Project no.: **SSPE-CT-2006-44405**

Project acronym: RIVERS

"Resistance of Influenza Viruses in Environmental Reservoirs and Systems"

Instrument: Specific Targeted Research Project

Thematic Priority 8.1B

Periodic Activity Report

1st reporting period

Period covered: From **01.02.2007** to **31.07.2008 (M1-M18)**

Date of preparation: 5th November 2008

Start date of project: 01 February 2007 **Duration**: 36 months

Project coordinator: **Jean-Claude MANUGUERRA**
Project coordinator organisation: **Institut Pasteur, Paris (France)**

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PUBLISHABLE EXECUTIVE SUMMARY

The surge of the global avian Influenza epizootic caused by the genotype Z high pathogenic avian Influenza virus (HPAIV) has posed numerous questions, in particular to risk managers and policy makers. Scientific knowledge is thin on many aspects of the ecology and environmental properties of HPAIVs, in particular H5N1. Virus survival, a key element in control strategies, is an illustration of this paucity of knowledge. Data from the literature on AIV survival are rather limited, often very old and sometimes not confirmed from one study to another or even contradictory. The results obtained with various sub-types of Influenza A viruses cannot be extrapolated to the current A(H5N1) viruses before a careful consideration. Further, few information is provided regarding the survival of IVs in the air and surfaces. Meantime, no standardised protocols exist to detect AIVs in waters, in the air or in/on solid matrices. Ideally, the virus detection technique to be used should be sensitive, quantitative, rapid and applicable in routine before or after a standardised sampling method, including or not concentration. Under this project, 9 institutions directly involved in AIV, of which 3 from Asian countries, (listed in the table below) have joined forces in order to investigate the prevention and control of Influenza outbreaks in animal population at present and at time of restocking.

The objective of our project, whose website URL is www.rivers-project.eu, is the prevention and control of avian Influenza A(H5N1) at times of epizootics, and of endemic but sustained viral circulation and at times of post crisis management through the provision of a corpus of data, which policy recommendations can be drawn from, by - gathering data on the survival of avian IVs, in natural environments and in experimental setting.

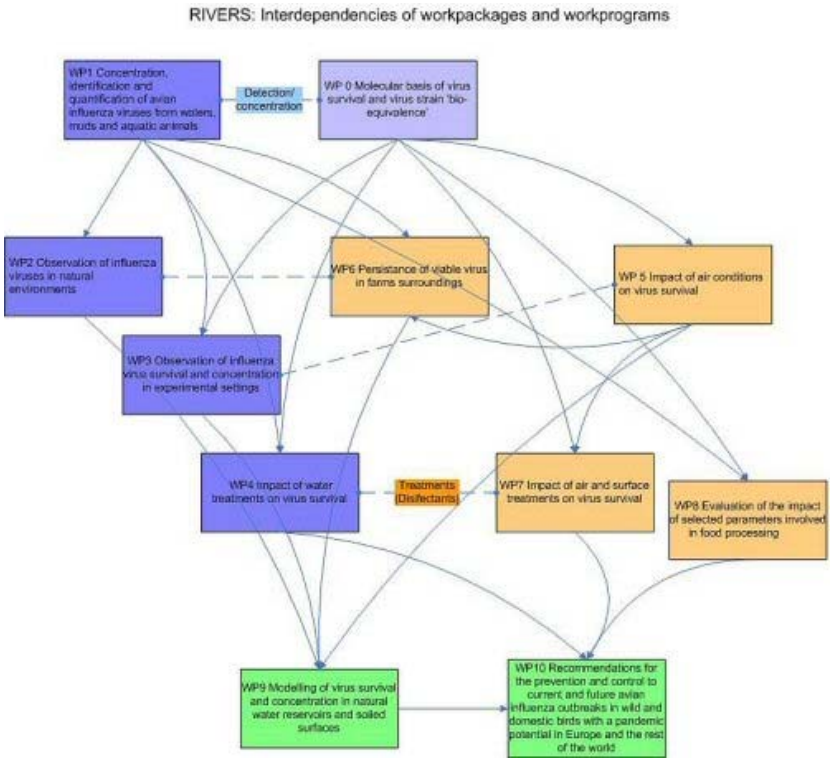
More specific objectives are: 1/ to understand the basis of virus survival from a virological viewpoint; 2/ to understand the impact of physical and chemical elements on virus survival; 3/ to evaluate the role of environmental reservoirs; 4/ to propose standardised protocols for the concentration and detection of AIVs in waters, including waste waters, and in different matrices including food; 5/ to provide a database together with analytical tools to allow the generation of evidence based guidelines for the prevention and control of Influenza outbreaks in animal and human populations, especially at times of restocking. Another specific objective is to elaborate models about the survival of avian Influenza virus in natural environments to demonstrate, in connection with other project and very recent published data, their perpetuation in nature both in biological and environmental reservoirs.

RIVERS Consortium (led by Partner 1, Institut Pasteur)

Partner	Participant Name	Organisation short name	Country	Scientific Leader
1 Coordinator	Institut Pasteur, Paris	IPP	FR	Jean-Claude MANUGUERRA jean-claude.manuguerra@pasteur.fr Institut PASTEUR F 75724 Paris cedex 15
2	Institutul Cantacuzino	IC	RO	Emilia LUPULESCU
3	The Stephan Angeloff Institute of Microbiology	MICB	BG	Angel S. GALABOV
4	Institut Pasteur du Cambodge	IPC	KH	Philippe BUCHY

5	Chinese Academy of Sciences - Pasteur Institute of Shanghaï	IPS	CN	Vincent DEUBEL
6	Centre de Coopération Internationale en Recherche Agronomique pour le Développement	CIRAD	FR	Flavie GOUTARD
7	Institut Pasteur de Lille	IPL	FR	Michèle VIALETTE
8	Interdisciplinary Centre for Mathematical and Computational Modelling	ICM	PL	Jan RADOMSKI
9	Wuhan Institute of Virology	WIV	CN	Ze CHEN

The RIVERS project is built on three workprograms: one focusing on virus in water, one focusing on virus in the air and on the surfaces and one workprogram dedicated to modeling and drawing guide lines. In the first two programs, two paired approaches are followed in parallel: observations in natural environments and in experimental settings.



Upstream of all work programs, RIVERS first focused on the concept of virus strain bioequivalence. The aim was to select a non High Pathogenic Avian Influenza (HPAI) virus strain, possibly not H5, giving the same survival profile as the tested model virus HPAIV H5N1 genotype Z strain. To this end, four strains of Influenza Virus (IV) were compared in relation to survival. Effects of temperature and salinity on the persistence of the four strains of IV in water were investigated. Data generated by RIVERS strongly suggest that IVs have the ability to persist in water for extended periods of time, even at 35°C. Viruses can theoretically persist in distilled water for more than 200 days at 4°C. At 25°C, IV has the ability to remain infectious until 40 to 50 days, depending on the strain. Results for salinity trials showed that salt impact

on virus survival was less important than that of temperature (at low temperature). Virus infectivity also persisted in saline waters for extended periods of time, and significantly decreased only for a salt concentration of 270 $g.L^{-1}$. However, even then, IVs were still infectious after 37 days.

Because of these results, we were not able to identify the "most resistant strain" in the environment since it varies with strains and conditions: strain A is more resistant in condition 1 and strain B is less resistant than A in condition 1 but more resistant in condition 2. Therefore, our data do not support the concept of bio-equivalence, except for identified specific set of conditions for one or more selected parameters.

- **Survival of Influenza A(H5N1) viruses in waters**

Concentration, identification and quantification of avian IVs **from waters, muds and aquatic animals**

RIVERS aims at developing quantitative methods for H5N1 virus detection. Concentration steps are often necessary to concentrate and extract viruses from clean or polluted waters, and from solid samples, such as mud or molluscs (gastropods and bivalves). Although a number of protocols have been published so far for IVs, no standard protocol is currently available to process quite large volume of water (100 L).

Optimised protocols for the concentration, identification and quantification of avian IVs from waters, muds and aquatic animals were developed in a multistep fashion. First quantitative detection methods by RT-PCR and cell culture have been developed to detect H5N1 as current detection techniques are often not sensitive enough, not only for direct analysis of heavily contaminated samples (such as duck ponds) and of solid matrices (sediments, biota, food), but also for analysis of lakes and especially for drinking water after a concentration step. An inventory of the conditions of real-time RT-PCR techniques used by the different partners within RIVERS was made. Kits providing the fastest and easiest way to purify viral RNA have been identified. A qRT-PCR protocol has been adopted as the consortium protocol and will be disseminated within it and beyond.

A particular emphasis was then put on the development of viral concentration procedures from surface waters**.** An inventory of methods used for concentrating infectious viral particles was first carried out. The aim was to select the most efficient method useable for large volumes of clean or dirty water. Rapidity and simplicity were also evaluated, since the method has to be applied by each RIVERS partner and if possible in the field. In RIVERS, two methods were evaluated: adsorption/elution on glass wool, as described in the French national standard method (AFNOR XP T90-451) usually used for the detection of enteroviruses, and viral adsorption/elution on a commercial electropositive filter. These methods could be used for primary concentrations, allowing concentration of large volumes of water.The use of a second round of concentration steps with chicken erythrocytes or with poly-ethylene glycol (PEG), in combination or not with filter concentration, was subsequently evaluated. Another task was to deal with the analysis of solid matrices (sediments, biota, and food). In this case, concentration steps are not necessarily the limiting factor. Strong matrix effects may occur with two main difficulties. Firstly, mud can trap infectious particles and secondly they can contain a lot of pollutants, potentially acting as polymerase inhibitors, thus lowering PCR performances. Such effects were studied and partially overcome.

(A)

(B)

(C)

(D)

Methodology and setup for filtration of surface water on glass wool (A and B) and on Argonide's electropositive filter (C and D).

The efficacy of a direct extraction protocol has been evaluated, based on a previously used protocol by RIVERS partners(Vong et al., 2008). As part of this program, a direct extraction protocol for Influenza A viruses from mud was optimised for small quantities of mud and proved to be efficacious, provided the virus quantity was high enough. The next step was its validation as well as a protocol for virus quantification in mud.

Observation of IVs in natural environments

Lakes most probably play a central role in virus transmission between birds and possibly constitute efficient relays for virus transmission from one year to the other one. If this role of virus conservatory is plausible in the North according to some data, no or very limited data are available both in the North and in the South. Moreover, available data deal with the contamination of lakes by AIVs but no data are specific of rivers and of the effect of water flow on virus dilution/transport. The role of biotic components of fresh water systems (ponds, lakes, rivers) was also investigated.

Water samples were collected in two large lakes in China: Dongting Lake on the Yangtze (Chang Jiang) River downstream of Wuhan (Hubei Province) and Qinghai Lake on the Yellow River (Huang He) where a severe epizootic occurred in 2005 and was an important point for the subsequent geographical spread of HPAI H5N1 viruses. Two methods associated with Influenza virus isolation from water samples have been established, with

which many strains of Influenza A virus of subtypes H5N1 and H9N2 have been isolated from the Dongting Lake samples. For comparison purpuses and in addition, samples from poultry farms and markets have also been collected, and H5N1 viruses were isolated. DNA sequencing and phylogenetic analysis of the isolates as well as pathogenicity evaluation of some isolates were performed. The potential role of aquatic organisms in the concentration and the conservation of AIVs in waters are now under investigation. Data generated by RIVERS revealed that some H5N1 strains isolated from lake water had close relationship with those from dead fowls found in farms.

In Cambodia, the team involved in RIVERS recently published (Vong S et al., EID, July 2008) data obtained from samples collected in 2007 showing that a very low load of virus was detected in few specimens of collected pond water. Further sample analysis is ongoing at the time of writing this report. Large volumes of water were sampled and kept at -80°C until testing by appropriate method to be delivered soon.

Environmental water samples have also been collected three weeks after outbreaks in the Tulcea area in Romania along the Danube River (e.g.: river and lakes waters). Samples will be analysed as soon as standard protocols are made available through RIVERS.

Observation of Influenza virus survival and concentration in experimental settings

As the experimental counterpart of the above mentioned observational approach focused on natural settings, data are being generated by the observation of strategic water collected in natural settings as detailed above in Cambodia. Uninfected natural waters collected in natural settings are being used to simulate environment conditions in water tanks such as aquarium and jars by controlled infection. Biological systems are progressively added to mimic natural environments. This is currently ongoing.

Before creating artificial aquatic biotopes, a list of the elements naturally existing in aquatic environment was established and physico-chemical characteristics of water during epidemics were recorded. Fortunately, only one outbreak in poultry occurred in Cambodia (and just at the beginning of the project).

Impact of water treatments on virus survival

In birds, AIVs replicate in the cells lining the intestinal tract and can be excreted in high concentrations in the faeces. Waters can be heavily contaminated and although viruses can be degraded and become non infectious, the titre, which might dramatically decrease never become null. In this first part of the project, work was focused on the impact of different chemicals in laboratory conditions on AIV survival in waters.

Disinfectants selection was based on several factors, such as, virus characteristics, method of action, cost and toxicity. The following four groups of disinfectants are being studied: chlorine and chlorine compounds; oxidizing agents such as pentapotassium bis(peroxymonosulphate) bis(sulphate); alcohol such as ethanol and different concentrations of isopropanol; glutaraldehyde. Globally, an effective inactivation was demonstrated by glutaraldehyde and pentapotassium bis(peroxy mono sulphate) even at 4 °C provided that the time of contact was prolonged from 20 min to 30 min.

To be closer to field situations, the impact of glutaraldehyde on AIV in association with organic materials was studied by infecting river water and standing water (lake or pond water) with H5N1 AIV. The preliminary data showed that a 2% glutaraldehyde preparation efficiently desinfected all the tested liquids at 20 °C or more, except in the case of the highest virus titres. As expected, the presence of organic materials decreased the inactivation

efficacy of glutaraldehyde at 37 °C for 40 hours. In these conditions, viable virus was still detectable in the tested water collected from a lake. Globally, preliminary results, in experimental conditions, showed that H5N1 AIV are sensitive to inactivation by glutaraldehyde and oxiding agents.

- **Survival of Influenza A(H5N1) viruses in air, surfaces and food products**

Impact of air conditions on virus survival

RIVERS also studies virus survival at various hygrometry and temperature conditions and this part has been divided into two main areas. The first one corresponds to the set up of a system where an aerosol is generated and submitted to controlled conditions such as temperature and hygrometry (Air Experimental System). The second area dealt with surfaces exposed to air and in particular with technical challenges to recover viruses from experimentally infected surfaces. As at the time of RIVERS launch, there was no standardised method for the recovery of IVs from smooth, rough or porous surfaces. A peculiar attention is being paid to mask surfaces. Virus survival on fabrics and other materials used in agricultural and industrial settings or in commercial planes are also being investigated.

As planned, the relevant types of surfaces to be tested as models were determined at the beginning of the study, after an extensive bibliographic search: glass as the prototype of non porous smooth surface, disposable weaved tissue handkerchiefs, disposable weaved filter material for FFP2 masks and disposable weaved surgical masks as rough and porous surfaces. Strategies for virus recovery from various surfaces are currently being developed in order to avoid virus loss during this operation.

A significant level of effort has been put to imagine how to elaborate protocols for the generation of aerosols made of viral suspensions. We worked on the conception and design of an Air Experimental System (AES): a BSL3 sealed climatic chamber with controlled temperature and humidity in which virus suspensions can be aerosolised and subsequently collected. The AES will allow us to simulate environmental conditions in the laboratory to evaluate Influenza virus survival in aerosols in a closed volume.

As planned, the Sartorius BioCollector 'Airport MD8' was evaluated in the real environment of passenger cabins of commercial aircrafts. The analysis by qRT-PCR of these air samples for Influenza A and B viruses and RSV showed that one was positive for Influenza B virus and one for RSV A. Virus culture using MDCK, Vero and /or MRC5 did not lead to virus isolation. These results illustrate that, in our real environment observational setting, the MD8 allows virus capture when present. The inability to recover viable virus might be due to the conditions in an environment very hostile to IVs with high air turn-over, efficient HEPA filters and very low humidity.

Persistence of viable virus in farms surroundings

First priority areas for samplings in natural environments were determined for African, Asian and European relevant countries where the project operates. Collections took place in Cambodia and Romania and sample analysis have been postponed until concentration and detection protocols are made fully available from other components of RIVERS to the whole consortium.

Impact of air and surface treatments on virus survival

Our first postulate was that, beside UV, inactivation of enveloped particles would first operate on the virus shell and not on the genome. Also, if any inactivation process would affect the genome integrity, it would be extremely difficult to differentiate this impact from that directed to the viral envelope. Our second postulate was that inactivation of virus would be graduated and dependant on time of exposure to the inactivating agent. Therefore, we have developed tools which would reproduce viral envelopes and which would allow easy quantification of virus replication. In order to avoid the use of infectious particles, we have engineered pseudoparticles containing H1 and N1 envelope proteins and a lentivirus defective RNA containing the reporter gene of luciferase to quantify the efficiency of peudovirus penetration and replication. This tool will also be used for molecular studies in other components of RIVERS. To quantify the efficiency of virus infection, real-time RT-PCR systems for M1, H1 and H5 genes were developed. Finally, protocols were established and equipment was bought to treat virus particles with an array of physical and chemical agents.

- **Modelling of virus survival and concentration in natural water reservoirs and soiled surfaces**

It was assessed during the first part of the project that no commercial software was available, which could even approximately fulfil the necessary requirements to establish an agent-based model of AIV survival in various environments. Therefore, it was decided to construct a modular system of various tools, acting on experimental data as soon as they become available from various component of RIVERS. In order to test and verify this conclusion, a simulatory model of the Influenza viral spread, (strain A/Panama/2007/99) infecting guinea pigs in laboratory-controlled conditions, was built. The experimental findings were reproduced by this model fairy well. It is expected, that due to a high similarity of the AIV H5N1 serotype to the one used by authors of the experimental study on guinea pigs, it would be fairly straightforward to repeat our simulations and modelling, as soon as data on H5N1 stability in various environments will be available from other component of RIVERS.

SECTION 1 – PROJECT OBJECTIVES AND MAJOR ACHIEVEMENTS DURING THE REPORTING PERIOD

OVERVIEW OF GENERAL PROJECT OBJECTIVES

The overall objective of the RIVERS STREP project is the prevention and control of avian Influenza A(H5N1) in animal population with the following objectives:

- gather data on the survival of avian IVs (AIV), in natural environments
- generate scientific knowledge about the survival of AIVs in experimental settings
- provide figures about the effect of various treatments either chemical (eg disinfectants) or physical on Influenza virus survival
- provide figures about the effect of various food processing on Influenza virus survival
- Elaborate models about the survival of AIV in natural environments to demonstrate, in connection with projects relevant to tasks 3 and 4 of the SSP-5B-Influenza call, their perpetuation in nature both in biological and environmental reservoirs.

The RIVERS project addresses avian influenza (AI) infection, epidemiology and control in situations that mimic the way the virus moves in natural environment and in controlled environment such as the farm yard. Virus survival will be examined through genetic and phenotypic characteristics of the virus in relation to the chemical and physical nature of those parameters that can affect virus structure that are likely to exist in the environment in which the virus is expected to be found. The concentration of the virus in particular locations will be a concern of this investigation. Coupled with these studies, further efforts will be expended in rapid detection of the virus and in the production of standard protocols for such diagnosis methods. International guidelines for the control and prevention (through virus inactivation and disinfection for example) of outbreaks in domestic birds but also in humans will thus benefit from the information generated by the project.

All members of this consortium (listed in the table below) have been selected on the basis of their track record and on their experience in the research fields addressed in this proposal. The consortium breaks into a central European laboratory based group, whose core belongs to the Pasteur institutes international network, and a collection of laboratories and institutes that are in countries where the disease has occured. All nine partners are extremely complementary in their technologies and research areas, each contributing a separate and crucial part of the overall project resources. Most of them have previously collaborated in a highly synergistic and productive way.

RIVERS Consortium (led by Partner 1, Institut Pasteur)

Partner	Participant Name	Organisation short name	Country	Scientific Leader
1 (Coordinator)	Institut Pasteur de Paris	IPP	FR	Jean-Claude MANUGUERRA
2	Institutul Cantacuzino	IC	RO	Emilia LUPULESCU
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MAIN ACHIEVEMENTS

The RIVERS project is built on three workprograms: one focusing on virus in water, one focusing on virus in the air and on surfaces and one workprogram is dedicated to modeling and drawing guide lines. In the first two programs, two paired approaches are followed in parallel: observations in natural environments and in experimental settings.

Upstream of all work programs, RIVERS has at first focused on the concept of virus strain bioequivalence. The aim was to select a non High Pathogenic Avian Influenza (HPAI) virus strain, possibly not H5, giving the same survival profile as the tested model virus HPAIV H5N1 genotype Z strain. We thus compared, in relation to survival, four strains of IV: the avian H5N1 genotype Z strain A/Duck/Cambodia/DK(4)/2006, the human H5N1 strain A/Hong Kong/156/97, and two human strains, A/Wyoming/3/2003 (H3N2) and A/NewCaledonia/20/99 (H1N1). Effects of temperature and salinity on the persistence of the four strains of IV in water were investigated. The four strains were submitted to various environmental parameters over time and tested for infectivity using a microtiter endpoint titration.

Data generated by RIVERS strongly suggest that IVs have the ability to persist in water for extended periods of time, even at 35°C. Viruses have the potential to persist in distilled water for more than 200 days at 4°C. At 25°C, IV remained infectious until 40 to 50 days, depending on the strain. Surprisingly, A/Hong Kong/156/97 virus seemed more resistant at 25°C than the other strains, still persisting over 40 days. At 35°C, total loss of infectivity was faster; no infectious virus could be detected after 20 days for H5N1 and H1N1 viruses, and 35 days for H3N2 virus.

Results for salinity trials showed that salt impact on virus survival was less important than temperature (at low temperature). Virus infectivity also persisted in saline waters for extended periods of time, and significantly decreased only for a salt concentration of 270 $g.L^{-1}$. However, IVs were still infectious after 37 days.

Sequencing of the surface proteins (HA, NA and M2) of each strain are in progress in order to identify critical residue or glycosylation site potentially involved in subtle variations between virus survival profile.

According to these results, we were not able to identify the most resistant strain in the environment since it varies with strains and conditions: strain A is more resistant in condition 1 and strain B is less resistant than A in condition 1 but more resistant in condition 2.Therefore, our data do not support the concept of bio-equivalence, except by identified specific set of conditions.

- **Survival of Influenza A(H5N1) viruses in waters**

Concentration, identification and quantification of AIVs from waters, muds and aquatic animals

RIVERS aims at developing quantitative methods for H5N1 virus detection. Concentration steps are often necessary to concentrate and extract viruses from clean or polluted waters, and from solid samples, such as mud or molluscs (gastropods and bivalves). Although a number of protocols have been published so far for IVs, no standard protocol is

currently available to process quite large volume of water (100 L). The physical and chemical features of IVs differ significantly from enteric viruses such as enteroviruses, for which there are such protocols. In RIVERS, emphasis was put on this important methodological aspect.

H5N1 detection by RT-PCR and cell-culture optimisation

Quantitative detection methods by RT-PCR and cell culture have been optimised to detect H5N1. Detection techniques may provide sufficient sensitivity, not only for direct analysis of heavily contaminated samples (such as duck ponds) and of solid matrices (sediments, biota, food), but also for analysis of lakes and especially for drinking water after a concentration step. First, an inventory of the conditions of real-time RT-PCR techniques used by the different partners within RIVERS was made. Kits providing the fastest and easiest way to purify viral RNA have been identified. Given the higher sensitivity of one of the methods, corresponding primers and TaqMan® probes targeting the hemagglutinin (H5), neuraminidase (N1) and Matrix (M) genes have been retained for the consortium protocol providing an improved one-step real-time RT-PCR assay.

Development of a viral concentration procedure from surface waters

An inventory of methods used for concentrating infectious viral particles was first made. The aim was to select the most efficient method useable for large volumes of clean or dirty water. Rapidity and simplicity were also evaluated, since the method has to be applied by each partner and if possible in the field. The use of adsorption/elution on electropositive filters, such as Virosorb 1-MDS (CUNO), was reported and seemed adapted to detect IVs in large volume of experimentally spiked tap water (Roepke et al., 1989) or of naturally contaminated surface water (Sivanandan et al., 1991). Given these references and characteristics of methods (efficiency to concentrate viruses and preservation of infectivity), two methods have been evaluated: adsorption/elution on glass wool, as described in the French national standard method (AFNOR XP T90-451), which is usually applied to the detection of enteroviruses, and viral adsorption/elution on a commercial electropositive filter. These methods could be used for primary concentrations, allowing concentration of large volumes of water. The use of a second round of concentration steps with chicken erythrocytes or with poly-ethylene glycol (PEG), in combination or not with filter concentration, was evaluated.

- **Validation of concentration and quantification methods on surface water**

Concentration and quantification methods previously developed for Influenza A virus subtype H1N1 have to be validated on artificially contaminated surface waters (clean or dirty waters) with Influenza A subtype H5N1. Influenza A virus (A/HongKong/156/97 (H5N1)) was used. The experimental design characterised by filtration, elution and concentration parameters in accordance with the above protocol (Task 2), was followed to determine nucleic acids recovery rates in eluates and precipitates. PCR inhibitions were observed in final viral concentrates. Five-fold dilutions of RNA extracts were at least needed to avoid reaction inhibition. The preliminary results confirmed the viral recovery rate obtained previously for H1N1 virus concentration in dirty surface water. Conditions used in the protocol, using glass wool filter and PEG precipitation, are thus adapted to Influenza A virus type H5N1 and allow to achieve satisfactory levels of virus recovery.

- **Development of an extraction procedure for solid samples (mud,biota)**

Another task dealt with the analysis of solid matrices (sediments, biota and food). The concentration step was not necessarily the limiting factor. Strong matrix effects may occur with two main difficulties. Firstly, mud can trap infectious particles and secondly it can contain a lot of pollutants, potentially acting as polymerase inhibitors lowering PCR performances. Such effects must be studied and overcome. Generally speaking, extraction methods are mainly based on elution methods for which different parameters could be changed, used in combination or not with the precipitation of viral particles in the presence of PEG, because the amount of virus present in the environment may be low. The work done by RIVERS showed that different methods, tested for extracting virus from mud, present a critical stage at the point of desorption of viral particles from the support. Three protocols, using elution buffer with borate, seemed to increase both quantities of infectious viruses and viral genomes recovered in eluates. H1N1 IV recovery rates ranged from 0.1 to 0.7% when following the tested protocols.

The effectiveness of a direct extraction protocol has been evaluated, based on a previously used protocol (Vong et al., 2008). Sterile water (0.5 mL) was artificially contaminated with virus in parallel to mud (0.5 g) and subjected to extraction procedure. Recovery efficiency of virus in mud was calculated in comparison with recovered virus in sterile water. The results suggested that infectious viral particles were trapped in the mud, and PCR inhibition occurred probably due to the complexity of matrices such as mud. As part of this programme, direct extraction step of IVs from mud could be used to extract viruses from small quantities of mud, provided the virus quantity is high enough.

Validation of extraction and quantification methods on mud

Concentration and quantification methods, previously developed for Influenza A virus type H1N1 have to be validated on artificially contaminated mud with Influenza A virus type H5N1. Further experiments will be performed with Influenza virus A H5N1 virus (A/HongKong/156/97) in combination with a PEG precipitation step. Maximal processable volume and detection threshold of this experimental design will be determined. In addition, because of observed PCR inhibitions in PEG precipitates, RNA purifications, using QIAamp Viral RNA Mini Kit (Qiagen) and QIAamp Ultrasens Virus Kit (Qiagen), might be compared in further experiments.

Observation of IVs in natural environments

Lakes most probably play a central role in virus transmission between birds and possibly constitute efficient relays for virus transmission from one year to the other one. If this role of virus conservatory is plausible in the North according to some data, no or very limited data are available. Moreover, available data deal with the contamination of lakes by AIVs but no data are specific of rivers and of the effect of water flow on virus dilution/transport. The role of biotic components of fresh water systems (ponds, lakes, rivers) were also investigated.

Water samples were collected in two large lakes in China: Dongting Lake on the Yangtze (Chang Jiang) River downstream of Wuhan (Hubei Province) and Qinghai Lake on the Yellow River (Huang He) where a severe epizootic occurred in 2005 and was an important point for the subsequent geographical spread of HPAI H5N1 viruses. Two methods associated with Influenza virus isolation from water samples have been established, with which many strains of Influenza A virus of subtypes H5N1 and H9N2 were isolated from the Dongting Lake samples. For comparison purpuses and in addition, samples from poultry farms and markets have also been collected, and H5N1 viruses were isolated. DNA sequencing and phylogenetic analysis of the isolates as well as pathogenicity evaluation of some isolates were performed. The potential role of aquatic organisms in the concentration and the conservation of AIVs in waters are now under investigation. Data generated by RIVERS revealed that some H5N1 strains isolated from lake water had close relationship with those from dead fowls found in farms.

In Cambodia, the team involved in RIVERS recently published (Vong S et al., EID, July 2008) data obtained from samples collected in 2007 showing that a very low load of virus was detected in few specimens. Further sample analysis is ongoing at the time of writing this report. Large volumes of water were sampled and kept at -80°C until tested by an appropriate method to be delivered soon.

Water environmental samples have also been collected three weeks after outbreaks in the Tulcea area in Romania along the Danube River (e.g.: river and lakes waters). Samples will be analysed as soon as standard protocols are made available through RIVERS.

Observation of Influenza virus survival and concentration in experimental settings"

As the experimental counterpart of the above mentioned observational approach focused on natural settings, data are being generated by the observation of strategic water collections detailed above. Uninfected natural waters collected in natural settings are being used to simulate environmental conditions in water tanks such as aquarium and jars by controlled infection. Biological systems are progressively added to mimic natural environments. This is currently ongoing.

Before creating artificial aquatic biotopes, a list of the elements naturally existing in aquatic environment was established and physico-chemical characteristics of water during epidemics were recorded. Fortunately, only 1 outbreak in poultry occurred (and just at the beginning of the project).

Impact of water treatments on virus survival

In birds, especially in *Anatidae* species, AIVs replicate in the cells lining the intestinal tract and can be excreted in high concentrations in the faeces. Waters can therefore be heavily contaminated and although viruses can be degraded and become non infectious, the titre might decrease dramatically without becoming null. In this first part of the project, work was focused on the impact of different chemicals in laboratory conditions on AIV survival in waters.

AIV vaccine strain, H5N1 – NIBRG – 14, with the haemagglutinin - HA and the neuraminidase – NA genes derived from A/Viet Nam/1194/2004 – clade 1 was used in the experiments reported here. High, medium and low concentrations of H5N1 were diluted in PBS, standing water and running water. The protein content, total number of live aerobic particles, heavy metals (lead) and pH of the two types of water were measured. The

selection of dsinfectants was based on several factors, such as virus characteristics, method of action, cost and toxicity. Based on the presence or absence of lipids on the virus and on the virus size, AIV belongs to category A, which includes all lipophilic enveloped viruses of intermediate to large size, susceptible to a wide variety of disinfectants. The following four groups are being studied:
chlorine and chlorine compounds containing 2.5 g sodium dichloroisocyanurate (NaDCC);
oxidizing agents: pentapotassium bis(peroxymonosulphate) bis(sulphate);
alcohols: ethanol and different concentration of isopropanol.
Aldehydes: glutaraldehyde.

An effective inactivation has been shown by glutaraldehyde and pentapotassium bis(peroxy mono sulphate) even at 4 °C if the time was prolonged at 20 min to 30 min.

To be closer to field situations, the impact of glutaraldehyde on AIV in association with organic materials was studied by infecting with H5N1 AIV river water and standing water (lake or pond water). The former had low protein content and a neutral pH, the latter had high protein content, high content of lead and a basic pH. The preliminary data showed that a 2% glutaraldehyde preparation efficiently desinfected all the tested liquids at temperatures of 20 °C or more, except with the highest virus titres. As expected, the presence of organic materials decreased the efficacy of glutaraldehyde at 37 °C for 40 hours, which resulted in viable virus still being detectable in water collected from a lake.

Globally, preliminary results, in experimental conditions, suggest that reverse genetic engineered H5N1 AIV are sensitive to inactivation by glutaraldehyde and oxiding agents.

- **Survival of Influenza A(H5N1) viruses in air, surfaces and food products**

Impact of air conditions on virus survival

RIVERS also studies virus survival at various hygrometry and temperature conditions and this part of the workprogram has been divided into two main parts. The first one corresponds to the set up of a system where an aerosol is generated and submitted to controlled conditions such as temperature and hygrometry (Air Experimental System). The second part of this WP dealt with surfaces exposed to air and in particular with technical challenges to recover viruses from experimentally infected surfaces. At the time of RIVERS launch, there was no standardised method for the recovery of IVs from smooth, rough or porous surfaces from the air. A peculiar attention is being paid to mask surfaces and virus survival. Virus survival on fabrics and other materials used in agricultural and industrial settings or in commercial planes are also being investigated.

From data actually collected by other components of RIVERS, the strain to be used in this set of experiments was decided upon: the strain most affected by environmental and experimental conditions among those tested. As planned, the relevant types of surfaces to be tested as models (rough and smooth, porous) were also determined at the beginning of the study, after an extensive bibliographic search: glass (watch glass) as the prototype of non porous smooth surface, disposable weaved tissue handkerchiefs, disposable weaved filter material for FFP2 masks and disposable weaved surgical masks as rough and porous surfaces. Strategies for virus recovery from various surfaces are currently being developed in order to avoid virus loss during this operation.

A significant level of effort has been put to imagine how to elaborate protocols for the generation of aerosols made of viral suspensions. We worked hard on the conception and design of a BSL3 sealed climatic chamber with controlled temperature and humidity in which virus suspensions can be aerosolised and subsequently collected. We involved three specialised company in this phase. The self contained BSL3 climatic chamber will allow us to simulate environmental conditions in the laboratory to evaluate IVs survival in aerosols in a closed volume. The range of conditions is 5 to 55°C for temperature and 20 to 98% for humidity.

To evaluate and validate air-biocollectors (assessment of the rate of physical output for virus collection), we based our work on the experience acquired in this field by the Sartorius Company. They previously performed assays to check the efficacy of their impact biocollectors and the behaviour of their gelatine filters with IVs using the A/Puerto Rico/8/34(H1N1) as a model. They also tested the virus ability to retain its infectivity during collection time lasting many minutes. A 15 minutes air collection, which allows the impact of the gelatine membrane of 337.5L, retained virus which remained infectious during this collection time. In addition, the reduction of the medium volume used to dissolve the membrane gave a better ratio virions/concentration which helped lowering the detection threshold to reach 10^2 infectious units/m^3. As planned in WP5, we evaluated the Sartorius BioCollector « Airport MD8 » in the real environment of the passenger cabins of commercial aircrafts by collecting 300 L of air in 6 min (50 L/min) using a 80 mm diameter gelatine membrane. The analysis by qRT-PCR of these air samples for Influenza A and B viruses and RSV showed that one was positive for Influenza B virus and one for RSV A. Unfortunately, virus culture using MDCK, Vero and /or MRC5 did not lead to virus isolation. These results illustrate that in our setting, the Sartorius BioCollector Airport MD8 allows virus capture when present. The inability to recover viable virus might be due to specific conditions in this type of environment hostile for enveloped viruses with high air turn-over, efficient HEPA filters and very low humidity.

- Persistence of viable virus in farms surroundings

First, priority areas for sampling in natural environments were determined for African, Asian and European countries in the project. Collections took place in Cambodia and Romania and sample analysis have been postponed until concentration and detection protocols are fully available from other components of RIVERS to the whole consortium.

- **Impact of air and surface treatments on virus survival**

Our first postulate was that, beside UV, inactivation of enveloped particles would first operate on the virus shell and not on the genome. Also, if any inactivation process would affect the genome integrity (RNA and all proteins associated with it), it would be extremely difficult to differentiate this impact from that directed at the viral envelope. Our second postulate was that inactivation of virus would be graduated and dependant on time of exposure to the inactivating agent. Therefore, we have developed tools which would reproduce viral envelopes and which would allow easy quantification of virus replication. In order to avoid the use of infectious particles, we have engineered pseudoparticles containing H1 and N1 envelope proteins and a lentivirus defective RNA containing the reporter gene of luciferase to quantify the efficiency of pseudovirus penetration and replication. This tool will be used for molecular studies in other components of the RIVERS project.

In parallel, stocks of infectious H1N1 virus were prepared to compare the effects of inactivation on both enveloped particles. To quantify the efficiency of virus infection, real-time RT-PCR systems for M1, H1 and H5 genes were developed. Finally, protocols were established and equipment was bought to treat virus particles with an array of physical and chemical agents.

- **Modelling of virus survival and concentration in natural water reservoirs and soiled surfaces**

It was assessed during the first part of the project that no commercial software was available, which could even approximately fulfill the necessary requirements to establish an agent-based model of AIV survival in various environments. Therefore it was decided to construct a modular system of various tools, acting on experimental data as soon as they will became available form other WP. In order to test and verify this conclusion, a simulatory model of the Influenza viral spread, (strain A/Panama/2007/99) infecting guinea pigs in laboratory-controlled conditions, was built. The experimental findings were reproduced by this model fairy well. It is expected, that due to a high similarity of the AIV H5N1 serotype to the one used by authors of the experimental study on guinea pigs, it would be fairly straightforward to repeat our simulations and modelling, as soon as data on H5N1 stability in various environments will be available from other component of RIVERS. In the meantime several different models of aerosol viral spread are currently at various stages of development, and their fruition is expected in the next nine to twelve months, perhaps earlier in dependency on other RIVERS components culminations, and their respective data availability.

As part of an effort to provide a methodological way of determining a cross-immunological distance between different viral strains or seroptypes, a novel variance maximization technique based on a Monte Carlo randomisation of synonymous codon replacements is being developed. It might be used to characterize variability of the viral receptor proteins: haemagglutinin and neuraminidase, in an alignment free manner – which is important for achieving a sufficient speed of comparisons, when performing very large scale supercomputer simulations. The method is of general use, and is potentially applicable to very many areas of genomic research.

In addition in 2008, analyses of the 2006 surveillance data in poultry in six provinces of Cambodia and of poultry mortality surveys in areas where H5N1 was identified either in humans or in poultry were analysed. A new epidemiological definition of H5N1-related mortality and signs and non-H5N1-related- in chicken and duck flocks with high sensitivity and specificity were generated. Poultry mortality characteristics and signs were compared between laboratory confirmed H5N1 infected flocks and non H5N1 ones. A space time analysis was carried out to determine plausible clusters of villages affected by H5N1. These results were subsequently included in a model that helps determining spatial and environmental risk factors associated with circulation of H5N1 viruses. Environmental parameters such as the land cover or the temporal variations of flooding will be taken into account by the analysis of time series of MODIS images. These results are currently compiled in the form of a report. A manuscript to be submitted to a peer-reviewed journal is in preparation.

SECTION 2 – WORK PACKAGE PROGRESS OF THE PERIOD

WORK PROGRAMME I: "SURVIVAL OF AVIAN IVS IN WATERS AND AQUATIC BIOLOGICAL SYSTEMS"

WPG LEADER: PHILIPPE BUCHY (INSTITUT PASTEUR DU CAMBODGE)

Work Package 0: "Molecular basis of virus survival and virus strain "bio-equivalence"
WP Leader: Partner 1 – Institut Pasteur Paris (India LECLERCQ)

Work Package objectives

1/ To determine whether the avian or mammalian origin of the cell hosting the virus influence IVs survival properties, for a given strain and given settings
2/ To determine whether there are different survival properties within the H5N1 subtype between virus strains. If so, are there any genetic determinants (number of possible glycosylation sites ….)?
3/ To determine whether the lipid bilayer of the virus envelope has an impact on virus survival
4/ To determine whether virus carbohydrate moieties are involved in virus survival
5/ To describe a model virus not requiring BSL3 conditions to work upon and make it available for other teams in and out of this project

Progress towards objectives

We have firstly focused on the concept of virus strain bioequivalence. The aim of the study was to select a non HPAI virus strain, possibly not H5, giving the same survival profile as the tested model virus HPAIV H5N1 genotype Z strain. This would allow not working in BSL3 conditions and would make the assay portable outside the EU if needed (H5 IVs being listed as double usage goods, they are very hard and lenghtly to export outside the EU). This deliverable was expected by others WPs for their own experiments. We then planned to realize the second part of the project that consists in studying the molecular basis of virus survival, by using data generated by bioequivalence.

We first compared in relation to survival four strains of IV: the avian H5N1 A/Duck/Cambodia/DK(4)/06, the human H5N1 strain A/Hong Kong/156/97, and two human strains, A/Wyoming/3/03 (H3N2) and A/NewCaledonia/20/99 (H1N1).

Effects of temperature and salinity on the persistence in water of the four strains of IV were investigated. The four strains were submitted to various environmental parameters over time and tested for infectivity using a microtiter endpoint titration.

Vials containing diluted virus in distilled water were evenly divided and stored at various temperatures. The 3 temperatures tested, 4°C, 25°C and 35°C, represented respectively cold, temperate and hot climate. In order to evaluate the effect of salinity, viruses were diluted in distilled water adjusted with sodium chloride and stored at 4°C until tested. Three salinities were evaluated: 5g.L^{-1} (Salinity of the Caspian Sea), 35 g.L^{-1} (average salinity of oceans) and 270 g.L^{-1} (salinity of the Dead Sea and salinity in the order of those used in some food processing see WP8). Samples were assayed over a 2 months period.

IVs had the ability to persist in water for extended periods of time, even at 35°C. Viruses had the ability to persist in distilled water for more than 200 days at 4°C. At 25°C, IVs remained infectious until 40 to 50 days, depending on the strain. Surprisingly, A/Hong Kong/156/97 virus seemed more resistant at 25°C than the other strains, still persisting over 40 days. At 35°C, the total loss of infectivity was faster; no infectious virus could be detected after 20 days for H5N1 and H1N1 viruses, and 35 days for H3N2 virus.

Results for salinity trials showed that the impact of salt on virus survival was less important than temperature. Virus infectivity also persisted in saline waters for extended periods of time, and significantly decreased only for a salt concentration of 270 $g.L^{-1}$. However, even at this level of salinity, IVs remained infectious after 37 days.

In the survival kinetics described above, the loss of infectivity was dramatic during the first 2 days of the experience for all strains, except for A/NewCaledonia/20/99 (H1N1). This initial behaviour was illustrated by a very short half life time compared to the length of time persistence. We thus tested if this initial sharp decrease was immediate or not by determining the $TCID_{50}$ titer at J0 just after having diluted the viruses in distilled water. The results showed that all strains except A/NewCaledonia/20/99 (H1N1) lost 2 to 3 log of infectivity immediately after water dilution, demonstrating a change of state of the viral particles, which has not been described as yet. Additional experiments were done with additional H3N2 and H1N1 virus strains. Results suggested that the initial sharp loss of infectivity was not an IV subtype dependant property. Besides IV strains following general trends, IV outliers, differing in their behaviour, were identified. For example, all H3N2 viral strains tested were not affected by water dilution except the A/Wyoming/3/2003 strain.

Sequencing of the surface proteins (HA, NA and M2) of each strain is in progress in order to identify critical residue or glycosylation sites potentially involved in subtle variations between virus survival profile.

According to these results, we were not able to identify the most resistant strain in a given environment. Persistence of Influenza A viral strains varied considerably with temperature and salinity. Altough IV strain behaviour was globally identical, outliers were identified making it impossible to consider strain bio-equivalence except for a specific set of conditions and for specific parameters.

The aim of the second part of the project was to study the molecular basis of virus survival, taking into account data obtained from previous experiments on bio-equivalence. Firstly, based upon data described above, we decided to work with lentiviral pseudoparticles expressing mutated HA and NA surface proteins, in order to free ourselves from BSL3 conditions and reverse genetic experiments (see progress in WP7).

Deviations from the project work programme, and corrective actions taken

There has been some delay in the development of the study for several reasons. Mainly, the M2 student in charge of the project arrived in October, 2007 and had many lessons during her training course. She really started working on the project at the beginning of March, 2008. She is now working full-time in the laboratory as a PhD student. Since June 2008, RIVERS has become one of the 3 top priorities of the IPP laboratory and three technicians are participating more to RIVERS as a whole, which helps WP0. Since June 2008, the strict compliance to 45 minutes weekly laboratory meetings implemented since March 2007 dedicated to RIVERS has also helped accelerating the pace of WP0.

For the second part of the project, and because we couldn't use low pathogenic IV as a model virus (as outlined by bioequivalence studies), we decided to use pseudoparticles containing HA and NA envelope proteins and lentivirus defective RNA as described by our partner IPS in the progress report for WP7 (see below). This tool reproducing viral envelopes and allowing easy quantification of virus replication has been recently engineered and improved by IPS. During a face to face meeting in Paris in September 2008, IPP obtained from IPS access to the plasmids necessary for engineering their own pseudoparticles which will be used to better understand the molecular basis of virus survival.

More globally, at the time of conceiving the project, not enough time has been allocated to this WP. Considering the new pace of work for WP0 and the concentrated resources poured in its achievement, WP0 should deliver what was planned originally with the time frame of RIVERS.

List of Deliverables (M1-M18)

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 0-1	Criteria for bio-equivalence in relation to virus survival between IV strains either within the A(H5N1) subtypes between HPAI and LPAI strains or within type A between A(H5N1) IVs and other subtypes such as A(H3N2) or human or avian-like A(H1N1) viruses	0	M6	M20	2	4	1
D 0-2	Experimental tools eg model virus not requiring BSL3 conditions to be used in other WPs	0	M6	M24	4	1	1
D 0-3	Method of virus viability other than virus titration of cell culture (TCID50 titre determination)	0	M5	M28	3	0	1
D 0-4	Knowledge on some determinants of virus stability	0	M6	M36	1	1	1
D 0-5	Peer-review scientific publications in international journals	0	M12	M33	2	0	1

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M0-1	Choice of a method to assess virus viability	0	M1-M2	M28	1
M0-2	Implementation of chick embryo fibroblasts culture (avian system) and SK93/2 cell propagation	0	M1-M2	M19	1
M0-3	Production of virus preparations on avian and mammalian systems	0	M2-M6	M36	1
M0-4	Preparation of viruses with diverse glycosylation and lipid patterns	0	M4-M6	M36	1
M0-5	Performance of survival tests on various 'avian' and 'mammalian' virus preparations	0	M3-M6	M25	1
M0-6	Definition of bio-equivalence between virus strains	0	M6	M24	1

Work Package 1: "Concentration, identification and quantification of avian IVs from waters, muds and aquatic animals"

WP Leader: Partner 7 – Institut Pasteur de Lille (Michele VIALETTE)

Work Package objectives

There is a need to understand the perpetuation of the virus and its survival in the environment, such as in water (lakes), in sediment, in biota and possibly in wastewaters (in case faecal contamination would occur). The aim is to evaluate the risks for poultry from contact with surface waters, or with contaminated drinking water inside premises, and the risks for humans from use of surface water in case of disinfection failure, or in absence of treatment (open shallow wells, roof-collected water,...), or from recreational waters (bathing, ...). In Work Package 1, the objective is to develop efficient and adapted detection and quantification methods for various matrices.

First of all, the development of quantitative detection methods for H5N1 virus was a prerequisite for WP2 (environmental contamination in tropical areas and Eastern Europe), 3 & 4 (lab experiments). In addition, concentration steps are necessary to concentrate and extract viruses from clean or polluted waters, and from solid samples, such as mud or molluscs (gastropods and bivalves). These methods are necessary for other WPs, in order to observe in the field the persistence of viable viruses in natural environments (WP2) and in farms surrounding (WP6), or in experimental setting to study virus survival and concentration (WP3) and to evaluate the impact of water treatments on virus survival (WP4).

Progress towards objectives

Optimisation of H5N1 detection by RT-PCR and cell-culture (Task 1)

Quantitative detection methods by RT-PCR and cell culture have been optimised to detect H5N1. Detection techniques must provide sufficient sensitivity, not only for direct analysis of heavily contaminated samples (such as duck ponds) and of solid matrices (sediments, biota, food), but also for analysis of lakes and especially for drinking water after a concentration step.

Madin Darby Canine Kidney cells (MDCK), obtained through the consortium, were used for the propagation of H5N1 virus and for measuring viral infectivity using a microtiter endpoint titration.

At the beginning of this WP, an inventory of the conditions of real-time RT-PCR techniques used by the different RIVERS partners was made.

Kits providing the fastest and easiest way to purify viral RNA have been identified. QIAamp Viral RNA Minikit® and QIAamp Ultrasens Virus kit have been retained for purification of viral RNA from viral sample of 140 µL and 1 mL, respectively. Given the higher sensitivity of the method, corresponding protocol, primers and TaqMan® probes targeting the hemagglutinin (H5), neuraminidase (N1) and Matrix (M) genes were retained as the consortium standar protocol (Slomka et al., 2007, Payungporn et al., 2005, Spackman et al., 2002).

Real-time one-step RT-PCRs were performed on Light Cycler 2.0. Successful detections of H5 and M genes were obtained using the Superscript III Platinium One-Step qRT-PCR system

(Invitrogen), following the manufacturer's recommendations. However, no signal was observed for the N1 gene. This is being worked upon by the consortium.

Characterisation of the M and H5 genes detection and quantification thresholds, and repeatability/reproducibility of the real-time RT-PCR were evaluated. The real-time RT-PCR specific of M and H5 genes could quantify down to $5.3\,10^2$ and $2.4\,10^2$ copies/5 µL, respectively.

In conclusion, the improved one-step real-time RT-PCR assay described here provides a rapid, specific and sensitive method to detect M and H5 genes.

Development of a viral concentration procedure from surface waters (Task 2)

An inventory of methods used for concentrating infectious viral particles was made. The aim was to select the most efficient method useable for large volumes of clean or dirty water. Rapidity and simplicity were also evaluated, since the method has to be applied by each partner and if possible in the field.

The use of adsorption/elution on electropositive filters, such as Virosorb 1-MDS (CUNO), had been reported and seemed adapted to detect IVs in large volume of experimentally spiked tap water (Roepke et al., 1989) or of naturally contaminated surface water (Sivanandan et al., 1991). Given these references and the characteristics of methods they describe (efficiency to concentrate viruses and preservation of infectivity), two methods have been evaluated: one is adsorption/elution on glass wool, as described in the French national standard method (AFNOR XP T90-451) and usually used for the detection of enteroviruses, and one is viral adsorption/elution on a commercial electropositive filter (NanoCeram® filter - ARGONIDE). These methods could be used for primary concentrations, allowing concentration of large volumes of water.

The use of a second round of concentration steps with chicken erythrocytes or with poly-ethylene glycol (PEG), in combination or not with filter concentration, was evaluated.

Adsorption/elution on glass wool or Argonide filters

Concentration method based on adsorption/elution 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 viral adsorption/elution on Argonide electropositive filter, have been evaluated in combination with a second concentration step with PEG.

Influenza A virus (A/Puerto Rico/8/34(H1N1) or PR8) was used as a model. A fractional experimental design, including 16 conditions, was used to study and quantify the individual effects of the predominant parameters. Each assay of concentration was realised in duplicate on surface waters sampled in a duck pond in Lille (Citadelle de Lille) from the same place but at different dates ("water block").

Experimental settings are illustrated below: Methodology and setup for filtration of surface water on glass wool (A and B) and on Argonide's electropositive filter (C and D).

(A)

(B)

(C)

(D)

To evaluate the effectiveness of the different concentration protocols, virus infectivity was evaluated by microtiter endpoint titration. Viral suspensions used for seeding were analysed and used as positive controls of each experiment. Infectious virus recovery efficiencies, calculated in comparison with positive controls, were evaluated for the 16 conditions tested in the experimental design.

Using the results, it was possible to identify factors having an impact on the final virus recovery and to select an "optimum" protocol.

To confirm the effectiveness of the selected concentration protocol, viral suspensions used for seeding (used as positive control of each experiment), elution and PEG precipitation samples were also analysed by quantitative RT-PCR specific of matrix M gene, to determine the viral genome copies number. Negative amplification (sterile water), RT-PCR inhibitors and RNA positive controls were included in each amplification series. No PCR inhibition was observed in final viral concentrates.

On the other hand, virus recovery results both by virus titration on cell culture and by genetic detection (RT-PCR), confirmed the conditions for best concentration of H1N1 IVs in

dirty surface water. The final protocol, using glass wool filter, increased the quantity of viruses in final concentrates and allowed to achieve about 1% of viral recovery.

Concentration using PEG was also studied by a second team within RIVERS for optimisation. Several methods have been employed to determine the viral concentration in water from natural sites of interest. PEG 6000 precipitation was found to be an effective concentration method that enhanced the chances for detecting human and animal viruses in environmental samples (including water samples).

Concentration steps with chicken erythrocytes

Influenza A virus type H1N1 (PR8) was used as a model. Four viral concentrations, ranging from 10 to 10^4 $TCID_{50}$, were used for experimental contamination of relatively dirty surface waters. The experimental design was described previously (Khalenkov et al., 2008). To evaluate the effectiveness of the concentration protocol, dilutions of viral suspensions used for seeding (used as Positive control of each experiment), and concentrated samples were analysed, by cell-culture titration and by quantitative RT-PCR, specific of M gene.

PCR inhibitions were observed in final viral concentrates; at least 2-fold dilutions were needed to avoid reaction inhibition. In addition, a decrease of detected RNA quantity seemed to be observed in concentrates. It could be explained by interference of erythrocytes with nucleic acid fixation on QiAamp spin column (Qiagen).

As part of this program, concentration step with chicken erythrocytes could be used to concentrate IVs from small volumes of surface waters, provided the virus quantity was high enough. This was explored by another team of the consortium. Some previous studies indicated that the virus adsorbed onto formalin-fixed erythrocytes and subsequently isolated in chicken embryos was an effective and simple method for concentration of AIVs from sometimes even large volumes of natural water. However, our study suggested that formalin-fixed erythrocytes concentration system could become not enough efficient for AIV greatly decreasing the sensitivity of AIV detection in waters. Optimisation was then undertaken in a multistep manner.

Red blood cells (RBCs) were separated from whole chicken blood Packed erythrocytes and diluted to a final concentration of 5%(v/v) by using Alsever's Solution containing 1% BSA(m/v) and stored at 4 before use. (The chickens used in this study were all healthy and didn't carry AIV). NaCl was added into the water samples to a final concentration of 0.9% under sterile conditions, and then 5 mL of 5% fresh chicken erythrocytes was added to each 50 ml of water sample and mixed gently. After incubation for 1 h at 4 °C, samples were centrifuged at 1500 rpm for 10minutes at 4 °C. After removing the supernatant, 1 mL of PBS containing antibiotics were added and the precipitations were resuspended. 0.3 mL of the resuspended erythrocytes was injected into allantoic cavity of 2 eggs per sample. At the end of the incubation period or upon embryo death, the allantoic fluids were tested for hemagglutinating activity and RT-PCR for farther validation of Influenza A virus.

Validation of concentration and quantification methods on surface water (Task 3)

Concentration and quantification methods previously developed for IAV subtype H1N1 had to be validated on artificially contaminated surface waters (clean or dirty waters) with Influenza A virus H5N1. To this end, Influenza A virus (A/HongKong/156/97(H5N1) or HK97) was used. The experimental design characterised by filtration, elution and concentration parameters in accordance with the above protocol (Task 2), was followed to determine

nucleic acids recovery levels in eluates and precipitates. PCR inhibitions were observed in final viral concentrates. Five-fold dilutions of RNA extracts were at least needed to avoid reaction inhibition. The preliminary results confirmed the level of viral recovery obtained previously for H1N1 virus concentration in dirty surface water. Conditions used in the protocol, using glass wool filter and PEG precipitation, are thus adapted to Influenza A virus type H5N1 and allow to achieve satisfactory virus recovery.

Development of an extraction procedure for solid samples (mud,biota) (Task 4)

Another question was the analysis of solid matrices (sediments, biota, and food). The concentration step is not necessarily the limiting factor. Strong matrix effects may occur with two main difficulties. Firstly, mud can trap infectious particles and secondly it may contain a lot of pollutants, potentially acting as polymerase inhibitors potentially lowering PCR performances. Such effects must be studied and overcome.

Extraction methods are mainly based on elution methods for which different parameters could be changed, used in combination or not with the precipitation of viral particles in the presence of PEG, because the amount of virus present in the environment may be low.

Development of an extraction method of Influenza A virus from mud based on virus elution

Accordingly, studied parameters of elution were defined. PR8 was used as a model. Each extraction assay was performed on muds sampled at different dates near a duck pond in Lille (Citadelle de Lille). In order to control virucidal effect of extraction methods on virus, sterile water (10 mL) was artificially contaminated with virus in parallel to mud (10 g) and subjected to each extraction procedure.

To evaluate the effectiveness of the extraction protocol, supernatant samples, corresponding to eluated viruses, were analysed by cell-culture titration or by quantitative M RT-PCR. Ten different protocols based on literature data were tested.

The work showed that different methods, tested for extracting virus from mud, go through a critical stage at the time of viral particle desorption the solid support. Three protocols, using elution buffer with borate, seemed to increase both the quantities of infectious viruses and viral genomes recovered in eluates. Recovery rates ranged from 0.1 to 0.7%.

Controls, corresponding to surface waters inoculated with viral suspensions and kept at room temperature during the viral concentration steps, were analysed in parallel and showed no virucidal effect of the tested material and of the duration of concentration methods. In addition, no virucidal effect of the extraction methods has been shown, when inoculated sterile water was treated in absence of enzymes during viral extraction steps. The results obtained for mud extraction also indicated that infectious viral particles were retained in the mud.

Development of a direct extraction method of Influenza A virus from mud

The effectiveness of a direct extraction protocol has been evaluated, based on a previously used protocol (Vong et al., 2008). Sterile water (0.5 mL) was artificially contaminated with virus in parallel to mud (0.5 g) and subjected to extraction procedure. The

efficiency of virus recovery from mud was calculated in comparison with virus recovered from sterile water.

While the effectiveness of extraction was total when virus was diluted in water, a decrease of detected RNA quantity was observed in mud extracts. This decrease could be explained by the interference of the matrix with nucleic acids. In addition, surface waters inoculated with the viral suspension, were analysed in parallel and showed PCR inhibition. A 50-fold dilution of the final viral extracts was needed to avoid PCR reaction inhibition when virus was extracted from 0,7 mL, while inhibitions were still observed in 50-fold dilutions, when the total volume was extracted.

These results suggest that infectious viral particles were trapped in the mud, and PCR inhibition did occur probably due to the complexity of matrices such as mud. As part of this program, direct extraction step of AIV from mud could be used to extract IVs from small quantities of mud, provided the virus quantity is high enough.

Validation of extraction and quantification methods on mud

Concentration and quantification methods, previously developed for Influenza A virus subtype H1N1 have to be validated on artificially contaminated mud with Influenza A virus subtype H5N1.

Influenza A virus (A/HongKong/156/97) (H5N1) was used. Two viral concentrations of about 10^3 and 10^6 $TCID_{50}$ were used for experimental contamination of muds. To validate the effectiveness of the extraction protocol, supernatant samples were analysed by quantitative RT-PCR, specific of matrix M gene, to quantify the number of copies of viral genome. Negative amplification (sterile water), RT-PCR inhibitors and RNA positive controls were included in each amplification series. No PCR inhibition was observed in supernatant extracts. This method seemed more efficient when applied to a weight of 10 g mud.

Further experiments will be performed with Influenza virus A H5N1 virus (A/HongKong/156/97) in combination with a PEG precipitation step. Maximal processable volume and detection threshold of this experimental design will be determined. In addition, because of observed PCR inhibitions in PEG precipitates, RNA purifications, using QIAamp Viral RNA Mini Kit (Qiagen) and QIAamp Ultrasens Virus Kit (Qiagen), might be compared in further experiments.

Deviations from the project work programme, and corrective actions taken

No major deviations occurred, except some slight delay in delivering some deliverable and the involvement of an additional partner (WIV) in this WP.

List of Deliverables (M1-M18)

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D1-1	Practical reports describing relevant and chosen methods for concentration, identification, and quantification of AIV in various aquatic environments	1	M18	M22	9	9	7

D1-2	Standard Operating Procedures (SOPs) available for other partners of the project	1	M18	M22	10	10	7

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M1-1	End of Task 1 [H5N1 detection by RT-PCR and cell-culture (Vero or NDCK line). Optimisation of methods]	1	M1-M4	M4	7
M1-2	End of Task 2 [Development of a concentration procedure for drinking/surface waters: selection among PEG (poly-ethylene-glycol), Virosorb (adsorption/elution at selected pH's), chicken erythrocytes concentration, and/or combination.] + Report	1	M2-M8	M18	7
M1-3	End of Task 3 [Validation of concentration and quantification methods on surface waters]	1	M3-M12	In progress: M20	7
M1-4	End of Tasks 4 [Development of an extraction procedure, for solid samples (mud, biota)]& 5 [Transfer of the various protocols to other participants, intercalibration] + Report	1	M4-M18	In progress: M22	7

Work Package 2: "Observation of IVs in natural environments"
WP Leader: Partner 9 – WIV (Ze CHEN)

Work Package objectives

1/ To determine the prevalence and the load of AIVs in waters, especially during Influenza seasons at the epicentre of HPAIV H5N1 in Asia, and to probe into their connection with the presence of virus in wild birds of the same area
2/ To determine whether the outbreaks of H5N1 HPAI along the main waterways coincide with the presence of AIVs in rivers
3/ To assess the potential role of aquatic organisms in the concentration and the conservation of AIVs in waters

Progress towards objectives

All tasks for the first period of this study, *i.e.* M1-M18, were well completed as scheduled. In particular, In China, water samples were collected from two large lakes: Dongting Lake and Qinghai Lake. Many strains of AIV belonging to the H5N1 and H9N2 subtypes were isolated from the Dongting Lake samples. In addition, samples from poultry farms and markets were also collected, and many H5N1 viruses were isolated. DNA sequencing and phylogenetic analysis of the isolates as well as pathogenicity evaluation of some isolates were performed. The work made in China revealed that some H5N1 strains isolated from lake water had close relationship with those from dead fowls at farms.

In Cambodia, the results of an investigation done prior to the start of the RIVERS project was recently published (Vong S et al., EID, 2008). Samples collected in 2007 have been analysed by RT-PCR. Very low viral load of AIVs were detected in few specimens demonstrating the persistence of low amount of H5N1 HPAI RNA in ponds after the end of outbreaks. The last passage on eggs of the specimens is ongoing. In addition, large volume of water were sampled and kept at -80°C until testing by appropriate method (WP2)

The potential role of aquatic organisms in the concentration and the conservation of AIVs in waters is still under investigation both in China and in Cambodia.

1. Sample collection

a. Determination of priority areas for samplings in natural environments (task 1)

In Asia, the priority areas for samplings were determined as follows:
1/ in China, the Dongting and Qinghai Lakes
2/ in Cambodia, one pond in the house surroundings of a patient who was contaminated by the H5N1 virus in 2007 as fortunatly, only 1 outbreak in poultry occurred just at the beginning of the project and no outbreak in poultry was detected so far in 2008.
In Europe, the priority areas for sampling were determined as follows:

1/ in Romania, the Tulcea area in the Danube delta in three places affected three weeks before (27/11/2008) by an H5N1 HPAI outbreak; water was collected from a well, bird feces, soil and animal food were collected in a farm backyard in Murighiol, water was collected from a Danube Delta lake and its immediate surroundings in Ghiolul Pietri and from the Danube River in Mahmudi.

2/ In France, the Somme bay and the Camargue region.

b. Water collection (task 3)

In China, a total of 525 water samples were collected from Dongting Lake water where migratory waterfowls lived or nested, in March, April and October, November, December 2007 and in February and March 2008. One hundred water samples were collected from Qinghai Lake in 2007. Water Samples were put into sterile bottles, the lids being sealed, and transported to the State Key Laboratory of Virology, Wuhan Institute of Virology, CAS, China, then stored at -80 °C until assayed.

In addition, a total of 4042 cloacal specimens of apparently healthy domestic fowls were collected from the markets in the Wuhan city of Hubei province, Yueyang city of Hunan province, Hefei city of Anhui province, Zhengzhou city of Henan province (including 3231 from chickens, 605 from ducks, 186 from pigeons and10 from geese).

In Romania, a total of 10 water samples were collected: 4 from a well in the backyard of a farm in the affected area in Murighiol, 3 from a lake of the Danube Delta at Ghiolul Pietri and 3 from the Danube River at Mahmudi.

In France, samples will be collected in the second part of the program.

2. Observation of IVs in natural environments (lakes)

In China, during spring and winter of 2007, and spring of 2008, eighteen H5N1 and nine H9N2 Influenza A viruses were isolated from the water of Dongting Lake by using chicken erythrocytes adsorption method for concentration Influenza A virus from water. And two H5N1 were isolated from dead domestic duck and dead chicken found in the Dongting Lake marsh respectively. One H9N2 was isolated from the fecal samples of migratory waterfowls that live throughout the winter in Dongting Lake marsh. The subtype of the isolated virus was identified by sequencing the HA and NA gene segments of the viruses.

In November 2007, domestic ducks and chickens died of H5N1 AIV temporally coinciding with the first group of migratory waterfowls that lived throughout the winter in Dongting Lake marsh or just pass through there. HA gene phylogenetic analyses of the isolated H5N1 AIV demonstrated that the viruses isolated from the domestic duck (A/duck/Dongtinglake/10/07(H5N1)) and chicken (A/chicken/Dongting lake/10/07(H5N1)) were genetically closely related to the viruses isolated from the water in the later months. This result strongly suggests that there might be some relation between the waterfowls' migration and the epidemic of the Influenza A virus among domestic fowls in Dong Lake area. This result also strongly suggests that the H5N1 avian IVs may have the ability to persist in Dongting Lake water over winter.

In addition, a virus isolated in March 2007 from Dongting lake waters (A/enviroment/Dongting lake/1-8/07(H5N1)) and a virus isolated in February 2008 from the same lake have the closest genetic relationship among all of the isolated viruses. In addition, both strains were genetically closely related to the virus A/common magpie/Hong Kong/05(H5N1). This result indicates that the resident birds living around the Dongting Lake marsh might be the perennial hosts of H5N1 AIVs. These resident birds may continually excrete the virus and pollute the environment.

Three H5N1 strains A/enviroment/Dongting lake/1-35/07(H5N1), A/enviroment/Dongting lake/1-90/07(H5N1) and A/enviroment/Dongting lake/1-91/07(H5N1) have farside genetically relationship comparing with the other isolates. In contrast, the three

strains of IVs have close genetic relationship with the virus A/domestic mallard/Hunan/67/2005.

In summary, the viruses isolated from the water of Dongting lake 'have much to do' with the resident birds and the domestic fowls. There is no convictive proof suggesting that migratory waterfowls have a direct relationship with the epidemic of avian Influenza A virus in the Dongting Lake area in spite of the epidemic of October 2007 being coincidental with the arrival at Dongting Lake marsh of the first group of migratory waterfowls.

HA gene phylogenetic analyses of the isolated H9N2 AIV demonstrated that H9N2 AIVs continually circulate in the region of Dongting Lake marsh. Moreover, the H9N2 AIVs isolated from the faeces of migratory waterfowls suggest that local H9N2 viruses might infect migratory waterfowls. Many strains of H9N2 viruses were isolated from the water of Dongting Lake suggesting that the polluted water might be a place of virus transmission.

In Cambodia, no viable H5N1 HPAI virus has been detected by real time RT-PCR in the water collected from the pond of a patient previously by the avian A(H5N1) IV.

In Romania, samples have been stored at -80°C until they are assayed for the detection of IVs, waiting for the deliverables from WP1 (tasks 1 to 3).

3. Observation of IVs in Poultry Market

During the surveillance of poultry for AIV in poultry markets in Hubei, Hunan, Henan and Anhui Provinces, central China in late 2006 and early 2007, 16 H5N1 AIV were isolated from 4,042 samples. The H5N1 isolates were all from chickens and ducks, with no isolates from pigeons, geese and breed waters. Based on genome sequencing and phylogenic analysis, our results showed that multiple genotypes of H5N1 viruses (A, B, C and D) were circulating in poultry markets. The evaluation of the relationship between these viral strains isolated on markets and the viral strains isolated in rivers are currently in progress in this study.

Deviations from the project work programme, and corrective actions taken

None

List of Deliverables (M1-M18)

N/A – Not Applicable

Deliverables D2-1, D2-2, D2-3, D2-4 and D2-5 will be prepared and delivered in the next 18 months.

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M2-1	Water sampling without concentration	2	M1-M12	Not applicable	9
M2-2	Water sampling with possible concentration (end of WP1 task 3)	2	M13-M33	M13	9
M2-3	Detection of AIV in water samples	2	M1-M6	M6	9
M2-4	Detection of viable viruses in water samples (end of WP0)	2	M7-M12	M7	9
M2-5	Detection and quantification of viable viruses in water samples with standardised protocols (end of WP1 task 3)	2	M13-M33	M32	9

Work Package 3: "Observation of Influenza virus survival and concentration in experimental settings"

WP Leader: Partner 4 – Institut Pasteur du Cambodge (Philippe BUCHY)

Work Package objectives

1/ To create artificial aquatic biotopes
2/ To observe virus survival and concentration in all components
3/ To test hypothesis of virus circulation in aquatic environments

Progress towards objectives

Before creating artificial aquatic biotopes, it was necessary to list the elements naturally existing in aquatic environments and to record physico-chemical characteristics of water during epidemics. Fortunately, only 1 outbreak in poultry occurred (and just at the beginning of the project). In 2008, no outbreak in poultry was detected so far. We used the water and the mud from one pond in the house of a patient who was contaminated by the H5N1 virus in 2007 to create artificial water settings in aquarium (task 1). Due to the late delivery of the BSL3+ animal facilities, the project was post-poned and the first experiments took place in July 2008. We built 8 aquariums that could fit into a BSL2 cabinet located in the A3+ laboratory.

Water used for the experiments was collected from the field (in a pond previously contaminated by H5N1). Water was analysed for chemical parameters, pH, temperature, etc.... and its bacterial content. Aquariums containing mud, plants, fish, shells, and snails were contaminated with H5N1 virus at a fixed EID_{50} value (task 2). The temperature of the water was controlled by individual water-heaters and the temperatures used were in the range of the temperatures observed in the field. Specimens are now analysed by quatitative real-time PCR and by egg inoculation according a specific method using a cocktail of antibiotics and antifungi. Results of this 1st serie of experiments are expected by October 2008 (3 passages on eggs are necessary to assess virus survival) and a second serie of experiments will start just after.

Hypothesis will be tested after the results of the 1st round

Deviations from the project work programme, and corrective actions taken

No significant deviation. Project was delayed by the absence of outbreak in 2008 and by the late delivery of BSL3+ animal facilities.

List of Deliverables (M1-M18)

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D3-1	Inventory of all the elements naturally existing in the aquatic environment in the countries where H5N1 virus is circulating.	3	M33	M33	15	8	4

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M3-1	Inventory of the elements naturally existing in the aquatic environment.	3	M1-M6	M6	4
M3-2	Record of the water characteristics (temperature, pH, TH ...) observed in countries during epidemic season.	3	M1-M6	M18	4
M3-3	Creation of artificial biotopes in BSL3 facilities.	3	M6-M18	M16	4
M3-4	Development of techniques for detection and study on viability of H5N1 virus after inoculation in artificial aquatic biotopes.	3	M6-M18	M16	4
M3-5	Technological transfer from and to other participating laboratories.	3	M6-M12	M18	4
M3-6	Detection and study on viability of H5N1 virus after inoculation in artificial aquatic biotopes.	3	M6-M24	M24	4

Work Package 4: "Impact of water treatments on virus survival"
WP Leader: Partner 2 – Institutul Cantacuzino (Emilia LUPULESCU)

Work Package objectives

The primary objective of the study is to find the best combinations of chemicals with conditions of pH, salinity, temperature on virus survival in water:
1/ To assess virus survival in various concentrations in various types of water after the treatment with various chemicals;
2/ To assess virus survival in various concentrations in various types of water after different physical treatments;
3/ To evaluate the impact on the virus viability of the combined action of chemicals and physical factors in water.

Progress towards objectives

In this first part of the project (tasks 5 and 6), the work was focused on the assessment of the impact on AIV survival of different chemicals in laboratory condition using phosphate buffer saline (PBS), standing water and river water based on a non-pathogenic avian reassortant virus model.

AIV vaccine strain H5N1 – NIBRG – 14 (originated from NIBSC), with haemagglutinin - HA and neuraminidase – NA genes derived from A/Viet Nam/1194/2004 – clade 1 was propagated in SPF embryonnated eggs in order to obtain a virus stock. Virus concentrations used in the experiments were calculated by 50% eggs infectious doses (EID50) and 50% tissue culture infectious doses (TCID50). High, medium and low concentrations of H5N1 were diluted in PBS, standing water and running water. The protein content, total number of viable aerobic particles, heavy metals (lead) and pH of the two types of water were measured.

Substrates to monitor the retention of infectivity of IV after different chemical treatment have been embryonnated chicken eggs (two or three passages) or certified MDCK cell line.

Virus detection was done by haemagglutination using 0.5% turkey erythrocytes or by RT-PCR using commercial kits. The estimation of the inactivation of the virus in MDCK cells by qRT-PCR was made comparing the number of copies in the treated product with the number detected 20 and 40 hours after inoculation.

Disinfectants selection was based on several factors, such as virus characteristics, method of action, cost and toxicity. Based on the presence or absence of lipids on the virus and on the virus size, AIV belongs to category A, which includes all lipophilic enveloped viruses of intermediate to large size, susceptible to a wide variety of disinfectants.

Disinfectants: four groups:

chlorine and chlorine compounds containing 2.5 g sodium dichloroisocyanurate (NaDCC);
oxidizing agents: pentapotassium bis(peroxymonosulphate) bis(sulphate);
alcohols: ethanol and different concentration of isopropanol.
aldehydes - glutaraldehyde;

Concentration, dilutions and time exposure were those recommended by the respective manufacturers. Sodium bisulphite was used for neutralization of the disinfectant.

Preliminary results

The impact of the four groups of disinfectants on the AIVvaccine strain survival in PBS was evaluated. The efficacy of clorine compounds and alcohols disinfectants tested was influenced by virus titers and temperature, decreasing at high and medium virus titers of the AIV at low temperature. An effective inactivation has been shown by glutaraldehyde and pentapotassium bis (peroxy mono sulphate) even at 4 °C if the time was prolonged at 20 min to 30 min.

Were included experiments about the impact of glutaraldehyde on AIV in association with organic materials. River water and standing water were artificially infected: the former having a low protein content (3.58 µg/ml) and a neutral pH (7.2) and the latter a high protein content (25.5 µl/ml) high content of lead (0.5 ppm) and a basic pH (9.6). A high efficacy of 2% glutaraldehyde in all the fluids at temperature above 20 °C was observed, except for the highest initial virus titre. Organic content decreased the efficacy of glutaraldehyde at 37 °C during 40 hours post infection: the virus was only detected in the lake water.

The estimation of surviving particles of AIV in tissue culture by qRT-PCR method at 40 hours post infection has had advantage vs. classical methods, eggs isolation or tissue culture titration in term of time. The test could be improved by using a RNase digestion in the product to destroy the nucleic acid which is not associated with viral particles after treatment with disinfectants and before inoculation on MDCK monolayer.

Based on the non-pathogenic avian reassortant model NIBRG-14, the results generated by this WP suggest that HPAI viruses in experimental conditions, might be sensitive to glutaraldehyde and oxiding agents.

Deviations from the project work programme, and corrective actions taken

Work (tasks 5 and 6) have been anticipated. As planned, the other tasks will start at M19.

List of Deliverables (M1-M18)

Deliverables D4-1, D4-2, D4-3 and D4-4 will be prepared and delivered in the next 18 months.

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D4-1	Approvable and standardized protocols for testing the effect of chemical and physical treatment of different types of water on Influenza virus survival	4	M19-M33	M19-M33	19	Not applicable	2
D4-2	Data on the efficacy of chemical and physical treatments of water for the reduction of virus load and virus survival	4	M19-M33	M7-M33	19	In progress	2
D4-3	Guidelines for treatments of different types of water aiming to reduce virus survival	4	M30-M33	M30-M33	4	Not applicable	2
D4-4	Scientific publications in peer-review journal	4	M30-M36	M30-M33	2	Not applicable	2

List of Milestones (M1-M18) - N/A – Not Applicable.

WORK PROGRAMME II
"*SURVIVAL OF AVIAN* IVS *IN AIR AND SURFACES*"
WPG LEADER: VIOREL ALEXANDRESCU (INSTITUTUL CANTACUZINO)

Work Package 5: "Impact of air conditions on virus survival"

WP Leader: Partner 1 – Institut Pasteur Paris (Ana-Maria BURGUIERE)

Work Package objectives

1/ To implement a strategy for virus recovery from various surfaces: non biological (rough and smooth, porous) including fabrics and filters for the detection and quantification of IVs.
2/ To evaluate virus survival on the above mentioned surfaces in the course of time, with various room temperature and hygrometry, in particular those found in poultry farms, in commercial planes.
3/ To evaluate virus survival in contained air charged with IV contaminated dropplets, in the course of time, with various room temperature and hygrometry, in particular those found in commercial planes.
Progress towards objectives

The specific objective of WP5- task 1 was to determine, from the data collected in WP0, the virus strain(s) to use in the set of experiments of the whole WP5. Because of safety issues, a human strain included in the current human vaccine would be preferred to be used in the aerosol box.

From data actually collected by WP0, we observed that IVs behave differently to environmental conditions according to specific strains, some of them being total outlier despite general trends (see WP0). We observed a rapid and sharp drop of virus titres after virus stock thawing and dilution in liquid medium for A/Wyoming/3/2003 (H3N2) whereas the loss of virus titre for strains such as A/New Caledonia/20/99 (H1N1) was much lesser in the same experimental conditions. Both Influenza A(H5N1) tested, A/Hong Kong/156/97 et A/Duck/Cambodia/DK(4)/06 had an intermediate behaviour between the above mentioned strains. Because one of the goals of WP5 is to provide protocols which allow the best possible virus recovery, we chose to use the strain most affected by environmental and experimental conditions: A/Wyoming/3/2003 (H3N2).

As planned in WP5-task2, we had to determine relevant types of surfaces to be tested (rough and smooth, porous). After bibliographic searches, we chose the following surfaces as models:

Glass (watch glass) as the prototype of non porous smooth surface
Disposable weaved tissue handkerchiefs, disposable weaved filter material for FFP2 masks and disposable weaved surgical masks as rough and porous surfaces. Moreover, we are in contact with a major worldwide airline company to try to obtain some fabrics used in passenger cabins in civil aeronautics.

As planned by WP5-task 3, we developed strategies for virus recovery from various surfaces in order to avoid virus loss during this operation.

For smooth and non porous surfaces, assays were carried out after sterilisation by autoclave (120°C, 30 min).

After complete drying (34 to 43 min at laboratory room temperature) of 50µL containing the equivalent amount in pfus as 1 mL of a A/Wyoming/3/2003 (H3N2) virus stock suspension with a titre of $1,25.10^5$ pfu/mL, simply scrapping the glass and adding ppi water allowed to recover the equivalent in pfus as 1 mL of a viral suspension with a titre of $8.08\ 10^4$ pfu/mL, losing 0.19 $\log_{10}$. The same experiment without drying the initial viral suspension allowed the total recovery of virus.

For rough and non porous surfaces, assays were carried out with or without prior sterilisation by autoclave (120°C, 30 min).

Weaved tissue handkerchieves: because its level is low, bacterial and fungal contamination of disposable handkerchieves is totally controlled by classic antibiotic and antifungal treatment of cell culture supernatants. This makes possible the use of non sterilised handkerchieves in these assays.

An array of types of assays was carried out to determine the best virus recovery method from tissues contaminated with 50µL containing the equivalent amount in pfus as 1 mL of a A/Wyoming/3/2003 (H3N2) viral suspension with a titre of $10^{7,5}$ $TCID_{50}$/mL.

1/ Using the mechanical effect of a mix of beads (silica 0.1mm, ceramic 1.4 mm and glass 4 mm) shaken during 5 or 2 min at 15Hz by a Retsch MM200 machine allowed to recover the equivalent of 1 mL of a viral suspension with a titre of $10^{2,43}$ $TCID_{50}$/mL, which corresponds to a loss of 4,76 $\log_{10}$.
2/ Using the mechanical effect of agitation during 15 or 30 min at 400 rpm by a Thermomixer allowed to recover the equivalent of 1 mL of a viral suspension with a titre of $10^{4,45}$ $TCID_{50}$/mL which corresponds to a loss of 2,75 $\log_{10}$.
3/ Using the mechanical effect of agitation during 15 or 30 min at 400 rpm by plate shaker allowed to recover :
a/ in the case of sterile handkerchieves after 15 min shaking, the equivalent of 1 mL of a viral suspension with a titre of $10^{4,45}$ $TCID_{50}$/mL which corresponds to a loss of 2,75 $\log_{10}$;
b/ in the case of non sterile handkerchieves after 15 min shaking, the equivalent of 1 mL of a viral suspension with a titre of $10^{4,50}$ $TCID_{50}$/mL which corresponds to a loss of 2,70 $\log_{10}$;
c/ in the case of sterile handkerchieves after 30 min shaking, the equivalent of 1 mL of a viral suspension with a titre of $10^{4,89}$ $TCID_{50}$/mL which corresponds to a loss of 2,31 $\log_{10}$;
d/ in the case of non sterile handkerchieves after 30 min shaking, the equivalent of 1 mL of a viral suspension with a titre of $10^{4,11}$ $TCID_{50}$/mL which corresponds to a loss of 3,09 $\log_{10}$;

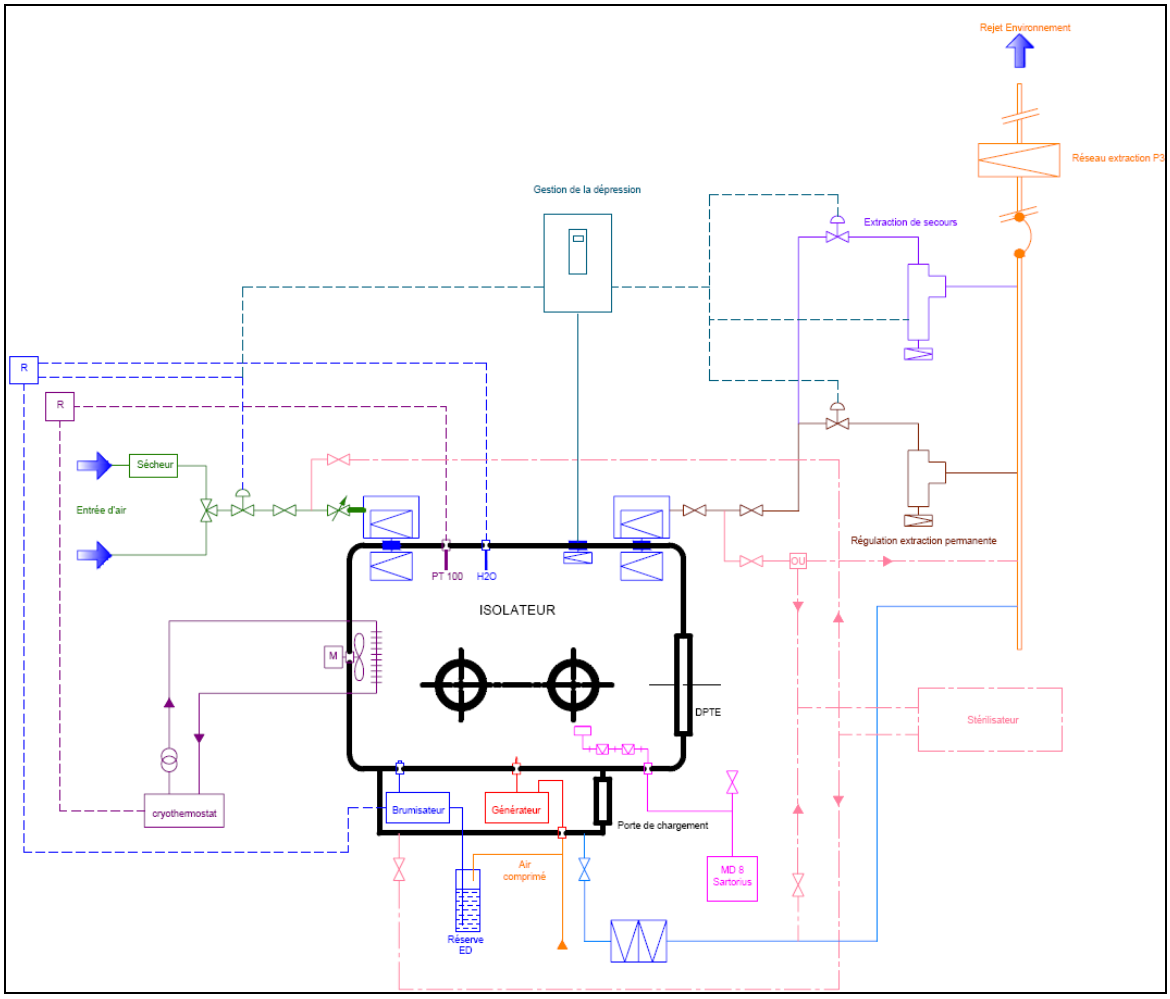
As non sterile samples do not contaminate the cell supernatant used to perform virus titration, all subsequent assays will be carried out without sterilising by autoclave, which might transform the properties of the handkerchief structure.

Detection and titration of virus infectivity in the course of time (from 5 min. to three days) at given temperatures (4°C, 18°C and 35°C) and at given hygrometry (10%, 50% et 90%) (Task 4) will be done as soon as experimental conditions are possible.

A significant level of effort has been put to imagine how to elaborate protocols for the generation of aerosols made of viral suspensions (task 5). We worked on the conception and design of a BSL3 sealed climatic chamber with controlled temperature and humidity in which virus suspensions can be aerosolised and subsequently collected. We involved three specialised companies in this phase: Ysebaert for the conception and the making of the core

box, Ecomesure for the aerosolgenerator and Sartorius for the bio-collector using impact on a gelatine membrane. The layout of the BSL3 self-contained climatic chamber is shown below.

The self contained BSL3 climatic chamber will allow us to simulate environmental conditions in the laboratory to evaluate IVs survival in aerosols in a closed volume. The range of conditions is 5 to 55°C for temperature and 20 to 98% for humidity.



General layout of the self contained climating chamber

To evaluate and validate air-biocollectors (assessment of the rate of physical output for virus collection) (WP5-task 6) we based our work on the experience acquired in this field by the Sartorius Company. They previously performed assays to check the efficacy of their impact biocollectors and the behaviour of their gelatine filters with IVs using the A/Puerto Rico/8/34(H1N1) (PR8) as a model. They also tested the virus ability to retain its infectivity during collection time lasting many minutes. A 15 minutes air collection, which allows the impact of the gelatine membrane of 337.5L, retained virus which remained infectious during this collection time. In addition, the reduction of the medium volume used to dissolve the membrane gave a better ratio Virions/concentration which helped lowering the detection threshold to reach 10^2 infectious units/m^3. Information about air renewal and circulation available for modern aircrafts indicates that the concentration in total particles is many logs above this calculated detection threshold.

As planned in WP5, we evaluated the Sartorius BioCollector « Airport MD8 » in the real environment of the passenger cabins of commercial aircrafts by collecting 300 L of air in 6 min (50 L/min) using a 80 mm diameter gelatine membrane. Eighty-six samples of air were collected during 14 long haul flights during the circulation of seasonal IVs and respiratory syncytial virus (RSV).

The analysis by qRT-PCR of these air samples for Influenza A and B viruses and RSV showed that one was positive for Influenza B virus and one for RSV A. Unfortunately, virus culture using MDCK, Vero and/or MRC5 did not lead to virus isolation. These results illustrate that in our setting, the Sartorius BioCollector Airport MD8 allows virus capture when present. The inability to recover viable virus might be due to the specific conditions in this virus hostile environment with high air turn-over, efficient HEPA filters and very low humidity.

Task 7 (Installation of the specific biocollector) and task 8 (Detection and titration of virus infectivity in the course of time in contained and ventilated air as collected by the above mentioned biocollectors), will be carried out as soon as the self contained BSL3 climatic chamber is available.

Deviations from the project work programme, and corrective actions taken

Due to the quick start of the project and the delay or difficulty in recruiting students and staff to work on this WP, both in IPP and IPS, this part of the program is behind schedule. In IPP, an MSc now PhD student is now fulltime on the RIVERS project and the teams both in IPP and IPS have been mobilised. In IPP, RIVERS is one of the 3 top priorities in the laboratory and permanent technicians and scientists are now concentrating their effort on WP0 and WP5. The current pace of work should guaranty the completion of WP5 within the time framework of RIVERS.

The cost of the specifically engineered self contained BSL3 climatic chamber with aerosol production and biocollection is much higher than anticipated before the intense work and numerous meetings held to design its specification. It is in the range of 70 000 €. We chose to use RIVERS money first for human resources and IPP is seeking complementing funding to have the climatic chamber engineered. The earliest time of availability would be M22. In the case of not getting additional funding from external sources, tasks 7 and 8 will be performed with alternative means, the consequence being that the array of parameters controlled and tested would be narrowed.

List of Deliverables (M1-M18)

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D5-1	Approvable and standardised protocols for Influenza virus recovery from various surfaces	5	M13	M25	3	3,5	1
D5-2	Integrated air system for respiratory virus survival in aerosols	5	M13	M33	5	1	1
D5-3	Data (database) about IV survival in the air and on various kind of surfaces and in various conditions	5	M17	M33	3	0	1
D5-4	Guidelines for recommendations	5	M17	M33	3	0	1

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M5-1	Choice of surfaces: sterilisation methods, preparation of experiment friendly surfaces	5	M4-M8	Choice: M12 Sterilisation: M20	1
M5-2	Availability of strategies for virus recovery from various surfaces	5	M8-M13	M22	1
M5-3	Availability of aerosol generator and choice of biocollector	5	M4-M13	M26	1
M5-4	Implementation of an air system to study virus survival	5	M13-M17	M27	1

Work Package 6: "Persistence of viable virus in farms surroundings"
WP Leader: Partner 3 – The Stephan Angeloff Institute of Microbiology (Angel GALABOV)

Work Package objectives

1/ To determine the prevalence and the load of AIVs in the earth and poultry commodities (including barns, litters, feeding bowl and water supplies) in the course of the seasons at the epicentre of HPAIV H5N1 in Asia (Cambodia and China) and in more recently affected areas of the world such as Europe (Romania and Bulgaria) around places where outbreaks occurred.
2/ To evaluate virus survival in body fluids and wastes (birds feaces, farm manure)
3/ To assess AIV survival in farms and villages
4/ To gather data for future modelling (WP9) about the potential role of earth and soiled agricultural equipment as reservoirs.

Progress towards objectives

1. Sample collection

a. Determination of priority areas for sampling in natural environments (task 1)
In Asia, the priority areas for samplings were determined as follows: in Cambodia, one pond in the house surroundings of a patient who was contaminated by the H5N1 virus in 2007 as fortunatly, only 1 outbreak in poultry occurred just at the beginning of the project and no outbreak in poultry was detected so far in 2008.
In Europe, the priority areas for sampling were determined as follows:
1/ in Romania, the Tulcea area in the Danube delta in three places affected three weeks before (27/11/2008) by an H5N1 HPAI outbreak: a farm backyard in Murighiol, a Danube Delta lake and its immediate surroundings in Ghiolul Pietri and the Danube River in Mahmudi.
2/ In France, the Somme bay and the Camargue region.
In Africa, the priority areas for samplings were those covered by the CIRAD GRIPAVI project.

b. Collection of earth, instruments, litter and food for farm animals (tasks 3 and 4)

In Cambodia, there was no such collection since no new outbreak was recorded after the operational starting point of the RIVERS project in IPC.

In Romania, 3 farm animal faeces samples, 6 soil samples and 3 animal food samples were collected: from the backyard of a farm in the affected area in Murighiol, 4 farm animal faeces samples and 5 soil samples were taken from the surroundings of a lake of the Danube Delta at Ghiolul Pietri and 2 farm animal faeces samples and 7 soil samples were taken from the surroundings of the Danube River at Mahmudi. For each sample, data regarding weather conditions (sunshine, air temperature, rainfall) were collected.

In France, samples will be collected in the second part of the program.

In Africa, the collection of samples will be reported in the final activity report.

2. Observation of IVs in farm and lakes and rivers sourroundings in affected areas

In Cambodia, results of an investigation done prior to the RIVERS project were recently published (Vong S et al., EID, 2008). Samples collected in 2007 were analyzed by RT-PCR. Very low viral ARN load of virus were detected in few specimens in water but soil specimens will be later retested using adapted extraction column to avoid false positives (inhibitors of PCR in the soil).

In Romania, samples have been stored at -80°C until they are assayed for the detection of IVs, waiting for the deliverables from WP1-task 4.

In Africa, the results of samplesanalysis will be reported in the final activity report.

Deviations from the project work programme, and corrective actions taken

No deviation was observed for this WP except a slight delay in sample collection and analysis but all deliverables of this WP should be available within the timeframe allocated to this WP.

List of Deliverables (M1-M18)

Deliverables D6-1, D6-2, D6-3 and D6-4 will be prepared and delivered in the next 18 months.

List of Milestones (M1-M18)

N/A – Not Applicable

Work Package 7: "Impact of air and surface treatments on virus survival"

WP Leader: Partner 5 – Chinese Academy of Sciences-Pasteur Institute of Shanghai (Vincent DEUBEL)

Work Package objectives

1/ To study the stability of viral strains of IVs dried on surfaces under several natural conditions
2/ To assess the efficacy of several physical and chemical treatments on virus inactivation.
3/ therefore better understand the maintenance and possible transmission of HPAIV virus in nature and
4/ propose the use of peculiar measures for preventing virus transmission in research laboratories, in hospitals, and in any natural condition.

Progress towards objectives

Our first postulate was that, beside UV, inactivation of enveloped particles would first operate on the virus shell and not on the genome. Also, if any inactivation process would affect the genome integrity (RNA and all proteins associated with it), it would be extremely difficult to differentiate this impact from that directed to the viral envelope.

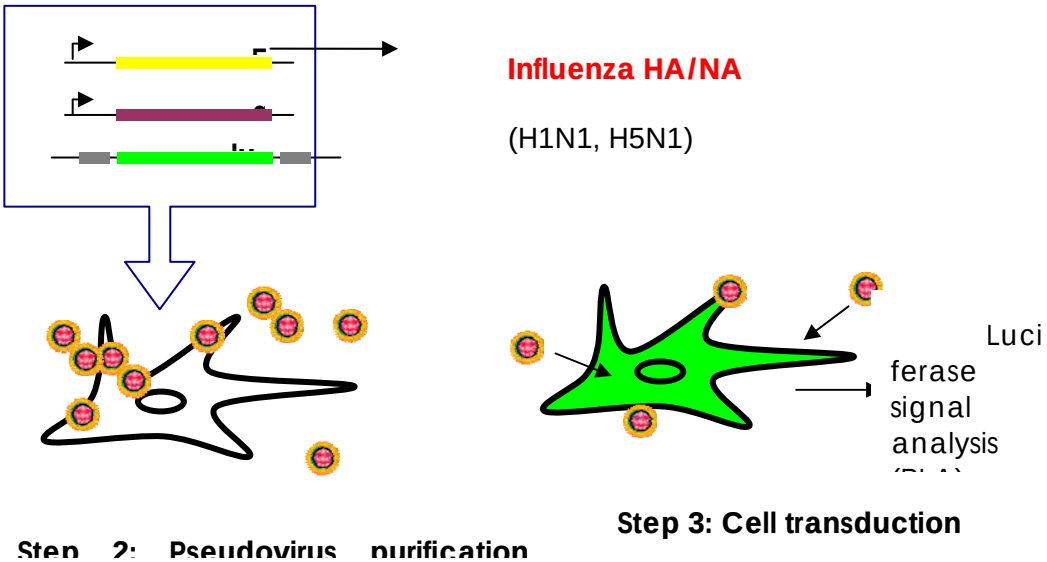
Our second postulate was that inactivation of virus would be graduated and dependant on time of exposure to the inactivating agent.

Therefore, we have developed tools which would reproduce viral envelopes and which would allow easy quantification of virus replication. In order to avoid the use of infectious particles, we have engineered pseudoparticles containing H1 and N1 envelope proteins and a lentivirus defective RNA containing the reporter gene of luciferase to quantify the efficiency of peudovirus penetration (transduction) and replication (figure below). This tool will also be used by WP0.

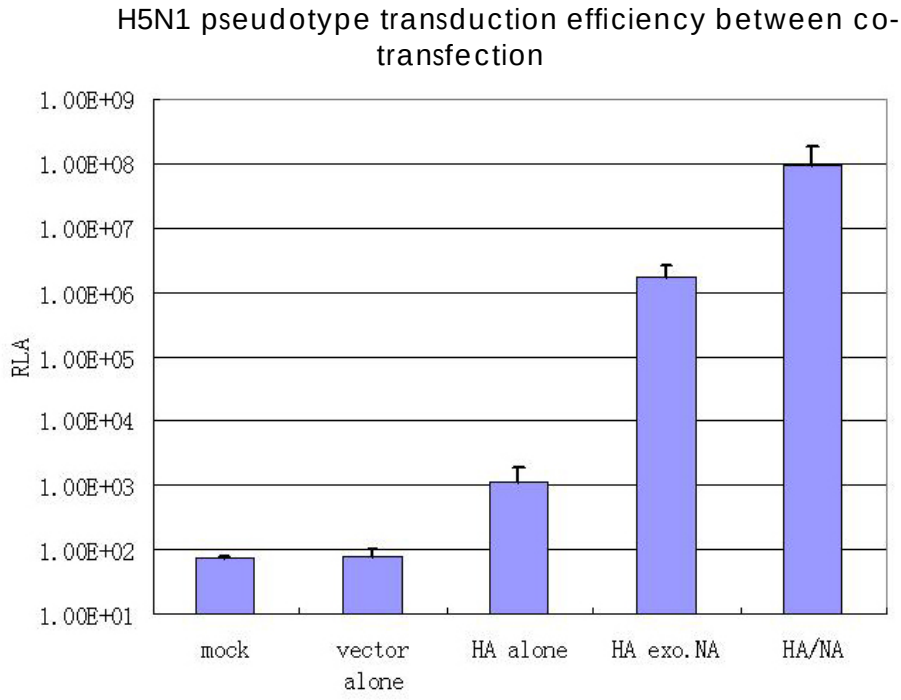
In parallel, we have prepared stocks of infectious H1N1 virus to compare the effects of inactivation on both enveloped particles. To quantify the efficiency of virus infection, we have developed real-time RT-PCR systems for M1, H1 and H5 genes (figure below, Wang et al., in revision).

Finally, we have established the protocols and bought the equipments to treat virus particles with an array of physical and chemical agents.

Figure 1. Retrovirus-vectored pseudoparticles



HA	NA	Relative Luciferase Activity (RLA)	
0.4	0	275	311
0.4	3.6	88	285
0.4	3.6	38709	32822
0.4	1.8	82871	75850
0.4	1.8	216363	297144
0.4	0.45	775093	707153
0.4	0.45	1268515	1850444



Deviations from the project work programme, and corrective actions taken

There has been some delay in the development of the study for several reasons: difficulties to find a suitable technician in virology, time to receive the ordered reagents and equipment, closure of the biosafety laboratory necessary for virus cultivation and virus stock production, setting up the pseudoparticle and real time systems. However, the tests are now being carried out and time will be caught up.

List of Deliverables (M1-M18)

Deliverables D6-1, D6-2, D6-3 and D6-4 will be prepared and delivered in the next 18 months.

List of Milestones (M1-M18)

N/A – Not Applicable

Work Package 8: "Evaluation of the impact of selected parameters involved in food processing"

WP Leader: Partner 7 – Institut Pasteur de Lille (Michèle VIALETTE)

Work Package objectives

This workpackage aims at evaluating the behaviour of avian IVs in animal material. Methods for detection and quantification of AIV in such materials are needed. It will consequently be possible to evaluate the impact of some food processing parameters on viruses. The ultimate goal would be the assessment of processing operation abilities to inactivate AIVs, and possibly recommendation of efficient methods.

Progress towards objectives

Animal materials involved in this project include bird faeces, blood, carcasses and food products. Naturally dead animals or experimentally infected animals or products have to be considered. Methods for extraction, detection and quantification of AIV in these materials have to be evaluated. With a suitable method, quantitative studies of the impact of food processing conditions on IVs will be possible. Temperature, pH, and salinity are key factors in controlling micro-organisms in food products. Thermal inactivation of viruses has proven an efficient decontamination method. Chicken is generally well cooked as are 'white' meats, however in some countries like France duck breast filet (magret de canard) is often served medium rare and the temperature at the centre of the piece of meat might not be sufficient for a long enough time. In addition in other part of the world, consumption of raw duck blood is possible and can be the source of infection. Controlling micro-organisms in food products is an increasing field of investigation, and other factors (such as pH and salt content) may positively interact with heat. It is thus essential to evaluate the effect of these factors on AIV control.

Two draft standards PR NF ISO 20837 and PR NF ISO 20838-September 2004 respectively define requirements relating to sample preparation and amplification for qualitative PCR detection of pathogens in food. Our methodological approaches have to be compatible with these existing standards for the detection and quantification of IVs in poultry meat. IPL, which has long been specialised in food microbiology and has been involved in the making of numerous standards, has been working on the evaluation and modelling of thermal inactivation of enteric viruses in fruit as influenced by pH and sugar, or the development of detection and quantification methods of micro-organisms in food products using various methods (titration, molecular biology).

In the field of food microbiology, there are a great number of standards (ISO, European standards (EN) or National (eg AFNOR)). Some of these standards stipulate general rules for food microbiological examination (NF ISO 7218 and NF ISO 7218/A1 - December 2001), others define the methodology for samples preparation (NF ISO 6887-1 of September 1999), some of them being very specific for meat and meat derived products (eg NF ISO 6887-2 of January 2004). Nevertheless these standards relate to the analysis of the bacteriological quality of food and the detection of a limited amount of given pathogens. Since June 2005, Standard NF IN ISO 22174 outlines general requirements and establishes definitions for the detection and identification of micro-organisms (either pathogens or not) in food by Polymerisation Chain Reaction (PCR).

A bibliography study is in progress. So far, no data concerning the real risk of food contamination and the recovery of respiratory viruses, such as avian IVs, in meat from duck, chicken, turkey, is available.

WP0 generated data which give some insight regarding parameters such as refrigerating temperature used to preserve food (+4°C) and such as high salinity used to keep food like meat in dry sausage for example. Indeed the amount of salt for meat preservation is about 200 G/L. WP0 suggest that IVs could survive with salinity as high as 270 G/L, at least at +4°C. Combining high salinity and higher temperatures might yield different results.

Deviations from the project work programme, and corrective actions taken

No deviation reported for this WP. A slight delay for completion at M28 might occur but every aspect should be delivered with the time of RIVER Swith the commitment of IPL and IPP.

List of Deliverables (M1-M18)

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D8-1	Intermediate report (every 6 months)	8	M16	M18	6	3	7

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M8-1	End of WP8 Task 1 [Evaluation of effect of pH levels, salt content and temperature on various survival/inactivation, in laboratory medium]	8	M10-M15		7
M8-2	Report	8	M12-M16	M18	7
M8-3	End of WP8 Task 2 [Study of the interaction of factors (temperature/pH, temperature/Aw, etc.) on the behaviour of various viral strains]	8	M13-M20	M20	7
M8-4	End of WP8 Tasks 3 [Development of inactivation predictive models] & 4 [Extraction of H5N1 virus from various food products (chicken, turkey,...)] + Report	8	M13-M23	M27	7
M8-5	End of WP8 Task 5 [Validation with contaminated food products, of the predictive data obtained from laboratory media] + Final report	8	M14-M28	M33	7

WORK PROGRAMME III
"*MODELLING AND RECOMMENDATIONS*"
WPG LEADER: JAN RADOMSKI (ICM)

Work Package 9: "Modelling of virus survival and concentration in natural water reservoirs and soiled surfaces"

WP Leader: Partner 8 – Interdisciplinary Centre for Mathematical and Computational Modelling (Jan RADOMSKI)

Work Package objectives

The objectives of WP9 are to build *in silico* possible conceptual models, and to implement them within computationally suitable environment. First, starting from many classes of simple, data driven, descriptive models concerned with the individual studies of the stability of avian IV strains in water reservoirs, and in laboratory-controlled conditions as well as the stability of AIV on dried surfaces, again under laboratory-controlled and natural conditions. The models of the efficacy of physical and chemical treatments on virus inactivation should be assessed at the next stage. Therefore, the objectives are twofold: foremost, as the final goal of the project, to build and validate as far as possible, a hierarchical, multi-scale agent-based simulation model of avian Influenza virus perpetuation, as well as its possible methods of deactivation. Secondly, as means to achieve the main goal, to build a series of partial models, each simulating partial aspect[s] of AIV behavior, stability and perpetuation in specific environments.

Progress towards objectives

It was assessed during the first part of the project that no commercial software is available, which could even approximately fulfill the necessary requirements to establish an agent-based model of AIV survival in various environments. Therefore it was decided to construct a modular system of various tools, acting on experimental data as soon as they will became available form other Workpackages. In order to test and verify this conclusion, a simulatory model of the Influenza viral spread, (strain A/Panama/2007/99) infecting guinea pigs in laboratory-controlled conditions, was built. The experimental findings were reproduced by this model fairy well (T.Zuk, F.Rakowski, and J.P. Radomski, "M*odel of* Influenza *virus spread as a function of temperature and humidity",* submitted to Computational Biology and Chemistry). We expect, that due to a high similarity of the avian IV H5N1 serotype to the one used by authors of the experimental study on guinea pigs, it would be fairly straightforward to repeat our simulations and modelling, as soon as data on H5N1 stability in various environments will be available from WP5. In the meantime several different models of aerosol viral spread are currently at various stages of development, and their fruition is expected in the next nine to twelve months, perhaps earlier in dependency on the WP5 culminations, and their respective data availability.

Work towards the deliverable D9-6 (Multi-scale agent-based simulation model of possible determinants for AIVs stability, perpetuation and deactivation) is progressing according to schedule. Already we have implemented a country-wide model

of social contacts, quintessential for an Influenza spread, due mainly to a daily commuting of population to their respective workplaces, schools, offices, etc. was implemented and tested. It shows very good agreement with all available census data, reproducing well local population densities, average times in transit, and other indicators decisive for assessment of social mobility. The model was implemented and tested for the case of Poland, however, should appropriate census data, as well as the data on necessary networks of schools, workplaces etc. be available, there exist a possibility to construct analogous models for any other EU country, provided a close collaboration with their respective census authorities will be secured (however, such an option lays outside both a scope, and a budget of the RIVERS project). The results were submitted: F. Rakowski, M. Gruziel, M. Krych, and J.P. Radomski, *"Very large scale social mobility model - a Monte Carlo countrywide simulation study for Poland"*, submitted to the Journal of Royal Statistical Society. The next stage of testing for our multi-scale, agent-based simulation platform, that is the most far reaching possibility of the actual study to model an Influenza spread scenarios, built upon the already described social mobility model, is currently at it's final stages, and the results will be described, and sent to print, within the next 3-4 months. It will then be possible to draw a final conclusion as to the validity of the underlying assumptions, and decision of the chosen software solutions (deliverable D9-1, and milestone M9-1). As the human infectivity data of the H5N1 serotype are as yet unknown, we are testing model assumptions derived mostly from available data on the H1N1 and the H3N2 serotypes; however, all necessary provisions have been made to support model's capacity towards inclusion of the H5N1 variant as well. As the simulation model is scalable, it will be rather easy to scale it down from a current capacity to simulate contacts of all 38 millions of individuals, towards any other objective on a smaller scale. Possibilities to scale it up, say, towards countries of the size of France or Germany are also included.

As part of an effort to provide a methodological way of determining a cross-immunological distance between different viral strains or seroptypes, we developed a novel variance maximization technique based on a Monte Carlo randomisation of synonymous codon replacements. It might be used to characterize variability of the viral receptor proteins: haemagglutinin and neuraminidase, in an alignment free manner – which is important for achieving a sufficient speed of comparisons, when performing very large scale supercomputer simulations. The method is of general use, and is potentially applicable to very many areas of genomic research (J.P. Radomski, and P.P. Slonimski, *"ISSCOR: Intragenic, Stochastic Synonymous Codon Occurrence Replacement – a new method for an alignment free genome sequence analysis"*, submitted to the Comptes Rendus Biologies.

The work is in progress (a joint effort of the ICM in Warsaw, and the Remote Sensing Unit of CIRAD in Monpellier) to develop new techniques of extending a methodology of multiple vote artificial neural networks self-learning classifiers to analyze possible habitats of preference for a wild and free ranging poultry on their migration routes, based upon satelite images. Possible finalization of this study is possible in the next twelve to sixteen months.

In addition to the work performed in ICM, IPC and CIRAD have been collaborating in the framework of RIVERS. In 2008, the Epidemiology Unit in IPC has finalized the analyses

of the 2006 surveillance data in poultry in six provinces of Cambodia and of poultry mortality surveys in areas where H5N1 was identified either in humans or in poultry. A new epidemiological definition of H5N1-related mortality and signs and non-H5N1-related- in chicken and duck flocks with high sensitivity and specificity were generated; we compared poultry mortality characteristics and signs between laboratory confirmed H5N1 infected flocks with non H5N1 ones. We applied the proposed definitions to the 2006 surveillance data and performed a space time analysis to determine plausible clusters of villages affected by H5N1. These results were subsequently sent to the CIRAD team led by Flavie Goutard, Montpellier to be included in a model that helps determining spatial and environmental risk factors associated with circulation of H5N1 viruses. Environmental parameters such as the land cover or the temporal variations of flooding will be taken into account by the analysis of time series of MODIS images. The IPC results are currently compiled in the form of a report. A review of the data currently available in the scientific literature about the Contribution of remote sensing for the detection and characterisation of water is ready to be submitted for publication in English and is based on a MSc Degree report. Furthermore, another publication in English is being prepared and focuses more specifically on the use of remote sensing for the study of risk factors linked to water in the H5N1 survival.

Deviations from the project work programme, and corrective actions taken

As it is understandable, up to day, only limited amounts of experimental data have been released by the other WPs of the project or are too recent. This current lack of exploitable data has been a crucial impediment towards achieving deliverables D9-2, D9-3, D9-4, and D9-5, as they are essential to characterize possible parameterisations, and the intended modelling studies leading to a hierarchy of modules simulating, and possibly determining AIVs stability, and maybe ways of its deactivation. As soon as respective data should become available, the modelling efforts will continue. All deliverables should be delivered with the time of RIVERS and were scheduled to occur from M1 to M33.

List of Deliverables (M1-M18)

Deliverables D9-1, D9-2, D9-3, D9-4, D9-5, D9-6 and D9-7 will be prepared and delivered in the next 18 months.

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D9-1	Assessment guidelines for appropriate analytical, algorithmic, and software tools optimally suited for modelling AIVs at various descriptive details levels, different temporal and spatial scales.	WP9	M1-M24	M24	4	3	8
D9-2	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in various water environments, laboratory-	WP9	M7-M33	M20-M33	4	3	8

	controlled and natural.						
D9-3	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in a dry state at various surfaces, laboratory-controlled and natural.	WP9	M7-M33	M24-M33	4	3	8
D9-4	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in air at laboratory-controlled environments.	WP9	M7-M33	M24-M33	4	2	8
D9-5	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in avian faeces and farm manure.	WP9	M7-M33	M24-M33	2	1	8
D9-6	Multi-scale agent-based simulation model of possible determinants for AIVs stability, perpetuation and deactivation.	WP9	M13-M33	M33	16	9	8
D9-7	Peer-review scientific publications in international journals.	WP9					

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M9-1	Choice of software tools for modelling of virus viability and perpetuation	9	M1-M12	M20	8
M9-2	Implementation of simulation models of AIVs perpetuation, viability and deactivation in various water environments	9	M7-M18	M20-M33	8
M9-3	Implementation of simulation models of AIVs perpetuation, viability and deactivation in a dry state	9	M7-M18	M24-M33	8
M9-4	Implementation of simulation models of AIVs perpetuation, viability and deactivation in air at various conditions	9	M7-M18	M24-M33	8
M9-5	Implementation of hierarchical, multi-scale agent-based simulation model of AIVs perpetuation and possible deactivation	9	M13-M33	M33	8

WORK PROGRAMME IV
"*EXPLOITATION AND DISSEMINATION OF THE RESULTS, PROJECT MANAGEMENT*"
WPG LEADER: JEAN-CLAUDE MANUGUERRA (INSTITUT PASTEUR, PARIS)

Work Package 10: "Recommendations from the prevention and control to current and future avian Influenza outbreaks in wild and domestic birds with a pandemic potential in Europe and the rest of the world"

WP Leader: Partner 1 – Institut Pasteur Paris (Jean-Claude MANUGUERRA)

Work Package objectives

To ensure the delivery of a final report to the EC that will include the scientific results and recommendations for prevention and control measures against avian Influenza A(H5N1) in Europe.

Progress towards objectives

As scheduled, this WP will start at the final phase of the RIVERS project.

Deviations from the project work programme, and corrective actions taken

None

List of Deliverables (M1-M18)

Deliverable D10-1 will be prepared and delivered in the next 18 months.

List of Milestones (M1-M18)

N/A – Not Applicable

Work Package 11: "Management of the RIVERS project"
WP Leader: Partner 1 – Institut Pasteur Paris (Jean-Claude MANUGUERRA)

Work Package objectives

The main objective is to animate research momentum by ensuring a collaborative activity between the partners. This will be achieved by applying proper administrative management to project progress, accountability and matters concerning dissemination of results:

Self-evaluation, feedback & decision-making- coordination of work planning (work-committee milestone meetings) preceding decision making points (executive meetings).
Contingency planning- to allow adequate reflection whereby concerted strategies aimed to pass unexpected negative results can be quickly implemented.

Progress towards objectives

The main activities during the first 18 months of activity have been related to the kick-off of the consortium embedding into the community, and detailed definition of joint efforts on the component disciplines. The specific efforts of the consortium are outlined as follows.

a/ Meetings

An important part of the RIVERS effort during start up has been the integration of the consortium. Number of partners had not collaborated prior to this project and there has consequently been an effort to establish a joint basis in terms of protocols and joint research problems. To facilitate this, the consortium has organised one plenary meeting; in addition, two partial small meetings involving teams from two or more partners have taken place as well as a teleconference.

a.1/ Consortium Kick-Off Meeting Paris – 12 and 13th Februrary 2007
During early 2007 a 2 day kick-off meeting for the consortium was organised at Institut Pasteur, Paris (France).
The objectives of the meeting were
• Introduction to the overall project for project staff
• Brief presentation of prior research at partner institutions
• Identification of joint research initiatives and discussion of first integration
• Planning for the first 18 months

The meeting was attended by all the involved partner teams. The beginning of the first day was used for brief presentations from each partner to provide a joint view of existing research competence.

The rest of the first day and the second day were devoted to a brief review of expected research to be performed for each of the workpackages.

The minutes of the meeting are avalaible on the RIVERS website.

a.2/ Teleconference on the 2nd July 2007

The agenda of this telephone meeting was to adopt the minutes of the kick off meeting held in February 2007, to present the RIVERS website and to collect comments about it, to review the actions WP by WP, to discuss management issues in particular the rules for allocating EU money to specific spending, to review intellectual property issues and material transfer agreement especially concenrning Influenza A(H5N1) virus sharing within the consortium.

Unfortunately, technical difficulties made the connection of most partners impossible except for ICM, IPP and IPL.

a.3/ M12 Partial European Meeting – 26th February 2008

This one day meeting was held in Institut Pasteur, Paris (France) and the agenda was to assess the progress for the first 12 months, to discuss on ongoing research, to plan the activities to be performed by end of M18, to try to define a date for the mid-term meeting and to talk about the interim activity and management report. One or more individual from the following partner attended the meeting: IPP, IPL, ICM, CIRAD, IC and MICB. IPC joined the meeting by telephone at the end of the morning to discuss about the current status of the work in IPC in particular regarding WP1 and WP3, about exchange of viral strains within the consortium and about management issues. The minutes have been circulated to the participants and are posted on the RIVERS website.

a.4/ M16 Partial Meeting – 23rd May 2008

This one day meeting was held in Institut Pasteur de Lille, Lille (France) and the agenda was to assess the progress for WP0 and WP1 to discuss on ongoing research, to plan the activities to be performed by end of M18, to try to define a date for the mid-term meeting and to talk about the interim activity and management report. One or more individual from the following partner attended the meeting: IPP, IPL and ICM.

b./ Infra-structure

To accommodate the research a rich infrastructure has been established within the consortium. This Includes:

WWW: A public www facility with information about the project and its activities was developed and implemented (www.rivers-project.eu and www.projet-rivers.eu). The facility has a private area only accessible to members by login and password with internal documents (i.e. meetings agendas, PowerPoint presentations and minutes ……), with a repository of scientific articles hard to find by some partners.

E-mail: Three major email lists are in place for sharing of information. One is purely for administrative activities and another includes all involved researchers in Europe and the third one all partners in Asia.

Visioconference: Because it proved difficult to organise a telephone conference using a Telecom company, we are implementing the use of web-based visioconference using the CNRS server facility through Institut Pasteur's IT department and the Ekiga freeware solution on personal computers using Windows or Linux or X Meeting on Apple

computers running on Mac OSX. IP telephone or camera based teleconferencing should provide a solution for interim working sessions between partners far away.

c./ Assessment of progress

Management is primarily organised by email discussions and bilateral telephone conversations. In addition conferences, meetings and lab visits have been organised for synchronisation. Overall good progress has been made and the project is progressing according to plans without major deviations except a slow start due to the sudden start of the project and the difficulties to recruit staff or students.

The project is required to set up a scientific advisory board. A list of potential candidates has been identified.

d./ Training and dissemination

A training session for the correct use of water filters used for concentration following the protocol deveopped in WP1 will be organised before the end of the project.

e./ Summary

In summary, the RIVERS project has been successfully initiated and although the start was slow the project is making good progress according to plans. In addition a number of related activities have been organised such as the programme kick-off and several new activities have been planned for the coming 18 month period.

Deviations from the project work programme, and corrective actions taken

No logo has been designed as yet. This will be corected by M20.

List of Deliverables (M1-M18)

Del. No.	Deliverable name	WP No	Date due	Actual/ Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D11-1/4	Management Activities	11	M1-M36	M1-M36	39	19	1

List of Milestones (M1-M18)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M11-1	Activation of Consortium internet site	11	M1-M36	M1-M36	1
M11-2	Reportings and organisation of meetings	11	M1-M36	M1-M36	1

SECTION 3 – CONSORTIUM MANAGEMENT

CONSORTIUM MANAGEMENT TASKS AND ACHIEVEMENTS

This has been treated as a WP (for details please see WP11).

The objective of a good management of project is to animate research momentum by ensuring a collaborative activity between the partners. This is being achieved by applying proper administrative management to project progress, accountability and matters concerning dissemination of results:

Self-evaluation, feedback & decision-making- coordination of work planning (work-committee milestone meetings) preceding decision making points (executive meetings).
Contingency planning- to allow adequate reflection whereby concerted strategies aimed to pass unexpected negative results can be quickly implemented.

The main activities during the first 18 months of activity have been related to the kick-off of the consortium embedding into the community, and detailed definition of joint efforts on the component disciplines. The specific efforts of the consortium are outlined as detailed in WP11 progress report.

An important part of the RIVERS effort during start up has been the integration of the consortium. Some partners had not collaborated prior to this project and there has consequently been an effort to establish a joint basis in terms of protocols and joint research problems. To facilitate this, the consortium has organised one plenary meeting; in addition, two partial small meetings involving teams from two or more partners took place as well as a teleconference.

To accommodate the research, a rich IT infrastructure has been established within the consortium, including a public and members only www facility with information about the project and its activities, email lists. Visionconferences are being considered.

Management is primarily organised by email discussions and bilateral telephone conversations. In addition conferences, meetings and labortory visits have been organised for synchronisation. Overall good progress has been made and the project is progressing according to plans without major deviations except a slow start due to the sudden start of the project and the difficulties to recruit staff or students.

The project is required to set up a scientific advisory board. As mentioned in the submitted project document, a list of potential candidates has been identified.

In summary, the RIVERS project has been successfully initiated and although the start was slow the project is making good progress according to plans. In addition a number of related activities have been organised such as the programme kick-off and several new activities have been planned for the coming 18 month period.

Contractors

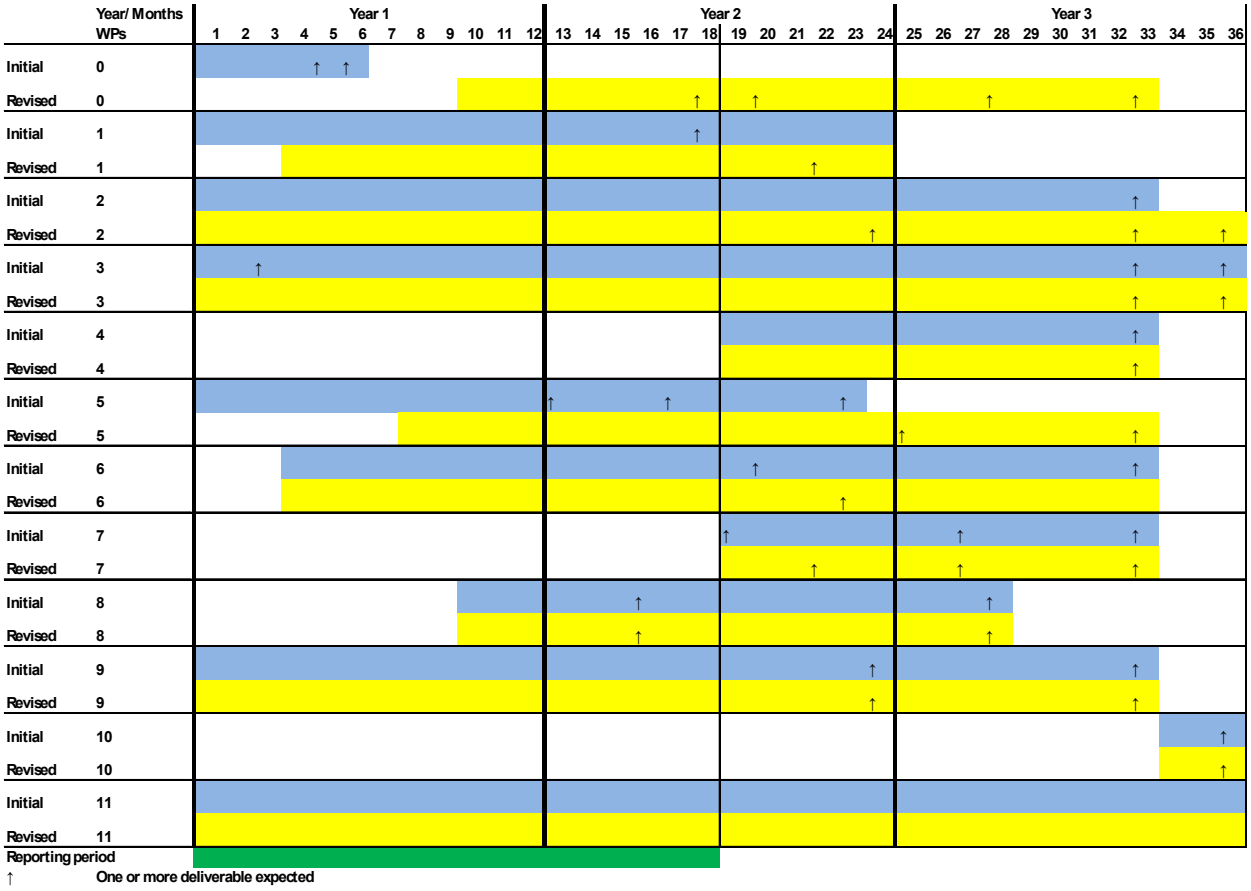
The list of contributors is as shown in the table below and there has been no change in responsabilities except that the WP0 leader was Jean-Claude Manuguerra (Partner 1) and has become India Leclercq (Partner 1):

RIVERS Consortium (led by Partner 1, Institut Pasteur)

Partner	Participant Name	Organisation short name	Country	Scientific Leader
1	Institut Pasteur de Paris	IPP	FR	Jean-Claude MANUGUERRA
2	Institutul Cantacuzino	IC	RO	Emilia LUPULESCU
3	The Stephan Angeloff Institute of Microbiology	MICB	BG	Angel S. GALABOV
4	Institut Pasteur du Cambodge	IPC	KH	Philippe BUCHY
5	Chinese Academy of Sciences - Pasteur Institute of Shanghaï	IPS	CN	Vincent DEUBEL
6	Centre de Coopération Internationale en Recherche Agronomique pour le Développement	CIRAD	FR	Flavie GOUTARD
7	Institut Pasteur de Lille	IPL	FR	Michèle VIALETTE
8	Interdisciplinary Centre for Mathematical and Computational Modelling	ICM	PL	Jan RADOMSKI
9	Wuhan Institute of Virology	WIV	CN	Ze CHEN

Project timetable and status

Globally at the time of reporting, RIVERS has reached a momentum compatible with the achievement of the objectives of each of the WPs within the time frame of the project and this inspite of the delay in delivering some WPs. In particular, WP0 and WP5 started in a much delayed fashion and the initial schedule was probably not realistic for WP0 (especially). However, having acknowledged this situation, the graphical representation below shows that a majority of deliverables are on time and that all of them should be delivered by M36.



Initial and revised timetable of the RIVERS project

➢ Person-Months Status Table

WP0

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	University Associate Professor	3
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.2
Frédéric FICHENICK	Laboratory technician	0.5
Gilberte CORALIE	Laboratory technician	0.5
Christophe BATÉJAT	Laboratory technician	0.2
Amélie DUBLINEAU	MSc student	5
Personnel funded by the project:		
-	-	-

WP1

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	University Associate Professor	0.3
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.2
Frédéric FICHENICK	Laboratory technician	0.3
Gilberte CORALIE	Laboratory technician	0.3
Christophe BATÉJAT	Laboratory technician	0.3
Ana Maria BURGUIÈRE	Medical microbiologist	0.3
Personnel funded by the project:		
-	-	-
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent personnel:		
Philippe BUCHY	Head Virology Unit	1
Personnel funded by the project:		
-	-	-
Participant 7 – Institut Pasteur de Lille (IPL)		
Permanent staff:		
Nathalie DEBOOSERE	Engineer	4
Anthony PINON	Engineer	1
Personnel funded by the project:		
Jessica GACHET	Lab Technician (Level 4)	16

WP2

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	University Associate Professor	0.1
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.2
Frédéric FICHENICK	Laboratory technician	0.2
Gilberte CORALIE	Laboratory technician	0.2
Christophe BATÉJAT	Laboratory technician	0.2
Amélie DUBLINEAU	MSc student	0.2
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
Alexandrescu Ion Viorel	MD, PhD	0.2

Lupulescu Emilia	MD, Scientist	0.2
Sbarcea Claudiu	MD, Scientist	0.1
Personnel funded by the project:		
-	-	-
Participant 3 – The Stephan Angeloff Institute of Microbiology (MICB)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
Angel S. GALABOV	Professor, MD, DSc	0.5
Ivan ZARKOV	Assoc. Professor, DVM, DSc Department of Virology, Faculty of Veterinary Medicine, Thracian University, Stara Zagora	3
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
Philippe BUCHY	Head Virology Unit	1
Horm Srey VISETH	Research Assistant	1
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
E. ALBINA	Scientist	0.6
S. HAMMOUMI	Scientist	2.9
P. Gil	Technician	2.9
Participant 9 – Wuhan Institute of Virology (WIV)		
Permanent staff:		
Ze Chen	Professor	12
Personnel funded by the project:		
Jianjun Chen	Researcher assistant	18
Quanjiao Chen	Researcher assistant	18
Huadong Wang	Researcher assistant	18

WP3

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 3 – The Stephan Angeloff Institute of Microbiology (MICB)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
Philippe BUCHY	Head Virology Unit	2
Horm Srey VISETH	Research Assistant	3
Ramona GUTIERREZ	Vet	3
Personnel funded by the project:		
-	-	-

WP4

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.05
Ana Maria BURGUIÈRE	Medical microbiologist	0.1
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
Alexandrescu Ion Viorel	Scientist, MD, PhD	0.48
Onu Adrian	Scientist, MD	0.80
Lupulescu Emilia	Scientist, MD, Leader WP4	1.31
Mihai Maria Elena	Scientist, Biologist	1.75
Baetel Alina Elena	Assistant Researcher, Biologist	0.53
Tecu Christina	Scientist, MD, PhD	0.50
Ionita Emil	Scientist, Biologist	0.09
Ustea Luiza	Technician	1.24
Burcus Mirela	Technician	1.24
Enache Nuti	Technician	1.24
Preda Florica	Technician	1.24
Laes Doina	Technician	0.63
Dobre Emilia	Technician	0.86
Parvu Violeta	Economist	0.13
Stefanescu Maria	Scientist, Biologist	0.13
Radu Dorel Lucian	General Director, MD, PhD	0.08

Personnel funded by the project:		
-	-	-

WP5

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana BURGUIÈRE	Medical microbiologist	1.5
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.2
India LECLERCQ	University Associate Professor	0.3
Frédéric FICHENICK	Laboratory technician	0.5
Gilberte CORALIE	Laboratory technician	0.5
Christophe BATÉJAT	Laboratory technician	0.5
Amélie DUBLINEAU	MSc student	1
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-

WP6

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana BURGUIÈRE	Medical microbiologist	0.1
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.1
India LECLERCQ	University Associate Professor	0.2
Frédéric FICHENICK	Laboratory technician	0.1
Gilberte CORALIE	Laboratory technician	0.1
Christophe BATÉJAT	Laboratory technician	0.1
Amélie DUBLINEAU	MSc student	0.2
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
Lupulescu Emilia	Scientist, MD	0.24

Sbarcea Claudiu Edward	Scientist, MD	0.42
Necula George	Assistant Researcher, Biologist	0.54
Personnel funded by the project:		
-	-	-
Participant 3 – The Stephan Angeloff Institute of Microbiology (MICB)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
Angel S. GALABOV	Professor, MD, DSc Department of Virology, MICB	5
Georgi GEORGIEV	Assoc. Professor, DMV, DSc Dept. Exotic and Dangerous Viral infections, National Diagnostic Research, Veterinary Institute, Sofia	5
Ivan ZARKOV	Assoc. Professor, DVM, DSc Department of Virology, Faculty of Veterinary Medicine, Thracian University, Stara Zagora	1
Lora SIMEONOVA	Research Scientist, MS Department of Virology, MICB	2
Lilyana WASSILEWA	Professor, PhD	1.5
Gabriela GUJGULOVA	Research Assistant, MS Dept. Exotic and Dangerous Viral infections, National Diagnostic Research, Veterinary Institute, Sofia	2.5
Lucia MUKOVA	Research Assistant, MS Department of Virology, MICB	1.5
Galya GEGOVA	Research Assistant, MS Department of Virology, MICB	1
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
Philippe BUCHY	Head Virology Unit	1
Horm Srey VISETH	Research Assistant	1
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
E. ALBINA	Scientist	0.1
P. GIL	Technician	2.6
Participant 9 – Wuhan Institute of Virology (WIV)		

Permanent staff:		
Tianxian Li	Professor	4
Personnel funded by the project:		
-	-	-

WP7

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana BURGUIÈRE	Medical microbiologist	0.3
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.1
Frédéric FICHENICK	Laboratory technician	0.1
Gilberte CORALIE	Laboratory technician	0.1
Christophe BATÉJAT	Laboratory technician	0.1
Amélie DUBLINEAU	MSc student	0.2
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
Vincent DEUBEL	Principal Investigator	1
Paul ZHOU	Principal Investigator	1
Cheguo CAI	Research Assistant	2
Wei WANG	Research Assistant	4
Yimei ZHENG	Administrative Assistant	1
Participant 9 – Wuhan Institute of Virology (WIV)		
Permanent staff:		
Hualin Wang	Associated professor	4
Personnel funded by the project:		
-	-	-

WP8

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana BURGUIÈRE	Medical microbiologist	0.1
Frédéric FICHENICK	Laboratory technician	0.1
Gilberte CORALIE	Laboratory technician	0.1

Christophe BATÉJAT	Laboratory technician	0.1
Amélie DUBLINEAU	MSc student	0.2
India LECLERCQ	University Associate Professor	0.2
Personnel funded by the project:		
-	-	-
Participant 7 – Institut Pasteur de Lille (IPL)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-

WP9

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana BURGUIÈRE	Medical microbiologist	0.2
Amélie DUBLINEAU	MSc student	0.2
India LECLERCQ	University Associate Professor	0.2
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0.3
Personnel funded by the project:		
-	-	-
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
Sirenda VONG	Head Epidemiology Unit	4
Personnel funded by the project:		
-	-	-
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
L. CHAMAILLE	Trainee	5
F. GOUTARD	Scientist	4
Participant 8 – Interdisciplinary Centre for Mathematical and Computational Modelling (ICM)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
Jan RADOMSKI	Senior Researcher	14
Franciszek RAKOWSKI	Research Assistant	5
Kamil WALAS	Student Trainee	2
Jan RADOMSKI	Senior Researcher	1/3
The 1/3 person-month reported here corresponds to time spent travelling, attending meetings, etc. (Management activities). No extra personnel costs were charged.		

WP10

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana BURGUIÈRE	Medical microbiologist	0
India LECLERCQ	University Associate Professor	0
Jean-Claude MANUGUERRA	Senior scientist, Head of Laboratory	0
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
Philippe BUCHY	Head Virology Unit	0
Personnel funded by the project:		
-	-	-
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
-	-	-
Participant 7 – Institut Pasteur de Lille (IPL)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 8 – Interdisciplinary Centre for Mathematical and Computational Modelling (ICM)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
Jan RADOMSKI	Senior Researcher	1/3
The 1/3 person-month reported here corresponds to time spent travelling, attending meetings, etc. (Management activities). No extra personnel costs were charged.		

WP11

Name	Position (professional category)	Person-Months spent on the RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate University Professor	0.5
Ana Maria BURGUIÈRE	Medical Biologist	1
Jean-Claude MANUGUERRA	Head of Laboratory	2
Sophie ABLOTT	Contract manager	5
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 3 – The Stephan Angeloff Institute of Microbiology (MICB)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
Philippe BUCHY	Head Virology Unit	1
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
D. MARTINEZ	Scientist	0.9
E. ALBINA	Scientist	0.3
C. HO	Financial Project Officer	0.6
Participant 7 – Institut Pasteur de Lille (IPL)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 8 – Interdisciplinary Centre for Mathematical and Computational Modelling		

(ICM)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
Jan RADOMSKI	Senior Researcher	1/3
The 1/3 person-month reported here corresponds to time spent travelling, attending meetings, etc. (Management activities). No extra personnel costs were charged.		
Participant 9 – Wuhan Institute of Virology (WIV)		
Permanent staff:		
Ze chen	Professor	2
Personnel funded by the project:		
-	-	-

Coordination Activities

(Comments on communication between partners, project meetings, possible cooperation with other projects/programmes, if any)

The kick off meeting was very successful in starting the collaboration between the RIVERS consortium as contact has then been properly established between teams from that point in time. Contract related and financial matters have dragged slightly and somewhat delayed the proper start of the project in some cases. Overall, communication between partners has been fairly good and also in a constructive matter. We felt that partial meetings and face to face meetings were useful and some were organised in Europe, one being scheduled in Asia between M20 and M22. A meeting involving all partners will soon be scheduled (M24 probably).

Because the avian virus pool and the set of viruses present in the environment are inter-related (or supposedly so), it is important to link RIVERS with other program. This is done in particular with the CIRAD led Gripavi (www.avian-influenza.cirad.fr/projects/research/gripavi) which focuses on Influenza virus circulation in wild and domestic birds and which is the channel through which CIRAD collects environmental samples for RIVERS (in Africa). Other links will be considered with other EU FP6 projects between M24 and M36.

List of meetings held during the period 01/02/2007- 31/07/2008

Meeting name	Meeting date and place	Participants
Kick-off meeting	12/02/2007 - Institut Pasteur, Paris, France	All
European partners meeting	26/02/2008 - Institut Pasteur, Paris, France	IPP, IPL, ICM, IC, MICB, CIRAD
Partial European partners meeting	23/05/2008 - Institut Pasteur de Lille, Lille, France	IPP, IPL, ICM

Other meetings, visits and teleconferences are detailed in the WP11 'progress towards achieving the objectives' section and in Annex section 3.

SECTION 4 – OTHER ISSUES

IF RELEVANT

Projects which were subject to requirements and/or recommendations concerning ethical issues.

NOT RELEVANT.

ANNEX – PLAN FOR USING AND DISSEMINATING THE KNOWLEDGE

SECTION 1 – EXPLOITABLE KNOWLEDGE AND ITS USE

Exploitable knowledge (description)	Exploitable product(s) or measure(s)	Sector(s) of application	Timetable for commercial use	Patents or other IPR protection	Owner & Other Partner(s) involved
Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable

SECTION 2 – DISSEMINATION OF KNOWLEDGE

The following table shows all dissemination activities undertaken by all RIVERS partners during the first reporting period (01/02/2007 to 31/07/2008).

Date	Type[1]	Type of audience[2]	Countries addressed	Size of audience	Partner responsible/ involved
28/02/2007 – 31/07/2008	RIVERS Website	General public and scientists	Worldwide	Unknown	IPP
14/03/2008	Seminar of the CINDEX group	Association of physicians for health at work of major French Companies	France	50 people	IPP

SECTION 3 – PUBLISHABLE RESULTS

List of RIVERS-related publications during the period 01/02/2007- 31/07/2008

PARTNER 1 – Institut Pasteur Paris (IPP)
As the data generating phases of WP0 and WP5 started with some delay, no publication has been produced as yet.
PARTNER 2 – Institutul Cantacuzino (IC)
As the data generating phases of the WP led by IC was due to start in the second half of the project, no publication has been produced during the first 18 months. However during M19, a first poster was presented at the Third European Conference on Influenza in Villamura, Portugal (13-17 September 2008) and will be reported in the final activity report.
PARTNER 3 – The Stephan Angeloff Institute of Microbiology (MICB)
As the data generating phases of WP6 started with some delay, no publication has been produced as yet.
PARTNER 4 – Institut Pasteur du Cambodge (IPC)
Vong S., Ly S., Mardy S., Holl D., Buchy P. (2008). Environmental contamination during Influenza A virus (H5N1) outbreaks, Cambodia, 2006. Emerg Infect Dis., 14:1303-1305
PARTNER 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)
As the data generating phases of the WP led by IPS was due to start in the second half of the project, no publication has been produced during the first 18 months except a manuscript sublitted for publication on Influenza pseudoparticles to be used in WP0 and WP7.

[1] *Press release (press/radio/TV), Media briefing, Conference, Exhibition, Publications, Project website, Posters, Flyers, Direct e-mailing, Film/Video, etc.*

[2] *General public, Higher education, Research, Industry, etc.*

PARTNER 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)
A big part of data produced by this partner is dependent on another program (GRIPAVI) and publication will be reported in the final activity report.
PARTNER 7 – Institut Pasteur de Lille (IPL)
As the data generating phases of WP1 and WP8 started with some delay, no publication has been produced as yet.
PARTNER 8 – Interdisciplinary Centre for Mathematical and Computational Modelling (ICM)
1- T.Zuk, F.Rakowski, and J.P.Radomski, "*Model of* Influenza *virus spread as a function of temperature and humidity",* submitted to the **Computational Biology and Chemistry** 2- F.Rakowski, M.Gruziel, M.Krych, and J.P.Radomski, *"Very large scale social mobility model - a Monte Carlo countrywide simulation study for Poland"*, submitted to the **Journal of Royal Statistical Society** 3--J.P.Radomski, and P.P.Slonimski, "ISSCOR: Intragenic, Stochastic Synonymous Codon Occurrence Replacement – a new method for an alignment free genome sequence analysis", submitted to the **Comptes Rendus Biologies**
PARTNER 9 – Wuhan Institute of Virology (WIV)
Characterization of H5N1 Influenza A viruses isolated from domestic green-winged teal ***Virus Genes*** revised paper with minor revision Characterization of H5N1 IVs Isolated from Migratory Birds in Qinghai Province of China in 2006 ***Avian Diseases*** 51:568–572, 2007 Circulation of multiple genotypes H5N1 avian IVs in central China during the year 2006-2007 In preparation Characterization of an avian Influenza virus of subtype H10N8 isolated from water of Dongting Lake In preparation

Collaborations, visits, training of people, exchange of materials

- **Visits**

Type of travel	Date
Meetings between ICM and CIRAD in CIRAD, Montpellier, France	20/03/07-25/03:07
Meetings between ICM and CIRAD in CIRAD, Montpellier, France	03-09-06/09:07
Meeting between ICM and IPL in in Inst. Pasteur, Lille, France	22.10.07
Meetings in Inst. Pasteur, Paris	30/10/07 and 08/11:07
Meeting in Inst. Pasteur, Paris	11/12/07
Preparation of RIVERS partial meeting by ICM and IPP in Inst. Pasteur, Paris	25/02/08

- **Exchange of material**

A HPAI (H5N1) strain isolated from a duck in IPC was given to IPP and subsequently to IPL. A strain of Influenza A (H5N1) isolated in humans in Hong Kong was transferred from IPP to IPL.