

## Isolation of a Zoonotic Pathogen *Kluyvera ascorbata* from Egyptian Fruit-Bat *Rousettus aegyptiacus*

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**ABSTRACT.** The Egyptian fruit-bat *Rousettus aegyptiacus* which had been raised at the private commercial aquarium in Seoul, Korea for indoor exhibition was found dead and submitted to College of Veterinary Medicine, Seoul National University for postmortem examination. A pure bacterium of *Kluyvera ascorbata* was isolated from the blood specimen. The isolation of *K. ascorbata* from fruit bat is very important, because it is the most infectious agent of the genus *Kluyvera* that cause serious diseases to animals and human. Fruit-bats which are distributed in pet shops through black-market in Korea although unproven become popular pet nowadays. This situation enhances chance of zoonosis. This paper describes the first isolation of *K. ascorbata* from the Egyptian fruit-bat.

**KEY WORDS:** *Kluyvera ascorbata*, *Rousettus aegyptiacus*, zoonotic.

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The genus *Kluyvera* is a small, flagellated, motile Gram-negative bacillus and belongs to the family *Enterobacteriaceae* [4]. There are three strains of *Kluyvera*: *Kluyvera ascorbata*, *K. cryocrescens* and *Kluyvera* species group 3, as reported by Farmer *et al.* [4]. *K. ascorbata* is the type of species for the genus that was not only been isolated and reported from different cases of human clinical infections: urinary tract infection, sepsis and bacteremia, diarrhea, soft tissue infection, cholecystitis, peritonitis and intra-abdominal abscess, pancreatitis, mediastinitis, urethrectal fistula [1, 3, 11–13], but was also isolated from subclinically infected primate Madagascan lemurs [10]. *K. ascorbata* is an uncommon but clinically very important pathogen. It is catalase positive and oxidase negative. It grows on MacConkey agar, ferments D-glucose with the production of acid and gas and is susceptible to many antibiotics [4]. *K. ascorbata* can be differentiated from *K. cryocrescens* by its positive ascorbate test, inability to grow at 5°C in a refrigerator, and smaller zones of inhibition around carbenicillin and cephalothin disks [4].

The Egyptian fruit-bat or Egyptian rousette *Rousettus aegyptiacus* (Family Pteropodidae) have a wide geographical distribution throughout Africa especially in Egypt and inhabit a wide range of habitats from lowlands to mountains, which makes it the most successful fruit-bat in the sub-order Megachiroptera [7]. They are light brown in color with darker brown wings. They have dark eyes, a long dog-like muzzle, large pointed ears and very soft fur. Accordingly, these bats are sometimes referred to as flying foxes, because they are small enough to handle and have familiar appearance like that of dogs or foxes. They can be found roosting

in natural caves and feeds on various juicy fruits [6]. Twenty four percent of all known mammalian species are bats which frequently act as vectors of lyssaviruses that play an important role in the epidemiology of rabies. Transmission of rabies from an infected bat may be via a bite but other routes are also apparently possible [9].

Recently, fruit-bat becomes a popular pet and is thought to be distributed in black-markets in Korea although unproven. One of the Egyptian fruit-bats (body weight=162 g, wingspan=615 mm and total length=16.5 cm) that was imported from Egypt to a pet shop in Korea was sold to a private commercial aquaria for indoor exhibition. It was later found dead and its body was submitted to the College of Veterinary Medicine, Seoul National University for postmortem examination. Upon examination, it was noted to have bite marks on both sides of the neck (Fig. 1-A, B), exposed foreleg (wing) bone and lung hemorrhages.

Sterile swabs from the blood specimen were streaked on Tryptic Soya Agar (TSA; Oxoid, Basingstone, Hampshire, England) and incubated at 25°C for 24 hr. Dense pure culture growth of single colonies of bacteria were recovered from blood and re-streaked again on the same fresh media to obtain the pure isolate. Gram staining and motility test were performed. Vitek System®2 (bioMérieux®, Marcy-l'Etoile, France) test was inoculated with the pure isolate and read as described by the identification kit for the characterization of the isolate. The result of the phenotypic bacterial identification was compared to reference strain from Bergey's Manual of Determinative Bacteriology [5]. The result showed that the isolate was catalase positive, positive for utilization of maltose, mannitol, mannose, sucrose, trehalose and ferments D-glucose (acid and gas production). It is oxidase negative and negative for H<sub>2</sub>S production, lipase and utilization of adonitol (Table 1). A test for hemolysis was also conducted in pure isolate using 5% sheep blood agar (Korea

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Fig. 1-A, B. Postmortem examination of Egyptian fruit-bat with bitten wounds (white arrows) on both sides of the neck (A: left bite mark, B: right bite mark).

Table 1. The characteristics of isolated strain as revealed by Vitek System<sup>®</sup>2 profile in comparison with the reference of *Kluyvera ascorbata* strain from Bergey's Manual of Determinative Bacteriology

Characteristics	Vitek System <sup>®</sup> 2	Bergey's Manual <sup>a)</sup>
Hydrogen sulfide production (H <sub>2</sub> S)	–	–
D-Glucose, acid production	+	+
D-Adonitol	–	–
Maltose	+	+
D-Mannitol	+	+
D-Mannose	+	+
Sucrose	+	+
Trehalose	+	+
Lipase	–	–
Oxidase	–	–

+: positive, –: negative.

a) According to Holt *et al.* [5].

Media, Korea). The isolate shows whitish hemolysis and it is  $\beta$ -hemolytic. It also showed 98% probability using the Vitek System<sup>®</sup>2 test. Based on these features and comparison with reference strain from the Bergey's Manual of Determinative Bacteriology indicates that the isolate is closely related to *K. ascorbata*. So far, there were no reports on the isolation of *K. ascorbata* in bat especially in fruit bat. Although, the origin and mode of infection of the *K. ascorbata* isolated from fruit bat in the present study is not known. It might be that the fruit bat died of septicemia because *K. ascorbata* was isolated from the blood of the fruit bat. Maybe the most likely route of infection was through the bitten wound in the neck of the fruit bat that was infected by this zoonotic pathogen. It was suspected that the wound was caused by bite from other live fruit-bats in the same cage that carries this zoonotic pathogen. Although the Vitek System<sup>®</sup>2 profile revealed the *K. ascorbata*, it was not possible to obtain an accurate identification of the *K. ascor-*

*bata* isolates using this rapid system. In addition, the genotypic identity of the present bacterial isolate was further confirmed using the 16S rRNA gene sequencing. Polymerase chain reaction (PCR) and sequencing were applied for the bacterial species confirmation. The representative 16S rRNA gene of the bacterial species was amplified by PCR using universal primers 518F and 800R [8]. The procedure used for the isolation and purification of genomic DNA from the sample was done by DNeasy<sup>®</sup> Tissue Kit (QIAGEN, Hilden, Germany). Sequencing of the purified PCR product was performed using ABI PRISM Big Dye TM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, U.S.A.) at the MacroGen Genomic Division, Korea. Electrophoresis of sequencing reactions was completed using automated ABI PRISM 3730XL DNA Sequencing System (Applied Biosystem, Foster, CA, U.S.A.). The rRNA sequence gene of the bacterial species obtained in this study was aligned with those of other same bacterial species available from GenBank database using the multiple alignment algorithms in the MegAlign package (Windows Version 3.12e; DNASTAR Software Package, Madison, WI, U.S.A.) and percentage sequence similarities were determined. The GenBank accession numbers of the same known bacterial sequences used were AJ627201, AJ627202, AJ627203, AF176559, AF176560, AF176566 and AF008579. The determined sequence consisted of about 738 nucleotides and was compared with the sequences of other known isolates of *K. ascorbata* available from GenBank. The 16S rRNA gene of *K. ascorbata* of the present study was identical and exhibited 99% sequence similarity with other strains of *K. ascorbata* available in the GenBank.

The susceptibility pattern of bacterial isolate to antimicrobial drugs was followed using the protocol of Baeck *et al.* [2] with additional test on antimicrobial drugs such as enrofloxacin (5  $\mu$ g), amoxicillin/clavulanic acid (30  $\mu$ g), oxytetracyclin (30  $\mu$ g), cefepime (30  $\mu$ g), cefotaxime (30  $\mu$ g) and chloramphenicol (30  $\mu$ g) (Becton, Dickinson and

Company, Baltimore, MD, U.S.A.). Antimicrobial susceptibility studies of *K. ascorbata* has shown resistance to amikacin, ampicillin, carbenicillin, cefixime, cefoperazone, colistin, gentamicin, kanamycin, nalidixic acid, neomycin, nitrofurantoin, ofloxacin, polymyxin b, tetracycline, tobramycin, trimethoprim, sulfamethoxazole/trimethoprim enrofloxacin, amoxicillin/clavulanic acid, oxytetracycline, cefepime, and chloramphenicol. It is sensitive to ciprofloxacin, norfloxacin, and cefotaxime. Based on the result of the antibiotic susceptibility test, ciprofloxacin, norfloxacin, and cefotaxime seem to be effective treatment against *K. ascorbata* infection.

Test for the qualitative detection of rabies virus antigen in the fruit-bat saliva and brain homogenates using the Rapid Rabies Ag Test Kit (Anigen, Hwasung, Kyunggi, Korea) was also conducted in accordance to the manufacturer's protocol. The result showed that the saliva and brain homogenate samples were negative for rabies virus antigen (data not shown). Even though, rabies virus antigen was not detected from the dead fruit bat, the test was also valuable to confirm if it is pathogen free or not, since fruit-bat can be a vector of rabies.

Two test groups of five ICR male mice (aged 2 and 12 weeks) were intraperitoneally injected with  $1.3 \times 10^8$  CFU/ml (dose volume 0.2  $\mu$ l) of *K. ascorbata*. Control group of 5 ICR male mice (aged 2 weeks) were intraperitoneally injected with HBSS. This experiment was performed in accordance with the guideline for animal experiment, College of Veterinary Medicine, Seoul National University, Korea. The result showed no mortality and no other clinical symptoms were observed all throughout the infection experiment.

Based on morphological, biochemical test and 16S rRNA gene sequencing, the present bacterial strain isolated from blood specimen was identified as a *Kluyvera ascorbata*. Since the submitted fruit-bat had bite marks on both sides of the neck and exposed foreleg (wing) bone, there was a chance of horizontal transmission from other bats who are carriers of this pathogenic and potentially dangerous strain of the genus *Kluyvera* through this route. Moreover, diagnosis and detection of this infrequent zoonotic pathogen from the fruit bat for the first time has also important roles. First, it can act as a reservoir of this pathogen that can be easily transmitted to human and produce clinical infection, although in the present study, infection experiment to mouse did not produced any mortality or human like clinical symptoms or infection. Thus, the present isolate can be considered as non-virulent to mammals or human. Secondly, this fruit-bat was imported to Korea through black market from Egypt and did not pass through quarantine system to check whether it is pathogen free or not. Many steps should be done in order not to threaten human health from many diseases of animals. It's good to lay out the appropriate education for pet breeder and pet lovers as well. Then, when animals are imported from other countries, it must be quarantined to prevent the spread of any infectious disease brought about by these animals. Animals which transferred

through unproven way may enhance the chance to cause zoonosis. Lastly, when some symptoms of disease are observed in these animals, it is better to send these animals to a veterinary laboratory for diagnostic examination whether alive or dead in order to prevent spread of zoonotic pathogen that cause human diseases. To our knowledge, this report was the first case of isolation of *Kluyvera ascorbata* in fruit-bat. However, its direct association to the death of fruit-bat is not certain.

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