolated from his blood and ascitic fluid, which were all proven to be *Y. pseudotuberculosis* with VITEK GNI card (bioMerieux Vitek, Hazelwood, MO, U.S.A.). To confirm the source of the infection, the mountain water in the spring which he drank was collected in twice a week, and the same kind of cooked wild vegetables which he usually had was cultivated on irgasan novobiocin (IN) agar as a selective media using the filtration and culture technique after alkaline treatment (Fig. 1). *Y. pseudotuberculosis* was also isolated in the spring water in twice and not in the food. For the identification of pathogenic *Y. pseudotuberculosis*, each isolate from the three sources was analysed into the *inv* gene for *Y. pseudotuberculosis* and *virF* gene for virulent plasmid by PCR. All strains were positive for the *virF* and *inv* genes (Fig. 2) and also positive for autoagglutination test which was closely related to the presence of 70-kb plasmid. For epidemiological study, each isolate from the three sources was analysed also with serotyping and restriction endonuclease analysis of virulence plasmid DNA (REAP) using *Bam*HI. All belonged to the serotype 4b and REAP pattern D (Fig. 3). The patient received cefotaxime therapy for five

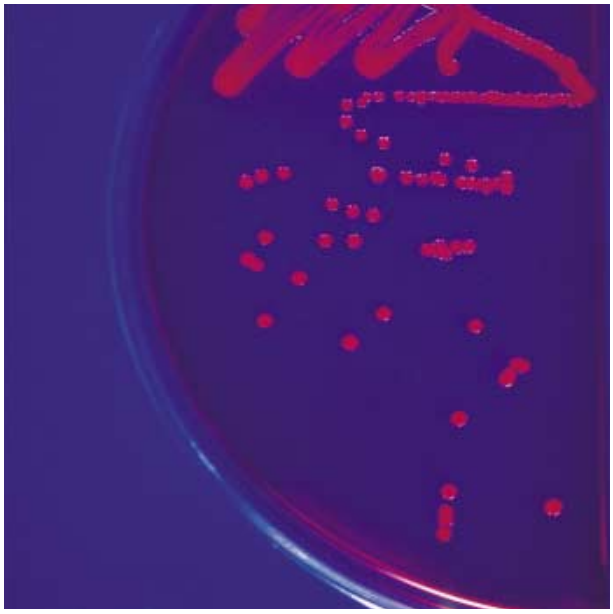

Fig. 1. Typical small round pink colored colonies of *Y. pseudotuberculosis* on IN agar.

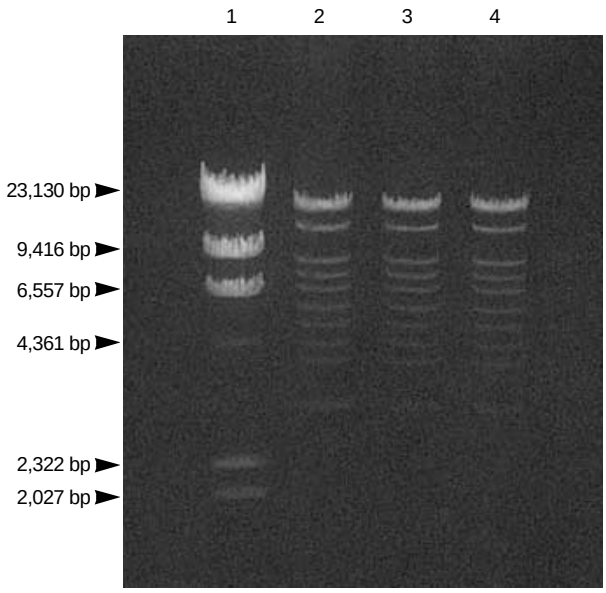

Fig. 3. Restriction endonuclease analysis of virulence plasmid DNA (REAP) with *Bam*HI.
Lane 1: DNA ladder marker, lambda DNA digested with *Hin*dIII; lane 2: *Y. pseudotuberculosis* (blood); lane 3: *Y. peudotuberculosis* (ascitic fluid); lane 4: *Y. pseudotuberculosis* (water).

days and was discharged with clinical improvement.

The isolation of *Y. pseudotuberculosis* from drinking water, food, and stool

To isolate *Y. pseudotuberculosis* from drinking water, the direct culture method of Fukushima (8, 9) was used. Two

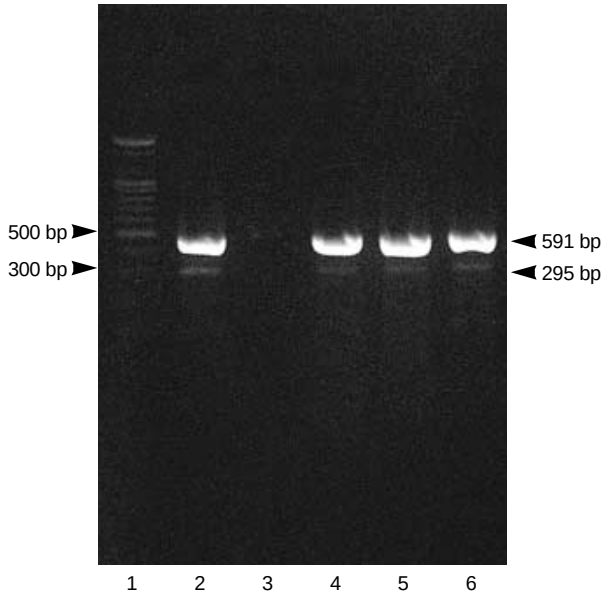

Fig. 2. *virF* gene (591 bp) and *inv* gene (295 bp) PCR.
Lane 1, DNA ladder marker; lane 2, positive; lane 3, negative; lane 4, *Y. pseudotuberculosis* (blood); lane 5, *Y. pseudotuberculosis* (ascitic fluid); lane 6, *Y. pseudotuberculosis* (water).

liters of water sample was filtered with suction and through a 0.45-μm filter paper (Millipore, Bedford, MA, U.S.A.). The filter paper was treated with 0.05 M NaOH in normal saline for 30 sec, and then directly spread on a plate of IN agar containing 2.5 mg/L novobiocin (Sigma Chemical Co., St Louis, MO, U.S.A.) in a Yersinia selective agar base (Difco Laboratories, Detroit, MI, U.S.A.). The plates were incubated for 48 h at 25℃. To isolate *Y. pseudotuberculosis* from food, 1 liter of sterile distilled water was add to 200 g food and throughly mixed. After 30 min standing at room temperature, the supernatant was treated with the same method as the water. To isolate *Y. pseudotuberculosis* from stool, the stool was directly applied on the IN agar (10). The plates were incubated for 48 h at 25℃. The colonies on the IN agar were identified with Gram staining and the VITEK GNI card (bioMerieux Vitek, Hazelwood, MO, U.S.A.). The identified colonies were subcultured on the IN agar.

Serotyping and autoagglutination

Y. pseudotuberculosis isolates (on the IN agar plate) were serotyped by slide agglutination with rabbit anti-*Y. pseudotuberculosis* serotypes 1, 2, 3, 4, 5, and 6 sera (Denkaseiken Co., Tokyo, Japan) and then with rabbit anti-*Y. pseudotuberculosis* serotypes 1a, 1b, 2a, 2b, 2c, 4a, 4b, 5a, and 5b sera (Dr Fukushima's Lab, Public health institute, Shimane Prefecture, Japan).

The isolates on the IN agar plate were studied for autoagglutination. The test was performed as described by Laird and Cavanaugh and was believed to be closely related to the

presence of 70-kb plasmid (11). Each isolate was inoculated to 2 tubes of tryptic soy broth (Difco Laboratories, Detroit, MI, U.S.A.) and then incubated for 24 hr, one tube at 37℃ and the other at 25℃.

virF gene and *inv* gene PCR

To prepare template DNA, the method of Nakajima et al. (12) was used. We selected the primer for the *virF* gene used by Wren and Tabaqchali (13) and the primer for the *inv* gene used by Nakajima et al. (12). Amplification was performed in a 20-μL reaction mixture containing the following: 0.2 μM of each primer; 200 μM (each) dATP, dCTP, dTTP, and dGTP; 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 40 mM KCl; 1 unit of *Taq* polymerase (Perkin-Elmer, Cetus, CT, U.S.A.); and 1 μL of DNA sample. Thirty cycles of amplification were preformed in a DNA thermal cycler (GeneAmp PCR 9600 system, Perkin-Elmer, Cetus, CT, U.S.A.). Each cycle consisted of the followings: predenaturation at 94℃ for 1 min, 30 cycles of denaturation at 94℃ for 1 min, primer annealing at 55℃ for 2 min, extension at 72℃ for 1 min (14). The size of the amplified DNA fragments were 295 bp and 591 bp for the *inv* and *virF* genes respectively (12-14).

Restriction endonuclease analysis of virulence plasmid DNA (REAP)

The plasmid DNA was prepared by the alkaline lysis method by Kaneko and Maruyama (15). Plasmid DNA isolated from each strain was digested with restriction endonuclease *Bam*HI (15-18). The enzyme digestions of the plasmid DNA and gel visualization were done with the method by Fukushima et al. (16-18). The REAP pattern was determined according to the classification of Fukushima et al. (16) and confirmed by Dr. H. Fukushima.

DISCUSSION

Y. pseudotuberculosis causes sporadic infections and epidemiologic outbreaks in human and is widely distributed among domestic pets, farm animals, and wild animals (19, 20). Wildlife animals are thought to be the principal reservoir of infection of *Y. pseudotuberculosis* (21, 22). *Y. pseudotuberculosis* could be transmitted to humans through drinking water and food contaminated by feces from wildlife and domestic animals (9, 21). Unchlorinated drinking water from wells, springs, and streams in mountainous areas contaminated by a wild animal was considered as a important route of the *Y. pseudotuberculosis* infections (20). In Japan almost all the clinical cases of *Y. pseudotuberculosis* infections occurred in children living in mountainous area and their family who drank untreated mountain stream, well, and river water (21, 22).

The epidemiologic link among human, wildlife animal and environment substances was examined by serotyping and REAP (21, 22). In the present case, the food that the patient and his one and only colleague had been boiled or fried. He recently changed his habit to drinking the mountain spring water without any treatment because of the belief of good for his health. His colleague monk did not drink it but enjoyed tea. To our regret, we could not collect the food (he had boiled rice and cooked wild vegetables) which he had before symptom onset. So we collected the same kinds of cooked wild vegetables that he had taken. We could isolate a *Y. pseudotuberculosis* strain, which had identical REAP pattern and serotyping with the strains isolated from patient's blood and ascitic fluid, in the mountain spring water only. So, all these evidences supported that the mountain spring water was contaminated with a *Y. pseudotuberculosis* and was the source of the infection. In addition, each strain had 70-kb virulence plasmid which is essential for virulence and differentiates pathogenic from nonpathogenic Yersinia (10, 12). It have been reported that there were differences in the geographic distribution of serogroups and REAP patterns of *Y. pseudotuberculosis* (16, 18). Fukushima et al. reported that almost all strain of serogroup 1b from Europe belonged to REAP pattern E, but almost all strain of serogroup 1b from eastern Asia (Japan, Far-east Russia) belonged to REAP pattern D (16). In Korea, Paik et al. analysed 49 strains from human stool with *Y. pseudotuberculosis* gastroenteritis and their drinking water in Dobong-gu, Seoul, Korea and reported that almost all isolates belonged to serotype 15 REAP pattern B (59.2%) or serotype 4b REAP pattern D (38.8%) (10). These findings demonstrated a close link between human *Y. pseudotuberculosis* infection and contamination of water with *Y. pseudotuberculosis*. In the present case, the strain was belonged to the serotype 4b and REAP pattern D.

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