

Evaluation of elution methods for recovery of highly pathogenic avian influenza (H5N1) virus from infected duck feathers

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ABSTRACT

In view of the tenacity of viruses in the feathers, many workers have suggested the importance of using feathers as the diagnostic material for epidemiological surveillance of highly pathogenic avian influenza (HPAI) H5N1 virus infections. In this study, we have compared the efficiency of two different processing methods, immersion method and trituration method for elution of HPAI H5N1 virus from the feathers of avian influenza negative ducks in terms of virus isolation in 9-to 11-day-old embryonated specific pathogen free chicken eggs and viral RNA detection by haemagglutinin gene based real-time RTPCR. The recovery of virus by immersion method in terms of percent infectivity in 3 replicates was 96.67, 93.33 and 96.67, whereas in trituration method, percent infectivity was 26.67, 20 and 16.67. In real-time RTPCR, viral RNA could be detected in 17 out of 18 samples by immersion method and from only 2 out of 18 samples by trituration method. The study revealed that the immersion method gave higher viral infectivity percentage and could also be easily detected by real-time RTPCR. We conclude that immersion method of virus elution could be useful for processing of shed duck feathers present in the environment for epidemiological screening against HPAI H5N1 virus infections.

Key words: Duck feathers, H5N1 subtype, Highly pathogenic avian influenza, Real-time RTPCR, Virus isolation

In domestic ducks, fecal–water–oral route plays a significant role in transmission of avian influenza virus in other poultry species because of higher level of virus excretion from their cloaca than from the respiratory route (Markwell and Shortridge 1982). The progressive dilution of infectious feces in water could decrease likelihood of virus/host interactions implying that there could be some natural mechanisms that concentrate AIVs from water onto bird bodies, particularly feathers. These contaminated feathers lead to spread of infections, but shed feathers may also harbor viruses for long periods of time (Yamamoto *et al.* 2008). Feather swabs collected from experimentally preened birds were found positive by virus isolation roughly for one month (Delogu *et al.* 2012). Many workers suggested importance of using feathers as the diagnostic material for epidemiological surveillance (Nemeth *et al.* 2009, Busquets *et al.* 2010, Lebarbenchon *et al.* 2013). However, to use infected feathers for diagnosis without compromising sensitivity, it is important that elution of virus

from such feathers during processing is efficient. Recovery of H5N1 HPAI viruses for isolation and diagnosis from duck feathers is carried out either through trituration or by removing viral particles adsorbed on feather surfaces by immersion. However, even though some studies have compared the results of both methods with reference to virus isolation, no comparable literature could be retrieved for real-time RTPCR. In this study, we compared efficiency of immersion and trituration methods for diagnosis of H5N1 avian influenza viruses from duck feathers by both virus isolation and real-time RTPCR.

MATERIALS AND METHODS

Source of virus: Highly pathogenic avian influenza (H5N1) virus (A/Crow/India/11TI 16/2011) accessed from repository of HSADL, Bhopal was used in this study. The virus was propagated in embryonated-specific pathogen free chicken eggs (ECEs) and EID₅₀ of the infective allantoic fluid was estimated as per Reed and Muench (1938). The EID₅₀ of the stock virus was estimated to be 10^{8.05}/ml.

Duck feather samples: Feathers from ducks were collected from 6 different locations in Kerala, West Bengal and Madhya Pradesh. To ensure that feathers being used for the study are free from avian influenza virus, feathers were screened for the presence of influenza A virus by real-time RTPCR using influenza A matrix gene specific primer

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and probe (Payungporn *et al.* 2006) and those tested negative were used for further studies.

Spiking of the virus in duck feathers: Spiking of H5N1 virus was carried out as per (Delogu *et al.* 2010) without modification.

Sample processing for recovery virus from feather sample: The protocols for immersion (Delogu *et al.* 2010) and trituration (Nemeth *et al.* 2009) were used without modification. The supernatant was collected in a set of sterile vials for virus isolation and RNA extraction. The experiment was replicated thrice.

Isolation of virus from the samples: The isolation of H5N1 HPAI virus was carried out in 9- to 11- day-old embryonated specific pathogen free chicken eggs and confirmed by haemagglutination test with the harvested amnioallantoic fluid from the infected eggs as per OIE (2008). The HA-negative allantoic fluids were repassaged up to third passage and tested for HA before considering the allantoic fluids to be negative for virus isolation.

Extraction of viral RNA: Viral RNA mini kit was used to extract viral RNA from 140 µl of the eluted sample as per the manufacturer's protocol.

Real-time RTPCR: Real-time RTPCR was carried out with RNA extracted from the treated feather samples and H5 specific primers and probe for HA gene (Spackman *et al.* 2002) using real-time PCR system.

Statistical analysis: The persistence of virus was determined by calculating the percent infectivity (Lu *et al.* 2003) as determined by the percentage of infected (haemagglutination positive) embryos out of the total number of embryos inoculated. The variables considered for the statistical analysis consisted of percent infectivity as dependent variable and time as the independent variable.

RESULTS AND DISCUSSION

Replication of HPAI H5N1 virus in feather epidermal cells of domestic ducks could result in the possibility of viral release from feathers and may result in environmental persistence of viruses (Yamamoto *et al.* 2008). Hence, feathers of infected waterfowl may have an epidemiological importance for avian influenza (H5N1) outbreaks. The feathers offer numerous advantages to surveillance

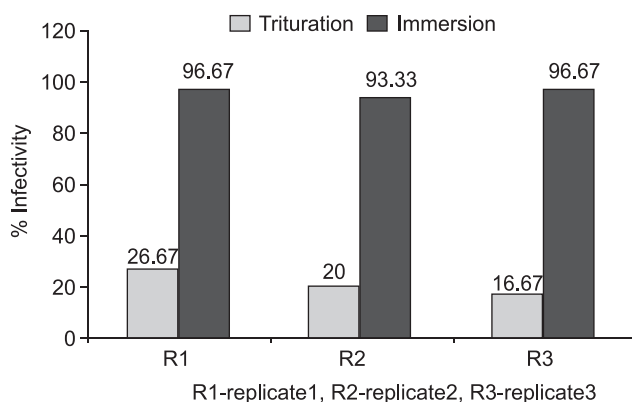


Fig. 1. Comparison of percent infectivity of feather samples processed by immersion and trituration methods.

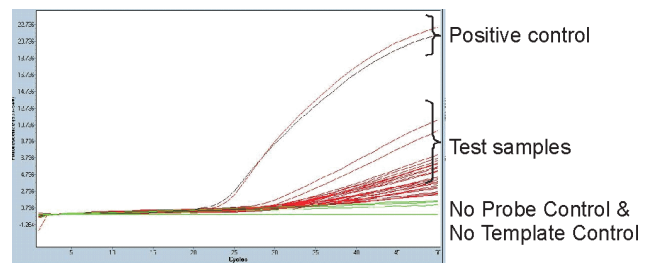


Fig. 2. Detection of viral RNA in feather samples by real-time RTPCR.

programs, viz. ease of collection, simpler storage and shipping conditions, longer period of detectability compared with tissues and lower biosafety risk. Further, these can also be used for morbidity surveillance and diagnosis in individual birds due to the ease in their collection from live birds.

In order to ensure optimum recovery of the virus from the virus spiked feathers used in the present study, 2 different methods were evaluated: immersion method and trituration. The evidence of virus recovery was studied both by virus isolation in embryonated chicken eggs as well as in real-time RTPCR. The recovery of virus by immersion method in terms of percent infectivity in 3 replicates was 96.67, 93.33 and 96.67, respectively, whereas in trituration, percent infectivity was 26.67, 20 and 16.67 (Fig. 1) respectively. In real-time RTPCR viral RNA (Fig. 2) could be detected in 17 out of 18 samples (Table 1) included in 3 replicates of the virus eluted by immersion method. In contrast, in trituration method used for virus elution, viral RNA could be detected from only 2 out of 18 samples. Comparative evaluation revealed that immersion method gave higher viral infectivity percentage in ECEs and could also be easily detected by real-time RTPCR. In contrast, in trituration method, virus recovery was lower in embryonated chicken eggs and viral RNA was also not detected by real-time RTPCR. One of the reasons for lower recovery by trituration method could probably be the release of inhibitors from the feathers due to mechanical shearing of feather calami during grinding (Nemeth *et al.* 2009). The feathers used in our present study were collected from the ducks that were free from avian influenza virus infection and hence there was a very little likelihood of the virus being present in internal structures of feathers, particularly the feather epidermal cells except for a little quantity entering internal structures of feather during spiking of the virus. Major proportion of virus concentration in our study would be on the surface of the feather on shaft following adsorption of virus on feather surface during virus spiking. Consequently, possibility of immersion method being more efficient in detaching adsorbed virus from feather surface in our study cannot be ruled out. In this scenario, while trituration method could be useful while processing feathers derived from infected birds, immersion method could be more beneficial in processing feathers that have been externally contaminated by virus present in certain ecological niches, viz. soil and other solid waste in the

vicinity of duck farms or water sources such as duck ponds or lakes. There are some reports of birds carrying viruses on their feathers in spite of these birds testing negative for virus in their cloaca and trachea (Rose *et al.* 2006). Among the various abiotic factors present in environment, feathers shed in the vicinity of farms and ponds could play a major role in spreading the infection particularly because of the ability of feathers to adsorb viruses more easily on its surface because of preen oil present in these feathers. Reports suggested that preen oil has a capacity to adsorb avian influenza virus from the infected water sources and may result in contamination of duck plumage when they are in water and these preened bodies are an ecologic link between aquatic birds and the environmental persistence of AIVs (Delogu *et al.* 2010). In conclusion, immersion method evaluated in the present study could be especially useful in processing feather samples collected from environment, particularly for screening environmental samples from farms suspected for influenza outbreak.

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