Journal of Virological Methods 170 (2010) 173–176

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Development and validation of a commercial real-time NASBA assay for the rapid confirmation of influenza A H5N1 virus in clinical samples

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Article history:
Received 29 April 2010
Received in revised form 5 August 2010
Accepted 13 September 2010
Available online 21 September 2010

Keywords:
Real-time NASBA
Influenza A H5N1 virus
Respiratory samples

ABSTRACT

A real-time NASBA assay for the specific confirmation of influenza A H5N1 infection was developed and evaluated using proficiency panels distributed to the UK influenza network of laboratories and clinical samples received through the Chinese National Influenza Centre in Beijing. The aim of the proficiency panels was to determine the sensitivity and specificity of the assay on a range of influenza virus types and subtypes including different clades of influenza A H5 viruses. The assay was then evaluated using 19 clinical samples obtained from seven confirmed human cases of influenza A H5N1 infection in China. The assay was shown to have a level of sensitivity of 0.01 TCID50 and 10 copies/μl using RNA transcripts of the A/VietNam/1194/2004 H5N1 virus. During the evaluation the assay successfully detected H5N1 viruses known to infect humans from clades 1, 2.1, 2.2 and 2.3 as well as low pathogenic H5N3 avian influenza viruses. The clinical utility of the real-time NASBA assay was proven on a range of clinical samples from patients with confirmed H5N1 infection collected during 2005 and 2006. The real-time NASBA assay was demonstrated to be sensitive and rapid allowing for same day confirmation of a H5N1 infection direct from clinical samples.

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Since the emergence of pandemic influenza A (H1N1) 2009 virus there has been a reduction in the media coverage of human infections with highly pathogenic avian influenza A (H5N1) viruses. It is well recognised that H5N1 remains a potential pandemic virus through adaptation via genetic drift to infect humans preferentially or via re-assortment with a human influenza A virus if a dual infection occurs (WHO writing committee, 2008). H5N1 continues to circulate and outbreaks have occurred in birds throughout Asia and into Europe and Africa with the viral H5 haemagglutinin evolving into phylogenetically distinct clades and subclades (Webster and Govorkova, 2006). Reports to WHO show that human cases continue to occur and although the vast majority are associated with contact with infected birds there are a few reports of limited human to human transmission (Wang et al., 2008). Human H5N1 infection often leads to a severe systemic infection which is associated with a high mortality rate. During 2009 into early 2010 there were 84 human cases of H5N1 virus reported in Egypt, Indonesia, Vietnam, and China. The overall mortality rate was 46%, which remains greater than any pandemic human influenza A (WHO website accessed March 2010). For this reason, rapid diagnosis and global surveillance of H5N1 influenza A is still as important today as when the number of reported cases peaked at 115 with an associated mortality of 69% in 2006 (WHO writing committee, 2008; WHO website accessed March, 2010).

H5N1 infection in returning travellers from an endemic region, or infection in poultry with onward transmission to poultry workers, are considered to be the major routes of introduction of H5N1 to non-endemic regions such as Western Europe and the Americas. Once this occurs; a rapid confirmation of infection is required to ensure appropriate management of the patient and potential contacts (Curran et al., 2007; Ellis et al., 2007; HPA guidelines accessed March, 2010). Assays designed to detect and confirm H5N1 virus must be rapid, sensitive and specific for all known circulating clades. Molecular techniques are preferable to traditional laboratory techniques such as culture for the detection of H5N1 in clinical samples, with most current molecular assays developed using real-time RT-PCR methods (Ellis et al., 2007). An alternative molecular method for the amplification and detection of respiratory viruses is real-time nucleic acid sequence based amplification (NASBA) the

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doi:10.1016/j.jviromet.2010.09.014

detailed method of which has been described previously (Leone et al., 1998; Moore et al., 2004; Fox et al., 2009).

The NucliSens EasyQ Influenza A H5 and N1 NASBA assay (bioMérieux, Grenoble, France) was developed as a rapid and specific sub-typing assay for the detection of H5N1 virus in patients positive for influenza A who have travelled to or live in an H5N1 endemic regions and have been in close contact with sick-poultry or confirmed human H5N1 cases.

The assay provides primer and probes to detect H5 and N1 in separate reactions in combination with the NucliSens EasyQ basic kit version 2 on the EasyQ analyser. It was developed using sequence alignment of the haemagglutin and neuraminidase genes of all known H5 viruses circulating in 2004. Sensitivity of the assay was determined using in vitro RNA transcripts of the reference strain A/Vietnam/1194/2004 and serial dilutions of cultured H5N1virus with known TCID50.

Specificity of the assay was determined by performing the assay on non H5 strains of influenza A, influenza B, RSV, parainfluenza and adenovirus (Telles et al., 2007).

Further assay validation and specificity testing was performed by the Public Health Wales Cardiff Laboratory as part of the UK Influenza Laboratory Network (Curran et al., 2007). Validation of the NucliSens H5N1 NASBA assay was performed using influenza proficiency panels containing inactivated strains of influenza A and B from a range of hosts (human, avian, equine and swine) provided by the Health Protection Agency (HPA), Centre for Infections (CfI), London. The panels were sent to each laboratory within the network simultaneously and preliminary results were expected to be returned within 6 h of panel receipt. An aliquot of inactivated A/Duck/Malaysia/F119/3/97 (H5N3) culture fluid was provided by CfI to all laboratories in the UK network performing H5N1 testing for use as a H5 positive run control.

In Wales; four samples from suspected human cases of H5N1 infection were also tested in addition to the proficiency panels. Each suspected case fulfilled the UK Health Protection Agency (HPA) case definition for a suspected H5N1 infection (HPA guidelines accessed March, 2010). Each patient was sampled using dry respiratory swabs, as previously described to reduce the risk of infection during transportation to the laboratory and during sample processing (Moore et al., 2008).

All samples, proficiency panels and controls were processed in a category III facility. Guanidinium thiocynate based lysis buffer (bioMérieux, Grenoble, France) was used to inactivate any virus present in 200 μl of sample. Nucleic acid extraction was performed on the NucliSens automated EasyMag system and eluted into 60 μl following manufacturers instructions. The entire extraction procedure for up to 24 samples following sample processing was completed in 40 min. All samples were tested by a generic influenza A assay, an influenza B assay and a full respiratory screen using real-time NASBA to rule out other causes as described previously (Moore et al., 2008). All Influenza A positive samples were sub-typed to seasonal H1, H3, pandemic H1 and H5 using real-time molecular assays developed by CfI (Ellis et al., 2007; Moore et al., 2008). The NucliSens H5N1 real-time NASBA assay was used as the second line test to confirm an H5 positive sample as it allowed discrimination between H5N1 and any other neuraminidase sub-type, e.g. N3.

Due to the small number of human H5N1 cases each year, validation of any H5N1 assay using clinical samples is challenging, particularly in nonendemic regions. A clinical validation of the real-time NASBA assay was performed in China on 19 respiratory samples collected from seven confirmed cases of H5N1 infection between 2005 and 2006. The clinical validation included five sequential samples collected from a single patient. The samples tested included throat swabs, tracheal secretions, bronchoalveolar lavages and lung tissue. All samples were cultured for H5N1 virus by isolation in specific pathogen free (SPF) class eggs and confirmed by in-house PCR targeting the haemagglutinin of H5 viruses. Cases 1–6 were tested by a traditional block-based RT-PCR with end-point gel detection and case 7 was tested by real-time RT-PCR as described previously (Yu et al., 2008; Zou et al., 2007). Nucleic acid extraction was performed on 200 μl of clinical sample using the RNeasy Mini Kit (Qiagen, Germany), with elution into a 50 μl volume of nuclease free water following the manufacturers instructions. All sample extracts were stored at −20 °C and tested retrospectively by real-time NASBA following 1 year of storage.

The NASBA reaction was performed in both centres on 5 μl of extracted nucleic acid as described previously using the basic kit version 2 with a KCl concentration of 70 mM (Moore et al., 2004). Amplification and simultaneous detection was performed using the NucliSens EasyQ analyser with a wild-type (FAM) threshold of 1.2 for each of the targets (Telles et al., 2007).

During development, the lower level of sensitivity for the assay was shown to be 10 copies/μl of A/Vietnam/1194/2004 RNA transcript. For manufacturing and stability purposes each batch of primers and probe mix was tested at 0, 6, 12 and 18 month intervals on 42 replicate reactions of the RNA transcript at 20 copies/μl for both H5 and N1. As each primer and probe batch is only released if the reactions are positive in all of the replicates, the assay has consistently been shown to be 100% sensitive at 20 copies/μl and is stable for 18 months.

Serial dilutions of cultured A/Turkey/582/06 (H5N1) at stock concentration of 10^{763} $TCID^{50}$, A/Turkey/073/06 (H5N1) at a stock concentration of 10^{78} $TCID^{50}$ and A/VietNam/1194/04 (H5N1) (no data on $TCID^{50}$) were also tested in triplicate. The consistent level of sensitivity was at a dilution of 10^{-7} in both the H5 and N1 assays for the Turkey strains and 10^{-7} in the H5 assay and 10^{-8} In the N1 assay for the Viet Nam strain.

The results of the proficiency panels are summarised in Table 1, the H5 assay was shown to be sensitive and specific for a range of H5 viruses from clades 1, 2.1, 2.2 and 2.3 as well as low pathogenic avian strains of H5N3. The N1 assay was relatively specific although some cross reactivity was noted in samples containing high titre pandemic (H1N1) 2009 viruses, an avian H6N1 virus and a swine lineage H1N1 virus.

Of the four clinical samples received for H5N1 testing in the Cardiff laboratory all were negative for influenza A. The ease in assay set-up meant that the influenza A screening tests, H5 specific RT-PCR and the H5N1 real-time NASBA assays could be run simultaneously reducing the time to confirmed result significantly. The average turnaround time from sample receipt to result reporting was 4 h.

The clinical validation performed in China demonstrated that samples positive by egg culture and/or H5 RT-PCR were also positive by both the H5 and N1 NASBA reagents despite long term storage of the RNA extracts (Table 2).

Compared to the RT-PCR H5 specific assays used for screening in both the UK and China the real-time NASBA H5 assay was demonstrated to have equal sensitivity during the validation. The N1 assay was shown to be marginally more sensitive than the H5 assay during development and during the clinical validation.

The development and validation data from both the UK and China thus demonstrates that the NucliSens H5N1 real-time NASBA assay to be rapid, sensitive and specific for confirming the presence of H5N1 virus in clinical samples. As the assay is available for research use only its use is limited to being a second line confirmatory test despite having comparable sensitivity to the front-line H5 assay being used routinely by the two evaluation centres. The added benefit of the H5N1 reagents is the ability to rapidly subtype to the N1, thus confirming a H5N1 infection. The limited cross reaction seen with the N1 assay is not surprising due to considerable sequence identity between N1 viruses of avian lineage which includes the N1 of the pandemic influenza (H1N1) 2009 virus.

Table 1
The results of the real-time NASBA H5N1 assay on a range of influenza A strains received through the Chinese National Influenza Centre* and through the UK National Influenza Laboratory Network**.

Influenza A strain (H5N1 Clade)	H5 RT-PCR result	H5 NASBA result	N1 NASBA result
A/Guangdong/2/1991 (H3N2) *	–	–	–
A/Anhui/318/2003 (H3N2)*	–	–	–
A/Wisconsin/67/05 (H3N2)**	–	–	–
A/Brisbane/10/2007 (H3N2)**	–	–	–
A/Perth/16/2009 (H3N2)**	–	–	–
A/Swine/Italy/1477/96 (H3N2)**	–	–	–
A/Equine/Miami/63 (H3N8)**	–	–	–
A/Jingshen/1/1956 (H1N1)*	–	–	–
A/Gui/387/2002 (H1N1)*	–	–	–
A/New Caledonia/20/99 (H1N1)**	–	–	–
A/Solomon Islands/3/2006 (H1N1)**	–	–	–
A/Brisbane/59/2007 (H1N1)**	–	–	–
A/England/195/2009 (H1N1)**	–	–	–
A/Aragon/3218/2008 (H1N1)**	–	–	–
A/England/935240/2009 (H1N1)**	–	–	+
A/Scotland/8/2009 (H1N1)**	–	–	+
A/Swine/IV/1455/99 (H1N1)**	–	–	+
A/Turkey/England/198/2009 (H6N1)**	–	–	+
A/Eng/481/06 (H7N3)**	–	–	–
A/Av/1306/2007 (H7N2)**	–	–	–
A/Chicken/HK/G9/97(H9N2)*	–	–	–
A/HK/1073/99 (H9N2)**	–	–	–
A/Duck/Malaysia/F119/3/97(H5N3)**	+	+	–
A/Teal/Eng/06 (H5N3)**	+	+	–
A/Vietnam/1194/2004 (H5N1)** (Clade 1)	+	+	+
A/Indonesia/5/05 (H5N1)** (Clade 2.1)	+	+	+
A/Turkey/Turkey/05 (H5N1)** (Clade 2.2)	+	+	+
A/Anhui/2005 (H5N1)** (Clade 2.3)	+	+	+
A/Guangdong/1/2006 (H5N1)* (Clade 2.3)	+	+	+
A/Heishan/1/2006 (H5N1)* (Clade 2.3)	+	+	+

Conversely, it has been shown that assays designed to detect the pandemic H1N1 2009 neuraminidase gene will often cross react into the avian influenza A N1 viruses (Ellis et al., 2009). However, there is no evidence of the assay detecting seasonal human H1N1 influenza viruses and as the N1 assay would never be used in isolation to confirm the sub-type of an influenza A positive sample the limited cross reactivity observed should not be problematic.

With such a limited number of reported human infections with H5N1 virus globally, there is increased need to ensure that appropriate testing strategies are in place that will rapidly identify and confirm an infection. Recent data from China has shown that viruses from the Asian H5N1 2.3 clade have a predilection for infecting the lower respiratory tract with severe illness and prolonged viral shedding in survivors (Yu et al., 2008). This data is supported by this clinical validation with respect to cases 2, 5 and 7. Case 2 had H5N1 virus detected only in the lower respiratory tract and prolonged viral shedding was demonstrated with the detection of H5N1 virus in samples collected 12 and 14 days post symptom onset in cases 5 and 7 respectively. Whether it is adequate to collect swabs from the upper respiratory tract only from cases that may

Table 2
Results of the real-time NASBA H5N1 assay on clinical samples received from confirmed H5N1 infected Chinese patients 2005–2006.

Case	Sample type	Time since onset (days)	H5 NASBA result	N1 NASBA result	H5 RT-PCR (c_t value if available	Virus isolation
1	Throat swab	7	+	+	+	+
	Throat Swab	8	+	+	+	+
2	Throat Swab	10	–	–	–	–
	Throat Aspirate	10	+	+	+	+
	Tracheal Aspirate	10	+	+	+	+
	Lung tissue	10	+	+	+	+
3	Throat swab	6	+	+	+	+
	Mouth wash	6	–	+	–	–
4	Tracheal aspirate	9	+	+	+	+
	Tracheal aspirate	9	+	+	+	+
5	BAL	12	+	+	+	+
6	Throat swab	9	+	n/t	+	+
	Throat swab	9	–	+	–	–
	Tracheal aspirate	9	+	n/t	+	+
7	Tracheal aspirate	8	+	n/t	+ (29.8)	+
	Tracheal aspirate	9	+	n/t	+ (29.8)	+
	Tracheal aspirate	14	+	n/t	+ (28.3)	+
	Tracheal aspirate	20	–	n/t	–	–
	Tracheal aspirate	27	–	n/t	±(39)[a]	–

BAL: Bronchoalveolar lavages. n/t: insufficient RNA extract to test.
[a] Weakly reactive with late c_t value not confirmed by other assays.

have acquired infection from areas where clade 2.3 viruses circulate is questionable especially as they often present greater than 7 days post symptom onset.

Overall, the NucliSens H5N1 real-time NASBA assay has been proven to be sensitive in detecting all circulating clades of H5 virus known to infect humans from both cultured virus and directly from clinical material, including difficult to process lower respiratory tract samples and lung tissue. When used as a second line test in combination with a sensitive generic influenza A assay (with an internal control such as RNaseP) and an alternative H5 specific assay; H5N1 infection can be detected rapidly and confirmed within 4 h of sample receipt in the laboratory.

Acknowledgements

We would like to thank the Chinese provincial CDC including Anhui CDC, Zhejiang CDC, Fujian CDC and Sichuan CDC for providing the clinical samples for the study, the staff in the Molecular Diagnostics Unit, UHW for performing the H5N1 diagnostic service in Wales and the Health Protection Agency Virus Reference Laboratory, London for providing the characterised influenza proficiency panels. We especially thank the Chinese Shenzheng city CDC for providing the sequential samples from the H5N1 positive patient.

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