Avian influenza surveillance: on the usability of FTA® cards to solve biosafety and transport issues

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Abstract

Many zoonotic diseases have birds as their natural hosts. Waterfowl are the natural hosts of avian influenza viruses (AIVs) and most avian influenza infections of wild birds appear mild, with infected inviduals displaying no or few symptoms. It is clear that the epidemiology of avian influenza cannot be fully understood without taking the ecology of its hosts into account. However, large scale studies and surveillance are still hampered by issues about preservation, transport and storage of AIVs, including bio-safety regulations and maintaining samples. This complicates the possibilities of the many small projects across the world if they are not done within the framework of one of the few big projects. Here, evidence is provided of the potential for using Whatman FTA® cards as a new preservation method to solve the above mentioned issues. Its efficiency is comparable to that of a standard method in virology, and saves time and money. In both large scale AIV sampling and small scale independent projects this method might be the means by which the field of the AIV ecology will be lifted beyond the constraints of difficult and expensive sampling, storage and laboratory facilities.

Key words: Avian influenza virus, avian influenza sampling, FTA card, management, viral RNA.

Many zoonotic diseases have birds as their natural hosts (Shiina *et al.* 2004; Burt 2005). For example, waterfowl are the natural hosts of avian influenza viruses (AIVs; Webster *et al.* 1992) and there are a few reports of virulent epizootics in populations of wild birds and other wild animals (Li *et al.* 2004a). AIVs are known to infect other hosts such as poultry, domestic livestock and humans (Cavanagh 2005; Olsen *et al.* 2006) and may cause significant economic losses (Serratosa *et al.* 2007). Highly virulent variants of AIVs have been recorded in many non-native hosts. The role of ducks and other wildfowl in the origin and spread of low and high pathogenic strains of avian influenza is debated (Chen *et al.* 2006; Olsen *et al.* 2006).

Influenza viruses are a "genus" within the Orthomyxoviridae family of viruses. They have a segmented negative sense single-stranded RNA-genome. The influenza A virus can infect a wide variety of host species including birds, pigs, horses, seals, minks, whales and humans. The AIV genome consists of eight RNA segments. Viral subtypes are classified according to two of the encoded genes: the hemagglutinin (HA) gene and the neuraminidase (NA) gene (Webster *et al.* 1992). These genes code for surface proteins that play a key role in host recognition and initial infection. Sixteen HA and nine NA "subtypes" are recognised, amounting to 144 (= 9 × 16) possible subtype combinations (Fouchier *et al.* 2005). These are described as, for instance, H5N1 (subtype '5' of HA, and subtype '1' of NA). Until recently, the classification used to rely on immunoassays using standard procedures (Salk 1944; Aymard-Henry *et al.* 1973; Rimmelzwaan *et al.* 1998). Nowadays it is also possible to determine the nucleotide sequence of the virus genome using reverse transcriptase polymerase chain reaction (RT-PCR) with a set of universal primers for all genes and all subtypes (Hoffmann *et al.* 2001). The cDNA (complementary DNA) sequence obtained by this process can be identified with databases like GenBank (Benson *et al.* 2009).

Due to their tendency to feed in shallow waters and to congregate in large numbers, dabbling ducks are considered as one of the main vectors in avian influenza dispersal (Webster *et al.* 1992). Moreover, some ducks may show no clinical signs when infected with AIVs (Kida *et al.* 1980), though recent studies report subtle influences of infection on the migration and feeding behaviour of swans (van Gils *et al.* 2007) and Mallard (Latorre-Margalef *et al.* 2009). Therefore, as main vectors that survive most avian influenza infections, wild ducks and many other wildfowl would be a prime target for managers to monitor the potential spread of strains of highly pathogenic AIVs.

The importance of the ecological aspects of host biology, such as migration, and its consequences for the dispersal of AIV have led to the fusion of virology and ecology into many highly interesting projects (*cf.* Feare & Yasue 2006; Gilbert *et al.* 2006; Wallensten *et al.* 2006; Munster *et al.* 2007; Weber & Stilianakis 2007; Winker *et al.* 2007; Koehler *et al.* 2008). Still, the ecological research to aid the understanding of the host-pathogen system "AIV and wild birds" has not been utilised to its full potential. The field of research is hampered by the fact that working with AIV may

require biosafety precautions. Standard sampling and storage during avian influenza surveillance is bound to the availability of nearby deep freezers and transport of samples is subjected to strict regulations. Analysis can only take place in specialised laboratories. These facts make avian influenza research almost impossible if not conducted within the infrastructure of one of the few big collaborative projects. Hence, important contributions from the many smaller ecological projects may be missed (Bin Muzaffar *et al.* 2006; Cromie *et al.* 2006).

Here, a possible solution for this problem is examined: a method to sample, store and analyse potential AIV containing samples. This method does not require immediate deep freezing. The issue of preserving RNA viruses for later analysis (Munster *et al.* 2009) has been addressed several times already in similar fields (Li *et al.* 2004b; Moscoso *et al.* 2005; Ndunguru *et al.* 2005; Perozo *et al.* 2006; Purvis *et al.* 2006; Inoue *et al.* 2007; Nuchprayoon *et al.* 2007; Picard-Meyer *et al.* 2007; Muthukrishnan *et al.* 2008). The so-called FTA cards® (Whatman®) are used to preserve AIV RNA on a dry storage basis. The chemicals in the FTA (Flinders Technology Associates) card render pathogens inactive upon contact (Rogers & Burgoyne 1997) and transport can be arranged safely with only few further biosafety measures to be taken. FTA cards would therefore also be suitable for working with highly pathogenic strains of AIV. Proof of the potential of this principle is given in this short communication. The basis of this method is the isolation of the RNA followed by a one-step RT-PCR. The establishment of these protocols will be possible in any molecular laboratory, without the need for further biosafety measures. Samples can be mailed by normal postal services. Both sampling and analysis will be available to any molecular ecologist, thereby facilitating further scientific progress. This holds new possibilities for innovative studies in the fields of, for instance, molecular ecology, host-pathogen interactions or ecological immunology.

Methods

Wild Mallard were caught in a duck trap at Ottenby Bird Observatory, Sweden (56°12'N 16°24'E), and cloacal samples were taken for AIV detection. Detailed information about trapping, sampling techniques and methodology are described by Wallensten *et al.* (2007). Of these, one avian influenza isolate subtype H5N2 from 2004 was tested for the usability of Whatman FTA® cards. A volume of 125 µl of the allantoic fluid of an infected embryonated chicken egg (equalling 48 HA units as measured by standard titration) were applied to an FTA card (Burgoyne 1996). The dried sample on the FTA card was shipped at ambient temperatures for five days. Three 2 mm punches from this FTA card were incubated with RNA rapid extraction solution (Ambion) for 20 min at room temperature. RNA isolation was carried out with the MagMAX Viral RNA Isolation Kit (Ambion) according to the manufacturer's protocols. In short, RNA is captured by paramagnetic beads and washed in several steps to assure maximal purity, since biological samples from bird faeces would likely contain different PCR inhibitors.

The RNA was eluted into 50 µl elution buffer as provided by the kit. Three 2 mm punches from an untreated FTA card were carried along as negative extraction control; that is, to determine any contamination of the laboratory's tools or devices with AIV material. For RT-PCR detection we used the one-step Access RT-PCR System (Promega) – *i.e.* where reverse transcription into cDNA and PCR amplification is carried out in one tube – following a protocol adjusted from Fouchier *et al.* (2000). Stock solutions of 0.5 µl with 100 mM of the primers M52C and M253R (Fouchier *et al.* 2000) were used in reactions containing 10 µl AMV/*Tfl* 5× buffer, 1 µl dNTPs, 7 mM $MgSO_4$, 5U AMV reverse transcriptase and *Tfl* Polymerase each. A volume of 5 µl of isolated template RNA was added and the reaction volume adjusted to 50 µl with nuclease-free water. To exclude the possibility of AIV contamination and carry over in the RT-PCR kit chemicals, a negative control as additional sample with nuclease-free water as template was included. To test if the RT-PCR reaction works as expected, a positive control reaction is provided by the kit with its own primers.

RT-PCR commenced with an initial reverse transcription of 45 min at 45°C, followed by 2 min initial denaturation at 94°C and 40 cycles of: 94°C for 1 min, 45°C for 1 min, and 68°C for 2 min. An additional 7 min elongation at 68°C concluded the amplification. Amplicons (the amplified targeted fragments of the PCR reaction) were visualised on agarose gel stained with ethidium bromide and purified from it using the Millipore Montage DNA Gel Extraction Kit (Range 100–10,000 bp, Millipore Montage) as described in the kit manual. Cycle sequencing of the amplified Matrix gene fragment was carried out with ABI Big Dye 3.1 chemistry in 10 µl reactions containing 10–20 ng gel-purified template cDNA, 1.75 µl 5× dilution buffer, 0.5 µl Big Dye V3.1 premix, 1 µl forward primer (M52C, 10 mM), and ddH_2O. Cycling conditions were 1 min initial denaturation, followed by 25 cycles of: 10 s at 96°C, 5 s at 45°C and 4 min at 60°C. Samples were analysed on an ABI 3730 capillary sequencer. Wherever possible, preparation of reactions and handling of reagents was carried out under a fume hood and using RNAase-free barrier pipette tips. Care was taken to ensure that pre-PCR steps were carried out in a different room to the one in which the PCR and gel steps (post-PCR) were carried out, to avoid aerosol contamination of the laboratory.

In Wallensten *et al.* (2007), 125 µl of the same isolate was used for direct RNA extraction and for determining avian influenza subtypes, using standard protocols. Extraction was carried out using the MagAttract Virus Minikit (Qiagen) on an M48 extraction robot (Qiagen). Virus detection was performed by RRT-PCR (real-time reverse transcription polymerase chain reaction) for the presence of the matrix gene (Spackman *et al.* 2002), and the test proved to be positive (Wallensten *et al.* 2007).

Results

RT-PCR was successful for the positive sample extraction as well as for the positive RT-PCR kit control (Fig. 1), and amplicons

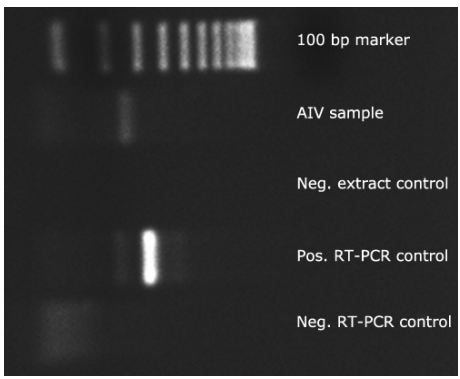

Figure 1. Agarose gel with RT-PCR products of the avian influenza virus (AIV) sample along with negative extraction control, and a positive and negative RT-PCR control (see main text). The AIV matrix gene (244 bp) amplicon appears between the 200 and 300 bp markers as expected.

were of the expected size (244 bp). Both negative extraction and negative RT-PCR control displayed no band on agarose gel, indicating that there were no contamination issues during the working procedures. A slight shadow below 100 bp in size indicates the possibility that primer dimer – an artefact of PCR wherein primers act as their own templates to make a small PCR product and appear faintly on an electrophoresis gel – might have been formed. The occurrence of multiple bands in the positive reaction control is described by the user manual of the RT-PRC kit and is a normal sign of good amplification. The sequencing of the amplicon yielded a 95 bp good quality cDNA sequence (TCT TTA GCC ATT CCA TGA GAG CCT CGA GAT CTG TGT TTT TCC CTG CAA AGA CAT CTT CAA GTC TCT GCG CGA TCT CGG CTT TGA GGG GGC CTG AC). The chromatogram of the sequencer covered the whole fragment, however. A BLAST search against the National Centre for Biotechnology Information (NCBI) nucleotide database (blastn, Zhang *et al.* 2000; http://blast.ncbi.nlm.nih.gov/Blast.cgi) identified this fragment as being an AIV matrix gene fragment with 100% sequence identity and with 100% sequence coverage on comparison with several AIV isolates.

Discussion

The study provides evidence that a new technology of viral RNA detection can be used to process samples containing AIV. The detection was successful without maintaining a cold chain for preserving the samples or involving unduly complicated biosafety measures regulations, and was comparable to a standard and fully validated RRT-PCR (Spackman *et al.* 2002). In our RT-PCR experiment the slight shadow below 100 bp in size could indicate primer dimer. This might be due to the one-step nature of the RT-PCR protocol, where polymerase and primer are present in the reaction mix at lower temperatures already for some time during reverse transcription. This possible issue is easily solved by purification from agarose gel by excising only the relevant amplicon for subsequent sequencing. Sequencing of the 244 bp amplicon yielded 95 bp of high quality cDNA sequence. The chromatogram of the remaining fragment was too weak for analysis in this trial. Since only one test run has been conducted so far there is potential to develop this work further. The amplified fragment is also larger than one previously

reported where alternative preservation methods were used (< 200 bp in ethanol; Wang *et al.* 2008).

Since it has recently been shown that RNA fragments of > 700 bp in size can be amplified successfully in other systems (Muthukrishnan *et al.* 2008) we assume that storage of avian influenza samples on FTA cards has the potential to be superior to the ethanol fixation method if primers for larger fragments are used. Some studies tested the sensitivity (*e.g.* RNA quantity) required for detection. They reported the detection of a positive signal even after many-fold dilutions (Perozo *et al.* 2006) or for only 0.1 fg of RNA template (Rogers & Burgoyne 2000), and after storage at ambient temperatures for > 2 weeks. Others claim that RNA on FTA cards is stable even after six months of storage under ambient conditions (Rogers & Burgoyne 2000). Whether these methods are applicable under fieldwork conditions remains to be tested. In natural samples like faeces or oral/cloacal swabs there is also the chance that AIV is present in lower concentrations than tested here. This poses the risk of not detecting an avian influenza infection when there actually is one (*i.e.* a false negative). In particular the effects of storage time and temperature, as well as sensitivity at lower concentrations and contamination through faecal material, would need attention in such a systematic test. Recent studies have however shown that PCR is more sensitive than traditional methods, even when AIVs are only present as unviable particles (Runstadler *et al.* 2007). This also makes detection possible when infection is almost cleared by immune response. To this point, cloacal samples were not tested directly in the present study but it seems that the use of FTA cards in large scale AIV sampling may be the means by which the field of AIV ecology can be lifted beyond the constraints of difficult sampling, storage and laboratory facilities.

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