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NOTE

Bacteriology

A case report on disseminated *Rhodococcus equi* infection in a Japanese black heifer

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ABSTRACT. *Rhodococcus equi* was isolated from the granulomatous lesions of the lung, kidney, liver, and hepatic, mesenteric, and abomasum lymph nodes of a Japanese black heifer. *R. equi* isolates were analyzed by polymerase chain reaction for virulence-associated protein genes. The *vapN* gene was detected in all the isolates examined. This is the first report in which *vapN*-positive *R. equi* was isolated from cattle in Japan.

KEY WORDS: cattle, *Rhodococcus equi*, VapN, virulence plasmid

Rhodococcus equi is a Gram-positive pathogen that causes severe pulmonary abscesses and purulent enterocolitis in foals [13]. *R. equi* is widespread in the environment in grazing farms and common in the feces of farm animals including cattle, swine, horses, and others [16]. *R. equi* is also the opportunistic pathogen that causes a disease similar to tuberculosis in human patients with AIDS [18, 22, 23].

The pathogenicity of *R. equi* is associated with the presence of a large virulence plasmid [17]. *R. equi* causes pyogranulomatous pneumonia and secondary ulcerative enterocolitis accompanied by a high mortality rate in young foals worldwide [20]. Isolates from the lesions of infected equine hosts possess a circular plasmid (pVapA), encoding virulence-associated protein A (VapA), a 15–17 kDa surface protein [10, 11, 17, 21]. *R. equi* has also been isolated from the porcine submaxillary lymph nodes with granulomatous lesions as well as from apparently healthy pigs. The strains isolated from the pig and wild boar often carry another type of virulence plasmid (pVapB) encoding VapB, a VapA variant [7–9, 19].

Recently, a novel linear virulence plasmid encoding VapN (pVapN) was characterized in cattle, goat, and human isolates [1, 14, 15, 24]. In cattle, *R. equi* has low pathogenicity and lesions are mostly confined to a single lymph node [5]. Herein, we report a case of disseminated infection with *R. equi* carrying pVapN in cattle.

A 19-month-old Japanese black heifer was brought to a slaughterhouse and inspected on December 14, 2015. The heifer appeared depressed and had anorexia while at the fattening farm. There was no particular abnormality in ante-mortem inspection. The biochemical measurement of a blood sample was done using SPOTCHEM™ EZ analyzer (ARKRAY Co., Ltd., Kyoto, Japan). Serum total bilirubin was 0.6 mg/d*l*. Blood urea nitrogen (BUN) was 16 mg/d*l*. To detect antibody against bovine leukemia virus, passive hemagglutination reaction (PHA) was performed according to the manufacture's instruction using the Bovine Leucosis Antibody Assay Kit (Nisseiken Co., Ltd., Tokyo, Japan), and the result was negative.

In post mortem inspection, multifocal small yellow nodules were observed in the lungs. On sectioning, the nodules oozed small amounts of yellow materials (Fig. 1). Hilar, hepatic, mesenteric, and

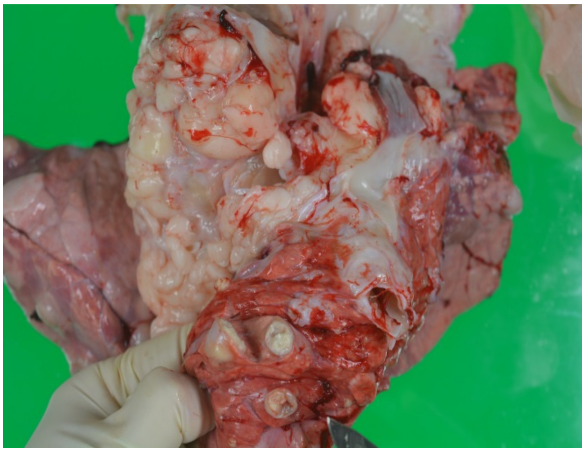

Fig. 1. Lung. Multifocal small yellow nodules are detected. Cut surface of the mass containing yellow materials encapsulated by a thick white septum.

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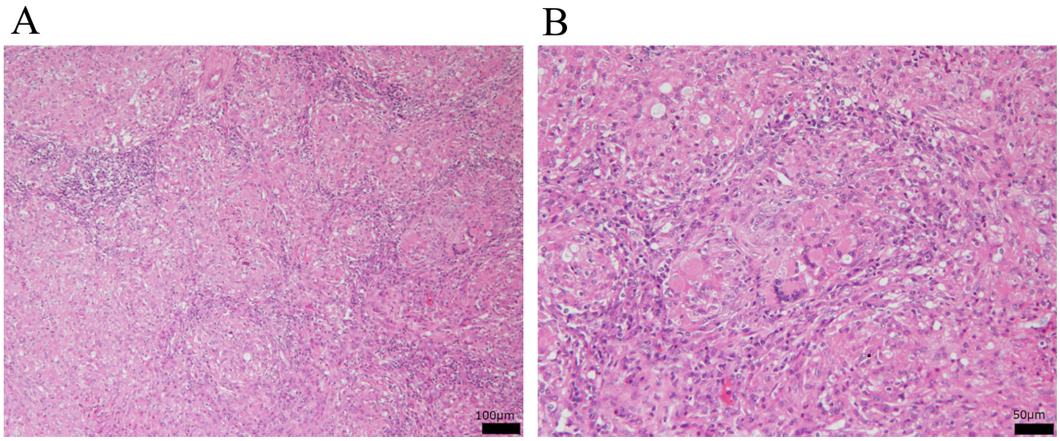

Fig. 2. (A) Hepatic portal lymph nodes. Multiple variably sized caseating granulomas contain areas of necrosis in medulla. HE stain. Bar=100 μm. (B) Hepatic portal lymph nodes. Granuloma is composed of epithelioid cells and multinucleated giant cells. HE stain. Bar=50 μm

Table 1. Primers used in this study

Primer	Sequence (5′→3′)	Reference
Primer1	GACTCTTCACAAGACGGT	[23]
Primer2	TAGGCGTTGTGCCAGCTA	[23]
Primer3	AACGTAGTCGCGGTGAGAA	[23]
Primer4	ACCGAGACTTGAGCGACTA	[23]
vapN F	CGCTTTTATCGAGGGCACTC	This study
vapN R	TTTGCCAGGTCTTGCGAATG	This study

abomasum lymph nodes were markedly enlarged. Hepatitis, rumenitis, reticulitis, omasitis, abomasitis, enteritis, and colonitis were observed. The hilar lymph nodes were enlarged to about 11 cm by 7 cm; the hilar and other enlarged lymph nodes were solid and carneous. The mesenteric lymph nodes were liquefied in the central part, and other lymph nodes were observed to be white, gritty foci on a cut surface.

Stamp samples of the hilar, hepatic, mesenteric, and abomasum lymph nodes were stained with Modified Giemsa, Gram and Ziehl-Neelsen stainings. There were a few mature lymphocytes without atypia in stamp samples stained with Modified Giemsa staining. Gram-positive rod-shaped bacteria were identified in all the stamp samples. The results of Ziehl-Neelsen staining were negative in all the stamp samples. In Hematoxylin and Eosin staining samples, the hilar, hepatic, and abomasum lymph nodes contained many foci of necrosis and multiple variably-sized granulomas in the medulla (Fig. 2A). The boundary between cortex and medulla was unclear. The central zones of granulomas sometimes contained irregular mineralized foci and were delimited by neutrophils, numerous epithelioid macrophages, and occasionally Langhans giant cells surrounded by fibrous connective tissue (Fig. 2B). Scattered lymphocytes and plasma cells were identified in the marginal zones of the granulomas. Macrophages and Langhans giant cells that had abundant and foamy cytoplasms occasionally contained coccobacilli positively stained with Gram staining. The mesenteric lymph nodes exhibited severe necrosis.

Samples of the liver, kidney, lung, and hilar, hepatic, mesenteric, and abomasum lymph nodes were plated on 5% sheep blood agar plates and cultured under anaerobic and aerobic conditions. Salmon pink colonies were visible about 4 days after aerobic incubation at 37°C. Colonies were mucoid and coalescing in teardrop form on the agar. The colony morphology was identical in the isolates from the liver, kidney, lung, and each lymph node. The organism was a Gram-positive, pleomorphic coccobacillus, varying from coccoid to bacillary depending on growth conditions.

The isolated strains were catalase-positive, oxidase-negative, urease-positive, and nitrate reduction-positive. The strains produced phosphatase and α-glucosidase, failed to oxidize or ferment any carbohydrates, and were non-proteolytic. Since these results suggested the strains could be *R. equi*, we performed the CAMP test and PCR amplification of the *R. equi*-specific *choE* gene. The CAMP test was performed on sheep blood agar plates and *Staphylococcus aureus* was used as the indicator strain. The primer sequences and PCR conditions are described elsewhere [12]. The isolates were all CAMP test-positive and had a 959 bp DNA amplicon in PCR of the *choE* gene (data not shown). Therefore, we concluded that the isolates derived from the cattle in this study were *R. equi*.

The presence of virulence plasmid specific markers, *vapA, vapB*, and *vapN* were examined by PCR using primer pairs shown in Table 1. PCR thermal cycler steps for each gene were identical: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. All the isolates were *vapN*-positive but *vapA*- and *vapB*-negative (Fig. 3). The DNA fragment amplified by *vapN* primers

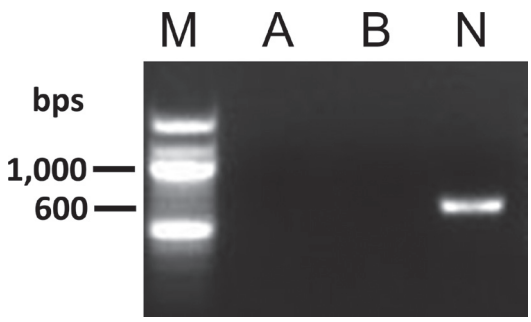

Fig. 3. Amplification of *vap* genes by PCR. The genomic DNA extracted form one representative strain derived from the Japanese black heifer was used as a template. The primer pairs that specifically amplify *vapA* (primer 1 and primer 2), *vapB* (primer 3 and 4) and *vapN* (*vapN*-F and *vapN*-R) were used. Lane M: 100 bp ladder, Lane A: Result of PCR using *vapA* primers, Lane B: Result of PCR using *vapB* primers, Lane N: Result of PCR using *vapN* primers. A single band of 638 bp was observed in lane N.

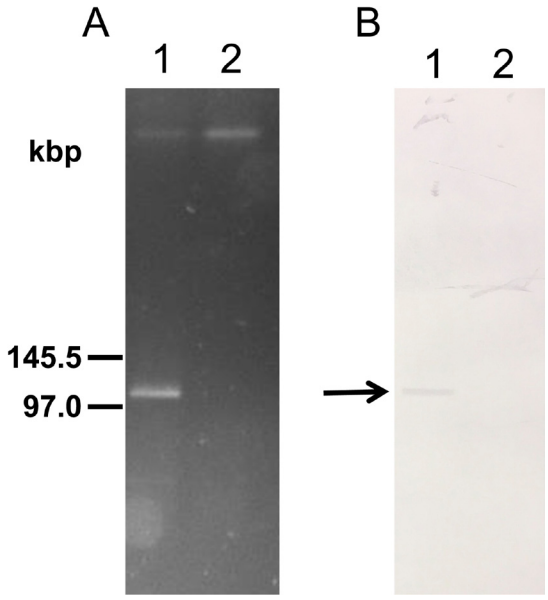

Fig. 4. Detection of pVapN by PFGE. (A) Genomic DNA of one representative strain derived from the Japanese black heifer (lane 1) and a strain possessing pVapA, ATCC33701 (lane 2). pVapN is observed as a distinct band of approximately 120 kb in lane 1. (B) Southern blot analysis. Resolved DNA fragments by PFGE were transferred onto a nylon membrane. pVapN was detected by using *vapN*-specific probe (arrow).

was sequenced using BigDye Terminator v1.1 (Thermo Fisher scientific, Waltham, MA, U.S.A.) according to the manufacturer's instructions. The nucleotide sequence of *vapN* gene (Genbank accession No. LC375161) was identical to that of *vapN* gene reported previously (Genbank accession No. KF439868) except for substitution of one base pair (343C>A).

To detect the linear plasmid encoding VapN, pulse-field gel electrophoresis (PFGE) and Southern blotting were carried out. PFGE was accomplished according to a method described elsewhere [24]. Southern hybridization was carried out using DIG High Prime DNA Labeling and Detection Starter Kit 1 (Merck, Darmstadt, Germany) according to the manufacturer's instructions. To prepare a probe, the *vapN* gene was amplified by PCR using a pair of primers used for detection of *vapN*. In the PFGE gel, an approximate 120 kb band was detected in *vapN*-positive strains but not in the strain harboring pVapA (Fig. 4A). Southern blot analysis showed that the *vapN* probe hybridized with this band (Fig. 4B). These data suggested that the *vapN*-positive strains isolated in this study possessed pVapN.

In the present study, the *R. equi*-carrying *vapN* gene was isolated from a Japanese black heifer that had granulomatous lesions detected in the slaughterhouse post mortem inspection. This is the first case report of *vapN*-positive *R. equi* from cattle in Japan. To date, the prevalence of *R. equi* in cattle in Japan remains unknown. Flynn and colleagues [5] reported that *R. equi* was isolated from 3.9% of the lymph node lesions of cattle examined in Ireland [5]. However, VapA and VapB protein were not detected by immunoblot assays using monoclonal antibodies against VapA or VapB in the Irish bovine isolates. Further, the bovine isolates in that study were negative for *vapA* or *vapB* genes by PCR. Therefore, it had been thought that the bovine isolates did not possess a virulence plasmid. In the present study, we found that the primers used in the previous study by Flynn and colleagues did not amplify the *vapN* gene of the strains isolated in this study. Also, when immunoblot was carried out using monoclonal antibodies against VapA or VapB as described previously [5], we confirmed that no signal was detected in *vapN*-positive isolates, presumably due to poor cross-reactivity with VapN. Furthermore, in Japanese bovine strains that were *vapN*-positive, the linear plasmid encoding *vapN* was not detected by conventional methods used for the isolation of the circular plasmid-encoding *vapA* or *vapB*. In a recent report from Brazil, *R. equi* was isolated from 31% of the bovine lymph nodes with signs of lymphadenitis, and 41.9% of those isolates were *vapN*-positive [14]. Therefore, in the previous studies [5], we believe that they failed to detect *vapN*-positive *R. equi* although it was included in the strains examined.

The present study reports a case of disseminated infection caused by *vapN*-positive *R. equi* in cattle. To the authors' knowledge, disseminated infection with *R. equi* in cattle has not been reported until now. In previous studies by Flynn and colleagues [5], almost all of the 264 cases of *R. equi* granulomas were restricted to a single lymph node. *R. equi* produces granulomatous lesions in the submaxillary lymph nodes of pigs and wild boars without any accompanying clinical symptoms [8, 9, 19]. Accordingly, it is thought that pathogenesis caused by *R. equi* in cattle is similar to that in the porcine host. Conversely, some cases of disseminated *R. equi* infection have been reported in goats [2–4, 6, 15]. The present case is similar to the previous, caprine cases. However,

judging from the rarity of reported cases of disseminated *R. equi* infection in cattle, some predisposing factors such as stress and immunosuppression might affect the host's susceptibility.

In this study, *vapN*-positive *R. equi* was isolated from cattle in Japan. Although it is thought that *R. equi* generally has low pathogenicity in cattle [5], exposure to some predisposing factors could result in disseminated infection. Further investigation is necessary to examine the pathogenesis and prevalence of bovine rhodococcosis in Japan.

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