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Stability of influenza virus as evaluated by integrity of its RNA

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Summary. – Various methods of handling samples of avian influenza prior to detecting influenza viruses can significantly influence both, the detection of the virus and the quantification of viral nucleic acids. The quantity of influenza viral RNA remaining in different collecting buffers and kept at temperatures of -20°C, +4°C or +22°C for various lengths of time, was determined. The quantity of viral RNA remained the same for 120 days at -20°C, but decreased when the samples were stored at either +4°C or +22°C. The quantity of RNA was influenced by the composition of the collecting buffer. The influenza virus sample that is to be used for RNA quantification can be stored at +4°C and freeze and thaw cycles should be avoided during transport. Our results clearly indicate that the quality and quantity of influenza virus nucleic acid depends on the chemical composition of used buffer and also that the samples can be protected from degradation even if they are not stored at ultra-low temperatures. However, repeated thaw and freeze cycles will damage viral RNA even if kept in stabilizing buffers.

Keywords: influenza virus; degradation; RNA; buffer

Influenza viruses are enveloped, negative-stranded RNA viruses with 8 segments of nucleic acid, and each segment encodes one or two proteins (Webster *et al*., 1992). The main surface glycoproteins, hemagglutinin and neuraminidase determine antigen variability and are used for influenza type A subtyping (Marschall *et al*., 1995). Hemagglutinin and neuraminidase primers are used for PCR detection of circulating influenza A virus subtypes but are not suitable for animal types of influenza A viruses. Therefore, it is more acceptable to use more conserved genes, like gene for matrix protein M1 located on segment 7 (Fouchier *et al*., 2000) or non-structural protein NS located on segment 4 (Ellis and Zambon, 2002). The most sensitive method for influenza nucleic acid detection and determination is reverse transcription and subsequent PCR (Boivin *et al*., 2004). The examination of influenza samples is performed immediately after occurrence of death or clinical symptoms and is done to prevent the spread of the disease (Layne *et al*., 2001). It is always necessary to transport samples, often over great distances, from the site of infection to a relevant diagnostic facility. Such samples require immediate processing or storage at an ultra-low temperature in order to ensure the integrity of the viral RNA (Frisbie *et al*., 2004). When samples are collected at remote location it is often impossible to store them at an ultra-low temperature. Often only a refrigerator is available when samples are collected in the form of cloacal swabs, for example when ringing birds outdoors (Killian, 2008).

Molecular biology studies are dependent on obtaining high-quality RNA. For the detection of virus in collected samples it is necessary to keep the samples in conditions that preserve the virus (Evers *et al*., 2007). Various protocols have been developed to overcome the difficulties experienced with test reproducibility, infectivity of samples, and the gradual degradation of biological material (Uhlenhaut and Kracht, 2005). Various ways of conserving viral nucleic acid have been tested. For example, the AVL buffer (lysis buffer for viral nucleic acid purification, Qiagen) stabilizes RNA and inactivates the infectiousness of the virus (Blow *et al*., 2008). There are other commercial products used for

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Abbreviation: RT-PCR = reverse transcription PCR

stabilizing RNA in tissues, such as RNAlater (Forster *et al.*, 2008). Nevertheless, this compound is not able to preserve native RNA in a sample for a long period of time. Despite this, RNAlater can preserve the infectiousness of the virus (Uhlenhaut and Kracht, 2005). In many cases there is a need to ensure non-infectiousness of a sample prior to its transportation, and therefore it is necessary to look for new ways to stabilize a sample. Therefore, in order to inactivate influenza viruses, formaldehyde (or beta-propiolacton) is incorporated in the buffer. The Rnases in samples of tissues preserved in alcohol (95% ethanol and 5% acetic acid) are usually inactivated. This RNA preparation can be kept at -20°C or +4°C for some time. Repeated tests with mRNA show that when kept at +20°C the RNA is fully degraded (Esser *et al.*, 1995). Another possibility is to use commercial kits, to stabilize DNA and RNA that are based on FTA® (Picard-Meyer *et al.*, 2007). Nevertheless, the classical way of analyzing the liquid from a cloacal swab is to spread the sample on a FTA® card, from which a sub-sample can be obtained by cutting the card. Usually a low titer of virus cannot be detected using the PCR test (Walsh *et al.*, 2008). Viral RNA (and DNA) can be detected after a long time in samples of tissues (e.g. spots of blood) preserved in, for example, paraffin wax or formalin. However, these are samples from tissues with much higher viral titer than in cloacal swabs (Solmone *et al.*, 2002) or viruses less susceptible to degradation (Campos *et al.*, 2008). The objective of this work was to develop a buffer that will prevent RNA from deteriorating even at high temperatures so that it is possible to send samples by mail or convey unfrozen samples to a laboratory for analysis.

The influenza virus A/Puerto Rico 8/34 H1N1 (10–8 ID_{50}/ml), from the National Accredited Testing Laboratory for Influenza at National Institute of Public Health in Prague was used as the standard virus. This virus was diluted by 1:5,000 in 12 different test buffers (Table 1). Samples were stored at different temperatures (-20°C, +4°C, +22°C) and for different periods of time (Table 1). Aliquots of the samples were made in order to avoid repeated freeze and thaw cycles. Viral samples in the buffers 3-cB and TE, with the same volume of 1 ml, were used to determine the effect of freeze and thaw cycles. One cycle consisted of freezing the sample at -20°C for 1 hr and then thawing it for 1 hour at a room temperature of +22°C. The viral RNA was isolated using a QIAamp DNA mini kit (Qiagen), according to the manufacturer's instructions, and stored at -70°C. The cDNA was prepared according to manufacturer´s instructions using MMLV reverse transcriptase (Fermentas) primed with random hexamers. The cDNA samples were used as a template for RT-PCR with primers specific to the influenza matrix gene (M52C, M253R) with an amplicon size of 244 bp (Fouchier *et al.*, 2000).

Our results show that, RNA of influenza virus A/H1N1/Puerto Rico 8/34 (10–8 ID_{50}/ml) in TE buffer was deteriorated within 2–5 days (data not shown). Comparing the storage of the samples at different temperatures, we have proved that the viral RNA was most stable when kept at -20°C than at either +4°C or +22°C. At +22°C virus kept in the TE buffer remained stable for four days.

By comparing the stability of RNA in 12 different storage buffers, the most effective in preserving RNA for the longest time were buffers 3-cB and 5-cB (Table 1).

The stability of RNA was also higher when the pH was reduced to 4 by ammonium sulphate and ammonium hydrogensulphate, as in buffer 8-cB.

In order to obtain the purest and highest quality RNA the activity of RNase released from the cells during lysis needs to be reduced. Usually RNase inhibitors, such as the inhibitor of placental ribonuclease or vanadyl-ribonucleoside complexes were added to samples containing low concentrations of RNase. A much stronger inhibitor was needed when isolating RNA from tissues that contain higher quantities of RNase.

Table 1. Duration of stability of viral RNA at different temperatures (days) and in different buffers

Buffer		Duration of stability of viral RNA at different temperatures (days)		
		+22°C	+4°C	-20°C
TE	10 mmol/l Tris pH7, 1 mmol/l EDTA	4[a]	8	120[b]
3-cB	3 mol/l $(NH_4)_2SO_4$, 1 mol/l urea, 1% proteinase-K, 1% formaldehyde	8	32	120[b]
5-cB	3 mol/l $(NH_4)_2SO_4$, 1% triton-X100, 1% formaldehyde	8	32	120[b]
6-cB	2 mol/l $(NH_4)_2SO_4$, 2 mol/l betaine, 1% triton-X100, 1% formaldehyde	4	16	96
7-cB	2 mol/l $(NH_4)_2SO_4$, 1% triton-X100, 1% proteinase-K, 1% formaldehyde	6	16	120[b]
8-cB	3 mol/l $(NH_4)_2SO_4$, 2 mol/l $(NH_4)_2SO_4$, 1 mol/l urea, 1% formaldehyde	6	32	96
10-cB	98% ethanol, 1% formaldehyde	8	12	96
12-cB	70% ethanol, 1% triton-X100, 25 mmol/l potassium citrate, 1% formaldehyde	4	12	96

[a]The experiment was duplicated and results were averaged. [b]The experiment ended on 120th day.

Comparison of the results obtained when samples were repeatedly frozen and thawed indicates that repeated freeze and thaw cycles during the transport of samples affects the stability of RNA. The results (data not shown) indicate that, despite the samples being stored in a stabilization buffer, the quality of influenza RNA is significantly reduced by five repeated freeze and thaw cycles.

The objective of this work was to develop a new method and set of reagents for the conservation of viral RNA in cloacal swabs kept at temperatures above freezing for extended periods of time (days to weeks), which would make it easier to transport samples to the laboratory for RNA isolation. The successful detection of influenza viruses greatly depends on how samples are collected, their quality just before processing and the way in which the samples are stored and handled during the transport. The time between collecting a sample and processing it, the number of times the sample is frozen and thawed, and the original titer of the virus in the sample is crucial (Killian, 2008). The way in which the viral sample is subsequently treated is also important. We assume that current media for RNA protection work on the principle of salting out by the effect of high concentrations or high levels of specific salts. This will affect proteins from tissue cells that protect RNA. The efficiency of protecting RNA by precipitating proteins, by high concentration of salt and a high pH, is highly complex because some combinations of salts of certain ionic strength and pH salt out more efficiently than others. The typical RNA preservation medium has pH between 4 and 8. At a pH of 5.5 the activity of RNase is as low as 10%, but still this is enough to degrade the samples quickly. However, it is important to maintain a low pH because at high pH levels RNase activity returns. The effect of ammonium sulphate, present in the buffer, is to lower the pH to 5.5 which will reduce the activity of RNase (Allewell and Sama, 1974; Lin and Brandts, 1987). Repeated tests with ammonium sulphate showed that it is effective at high concentrations, however these concentrations salted out other components from the solution (e.g. originally 1% SDS). The stability of RNA was measured indirectly by isolating the conserved RNA followed by reverse transcription and PCR, which resulted in a 244 bp product. The original RNA, in the storage buffer, can remain intact or it may be partially degraded. However, the PCR test will give a positive reaction even if the RNA is partially degraded.

The results presented, clearly indicate that it is important to determine the optimal composition of the collection buffer. Routine screening and recording the occurrence of various agents is a very important part of human and veterinary disease prevention programs, particularly those aimed at combating potential national pandemics.

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