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NOTE

Pathology

Septicemic invasive *Klebsiella pneumoniae* infection in a cynomolgus monkey (*Macaca fascicularis*) with severe diffused suppurative meningoencephalitis

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ABSTRACT. A 2-year-old male cynomolgus monkey (*Macaca fascicularis*) showed neurological symptoms during quarantine for importation into Japan, and was euthanized due to poor prognosis. Gross anatomical examination revealed a hemorrhagic lesion around the lateral ventricle in the cerebrum. Histologically, severe diffused suppurative meningitis and ventriculitis were detected with numerous Gram-negative bacilli in the cerebrum. Immunohistochemically, the bacilli were positively stained with an antibody against *Klebsiella pneumoniae*. The bacterium was isolated from the liver, and it was confirmed to be *K. pneumoniae* by 16S rDNA sequencing. The isolate displayed a hypermucoviscosity phenotype, was positive for the *rmpA* and k_2A genes, and demonstrated multidrug resistance. These results suggest that invasive *K. pneumoniae* can cause septicemic infection, characterized by severe diffused suppurative meningoencephalitis in monkeys.

KEY WORDS: invasive *Klebsiella pneumoniae*, meningoencephalitis, monkey

Klebsiella pneumoniae is a Gram-negative, facultative anaerobic, non-motile bacillus and belongs to the family *Enterobacteriaceae*. It is a common hospital-acquired pathogen, causing septicemia, pneumonia and urinary tract infections in humans [16]. Recently, invasive *K. pneumonia* infection was reported to cause liver abscesses, which were occasionally complicated by bacteremia, meningitis and endophthalmitis. It was first reported in Taiwan [21] and has subsequently been reported in other Asian and Western countries [3, 9, 10]. The bacterium is also a community-acquired pathogen [10, 11]. Invasive strains are associated with the hypermucoviscosity (HMV) phenotype, and the determination of HMV phenotype is based typically on the results of a positive string test [6, 8, 11]. In addition, such strains have one or two potentially important genes: *rmpA* (a regulator of the mucoid phenotype), which is known as an extracapsular polysaccharide synthesis regulator [13], and *magA* (mucoviscosity-associated gene A), which causes hypermucoviscosity and is restricted to the gene cluster of the *K. pneumoniae* capsule serotype K1 [5, 6, 19, 23]. k_2A (K2 capsule-associated gene A) determines the capsule serotype K2 [1, 24].

K. pneumoniae has caused the disease in both Old and New World primates [7, 15, 18]. Recently, multisystemic abscesses associated with invasive *K. pneumoniae* were reported in African green monkeys (*Chlorocebus aethiops*) in the U.S.A. [20]. The report indicated that a cerebellar abscess was detected only in a deceased adult female monkey [20]. Although *K. pneumoniae* was identified by immunohistochemical analysis, bacteriological examination, including culture, serotyping and polymerase chain reaction (PCR), was not performed in the case [20]. Furthermore, there is no other useful information regarding the pathogenesis of invasive *K. pneumoniae* in monkeys [22]. To the best of our knowledge, *K. pneumoniae* expressing the HMV phenotype has not been reported to cause suppurative meningoencephalitis in nonhuman primates.

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This report describes the clinical, microscopic and bacteriological characteristics of an imported cynomolgus monkey (*Macaca fascicularis*) with unique severe diffused suppurative meningoencephalitis.

Seven hundred and twenty cynomolgus monkeys were imported from Cambodia into Japan by air for experimental use. At the time of arrival, all monkeys appeared healthy and were treated with fosfomycin on the first three days of legal quarantine for importation to prevent dysentery. No clinical abnormalities were present until the eighth day of quarantine. However, on the ninth day, a 2-year-old male monkey displayed hypodynamia and anorexia, and the animal appeared to be lame in the right arm and leg. On the tenth day, it showed recumbence, torticollis, nystagmus, and the light reflex disappeared. Eventually, it was euthanized by the pentobarbital anesthesia and hemospasia, because the body temperature decreased. No clinical abnormalities were observed in the remaining 719 monkeys.

A necropsy was performed, and tissue samples from liver, spleen, kidney, heart, lung, stomach, intestines, cerebrum, cerebellum, mesencephalon, pons and medulla oblongata were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Tissue sections (approximately 3 μm thick) were stained with hematoxylin and eosin (HE) and Gram stain for histological examination.

For immunohistochemical analysis, a rabbit polyclonal antibody against *K. pneumoniae* ATCC #43816 (ab20947, Abcam Plc., Cambridge, U.K.) was used at a dilution of 1 in 1,024 with a commercial kit (*N*-Histofine Simple Stain MAX PO®; Nichirei Bioscience Inc., Tokyo, Japan).

The liver, spleen and blood were used for bacterial isolation. To determine HMV phenotype, the string test was performed by passing a standard bacteriological loop through a colony. Mucoviscous string forms greater than 5 mm were determined to be positive results.

For genetic tests, genomic DNA was extracted from the paraffin block of the cerebrum and bacterial colonies using a DNA extraction kit (DEXPAT; TAKARA BIO Inc., Kusatsu, Japan and InstaGene Matrix; Bio-Rad Laboratories, Hercules, CA, U.S.A.). A ~500 bp region of the 16S ribosomal RNA gene (16S rDNA) region was amplified and sequenced using a MicroSeq 500 16S rDNA PCR/Sequencing Kit (Applied Biosystems Life Technologies, Carlsbad, CA, U.S.A.). Therefore, to determine if the pathogen exhibits a capsular serotype, PCRs were performed using *magA*-specific primers (serotype K1) (forward: 5′-GGTGCTCTTTACATCATTGC-3′, reverse: 5′-GCAATGGCCATTTGCGTTAG-3′) and the k_2A-specific primers (serotype K2) (forward: 5′-CAACCATGGTGGTCGATTAG-3′, reverse: 5′-TGGTAGCCATATCCCTTTGG-3′) [24, 25]. The virulence-associated gene *rmpA* was also detected using the *rmpA*-specific primers (forward: 5′- ACTGGGCTACCTCTGCTTCA-3′, reverse: 5′-CTTGCATGAGCCATCTTTCA-3′) [25]. The expected PCR products of *magA*, k_2A and *rmpA* were 1,282, 531, and 535bp in size, respectively.

To test antibiotic susceptibility, the disk diffusion method was performed on isolates using antibiotic disks (SN disk; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan and Sensi disk; Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.). The tested antibiotics were fosfomycin, benzylpenicillin, ampicillin, cefazolin, cefotaxime, tetracycline, colistin, lincomycin, clindamycin, gentamycin, nalidixic acid, ciprofloxacin and norfloxacin.

Grossly, a hemorrhagic lesion was detected around the lateral ventricle in the cerebrum (Fig. 1a). The lesion was more severe in the left caudate nucleus. No gross abnormalities were found in the other organs.

Histologically, severe diffused suppurative meningitis (Fig. 1b) and ventriculitis (Fig. 1c) were detected in the cerebrum. In the parenchyma around the lateral ventricle, the lesions were also characterized by diffused infiltrations of neutrophils and macrophages. Numerous Gram-negative bacilli were detected in the lesions (Fig. 1c inserted figure) and some were detected in the macrophages, and the bacilli were positively stained with the antibody against *K. pneumoniae* (Fig. 1d). Moderate hemorrhage was detected around the infiltrations. In the other organs, neutrophilic infiltrations were also detected in the splenic red pulp and in the hepatic sinusoid.

The sequencing of the amplified 16S rDNA region of bacterial DNA extracted from the paraffin block of the cerebrum was confirmed as *K. pneumoniae*. Gram-negative bacilli were isolated from the liver, which were non-hemolytic, catalase-positive and oxidase-negative, and had an HMV phenotype (Fig. 2). The sequencing of the amplified 16S rDNA region of the isolate confirmed the pathogen as *K. pneumonia* (ATCC 10031, 99.9% identity), and it was positive for the k_2A and *rmpA* gene but negative for the *magA* gene. Furthermore, it demonstrated resistance to benzylpenicillin, ampicillin, colistin, lincomycin and clindamycin.

The present case was diagnosed as septicemic infection with severe diffused suppurative meningoencephalitis caused by *K. pneumoniae*, because Gram-negative bacilli in the suppurative lesions were positively stained with the antibody against *K. pneumoniae* and the sequence of 16S rDNA region of the DNA extracted from the paraffin block of the cerebrum corresponded with *K. pneumoniae*. Furthermore, the bacteria were isolated from the liver, and the isolate was considered as an invasive strain, because it expressed the HMV phenotype and its serotype was genetically determined to be K2.

In humans, invasive *K. pneumoniae* infection predominantly causes liver abscess [3, 4, 6, 10], and it is rarely associated with meningitis [12, 14, 17]. In nonhuman primates, there is only one report of invasive *K. pneumoniae* causing disease [20]. In the previous report [20], invasive *K. pneumoniae* infection characterized by the HMV phenotype was isolated from multisystemic abscesses in African green monkeys and was genetically $rmpA^{+}/magA^{-}$. *K. pneumoniae* antigen was detected in the cerebellar abscess in one dead monkey, but bacteriological culture was not conducted in the previous report [20]. In the present case without being found abscesses in the abdominal cavity, it was extremely characteristic, because the primary lesion was located in the brain and suppurative meningoencephalitis was caused by invasive *K. pneumoniae* infection. The isolate in present case demonstrated the HMV phenotype and the same genetic pattern, although the relationship between the genetic pattern and meningoencephalitis has not been clarified.

K. pneumoniae in this case demonstrated multidrug resistance. As we did not investigate the presence of *K. pneumoniae* in

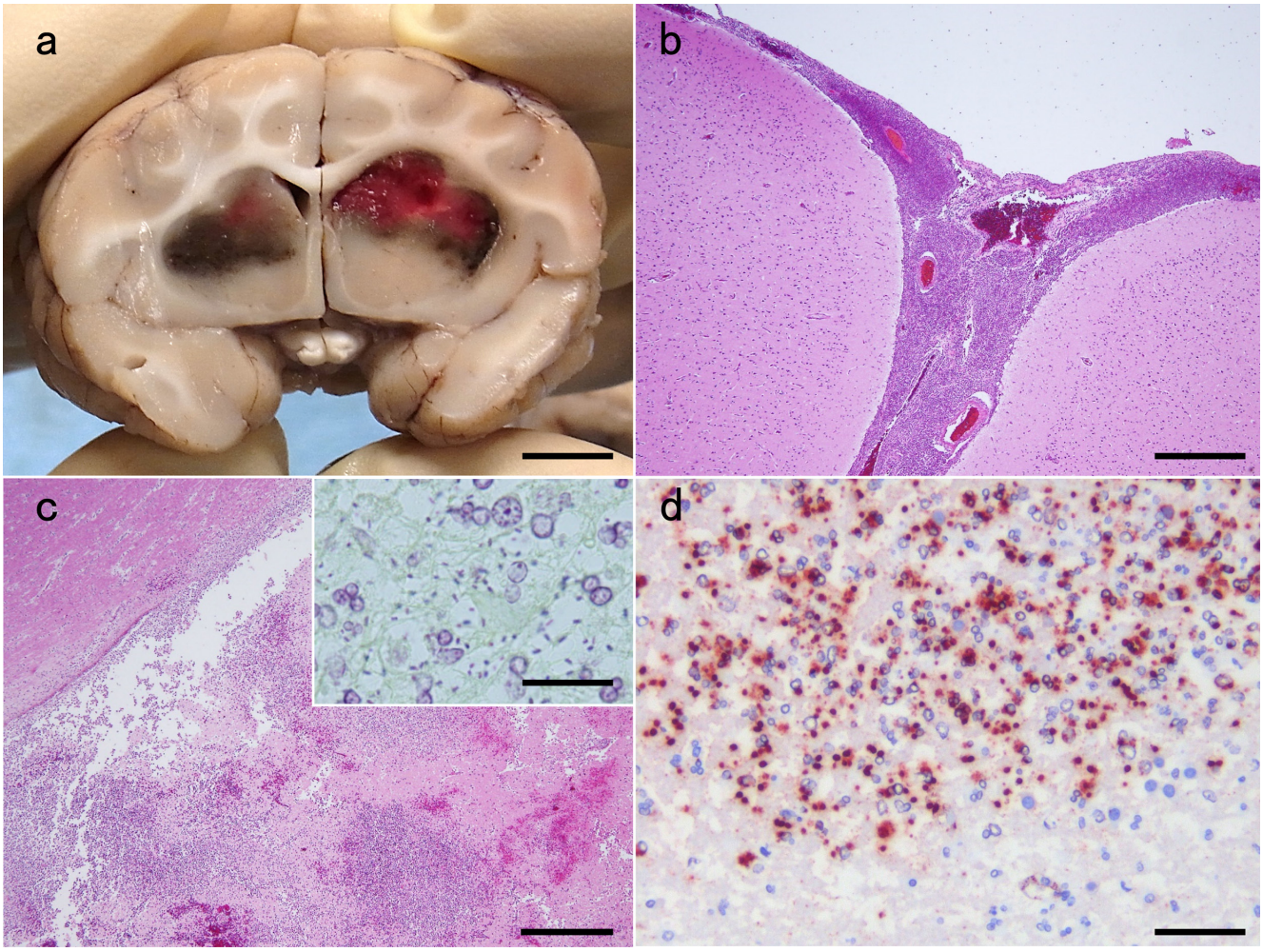

Fig. 1. a. Cerebrum, a cross-section. Hemorrhagic lesion was detected around the lateral ventricle. Bar=1 cm. b. HE staining of the cerebrum showing severe suppurative meningitis. Bar=500 μm. c. HE staining of cerebral ventricle showing severe suppurative ventriculitis with moderate hemorrhage. Bar=500 μm. The inserted figure is Gram staining of parenchyma around the lateral ventricle showing infiltrations of macrophages with numerous Gram-negative bacilli. Bar=20 μm. d. Immunohistochemistry counterstained with hematoxylin of the cerebrum showing that bacilli in the diffused infiltrations react with an antibody against *Klebsiella pneumoniae*. Bar=50 μm.

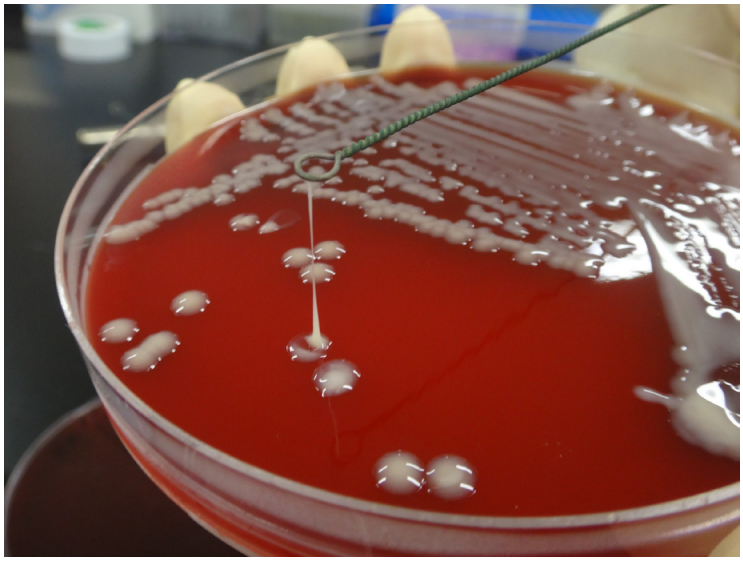

Fig. 2. Positive string test. The HMV phenotype of *K. pneumoniae* is defined by the test, and mucoviscous string forms greater than 5 mm were determined to be positive results.

the remaining monkeys, the dissemination of the infection was not clarified. However, since monkeys for experimental use, typically, are not administered many antibiotics, the multidrug resistant *K. pneumoniae* likely is transmitted from a human to the monkey in the feeding environment before or after transport. The prevalence of the HMV phenotype *K. pneumoniae* in a research colony and in wild-caught nonhuman primates was reported in the U.S.A. [2, 22], but there are no previous reports in Asian countries, including Cambodia and Japan. From the viewpoint of microbial control of laboratory animals, we believe it is useful to investigate the presence of the bacteria and to determine whether the isolate shows drug sensitivity and expressed the HMV phenotype.

In conclusion, this is the first report of invasive *K. pneumoniae* meningoencephalitis in nonhuman primates. Further investigation is necessary to clarify the means of transmission to the cerebrum and the virulence factor. In any case, more attention will be necessary for control measures to prevent infectious disease caused by invasive bacteria, such as *K. pneumoniae*, from laboratory monkeys in the future.

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