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Evaluation of a rapid isothermal nucleic acid amplification kit, Alere™ i Influenza A&B, for the detection of avian influenza viruses

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ABSTRACT

Rapid and accurate diagnosis of influenza virus infection is essential for quick responses for both human and animal health. The Alere™ i Influenza A&B is a novel isothermal nucleic acid amplification kit that can detect and differentiate between influenza A and B viruses in human specimens in approximately 15 min. In the present study, the performance of the Alere™ i Influenza A&B kit was evaluated for its ability to detect avian influenza virus in chickens. The kit was able to detect representative avian influenza virus strains (hemagglutinin subtypes H1–H16, including the recently isolated H5 and H7 highly pathogenic avian influenza viruses), and the detection limit of the kit for these viruses varied between $10^{-1.4}$–$10^{2.1}$ 50% egg-infective dose per test, which is higher than the analytical sensitivity of the antigen detection immunochromatography kit ESPLINE® A INFLUENZA. In experimentally infected chickens inoculated with a highly pathogenic avian influenza virus strain A/chicken/Hokkaido/002/2016 (H5N6), viral RNA was detected in the tracheal and cloacal swabs. These results indicate that this kit has the potential to be used as a rapid screening test of influenza A virus infection in chickens.

1. Introduction

Influenza A virus (IAV) has been classified into different subtypes based on the antigenic differences of its surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), in which 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) were reported (World Organisation for Animal Health, 2017). Avian species including chickens, are susceptible to infections of low pathogenic avian influenza virus (LPAIV) and highly pathogenic avian influenza virus (HPAIV) (Lee and Saif, 2009; Hiono et al., 2017; World Organisation for Animal Health, 2018).

Detection of avian influenza virus (AIV) is performed by standard methods such as virus isolation, viral gene detection and viral antigen detection (World Organisation for Animal Health, 2017). Virus isolation using embryonated chicken eggs remains a sensitive method that can provide decisive evidence of the infection. However, virus isolation is time-consuming and requires a highly equipped laboratory to subtype influenza viruses. PCR-based methods, such as conventional reverse transcription-polymerase chain reaction (RT-PCR), real-time RT-PCR (qRT-PCR) and gene sequencing allow rapid and sensitive gene detection as well as characterization of influenza viruses within as short period. Therefore, PCR-based methods are widely used for both surveillance and diagnostic purposes of IAV (World Organisation for Animal Health, 2017, 2018); however, these assays require sample processing such as RNA isolation before the reaction, molecular diagnostic laboratory, and temperature-sensitive reagents (Okamatsu et al., 2016). Antigen capture immunochromatographic (IC) test can be used for the early detection of infection of AIV in suspected cases on site (Bai et al., 2006; Manzoor et al., 2008); however, the analytical sensitivity of IC test is much lower than virus isolation and qRT-PCR (Okamatsu et al., 2016; Chiarella et al., 2016).

In recent years, several companies have developed simpler and more rapid diagnostic kits for the diagnosis of seasonal influenza in hospitals. One of the rapid molecular diagnostic assays that has recently been developed is Alere™ i Influenza A&B that could provide the qualitative detection and characterization of influenza A and influenza B viruses via amplification of nucleic acid under isothermal condition (Hurtado et al., 2015; Ménová et al., 2013). The kit has been deployed

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Table 1
Detection of avian influenza viruses by Alere™ i Influenza A&B kit and detection limit of the kit.

Viruses	Alere™ i Influenza A&B[a]	Detection limit (EID_{50} /test)[b]
Low pathogenic avian influenza virus		
A/duck/Tottori/723/1980 (H1N1)	A positive	$10^{0.9}$
A/duck/Hokkaido/17/2001 (H2N3)	A positive	$10^{-0.4}$
A/duck/Mongolia/4/2003 (H3N8)	A positive	$10^{1.4}$
A/duck/Czech/1956 (H4N6)	A positive	$10^{-0.9}$
A/duck/Pennsylvania/10218/1984 (H5N2)	A positive	$10^{0.6}$
A/duck/Hong Kong/960/1980 (H6N2)	A positive	$10^{1.4}$
A/seal/Massachusetts/1/1980 (H7N7)	A positive	$10^{0.6}$
A/turkey/Ontario/6118/1968 (H8N4)	A positive	$10^{1.1}$
A/turkey/Wisconsin/1966 (H9N2)	A positive	$10^{1.6}$
A/chicken/Germany/N/1949 (H10N7)	A positive	$10^{-0.1}$
A/duck/England/1/1956 (H11N6)	A positive	$10^{-0.4}$
A/duck/Alberta/60/1976 (H12N5)	A positive	$10^{-1.4}$
A/gull/Maryland/704/1977 (H13N6)	A positive	$10^{0.1}$
A/mallard/Astrakhan/263/1982 (H14N5)	A positive	$10^{-1.4}$
A/duck/Australia/341/1983 (H15N8)	A positive	$10^{-0.9}$
A/duck/Hokkaido/WZ/82/2013 (H16N3)	A positive	$10^{1.8}$
Highly pathogenic avian influenza virus		
A/duck/Vietnam/HU5-1571/2016 (H5N1)	A positive	$10^{2.1}$
A/chicken/Kumamoto/1-7/2014 (H5N8)	A positive	$10^{2.0}$
A/chicken/Hokkaido/002/2016 (H5N6)	A positive	$10^{1.9}$
A/duck/Japan/AQ-HE29-22/2017 (H7N9)	A positive	$10^{2.0}$

[a] Reactivity against $10^{6.0}$ EID_{50} of virus.
[b] Detection limits of the kit were indicated by EID_{50} of viruses.

in hospitals to diagnose human influenza in the US (Bell et al., 2014). The test is performed individually without pre-treatment of specimens and test result of a sample is available within 15 min from sample collection (Chiarella et al., 2016; Chapin and Flores-Cortez, 2015).

The present study aimed to evaluate the applicability of the Alere™ i Influenza A&B kit for the diagnosis of avian influenza (AI) in chickens by determining the sensitivity and specificity of this kit.

2. Materials and methods

2.1. Viruses

Overall, 20 AIV strains were tested for the determination of the sensitivity of the kit. These strains included sixteen LPAIVs of 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9), three H5 HPAIVs [A/duck/Vietnam/HU5-1571/2016 (H5N1), A/chicken/Hokkaido/002/2016 (H5N6) (Hiono et al., 2017) and A/chicken/Kumamoto/1-7/2014 (H5N8) (Kanehira et al., 2015)], and one strain of H7N9 HPAIV [A/duck/Japan/AQ-HE29-22/2017 (H7N9) (Shibata et al., 2018)] (Table 1). Additionally, 14 other avian viruses (Table 2) were used to evaluate the specificity of the kit.

For the virus propagation, each of the AIV strains was diluted with serum-free minimal essential medium (MEM) and inoculated into 10-day-old specific antibodies negative embryonated chicken eggs which derived from conventionally raised hens (World Organisation for Animal Health, 2017). After the incubation at 35 °C for 36–48 h, the allantoic fluids containing viruses were harvested and stored at −80 °C until further use.

2.2. Virus titration

Embryonated chicken eggs were also used for virus titration of 50% egg-infective dose (EID_{50}). According to the method (World Organisation for Animal Health, 2017), 10-fold serial dilutions of the virus with PBS were inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs, followed by incubation at 35 °C for 36–48 h. Thereafter, the allantoic fluids were tested using hemagglutination test, and the virus infectivity titers were calculated as EID_{50} per milliliter (EID_{50}/mL) by the Reed and Muench method (Reed and Muench, 1938).

Table 2
Reactivity of Alere™ i Influenza A&B kit with other avian viruses.

Viruses	Strain name	Alere™ i Influenza A&B
APMV	NDV/LaSota	Negative
	NDV/B1 Hitchner	Negative
	APMV-2/Chicken/Yucaipa/1956	Negative
	APMV-3/Turkey/Wisconsin/1968	Negative
	APMV-4/Duck/Mississippi/320/1975	Negative
	APMV-5/Budgerigar/Japan/TI/1975	Negative
	APMV-6/Duck/Hong Kong/199/1977	Negative
	APMV-7/Duve/Tennessee/4/1975	Negative
	APMV-8/Goose/Delaware/1053/1976	Negative
	APMV-9/Duck/New York/22/1978	Negative
	APMV-10/Penguin/Falkland Island/324/2007	Negative
IBV	B-42	Negative
ILTV	NS-175	Negative
IBDV	Saga	Negative

Abbrevation: APMV, avian paramyxovirus; NDV, Newcastle disease virus; IBV, infectious bronchitis virus; ILTV, infectious laryngotracheitis; IBDV, infectious bursal disease virus.

Madin-Darby canine kidney (MDCK) cells were only used for the determination of the virus infectivity of the tissue samples of dead chickens; the cells maintained in Eagle's minimum essential medium supplemented with 0.3 μg per milliliter (mg/mL) of L-glutamine, 100 Unit per milliliter (U/mL) of penicillin G, 0.1 mg/mL of streptomycin, 8 mg/mL of gentamicin and 10% fetal bovine serum. Ten-fold serial dilutions of viruses with serum-free MEM were inoculated into MDCK cells incubated at 37 °C under 5% CO_2 atmosphere. After 48–72 h of virus inoculation at 35 °C, the cytopathic effects (CPE) of the cells were observed and 50% tissue culture infective dose ($TCID_{50}$) was calculated (Reed and Muench, 1938).

2.3. Evaluation of the specificity and sensitivity of the Alere™ i Influenza A&B kit

A rapid isothermal nucleic acid amplification kit, Alere™ i Influenza A&B (Alere, Inc. Waltham, MA, USA), was evaluated for the rapid diagnosis of AI in chickens. The kit amplifies RNA by nicking enzyme amplification reaction (NEAR) that is usually performed at 55–59 °C

(Ménová et al., 2013). The kit set for single run contained a sample receiver, transfer cartridge, and test base. The tests were performed using the device under isothermal condition, and the sample was tested individually according to the manufacturer's instructions. Briefly, first, the sample receiver containing the elution buffer was attached to the device and heated for 3 min. Thereafter, 200 microliter (μL) of the sample was directly added into the heated elution buffer. The transfer cartridge was attached to the receiver to take a small portion of the sample mixture and transferred to the test base which contained the reaction mixture for isothermal amplification. Subsequently, the device was subjected to RNA amplification, and the result was presented on its display after 10 min while approximately 15 min was required for testing a single sample. The detection limit was calculated by showing the lowest virus titer detectable by the Alere™ i Influenza A&B assay, and it was expressed as EID_{50} per test (EID_{50}/test). Moreover, the detection of AIVs using the Alere™ i Influenza A&B kit was compared with the rapid diagnostic IC kit for IAV, ESPLINE® A INFLUENZA (FUJIREBIO, Inc. Tokyo, Japan), which was authorized by the Ministry of Agriculture, Forestry and Fisheries, Japan for the screening test of AI. The procedure of the IC kit described in the study by Bai et al. (2005) was followed. Accordingly, 30 μL of the sample suspension was dropped onto the IC kit. After 15 min of incubation at the room temperature, the results were observed. A single blue line in the control line represented a negative test, whereas double blue lines in the control which represented a positive test.

In addition, to evaluate the specificity of the kit, overall 224 tracheal and cloacal swabs were collected from total 83 chickens (*Gallus gallus, Julia*), 8 Muscovy ducks (*Cairina moschata*) and 8 domestic ducks (*Anas platyrhynchos domesticus*). In total, 134 swab samples were collected from apparently healthy chickens, 90 swab samples were collected from experimentally infected chickens, Muscovy ducks and domestic ducks. All swab samples were suspended in 2 mL of transport medium (TM) (MEM containing 10,000 U/mL of penicillin G, 10 mg/mL of streptomycin, 0.3 mg/mL of gentamicin and 0.5% bovine serum albumin), followed by test using the Alere™ i Influenza A&B assay. AI-free samples were also confirmed to be negative with IAV by virus isolation with 2 passages using embryonated chicken eggs (data not shown). The results were statistically analyzed with Cohen's kappa.

2.4. Virus detection from swabs and tissue homogenates of chickens experimentally inoculated with H5N6 HPAIVs

Four 6-week-old chickens were obtained from Hokkai Starchick (Hokkaido, Japan). The chickens were intranasally inoculated with 100 μL of the virus suspension containing $10^{6.0}$ EID_{50} of A/chicken/Hokkaido/002/2016 (H5N6) (Hiono et al., 2017). Each of the chickens was housed in a self-contained isolator unit (Tokiwa Kagaku, Tokyo, Japan) at the Biosafety Level 3 (BSL-3) facility of the Faculty of Veterinary Medicine, Hokkaido University, Japan. All chickens were monitored daily following virus inoculation.

Tracheal and cloacal swabs were collected from the chickens twice per day until death. The swabs were suspended in 2 mL of TM and stored at −80 °C until testing by virus isolation using embryonated chicken eggs, the Alere™ i Influenza A&B kit, and the IC kit. The tissue samples of the brain, trachea, lung, kidney, and colon were collected from dead chickens and homogenized in TM according to the method described in the supplemental materials section. The tissue homogenates were stored at −80 °C until testing by virus isolation using MDCK cells, the Alere™ i Influenza A&B kit and IC kit.

3. Ethics statements

All animal experiments were authorized by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval numbers: 13-0108) and all experiments were performed following the guidelines of the committee. The BSL-3 biosafety facility of the Faculty of Veterinary Medicine, Hokkaido University is permitted to store and use HPAIVs by The Ministry of Agriculture, Forestry and Fisheries, Japan in accordance with the Act on Domestic Animal Infectious Diseases Control (Permission number: 19)

4. Results

4.1. Gene detection of AIVs by the Alere i Influenza A&B kit

The sensitivity of the Alere™ i Influenza A&B kit was evaluated using 20 strains of IAVs including sixteen different representative strains of LPAIVs [16 HA subtypes (H1–H16) and 9 NA (N1–N9)] and three H5 and one H7 subtypes of HPAIVs (Table 1). Following virus propagation using embryonated chicken eggs, 10-fold serial dilutions of the allantoic fluid containing the virus were measured for their infectivity titer and performed for Alere™ i Influenza A&B test. The detection limits of the kit were expressed in EID_{50}/test. The results revealed that the kit could detect viral RNA of different subtypes of AIVs with the lower detection limit ranged in between $10^{-1.4}$–$10^{2.1}$ EID_{50}/test. In addition, the Alere™ i Influenza A&B kit could detect the recent H5 and H7 HPAIVs within the same detection limits.

Further, the specificity of the kit was also evaluated on 14 avian strains of avian paramyxoviruses (APMV), including Newcastle disease viruses (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), and infectious bursal disease virus (IBDV). The Alere™ i Influenza A&B kit did not show any reactivity with these samples (Table 2).

4.2. Detection of virus genes in swabs and tissue homogenates of chickens experimentally inoculated with H5N6 HPAIV

The applicability of the kit for the diagnosis of H5N6 HPAIV infection in the chickens was evaluated and compared with the IC kit, ESPLINE® A INFLUENZA. For experimental infection, all chickens started showing clinical signs on 1–1.5 days post-infection (d.p.i), and

Table 3
Comparison of virus detection from swab samples of experimentally inoculated chickens with H5N6 HPAIV.

Day post-infection	Chicken 1[a]		Chicken 2		Chicken 3		Chicken 4	
	Tracheal	Cloacal	Tracheal	Cloacal	Tracheal	Cloacal	Tracheal	Cloacal
0.5	- / - / < 0.8	- / - / < 0.8	- / - / < 0.8	- / - / < 0.8	+ / - / < 0.8	- / - / < 0.8	+ / - / 1.3	- / - / < 0.8
1.0	+ / - / 2.0	- / - / < 0.8	+ / - / 1.8	- / - / < 0.8	- / - / < 0.8	- / - / < 0.8	+ / - / 2.0	- / - / < 0.8
1.5	+ / + / 5.3	+ / - / 3.6	+ / - / 4.6	+ / - / 3.3	+ / + / 3.3	+ / - / 4.0	+ / + / 4.8	+ / + / 4.6
2.0	+ / + / 4.6[b]	+ / - / 3.8[b]	+ / + / 6.0[b]	+ / + / 3.0[b]	+ / + / 4.6[b]	+ / + / 4.6[b]	+ / + / 5.3	+ / + / 3.3
2.5	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND	+ / + / 4.3[b]	+ / + / 6.5[b]

[a] Result of Alere™ i Influenza A&B kit, ESPLINE kit and EID_{50} titer ($\log_{10}$ EID_{50}/ml) were indicated: + ; viral antigen or RNA detected. - ; viral antigen and RNA have not detected. ND; sample was not tested by the assay, because the chicken was died at previous day of post infection.
[b] Chicken died.

the last chicken died on 2.5 d.p.i. Overall, 34 swabs and 20 tissue samples were collected from chickens that were experimentally inoculated with A/chicken/Hokkaido/002/2016 (H5N6) (Table 3 and Supplemental Table 1) and following performed virus titration, the Alere™ i Influenza A&B kit and the IC kit.

Regarding tracheal and cloacal swab samples, virus titers varied in the range from under the detection limit to $10^{6.0}$ EID_{50}/mL and $10^{6.3}$ EID_{50}/mL, respectively, at 0.5 and 2.5 d.p.i. The Alere™ i Influenza A&B kit could detect viral RNA from all chickens between 0.5 and 1.5 d.p.i (Table 3). Moreover, the Alere™ i Influenza A&B kit and the IC kit could detect the genes or the proteins of IAV from the swabs with virus titer over than $< 10^{0.8}$ EID_{50}/mL and $10^{3.3}$ EID_{50}/mL, respectively. For the tissue homogenates, total 20 tissue samples were collected from the 4 dead chickens and tested using the Alere™ i Influenza A&B kit and the IC kit. Both kits showed all positive results for IAV in the tissue samples on experimental infection by H5N6 HPAIV. In addition, the virus titer ranged between $10^{7.0}$ and $10^{9.6}$ $TCID_{50}$ per gram tissue (Supplemental Table 1).

For the applicability in a field, the performance of the Alere™ i Influenza A&B kit was evaluated using tracheal and cloacal swabs which were collected from apparently healthy chickens, and experimentally infected birds; chickens, Muscovy ducks, and domestic ducks with HPAIV, including the samples mentioned in Table 3 were included. Results indicated that the kit does not detect viral RNA of IAV from these tracheal and cloacal swabs as the result without a non-specific detection in the field swab samples. From these results, the sensitivity and specificity of the Alere™ i Influenza A&B kit for detecting HPAIVs were 100% and 99.3%, respectively, (Table 4).

5. Discussion

Virus isolation is the recommended method for the diagnosis of AI to provide an accurate result with high sensitivity. In recent years, more rapid tests including PCR-based methods have been developed and used for the diagnosis. The Alere™ i Influenza A&B assay provides isothermal nucleic acid amplification for the qualitative detection by NEAR (Ménová et al., 2013) and differentiation of both influenza A and influenza B viruses within 15 min without specific laboratory equipment and techniques other than the Alere i device (Hurtado et al., 2015; Chiarella et al., 2016). The target gene segments of the Alere™ i Influenza A&B kit are PB2 gene of influenza A and PA gene of influenza B virus (Alere Scarborough, Inc, 2015); and these target regions are highly conserved in AIVs of all 16 HA subtypes (Obenauar et al., 2006), but not in other avian viruses. In addition, the device qualitatively measured fluorescently-labeled target RNA during NEAR described by Bell et al. (2014). In the present study, the Alere™ i Influenza A&B kit was evaluated for its applicability to detect AIVs as the kit has not been previously evaluated using animal samples. In the present study, the kit could detect representative strains [HA subtypes (H1–H16), NA subtypes (N1–N9)] of LPAIV and HPAIV with the lower virus titer, and consequently, the detection limit was expressed in the range of $10^{-1.4} - 10^{2.1}$ EID_{50}/test (Table 1). This detection limit was similar as that in other RNA detection methods including qRT-PCR, loop-mediated isothermal amplification (LAMP) and nucleic acid sequence based amplification (NASBA) (Bell et al., 2014; Lau et al., 2004).

To further assess the performance of the Alere™ i Influenza A&B kit, samples from the chickens experimentally inoculated with A/chicken/Hokkaido/002/2016 (H5N6) were tested. We used the kit on swab samples from birds since these samples are frequently used to detect AIV in the field and are easily prepared without risk of contamination. The kit could detect viral RNA of IAV on tracheal swab at 0.5 day and on cloacal swab at 1.5 days post-infection (Table 3), suggesting that the kit has the capacity to detect AIV infection from the upper respiratory organ as fast as qRT-PCR reported previously (Bell et al., 2014). In one swab sample, the kit showed positive result, albeit the virus isolation was under the detection limit. It is possible that viral RNA in the sample was detectable by the kit although the virus protein and infectious virus particle were undetectable by IC kit and virus isolation, respectively. For the kit specificity, the kit did not show non-specific results with avian influenza virus-free swab samples which were collected from healthy chickens raised in farms and in an animal facility (Table 4). These results suggest that the Alere™ i Influenza A&B kit can be used to screen samples for H5N6 HPAIV infection in chickens at various stages of infection.

Most countries adopt a stamping-out policy to eradicate highly pathogenic avian influenza (HPAI) in poultry within a short time period. To accomplish this strategy, rapid detection and accurate identification of HPAI cases are critical activities to control the disease spread. In countries, like Japan, which has only experienced sporadic outbreaks of LPAI and HPAI, screening tests on samples from dead poultry with suspected AIV infection can further accelerate control measures and decision making. Therefore, the IC kit, which detects nucleoprotein (NP) of AIVs, is authorized in Japan and is applied to dead birds for screening test in farm-side by local veterinarians. In the fact, HPAI outbreaks have been successfully eradicated in Japan without transmission of HPAIV to the surrounding farms (Saito et al., 2015; Kanehira et al., 2015; Takemae et al., 2017). In present study, the Alere™ i Influenza A&B kit was evaluated which has comparable performance to that of qRT-PCR-based assays (Bell et al., 2014; Bai et al., 2005; Tsukamoto et al., 2010; Lee and Suarez, 2004). Moreover, the kit could provide the benefits of simple and rapid detection of IAVs in a field without laboratory facility, particularly in far-off areas and in other developing countries where the diagnostic laboratory is not enough trained. However, the assay has limitations in that false positives may occur in samples (Hurtado et al., 2015; Bell et al., 2014), our test performance was not conducted in a fields to check actual condition of the kit usage, and cost per each test is higher compared with virus isolation or antigen detection kit (Davis et al., 2017; Barthelemy et al., 2017).

In the present study, the reactivity of the Alere™ i Influenza A&B kit was analyzed against LPAIVs, HPAIVs, and other avian viruses. Moreover, the kit was evaluated on the samples from experimentally inoculated chickens with A/chicken/Hokkaido/002/2016 (H5N6) and healthy chickens from the field. These results indicate that the Alere™ i Influenza A&B kit has potential for the detection of AIVs, including H5 and H7 HPAIVs. Furthermore, the kit is easy to handle and suitable to use on site, therefore, the kit is the applicable screening tools in a field for rapid diagnosis of AIV infection in chickens.

Table 4
Comparison of sensitivity and specificity of Alere™ i Influenza A&B kit and virus isolation method.

Alere™ i Influenza A&B	Virus isolation (Tracheal and cloacal swab)		Sensitivity (%) (95% CI)[a]	Specificity (%) (95% CI)[a]
	Positive	Negative		
Positive	78	1	100% (100%)	99.3%
Negative	0	145		(99.3 ± 0.01%)

[a] The data are analyzed by Cohen's kappa statistically. Abbrevation: CI, confidence interval.

Conflict of interest

The present study was supported by Alere Medical Co., Ltd, Tokyo, Japan, which provided the Alere™ i Influenza A&B kit for this study. The source of support had no influence on the analyses or interpretation of the results presented in this paper. All authors have declared that no conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.01.004.

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