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LETTER

Simultaneous detection of duck hepatitis A virus types 1 and 3, and of duck astrovirus type 1, by multiplex RT-PCR

Dear Editor,

Duck virus hepatitis (DVH) is caused by at least three different RNA viruses, including duck hepatitis A virus (DHAV), duck astrovirus type 1 (DAstV-1), and duck astrovirus type 2 (DAstV-2). The first of these, DHAV, has been classified into three serotypes by neutralization tests: type 1 (DHAV-1), type 2 (DHAV-2), and type 3 (DHAV-3) (Wang L, et al., 2008). Two of these serotypes, DHAV-1 and DHAV-3, together with DAstV-1, have been isolated from various different regions of China in recent years (Chen L, et al., 2012; Chen L L, et al., 2013; Ding C, et al., 2007; Fu Y, et al., 2008, 2009; Gao J, et al., 2012; Wang L, et al., 2008; Wang X, et al., 2011; Xu Q, et al., 2012).

In ducklings, it is difficult using conventional diagnostic methods to determine which particular pathogen is responsible for an incidence of DVH, because the clinical indications and gross pathological changes are similar for all three types of duck virus hepatitis (DHV) infections. A number of laboratory methods for the molecular detection of DHV infection have been developed recently and are now in common use (Cheng A C, et al., 2009; Chen L L, et al., 2013; Huang Q, et al., 2012; Kim M C, et al., 2007, 2008; Yang M, et al., 2008); however, they all share a major disadvantage in that their ability to distinguish between DHAV-1, DHAV-3, and DAstV-1 in a single reaction is limited. In this study, therefore, a multiplex reverse transcription polymerase chain reaction (RT-PCR) assay has been developed that can detect and distinguish DHAV-1, DHAV-3 and DAstV-1 simultaneously.

Three pairs of primers for DHAV-1, DHAV-3, and DAstV-1 were selected on the basis of whole-genome sequence alignments of DHV sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov). To identify and select suitable primers, 57 DHAV-1 sequences, 20 DHAV-3 sequences, and 6 DAstV-1 sequences were aligned. The primer pairs for the three viruses [DHAV-1F: 5'-GATGTGGCAY(T/C)GTTGTY(T/C)AAY(T/C)CGA-3'; DHAV-1R: 5'-CTGATGTD(G/A/T)CCAG GR(A/G)ATTGGTCG-3'; DHAV-3F: 5'-GAGCCA GAA TTGGAATGGACACA-3'; DHAV-3R: 5'-CATACT TR(G/A)CCACCAACTGCCAATC-3'; DAstV-1F: 5'-ATGGCCCAGAGCGGTGAAAA-3'; and DAstV-1R: 5'-GCCAGGTGTCAACAATCATGC-3'] were designed to amplify 570 bp, 1099 bp and 898 bp fragments, respectively. All of the primers were synthesized by Sangon (Shanghai, China).

DHAV-1 strain LY0801 (accession no. FJ436047), DHAV-3 strain SD1101 (accession no. JQ409566), and DAstV-1 strain WF1201 (accession no. JX439643) were selected as reference isolates (Chen L L, et al., 2013; Xu Q, et al., 2012). The three viruses were propagated in healthy 1-day-old ducklings by intramuscular injection. At 24 h post-inoculation, the ducklings began to die and their livers were collected. RNA was then extracted using an EZNA Viral RNA Kit (Omega Bio-Tek, Doraville, GA, USA), according to the manufacturer's instructions. Reverse transcription (RT) was carried out using a RevertAid First Strand cDNA Synthesis Kit (Fermentas/Thermo Scientific, Waltham, MA, USA), according to the manufacturer's directions. The reaction mixture contained 8 μL of RNA and 1 μM DHAV-1F, DHAV-3F and DAstV-1F primers, in a final volume of 20 μL. The RT step was carried out at 42°C for 60 min and was followed by a 5 min period at 70°C. Finally, a multiplex PCR assay was carried out in a volume of 50 μL in a reaction mixture comprising 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.4 μmol/L DHAV-1F/R, 2.5 μmol/L DHAV-3F/R, 3 μmol/L DAstV-1F/R, 2.0 U of Taq DNA polymerase, and 3 μL of cDNA. The eventual optimized cycling conditions were: 95°C for 5 min; followed by 32 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and finally 72°C for 10 min. The amplified products were electrophoresed on a 0.8% agarose gel and visualized with ethidium bromide.

Next, the detection sensitivity of the multiplex RT-PCR procedure was determined, using both total tissue RNA and viral genome equivalents. In the case of total tissue RNA, the PCR experiment was performed with serial 10-fold dilutions (1 μg–1 pg) of total RNA extracted from liver samples that had been artificially infected with DHAV-1, DHAV-3, and DAstV-1. The detection sensitivity was observed to be 10 pg of total RNA for each of the three types of viruses (Figure 1A). In the case of viral genome equivalents, cRNA standards for DHAV-

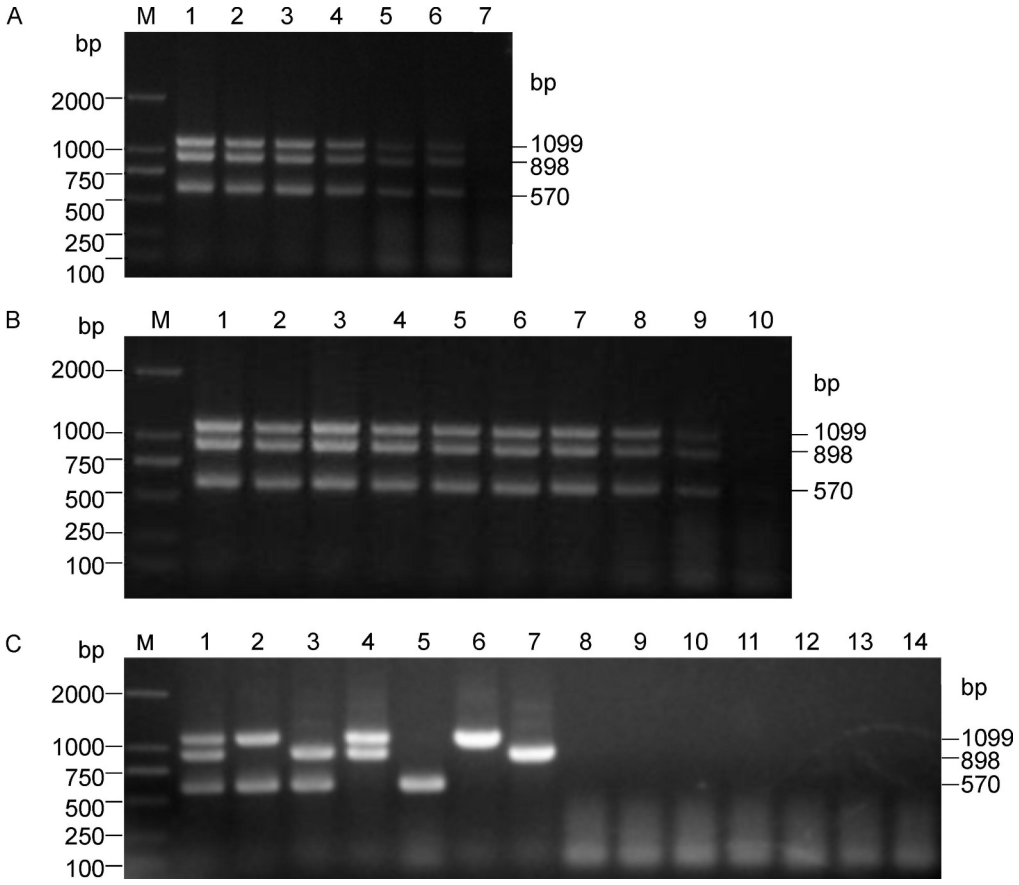

Figure 1. Sensitivity and specificity of the multiplex RT-PCR. A: Sensitivity of the multiplex RT-PCR using total liver tissue RNA as a template. M, DNA marker; 1, 1 μg; 2, 100 ng; 3, 10 ng; 4, 1 ng; 5, 100 pg; 6, 10 pg; and 7, 1 pg. B: Sensitivity of the multiplex RT-PCR prepared from 10^{10}–10^{1} copies of cRNA for each of DHAV-1, DHAV-3, and DAstV-1 used as a starting template. M, DNA marker; 1, 10^{10} copies; 2, 10^{9} copies; 3, 10^{8} copies; 4, 10^{7} copies; 5, 10^{6} copies; 6, 10^{5} copies; 7, 10^{4} copies; 8, 10^{3} copies; 9, 10^{2} copies; and 10, 10^{1} copies. C: Multiplex PCR-amplified products from purified DNAs/RNAs of known duck pathogens. M, DL2000 DNA marker; 1, DHAV-1, DHAV-3, and DAstV-1; 2, DHAV-1 and DHAV-3; 3, DHAV-1 and DAstV-1; 4, DHAV-3 and DAstV-1; 5, DHAV-1; 6, DHAV-3; 7, DAstV-1; 8, negative control (healthy duckling liver sample); 9, avian influenza virus; 10, Newcastle disease virus; 11, duck plague virus; 12, duck circovirus; 13, *Riemerella anatipestifer*; and 14, *Escherichia coli*.

1, DHAV-3, and DAstV-1 were constructed, starting with three pairs of primers designed for this purpose [DHAV-1 comFSP6 5'-CCCAAGCTT*ATACGATTTAGGTGACACTATAG*GATGTGGCAY(T/C)GTTGTY(T/C)AAY(T/C)CGA-3'; DHAV-1 comR 5'-CGGGATCCCTGATGTD(G/A/T)CCAGGR(A/G)ATTGGTCG-3'; DHAV-3 comFSP6 5'- CCCAAGCTT*ATACGATTTAGGTGACACTATAG*GAGCCAGAATTGGAATGGACACA-3'; DHAV-3 comR 5'-CGGGATCCCATACTTR(G/A)CCACCAACTGCCAATC-3'; DAstV-1 comFSP6 5'-CCCAAGCTT*ATACGATTTAGGTGACACTATAG*ATGGCCCAGAGCGGTGAAAA-3'; and DAstV-1 comR 5'-CGGGATCCGGAGCATGCACAAGTGTTCATCA-3'; the SP6 promoter sequence is indicated in italics]. The *in vitro* transcription of the RNAs for DHAV-1, DHAV-3, and DAstV-1 was carried out using the RiboMAX Large scale RNA Production System-SP6 and T7 kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Using 10-fold serial dilutions (10^{10}–10^{1} copies) of the synthetic cRNA mixture as template, the lowest detection limit of the multiplex RT-PCR was determined to be 10^{2} copies for each of the three types of viruses (Figure 1B).

The specificity of the multiplex RT-PCR was ascertained by testing it against a range of other duck pathogens that can be present during multiple infections with DHAV-1, DHAV-3, and DAstV-1, including duck plague virus, avian influenza virus (H9N2), Newcastle disease virus, the complete genomic DNA of duck circovirus strain FJ0601, *Riemerella anatipestifer* and *Escherichia*

coli. All of these other pathogen samples tested negative, except in the case of admixture with one (or more) of DHAV-1, DHAV-3, and DAstV-1 (Figure 1C).

A total of 48 clinical liver samples with suspected viral hepatitis infection were then tested in order to evaluate the performance of the multiplex RT-PCR assay. In addition, a simplex RT-PCR assay, viral culture, and a neutralization test were each undertaken on the 48 samples, as previously described (Chen L L, et al., 2013). The primers used in the simplex RT-PCR assays were the same as for the multiplex RT-PCR assay. Overall, eight samples were identified as co-infected with DHAV-1/DHAV-3, 1 sample was identified as co-infected with DHAV-1/DAstV-1, 3 samples were identified as co-infected with DHAV-3/DAstV-1, and 36 samples were shown to be positive for either DHAV-1 (10 samples), DHAV-3 (18 samples), or DAstV-1 (8 samples) alone. No sample was found to be co-infected with all the three viruses together. The results were confirmed by simplex RT-PCR, viral isolation, and a neutralization test – and with 100% agreement.

DVH is of economic importance in all duck-rearing regions of the world. In China, infection has been associated with three types of virus: DHAV-1, DHAV-3, and DAstV-1 (Chen L, et al., 2012; Chen L L, et al., 2013; Ding C, et al., 2007; Fu Y, et al., 2008, 2009; Gao J, et al., 2012; Wang L, et al., 2008; Wang X, et al., 2011; Xu Q, et al., 2012). Ducklings infected by any one of these three viruses can exhibit similar symptoms individually, such as a short incubation period, sudden onset, high mortality, opisthotonus, and enlarged liver with distinct ecchymotic hemorrhages. A presumptive diagnosis of DVH can be made based on the history and lesions, but it is difficult to ascertain which type (or types) of virus is causing the infection. In 2008, a multiplex PCR was developed to differentiate between the DHAV-1 and DHAV-3 strains (Kim M C, et al., 2008); and more recently, we developed an improved duplex RT-PCR method that facilitates the rapid and cost-effective laboratory detection of mixed infections caused by different DHAV-1 and DHAV-3 strains in ducklings (Chen L L, et al., 2013). Neither of these multiplex PCR assays could detect DAstV-1, however.

In the present study, a multiplex RT-PCR has been developed for the simultaneous detection of DHAV-1, DHAV-3, and DAstV-1 in clinical specimens. To the best of our knowledge, this is the first description of a multiplex RT-PCR that can detect the three types of DVH viruses. The multiplex RT-PCR method is DVH virus-specific and does not amplify genomic DNA or RNA from other duck pathogens. Its detection limit is estimated to be 10 pg of total liver tissue RNA, or 10^2 copies of each of DHAV-1, DHAV-3 and DAstV-1 viral RNA. Furthermore, a differential diagnosis of DHAV-1, DHAV-3, and DAstV-1 may be obtained in a single reaction within a few hours, whereas differentiation of the three viruses followed by cross-neutralization tests takes several days at least. This multiplex PCR method is therefore a rapid, effective and practical tool for the differential diagnosis of mixed infections with the three types of DVH viruses, and for the epidemiological surveillance of DVH.

FOOTNOOTES

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All the authors declare that they have no competing interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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