Institut Pasteur de Lille	PROCEDURE	N°	P002RIVERS
	Concentration of avian *Influenza* A viruses from surface water	Version	2
		Date	16/03/2009

This paper describes procedures for the concentration of avian Influenza viruses from surface water that can be used in case of suspicion of H5N1 contamination. The following testing strategy is suggested:

- Virus adsorption/elution by filtration
- Virus concentration by PEG precipitation.

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1. AIM

This procedure describes methods for the concentration of avian *Influenza* A viruses (AIVs) from surface water by an adsorption/elution method based on a filtration step, followed by a concentration step.

The aim was to select the most efficient method useable for large volumes of clean or dirty water, but rapidity and simplicity were also evaluated. Primary concentration allows concentration of large volumes of water, and secondary concentration was used to concentrate infectious particles in volumes as small as possible, in combination with a primary concentration.

The primary concentration method is based on an adsorption step on glass wool, followed by an elution step with a beef extract solution at alkaline pH, as described in the French national standard method (XP T90-451) and used mainly for enteroviruses, in combination with a second concentration step with poly-ethylene glycol. Parameters of filtration and elution were defined according to conditions used for detection of enteroviruses in waters for adsorption/elution on glass wool. Parameters of PEG precipitation were defined based on conditions described by Dubois *et al.* (2007).

Quantitative detection methods by RT-PCR have been optimised to detect H5N1 in concentrated water samples (described in Procedure P001RIVERS).

2. MATERIAL

2.1. EQUIPMENT

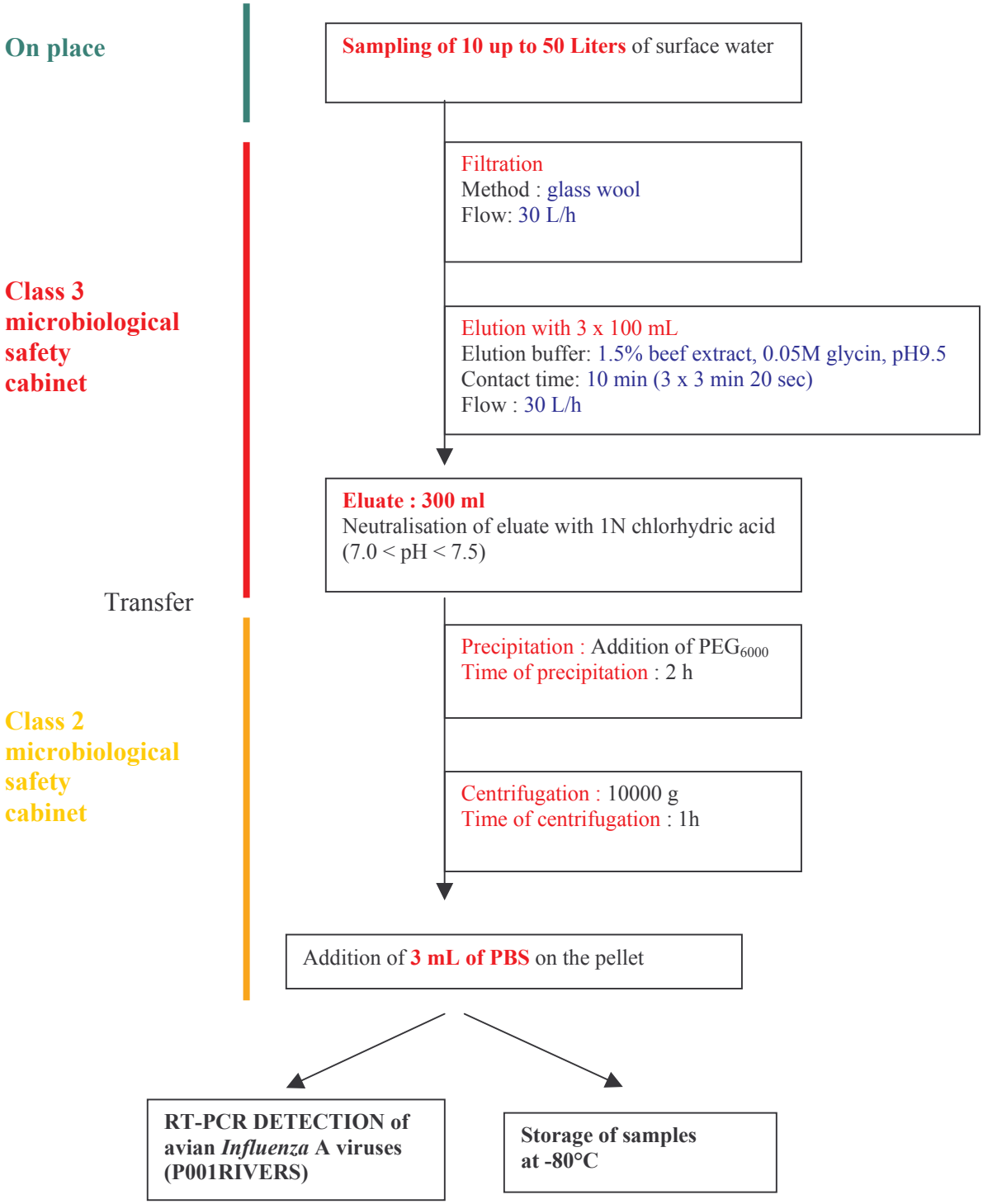
Equipment	Supplier	Reference
Inox cartridges	Sartorius	16249
Sodocalcium Glass wool	Saint-Gobain	Rantigny 725
Peristaltic pump	Masterflex-Bioblock	B39618
Pump head	Masterflex-Bioblock	B89476
Tube	Masterflex-Bioblock	Silicone platinium 96410

2.2. REAGENTS

Reagent	Supplier	Reference
Beef extract	DIFCO	1653R
Glycin	Sigma	G7126
Poly-Ethylen-Glycol (PEG)	VWR Merck Eurolab	26603293
HCl	Merck	/
NaOH	Sigma	/
DPBS	GIBCO	14190

Dubois, E., et al. (2007). "Detection and quantification by real-time RT-PCR of hepatitis A virus from inoculated tap-waters, salad vegetables, and soft fruits: characterization of the method performances". Int. J. Food. Microbiol. 117:141-9).
National standard method XP T90-451 "Recherche des entérovirus – Méthode par concentration sur laine de verre et détection par culture cellulaire". AFNOR 1996.

3. SYNOPSIS


On place
Sampling of 10 up to 50 Liters of surface water
Filtration
Method : glass wool
Flow: 30 L/h
Class 3 microbiological safety cabinet
Elution with 3 x 100 mL
Elution buffer: 1.5% beef extract, 0.05M glycin, pH9.5
Contact time: 10 min (3 x 3 min 20 sec)
Flow : 30 L/h
Eluate : 300 ml
Neutralisation of eluate with 1N chlorhydric acid
(7.0 < pH < 7.5)
Transfer
Precipitation : Addition of PEG6000
Time of precipitation : 2 h
Class 2 microbiological safety cabinet
Centrifugation : 10000 g
Time of centrifugation : 1h
Addition of 3 mL of PBS on the pellet
RT-PCR DETECTION of avian Influenza A viruses (P001RIVERS)
Storage of samples at -80°C

4. PROCEDURE

4.1. PREPARATION OF REAGENTS AND MATERIAL

4.1.1. Elution buffer

Weigh 15 g of Beef extract in an Erlenmeyer flask.
Add 3.76 g of glycin, 900 mL of ultrapure water.
Put under magnetic agitation.
Adjust pH at 9.5 with a solution of NaOH 5M.
Add ultrapure water QS 1 L.
Autoclave 20 minutes at 120°C.

4.1.2. NaCl-PEG$_{6000}$ solution

Weigh 75 g of NaCl in an Erlenmeyer flask.
Add progressively 500 g of PEG $_{6000}$.
Add warm ultrapure water QS 1 L.
Put under magnetic agitation until total dissolution.
Autoclave 20 min at 120°C.

4.1.3. Glass wool filter

3 x 16.5 g of wet glass wool are compacted into a stainless steel cartridge. A chemical sterilisation is realised with successive addition of 200 mL HCl 1N, 500mL H_2O, 200 mL NaOH 1N and finally sufficient water to obtain a pH between 7 and 7.5.

4.2. PRIMARY CONCENTRATION BY FILTRATION

4.2.1. Sampling

A volume of 10 up to 50 litres of surface water is taken (by sampling point) and transferred into 10 L jerrycans.

4.2.2. Filtration

This technique refers to the norm AFNOR XPT 90-451, March 1996.
Surface water sample is filtered on a glass wool cartridge with a peristaltic pump, with an approximate flow of 30 litres / hour (pictures in Annex).

NB: Be careful not to dry the cartridge.

4.2.3. Elution

An elution is conducted in 3 steps (3 x 100 mL), at a flow of 30 L/h with a buffer composed of 0.05 M of Glycin / 1.5% of Beef extract at pH 9.5. The contact time between the buffer and the cartridge is 10 minutes (3 contacts of about 3 minutes and 20 seconds).

First pump 100 mL buffer into the cartridge, stop the pump and wait for 3 min and 20 sec. Do it a second time with another 100 mL buffer. Then do it a third time, but wait for the air bubble to reach the upper part of the cartridge to stop the pump. Then wait for 3 min and 20 sec to start the pump again (and collect the eluate in a flask). Total eluate volume is then approximately 400 mL (corresponding to 300 mL buffer and about 100 mL residual water).

Eluate is neutralised with an HCl 1N solution to obtain a pH between 7 and 7.5. Eluate is divided in 2 parts in centrifugation bottles.

4.2.4. Decontamination

The stainless steel cartridge is disassembled. The cartridge and the glass wool are separately decontaminated with an adapted and validated disinfectant. Glass wool is eliminated and cartridge is rinsed before a new filter preparation.

4.3. SECONDARY CONCENTRATION BY PRECIPITATION

4.3.1. Precipitation with PEG_{6000} solution

The PEG_{6000} solution is added to the eluate in the 2 centrifugation bottles: a volume corresponding to a quarter of the neutralised eluate volume (approximately 50 mL by bottle) is added gently with a magnetic shaking. The mixture is incubated at 4°C for 2 hours.

4.3.2. Centrifugation

The mixture is centrifugated at 10000 g for 1h at 4°C. The supernatant is removed and the pellet is recovered and dissolved with 3 mL of PBS buffer.
Concentrated water sample is stored at -80°C.

5. QUANTITATIVE DETECTION OF M AND H5 GENES

Concentrated water sample is extracted and tested by RT-PCR following the procedure for the detection of M and H5 genes by real-time RT-PCR (Procedure P001RIVERS).

6. VIRAL DETECTION THRESHOLD

The detection threshold corresponds to the limit log-number of infectious particles under which no RT-PCR detection was obtained. The real-time RT-PCR specific of M and H5 genes can detect down to **10^3 $TCID_{50}$ / filtered volume for H5, and 62 $TCID_{50}$ / filtered volume for M.**

7. ANNEX

Experimental setting for filtration of surface water on glass wool is illustrated in Figures A and B.

(A) Filtration step :

(B) Elution step :