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Detection of Animal Viruses Using Nucleic Acid Sequence-Based Amplification (NASBA)

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Abstract: As seen in recent avian influenza outbreaks in Asia, prevention is the key to fighting infectious disease successfully. Efficient disease surveillance systems on the basis of molecular diagnostics will help monitor the emergence of viruses in the early stage and thus prompt containment measures can be in place to minimize disease spread. Here we describe and review molecular diagnostics focusing on nucleic acid sequence-based amplification (NASBA) technology in detecting viruses causing animal diseases, such as avian influenza, foot-and-mouth disease, and Newcastle disease. NASBA offers high sensitivity, specificity, accuracy, and speed of availability of results, and NASBA would be the most applicable molecular diagnostics for disease surveillance and control.

INTRODUCTION

Viruses are known to expand their range of hosts by mutation, with humans being unfortunate in becoming haphazard victims of viral mutation when humans and animals co-exist in close proximity. Outbreaks of avian influenza H5N1 infection since 1997 and the emergence of SARS (severe acute respiratory syndrome) in 2003 have raised serious concern of possible animal-to-human transmission of viruses through contact of humans with birds, poultry and other livestock. Many Asian countries have experienced the loss of human lives and devastation to their economy brought about by H5N1 outbreaks, and the need to develop rapid and sensitive diagnostic methods to detect such viruses is vital. Rapid detection tests for viruses will allow timely surveillance of possible sources of diseases and immediate actions could be taken to

contain the diseases and prevent outbreaks [1].

Current virus identification methods include conventional serology (such as antigen detection and antibody testing), virus culture (usually considered as the gold standard), and nucleic acid-based testing (NAT) (such as real-time PCR and nucleic acid sequence-based amplification (NASBA)). Blood-based serological methods may reveal some of the infection history of the test subject, but these methods suffer from lack of sensitivity and frequent false positives. Culturing live virus usually provides unambiguous results, but culturing often requires a longer time, and negative culture results do not necessarily mean absence of the virus tested. The shortcomings of these traditional methods may cause delay or mis-interpretation of results and lead to spreading of the viruses. From the experience of recent H5N1 outbreaks, diagnostic methods offering speed, specificity, and high sensitivity will be particularly important to the front line surveillance of infectious diseases. NAT are molecular diagnostic tools that can be used in disease surveillance to achieve early and sensitive detection of viruses. The main NAT streams are the PCR-based technologies, particularly real time PCR, and NASBA-based technologies.

Preventing global outbreaks of infectious diseases should be a mandate for all countries as globalization and advanced transportation systems bring the whole world closer by promoting frequent mobility of people and livestock. Exemplified by the SARS coronavirus spread from China to many parts of the world, governments and laboratories of different countries bear the responsibility to share disease surveillance information and technology available in monitoring disease outbreaks. Prevention is the key to fighting infectious diseases successfully, and efficient disease surveillance systems will help monitor the emergence of viruses and prompt containment measures to minimize disease spread.

PRINCIPLE OF NASBA

NASBA, developed in 1991, is an RNA-based amplification technology [2]. It is a continuous, isothermal, and enzyme-based method for the amplification of nucleic acids [3] which can serve as an alternative to PCR. NASBA uses a mixture of reverse transcriptase, ribonuclease-H, and RNA polymerase and two target-specific primers. The DNA oligonucleotide primers are specially designed with forward primer containing a 5' extension carrying the promoter sequence for bacteriophage T7 DNA-dependent RNA polymerase, and a reverse primer containing a 5' extension with a sequence complementary to a DNA detection probe labelled with a ruthenium-based electro-chemiluminescent (ECL) tag. Amplification takes place with the 5' primer extensions fully incorporated into the amplified products allowing both highly efficient production of complementary RNA template (directed by RNA polymerase) and specific detection by a ruthenium-containing probe. This method of detection requires the use of a chemi-luminenscence detector. Under optimum conditions, it is possible to achieve 10^{12}-fold amplification in 1.5 hours, versus the 10^{9}-fold amplification accomplished by PCR [4].

NASBA has the edge in amplifying specific single-stranded RNA targets without the need for nucleic acid denaturation among common contaminants such as genomic DNA, heparin, EDTA, citrate, haemoglobin, albumin, and lipids [3]. Moreover, the end product of amplification is RNA, which tends to be unstable under normal

environmental conditions, thus minimizing the possibility of carry-over contamination of equipment from previous experiments. There are numerous NASBA-based diagnostic tests for viruses such as human immunodeficiency virus type 1 (HIV) [3], cytomegalovirus (CMV) [5], hepatitis C virus [6], and papillomavirus type 16 [7]. Recently, we have proved the applicability of NASBA technology in the surveillance of animal diseases by successful development of NASBA-based methods to detect a number of animal viruses including avian influenza virus (AIV) and foot-and-mouth disease virus (FMDV) [8-11]. The use of this isothermal method renders the purchase of expensive and maintenance-requiring PCR thermocycler (conventional or real-time type) unnecessary, since a simple temperature-calibrated water bath will be able to provide the isothermal environment.

NASBA-Based Animal Virus Detection System

As cross-species viral infections are becoming more common, there is an imperative need for detecting animal viruses to control potential infection in livestock. With the high sensitivity and specificity offered by the NASBA technology, we have successfully developed various methods for AIV [8-10], FMDV [11], Newcastle disease virus, classical swine fever virus, and porcine reproductive and respiratory syndrome virus [unpublished data]. Various public and private laboratories in collaboration with us have started to use our NASBA-based methods for routine testing and surveillance of AIV and FMDV on their farm animals and poultry [personal communications]. The ultra-high sensitivity of the NASBA method to detect AIV H5N1 has been proved to be comparable to virus isolation [12]. The high sensitivity achieved by NASBA may be contributed by the direct incorporation of reverse transcription and highly efficient in vitro transcription by T7 RNA polymerase into one isothermal amplification reaction, whereas the reverse transcription step at a different temperature precedes PCR amplification involving more temperature changes for RT-PCR.

Avian Influenza Virus (AIV)

All AIVs belong to influenza type A, which can be further classified according to the antigenicity of two envelope glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Currently there are 15 subtypes of HA (H1-15) and nine subtypes of NA (N1-9) [8]. H5N1 and H7N2 are the two most dangerous subtypes of AIV strains known in terms of flock mortality and potential for virulence in humans. They belong to the category of highly pathogenic avian influenza (HPAI) with a mortality rate reaching 100%, while low pathogenic avian influenza (LPAI) strains cause less serious infections mainly in the respiratory and intestinal tracts [8].

The conventional method for diagnosis of avian influenza is through virus culture in embryonated fowl's eggs, albeit sensitive but labour-intensive and time-consuming [13]. Virus antigen detection techniques such as enzyme-linked immunoassay and immunofluorescence are relatively fast but less sensitive and less specific than virus isolation. Both RT-PCR and NASBA are highly sensitive and rapid diagnostic methods. We have previously conducted a study to compare the NASBA method with virus culture in the detection of AIV H5N1 strain, demonstrating equivalent sensitivity of the NASBA method to virus culture (Table 1, [12]). The study elegantly shows the

applicability of a four-hour NASBA method in detecting H5N1 with equivalent sensitivity to the "gold standard" virus culture approach, which takes days to be completed [12].

In addition to high sensitivity, NASBA is an effective way to test specifically for the presence of H5N1 virus in poultry vaccinated with attenuated AIV [14]. Conventional serology-based detection methods could not distinguish whether the presence of H5N1 antibodies is due to induction by vaccine or from natural immunity after past infection. NASBA detection of H5N1 using throat or cloacal swab samples targets H5N1 nucleic acid (RNA) to reflect the current presence of viral load and therefore is unaffected by vaccination.

Table 1: Comparable sensitivity of NASBA and virus culture in the detection of AIV H5N1.

Sample	**NASBA**[a]	**Virus culture**
10^{-1} dilution	Positive/positive	Positive
10^{-2} dilution	Positive/positive	Positive
10^{-3} dilution	Positive/positive	Positive
10^{-4} dilution	Positive/positive	Positive
10^{-5} dilution	Positive/positive	Positive
10^{-6} dilution	Positive/negative[b]	Positive
Negative Control	Negative	Negative

[a] Replicate analyses were performed on serially diluted aliquots of A/chicken/Hong Kong/1000/97 (H5N1) with known viral titre [12] using NASBA H5N1 method or virus culture.

[b] The limit of detection in these NASBA assays is probably at 10^{-6} dilution.

Foot-and-Mouth Disease Virus (FMDV)

FMDV is a single-stranded RNA virus belonging to the family of *Picornaviridae*, including other members such as swine vesicular disease virus (SVDV), Coxsackie virus, and poliovirus [11]. FMDV can be classified into seven antigenically and genetically distinct serotypes, A, O, C, Asia-1, SAT-1, SAT-2, and SAT-3 [11]. Infection by this virus can be symptomatic or asymptomatic, and for the asymptomatic ones, both vaccinated and unvaccinated animals could be found [15,16]. It is thus important to develop an FMDV detection method to rapidly and sensitively identify genuine FMDV carriers from vaccinated animals [11], or the spread of FMDV amongst livestock will pose enormous economic disaster to the livestock industry.

We have been able to detect the major seven serotypes of FMDV using the NASBA method [11]. Recently, NASBA methods for specifically serotyping FMDV A, O, and Asia 1 have been developed in our laboratory. Table 2 summarizes the high specificity exhibited by NASBA in distinguishing A, O, or Asia 1 serotype, showing no evidence of cross hybridization of the specific primers/probes among these three closely related serotypes. Therefore, the FMDV NASBA method can be applied to generally screen for the presence of FMDV carrier animals (using the universal primers/probe for seven serotypes) and serotyping of A, O, or Asia 1 can be performed to study the prevalence and epidemiology of various FMDV serotypes in the infected regions. The use of FMDV A/O/Asia 1 serotyping NASBA may be particularly useful for tracing the outbreak origin as seen in the recent FMDV outbreak in China.

Table 2: Specificity of FMDV NASBA method for serotyping A, O, and Asia 1[a].

	FMDV A RNA	**FMDV O RNA**	**FMDV Asia RNA**
FMDV A NASBA	Positive	Negative	Negative
FMDV O NASBA	Negative	Positive	Negative
FMDV Asia 1 NASBA	Negative	Negative	Positive

[a] RNA samples extracted from FMDV A, O, and Asia 1 were subjected to NASBA amplification using primers/probes specific to A, O, or Asia 1.

Newcastle Disease Virus (NDV)

Newcastle disease is a contagious and widespread avian disease that affects most species of birds. It is caused by the avian paramyxoviridae serotype 1 (APMV1), also known as the Newcastle disease virus (NDV). Its negative-sense RNA genome contains six genes encoding an RNA-directed RNA polymerase, haemagglutinin-neuraminidase protein, fusion protein, matrix protein, phosphoprotein, and nucleoprotein from 5' to 3' direction [17]. NDV is classified according to its virulence. Lenogenic strains display low virulence, mesogenic isolates display intermediate virulence, while velogenic ones are the most virulent [17]. Velogenic strains are further classified as neurotropic or viscerotropic depending on the virus's clinical manifestation. Unvaccinated flocks infected with velogenic strains of NDV often have 100% mortality rate, and this is a threat to commercial poultry industries as a huge number of infected poultry might be decimated with an NDV outbreak. Turkeys, pigeons, and caged birds such as cockatiels, budgies, and amazons are also prone to infection. The Office International des Epizooties (OIE) has in fact designated the velogenic strains as List A reportable viral agents, requiring federal reporting, strict quarantine measures and trade embargoes [18].

We have once again applied the NASBA technology to detect NDV isolates rapidly and reliably of a broad genetic lineage [manuscript submitted]. NDV NASBA shares similar sensitivity to virus isolation and demonstrates high specificity only to NDV strains.

Classical Swine Fever Virus (CSFV) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

In the swine farming industry, two commonly seen swine diseases, classical swine fever and porcine reproductive and respiratory syndrome, can lead to economically significant loss, as seen in reduced pork production and decreased piglet population. These two diseases are highly contagious and have high mortality rates. They are among the most serious forms of porcine illnesses known, and have caused devastation to the economy in countries affected by these diseases. Rapid and sensitive diagnostics, such as NASBA, would allow early detection of these diseases to minimize spreading.

Detection of NASBA amplicons by Enzyme-linked Oligonucleotide Capture (EOC)

NASBA with ECL detection, being highly sensitive, specific, and accurate for nucleic acid amplification, allows rapid diagnosis of viral infections in animals and humans. However, the high cost of the equipment to perform electrochemiluminescent (ECL) detection may make this method less affordable to laboratories with a limited testing budget. In an attempt to enhance the applicability of NASBA technology, an enzyme-linked oligonucleotide capture (EOC) detection method making use of standard ELISA plate readers has been developed. The EOC detection procedure requires no extra equipment investment and would make the highly sensitive NASBA method an affordable technology in many laboratories. In the EOC detection method, NASBA amplicons are immobilized by hybridization to a biotinylated oligonucleotide capture probe bound to a streptavidin-coated surface. Detection is then mediated by a digoxigenin (DIG)-labelled detection probe and an anti-DIG antibody-alkaline phosphatase conjugate, which provides the colorimetric end-product.

The use of common 96-well microtitre plate spectrophotometers allows relatively inexpensive and high throughput analyses of NASBA amplicons, thus providing a viable option for highly sensitive and specific molecular diagnostics without the need to bear high machine costs as seen in real-time PCR equipment. Table 3 shows the equivalent magnitude of detection exhibited by NASBA/ECL and NASBA/EOC methods using primers/probes specific for all FMDV serotypes on FMDV field samples. NASBA/EOC will allow a more versatile application of this highly sensitive detection assay in locations where direct testing of field samples is crucial to monitor livestock crossing country borders, since only simple equipment, such as water bath and ELISA plate reader, are required.

Ease of Standardizing NASBA over PCR Technology

NASBA is a continuous, isothermal (usually at 41°C) and enzyme-based method [2]. NASBA experiments require equipment as simple as a water bath, and there is no need for expensive thermocyclers, which become obsolete when newer models are marketed. Without specialized equipment, the entire NASBA reaction can be performed with a water bath at one temperature, regardless of laboratories, regions, or even laboratory technicians. Because no specialized machine is required for the NASBA, developing countries and budget-restricted laboratories can afford to include NASBA-based molecular diagnosis among their routine disease surveillance detection systems. NASBA is able to deliver accurate, consistent, and reliable results with high sensitivity. NASBA may equal or even surpass real-time PCR in sensitivity; in our numerous experiments with the NASBA method, NASBA has consistently demonstrated a sensitivity level exceeding that of real-time PCR in detecting avian influenza A virus (Table 4). Moreover, because of the isothermal nature, NASBA methods can be standardized easily. On the contrary, although real-time PCR is currently the commonly used molecular method in the detection of various viruses, the amplification phase involves stepwise changes in temperatures. Different brands and types of thermocyclers (laser or white lamp based), primers, probes (various fluorescent labels and quenchers), *Taq* DNA polymerase ("hot-start" or "cold-start"), different reaction additives (passive reference), and different real-time detection chemistry are commonly seen in real-time PCR. Temperature transition rates to achieve denaturation, annealing, and extension also depend on the machines used. All these factors contribute to variations of real-time

Table 3: Applicability of NASBA/EOC method to field samples of FMDV compared to NASBA/ECL method.

Sample number[a]	ECL result	EOC result
400	Positive	Positive
409	Positive	Positive
415	Positive	Positive
418	Positive	Positive
420	Positive	Positive
423	Negative	Negative
426	Negative	Negative
428	Positive	Positive
445	Positive	Positive
461	Negative	Negative
486	Positive	Positive
488	Positive	Positive
Positive control	Positive	Positive
Negative control	Negative	Negative

[a] FMDV RNA field samples (RNA samples obtained from the Institute of Animal Health, UK as part of the collaboration to evaluate the FMDV NASBA method) were subjected to NASBA amplification using standard NASBA protocol. The NASBA amplicon samples were subsequently detected by the ECL or EOC methods. Signals higher than the cut-off values were considered "Positive" whereas those below the cut-off values were considered "Negative". Cut-off value is determined by the mean value of signals obtained from negative controls ± k*S.D. where k is a constant.

Table 4: Comparison of NASBA and real-time PCR in detecting avian influenza A virus (AIV) (all subtypes).

Sample[#]	AIV NASBA	Taqman real-time PCR
10^{-1}	Positive	Positive
10^{-2}	Positive	Positive
10^{-3}	Positive	Positive
10^{-4}	Positive	Positive
10^{-5}	Positive	Negative
10^{-6}	Negative	Negative

[#] An AIV sample of known viral titre was serially diluted 10-fold and extraction was carried out to isolate the AIV RNA. RNA samples were subjected to AIV NASBA [8] and AIV real-time PCR [using a TaqMan real-time PCR method].

PCR at inter-laboratory and intra-laboratory levels. In the event of a disease outbreak, there is the possibility of inconsistent or even contradictory results due to the intrinsic differences in real-time PCR settings, thus impeding testing speed and timely quarantine or treatment actions when tested samples constantly require confirmation by other laboratories. Sample confirmation and inter-laboratory comparisons have been particularly seen recently with frequent H5N1 outbreaks in Southeast Asian regions. For real-time PCR-based nucleic acid testing methods, variations in reaction temperatures, signal-generating chemistry and detection technologies, and use of different reagents, will render standardization of PCR conditions almost unfeasible.

One unique and valuable feature of NASBA is its ability to resist interference from inhibitory substances commonly present in biological samples, especially faeces. Our in-house evaluation has demonstrated that RNA extracted from positive viral material spiked into faeces-containing samples can be detected by NASBA, but not real-time PCR [unpublished data]. Our evaluation indicates that NASBA technology is highly applicable in clinical and veterinary settings when faecal materials are among one of the most accessible types of samples.

The control of the spreading of diseases in Asia has been a difficult task, as public healthcare systems and policies vary among Asian countries and the detection technology is far beyond the current needs [19]. The World Health Organization, Food and Agriculture Organization of the United Nations (FAO), and the Office International des Epizooties (OIE) have been pleading the need for the surveillance, early warning, detection, and reporting of diseases. Despite the wide recognition of real-time PCR technology, the high machine cost usually prohibits its use for nation-wide surveillance and front-line screening. Instead of being the method of first choice, real-time PCR becomes restricted to certain central or reference laboratories and only serves as an alternative method. Also, the intrinsic difficulties in standardizing real-time PCR methods will render this technology less likely to serve the purpose of unified surveillance programmes. NASBA-based technology can provide a good option in controlling and fighting against disease outbreak.

Conclusions

Molecular diagnostic tools for virus detection have been widely developed and used for human diseases. While these techniques are usually less readily available for animals, pioneering research and development in animal virus detection would contribute tremendously to our knowledge and monitoring of modern infectious diseases caused by animal viruses. NASBA, because of its sensitivity, specificity, accuracy, and rapid availability of results, would be a good method of choice for disease surveillance and control.

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