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External Quality Assessment for Avian Influenza A (H7N9) Virus Detection Using Armored RNA

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An external quality assessment (EQA) program for the molecular detection of avian influenza A (H7N9) virus was implemented by the National Center for Clinical Laboratories (NCCL) of China in June 2013. Virus-like particles (VLPs) that contained full-length RNA sequences of the hemagglutinin (HA), neuraminidase (NA), matrix protein (MP), and nucleoprotein (NP) genes from the H7N9 virus (armored RNAs) were constructed. The EQA panel, comprising 6 samples with different concentrations of armored RNAs positive for H7N9 viruses and four H7N9-negative samples (including one sample positive for only the MP gene of the H7N9 virus), was distributed to 79 laboratories in China that carry out the molecular detection of H7N9 viruses. The overall performances of the data sets were classified according to the results for the H7 and N9 genes. Consequently, we received 80 data sets (one participating group provided two sets of results) which were generated using commercial ($n = 60$) or in-house ($n = 17$) reverse transcription-quantitative PCR (qRT-PCR) kits and a commercial assay that employed isothermal amplification method ($n = 3$). The results revealed that the majority (82.5%) of the data sets correctly identified the H7N9 virus, while 17.5% of the data sets needed improvements in their diagnostic capabilities. These "improvable" data sets were derived mostly from false-negative results for the N9 gene at relatively low concentrations. The false-negative rate was 5.6%, and the false-positive rate was 0.6%. In addition, we observed varied diagnostic capabilities between the different commercially available kits and the in-house-developed assays, with the assay manufactured by BioPerfectus Technologies (Jiangsu, China) performing better than the others. Overall, the majority of laboratories have reliable diagnostic capacities for the detection of H7N9 virus.

Over the past few months, the avian influenza A (H7N9) virus has infected at least 132 people in China, of whom 43 have died (as of 17 July 2013). Despite the abruptly reduced number of H7N9 infections occurring after April 2013 (1), concerns were once again raised when a woman was diagnosed with severe infection on 20 July 2013 in Beijing. To date, the epidemiological and pathogenic characteristics of the H7N9 virus have not yet been elucidated. Initial epidemiological and clinical evidence indicated that the H7N9 virus rarely infected humans but was capable of causing severe diseases following infection (2). H7N9 infection has often presented with severe respiratory illness, but some cases developed acute respiratory distress syndrome and multiorgan failure (3–5). Studies on human airway epithelial cells and animal models indicated that the H7N9 virus exhibits high pathogenicity and has a robust replication capacity but limited transmissibility (6, 7). The pandemic potential of the H7N9 virus continuously poses a threat to public health. Efforts to contain H7N9 infection heavily rely on its rapid and effective detection.

For detection of the H7N9 virus, the WHO developed a reverse transcription-PCR (RT-PCR) protocol (8), and the Chinese Center for Disease Control and Prevention (CDC) also provided primer-probe sets for H7N9 detection. In addition, several commercial kits are available for this purpose. However, since molecular detection has been broadly applied, this may present issues with quality control due to variations in laboratory capacities, particularly when it is the first time this novel strain of influenza virus has acquired human adaptation capabilities. The lack of standardization of the commercially available assays and laboratory-developed assays also causes difficulties in comparing results from the different laboratories. It is apparent that there is a need for quality assurance within the context of H7N9 virus diagnostics. The essential role of external quality assessment (EQA) in quality management and accreditation schemes has long been recognized (9). It ensures that laboratories maintain robust laboratory diagnostic capacities. The H7N9 epizootic provides us with an opportunity to learn about the issues in the laboratory diagnosis of H7N9 infection. The National Center for Clinical Laboratories (NCCL) previously conducted an EQA program on RNA viruses, such as the human enterovirus 71 (HEV71) and the coxsackievirus A (CA16), which highlighted significant interlaboratory differences in diagnostic sensitivities (10). We wondered if these issues were also present with H7N9 virus detection.

To this end, the first nationwide EQA study was implemented by the NCCL to provide information about the proficiency of diagnosis in laboratories from the Chinese Influenza Laboratory Network or in other designated hospitals.

MATERIALS AND METHODS

Preparation of samples. The RNA sample of strain A/Shanghai/2/2013 (H7N9) was kindly provided by the CDC of China. Four target segments

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TABLE 1 Composition of the EQA panel and results for molecular detection of samples

Sample no.	Concn of armored RNAs in samples (copies/ml)				Classification	No. of correct/total no. tested (%) for:		
	HA	NA	MP	NP		HA	NA	Data
H7N9-1301	1.3×10^7	8×10^6	2.4×10^6	1.3×10^7	Positive	78/80 (97.5)	73/80 (91.3)	73/80 (91.3)
H7N9-1302	Negative	Negative	Negative	Negative	Negative	80/80 (100)	80/80 (100)	80/80 (100)
H7N9-1303	1.3×10^6	2.2×10^5	6×10^5	1.3×10^4	Weakly positive	75/80 (93.8)	67/80 (83.8)	67/80 (83.8)
H7N9-1304	Negative	Negative	Negative	Negative	Negative	80/80 (100)	80/80 (100)	80/80 (100)
H7N9-1305	6.5×10^6	8.8×10^6	2.4×10^6	5.2×10^6	Positive	79/80 (98.8)	76/80 (95)	76/80 (95)
H7N9-1306	9.2×10^6	8.8×10^5	2.4×10^5	1.3×10^6	Positive	78/80 (97.5)	70/80 (87.5)	70/80 (87.5)
H7N9-1307	5.2×10^8	8.8×10^7	2.4×10^8	6×10^7	Positive	79/80 (98.8)	78/80 (97.5)	77/80 (96.3)
H7N9-1308	Negative	Negative	1.3×10^7	Negative	Negative	79/80 (98.8)	80/80 (100)	79/80 (98.8)
H7N9-1309	6.5×10^6	8.8×10^6	2.4×10^6	5.2×10^6	Positive	78/80 (97.5)	75/80 (93.8)	75/80 (93.8)
H7N9-1310	Negative	Negative	Negative	Negative	Negative	78/80 (97.5)	79/80 (98.8)	78/80 (97.5)

of H7N9, encoding hemagglutinin (HA), neuraminidase (NA), matrix protein (MP), and nucleoprotein (NP), were amplified using RT-PCR. The primers that were used are listed in Table S1 in the supplemental material. Thereafter, gel-purified RT-PCR products were subcloned into the BglII/KpnI and KpnI/PacI sites of the pACYC-MS2 vector.

The MS2 virus-like particles (VLPs) were expressed and purified as in previous studies in our laboratory (10–12). Briefly, the recombinant plasmids pACYC-MS2-HA, pACYC-MS2-NA, pACYC-MS2-MP, and pACYC-MS2-NP were transformed into the *Escherichia coli* strain BL21(DE3). Subsequently, MS2 VLPs were expressed by addition of 1 mmol/liter isopropyl-β-D-thiogalactopyranoside to the bacterial cultures and incubation for 16 h. The cell pellets were harvested by centrifugation and lysed by sonication on ice. After elimination of the *E. coli* genome, cell debris was removed by centrifugation and MS2 VLPs were purified using Sephacryl S-200 gel exclusion chromatography (BioLogic DuoFlow chromatography system). The products were aliquoted and examined by agarose gel electrophoresis (1%) with ethidium bromide staining. Armored RNAs encapsulating the HA, NA, MP, and NP genes of the H7N9 virus were verified by RT-PCR.

Evaluation of armored RNAs. The stabilities of all four types of armored RNAs (AR-HA, AR-NA, AR-MP, and AR-NP) in Dulbecco's modified Eagle medium (DMEM) were examined. Initially, the RNAs of MS2 VLPs were isolated using a QIAamp viral RNA minikit (Qiagen) according to the manufacturer's instructions and were quantified by an absorbance-based nucleic acid quantification method (NanoDrop 2000; Thermo). The concentration of each sample (copies/ml) was estimated using the following formula: $(6.02 \times 10^{23}$ copies/mol) × armored RNA mass (g/ml)/molar mass (g/mol) = concentration (copies/ml). Each quantified MS2 VLP was diluted with DMEM to yield two different concentrations. For each stability study, a single batch was separated into several 1.0-ml (the volume sufficient for RNA detection) aliquots representing individual time points. The samples were separately incubated at −20°C, 4°C, room temperature, and 37°C and were analyzed at different time periods. The samples were removed at each time point (1 day, 3 days, 1 week, 2 weeks, 1 month, and 2 months) and stored at −80°C until the completion of the experiment. All samples were quantified in duplicate using the avian influenza virus (H7N9) real-time RT-PCR kit (Beijing Sino-MDgene Technology Co., Ltd., Beijing, China), according to the manufacturer's protocol.

Organization of the EQA. To assess the proficiency of the laboratories in detecting H7N9 by nucleic acid amplification, a coded panel ($n = 10$) consisting of six samples positive for the H7N9 virus, one sample (no. H7N9-1308) containing only the influenza A target to simulate an "unsubtypeable" influenza A result, and three negative samples that contained no virus were included (Table 1). The test samples were shipped on ice to the participating laboratories. The participating groups were asked to use their routine setup for molecular assays that are used at their respective laboratories for the diagnosis of H7N9 infections. The results were returned to the NCCL via e-mail or fax within 2 weeks of the receipt of the test panel. For reporting the EQA results, the laboratories were requested to submit the results of H7 and N9 gene testing and other details of the assay used (basic information about detection method and manufacturer, as well as the extraction procedure), while the results of MP and NP gene tests were submitted when conducted.

Statistical analysis. The results were classified as competent (100% correct responses), acceptable (only one false-positive result), or improvable (false-negative result and/or several false-positive results).

All data analyses were performed using SPSS version 16.0 for Windows. Comparisons of the sensitivities for different parameters were performed by applying Pearson's chi-square test or Fisher's exact test when appropriate. P values of <0.05 were regarded to be statistically significant.

RESULTS

Construction and evaluation of the panel. VLPs encapsulating the HA, NA, MP, and NP mRNAs were constructed successfully (see Fig. S1 in the supplemental material) and were validated by sequencing to contain full-length target sequences. The concentrations of the VLPs were 3×10^{12} to 12×10^{12} copies/ml. The armored RNA was demonstrated to resist RNase and DNase treatment (see Fig. S2 in the supplemental material). As expected, the data obtained from stability analyses revealed that at concentrations of 10^9 and 10^6 copies/ml, the VLPs were stable for at least 2 weeks at 37°C, 4 weeks at room temperature, and 2 months at both 4°C and −20°C. These were consistent with the previously reported results (11). Stability was defined as a decrease of <0.5 $\log_{10}$ copies/ml compared to the control that was stored at −80°C for the entire duration.

Before distribution, we validated the panel samples using different commercially available kits. Each sample was tested three times by kits manufactured by Shanghai ZJ Bio-Tech Co. Ltd. (for H7 and N9 gene detection), Daan Gene Co. Ltd. (for H7 and N9 gene detection), and Beijing Sino-MDgene Technology Co., Ltd. (for H7, N9, MP, and NP gene detection). All tests successfully confirmed the positive and negative results of each sample.

Participating groups and methodologies. Participation was open to all accredited laboratories in the Chinese Influenza Laboratory Network in mainland China. Seventy-nine laboratories from 21 provinces, municipalities, or autonomous regions (42 from Beijing, 4 from Shanghai, 3 from Tianjin, 1 from Shandong, 2 from Anhui, 2 from Guangdong, 4 from Jiangsu, 1 from Fujian, 1 from Ningxia, 1 from Liaoning, 3 from Yunnan, 1 from Hubei, 1 from Shaanxi, 1 from Gansu, 3 from Inner Mongolia, 1 from Shanxi, 1 from Zhejiang, 1 from Sichuan, 1 from Guangxi, 4 from Xinjiang, and 1 from Qinghai), including 48 from National Influenza Surveillance Network (NISN), 28 from sentinel hospitals or

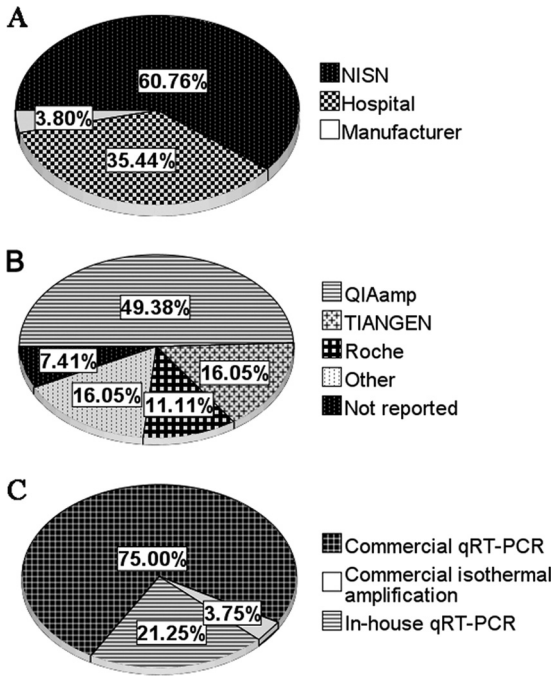

FIG 1 (A) Composition of participating laboratories. Forty-eight (60.76%) laboratories belong to the National Influenza Surveillance Network (NISN), 28 (35.44%) laboratories are sentinel hospitals or designated hospitals for H7N9 infection, and three (3.80%) are from diagnostic reagent manufacturers. (B) A wide range of nucleic acid extraction kits were applied: from QIAamp (40/80 [49.38%]), the RNeasy minikit and the QIAamp viral RNA minikit; from TIANGEN (13/80 [16.05%]), the TIANamp RNA kit for virus detection; from Roche (9/80 [11.11%]), MagNA Pure LC total nucleic acid isolation kit and the MagNA Pure LC2.0 automatic extractor; and other (13/80 [16.05%]) RNA recovery kits provided by qRT-PCR kits manufacturers. (C) Methodologies used by participating groups in the present EQA: commercially available reverse transcription-quantitative PCR (qRT-PCR) kits, 60/80 (75%); commercially available isothermal amplification assay, 3/80 (3.75%), and in-house-developed qRT-PCR assays, 17/80 (21/25%).

designated hospitals for H7N9 infection, and 3 from diagnostic reagent manufacturers, participated in this first nationwide H7N9 EQA program (Fig. 1A).

A range of commercial nucleic acid extraction kits were applied (Fig. 1B). Overall, 80 completed data sets were generated using commercially available reverse transcription-quantitative PCR (qRT-PCR) kits ($n = 60$) and an isothermal amplification assay ($n = 3$); the remainder ($n = 17$) were in-house-developed qRT-PCR assays (Fig. 1C). One laboratory presented two sets of results, which were generated by qRT-PCR and the isothermal amplification assay.

Performance of laboratories. Of the 80 completed data sets, the performances were found to be competent in 66 (82.5%) analyses, no data set met the criteria with acceptable results, and 14 (17.5%) results implied that there was room for improvement regarding H7N9 virus detection.

We next compared the performance of H7N9 detection among the different participating groups. For this analysis, the results from the manufacturers were excluded because of their limited number ($n = 3$). The results revealed that there was no statistical difference in testing performance between NISN laboratories and hospital laboratories ($P = 0.166$).

In total, 54 false-negative results (54/960 [5.6%]) were reported by 12 laboratories (Table 1). The individual sensitivities of detection for the HA and NA genes were 97.3% (467/480) and 91.5% (439/480), respectively. We observed a decline in testing accuracy corresponding with decreased concentrations for both HA and NA detection. At the concentration of 2.2×10^5 copies/ml of NA (no. H7N9-1303), which is close to the detection limits of most commercial kits, the sensitivity even decreased to 83.3%. On the other hand, only four (4/640 [0.6%]) results were false positives. In the current study, duplicate samples were also included to examine the reproducibility of H7N9 virus detection. The results revealed no differences in the accuracies of both H7 and N9 gene detection ($P > 0.05$).

We next assessed the performances of different assays. The commercially available assay manufactured by BioPerfectus Technologies (Jiangsu, China) was the most widely used by the participating groups and performed better than the commercial assays from Beijing Kinghawk Pharmaceutical Co. Ltd. (Beijing, China), Shanghai ZJ Bio-Tech Co. Ltd. (Shanghai, China), Daan Gene Co. Ltd. (Guangzhou, China), and Shanghai Huirui Biotechnology Co., Ltd. (Shanghai, China), as well as the in-house-developed assays (Table 2). The sensitivity of the assay from Beijing Kinghawk Pharmaceutical Co. Ltd. (Beijing, China) was relatively low in this EQA, particularly for the detection of the N9 gene.

In-house-developed assays showed a decreased sensitivity for the N9 gene. It is noteworthy that three participating groups applied one commercial kit using the isothermal amplification platform. Intriguingly, one participating group that presented two data sets applied both a commercially available kit using the isothermal amplification method and an in-house-developed assay. However, the in-house assay failed to detect three NA genes (sample no. H7N9-1301, H7N9-1303, and H7N9-1306), whereas the commercial kit successfully detected all target sequences.

The results for the MP gene (for subtyping influenza viruses) were reported by 19 participating groups. All MP results from samples H7N9-1301 to H7N9-1310 were correctly reported except for one false-negative result (see Table S2 in the supplemental material). Of these 19 data sets, two were classified as improvable (one missed the N9 gene of sample H7N9-1303, and another missed the H7, N9, and MP genes of sample H7N9-1303) (see Table S2 in the supplemental material).

Only one participating group submitted the results for the NP gene; this data set was generated by using assay F, which allows for the detection of the H7, N9, MP, and NP genes. The results were optimal, except for one NP gene at a concentration of 1.3×10^4 copies/ml that was undetected.

DISCUSSION

Here, we describe a blind study performed in mainland China that aimed to evaluate whether laboratories carry out the most stringent laboratory procedures to preclude false-positives and false negatives in the detection of the H7N9 virus. VLPs encapsulating the genome fragments of the H7N9 virus were expressed as described in Materials and Methods. Since the H7 avian viruses have adapted to human hosts (2, 13), the results that presented only with H7 were not identified as H7N9 virus positive.

The results of the current EQA, as expected, show that most of the laboratories (82.3% of data sets) met the criteria for the accurate detection of H7N9 virus. False-positive results, which are always ascribed to contamination or the nonspecific hydrolysis of primers, were rare (0.6%). However, problems mainly existed in

TABLE 2 Comparison of sensitivities and specificities of different assays

Assay	No. of data sets	H7 gene		N9 gene	
		No. of correct positive results/total no. of positive results (sensitivity [%])	No. of correct negative results/total no. of negative results (specificity [%])	No. of correct positive results/total no. of positive results (sensitivity [%])	No. of correct negative results/total no. of negative results (specificity [%])
BioPerfectus[a]	40	240/240 (100)	159/160 (99.4)	237/240 (98.8)	160/160 (100)
Kinghawk[a]	9	43/54 (79.6)	35/36 (97.2)	40/54 (74.1)	35/36 (97.2)
ZJ Bio-Tech[a]	5	29/30 (96.7)	20/20 (100)	26/30 (86.7)	20/20 (100)
Daan Gene[a]	4	24/24 (100)	16/16 (100)	22/24 (91.7)	16/16 (100)
Ipe-bio[b]	3	18/18 (100)	12/12 (100)	18/18 (100)	12/12 (100)
Sino-MDgene[a]	1	6/6 (100)	4/4 (100)	6/6 (100)	4/4 (100)
Huirui[a]	1	5/6 (83.3)	4/4 (100)	5/6 (83.3)	4/4 (100)
In-house[c]	17	102/102 (100)	67/68 (98.5)	85/102 (83.3)	68/68 (100)
Total	80	467/480 (97.3)	317/320 (99.1)	439/480 (91.5)	319/320 (99.7)

[a] Six commercial TaqMan real-time RT-PCR kits for H7N9 virus detection: BioPerfectus (BioPerfectus Technologies, Jiangsu, China), Kinghawk (Beijing Kinghawk Pharmaceutical Co. Ltd, Beijing, China), ZJ Bio-Tech (Shanghai ZJ Bio-Tech Co. Ltd, Shanghai, China), Daan Gene (Daan Gene Co. Ltd, Guangzhou, China), Sino-MDgene (Beijing Sino-MDgene Technology Co., Ltd, Beijing China), and Huirui (Shanghai Huirui Biotechnology Co., Ltd).
[b] One commercial kit employed the isothermal amplification method: Ipe-bio (Beijing Ipe-bio Technologies Co., Ltd, Beijing, China).
[c] In-house, in-house-developed qRT-PCR assay for H7N9 virus detection recommended by the CDC of China.

diagnostic sensitivity, particularly for the detection of the N9 gene. At the concentration of 2.2×10^5 copies/ml, there were 16.2% analyses that failed to detect the N9 gene. An alternative approach for controlling false negatives is to introduce weakly positive controls (not transcribed RNA controls provided by manufacturers) to monitor procedures, RNA recovery, and qRT-PCR. Armored RNA is a well-suited candidate as a positive control. Comparing to false positives, special attention should be paid to false negatives, because delayed treatment might be fatal for patients infected with the H7N9 virus. It was noteworthy that there were 19 participating groups, most of which came from NISN, that carried out tests for the detection of the influenza A target before H7N9. The results of these participating groups were better than those of groups that did not perform detection of the influenza A virus target.

Commercial assays were widely employed in this EQA. The performance of the assay from BioPerfectus Technologies (Jiangsu, China) was better than that of other commercial kits and the in-house-developed assays (see Results and Table 2 for details). We noticed that one assay (from Beijing Ipe-bio Technologies Co., Ltd., Beijing, China) employed the isothermal amplification method, which acquired a hit rate of 100% for detection of the H7 and N9 genes. However, we cannot draw the conclusion that this assay is better because of the limited number of participating groups that utilized this assay.

A wide range of materials, such as transcribed RNA synthesis, virus lysates, and gamma ray- or Triton X-100-inactivated influenza viruses, have been applied to carry out EQA programs for influenza virus (14, 15). In the present study, we used armored RNA containing the HA, NA, MP, and NP genes of the H7N9 virus as a surrogate. Armored RNA is used extensively as a vaccine candidate and gene therapy vector. It has unparalleled advantages, such as stability, RNase resistance, noninfectivity, and long RNA sequence package capability, which make it an ideal candidate as a virus substitute for a proficiency panel (for monitoring RNA recovery, reverse transcription, and amplification processes) (16). In addition, armored RNA can be prepared in large amounts using a prokaryotic expression system.

In conclusion, most participating laboratories adequately performed the steps for H7N9 virus detection. The results underline a requirement for further laboratory training in the management of samples with low concentrations of virus or that are of poor quality. Furthermore, in the future, more laboratories should be encouraged to participate in quality assessments. We plan to include more subtypes of the influenza virus (such as H1N1, H3N2, H5N1, and FluB) and other candidates for EQA materials to develop a continuing EQA frame for influenza viruses.

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REFERENCES

1. **Xu C, Havers F, Wang L, Chen T, Shi J, Wang D, Yang J, Yang L, Widdowson MA, Shu Y.** 19 August 2013. Mornitoring avian influenza A(H7N9) virus through national influenza-like illness surveillance, China. Emerg. Infect. Dis. [Epub ahead of print.] doi:10.3201/eid1908.130662.
2. **Morens DM, Taubenberger JK, Fauci AS.** 2013. H7N9 avian influenza A virus and the perpetual challenge of potential human pandemicity. mBio. **4**(4):e00445–13. doi:10.1128/mBio.00445-13.
3. **Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, Xiang N, Chen E, Tang F, Wang D, Meng L, Hong Z, Tu W, Cao Y, Li L, Ding F, Liu B, Wang M, Xie R, Gao R, Li X, Bai T, Zou S, He J, Hu J, Xu Y, Chai C, Wang S, Gao Y, Jin L, Zhang Y, Luo H, Yu H, Gao L, Pang X, Liu G, Shu Y, Yang W, Uyeki TM, Wang Y, Wu F, Feng Z.** 24 April 2013. Preliminary report: epidemiology of the avian influenza A (H7N9) outbreak in China. N. Engl. J. Med. [Epub ahead of print.] doi:10.1056/NEJMoa1304617.
4. **Wang Y, Zhang Y, Wang H, Zhou Z, Zhou Z, Gu Q, Wu C, Zhu B.** 2013. Sharing insights and H7N9 patient clinical data. Am. J. Respir. Crit. Care Med. **188:**115–117.
5. **Morens DM, Taubenberger JK, Fauci AS.** 2013. Pandemic influenza viruses–hoping for the road not taken. N. Engl. J. Med. **368:**2345–2348.
6. **Watanabe T, Kiso M, Fukuyama S, Nakajima N, Imai M, Yamada S, Murakami S, Yamayoshi S, Iwatsuki-Horimoto K, Sakoda Y, Takashita E, McBride R, Noda T, Hatta M, Imai H, Zhao D, Kishida N, Shirakura M, de Vries RP, Shichinohe S, Okamatsu M, Tamura T, Tomita Y,**

Fujimoto N, Goto K, Katsura H, Kawakami E, Ishikawa I, Watanabe S, Ito M, Sakai-Tagawa Y, Sugita Y, Uraki R, Yamaji R, Eisfeld AJ, Zhong G, Fan S, Ping J, Maher EA, Hanson A, Uchida Y, Saito T, Ozawa M, Neumann G, Kida H, Odagiri T, Paulson JC, Hasegawa H, Tashiro M, Kawaoka Y. 2013. Characterization of H7N9 influenza A viruses isolated from humans. Nature **501:**551–555.

7. **Belser JA, Gustin KM, Pearce MB, Maines TR, Zeng H, Pappas C, Sun X, Carney PJ, Villanueva JM, Stevens J, Katz JM, Tumpey TM.** 2013. Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. Nature **501:**556–559.
8. **World Health Organization.**2013. Real-time RT-PCR protocol for the detection of avian influenza A(H7N9) virus. WHO Collaborating Center for Reference an Research on Influenza at the Chinese National Influenza Center, Beijing, China. See http://www.who.int/influenza/gisrs_laboratory/cnic_real time_rt_pcr_protocol_a_h7n9.pdf.
9. **Valentine-Thon E.** 2002. Quality control in nucleic acid testing—where do we stand? J. Clin. Virol. **25**(Suppl 3):S13–S21.
10. **Song L, Sun S, Li B, Pan Y, Li W, Zhang K, Li J.** 2011. External quality assessment for enterovirus 71 and coxsackievirus A16 detection by reverse transcription-PCR using armored RNA as a virus surrogate. J. Clin. Microbiol. **49:**3591–3595.
11. **Wei Y, Yang C, Wei B, Huang J, Wang L, Meng S, Zhang R, Li J.** 2008. RNase-resistant virus-like particles containing long chimeric RNA sequences produced by two-plasmid coexpression system. J. Clin. Microbiol. **46:**1734–1740.
12. **Zhan S, Li J, Xu R, Wang L, Zhang K, Zhang R.** 2009. Armored long RNA controls or standards for branched DNA assay for detection of human immunodeficiency virus type 1. J. Clin. Microbiol. **47:**2571–2576.
13. **Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD.** 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute distress syndrome. Proc. Natl. Acad. Sci. U. S. A. **101:**1356–1361.
14. **Gao R, Gao Y, Wen L, Shao M, Zou S, Li C, Yang L, Li X, Wang W, Shu Y.** 2011. Development and implementation of the quality control panel of RT-PCR and real-time RT-PCR for avian influenza A (H5N1) surveillance network in mainland China. BMC Infect. Dis. **11:**67. doi:10.1186/1471-2334-11-67.
15. **Thompson CI, Lackenby A, Daniels RS, McCauley JW, Pereyaslov D, Broberg EK, Meijer A, Zambon MC.** 2013. Evaluation of influenza virus antiviral susceptibility testing in Europe: results from the first external quality assessment exercise. J. Clin. Virol. **56:**212–218.
16. **Hietala SK, Crossley BM.** 2006. Armored RNA as virus surrogate in a real-time reverse transcriptase PCR assay proficiency panel. J. Clin. Microbiol. **44:**67–70.