NOTE *Virology*

Evaluation of Antigen Detection Kits for Diagnosis of Equine Influenza

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ABSTRACT. In this study, we evaluated whether five rapid antigen detection kits for human influenza could be used for the diagnosis of equine influenza (EI). Limiting dilution analyses showed that Directigen Flu A+B and ESPLINE INFLUENZA A&B-N had the highest sensitivities to equine-2 influenza viruses (EIVs) among the kits investigated. From the results of virus detection in nasal swabs taken from horses infected with EIV, these two kits could produce positive results in reasonable agreement with those obtained by virus isolation or RT-PCR, suggesting that these kits could be useful for rapid diagnosis of EI in the field. However, from the viewpoint of specificity for EIV, Espline seems to be superior to Directigen.

KEY WORDS: diagnosis, equine influenza, rapid antigen detection.

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Many outbreaks of equine influenza (EI) have been caused by equine-2 influenza viruses (EIVs, H3N8). This disease is an acute respiratory disease characterized by pyrexia, depression, coughing and nasal discharge and is often complicated by secondary bacterial infections that can lead to pneumonia and death [12]. EIVs continue to circulate worldwide with the exception of a small number of countries, such as New Zealand and Iceland, where EI has never been recorded [12]. Traditionally, the gold standard for the diagnostic method of EI has been virus isolation from nasopharyngeal swabs using embryonated hen's eggs and/or MDCK cells [12, 13]. In recent years, RT-PCR has been introduced for diagnosis of EI. However, these established techniques are time consuming and require expensive equipment and reagents, as well as technical expertise. Since EI is highly contagious and introduction of a single infected horse can result in explosive spread of virus in horses [12, 13], the established diagnostic methods are not sufficient for rapid isolation of infected animals from healthy animals in veterinary practice. In addition, since early initiation of antiviral treatment for horses infected with EI by neuraminidase inhibitor is required to obtain a satisfactory outcome, an easy and rapid diagnostic system for EI in the field is strongly required for minimizing the spread of EIV among horses when EI occurs [14].

Recently, several rapid antigen detection (RAD) kits have become commercially available in Japan [5]. These RAD kits are designed to detect the nucleoprotein (NP) of the genuses influenza A and B viruses, without requiring additional reagents, equipment or degree of expertise [15]. Since EIV is classified into the species *Influenza A virus* [12], it is anticipated that the RAD kits would work for rapid diagnosis of EI. The Directigen Flu A (Becton-Dickinson) and Binax NOW Flu A test kits (Binax, Scarborough, ME, U.S.A.) have been reported to be useful for diagnosis of EI [4, 9]. However, these reports were based on the sensitivities for old EIV strains (A/equine/Kildare/89 or A/equine/Sussex/89). Although several RAD kits are available, the performances of RAD kits for diagnosis of EI, except for these two kits, are poorly understood. The main object of this study was to evaluate the performances of plural RAD kits on the basis of the sensitivities of each kit to recent EIV isolates and then to compare the utilities of the kits for diagnosis of EI with virus isolation using embryonated hen's eggs and RT-PCR [11].

First, in order to evaluate the sensitivities of the RAD kits for EIVs, we compared the detection limits of the five RAD kits that were commercially available in Japan at the beginning of the experiment (as of June in 2006) for A/equine/South Africa/4/03 (SA/03) and A/equine/Avesta/93 (Avesta/93). The features of the RAD kits in this note are summarized in Table 1. Since SA/03 is classified into the Florida sublineage, which is predominant in the world, this strain is currently recommended as a vaccine strain by the Office International des Epizooties (OIE) [3, 7]. Avesta/93 is of European lineage and is also recommended as a vaccine strain by OIE [3, 7]. These EIVs were propagated in the allantoic cavity of 10-day-old embryonated hen's eggs. The harvested allantoic fluids were centrifuged at 1,500 × g for 15 min, and the supernatants were used as the virus stocks without further purification in this study. The titers of the virus stocks of SA/03 and Avesta/93 were $10^{7.3}$ 50% egg infectious dose (EID_{50})/200 μl and $10^{5.5}$ EID_{50}/200 μl, respectively. Serial ten-fold dilutions of each virus stock were made in lactose broth medium (Nissui, Tokyo, Japan) containing 0.004% (w/v) gentamicin sulphate (Gentacin injection, Schering-Plough, Osaka, Japan; LBM), and 200 μl of each these viral dilutions was mixed with the extraction solution bundled with each RAD kit in this study. The procedures for antigen detection were conducted according to the manufacturer's instructions of each RAD kit. The detection limits of the RAD kits for two of the EIV strains, SA/03 and Avesta/93, are represented in Tables 2 and 3, respectively. Directigen Flu A+B (Directigen) and

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Table 1. The characteristics of the rapid antigen detection kits used in this note, RT-PCR and virus isolation

Method	Principle of kit	Approximate duration of test (min)
Quick S-influ A•B (Denka Seiken)[a)]	Membrane immunoassay	15
QuickVue Rapid SP influ (DS Pharma Biomedical)[a)]	Lateral-flow immunoassay	15
ESPLINE INFLUENZA A&B-N (Fujirebio)[a)]	Lateral-flow immunoassay	15
Capillia Flu A+B (Alfresa)[a)]	Lateral-flow immunoassay	15
Directigen Flu A+B (Becton-Dickinson)[a)]	Membrane immunoassay	8
RT-PCR[b)]		240
Virus isolation[b)]		3 days

a) Name of rapid antigen detection kit (distributor)
b) See text for details.

Table 2. Detection limits of the five RAD kits for A/equine/South Africa/4/03[a)]

Method	Dilution				
	×1	×10	$\times 10^2$	$\times 10^3$	$\times 10^4$
Quick S-influ A•B	+	+	+	–	–
QuickVue Rapid SP influ	+	–	–	–	–
ESPLINE INFLUENZA A&B-N	+	+	+	+	–
Capillia Flu A+B	–	–	–	–	–
Directigen Flu A+B	+	+	+	+	–

a) Serial ten-fold dilutions of the virus stock ($10^{7.3}$ EID_{50}/200 μl) were made in lactose broth medium containing 0.004% (w/v) gentamicin sulphate, and 200 μl each of these viral dilutions was mixed with the extraction solution bundled with each RAD kit.

Table 3. Detection limits of the five RADs for A/equine/Avesta/93[a)]

Method	Dilution			
	×1	×10	$\times 10^2$	$\times 10^3$
Quick S-influ A•B	+	+	–	–
QuickVue Rapid SP influ	+	–	–	–
ESPLINE INFLUENZA A&B-N	+	+	+	–
Capillia Flu A+B	–	–	–	–
Directigen Flu A+B	+	+	+	–

a) Serial ten-fold dilutions of the virus stock ($10^{5.5}$ EID_{50}/200 μl) were made in lactose broth medium containing 0.004% (w/v) gentamicin sulphate, and 200 μl each of these viral dilutions was mixed with the extraction solution bundled with each RAD.

ESPLINE INFLUENZA A&B-N (Espline) exhibited the highest sensitivities to EIVs among the RAD kits tested in this study, and thus were chosen for the further study.

Subsequently, in order to compare the sensitivities for EIV detection from nasal swabs of the two RAD kits (Directigen and Espline) with those of virus isolation and RT-PCR, three two-year-old horses were infected with $10^{8.6}$ EID_{50} in 20 m*l* of SA/03 using an ultrasonic nebulizer (Soniclizer 305, ATOM, Tokyo, Japan). Nasal swabs were collected for seven consecutive days after infection as described by Mumford *et al.* [6]. The swabs were immersed in 2.5 m*l* of LBM and centrifuged at 1,500 × g for 15 min. The supernatants were divided into aliquots and stored below –70°C until analyzed. For virus isolation, 200 μl of the supernatants diluted 1:10 (v/v) in LBM were injected into the allantoic cavities of 10-day-old embryonated hen's eggs (four eggs per sample). The allantoic fluid was harvested after 72 hr of incubation at 34°C and tested by hemagglutination (HA) using 0.5 % hen's red blood cells. If HA was observed, quantification assays were carried out to determine the viral titers of the nasal swab specimens [10]. Viral RNA was extracted from 100 μl of each sample using a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Tokyo, Japan) and was eluted in 100 μl of 10 mM Tris-HCl and 1 mM EDTA at pH 8.0 (TE). Amplification was performed by a PRISM 7,000 sequence detection system (Applied Biosystems, Tokyo, Japan) with TaqMan EZ RT-PCR Core Reagents (Applied biosystems, Tokyo, Japan). Forty five μl RT-PCR mix consisting of 8 μl of 25 mM $Mn(OAc)_2$; 10 μl of 5 × EZ RT-PCR buffer; 2 μl of *rTth* polymerase (2.5 unit/μl); 1.5 μl each of 10 mM dATP, 10 mM dCTP, 10 mM dGTP and 20 mM dUTP; 0.5 μl each of 20 μM LP/1/93-1067F primer, 20 μM LP/93/-1137R primer, 1 μl of 5 μM LP/1/93-1095T probe and 17 μl of TE were used for RT-PCR. In this study, 5 μl of the sample elution was added to the RT-PCR mix. The primer set, probe and thermal cycle conditions were previously described by Sugita and Matsumura [11].

The results are summarized in Table 4. EIV was first isolated on day 1 post-infection and persisted for a mean period of 6.7 days. The peak virus titers were observed on day 2 post-infection in all horses and ranged from $10^{3.0}$ to $10^{3.2}$ EID_{50}/200 μl. The two RAD kits were not as sensitive as virus isolation or RT-PCR. In particular, these kits were less sensitive in early- and late- infection when a small amount of virus was shed. However, the two kits were capable of producing positive results in reasonable agreement with those obtained by virus isolation or RT-PCR during most of the period of virus shedding from days 2 to 5 or 6 post-infection. Despite disagreement between the results of the RAD kits on day 7 post-infection for Horse 2, these two kits seemed to have almost equal sensitivities to the EIVs in the

Table 4. Detection of virus by isolation, RAD kits and RT-PCR in nasal swabs from horses infected experimentally with A/equine/South Africa/4/03

Horse and day post-infection	Virus isolation	Titer[a)]	ESPLINE INFLUENZA A&B-N	Directigen Flu A+B	RT-PCR
Horse 1					
0	–		–	–	–
1	+	≤1.3	–	–	–
2	+	3.0	+	+	+
3	+	1.7	+	+	+
4	+	2.0	+	+	+
5	+	1.5	+	+	+
6	+	≤1.5	–	–	+
7	+	≤0.7	–	–	–
8	–		–	–	–
Horse 2					
0	–		–	–	–
1	+	1.5	–	–	–
2	+	3.2	+	+	+
3	+	1.7	+	+	+
4	+	1.7	+	+	+
5	+	2.0	+	+	–
6	+	2.0	+	+	+
7	+	1.5	+	–	+
8	–		–	–	–
Horse 3					
0	–		–	–	–
1	+	2.3	–	–	+
2	+	3.0	+	+	+
3	+	2.3	+	+	+
4	+	≤1.5	+	+	+
5	+	2.0	+	+	+
6	+	1.7	–	–	+
7	–		–	–	–
8	–		–	–	–

a) Log EID_{50}/200 μl.

nasal swab samples.

Due to its rapid transmission, once a horse infected with EI is detected, prompt isolation of the horse from other healthy horses is required. Therefore, it is essential that such RAD kits are highly specific for influenza virus. To test the specificities of Directigen and Espline, nasal swab specimens from 93 Thoroughbred racehorses with acute pyrexia (above 38.5°C) kept at the Ritto and Miho Training Centers of the Japan Racing Association (Shiga and Ibaraki Prefectures, respectively, Japan) were analyzed. Because no occurrence of EI has been reported in Japan since 1972 and there was no outbreak of flu-like illness at these centers during collection of the specimens (from January to May of 2007) [2], it was thought that these specimens did not contain EIV. All the results with Espline were negative. In contrast, 2 samples gave positive results for both influenza A and B viruses with Directigen. The results of these samples with RT-PCR for EIV were negative. It is generally accepted that influenza B virus infection is restricted to humans and seals [8]. Based on the above, the positive results for the influenza A and B viruses given by Directigen were thought to be false.

Moreover, to examine whether these kits cross-react with equine respiratory pathogens other than EIV, equine herpesvirus (EHV)-1 ($10^{5.6}$ PFU/200 μl), EHV-4 ($10^{6.5}$ PFU/200 μl), 4 strains of *Streptococcus equi* subsp. *equi* [*S. equi*, $\geq 10^{6.0}$ colony forming unit (CFU)/m*l*] and 4 strains of *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*, $\geq 10^{6.0}$ CFU/m*l*) were tested using these kits. The results are summarized in Table 5. The results with Espline for all pathogens tested were negative. In contrast, one strain (6708) of *S. zooepidemicus* produced a positive result for influenza A virus and 3 strains (W60, 6708 and 6729) of *S. zooepidemicus* produced positive results for influenza B virus with Directigen. *S. zooepidemicus* is a common commensal organism in the equine tonsil and nasopharynx and is often associated with an opportunistic respiratory infection [1]. Although we did not isolate *S. zooepidemicus* from the two nasal specimens that produced false positive results with Directigen, *S. zooepidemicus* might have played a role in those results. Further study is be needed to clarify this point. Thus, the positive results with Directigen should be judged by careful consideration of the possibility of a false positive reaction.

In conclusion, although a negative result does not preclude the possibility of EIV infection, Espline and Directi-

Table 5. Cross-reactions with equine respiratory pathogens other than EIV for Directigen Flu A+B and ESPLINE INFLUENZA A&B-N

Pathogen (strain)[a]	Directigen Flu A+B		ESPLINE INFLUENZA A&B-N	
	A	B	A	B
EHV-1 (89c25p)	–	–	–	–
EHV-4 (TH20p)	–	–	–	–
S. equi (CF32)	–	–	–	–
S. equi (Hidaka)	–	–	–	–
S. equi (NCTC)	–	–	–	–
S. equi (lex)	–	–	–	–
S. zooepidemicus (W60)	–	+	–	–
S. zooepidemicus (6708)	+	+	–	–
S. zooepidemicus (6729)	–	+	–	–
S. zooepidemicus (7)	–	–	–	–

a) See text for virus titers and numbers of bacteria.

gen would be useful supplementary tests for initial diagnosis of an outbreak of EI, especially where this may occur in the absence of expert and/or laboratory facilities. However, from the viewpoint of specificity for EIV, Espline seems to be superior to Directigen.

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