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RNAlater® is a viable storage option for avian influenza sampling in logistically challenging conditions

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ABSTRACT

Surveillance of wild birds is critical in monitoring for highly pathogenic avian influenza A viruses (AIVs). However, a successful surveillance regime requires proper treatment of samples in the field – rapid placement of samples in −80 °C and subsequent maintenance of cold-chain. Given the logistical difficulties of this, many avian taxa and/or geographic locations are not sampled, or, when sampled may result in false negatives due to poor sample treatment in the field. Here, we assessed the utility of RNAlater® as a stabilization agent for AIV sampling. We found no difference in real time PCR performance between virus transport media at optimal conditions and RNAlater® at −80 °C, −20 °C, 4 °C or room temperature up to two weeks, at either low or high virus load. Not only was RNAlater® useful in comparison of spiked samples or those from duck experiments, it was employed successfully in a field study of backyard birds in China. We detected AIV in cloacal and oropharyngeal samples from chickens and a sample with a low Cq was successfully subtyped as H9, although sample storage conditions were suboptimal. Thus, despite limitations in downstream characterization such virus isolation and typing, RNAlater® is a viable option for AIV sampling under logistically challenging circumstances.

1. Introduction

Avian influenza A virus (AIV) surveillance has proven critical in monitoring for the introduction and spread of highly pathogenic strains of AIV that cause morbidity and mortality in poultry, and are a severe risk for public health (Bevins et al., 2014; Keawcharoen et al., 2008; Liu et al., 2005; Verhagen et al., 2015). In terms of wild birds, global surveillance strategies heavily target waterfowl (*Anseriiformes*) as these are known reservoirs of influenza A viruses, and given high prevalence, allow for the largest number of viruses to be recovered and subsequently analyzed (Alexander, 2000; Bevins et al., 2014; Brown et al., 2014; Olsen et al., 2006; Olson et al., 2014; Runstadler et al., 2013). These surveillance schemes often utilize existing frameworks to allow access to wild birds (e.g. Bevins et al., 2014), and often rely on the usage of vapour shippers or liquid nitrogen dewers to attempt to maintain cold-chain, which is imperative in preserving the viruses for traditional analysis, particularly virus isolation and typing (Latorre-Margalef et al., 2016; Munster et al., 2009). Indeed, laboratories with the highest isolation efficiencies often are able to freeze samples at −80 °C within a few hours of collection, and minimize freeze-thaw cycles (e.g. Latorre-Margalef et al., 2016; Munster et al., 2009). While these successful strategies allow for the effective targeting of known hosts, in order to better understand the host range and ecology of AIV, birds in more remote locations need to be accessed (Hoye et al., 2010; Lang et al., 2016; Runstadler et al., 2013). In these remote locations, equipment such as shippers or dewers are critical to maintain cold-chain, but are logistically challenging to transport, which may severely hamper the ability to detect AIV under those circumstances. For example, many seabirds, some of which may be a component of AIV ecology, breed on very remote islands, to which it is almost impossible to bring any type of cold storage, resulting in an added difficulty in doing appropriate surveillance of this avian group (Lang et al., 2016; Wille et al., 2014). Indeed, there has been serological evidence for AIV in Antarctic avifauna for over 30 years, but it is only recently that

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viruses have been detected by real-time reverse transcriptase PCR (rRT-PCR) (Hurt et al., 2014; Lang et al., 2016; Morgan and Westbury, 1981; Wallensten et al., 2006). One hypothesis for the lack of success in detecting viruses in Antarctic penguins is that maintaining cold-chain has not been done efficiently (Lang et al., 2016).

Currently viral transport media (VTM) is the gold standard for AIV sampling, with various formulations being used including those based on Hanks Balanced Solution (e.g. Latorre-Margalef et al., 2014) or brain-heart infusion broth (BHI) (e.g. Hurt et al., 2014). VTM is ideal when used in an environment where cold-chain can be maintained as it allows for culture of viruses, but may not be ideal in suboptimal temperature conditions. Alternative methods have been utilized, including ethanol (Runstadler et al., 2007; Wang et al., 2008), other alcohol based preservatives (Evers et al., 2007), and even FTA cards (Kraus et al., 2011). RNAlater®, which is a stabilization agent, allows for the protection of RNA in conditions where immediate access to −80 °C freezers, vapour shippers or liquid nitrogen dewers is not possible. It has not been frequently used in AIV surveillance, however, RNAlater® has been used in other studies of viruses (e.g. Blacksell et al., 2004; Forster et al., 2008; Korves et al., 2010). In this study we aimed to assess the utility of RNAlater® as a media for storage of AIV in suboptimal sampling conditions using current laboratory workflows. Specifically, we assessed the ability to detect AIV at both high and low titres in a range of storage temperatures. Finally, we also screened field samples collected in backyard birds in China, with positive results, demonstrating the usage in both laboratory and field settings.

2. Methods

2.1. Ethics statement

Sample collection from birds in the animal experiment was performed in accordance with regulations provided by the Swedish Board of Agriculture and were approved by the Ethical Committee on Animal Experiments in Uppsala (permit number C20/14). Samples in the field study were collected in accordance with permission from the management of Wuzhizhou island, China.

2.2. General sampling and screening strategies

Samples were collected with a sterile tipped applicator and placed in 1 mL of media, either RNAlater® (Ambion, ThermoFisher) or VTM (Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/mL penicillin, 200 mg/mL streptomycin, 100 U/mL polymyxin B sulfate, 250 mg/mL gentamicin, and 50 U/mL nystatin; Sigma). Samples from the duck experiment (see section 2.4) and China (see Section 2.5) were extracted using the QiaAMP Viral RNA Mini Kit (Qiagen, Qiagen, Hilden, Germany), selected following an assessment of extraction kit compatibility with RNAlater® (see Section 2.3). Following extraction, samples were assayed by real time reverse transcriptase PCR (rRT-PCR) for AIV using previously published methods (Spackman et al., 2002). Briefly, AIV was screened using a rRT-PCR assay targeting a short region of the matrix gene with the One Step RT-PCR Kit (Qiagen) and cycle threshold (Cq) cutoff of 40 was used.

2.3. Evaluation and validation of extraction methods

The extraction kit used for the duck experiment and the field study was selected based on 3 main criteria: (1) equal performance between RNAlater® and VTM in optimal conditions, (2) the performance of VTM had to be similar to the current method used in our research program (Maxwell 16 Instrument and Viral Total Nucleic Acid Purification Kit; Promega, Madison, USA), (3) and an extraction method used previously in the literature with RNAlater® (here, QiaAMP Viral RNA Kit). We spiked both RNAlater® and VTM with virus isolate A/Mallard/Sweden/101663/2010(H4N6) at different concentrations, and immediately stored the spiked samples at −80 °C for 2 days prior to extraction, to allow for a freeze–thaw cycle.

2.4. Samples from IAV experimental infection study in a Mallard model

To assess the efficacy of RNAlater® across different treatments, at both high and low viral load, we collected samples from an AIV experimental infection study in ducks (hereafter "duck experiment") used to assess antiviral resistance (e.g. Gillman et al., 2015). The first generation of ducks were artificially infected with A/Mallard/Sweden/51833-H274Y/2006 (H1N1) (Järhult et al., 2011), but all ducks used in this study were naturally infected by their conspecifics. Samples were collected from ducks at 2dpi (representing the highest viral load) and 5dpi (low viral load), from three different generations of ducks (n = 5 for each treatment). Seven swab samples were collected from each duck fecal deposit and placed in different storage conditions. The storage conditions were −80 °C, 1 week at −20 °C, 4 °C or room temperature before being placed in −80 °C, and 2 weeks at −20 °C, 4 °C or room temperature before being placed in −80 °C. An additional swab was placed in VTM and stored at −80 °C within 2 h of collection as a control, representing optimal storage conditions and the current standard for duck experiment sample collection.

2.5. Field study

To assess RNAlater® in field samples, backyard food production birds were sampled from Wuzhizhou Island (18°18′44″N 109°45′58″E) and Sanya city (18°15′12″N, 109°30′13″E), China. Paired samples were collected from each individual – an oropharyngeal and a cloacal sample, and placed in 1 mL of RNAlater®. Following collection, all samples were placed on ice packs for up to 12 h, until placed in temporary storage prior to shipment. Samples were in temporary storage for 5–7 days and conditions varied but included either refrigeration (4–12 °C) and/or use of a conventional freezer (approximately −20 °C). Samples were shipped on ice packs and subsequently stored in −80 °C until analysis.

In order to further assess the utility of RNAlater® as a preservative, we attempted to subtype two samples with the lowest Cq values. Briefly, RNA was re-extracted using the MagNA Pure 96 Instrument and Viral Nucleic Acid Small Volume kit (Roche, Mannheim, Germany) and cDNA was subsequently synthesized using Superscript IV Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA) using a combination of random hexamers (Life Technologies) and Uni12 (Hoffmann et al., 2001). The PCR was done using Amplitaq DNA polymerase (Life Technologies) using previously published subtype specific primers targeting short fragments of the HA (Wang et al., 2008) and NA (Qiu et al., 2009). PCR products were sequenced by Macrogen Inc. (Amsterdam, The Netherlands) for confirmation.

2.6. Statistics

Variation in Cq values across treatments were tested using ANOVA, following a test to ensure normal distribution of residuals. Prevalence comparisons between oropharyngeal and cloacal samples in the field study were compared using Fisher Exact Tests. P values of < 0.05 were taken to indicate a significant difference in the compared rates. Statistics were done using GraphPad Prism v.6.03 (GraphPad Software Inc, San Diego, CA).

3. Results

3.1. Compatibility of RNA extraction methods with RNAlater®

Spiked samples were extracted with a magnetic bead based-system which is routinely used in-house to detect AIV from VTM (Maxwell 16 Instrument and Viral Total Nucleic Acid Purification Kit; Promega,

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