Serological evidence of ebolavirus infection in bats, China

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Abstract
Background: The genus *Ebolavirus* of the family Filoviridae currently consists of five species. All species, with the exception of *Reston ebolavirus*, have been found in Africa and caused severe human diseases. Bats have been implicated as reservoirs for ebolavirus. *Reston ebolavirus*, discovered in the Philippines, is the only ebolavirus species identified in Asia to date. Whether this virus is prevalent in China is unknown.

Findings: In this study, we developed an enzyme linked immunosorbent assay (ELISA) for ebolavirus using the recombinant nucleocapsid protein and performed sero-surveillance for the virus among Chinese bat populations. Our results revealed the presence of antibodies to ebolavirus in 32 of 843 bat sera samples and 10 of 16 were further confirmed by western blot analysis.

Conclusion: To our knowledge, this is the first report of any filovirus infection in China.

Keywords: Ebolavirus, Antibody detection, Bats

Findings
Filoviruses are associated with acute fatal hemorrhagic diseases of humans and/or nonhuman primates when they spill over from their wildlife reservoir hosts. The family consists of two genera: *Marburgvirus* and *Ebolavirus* [1,2]. Five species of ebolavirus have been identified: *Ivory Coast ebolavirus*, *Sudan ebolavirus*, *Zaire ebolavirus* (EBOV), *Reston ebolavirus* (RESTV) and *Bundibugyo ebolavirus*. RESTV is the only known filovirus that does not cause severe disease in humans; however, it can be fatal in monkeys [3]. In 2009, infection of domestic pigs by RESTV was reported in the Philippines [4]. It was speculated that RESTV infected monkeys and pigs from an as yet unidentified host. Bats have been implicated as reservoirs for Marburgvirus [5] and Ebolavirus [6] in Africa and Asian country, the Philippines [7]. Previously, we have detected antibodies to the severe acute respiratory syndrome virus [8] and henipavirus [9] in bat sera in China. In this study, we conducted a surveillance study for the presence of ebolavirus in Chinese bat populations.

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Considering the close geographical relationship of Chinese and Philippine bats, Reston-NP was used for the initial screening. ELISA plates were coated with the recombinant Reston-NP at approximately 100 ng/well and bat sera were tested in triplicates at a dilution of 1:100, followed by detection with horseradish peroxidase (HRP) conjugated Protein A/G (Pierce) at 1:20,000. Samples with a mean optical density 2.1-fold or higher than that of the negative control (OD\textsubscript{450} value: 0.19) were considered positive. Positive serum samples were retested at dilutions of 1:100, 1:400, and 1:1600 against both Reston-NP and Zaire-NP (Figure 2).

A summary of the initial screening results is shown in Table 1. Of the 843 bat sera screened for antibodies to Reston-NP by ELISA, 32 were positive. These were from 10 of the 23 bat species collected from 5 different locations. Among the positive sera, 17 had OD\textsubscript{450} readings higher than 0.19 at 1:400 and 3 at 1:1600 (Figure 2). These positive sera were further tested with the Zaire-NP protein and 25 were positive, and 10 samples had OD\textsubscript{450} values higher than 0.19 at a 1:400 and 4 at a 1:1600. Sixteen bat sera with sufficient remaining quantity were further confirmed by western blot analysis with the recombinant NP expressed in Escherichia coli, and 10 were reactive to both the Reston- and Zaire-NP proteins (Figure 3, Table 2).

A surrogate virus neutralization test was conducted using a recombinant env HIV-1 virus containing the luciferase reporter gene pseudotyped with spike glycoprotein proteins (GP) of EBOV (Zaire-GP) or RESTV (Reston-GP [11]). The plasmid encoding Zaire-GP (L11365) was kindly provided by Prof. Lijun Rong (University of Illinois at Chicago, USA). The Reston-GP gene was synthesized based on the RESTV genome sequence (FJ621583). Serum was serially diluted at 1:20 to 1:640 in 30 μl of medium and mixed with 30 μl of pseudovirus solution. The mixture was incubated for 1 h at 37°C and subsequently added, in triplicate, to 293T cells grown in a 96-well plate. The plate was incubated for 1 h at 37°C before being replenished with fresh medium and incubated for 48 h. Cells were lysed in 30 μl of lysis reagent (Progema) and luciferase activity was measured using a
Luciferase assay kit (Promega). None of the positive bat serum inhibited entry of Reston-GP or Zaire-GP pseudotyped virus.

The Invitrogen OneStep RT-PCR Kit was used to screen ebolavirus RNA using the universal primers against the Filovirus L gene or N gene as described previously [12,13]. RNA was extracted using the QiAamp Viral RNA Mini Kit (Qiagen) following the manufacturer’s instructions. No filovirus-specific RNA was detected by one step RT-PCR among 143 tissue samples (spleen, liver or fecal swab) tested, therefore, virus isolation was not attempted.

In this paper, we presented serological evidence of ebolavirus infection in several bat populations in China. To our knowledge, this is the first report of any ebolavirus infection in this part of the world. The most significant prevalence of ebolavirus antibody was found among the Rousettus leschenaulti, Pipistrellus pipistrellus and Myotis species. Several serum samples have relatively high titer to both Reston-and Zaire-NP.

There are several possibilities to account for the failure in detecting neutralizing antibodies. In general, bats seem to produce lower level of neutralizing antibodies in response to viral infection, possibly due to the lower affinity of the bat antibodies [14]. Alternatively, it is possible that one or more as-yet-unknown ebolaviruses are circulating among the bat populations sampled in this study, producing antibodies cross-reactive with, but not neutralizing EBOV or RESTV. Also, a new ebolavirus species closely-related with EBOV but not the RESTV might be missed during the initial screening using Reston-NP solely. An initial screening for ebolavirus antibodies should be conducted by using ELISA with a 1:1 mixture of recombinant NP of EBOV and RESTV to detect wider range of ebolavirus species. Recently, a genetically distinct filovirus was found in dead insectivorous bats in Spain [15], suggesting that filoviruses have a wider host range and geographical

| Bat species                  | No. positive / No. tested (percent) |
|-----------------------------|-----------------------------------|
| **Megachiroptera**          |                                   |
| Rousettus leschenaulti      | 11/126 (8.73%)                    |
| Cynopterus sphinx           | 2/2 (100%)                        |
| **Microchiroptera**         |                                   |
| Hipposideridae              |                                   |
| Hipposideros Pomona         | 3/39 (7.69%)                      |
| Hipposideros spp.           | 1/15 (6.67%)                      |
| Hipposideros cineraceus     | 0/11                              |
| Hipposideros armiger        | 0/41                              |
| Hipposideros larvatus       | 0/21                              |
| **Rhinolophidae**           |                                   |
| Rhinolophus affinis         | 1/69 (1.45%)                      |
| Rhinolophus ferrumequinum   | 0/15                              |
| Rhinolophus sinica          | 0/6                               |
| Rhinolophus pusillus        | 0/14                              |
| Rhinolophus pearsoni        | 0/3                               |
| Rhinolophus spp.            | 0/15                              |
| **Vespertilionidae**        |                                   |
| Miniopterus schreibersii    | 2/23 (8.7%)                       |
| Pipistrellus pipistrellus   | 4/35 (11.43%)                     |
| Myotis ricketti             | 4/83 (4.82%)                      |
| Myotis dauvidii             | 0/5                               |
| Myotis chinensis            | 0/6                               |
| Myotis daubentoni           | 0/24                              |
| Myotis jimbarinus           | 0/2                               |
| Myotis spp.                 | 3/118 (2.54%)                     |
| Scotophilus kuhli           | 1/25 (4%)                         |
| Unknown                     | 0/2                               |
| **Total**                   | 32/843 (3.8%)                     |

Table 1 Detection of antibody to RESTV nucleocapsid protein by ELISA

Figure 3 Western-blot analysis of ELISA positive serum samples with recombinant Reston-NP and Zaire-NP expressed in E. coli. The polyclonal antibody against the full-length nucleocapsid protein of RESTV was used as positive control. Sample no. 2195 was ELISA negative and used as negative control. The other 5 samples were western blot positive. Note: western blot no. 1487, 1552, 1689, 1973 and 2166 is not presented due to the absence of signal in the scanned photograph.
location than previously thought. The unsuccessful identification of ebolavirus-related genes in the samples is likely attributable to the often low-level of virus replication, the similarly transient nature of the infection in bats or the sequence mis-match of the PCR primers used and the target sequence of the potential unknown ebolavirus genomes.

There are approximately 120 species of bats distributed throughout China. Bat species in the genera Rousettus, Hipposideros, Myotis, Miniopterus and Pipistrellus naturally reside in trees, buildings and caves that can be in close proximity to human residential areas, increasing the potential of zoontic transmission from bats to humans. There is evidence of human infection of ebolavirus in central Africa as a result of direct bat-to-human transmission [16]. In the Guangdong and Hainan provinces of China, local populations customarily eat large bats, such as Leschenault’s rousette. The preliminary results presented in this study highlight the need to continue and expand surveillance for ebolavirus or related viruses, and virus abbreviations.

Table 2 Determination of ELISA titer and western blot reactivity against the truncated nucleocapsid proteins of RESTV (Reston-NP) and EBOV (Zaire-NP)

| Bat species and sample ID | Titer in ELISA | Western blot |
|--------------------------|----------------|--------------|
|                          | Reston-NP      | Zaire-NP     | Reston-NP | Zaire-NP |
| Rousettus leschenaulti (no:1263) | 100 | 100 | N | N |
| Rousettus leschenaulti (no:1276) | 400 | 400 | N | N |
| Cynopterus sphinx (no:1467) | 400 | 400 | + | + |
| Hipposideros Pamaona (no:1552) | 100 | 100 | + | + |
| Rousettus leschenaulti (no:1688)* | 100 | 100 | + | + |
| Rousettus leschenaulti (no:1689) | 100 | 100 | + | + |
| Pipistrellus pipistrellus (no:1973) | 400 | 400 | + | + |
| Pipistrellus pipistrellus (no:1977)* | 400 | N | + | + |
| Myotis spp. (no:2114)* | 400 | 400 | + | + |
| Rousettus leschenaulti (no:2158) | 100 | 100 | N | N |
| Rousettus leschenaulti (no:2166) | 100 | N | + | + |
| Rousettus leschenaulti (no:2169)* | 100 | 100 | + | + |
| Rousettus leschenaulti (no:2183) | 400 | 400 | N | N |
| Rousettus leschenaulti (no:2190) | 100 | 100 | N | N |
| Rousettus leschenaulti (no:2211) | 100 | 400 | N | N |

+: positive; N: no signal was detected; *: The results of western blot are shown in Figure 3.

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References
1. Barrette RW, Xu L, Rowland JM, McIntosh MT: Current perspectives on the phylogeny of Filoviridae. Infect Genet Evol 2011, 11:1514–1519.
2. Kuhn JH, Becker S, Eibl H, Geissbaur T, Johnson KM, Kawacka Y, Lipkin WI, Negredo AI, Netesov SV, Nichol ST, et al: Proposal for a revised taxonomy of the family Filoviridae: classification, names of taxa and viruses, and virus abbreviations. Arch Virol 2010, 155:2083–2103.
3. Miranda ME, Kisazek TG, Retuya TJ, Khan AS, Sanchez A, Fullerton CF, Rollin PE, Calico AB, Manalo DL, Rosos MC, et al: Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. J Infect Dis 1999, 179(Suppl 1):S115–S119.
4. Barrette RW, Metwally SA, Rowland JM, Xu L, Zaki SR, Nichol ST, Rollin PE, Towner JS, Shieh WJ, Batten B, et al: Discovery of swine as a host for the Reston ebolavirus. Science 2009, 325:204–206.
5. Towner JS, Pourrut X, Albarino CG, Nkogue CN, Bird BH, Grard G, Kisazek TG, Gonzalez JP, Nichol ST, Leroy EM: Marburg virus infection detected in a common African bat. PLoS One 2007, 2:e764.
6. Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, Dellicat A, Pawaeska JT, Gonzalez JP, Swanepoel R: Fruit bats as reservoirs of Ebola virus. Nature 2005, 438:575–576.
7. Taniuchii S, Watanabe S, Masangkay JS, Omatso T, Ikagami T, Alviola P, Ueda N, Ika K, Fujii H, Ishii Y, et al: Reston Ebolavirus antibodies in bats, the Philippines. Emerg Infect Dis 2011, 17:1559–1560.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JY and YZ independently performed protein expression and ELISA. JL performed the RNA extraction and RT-PCR. YZ provided samples and information about bats. LW and ZS conceived the project and provided overall scientific oversight. All authors contributed to the preparation of the final manuscript. All authors read and approved the final manuscript.
8. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, et al: Bats are natural reservoirs of SARS-like coronaviruses. Science 2005, 310:676–679.

9. Li Y, Wang J, Hickey AC, Zhang Y, Li Y, Wu Y, Zhang H, Yuan J, Han Z, McEachern J, et al: Antibodies to Nipah or Nipah-like viruses in bats, China. Emerg Infect Dis 2008, 14:1974–1976.

10. Marsh GA, Haining J, Robinson R, Food A, Yamada M, Barr JA, Payne J, White J, Yu M, Bingham J, et al: Ebola Reston virus infection of pigs: clinical significance and transmission potential. J Infect Dis 2011, 204(Suppl 3):S804–S809.

11. Manicassamy B, Wang J, Jiang H, Rong L: Comprehensive analysis of ebola virus GP1 in viral entry. J Virol 2005, 79:4793–4805.

12. Ogawa H, Myamoto H, Ebihara H, Ito K, Morikawa S, Feldmann H, Takada A: Detection of all known filovirus species by reverse transcription-polymerase chain reaction using a primer set specific for the viral nucleoprotein gene. J Virol Methods 2011, 171:310–313.

13. Panning M, Laue T, Olschlager S, Eickmann M, Becker S, Raith S, Courbot MC, Nilsson M, Gopal R, Lundkvist A, et al: Diagnostic reverse-transcription polymerase chain reaction kit for filoviruses based on the strain collections of all European biosafety level 4 laboratories. J Infect Dis 2007, 196(Suppl 2):S199–S204.

14. Baker ML, Tachedjian M, Wang LF: Immunoglobulin heavy chain diversity in Pteropid bats: evidence for a diverse and highly specific antigen binding repertoire. Immunogenetics 2010, 62:173–184.

15. Negredo A, Palacios G, Vaquero-Morron S, Gonzalez F, Dapazao H, Molero F, Juste J, Quejéjas J, Savij N, de la Cruz Martinez M, et al: Discovery of an ebolavirus-like filovirus in europe. PLoS Pathog 2011, 7:e1002304.

16. Pourrut X, Delicat A, Rollin PE, Ksiazek TG, Gonzalez JP, Leroy EM: Spatial and temporal patterns of Zaire ebolavirus antibody prevalence in the possible reservoir bat species. J Infect Dis 2007, 196(Suppl 2):S176–S183.

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