JOURNAL OF CLINICAL MICROBIOLOGY, July 2010, p. 2575–2577 Vol. 48, No. 7
0095-1137/10/$12.00 doi:10.1128/JCM.01979-09

Development of Microsphere-Based Multiplex Branched DNA Assay for Detection and Differentiation of Avian Influenza Virus Strains▽†

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Received 6 October 2009/Returned for modification 6 April 2010/Accepted 5 May 2010

We developed and evaluated a multiplex branched DNA assay for the detection and subtyping of avian influenza (AI) virus strains. The assay successfully detected all 94 AI virus strains of 15 different hemagglutinin (HA) subtypes tested while simultaneously differentiating 24 North American H5, 11 Eurasian H5, and 11 H7 strains. Our study demonstrates for the first time that a branched DNA method can detect targets that show a great amount of sequence variation.

Avian influenza (AI) viruses are type A influenza viruses of the *Orthomyxoviridae* family (15). Influenza virus is typed based on the serological reactions of internal proteins (nucleoprotein and matrix 1) and is further classified into 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes, respectively. Among the 16 HA subtypes, detection of H5 and H7 subtypes has been emphasized because of their outbreak history and potential of becoming highly pathogenic AI (HPAI) viruses (14). In addition to the economic impact that HPAI virus can cause, the viruses have also been considered to be a public health threat since direct transmission from birds to humans has been documented (8, 14, 15). Thus, a prompt and accurate identification of AI virus strains, especially H5 and H7 subtypes, is essential for controlling further spread to domestic flocks as well as to humans. Real-time reverse transcriptase PCR (RRT-PCR) has been successfully applied for the detection of AI virus strains and differentiation between H5 and H7 subtypes, reducing time and labor compared to that needed for the traditional virus isolation in embryonating chicken eggs and subsequent hemagglutination inhibition (HI) testing (9, 10). However, pure RNAs are required to run RRT-PCR, and the limited multiplexing capacity of the assay further increases the cost as well as the assay time.

In this study, we applied branched DNA (bDNA) signal amplification technology (sandwich nucleic acid hybridization assay) to the suspension array platform for the detection and simultaneous differentiation of H5 and H7 subtype AI virus strains. bDNA technology is the basis of a clinically proven viral load test and has been in practice for over a decade in drug discovery and development applications (3, 6, 11, 12). bDNA technology, the basic approach being similar to that of an enzyme-linked immunosorbent assay (ELISA), was initially developed on a 96-well plate platform to detect target agents with conserved sequences. Each well was coated with one kind of "capture" molecule which allowed detection of only a single target in a sample at a time, and the probes were designed based on the consensus sequence of the target agents. Recently xMAP technology of 100 sets of beads in a liquid suspension array has been combined with bDNA technology to raise the multiplexity up to 100 at a time per well (5, 13, 16).

The multiplex bDNA assay, also known as QuantiGene Plex assay (Affymetrix, Fremont, CA), starts with developing target-specific probes. Two kinds of probes, capture extenders (CE) and label extenders (LE), were designed for Matrix (M), Eurasian H5 (EA-H5), North American H5 (NA-H5), and H7 subtypes, respectively. CE links between bead and the target gene (viral RNA in our case), and LE attaches bDNA molecules (tree-like structures of oligonucleotides which contain hybridization sites for 400 biotinylated label molecules) to each target gene, which leads to the formation of a bead-target-bDNA complex (12). For designing the target-specific part of CE and LE probes, all the sequences available in GenBank, including recent wild bird isolates, were retrieved for M, H5, and H7 genes. A large sequence variation between strains even in the same subtype was observed after alignment with the MegAlign program (LaserGene, Madison, WI). Thus, instead of generating a single consensus sequence directly from a large number of diverse sequences, we classified sequences into several phylogenetic subgroups per gene and selected a minimum number (2 to 8 sequences depending on the level of sequence variation) of representative strains per group that covered the sequence diversity within the subgroup. The number of sequences analyzed, number of subgroups, and total number of probes used for each target are shown in Table S1 in the supplemental material. The conserved regions in the selected sequences were identified manually, and all of them were screened using the ProbeDesigner software (Chiron Diagnostics, Walpole, MA) (1) to check whether they qualified as CE and/or LE probes. To minimize nonspecific hybridization (NSH) events, which elevate assay background, candidate probes were further screened for complementarity with generic sequences present in the assay. All the probes from each group that met the conditions were pulled together as one

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† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 19 May 2010.

probe set for each gene. All the probes (total of 302 probes [54 for M, 87 for EA-H5, 84 for NA-H5, and 77 for H7]) were manufactured by IDT (Integrated DNA Technologies, Coralville, IA). Reagents used in the assay, including bDNA molecules (preamplifier, amplifier) were included in the QuantiGene Plex 2.0 kit (Affymetrix). Four sets of beads, probe sets, and samples in a total volume of 100 μl were incubated on a 96-well hybridization plate for 16 h at 55.5°C in a Vortemp (Labnet, Berkshire, United Kingdom). The complete content was transferred and filtered through a 96-well filter plate and washed three times with wash buffer by using a vacuum manifold (Bio-Rad, Richmond, CA). A 100-μl volume of preamplifier solution was added to each well, and incubation was continued for an hour at 51.5°C in Vortemp. After washing twice, amplifier solution was added and incubated for an hour at 51.5°C. Label probe was added after washing two times, and this mixture was incubated for another hour at 51.5°C. Streptavidin-*R*-phycoerythrin (SAPE) solution was added, and the plate was incubated for 30 min at room temperature. After washing the plate, the beads were suspended in SAPE wash buffer and analyzed by the Bio-Plex 200 suspension array system (Bio-Rad). One hundred beads were read per gene in a sample, and the result was shown as the mean fluorescence intensity (MFI) of the total fluorescence per analyte. By determining the amount of fluorescence signal and the identity of the beads, the amount of each target RNA present in a sample was evaluated. A cutoff value was calculated as the average background signal plus three times the standard deviation.

Initially, the assay was performed with RNA samples extracted from infectious allantoic fluid containing influenza viruses (in the titer range of 10^5 to 10^8 50% egg infective doses [EID_{50}]/ml) by using Qiagen's RNeasy mini kit according to the manufacturer's instructions. The assay successfully detected all 94 AI virus strains of 15 different HA subtypes tested, while differentiating 24 NA-H5, 11 EA-H5, and 11 H7 strains simultaneously (see Table S2 in the supplemental material). A single isolate, CK/PA/1370/83 (H5N2), reacted against both EA-H5 and NA-H5 probes. This may due to the overlapping sequence similarity between EA-H5 and NA-H5 sequences present in this relatively old isolate. The MFI for the M gene was 28,435, most of them ranging around an MFI of 29,000 to 30,000 (cutoff value, 51.5). Eleven H7 subtype strains were tested, and all of them tested positive with strong fluorescent signals (MFI, 25,266; cutoff value, 19.75). Twenty-five strains of NA-H5 subtype and 11 strains of EA-H5 were tested, and the MFIs were 24,630 (cutoff value, 39.5) and 4,441 (cutoff value, 25.13), respectively. The lower MFI value with a few EA-H5 strains may be due to RNA degradation from repeated freezing and thawing since we do not have a biosafety level 3 (BSL-3) facility to maintain highly pathogenic foreign-origin live virus to extract fresh RNA in each experiment.

The detection limit determined with *in vitro*-transcribed RNA (9) was 100 fg for M and 1 pg for H5 and H7 probes. The detection limit, which was determined with viral RNA from three influenza strains of known titer, was approximately 10^3 to 10^4 EID_{50}. The analytical sensitivity of the new assay is approximately 10 times lower in *in vitro*-transcribed RNA and 100 to 1,000 times lower in extracted viral RNA samples than that of RRT-PCR (9). No difference was observed in sensitivity between the monoplex and 4-plex assay for each gene. To assess the intra- and interassay reproducibility, three different strains were tested in triplicate in three different runs performed on different days. The intraplate percent coefficient of variation (%CV) was below 1, and interplate %CV remained below 5, proving the high precision and reproducibility of the assay.

To further test the practical applicability of the assay, 12 nasal ($n = 6$), tracheal ($n = 3$), and cloacal ($n = 3$) swabs from experimentally infected chickens, turkeys, and pigs were tested without RNA extraction (see Table S3 in the supplemental material). The swabs were treated with lysis mixture (Panomics) and proteinase K before the assay. All the samples were tested AI virus positive, with MFIs ranging from 158 to 17,882 (cutoff value, 51.5), and the assay correctly subtyped the three H5 samples included in the test. The 12 swab samples were also tested with quantitative RRT-PCR and converted to 50% infective doses as previously described (7). The titer ranged between $10^{4.50}$ and $10^{5.83}$ EID_{50} (or $10^{5.68}$ and $10^{8.28}$ 50% tissue culture infective doses [$TCID_{50}$]), and individual sample information is shown in Table S3 in the supplemental material.

Allantoic fluids from the same viruses we used for evaluating detection limit were tested without the RNA extraction step as well, and sensitivity similar to that with the purified RNA samples was observed (data not shown).

The assay showed almost 100% diagnostic sensitivity and specificity (with the exception of the CK/PA/1370/83 isolate, which showed a positive signal for both H5 probes), with no false-positive result. Each probe set detected RNA only from strains of their respective subtypes. This is largely due to the specific matching condition between probes and their target genes; probe binding to targets occurs only when there are less than two mismatching nucleotides (data not shown). With the nature of influenza virus constantly evolving via antigenic drift, which is apparent in their sequence diversity within the same subtype, targeting multiple sites will reduce the chance of getting a false-negative result. Furthermore, our study demonstrates for the first time that a bDNA method can be applied to detect targets that show a great amount of sequence variation. This was accomplished through a new method for designing probes. The detection limit was around 10^3 to 10^4 EID_{50} of virus, which is good analytical sensitivity compared to other rapid hybridization based assays (2, 4); however, it may not be adequate for direct application to field samples. There are ways to further increase the sensitivity: optimizing the number of probes, updating the bDNA molecules to have more binding sites for fluorescent molecules, and using newly available magnetic beads instead of polystyrene beads, which can increase the sensitivity through incorporating a more efficient washing process. Considering the flexibility, ability to multiplex, and the automated high-throughput capabilities, the multiplex bDNA assay has great potential in the rapid diagnosis of influenza virus strains which have multiple HA and NA subtypes.

We thank David Suarez (Southeast Poultry Research Lab, Athens, GA) for providing inactivated virus and RNA samples of highly pathogenic and low-pathogenicity strains of foreign origin.

This work was supported in part by the United States Department of Agriculture, CSREES AI-CAP grant, and the Ohio Agricultural Research and Development Center Research Enhancement Competitive Grants Program.

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