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Short communication

Quantitative measurement of influenza virus replication using consecutive bronchoalveolar lavage in the lower respiratory tract of a ferret model

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The ferret is an established animal model of influenza virus infection. Although viral replication in the upper respiratory tract is usually measured with consecutively collected nasal washes, daily evaluation of viral replication in the lung is limited because a large numbers of ferrets need to be sacrificed at consecutive time points. To overcome this limitation, we performed a virus quantification assay using bronchoalveolar lavage (BAL) fluid. This non-invasive BAL technique allows consecutive quantification of virus replication in the lungs of living ferrets. Our method can be used for the longitudinal evaluation of virus tropism in the lower respiratory tract.

Keywords: bronchoalveolar lavage, ferret, infection, influenza

The ferret (*Mustela putorius furo*) has been identified as an appropriate animal model of influenza A virus infection in humans. This is because ferrets are sensitive to natural infection, and develop clinical symptoms along with infection-associated lesions that mimic those in humans [1,6]. Ferrets also have a respiratory physiology similar to that of humans. Moreover, influenza A viruses exhibit similar patterns of binding to the sialic acid influenza virus receptors that are distributed throughout the respiratory tract in both species. In addition, similar clinical and virological features are observed in ferrets and humans following influenza virus infection including fever, nasal secretion, coughing, gastrointestinal complications, serum abnormalities, neurological complications, weight loss, lethargy, lymphopenia, hypercytokinemia, and virus transmission to susceptible contacts [1]. Therefore, ferrets have been used to study the pathogenesis and transmission of influenza viruses with pandemic potential. For instance, virus pathogenicity and transmissibility of the swine-origin pandemic 2009 A (H1N1) virus were evaluated using ferret models [4,7,8]. The efficacy of novel influenza vaccine candidates has also been measured in ferrets [2,3].

When using ferret models, virus infection is largely assessed based on quantification of shed virus in the respiratory tract following viral inoculation. Most previous studies examined virus replication using consecutively collected nasal washes and oropharyngeal swabs to measure viral loads in the upper respiratory tract. Longitudinal evaluation of virus replication in the lungs could allow the characterization of virus tropism and invasiveness. However, daily evaluation of virus replication in the lower respiratory tract is limited because large numbers of animals need to be sacrificed at consecutive time points to collect lung tissues.

Analysis of bronchopulmonary secretions is widely used to diagnose lower respiratory tract diseases in small animals. These secretions can be harvested by bronchoalveolar lavage (BAL). Additionally, the cellular composition and microbial contents in bronchoalveolar lavage fluid (BALF) can be further examined. In the present study, we quantified viral load in BALF collected from ferrets infected with influenza A virus. In addition, we evaluated the safety of the BAL procedure in ferrets using histopathology and measuring pro-inflammatory cytokine concentrations 3 days after BAL was performed.

Female ferrets (*Mustela putorius furo*) that were 5 months old and seronegative for antibodies against influenza A virus were used for the study. Animal housing and handling were performed under biosafety level (BSL)-2+ conditions approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University (Korea). General anesthesia was induced by injection with 7 mg/kg

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body weight Zoletil (Virbac, France) and 3 mg/kg body weight Xylazine-HCl (Rompun; Bayer HealthCare, Germany) during sample collection.

Three ferrets were intranasally challenged with a $10^{7.0}$ 50% egg infectious dose (EID_{50}) of A/canine/Korea/LBM412/2008(H3N2) virus as previously described [5]. All ferrets underwent a daily physical examination. Virus shedding was monitored 1, 3, 5, and 7 days post-inoculation (dpi) in collected nasal washes and BALF. The nasal cavity was washed with sterile phosphate-buffered saline (PBS). Xylocaine spray (AstraZeneca, UK) was topically administered in the oropharynx during the BAL procedures. As shown in Fig. 1, a sterile 3.0-mm endotracheal tube was placed in the trachea using the lighted guide of a laryngoscope, and a 6 French feeding tube was inserted through the lumen of the endotracheal tube. After each administration of 10 mL sterile PBS, the BAL fluid was retrieved by gentle suction. The total volume of recovered BALF was typically 5 ~ 7 mL.

Total RNA was extracted from 0.2 mL of each collected nasal washes and BALF using RNeasy Mini Kits (Qiagen, USA) according to the manufacturer's protocol. Viral M gene RNA was quantified by real-time reverse transcriptase PCR (rRT-PCR) according to the cycle threshold (Ct) value as previously described [9]. To extrapolate the Ct values to measure infectious units, samples of A/canine/Korea/LBM412/2008(H3N2) virus with known titers (EID_{50}) were serially diluted 10-fold using PBS. Viral RNA was extracted from these solutions and quantified by rRT-PCR. To generate a standard curve, Ct values for each viral dilution were plotted against viral titers. The resulting standard curve strongly correlated ($r^2 > 0.99$) and was used to convert Ct values into EID_{50} values.

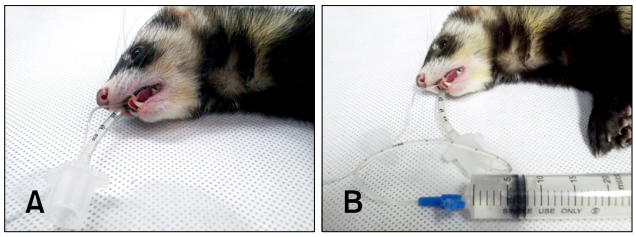

Fig. 1. Collection of bronchoalveolar lavage fluid (BALF) from the ferret model. (A) A sterile 3.0-mm endotracheal tube was placed in the trachea using the lighted guide of a laryngoscope. (B) A 6 French feeding tube was inserted through the lumen of the endotracheal tube. After each administration of 10 mL sterile phosphate buffered saline, BALF was retrieved by gentle suction.

Three additional ferrets were used to evaluate BAL safety. Histopathological analysis and measurement of pro-inflammatory cytokine levels were conducted using lung tissues collected 3 days post-BAL. Briefly, lung tissues were fixed in 10% neutral-buffered formalin, processed, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and examined by light microscopy. For cytokine analysis, the mRNA levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were measured as previously described [10].

In our previous study, A/canine/Korea/LBM412/2008 (H3N2) virus was found to replicate efficiently in the respiratory system of inoculated ferrets [5]. To measure the viral load in the lower respiratory tract of ferrets, three ferrets were euthanized 3 dpi. Robust virus replication was evident in the lower respiratory tract. Although these results demonstrated the replication capacity of this virus in the lower respiratory tract of ferrets, viral particles were quantified in collected lung tissues only from ferrets sacrificed 3 dpi. In the present investigation, we quantified the virus load in BALF collected from ferrets 1, 3, 5, and 7 dpi. This was done to make consecutive measurements of virus replication in the lower respiratory tract using the same ferrets instead of sacrificing the animals at multiple time points. As shown in Table 1, the challenge virus was detected in BALF from all H3N2-inoculated ferrets. Viral shedding from the H3N2-inoculated ferrets was detected from 1 to 7 dpi. Based on findings from our previous study, virus titers in the lung tissues of H3N2-inoculated ferrets at 3 dpi range from 3.0 to 4.2 EID_{50}/g [5]. Virus titers in the BALF collected from ferrets 3 dpi in the current study were higher than those in lung tissue analyzed in our previous investigation. Because the method we used for the present study involved repeated BALF collection from the same ferret rather than sacrificing multiple animals at different

Table 1. Excretion of challenge virus in the nasal wash and BALF samples

Ferret number	Virus titers in nasal washes*				Virus titers in BALF*			
	1 dpi	3 dpi	5 dpi	7 dpi	1 dpi	3 dpi	5 dpi	7 dpi
1	7.2	7.7	6.6	4.1	5.2	6.9	5.9	4.1
2	7.3	7.5	7.0	5.7	4.2	3.2	6.3	3.4
3	7.5	7.1	7.0	5.0	5.6	6.8	5.4	3.7

*Log EID_{50} equivalents were determined by real-time reverse transcription (rRT)-PCR. dpi: days post-inoculation.

time points, data collection could be refined and the number of ferrets required may be reduced. In addition, the replication capacity of the virus in each ferret can be consecutively analyzed, thus generating more detailed and relevant data for each ferret.

Although BAL is widely used to diagnose lower respiratory tract diseases in humans and animals, we also conducted a histopathological analysis and measured pro-inflammatory cytokine levels after BALF collection to determine whether the BAL procedure induces injury or inflammation in lung tissues. No pathological changes in the lung tissues from any ferret were uncovered by histopathological examination. Similarly, neither IL-6 nor TNF-α mRNA levels were elevated after the single BAL procedure. The outcome of influenza virus infection in animal models depends on multiple parameters including the extent of virus-associated pathology in the respiratory tract as well as immune and inflammatory responses. The present results indicated that the BAL procedure we performed did not affect lung pathology or induce an inflammatory response. Therefore, BAL can be safely performed for influenza studies using ferret models. However, the safety of multiple BAL procedures is unclear since we conducted a histopathological analysis and pro-inflammatory cytokine measurements only after a single collection of BALF in the current study.

A detailed assessment of pulmonary lesions can be made by pathological examination after the animals are euthanized. In order to identify ferrets with pulmonary lesions and perform longitudinal studies to monitor disease progression in infected ferrets, a non-invasive *in vivo* assessment of the lungs without sacrificing the ferrets is required. Veldhuis Kroeze *et al*. [12] recently reported that computed tomography (CT) scanning facilitates a longitudinal evaluation of influenza-induced pulmonary lesions in living animals. In addition, this group successfully used a consecutive CT scanning technique to evaluate the efficacy of an influenza vaccine using a ferret model [11]. In these previous studies, consecutive daily imaging overcame the limitations associated with necropsy performed at predefined time points after infection and enabled repeated evaluation of lung pathology in real time. CT scanning and BAL techniques in a ferret model would facilitate the consecutive analysis of pulmonary pathology and virus replication without requiring the sacrifice of a large number of ferrets at different time points.

In conclusion, results of the current pilot study demonstrated that a non-invasive BAL procedure allows the consecutive quantification of influenza virus replication in the lower respiratory tract of living ferrets. Performing BAL techniques in ferret models can enable the longitudinal evaluation of virus replication in the lungs to examine virus tropism and invasiveness. Furthermore, this methodology can be used in vaccine and anti-viral drug efficacy studies.

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Conflict of Interest

There is no conflict of interest.

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