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Project acronym: RIVERS

"Resistance of Influenza Viruses in Environmental Reservoirs and Systems"

Instrument: Specific Targeted Research Project

Thematic Priority 8.1B

Periodic Activity Report

2nd Reporting Period

Period covered: From **01.08.2008** to **31.01.2010 (M19-M36)**

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Project coordinator: Jean-Claude MANUGUERRA

Project coordinator organisation: Institut Pasteur, Paris (France)

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PUBLISHABLE EXECUTIVE SUMMARY (PES)

The surge of the global avian Influenza epizootic caused by the genotype Z high pathogenic Avian Influena 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 http://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 AIV 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.

As the influenza pandemic due to a swine origin A(H1N1) influenza virus started to unfold, our project was hindered by most partners being involved in the laboratory identification of the novel virus in their respective country. Moreover, as soon as this outbreak started, the RIVERS project coordinator decided after asking for permission to the EU to incorporate the new A(H1N1)v virus in its experiments.

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

Table PES-1 – RIVERS Consortium

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.

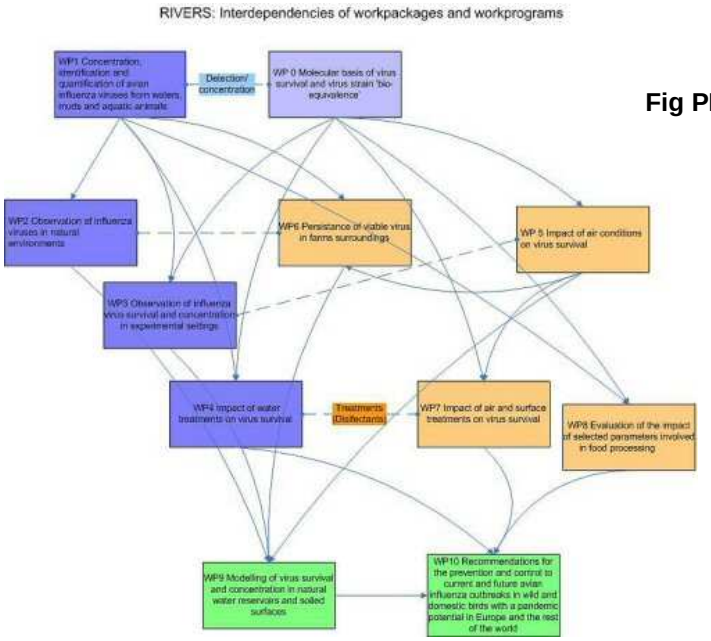


Fig PES-1

WPG 1 - Survival of Influenza A(H5N1) viruses in waters

WP0- Molecular basis of virus survival and virus strain "bio-equivalence

Our first objective was to establish criteria for bio-equivalence in relation to virus survival between Influenza Virus (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. As described in the Periodic Activity Report M1-M18, we were not able to identify the most resistant strain in a given environment.

To determine whether the avian or mammalian origin of the cell hosting the virus influence IVs survival properties, we planned to work with CE (CE) fibroblasts (avian system) and SK93/2 cells (mammalian system). Because CE fibroblasts were not easily cultivable for extended periods of time, during the M19-M36 period, we decided to work with different avian cell lines, i.e. DF1 and QT6 cells derived from chicken and quail fibroblasts respectively. For mammalian cells, we used the well characterized MDCK cell line to grow influenza viruses. H5N1 viruses such as A/Hong Kong/156/97 (H5N1) –human- and A/Duck/Cambodia/2006 (H5N1) –avian-were grown on MDCK, QT6 and DF1 cells. Human viruses, A/New Caledonia/20/99 (H1N1) and A/Wyoming/03/2003 (H3N2) were also grown on MDCK cells as well as on avian cells in order to compare their survival kinetics in different environmental conditions. Survival experiments in water with those different viruses are currently in progress.

In addition, at the beginning of the A(H1N1) outbreak in April 2009, we decided to evaluate the survival of the new pandemic virus in the environment. Influenza A virus survival in different environmental conditions is dependent on virus isolates even within the same subtype. Thus, the results obtained with various subtypes could not be extrapolated without careful consideration, to the current H1N1 pandemic (H1N1pdm) virus. Our results showed that this virus has the ability to persist in water and on glass surface for extended periods of time (see WP5 for data obtained on a smooth surface). At low salinity levels (0 and 5 ppt), maximum survival times in water varied between 400 and 1800 days. Increasing environmental temperature and salinity level had a strong negative effect on the survival of the H1N1pdm virus which retained its infectivity no more than 2 days at 35℃ and 270 ppt of salt. However, for lower levels of salinity, mostly encountered in the environment, the virus remained infectious for at least 10 days in water. Our results are in agreement with previously published data generated with others influenza virus subtypes especially those generated by WP8. Additional experiments suggested that external viral structures in direct contact with the environment must be involved in virus loss of infectivity. All the data generated by this study are described in a full research paper entitled "Long-lasting Persistence of the Novel Pandemic Influenza A(H1N1) Virus in the Environment" which has recently been

submitted for publication. The article will be available on RIVERS website as soon as it is acceped for publication. This work has been presented as an oral communication at the Journées Francophones de Virologie, March 2010.

The main aim of WP0 was to study the molecular basis of virus survival. Firstly, based upon data described above, we decided to work with lentiviral pseudoparticles expressing mutated HA and NA surface proteins. This choice was 'validated' by the results briefly mentioned above suggesting that external viral structures in direct contact with the environment must be involved in virus loss of infectivity and in order to free ourselves from BSL3 conditions and reverse genetic experiments. We first decided to focus on the role of HA glycosylation and composition of the lipid bilayer on virus survival. Lentiviral pseudoparticles system required technical adjustments and is now currently available. Survival experiments with this system are currently in progress.

WP-1 Concentration, identification and quantification of avian IVs from waters, muds and aquatic animals

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. MDCK cells 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® has been retained for purification of viral RNA from 140 µL of viral sample. 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 standard protocol (see Table WP1-1).

Within WP2 and WP6, CIRAD (Partner 6) has adapted and validated four quantitative real-time PCR assays for AIV detection (type A, H5, H7, N1). The performance of the PCR assays was assessed on several thousands of swab samples collected on wild and domestic birds in Africa and also on bird faeces collected in the field. In conclusion, the improved one-step real-time RT-PCR assay described here provides a rapid, specific and sensitive method to detect M, H5 and N1 genes. A procedure was written and disseminated to different RIVERS partners (P001RIVERS - RT-PCR detection of avian Influenza A viruses H5N1).

Development of a viral concentration procedure from surface waters (Task 2) and Validation of concentration and quantification methods on surface water (Task 3)

Following the main achievements of the M1-M18 period reported previously, the procedure for concentration of AIVs from water was finally validated by using it to analyse environmental waters, sampled in Cambodia and in France (see WP2).

Possibly contaminated waters were previously sampled by IP Cambodia, in ponds and lakes near contaminated villages and farms, during H5N1 outbreaks in 2007 and 2008. These samples were kept at 4°C and then were analysed. Moreover, waters were sampled in 2009 from two places which were involved in previous outbreaks in 2006. Unfortunately, no Influenza virus was detected. During autunm migration in 2009, waters from Dombes ponds (Ain, France), where many birds had tested positive to highly pathogenic H5N1 in early 2006, were analysed in IPL. This method allowed the detection by RT-PCR of Influenza A virus (M gene detection) in several ponds in November, but none belonged to the H5N1 subtype. An oral communication « Optimisation et validation d'une méthode de concentration et de détection de virus Influenza A dans l'eau de surface, basée sur les méthodes utilisées pour la détection des virus entériques » was submitted to Journées Francophones de Virologie, March 2010.

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.

<u>Development of a direct extraction method of Influenza A virus from mud</u>

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 was high enough. Firstly, the effectiveness of a direct extraction protocol has been evaluated, based on a previously used protocol. Sterile water was artificially contaminated with virus in parallel to mud and subjected to extraction procedure. The efficiency of virus recovery from mud

was calculated in comparison with virus recovered from sterile water. Our 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.

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

Extraction methods are mainly used for the detection of enteric viruses in sludge from wastewater treatment plants. They are 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. Previously, experiments were performed to evaluate ten different protocols based on literature data with Influenza H1N1 virus (PR8), used as a model then with A(H5N1) virus strains. As an end result of this task, an operating procedure was written and diffused to different RIVERS partners (P003RIVERS - Procedure for concentration of AIVs from mud).

A poster « Optimisation et validation d'une méthode d'élution/concentration et de détection de virus Influenza A, H5N1, à partir de boues » was submitted to Journées Francophones de Virologie, Mars 2010. A publication (probably for submission to "Applied and Environmental Microbiology") will be written in 2010.

WP-2 Observation of IVs in natural environments

In China

The research in M19-M36 contained five parts including the three following.

1- Sample collection and virus isolation. We continued to collect the water samples, fecal samples, cloacal samples of the backyard poultry in Dongting lake wetland and in the living poultry market in the city around the wetland;

2- DNA sequencing and phylogenetic analysis of the whole genomes of the selected isolates. The whole genomes of the selected isolated viruses were sequenced and the phylogenetic analysis was undertaken.

In order to study the molecular characteristics and the genetic relationship between the isolates in this study and other H5N1 viruses, the whole genome of 14 of the H5N1 isolates were sequenced, based on which the phylogenetic trees were generated. The full-genome sequences of the 14 H5N1viruses isolated in this study are available from GenBank. In addition, the whole genomes of 8 viruses of the H9N2 subtype isolated in this study were sequenced and all sequences are available from GenBank. The nucleotide sequences for the viral genome of the H10N8 isolate have been submitted to GenBank and are available under accessing numbers. Phylogenetic analysis of the H5N1 viruses showed that the viruses isolated in Dongting Lake wetland in various years, even in various seasons of the same year, were of different sublineages or clades, suggesting multiple introductions of influenza viruses in this region.

3 - Observation of persistence of AIV H5N1 and H9N2 in natural water. The duration of persistence of two subtypes of AIVs (AIVs) H5N1 and H9N2 was studied in four natural waters of China. Among them, Qinghai Lake, Poyang Lake and Dongting Lake are important habitats for migratory birds, and we also took the Yangtze River in Wuhan section (table WP2-1). The infectivity of the AIVs was tested throughout the sample period and the TCID50 was calculated using Reed-Muench method. Then, we also calculated the equation of the viral TCID50 regression at various temperatures and evaluated the duration of persistence, which provided insights into the pandemic risk of the AIVs in the natural water. In addition, the arameters of the natural water of Dongting, Poyang, Changjiang (Fig. WP2-1) and Qinghai water have been tested (table WP2-1).

We regularly tested the infectivity of the H5N1 virus and the H9N2 virus in four natural water samples at different temperatures. The results derived from regression models (Fig.WP2-2 and Fig.WP2-3) showed that the temperature could impact the persistence of AIVs in water significantly and the presence of microorganisms in water had a negative effect on viral persistence in water, but not significantly. At 4℃ and16℃, the AIVs had a much longer duration of persistence in freshwater lakes Dongting, Poyang, Yangtze River) than in salted lakes (Qinghai), which showed that high salinity can inhibit the persistence of the AIVs. At 4℃, H5N1 and H 9N2 viruses could retain their infectivity for at least 30 days in the Milli-QH2O and the freshwater lakes, whereas they only survived 20 days in salted lake.

The Dongting Lake and Poyang Lake are important habitats and overwintering areas along the East Asia migration route for migratory birds. The low-temperature environment in this region allows the AIVs to survive in water for a long time, which greatly increase the probability for other migratory birds or poultry to be infected with influenza viruses still present in the same waters.

Qinghai Lake is located in highland areas in northwest China and the low-temperature is all year round. In 2005 the first time outbreak of the highly pathogenic avian influenza H5N1 virus in migratory birds in Qinghai Lake has

aroused the concern of many researchers around the world. Some researchers have speculated the mode of transmission of the AIV in migratory birds in Qinghai Lake, but few studies have reported the approach of the virus transmitted through the Qinghai Lake water. Our research have confirmed that the virus could persist in Qinghai Lake about 20 days, even at 16℃ and was al so able to survive 7 days or so, which strongly suggested that the AIVs could be transmitted in the migratory birds through the fecal-contaminated water on shared aquatic habitats.
Dongting Lake and Poyang lakes frequently exchange water with the Yangtze River, so in autumn and winter, the contaminated water of the Dongting Lake and Poyang Lake is likely to enter the Yangtze River and is carried to the wider region along the Yangtze River. Our results showed that the AIVs can persist for at least 30 days at 4℃, even at the higher temperature of 16℃, the AI Vs were still able to survive more than 10 days which also proved theoretically that the non-biological factors (flowing water) could be a mediator for the rapid spread of the influenza virus.

In Cambodia

In response to the notification of a confirmed case of influenza subtype H5N1 infection in humans or poultry, we studied the environmental contamination by the H5N1 virus. The investigation was conducted in some households and their surroundings in the vicinity of the index household. Environmental specimens such as mud, pond water, water plants, soil, insects, aquatic animals and swabs were collected. Swabs were placed in virus transport medium; all environmental samples were transported at 4℃ within 36 hours to Instit ut Pasteur in Cambodia for subtype H5N1 testing by real-time quantitative RT-PCR (qRT-PCR) and for virus isolation after inoculation into embryonated chicken eggs. Each year from 2007 until now, the environmental investigations were conducted in the location with influenza virus (H5N1)–associated outbreaks.

H5N1 outbreak in 2009 and 2010

In December 2009, one confirmed human case of H5N1 was reported from the province of Kampong Cham, in a village where a previous H5N1 outbreak had already been reported in 2007. This case was non fatal. A total of 82 specimens were collected in the location surrounding the index household with 37 out of these are now in process of analysing to investigate the possible environmental contamination of the H5N1 virus. At least 11 samples were H5 gene positive. These positive samples are currently processed for viral isolation.

At the beginning of 2010 in Takeo province, which is located in the south next to the Vietnamese border, an outbreak of H5N1 AIV was reported in poultry. This outbreak caused thousands of poultry deaths in a single village. The sampling process was conducted in the surroundings of the households and in the field of villagers. A total of 62 environmental specimens were collected and out of these 28 were analysed by qRT-PCR. Of these 28 specimens 12 were H5 gene positive. These positive samples are currently processed for viral isolation

WP-3 Observation of Influenza virus survival and concentration in experimental settings

A series of 3 experiments have been conducted in 1/ aquaria and 2/ in jars with fish.

Experiments with aquaria: In these experiments, 3 to 10 aquaria of various volumes were used.

The artificial aquatic biotopes were created using natural water and mud collected from natural spots where outbreaks of A(H5N1) HPAI have been reported.

The first experimental settings in aquaria showed the absence of viral RNA detectable in all specimens of mud, plants and aquatic animals. This may suggest that the virus quantity used at 10^7 EID50 in 20 litres of pond water in aquarium was insufficient to infect the water and other materials in it. Another possible explanation could be that the environmental conditions (temperature,…) applied were not favourable for the virus survival.

In the second series of experiments with duck faeces in the aquaria, no infectious virus could be detected in any type of water at day 3 and day 7 post-infection(PI), even though the viral RNA was present in these specimens. The quantity of viral RNA detected in the same type of water was not different at 32ºC and at 25ºC. However, the amount of viral RNA detected seemed to differ with the type of water (lake or distilled water): viral RNA was more detected in distilled water then in lake water. In this experiment, the survival of H5N1 in water was not demonstrated even though viral RNA was detected in the water until at least 3 days after viral inoculation. The contamination of aquatic plants and animal has not been detected.

In the third series of experiments, aquaria containing rain water and molluscs but no mud were tested at a temperature of 25℃. The organs taken from mollus cs of the infected aquarium on days 2, 3, 4 and 6 were all

H5 gene positive. Furthermore, infectious viral particles were successfully isolated from these specimens. However, in one aquarium (infected), because of the rapid death of the molluscs, no viral detection could be done later than 6 days PI. Daily deaths were observed, and all molluscs had died by day 6. As for the water, the analysis of the water sampled from the infected aquarium showed that infectious virus particles could be isolated on day 3 PI.

Experiments with jars with fish in them: Three different experimental settings wre used in these 3 series of experiments conducted in jars with fish.

The 20-day long first experiment was done by using 14 jars containing 500 mL of rain water each, with one fish and one tadpole in each jar. Of these jars, two were used as negative controls in which the water was not infected and the 12 other jars were infected with the H5N1 virus A/Ck/CAM/LC1AL/07 strain. Fish and tadpoles were collected from two jars on days 1, 3, 7, 11, 15 and 20 (until all jars were empty) and dissected to collect different organs. Tadpoles appeared not to be very resistant in these experimental conditions. Many of them died at day1-day4 and only two of them survived until day 13 and day 14. We collected 98 samples on fish, 15 samples on tadpoles and 134 water samples. The analysis of the specimens collected on the tadpoles showed the presence of viral RNA in these samples from day 1 until day 14. The viral RNA load was similar in all tadpole organs. Infectious viral particles could only be detected by viral isolation on the specimens of gills and skin on day 1 of the experiment. Viral RNA was detected in different organs of fish during 15 days with a quantity of 10^4-10^5 copies/g. However, infectious viral particles were isolated in specimens of fish only on day1 of the experiment. The quantity of viral RNA seemed to be slightly higher in the gills than in the other organs of the fish. Globally, aquatic animals such as fish and tadpoles were contaminated by the virus from the infected water and carried the infectious virus only for 1 day although viral RNA was detected at least for two weeks.

The second experiment lasted 12 days and consisted in using 14 jars containing 500 ml of rain water each, with one male fish in each of 7 jars and one female fish in each of the remaining 7 jars. In each 7-jars-group, two were used as negative controls (non-infected). In 5 of the jars containing male fish, the water was infected on day0 by an inoculation of avian H5N1 virus. These male fish were kept in the infected water for 5 days and were then transferred to 5 new jars containing non-infected rain water for 1 day. They were then finally put for 6 days in 5 of the jars containing female fish. The male fish in the 2 control jars were also put in the 2 control jars containing female fish. This experiment showed that fish in infected water were contaminated by the virus. Even though the survival of infectious virus in fish was not demonstrated, different amounts of viral RNA were detected in different organs. Furthermore, the contaminated male fish may have transmitted the virus to the female fish, since viral RNA was also detected in different female fish organs. The survival of the virus in water was confirmed for only 2 days although the viral RNA was detected at least for 5 days.

The third experiment series also lasted 12 days and consisted in using 16 jars containing 500 ml of rain water each, with one male fish in each of 10 jars and one female fish in each of the remaining 6 jars. In both groups (male and female), 2 jars were used as negative controls (non-infected). In 8 of the jars containing male fish, the water was infected at day0 by a viral inoculation of avian H5N1 virus. On days 1, 3, 5 and 7 post-viral inoculation, one male fish was taken out of an infected jar and dissected. Another four male fish in infected water were kept for 5 days in the jars, were then transferred to 5 new jars containing non-infected rain water for 1 night, and were finally put into 4 of the jars containing female fish. On days 1, 3, 5, and 6 post-exposure, one male and female were collected from each of these jars to be dissected. The male fish in the control jars were also put into the jars containing female fish.

A total of 147 specimens were collected and analysed: 21 specimens of water from the jars, 27 specimens of fish faeces and 99 specimens of fish organs. The presence of viral RNA was detected in 22 out of 99 specimens of fish organs. This positive detection was found only in some organs of the infected fish collected from the infected jars. The viral RNA detected seems to be in higher quantity in the gills. Positive samples (for RNA detection) were then tested for viral isolation, but no infectious particle could be isolated. All male fish kept for 5 days in infected water then a few days (d1, d3, d5 and d6) in non-infected water with female fish from non-infected water were all negative for viral RNA. And all exposed female fish were also negative for viral RNA. So no further analysis by viral isolation was done on these specimens.

The specimens of fish faeces collected (day1 to day 5) from the jar containing infected water were positive for viral RNA. Moreover, infectious viral particles could be isolated from two specimens collected on day 3 PI. Further analyses performed on the fish faeces collected on days 1,3, 5 and 6 post-exposure, from jars where the exposure part of the experiment took place, showed that only one specimen collected on day 3 post-exposure was positive for viral RNA detection with no infectious particle isolated. The rest of these fish faeces specimens were negative for viral RNA. Viral RNA was detected in the water directly inoculated with virus. Results of viral isolations performed on these samples are pending.

WP-4 Impact of water treatments on virus survival

Our work continued to assess the impact of the aldehydes agents against the AIV in standing and running water. The action of the UV light was assessed also. Viability of the influenza virus was tested by qRT-PCR at 72 hours after inoculation of samples in MDCK cells. The results suggest that glutaraldehyde shows a good efficacy of destroying virus infectivity in relation with environmental temperature. Ultraviolet radiation light has been proven to inactivate AIVs in 10 - 20 min in experimental condition if the viral loading is 10^3 $TCID_{50}$/0.1 ml and the effective dose of irradiation is at least 3000 J/m^2.

The persistence of the infective viral particles of the same influenza reassortant was evaluated in three kinds of water: rivers, lake and Black Sea water, and PBS as control, in accordance with their main characteristics, at three temperatures: 4-8 ^oC; 22 ^oC and 35 ^oC. Viability of the influenza virus was tested periodically by microELISA technique on MDCK cells. Our results show that the viability of the virus is maintained at least 20 days at low temperature and 12 days postinfection at higher temperature.

WPG 2 Survival of Influenza A(H5N1) viruses in air, surfaces and food products

WP-5 Impact of air conditions on virus survival

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, as rough and porous surfaces. Moreover, we obtained from a major worldwide airline company 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. Assays were preformed in triplicate and several conditions of time and speed, in order to validate the best method for virus recovery, were tested.

On porous surfaces: The ratios of virus recovery on weaved tissue handkerchiefs were estimated by $TCID_{50}$/mL and for fabrics used in passenger cabins in civil aeronautics by $TCID_{50}$/mL and qRT-PCR (M gene). For viral recovery on weaved tissue handkerchiefs we chose 30 seconds at 4(meter per second) (M/S) conditions. In fact these conditions allowed recovery of virus with a loss of $10^{0.59}$. For fabrics used in passenger cabins we chose 30 seconds at 6M/S conditions. In fact these conditions allowed recovering virus with a loss of $10^{3.79}$.

On smooth surfaces: Detection and titration of virus infectivity in the course of time (from 5 min. to three days) at given temperatures (4℃, 25℃ and 35 ℃) (Task 4) were carried out after using the strat egy previously chosen for virus recovery on watch glasses.

The assay with Influenza H5N1 virus was performed at 3 temperatures (4℃, 25 and 35℃) and survival evaluation was performed by titration by $TCID_{50}$/mL and compared with qRT-PCR for M gene after 1 day, 2 days and 8 days.

As already explained in Work Package 0 progress, we decided to study the survival of the new A(H1N1) pandemic virus in the environment, because Influenza A virus survival in different environmental conditions is dependent on virus isolates even within the same subtype. Our results showed that this virus has the ability to persist on glass surface for extended periods of time. On smooth nonporous surface, the H1N1pdm virus retained its infectivity for at least 4 days at 35°C and up to 56 days at 4℃. All the data generated by this study are described in a full research paper entitled "Long-lasting Persistence of the Novel Pandemic Influenza A(H1N1) Virus in the Environment" which willhas recently been submitted for publication.

Currently, the scientific community has still a limited knowledge on many aspects of the ecology and environmental properties of influenza viruses in urban settings. Studies on the survival of influenza viruses in the atmospheric environment and on inert surfaces are rather limited and difficult to interpret because of the methodological difficulties. Further studies are needed in order to develop suitable control strategies to prevent risk of infection. Consequently, conducting environmental studies on the resistance of influenza viruses has been an important focus of WP5. In this context, the underground public transportation in Paris appeared as an

essential sampling site because of its representativeness of the urban environment and its convenience for sampling (confined space, dry air and high density of population). The aim of the present study was to determine whether respiratory viruses, particularly human influenza viruses or respiratory syncytial virus, could be detected in the air and on inert surfaces of subway trains at the peak of the influenza virus epidemic in metropolitan France. Samples were taken twice a week throughout the active circulation of influenza viruses in the northern hemisphere, during rides within the busiest lines of the Parisian subway network. A standalone portable device was used to collect air samples on gelatin membranes. Surface smears were taken on door handles and bars of the subway cars using nylon swabs. A total of 72 air samples and 150 surface samples were collected. Searches for influenza viruses or respiratory syncytial viruses were performed by standardized quantitative and classical RT-PCR methods. Analysis revealed that one air sample was positive for influenza A virus. Unfortunately, virus culture using MDCK cells did not lead to virus isolation.

WP-6 Persistence of viable virus in farms surroundings

In Asia (Cambodia)

As explained for WP2, in Cambodia, in response to the notification of a confirmed case of influenza subtype H5N1 infection in humans or poultry, the environmental contamination by the H5N1 virus was studied. The investigation was conducted in households surroundings in the vicinity of the index household. Environmental specimens such as mud, pond water, water plants, soil, insects, aquatic animals and swabs were collected and processed as indicated in WP2. each year since the beginning of the RIVERS project and until now, the environmental investigations were conducted in the location with influenza virus (H5N1)–associated outbreaks.

The main activities carried out during the second 18-months period were related to WP6 and as well as 2 and 9. Two directions were followed (1) improvement and standardisation of the molecular detection methods for AIV and (2) contribution to the development of models for virus survival in its natural environments.

Within the framework of this project, the following activities were undertaken in Montpellier and in Cambodia in coordination between the Pasteur Institute of Phnom Penh and the UR AGIRs of CIRAD:

- Publication of an article in the International Journal of Applied Earth Observation and Geoinformation, Tran, A., et al., Remote sensing and avian influenza: A review of image processing methods for extracting key variables affecting AIV survival in water from Earth Observation satellites. Int. J. Appl. Earth Observ. Geoinform. (2009), doi:10.1016/j.jag.2009.09.014 Recent studies have highlighted the potential role of water in the transmission of AIVs and the existence of often interacting variables that determine the survival rate of these viruses in water; the two main variables as studied as shown in the other WPS of the RIVERS project are temperature and salinity. Remote sensing has been used to map and monitor water bodies for several decades. In this paper, we review satellite image analysis methods used for water detection and characterization, focusing on the main variables that influence AI virus survival in water. Optical and radar imagery are useful for detecting water bodies at different spatial and temporal scales. Methods to monitor the temperature of large water surfaces are also available. Current methods for estimating other relevant water variables such as salinity, pH, turbidity and water depth are not presently considered to be effective.

- *Our work in 2008 allowed us to identify environmental indicators in connection with floods and landuse, from MODIS data. These indicators are linked to epidemiological data in order to identify risk factors for the occurrence of avian influenza cases. A model to detect water surfaces and periods of floods has been created.*
- *Field data, in Cambodia in several spots known for previous H5N1 outbreaks, have been collected between March and August 2009 (before and at the end of rainy season), in order to validate the model.*
- *June 2009: a mission of modeler war organized in Cambodia in order to work on the H5N1 data available in IPC.*
- *October 2009: start of the analysis of correlation between environmental conditions linked to water and the number of H5N1 cases in Cambodia. This analysis should be finished end June 2010.*

In Europe (Bulgaria)

Prevalence of AIV in Bulgaria

Samples studied were collected from fresh faeces and cloacal swabs from mule ducks and fecal materials from *Anser albifrons* and *Branta ruficollis* along *Via Pontica.* Samples from mule ducks from the following regions in Bulgaria were collected in a specific schedule and were sent for laboratory diagnostics:

1/ Regions of Stara Zagora, Haskovo, Dobrich and Yambol– samples collected and sent five times monthly in the period November 2008 – March 2009. 2/ Regions of Plovdiv and Pazardjik– samples collected and sent two times monthly in the period March 2009 – April 2009.

In the period November 2008 -2009, 22 low-pathogenicity strains of Influenza A viruses were isolated and laboratory confirmed in Bulgaria. All the strains H4N6, H3N2 and H4N2 were isolated from samples from mule ducks, imported from France.

Persistence of AIV in cloacal samples

The low-pathogenic avian influenza A virus (LPAIV) of the H6N2 subtype obtained from a mallard duck was used. Allantoic fluid was collected after inoculation of LPAIV (H6N2 subtype) from 5 to 9-day old CEs (CE). Embryos were observed daily for 120 hours. Allantoic fluid derived from them was explored by haemagglutination assay (HA). Samples with haemagglutin titres of 1:128 were stored at -84℃ until use d in the experiment. Nine mallard ducklings 30–day-old and were used in this experiment. 7 ducklings were intravenously infected with 100 µL allantoic fluid from infected CE while 100 µL allantoic fluid from intact CE was intravenously injected to the other birds (uninfected control group, n = 2). The 2 groups of infected and uninfected birds were kept separately in 4 x 4 m rooms at 1.8 m feeding and watering front, 20℃ and 7 0% humidity.

Fecal swabs from all infected and uninfected birds were collected on day 5 post infection (P.I.) and were stored in riginal form without any further processing. In intervals of two days (2, 4, 6, 8, 10, 12, 14, 16, 18, 20) parts of fecal samples were taken and infectious vitus titers were evaluated by cultivation in CE. Control birds faeces gave always negative results for H6N2 subtype virus for the whole experimental period. The period of avian influenza A virus H6N2 subtype tenacity varied from all infected feces between the 2 to the 8 P.I. days (Table WP6-4).

In the present study, the LPAIV H6N2 subtype virus was successfully isolated from feces from previously intravenously infected ducklings for period to 8 days. Experiments were preformed in specific conditions of storage of fecal swabs and normal bacterial microflora. These parameters of humidity and temperature were considered as optimal for housing of poultry. The period of storage is similar to data published by Lu et al. (2003) – 7 days but these studies were performed keeping temperature without monitoring of humidity. The infectious virus titer decreased sharply after day 4. The transmittion of infection during housing of domestic ducks in closed poultry farms is influenced not only by the period of persistence of virus in feces but by the contact of susceptible birds with faeces infected with virus.

WP-7 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 which was conforted by the results and conclusions from WP0 between M19 and M36. 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, during the M1-M18 period, we had 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 (see first interim report). This tool is now also used by WP0. During the M19-M36 period, delays for this WP were due to the overload of work linked to the A(H1N1) pandemic.

WP-8 Evaluation of the impact of selected parameters involved in food processing

Poultry meats can be contaminated by differents contamination routes. Some data concerning chicken and duck thigh and breast naturally infected by H5N1 were available and were considered when we designed our experiments.

Extraction and detection of AIV in poultry meat (Task 3)

To study survival/inactivation of AIV in poultry products, a protocol for extraction and detection of AIV from poultry meat was based on previously described methods. Recovery of two strains H5N1 and H1N1 was

validated. Preliminary tests were carried out in order to determine conditions to remove cytotoxicity and titration interference, when meat was 50-diluted before titration on cells, and to obtain a good virus recovery from meat. No significant decrease of infectious titres was immediately observed when virus was added in raw or cooked meat with or without NaCl. About 2 10^5 $TCID_{50}$ / ml were recovered when 8 10^5 was inoculated. No cytotoxicity and no interference were observed in the previously described conditions.

Evaluation of individual effect of main parameters, involved in cooking of poultry meats, and their interaction on survival of H5N1 in poultry matrices (Task 1 and 2)

This workpackage consists in evaluating the impact of parameters involved in cooking of poultry meats. Based on results obtained with enteric viruses, chemical and physical factors, such as pH, divalent cations and other environmental solid particles have been reported to substantially decrease sensitivity of viruses to heat inactivation treatment. Influence of pH, salinity and temperature on Influenza virus infectivity in water had been investigated by previous studies. Influenza virus infectivity decreased in water with low pH from 8,6 to 5,8, increased temperature from 4 to 37℃ and high salin ity (3%). Time required reducing infectivity decreased with low pH, increased temperature, and high salinity.

Based on the data available in the litterature, the impact of salt concentrations, ranging from 0 to 4%, on cooking of artificially infected poultry breast at pH 5.5 was evaluated.

With the suitable method for extraction and detection of AIV described in details in this report, quantitative studies of the impact of food processing conditions on AIV was possible. The thermal inactivation procedure was based on available data. Experiments were performed with a H5N1 strainSince thermal inactivation of Influenza viruses have been mainly investigated on chicken meat, four repetitions of experiments were performed in ground duck meat to obtain survival curves. One experiment was however carried out on ground chicken breast, to fit predictive model for poultry matrices. Viral inactivation curves were constructed on viral residual titres measured during the ramp-up period of the heating from 26℃ up to the targeted t emperature of 70℃.

WP-8 Modelling of virus survival and concentration in natural water reservoirs and soiled surfaces

It was already estabilished during the first stage of the project (D9-1) that no commercial software was available to even approximately fulfil the necessary requirements for an agent-based model of IV survival in various environments. Therefore it was decided to construct a modular system of various tools – to act on experimental data as soon as they will became available form other consortium partners.

Two main modules of the final simulation platform were designed, implemented and tested (D9-6, and M9-5). First module realized a country-wide model of social contacts, quintessential for a simulation of influenza spread has been implemented, Particular emphasis was put on contact patterns arising from daily commuting to schools, offices and other workplaces. In order to reproduce the map of contacts, we were using geo-referenced, individual-based models (IBMs). Within this framework, the set of different stochastic algorithms was employed, utilizing available aggregated data. Based on such model system, selected statistical analyses were performed, in particular the accessibility of schools, or location of emergency rescue service units. The results (*Journal of Artificial Societies and Social Simulation, **13** (2010) 13*) show a good agreement with available census data, reproducing well local population densities, average times in transit, and other indicators decisive for assessment of individual contacts and daily mobility patterns.

This platform then served as a base for the next stage – providing social context for a large-scale epidemiological simulations. The second main module involved a construction of an agent-based model for studying the effects of influenza epidemic in large-scale stochastic simulations, together with the resulting various scenarios of disease spread. Simple transportation rules were employed to mimic individuals' travels in dynamic route-changing schemes, allowing for variance of contacts, and their possiblr effects on infection spread. Parameter space was checked for stable behavior, especially towards the effective transmission rate variability. Although the model was based on quite simple assumptions, it allowed observe two different types of epidemic scenarios: characteristic for urban and rural areas. This differentiates it from the results obtained in the analogous studies for the UK or US, where settlement and daily commuting patterns are both substantially different and more diverse. The resulting epidemic scenarios from ABM-simulations were compared with simple, differential equations-based, SIR models – both types of our results have shown strong similarities and correlations (submitted to *Physica A*). The software platforms developed in both stages are currently used in the

next step of the project, employed to study various epidemic mitigation strategies. As the human infectivity data of the H5N1 are as yet unknown, we were testing model assumptions derived mostly from available data on the H1N1 and the H3N2 serotypes. The simulations are scaleable, thus it will be not particularly difficult to transform the model, from it's current capacity, towards countries of different sizes – provided that census data will be available at sufficiently detailed granulation.

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 avian IVs 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.

Table Sec2-1: 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

Main achievements

The main achievements of the first period of the RIVERS project drom M1 to M18 were detailed in the first interim report covering that period. For ths M19-M36 reporting period, the main achievements are detailed here after in this report. Briefly, a number of tools have been finalised or developed in particular protocols ready for dissemination dealing with influenza A(H5N1) virus detection in different kinds of waters, virus concentration methods and ARN extraction techniques from other matrices such as mud or animal derived product susch as chicken meat. Main progresses were also made in designing protocols for the recovery of AIV from smooth and porous surfaces as well as from the air either in natural settings or in experimental systems.

In addition a wealth of data have been collected and analysed from natural environments particularly in China, Cambodia and Bulgaria either ecological systems centred around waterway systems or in farm surroundings. Still ongoing, such data collection will provide critical data to the community and help in drawing guidelines and recommendations.

Despite epidemiological intelligence and microbiological surveillance systems or programs, pathogen emergence mostly occurs by surprise. Influenza A virus offers a prime example of such a situation: the world was bracing itself for a threat due to highly pathogenic A(H5N1) AIV in Asia and instead, it had to confront with a novel reassortant swine-origin influenza A(H1N1) virus (H1N1pdm) with pandemic potential on the American continent. Very few anticipated this situation and hence when the global alert spread worldwide about a new influenza A(H1N1) virus with a novel genetic make up in April 2009, all front line laboratories were taken off guard having to deal with the new virus while being already engaged in programs about A(H5N1) such as this one. We all had to postpone our programs because our involvement in public health issues and asked for a no cost six months extension to RIVERS, which was granted to us. In addition, with the agreement of the EU, RIVERS included the new virus A(H1N1) in the program.

Section 2 – Work Package progress of the period

WORK PROGRAMME I

"*Survival of AIVs 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 (Jean-Claude MANUGUERRA)

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

Our first objective was to establish criteria for bio-equivalence in relation to virus survival between Influenza Virus (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. This deliverable was expected by others WPs for their own experiments.

As described in the Periodic Activity Report M1-M18, we were not able to identify the most resistant strain in a given environment. Persistence of IV strains varied considerably with temperature and salinity. Although IV strains 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.

One of the objectives was to assess a method of virus viability other than virus titration of cell culture ($TCID_{50}$ titre determination). Up to the present, we used $TCID_{50}$ titration method to evaluate virus survival in different environmental conditions. Plaque forming unit assays were done at the beginning of the project but were no longer used for safety and feasibility reasons. As $TCID_{50}$ titration method was relatively time consuming and could introduce variations of 1 log due to the experimental procedure itself, we should test other method of viral viability assessment.

To determine whether the avian or mammalian origin of the cell hosting the virus influence IVs survival properties, we planned to work with CE fibroblasts (avian system) and SK93/2 cells (mammalian system). Because CE fibroblasts were not cultivable easily for extended periods of time, we decided to work with different avian cell lines, i.e. DF1 and QT6 cells derived from chicken and quail fibroblasts respectively. For mammalian cells, we used the so well characterized MDCK cell line, routinely used in our laboratory to grow influenza viruses.

H5N1 viruses such as A/Hong Kong/156/97 (H5N1) –human- and A/Duck/Cambodia/2006 (H5N1) –avian- were grown on MDCK, QT6 and DF1 cells.

Human viruses, A/New Caledonia/20/99 (H1N1) and A/Wyoming/03/2003 (H3N2) were also grown on MDCK cells as well as on avian cells in order to compare their survival kinetics in different environmental conditions.

At the beginning of the A(H1N1) outbreak in April 2009, we decided to evaluate the survival of the new pandemic virus in the environment. Influenza A virus survival in different environmental conditions is dependent on virus isolates even within the same subtype. Thus, the results obtained with various subtypes cannot be extrapolated without careful consideration, to the current H1N1 pandemic (H1N1pdm) virus. Our results showed that this virus has the ability to persist in water and on glass surface for extended periods of time (see WP5 for data obtained on a smooth surface). At low salinity levels (0 and 5 ppt), maximum survival times in water varied between 400 and 1800 days. Increasing environmental temperature and salinity level had a strong negative effect on the survival of the H1N1pdm virus which retained its infectivity no more than 2 days at 35°C and 270 ppt of salt. However, for lower levels of salinity, mostly encountered in the environment, the virus remained infectious for at least 10 days in water. Our results are in agreement with previously published data generated with others influenza virus subtypes.

Additional experiments suggested that external viral structures in direct contact with the environment must be involved in virus loss of infectivity.

All the data generated by this study are described in a full research paper entitled "Long-lasting Persistence of the Novel Pandemic Influenza A(H1N1) Virus in the Environment" which has recently been submitted for publication. The article will be available on RIVERS website as soon as it will be published.

An experiment over a long period (two years) evaluating infectivity by endpoint titration was started in order to have a more precise idea about the survival of four different viruses in water. The strains we used were A/New Caledonia/20/1999 (H1N1), A/Wyoming/03/2003 (H3N2), A/duck/Cambodia/(D4)KC/2006 (H5N1), A/Hong Kong/156/1997 (H5N1). Virus preparations were diluted in distilled water for injectable preparation. On the one hand, the effect of two temperatures (4°C and 25°C) was evaluated on viral survival and on the other hand the effect of four levels of salinity (0, 5, 35 and 270 part per thousand (ppt)) was evaluated at 4°C. Salinity was adjusted using sodium chloride. Saline concentrations were selected to represent natural saline environments. The values of 0, 5, 35 and 270 ppt respectively correspond to the average levels of salinity encountered in river freshwaters, the Baltic Sea, oceans and the Dead Sea.

After 380 days, infectious virus was still detectable whatever the strain at 4°C without salt. The virucidal activity which corresponded to a reduction of 4 $\log_{10}$ of the initial titer according to the European Standarts (NF EN 14476) was estimated using a linear regression model. Strains virucidal activity varied between 1 year and 3 years with lower persistence for A/Hong Kong/156/1997 (H5N1).

At 25°C, virucidal activity varied between 34 and 47 days. In contrast with 4°C, higher virucidal activity was obtained for A/Hong Kong/156/1997 (H5N1).

At 4°C and for a salinity level of 5 ppt, virucidal activity varied between one year and 2 years. For a salinity level of 35 ppt, virucidal activity ranged from three to seven months. At 270 ppt, it ranged from 2 to 3 months.

The effect of initial change on virus infectivity was evaluated for these same four strains and A/Texas/36/1996 (H1N1), A/Beijing/262/1995 (H1N1), A/Sydney/5/1997 (H3N2), A/Victoria/3/1975 (H3N2). Strains were diluted 1: 10 in distilled water for injectable preparation and thirty minutes after dilution an endpoint titration was performed. The difference between the titer obtained after defrosting of viral stock according to the dilution and the titer obtained after thirty minutes was calculated to evaluate the loss of infectivity. Infectivity loss was strain dependant but lower for both subtypes H1N1 and H3N2 than subtype H5N1. Infectivity losses obtained for subtype H5N1 were more variable from one experiment to the other.

In order to compare the effect of initial change according to the liquid medium source on virus infectivity, strains A/Beijing/262/1995 (H1N1) and A/duck/Cambodia/D4(KC)/2006 (H5N1) were diluted 1:10 in distilled water for injectable preparation, in water from duck pond and in cell culture medium. Water was extracted from artificial duck pond situated at Savigny le Temple (Seine-et-Marne, France) on April 5th, 2009. The duck pond had a diameter of 1 meter approximately and a depth of 50 centimetres. The GPS coordinates of the pond is: 48° 36'14'' N, 02° 33'47'' E with an altitude of 92 meters. A female and a male ornamental duck lived in this pound (canard mignon).

Fig- WP0.1: Water collection from small ponds in Ile-de-France

When the sample was taken, the external air temperature was of 17.4℃ and humidity was 43%. At the surface, water temperature was 11℃ and in-depth was 10℃. The UV Index was 4. A 0.5 centimetres of mud was present. A bacterial count was made using trypticase soy agar. Surface and depth water were tested in aerobe and anaerobe. Few bacteria were present.

Before to use water to evaluate viral persistence, water was first centrifuged during 15 minutes at 3000 rounds/minute at 4℃ and then filtered through a Sartorius filter (Minisart) of 0.45 µm. Infectivity losses were low whatever the conditions (distilled water, surface or depth water from duck pond and cell culture medium) and the strains.

The main aim of WP0 was to study the molecular basis of virus survival. 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. Results obtained with H1N1pdm virus suggested that external viral structures are involved in virus survival. We thus decided to focus on the role of HA glycosylation and composition of the lipid bilayer.

Lentiviral pseudoparticles system required technical adjustments and is now currently available. Experimental survival experiments with this system are currently in progress.

In the initial programm, we planned to grow A(H5N1) virus strains belonging to genotype Z and A(H5N1) strains belonging to other genotypes, on cell culture such as chick embryo fibroblasts and SK93-2 (a continuous human cell line derived from melanoma, which is susceptible to human and animal (including avian) IVs) in order to determine whether the lipid bilayer of the virus envelope has an impact on virus survival and whether virus carbohydrate moieties are involved in virus survival. Indeed, besides MDCK cells, we used two continuous avian cell lines: DF1 derived from chicken and QT6 derived from quails instead of fibroblasts from CEs and SK-93/2. After having implemented the new cell lines in our laboratory and adapted them to grow in the presence of trypsine, which is essential for non HPAI viruses, we started the approach by using two human virus strains A/ New Caledonia/20/99(H1N1) and A/Hong Kong/156/97(H5N1) as models before using strains of HPAI Genotype Z H5N1 viruses. We first made viral stocks and performed TICD50 titration using a combination of cell lines for

virus growth and for titration. Now the comparison of virus survival in liquid environment has started and is in good progress.

Deviations from the project work programme, and corrective actions taken

There has been some delay in the development of the study for several reasons. The PhD student in charge of the project really started working on the project at the beginning of March, 2008. Immediately after the beginning of the A(H1N1)v outbreak in April 2009, we decided to undertake survival experiments on A/Paris/2590/09 (H1N1)v strain in water and smooth surfaces as described above. The data collected are the object of the full research paper mentioned above and recently submitted.

For the main 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. Our partner in IPS gave us the expression plasmids for H5 and N1 proteins, but couldn't give us the lentiviral vectors required for the generation of pseudoparticles. We had to get the plasmids from other sources. We worked mainly with commercial plasmids but all the process required additional adjustments and experiments to be fully functional. We succeeded in generating pseudoparticles and we are now able to undertake survival experiments.

List of Deliverables M19-M36

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	2	1
D 0-2	Experimental tools eg model virus not requiring BSL3 conditions to be used in other WPs	0	M24	M42	4	6.5	1
D 0-3	Method of virus viability other than virus titration of cell culture (TCID50 titre determination)	0	M28	M42	3	0.2	1
D 0-4	Knowledge on some determinants of virus stability	0	M36	M42	1	1.9	1
D 0-5	Peer-review scientific publications in international journals	0	M33	M33-M42	2	1	1

List of Milestones

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	M42	1
M0-2	Implementation of chick embryo fibroblasts culture (avian system) and SK93/2 cell propagation	0	M1-M2	M?	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	M42	1
M0-5	Performance of survival tests on various 'avian' and 'mammalian' virus preparations	0	M3-M6	M42	1
M0-6	Definition of bio-equivalence between virus strains	0	M6	M24	1

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

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

<u>Optimisation of H5N1 detection by RT-PCR and cell-culture (Task 1)</u>

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® has been retained for purification of viral RNA from 140 µL of viral sample. 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 standard protocol (Table WP1-1).

Within WP2 and WP6, CIRAD (Partner 6) has adapted and validated four quantitative real-time PCR assays for AIV detection (type A, H5, H7, N1). The performance of the PCR assays was assessed on several thousands of swab samples collected on wild and domestic birds in Africa and also on bird faeces collected in the field. In addition, these assays were used in interlaboratory proficiency tests (2008 and 2009) organised by the European reference laboratory for avian influenza. From the results, it was concluded that the methods used at CIRAD were sensitive enough and reliable. During this period, CIRAD has also prepared a new set of calibrated, inactivated and lyophilised AIV isolates including H5 and H7 strains for further validation of detection methods. This panel was sent to partner 7 (IPL) and contacts were made for the use of PCR methods for AIV detection in biological material concentrated from water. A persistent problem with the sensitivity of N1 detection was encountered at IPL whereas the M and H5 detections were satisfactory. New primers and probe for the detection of N1 were provided by CIRAD to IPL. IPL provided CIRAD with a protocol for the collection and concentration of water collected in the field. However, it was not possible to apply this protocol during the campaign of sample collection in Mali, organised by CIRAD in 2009. Indeed, the sites where the samples had to be collected are too distant from the laboratory facilities to allow good preservation of huge volumes of water

samples. In addition, the prevalence of avian influenza in Malian birds is very low (0.1 – 0.2% for the 2008 and 2009 campaigns respectively) and H5N1 is absent, therefore, the chance to detect influenza virus in the water appears to be very low.

Table WP1-1 – Influenza A virus H5N1 RT-PCR primers and TaqMan® probes

Specificity	Primers/probe	Sequence (5'-3')	Reference
Influenza A virus Matrix (M) gene	Flu/A/M/25/+ Flu/A/M/124/- Flu/A/M/64/+prob	AGA TGA GTC TTC TAA CCG AGG TCG TGC AAA AAC ATC TTC AAG TCT CTG Fam-TCA GGC CCC CTC AAA GCC GA-Tamra	(Spackman et al., 2002)
Avian H5 gene	Flu/A/H5/1456/+ Flu/A/H5/1685/- Flu/A/H5/1637/+prob	ACA TAT GAC TAC CCA CAR TAT TCA G AGA CCA GCT AYC ATG ATT GC Fam-TCW ACA GTG GCG AGT TCC CTA GCA-Tamra	(Slomka et al., 2007)
Avian N1 gene	Flu/A/N1/539/+ Flu/A/N1/669/- Flu/A/N1/627/-probMGB	GTT TGA GTC TGT TGC TTG GTC TGA TAG TGT CTG TTA TTA TGC C Fam-TTG TAT TTC AAT ACA GCC AC-MGB	(Payungporn et al., 2005)

Real-time one-step RT-PCRs were performed on Light Cycler 2.0. Successful detections of M, H5 and N1 genes were obtained using the Superscript III Platinium One-Step qRT-PCR system (Invitrogen), following the manufacturer's recommendations.

Characterisation of the genes detection and quantification thresholds, and repeatability/reproducibility of the real-time RT-PCR were evaluated. The real-time RT-PCR specific of M, H5 and N1 genes could quantify down to 5.3 10^2, 2.4 10^2 and 6.4 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, H5 and N1 genes. A procedure was written and disseminated to different RIVERS partners (P001RIVERS - RT-PCR detection of avian Influenza A viruses H5N1).

Development of a viral concentration procedure from surface waters (Task 2) and Validation of concentration and quantification methods on surface water (Task 3)

Following the main achievements of the M1-M18 period reported previously, the procedure for concentration of AIVs from water was finally validated by using it to analyse environmental waters, sampled in Cambodia and in France.

Possibly contaminated waters were previously sampled by IP Cambodia, in ponds and lakes near contaminated villages and farms, during H5N1 outbreaks in 2007 (Ponhea Kreak in Kompong Cham) and 2008 (Kandl Steung in Kandal). These samples were kept at 4℃ and then were analysed. Moreover, waters were sampled in 2009 from two places which were involved in previous outbreaks in 2006 (Boeung Thom lake in Kompong Cham, and wet zone near Prey Trakhob village) Unfortunately, no Influenza virus was detected.

During autunm migration in 2009, waters from Dombes ponds (Ain, France), where many birds had tested positive to highly pathogenic H5N1 in early 2006, were analysed in IPL. This method allowed the

detection by RT-PCR of Influenza A virus (M gene detection), in concentration ranging from 3.10^1 to 9.10^3 $TCID_{50}$/liter, in several ponds in November, but none belonged to the H5N1 subtype (H5 gene detection).

A poster « Développement d'une technique d'adsorption - élution pour la concentration de virus *Influenza A* dans les eaux de surface » was presented in Journées Francophones de Virologie, April 2009.

An oral communication « Optimisation et validation d'une méthode de concentration et de détection de virus Influenza A dans l'eau de surface, basée sur les méthodes utilisées pour la détection des virus entériques » was submitted to Journées Francophones de Virologie, March 2010.

A publication (in "Applied and Environmental Microbiology") is in progress.

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.

Development of a direct extraction method of Influenza A virus from 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 was high enough.

Firstly, 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.

RNA PowerSoilTM Total RNA Isolation Kit (MOBIO Laboratories), designed to isolate total RNA from micro-organisms found in soil (up to 2 g), was evaluated. The protocol includes a step of mechanical lysis of a soil sample by beads agitated with a vortex or a grinder / homogenizer, and purification of RNA on columns of silica. The patent pending properties of the kit permit consistent removal of humic substances, fulvic acids, and other RT-PCR inhibitors from soil purified RNA. Diverse soil types, including compost, manure, estuary sediment, and other soil types high in organic content, have successfully provided biologically intact and RT-PCR amplifiable RNA using this kit. The RNA PowerSoilTM Total RNA Isolation Kit reliably provides RNA for experiments requiring qualitative and quantitative RT-PCR analysis. Mud and sterile water were artificially contaminated with Influenza H1N1 virus and subjected to extraction procedure. RNAs were purified from samples with RNA PowerSoilTM Total RNA Isolation Kit, according to the manufacturer's instructions. The efficiency of virus recovery from mud was calculated in comparison with theorically virus amounts introduced in mud, because of ineffectiveness of extraction when virus was diluted in water. First, PCR inhibitors were completely removed. The results showed that more than 20% infectious viral particles seemed be eluted from the mud. However the long time needed to purify RNA from soil (approximately 4 hours) and the use of a phenol/chloroform/isoamyl alcohol solution were a problem to be applied on H5N1 viruses in high biosafety lab.

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

Extraction methods are mainly used for the detection of enteric viruses in sludge from wastewater treatment plants. They are 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.

Previously, experiments were performed to evaluate ten different protocols based on literature data with Influenza H1N1 virus (PR8), used as a model. Each extraction assay was performed on 10 g of muds sampled at different dates near a duck pond in 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 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. The work showed that different methods, tested for extracting virus from mud, go through a critical stage at the time of viral particle desorption from 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.

Experiments were carried out with a highly pathogenic Influenza H5N1 virus (A/HK/156/97). Maximal processable volume and detection threshold of this experimental design were determined. An experimental protocol has been defined on the basis of the previous results and standardized methods for the detection of enteric viruses in sludge from wastewater treatment plants (Environmental Protection Agency). A viral elution step with different buffers, combined with a concentration step by precipitation with poly-ethylene glycol (PEG) was studied for the detection of a highly pathogenic Influenza H5N1 virus. Three elution buffers, such as Borate 0.1 M / Beef Extract 3% (pH 9.6), Glycine 0.05 M / Beef Extract 3% (pH 9.6) and Beef Extract 10% (pH 7), were selected because of their effectiveness for elution of enteric viruses. The conditions of PEG concentration, previously described for enteric viruses by Dubois et al. (2007), were used. The experiments were conducted on 42 grams of sludge sampled from an ornithological site and doped with viral quantities ranging from 10^3 and 10^6 $TCID_{50}$. The recovery efficiency was calculated for each elution buffer from infectious and genome titres measured in eluted and PEG-concentrated samples, respectively obtained by microtiter endpoint titration and matrix (M) gene-specific RT-PCR. Analyzed fractions after elution and after precipitation showed interference and inhibition, respectively during the extraction of nucleic acids (NA) and the molecular detection, probably related to compounds present in the starting matrix. Two NA purification kits, based on the use of silica gel-based membrane (QIAamp Viral RNA Mini kit-QIAGEN) and microspherical paramagnetic silica beads (MagMAX AI/ND Viral RNA Isolation Kit-AMBION), were compared. Beads have a large available binding surface and can be fully dispersed in solution, allowing thorough NA binding, washing and elution. NA purification was 1 log-increased with Ambion kit, when NA seemed to be trapped in membrane when the Qiagen kit was used, probably related to compounds present in the mud. Moreover, PCR additives, such as Bovine Serum Albumine (BSA) and protein 32 of T4 phage (T4gene32-ROCHE), usually removed inhibitors present in humic acids and feces, when they were added to the PCR reaction mixture. Where 2 µg T4gene32 added in reaction mix (100 ng/µl) eliminated the inhibitors in elution samples, inhibitors were still present in PEG samples, probably concentrated along the NA. However addition of T4gene32 improved significantly the amplification curves.

The infectious titres and RT-PCR results were used to calculate an optimal recovery rate. 30% of viral particles were recovered in the elution buffer, when Beef Extract 10% at pH 7 was used, and 10% in the PEG-pellet, when at least 10^4 $TCID_{50}$ were inoculated in mud. 50 µl of elution buffer. 2 µg T4gene32 were added in RT-PCR reaction mix (100 ng/µl).

The detection threshold (DT) of the elution step, corresponding to the limit of the amount of infectious particles below which no detection by RT-PCR can be obtained, was determined mathematically. DT of the elution step in the amount of mud treated was approximately $3{,}27.10^2$ $TCID_{50}$. Regarding the whole procedure of elution and concentration by precipitation with PEG, the molecular detection protocol in the presence of T4gene32 did not completely eliminate the inhibitors. Therefore, the optimized elution / concentration protocol allowed to detect approximately 10^3 $TCID_{50}$ H5N1 in an initial amount of mud over 50 g, adjusting the volume of elution buffer used.

A procedure was written and diffused to different RIVERS partners (P003RIVERS - Procedure for concentration of AIVs from mud). Experiments would be performed on natural contaminated muds in IP Cambodia to validate the detection method.

A poster « Optimisation et validation d'une méthode d'élution/concentration et de détection de virus Influenza A, H5N1, à partir de boues » was submitted to Journées Francophones de Virologie, Mars 2010.

A publication (probably in "Applied and Environmental Microbiology") will be written in 2010.

Deviations from the project work programme, and corrective actions taken

No major deviations occurred, except some slight delay in delivering some deliverables in this WP.

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP N°	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
1-1	Practical reports will be produced at the end of task 2 & 4 describing relevant and chosen methods for concentration, identification, and quantification of AIV in various aquatic environments	1	22	M28	0	4	7
1-2	Standard Operating Procedures (SOPs) available for other partners of the project	1	22	M28	0	9	7
1-3	Publications in peer review international journals	1	24	M24-42	0	0	7

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP n°	Date due	Actual/Forecast delivery date	Lead contractor
M 1-1	End of Task 1[H5N1 detection by RT-PCR and cell culture (Vero and MDCK line). Optimisation of methods]	1	M4	M22	7
M 1-2	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	M18	M28	7
M 1-3	End of Task 3 [Validation of concentration and quantification methods on surface waters]	1	In progress: M20	M36	7
M 1-4	End of Task 4 [Development of an extraction procedure, for solid samples (mud, biota)]& 5 [Tranfer of the various protocols to other participants, intercalibration] + Report	1	In progress: M22	M36-M42	7

Work Package 2: "Observation of influenza viruses 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

In China

The research in M19-M36 contains five parts. **1) Sample collection and virus isolation.** We continued to collect the water samples, fecal samples, cloacal samples of the backyard poultry in Dongting lake wetland and in the living poultry market in the city around the wetland; **2) DNA sequencing and phylogenetic analysis of the whole genomes of the selected isolates. 3). Pathogenicity evaluation of some isolates.** Some of the H5N1 viruses and H9N2 viruses have been chosen to test the pathogenicity in chickens and mice; **4) To assess the potential pathogenicity of the isolated low pathogenic virus to the mammals in mice.** The adaptation of the H10N8 strain in the mouse lung was carried out by serial lung to lung passages.The molecular mechanism involved in the enhanced virulence was analyzed ; **5)To assess the perpetuation of AIVs in natural water** by determining the duration of the persistence of H5N1 (highly pathogenic AIV) and H9N2 viruses (low pathogenic AIV) in different natural waters.

1) Sample collection and virus isolation

Sample collection has continuously been performed during every spring, autumn and winter. The sources of the sample included: the water contaminated by the feces of the aquatic fowls, the feces in the environment, the backyard poultry in the Dongtinglake wetland, the healthy domestic fowls in the markets in the cities around the Dongtingl lake. The virus isolation in these collected samples will be continued during the next step.

2) DNA sequencing and phylogenetic analysis of the whole genomes of the selected isolates

The whole genomes of the selected isolated viruse were sequenced and the phylogenetic analysis were undertaken.

In order to study the molecular characteristics and the genetic relationship between the isolates in this study and other H5N1 viruses, the whole genome of 14 of the H5N1 isolates were sequenced, based on which the phylogenetic trees were generated. The full-genome sequences of the 14 H5N1viruses isolated in this study are available from GenBank under accession nos: GU182139-GU182250. In addition, the whole genomes of 8 viruses of the H9N2 subtype isolated in this study were sequenced and all sequences are available from GenBank under accession nos: GU474547- GU474610. The Nucleotide sequences for the viral genome of the H10N8 isolate have been submitted to GenBank and are available under accessing numbers (GenBank) GQ290464—GQ290471 .

Phylogenetic analysis of the H5N1 viruses showed that the viruses isolated in Dongting Lake wetland in various years, even in various seasons of the same year, were of different sublineages or clades, suggesting multiple introductions of influenza viruses in this region.

The whole genomes of 8 of the all isoalted H9N2 viruses were sequenced, of which 8 gene segments were subjected to phylogenetic analysis. The results showed that all the isolates belonged to the same genotype, of which HA, NA and NS gene segments belonged to Chicken/Beijing/94-like, PB2, PB1, PA and NP gene segments to Chicken/Shanghai/F/98-like, and M gene to Quail/Hong Kong/G1/97-like. The H9N2 viruses

isolated in this study were generated by gene reassortants of Chicken/Beijing/94-like, Chicken/Shanghai/F/98-like and Quail/Hong Kong/G1/97-like viruses.

A H10N8 subtype influenza A virus was isolated from the water sample of Dongting lake wetland. Phylogenetic analysis indicated that the virus was generated by multiple gene segment reassortment, because each gene segment of the virus was of different origin.

The domestic ducks and chickens deaths due to H5N1 avian influenza(AI) virus in number in the autumn of 2007 and 2008 were consistent with the first group of migratory waterfowls that lived through the winter in the Dongtingn Lake marsh or just pass through there. The result suggested that the times, when AI virus appeared in domestic fowls in the autumns of 2007 and 2008 in Dongting Lake wetland, coincided with the periods of peak migratory birds' movement. Some H5N1 viruses isolated from water samples and domestic fowls in Dongting Lake wetland from 2007 to 2008 had close genetic relationship with the viruses isolated from the whooper swans in Japan and the dead wild birds in Hong Kong in the recent 2 years.The viruses isolated in this study might have been carried here by migratory birds, and introduced into the domestic fowls through the waterway. The mixed breeding of chickens, ducks and geese is very popular in households in this region; and some duck-breeding farms are built by Dongting Lake, in which ducks are fenced only by a fish net, so that the domestic ducks and the wild aquatic birds share the same water area frequently. Importantly, the viruses isolated from the water had great genetic similarity with those from the domestic fowls, providing the evidence that the waterway might be an important transmission pathway of the AI viruses between the domestic fowls and the wild aquatic birds in this region.

All the isolated H9N2 viruses belonged to the same genotype. During the study lasting for nearly one year, the viruses of this genotype were continuously isolated in this area, indicating that the viruses of this genotype might have become predominant in this area and these viruses were genetically stable to a certain degree, suggesting that these viruses are genetically stable and not a transient gene constellation. Two-way interspecies transmission of H9N2 viruses may also exist in Dongting Lake region. Influenza virus may be transmitted from chickens to domestic ducks because of the mixed raising of them and then the viruses were released into the water body by the infected ducks, resulting in the infection of chicken-derived virus in wild waterfowls using the same water area. Alternatively, viruses were carried here by wild waterfowls during migration and then released into water body, resulting in the infection of migratory birds-derived virus in domestic ducks sharing the same water area with the wild aquatic birds. And then the infected domestic ducks transmitted the viruses to chickens.

3) Pathogenicity evaluation of some isolates

All the selected H5N1 viruses killed the inoculated chickens within 24h and had an IVPI of 3.0, indicating that all the isolated viruses were highly pathogenic to chickens. However, the pathogenicity of the viruses to mice was significantly different. Some viruses caused systemic infection in mice, and viruses were detected in multiple organs including brain tissue, while other viruses were only replicated in the respiratory system of the mice.

Animal experiment showed low pathogenicity of the selected H9N2 viruses to chickens, though some chickens died after inoculation. The result showed that the H9N2 viruses isolated in this study were mainly replicated in the respiratory tracts of fowls, indicating that these H9N2 influenza viruses are mainly spread via the respiratory route. And the viruses showed no overt clinical signs to mice, but they could be replicated in murine lungs prior to adaptation.

Pathogenicity experiments showed that the H10N8 virus was lowly pathogenic to chicken. It could replicate efficiently in the mouse lung without prior adaptation, and the virulence to mouse increased rapidly during adaptation in mouse lung.

4) The potential pathogenicity of the isolated low pathogenic virus to the mamal animals in the mice

The H10N8 virus could be replicated efficiently in the mouse lung without prior adaptation, and the virulence for the mouse rapidly increased during adaptation in mouse lung. Sequence analysis of the genome of the virus from different passages showed that multiple amino acid changes were involved in the adaptation of the isolate to mouse, and the PB2-E627K substitution for mouse adaptation extended to H10N8 subtype influenza virus.

5) Observation of persistence of AIV H5N1 and H9N2 in natural water

The duration of persistence of two subtypes of AIVs (AIVs) H5N1 and H9N2 was studied in four natural waters of China. Among them, Qinghai Lake, Poyang Lake and Dongting Lake are important habitats for

migratory birds, and we also took the Yangtze River in Wuhan section (table WP2-1), using the Milli-QH_2O as a control. The infectivity of the AIVs was tested throughout the sample period and the $TCID_{50}$ was calculated using Reed-Muench method. Then, we also calculated the equation of the viral $TCID_{50}$ regression at various temperatures and evaluated the duration of persistence, which provided insight into the pandemic risk of the AIVs in the natural water. In addition, some parameters of the natural water of Dongting, Poyang, Changjiang (Fig. WP2-1) and Qinghai water have been tested (table WP2-1).

We tested the infectivity of the H5N1 virus ($10^{2.25}$TCID50) and the H9N2 virus ($10^{2.0}$TCID50) in four natural water samples at different temperatures regularly, for example, 4℃each 48 hours, 16℃each 24 hour, 28℃each 12 hours. The results derived from regression models (Fig.WP2-2 and Fig.WP2-3) showed that the temperature could impact the persistence of AIVs in water significantly and the microorganism in water had a negative effect on viral persistence in water, but not significantly. At 4℃ and16℃ the AIVs had a much longer duration of persistence in freshwater lakes (Dongting, Poyang, Yangtze River) than in salted lakes (Qinghai), which showed that the high salinity can inhibit the persistence of the AIVs. At 4℃, H5N1 and H9N2 viruses could retain their infectivity for at least 30 days in the Milli-QH_2O and the freshwater lakes, wheres they only survived 20 days in salted lake. Even at 16℃, the AIVs could retain their infectivity more than 10 days, whereas they only survived 7 days or shorter in salted lake. At the high temperature of 28℃, the inactive rate of the AIVs increased rapidly, H5N1 and H9N2 viruses could only survive about 5 days in Milli-QH_2O, 3 days in other waters, and without significant difference between the freshwater and salted water. The results in our study suggested that the infectivity of AIVs in water is associated with temperature and salinity of the water. Moreover, we also proved that the PH could influence the persistence of the AIVs in water.

The Dongting Lake and Poyang Lake are important habitats and overwintering areas along the East Asia migration route for migratory birds. The low-temperature environment in this region make the AIVs survive in water for a long time, which greatly increased the probability of the other migratory birds or poultry getting infected with influenza viruses still present in the same waters.

Qinghai Lake is located in highland areas in northwest China and the low-temperature is all year round. In 2005 the first time outbreak of the highly pathogenic avian influenza H5N1 virus in migratory birds in Qinghai Lake has aroused the concern of researchers around the world, many researchers have speculated the mode of transmission of the AIV in migratory birds in Qinghai Lake, but few studies have reported the approach of the virus transmitted through the Qinghai Lake water. Our research have confirmed that the virus could persist in Qinghai Lake about 20 days, even at 16℃ it was also able to survive 7 days or so, which strongly suggested the AIVs could be transmitted in the migratory birds through the fecal-contaminated water on shared aquatic habitats.

Dongting Lake and Poyang lakes frequently exchange water with the Yangtze River, so in autumn and winter, the contaminated water of the Dongting Lake and Poyang Lake is likely to enter the Yangtze River and is carried to the wider region along the Yangtze River. Our results showed that the AIVs can persist for at least 30 days at 4℃, even at the higher temperature of 16℃ , the AIVs were still able to survive more than 10 days which also proved theoretically that the non-biological factors (flowing water) could be a mediator for the rapid spread of the influenza virus. The length of the Yangtze River is about 6,300 km and it is the longest river in Asia and third longest in the world. Yangtze River region is thriving in population, poultry industry is developed, and the favorable geographical environment makes it become a natural habitat for many wild birds. Thus, when the contaminated water enters into the Yangtze River in these areas and cause the infections of poultry, it will bring huge economic losses to the poultry industry and a great danger to the wild birds, or even the threat to the human health of the Yangtze River region.

Table WP2-1. The parameters of the natural water of Dongting，Poyang, Changjiang and Qinghai water.

	QingHai Lake	DongTing Lake	PoYang Lake	ChangJiang River
PH	8.89	6.95	7.06	6.93
Conductivity（us/cm）	28220	1546	651	355
BOD5	69.9	44.4	6.62	9.9
MPN	3.2×10^3	5.9×10^6	6.9×10^6	3.4×10^5

Fig. WP2-1. Location of three lakes and Changjiang River in China

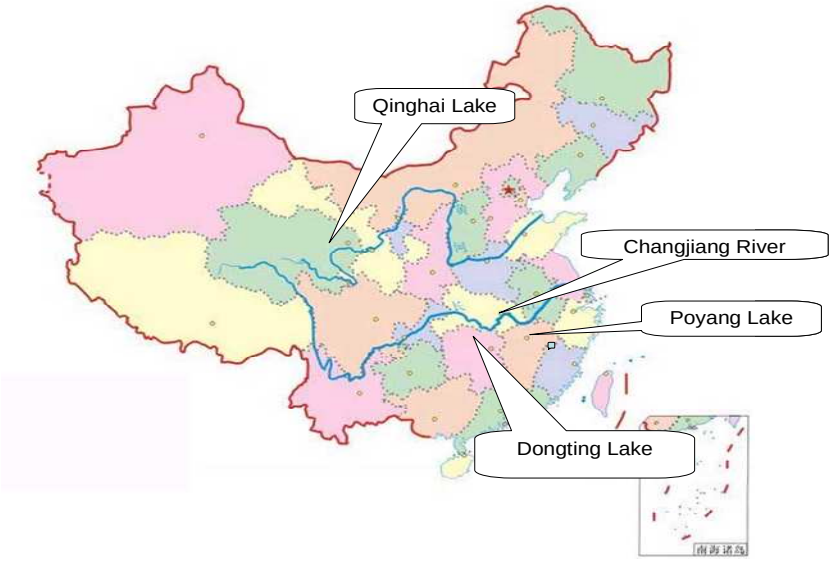


Fig. WP2-2. Regression models for persistence of HPAIV H5N1 in four natrual water at 4℃, 16℃ and 28℃

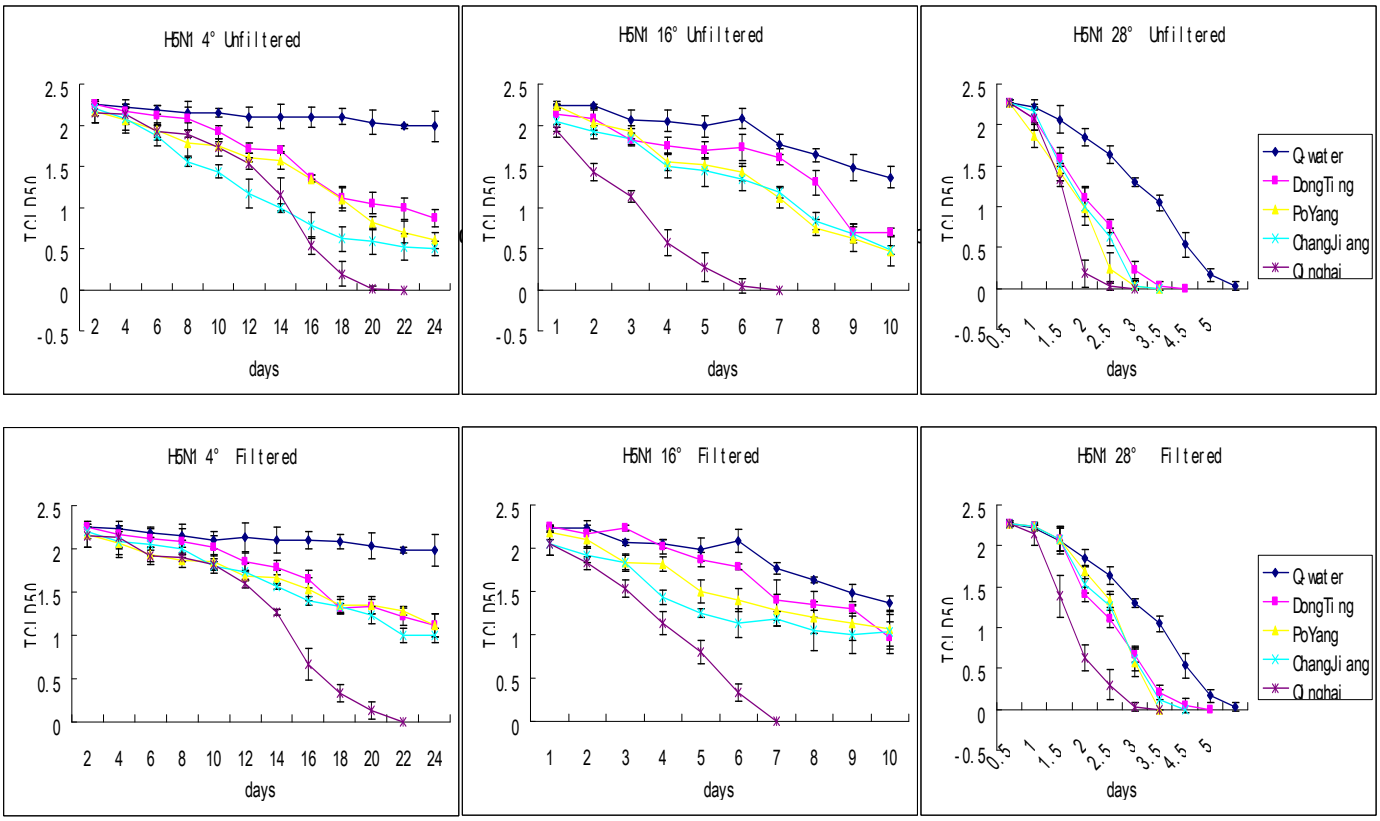
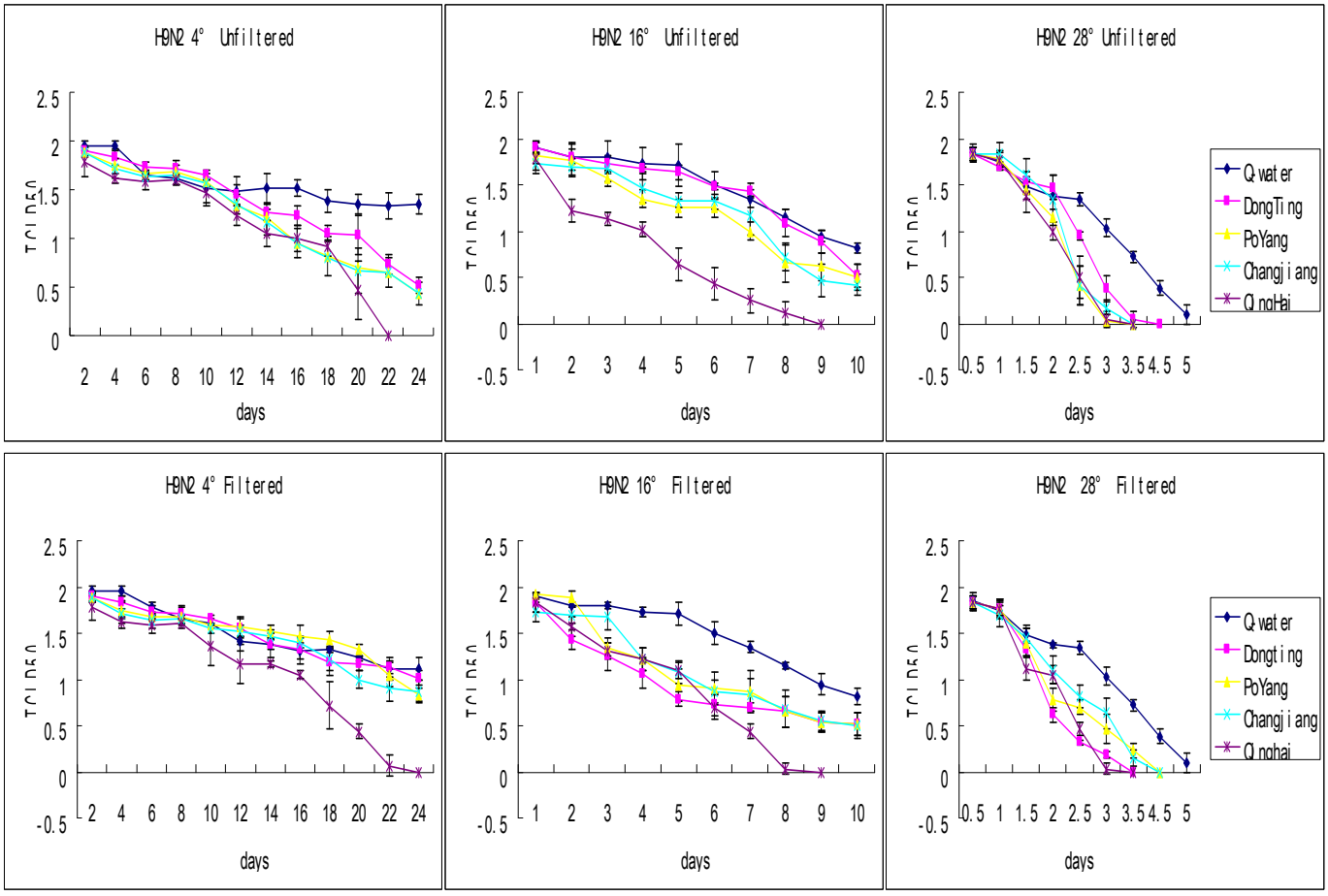


Fig. WP2-3. Regression models for persistence of HPAIV H9N2 in four natural water at 4℃, 16℃ and 28℃



Cambodia

In response to the notification of a confirmed case of influenza subtype H5N1 infection in humans or poultry, we studied the environmental contamination by the H5N1 virus. The investigation was conducted in some households and their surroundings in the vicinity of the index household. Environmental specimens such as mud, pond water, water plants, soil, insects, aquatic animals and swabs were collected. Swabs were placed in 1.5-mL virus transport medium; all environmental samples were transported at 4°C within 36 hours to Institut Pasteur in Cambodia for subtype H5N1 testing by real-time quantitative RT-PCR (qRT-PCR) after RNA extraction using the MagNa Pure Nucleic Acid Isolation Kit (Roche™) on the MagNa Pure LC machine (ROCHE™) and for virus isolation after inoculation into embryonated chicken eggs.

Each year from 2007 until now, the environmental investigations were conducted in the location with influenza virus (H5N1)–associated outbreaks.

1. H5N1 outbreak in 2010

At the beginning of 2010 in Takeo province, which is located in the south next to the Vietnamese border, an outbreak of H5N1 AIV was reported in poultry. This outbreak caused thousands of poultry deaths in a single village. The sampling process was conducted in the surroundings of the households and in the field of villagers. A total of 62 environmental specimens were collected and out of these 28 were analysed by qRT-PCR. Of these 28 specimens 12 were H5 gene positive with a quantity of virus varying from 10^3 to 10^5 RNA copies per ml and 10^2 to 10^5 RNA copies per gram. These positive samples are currently processed for viral isolation

2. H5N1 outbreak in 2009

In December 2009, one confirmed human case of H5N1 was reported from the province of Kampong Cham, in a village where a previous H5N1 outbreak had already been reported in 2007. This case was non fatal. A total of 82 specimens were collected in the location surrounding the index household with 37 out of these are now in process of analysing to investigate the possible environmental contamination of the H5N1 virus. At least 11 samples were H5 gene positive (10^2-10^3 RNA copies per ml and 10^2-10^4 RNA copies per gram). These positive samples are currently processed for viral isolation

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

Within the framework of this project, the following activities were undertaken in Montpellier and in Cambodia in coordination between the Pasteur Institute of Phnom Penh (Partner 4) and the UR AGIRs of CIRAD (Partner 6):

- Publication of an article in the International Journal of Applied Earth Observation and Geoinformation, Tran, A., et al., Remote sensing and avian influenza: A review of image processing methods for extracting key variables affecting AIV survival in water from Earth Observation satellites. Int. J. Appl. Earth Observ. Geoinform. (2009), doi:10.1016/j.jag.2009.09.014 Recent studies have highlighted the potential role of water in the transmission of avian influenza (AI) viruses and the existence of often interacting variables that determine the survival rate of these viruses in water; the two main variables are temperature and salinity. Remote sensing has been used to map and monitor water bodies for several decades. In this paper, we review satellite image analysis methods used for water detection and characterization, focusing on the main variables that influence AI virus survival in water. Optical and radar imagery are useful for detecting water bodies at different spatial and temporal scales. Methods to monitor the temperature of large water surfaces are also available. Current methods for estimating other relevant water variables such as salinity, pH, turbidity and water depth are not presently considered to be effective.
- Our work in 2008 allowed us to identify environmental indicators in connection with floods and landuse, from MODIS data. These indicators are linked to epidemiological data in order to identify risk factors for the occurrence of avian influenza cases. A model to detect water surfaces and periods of floods has been created.
- Field data, in Cambodia in several spots known for previous H5N1 outbreaks, have been collected between March and August 2009 (before and at the end of rainy season), in order to validate the model.
- June 2009: a mission of a modeler in Cambodia was organised in order to work on the H5N1 data available in IPC.
- In October 2009, started the analysis of the correlation between environmental conditions linked to water and the number of H5N1 cases in Cambodia. This analysis should be finished by the end of June 2010 (M41)

In France

During the 2009 autunmal bird migration, samples of water were collected from Dombes ponds (Ain, France), where many birds had tested positive to highly pathogenic H5N1 in early 2006. They were analysed by

IPL (Partner 7). This method allowed the detection by RT-PCR of Influenza A virus (M gene detection), in concentration ranging from 3.10^1 to 9.10^3 $TCID_{50}$/liter, in several ponds in November, but none belonged to the H5N1 subtype (H5 gene detection).

Deviations from the project work programme, and corrective actions taken

No major deviations occurred, except some slight delay in delivering some deliverables in this WP and except that water collection if France was done in Les Dombes instead of Camargue and Baie de Some by Partner 7 (IPL) instead of Partner 1 (IPP).

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP n°	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
2-1	Data on the prevalence of AIVs in waters (lakes, ponds and rivers) in the far East (China, Cambodia), Africa (Senegal, Mali) and Europe (Romania, Bulgaria, France)	2	33	M33-42	15	29	9
2-2	Data on variations in prevalence and in virus load in waters in the course of time throughout the year in tropical and non tropical regions of the world	2	33	M19-42	10	29	9
2-3	Data on variations in prevalence and in virus load along the stream of rivers (eg Yangtze River)	2	33	M19-M42	9	22.8	9
2-4	Data on gastropods and bivalves Molluscs regarding their potential role a sconcentrators and conservatories of AIVs in aquatic biotopes	2	24	M42	5	23.3	9
2-5	Publications in peer reviewed international journals	2	36	M42	5	9	9

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP No.	Date due	Actual/Forecast delivery date	Lead contractor
M 2-1	Water sampling without concentration	2	Not applicable	M42	9
M 2-2	Water sampling with possible concentration (end of WP1 task 3)	2	M13	M42	9
M 2-3	Detection of AIV in water samples	2	M6	M42	9
M 2-4	Detection of viable viruses in water samples (end of WP0)	2	M7	M42	9
M 2-5	Detection and quantification of viable viruses in water samples with standardised protocols (end of WP1 task 3)	2	M32	M42	9
M 2-6	Detection and quantification of viable viruses in solid samples (biota, mud) Task 3: Data analysis and transfer to WP9	2	M33	M42	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

Creating artificial aquatic biotopes and observation of AIV survival in experimental settings

Uninfected natural waters collected in natural settings were used to simulate environmental conditions in water tanks such as aquarium and jars by controlled infection. Biological systems were re-created to mimic natural environments. We realised 3 experimental settings of aquariums and 3 experimental settings with fish in jars to study: 1) the H5N1 virus survival in water, 2) whether the aquatic animals and plants can be contaminated by H5N1 virus in infected water, 3) whether fish may play a role in transmission of H5N1 virus from one to other.

3.1. First experimental setting in aquarium

3.1.1. Materials and methods

For this experiment of 15 days we used: 8 aquariums with a volume capacity of 29 litres, 8 thermostat elements and 8 thermometers for adjusting and measuring the water temperature in each aquarium. The artificial aquatic biotopes were created using natural water and mud collected from 2 ponds located in the site of H5N1 outbreak in 2007 (Kampong Cham province).The temperature, pH, conductivity, physico-chemical parameters and bacterial parameters of these waters were measured.

Out of a total of 8 aquariums, 4 contained only mud and water and another 4 aquariums contained mud, water, aquatic plants and aquatic animals such as fish (guppy), small snails, and shells from fresh water. For each type of water (big pond or small pond), 2 different temperature 34ºC and 32ºC were adjusted according to the temperature measured on the fields and another 2 different temperature 25ºC and 22ºC were adjust according to the variation of temperature on the season.

In each aquarium, 3 falcon tubes of 50 cc were closed tightly and let floating in. These tubes contained pond water inoculated with virus (tube 1), non-infected pond water (tube 2), and distilled water inoculated with virus (tube 3). The quantity of virus in tube 1 and tube 3 was 10^6 RNA copies.

All 8 aquariums were infected with a HPAI H5N1 Cambodian strain that has been isolated from chicken in 2007 (A/Ck/CAM/LC1AL/07). The quantity of virus used to infect the aquarium was chosen according to the amount of virus that has been detected in environmental specimens (Vong et al 2008). On day 0 (D0), each aquarium with 20 litres of water was inoculated with 10^7 EID50 of this H5N1 virus, which corresponds to 10^8 RNA copies.

Samples of water and mud were collected daily and samples of plant and aquatic animals were taken every 3 days. Different organs (gills, intestine, and muscle) were dissected from fish, snail or shells when possible.

3.1.2. Results

Table WP3-1: Specimens collected and analysed:

Sample type	Number	PCR for H5 gene	PCR for M gene	Viral Isolation on eggs
Water	128	Not done	Not done	Not done
Water from 3 tubes	384	Not done	Not done	Not done
Mud	128	All Negative	All Negative	All Negative
Fish	48	All Negative	All Negative	Not done
Fresh shell	33	All Negative	All Negative	Not done
Snail	65	All Negative	All Negative	Not done
Plant	24	All Negative	All Negative	Not done

The first experimental setting in aquarium showed absence of viral RNA detectable in all specimens of mud, plant and aquatic animals. This may suggested that the virus quantity used at 10^7 EID50 in 20 litres of

pond water in aquarium was insufficient to infect the water and other materials in it. Another possible explanation could be that the conditions (temperature,…) applied were not favourable for the virus survival.

3.2. Second experimental setting in aquarium

3.2.1. Materials and methods

A total of 6 aquariums were used for this 14-days experiment. Two aquariums contained only water and mud while another 4 aquariums contained water, mud, plants, and aquatic animals. The water and mud used in these aquariums was collected from a lake in a site where H5N1 outbreak had occurred in 2006. All physico-chemical and bacterial parameters of this water were measured. The 6 aquariums were separated in 2 groups of 3 aquariums. A temperature of 32ºC and 25ºC were adjusted for each group. In each group, two were infected and one was not infected.

In the 4 infected aquariums, 3 falcon tubes of 50 cc (as described above) were floating.

For this experimental setting, 4 aquariums were infected with duck faeces infected with 10^8 EID50 (10 times more then in the first experiment) of the H5N1 virus A/CAM/408008/2005 The quantity of virus used to infect the aquarium was chosen according to the amount of virus shed by an infected duck in natural conditions (Stallknecht et al., 1990). The presence of micro-organisms and the physical and chemical parameters of water were measured 3 times during the experiment.

The specimens of water, mud were collected daily and the specimens of plant and aquatic animals were taken every 3 days.

Specimens collected: 78 specimens of water from the aquarium, 60 specimens of water from the floating tubes, 84 mud samples, 66 samples of fish, 14 samples of shell, 54 samples of tadpoles and 18 plant specimens.

The analysis of all aquatic animals and plant specimens by qRT-PCR of H5 gene showed no RNA viral detected. The viral isolation was not done for these samples.

The qRT-PCR performed on the water samples collected from day 1 to day 7 showed that only the water specimens collected in the first 3 days were positive for H5 gene with values of 10^3, 10^2, 10^1 RNA copies per millilitres for days 1, 2 and 3 PI respectively. From day 4, the water specimens were all negative in qRT-PCR of H5 gene. The viral isolation performed on water specimens collected from day 1 to day 7 was all negative.

The water specimens of the floating tubes analysed on days 0, 3 and 7 PI showed that no infectious virus could be detected in any type of water at day 3 and day 7 PI, even though the viral RNA was present in these specimens. The quantity of viral RNA detected in the same type of water was not different at 32ºC and at 25ºC. However, the amount of viral RNA detected seemed to differ with the type of water (lake or distilled water): viral RNA was more detected in distilled then in lake water.

In this experiment the survival of H5N1 in water was not demonstrated even thought viral RNA was detected in the water until at least 3 days after viral inoculation. The contamination of aquatic plant and animal has not been detected.

3.3. Third experimental setting in aquarium

3.3.1. Materials and methods

In this experiment of 14 days, three aquariums containing 10 litres of rain water were tested at a temperature of 25°C. No mud was introduced in these aquariums. The first aquarium was infected with 5.10^8 EID50 of the H5N1 virus A/CAM/408008/2005 (10 times more then in the second experiment). A total of 25 molluscs were introduced in this infected aquarium at the same time as of viral inoculation. Then every 3 days, three of these molluscs were collected and dissected to collect different organs (gills, intestine, digestive gland, muscle). The second aquarium was not infected and contained 20 molluscs which were to be exposed to infected molluscs in a third aquarium (non-infected). On days 3, 5 and 7 PI, two molluscs from the first aquarium (infected) were taken out and placed with 4 molluscs from the second aquarium (non-infected) into a third aquarium (non-infected). Every two days after exposure of non-infected molluscs with infected molluscs, 1 infected and 2 non-infected molluscs were collected and dissected to collect organs. All molluscs organs were placed in viral transport medium (VTM) at -80°C until the analyses were performed. Samples of water were taken daily from the first and the third aquariums.

3.3.2. Results

We collected a total of 56 specimens:

- from the first aquarium (infected): infected molluscs (IM, n=28);
- from the second aquarium (non-infected): non-infected molluscs (NIM, n=4);

- from the third aquarium (exposure experiment): infected mollusc placed into the aquariums on days 3 (IM-Exp-3d, n=4) and 6 (IM-Exp-6d, n=4), and "contact" molluscs (originally non-infected) on days 3 (NIM-Exp-3d, n=8) and 6 (NIM-Exp-6d, n=8).

The organs taken from molluscs of the infected aquarium (IM) on days 2, 3, 4 and 6 were all H5 gene positive with a quantity of viral RNA detected in a range of 10^6-10^7 RNA copies per gram of organ. Furthermore, infectious viral particles were successfully isolated from these specimens. However, in this first aquarium (infected), because of the rapid death of the molluscs, no viral detection could be done later than 6 days PI. Daily deaths were observed, and all molluscs had died by day 6. As for the water, the analysis of the water sampled from the infected aquarium showed that infectious virus particles could be isolated on day 3 PI, with an amount of viral RNA detected in a range of 10^2-10^3 RNA copies per millilitre of water.

Viral RNA was detected in the infected molluscs' organs until 3 and 6 days after their being placed in non-infected water. But no infectious virus was successfully isolated from these specimens. As for the non-infected molluscs exposed to infected ones in the third aquarium, viral RNA could be detected in their organs on days 3 and 6 post-exposure. However, no viral isolation could be done from these organs. No infectious viral particle was found in the water samples collected from the third aquarium after it was exposed to infected molluscs, even though some viral RNA was detected at day 1 post-exposure.

In this experimental setting with conditions such as a temperature of 25°C, rain water, no mud and a concentration of H5N1 virus of 5.10^8 EID50 in 10 litres of water, the survival of H5N1 virus in water was observed during 3 days with the persistence of viral RNA at least 11 days after viral inoculation. In this infected water the molluscs were contaminated by the virus and carried infectious virus particles until 6 days after inoculation of the water. Furthermore the contaminated molluscs may transmit the virus to water and other molluscs. Here, we observed the presence of viral RNA in water and in molluscs' organs after they were exposed to the contaminated molluscs. The quantity of viral RNA detected in different organs of molluscs was not different.

3.4. first experimental setting with fish in jars

3.4.1 Materials and methods

This 20-days-experiment was done by using 14 jars containing 500 ml of rain water each, with one fish and one tadpole in each jar. Of these jars, two were used as negative controls in which the water was not infected and the 12 other jars were infected with 10^5 EID50 of the H5N1 virus A/Ck/CAM/LC1AL/07. The pH and temperature of the water were measured on the first day of the experiment. Fish and tadpoles were collected from two jars on days 1,3, 7, 11,15 and 20 (until all jars were empty) and dissected to collect different organs. During this experiment samples of 10 millilitre of water were collected daily from each jar.

3.4.2 Results

The temperature of the water measured in these jars was between 16 to 19ºC and the pH was 8.2. Tadpoles appeared not to be very resistant in these experimental conditions. Many of them died at day1-day4 and only two of them survive until day 13 and day 14. We collected 98 samples on fish, 15 samples on tadpoles and 134 water samples. The analysis of the specimens collected on the tadpoles showed the presence of viral RNA in these samples from day 1 (10^6 copies/g) until day 14 (10^4 copies/g). The viral RNA load was similar in all tadpole organs. Infectious viral particles could only be detected by viral isolation on the specimens of gills and skin on day 1 of the experiment.

Viral RNA was detected in different organs of fish during 15 days with a quantity of 10^4-10^5 copies/g. However, infectious viral particles were isolated in specimens of fish only on day1 of the experiment.

The quantity of viral RNA seemed to be slightly higher in the gills than in the other organs of the fish.

The analysis of the water samples collected from the jars on days 1 to 5 showed that viral RNA could be detected in these specimens with a quantity of 10^4 RNA copies per millilitre of water. Isolation of infectious particles was successful only for the samples collected on the first 2 days PI.

This experiment showed that the survival of avian H5N1 virus at a quantity of 10^5 EID50 in 500ml of rain water at a temperature of 16-19°C and a pH of 8.2 w as observed only for 2 days even though the viral RNA was present in the water for at least one week. The aquatic animal such as fish and tadpoles were contaminated by the virus from the infected water and carried the infectious virus only for 1 day although viral RNA was detected at least for two weeks.

3.5. Second experimental setting with fish in jars

3.5.1 Materials and methods

This experiment lasted 12 days and consisted in using 14 jars containing 500 ml of rain water each, with one male fish in each of 7 jars and one female fish in each of the remaining 7 jars. In each 7-jars-group, two were used as negative controls (non-infected).

In 5 of the jars (J1a-J5a) containing male fish, the water was infected on day0 by a viral inoculation of 10^5 EID50 of avian H5N1 virus (A/Ck/CAM/LC1AL/07). These male fish were kept in the infected water for 5 days (M-Inf1 - M-Inf5) and were then transferred to 5 new jars containing non-infected rain water for 1 day. They were then finally put for 6 days in 5 of the jars (J1b-J5b) containing female fish. The male fish (M-Ctrl1 – M-Ctrl2) in the 2 control jars (J6a-J7a) were also put in the 2 control jars (J6b-J7b) containing female fish (F-Ctrl1 – F-Ctrl2).

The pH and temperature of water were measured at day 1 of experiment. The fish from each jar were collected and dissected to collect different organs at day 12. During this experiment samples of 10 millilitres of water and of fish faeces were collected daily from each jar.

3.5.2. Results

The temperature and pH measured at day 1 of the experiment were of 18-20°C and 8,2 respectively. A total of 98 specimens of fish (male and female) and 77 specimens of water were collected. All fish specimens (M and F) tested were positive for viral RNA in different organs with a quantity of 10^3-10^6 RNA copies per gram. But no infectious virus could be isolated from these specimens.

Of all water specimens, 22 of them, collected from both groups of jars (J1a-J1-J1b and J2a-J2-J2b), were analysed. The survival of the virus in water was confirmed only for the 2 first days post-viral inoculation. However the presence of viral RNA was detected in the water (10^5 copies/ml) for 5 days after viral inoculation.

This experiment showed that fish in infected water were contaminated by the virus. Even though the survival of infectious virus in fish was not demonstrated, different amounts of viral RNA were detected in different organs. Furthermore, the contaminated male fish may have transmitted the virus to the female fish, since viral RNA was also detected in different female fish organs. The survival of the virus in water was confirmed for only 2 days although the viral RNA was detected at least for 5 days.

3.6. Third experimental setting with fish in jars

3.6.1 Materials and methods

This experiment of 12 days consisted in using 16 jars containing 500 ml of rain water each, with one male fish in each of 10 jars and one female fish in each of the remaining 6 jars. In both groups (male and female), 2 jars were used as negative controls (non-infected). In 8 of the jars containing male fish (J1a-J8a), the water was infected at day0 by a viral inoculation of 10^6 EID50 of avian H5N1 virus (A/Ck/CAM/LC1AL/07). On days 1, 3, 5 and 7 post-viral inoculation, one male fish (J1a-J4a) was taken out of an infected jar and dissected (M-inf1 – M-Inf4). Another four male fish in infected water were kept for 5 days (M-Inf5 - M-Inf8) in the jars (J5a-J8a), were then transferred to 5 new jars containing non-infected rain water for 1 night (J5-J8), and were finally put into 4 of the jars (J5b-J8b) containing female fish (F-exp1 – F-exp4) . On days 1, 3, 5, and 6 post-exposure, one male and female were collected from each of these jars to be dissected.

The male fish (M-Ctrl1 – M-Ctrl2) in the control jars (J9a-J10a) were also put into the jars (J9b-J10b) containing female fish (F-Ctrl1 – F-Ctrl2).

The pH and temperature of the water were measured at day 1 of experiment. During this experiment samples of 10 millilitres of water and of fish faeces were collected daily from each jar.

3.6.2. Results

The temperature and pH of water measured at day 1 of experiment were of 18-20°C and 8,2 respectively. A total of 147 specimens were collected and analysed: 21 specimens of water from the jars, 27 specimens of fish faeces and 99 specimens of fish organs. The presence of viral RNA was detected in 22 out of 99 specimens of fish organs with a quantity of 10^4-10^5 copies/g. This positive detection was found only in some organs of the infected fish (M-Inf1 – M-Inf4) collected from the infected jars. The viral RNA detected seems to be in higher quantity in the gills. Positive samples (for RNA detection) were then tested for viral isolation, but no infectious particle could be isolated.

All male fish kept for 5 days in infected water (M-Inf5-M-Inf8) then a few days (d1, d3, d5 and d6) in non-infected water with female fish from non-infected water were all negative for viral RNA. And all exposed female fish were also negative for viral RNA. So no further analysis by viral isolation was done on these specimens

The specimens of fish faeces collected (day1 to day 5) from the jar containing infected water were positive for viral RNA with a quantity of 10^4-10^6 copies/ml. Moreover, infectious viral particles could be isolated from two specimens collected on day 3 PI.

Further analyses performed on the fish faeces collected on days 1,3, 5 and 6 post-exposure, from jars where the exposure part of the experiment took place, showed that only one specimen collected on day 3 post-exposure was positive for viral RNA detection at a concentration of 5.10^E3 copies/ml with no infectious particle isolated. The rest of these fish faeces specimens were negative for viral RNA.

Viral RNA was detected in the water directly inoculated with virus. The quantity of viral RNA detected was 10^3- 10^4 RNA copies per millilitre of water tested. In water collected from the "exposure" jars, the detection of viral RNA was lower, at the quantity of 10^1- 10^2 RNA copies per millilitre of water. *Results of viral isolations performed on these samples are pending.*

Deviations from the project work programme, and corrective actions taken

No major deviations occurred, except some slight delay in delivering some deliverables in this WP.

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP N°	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 3-1	Inventory of all the elements naturally existing in the aquatic environment in the countries where H5N1 virus is circulating	3	M33	M42	11	8	4
D 3-2	Data and analysis	3	M33	M42	14	12.5	4
D 3-3	Peer-review scientific publications in international journals	3	M36	M42	3	2.25	4

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP N	Date due	Actual/Forecast delivery date	Lead contractor
M 3-1	inventory of the elements naturally existing in the aquatic environment	3	M6	M42	4
M 3-2	record of the water characteristics (temperature, pH, TH, …) observed in countries during epidemic season	3	M18	M42	4
M 3-3	creation of artificial biotopes in BSL3 facilities	3	M16	M42	4
M 3-4	development of techniques for detection and study on viability of H5N1 virus after inoculation in artificial aquatic biotopes	3	M16	M42	4
M 3-5	technological transfer from and to other participating laboratories	3	M18	M42	4
M 3-6	detection and study on viability of H5N1 virus after inoculation in artificial aquatic biotopes M24-M30: data analysis	3	M24	M42	4
M 3-7	final report - Peer-review scientific publications in international journal publication, spreading of the information (conferences, reports, …)	3	M18-36	M42	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 part of the project our work continued to assess the impact of the aldehydes agents against the AIV – reverse genetic reassortant (NIBRG – 14, clade 1) in standing and running water. The action of the UV light was assessed also. Viability of the influenza virus was tested by qRT-PCR at 72 hours after inoculation of samples in MDCK cells. The results suggest that glutaraldehyde shows a good efficacy of destroying of virus infectivity in relation with environmental temperature. Ultraviolet radiation light has been proven to inactivate AIVs in 10 - 20 min in experimental condition if the viral loading is 10^3 $TCID_{50}$/0.1 ml and the effective dose of irradiation is at least 3000 J/m^2.

The persistence of the infective viral particles of the same influenza reassortant (10^4 $TCID_{50}$) was evaluated in three kinds of water: rivers, lake and Black Sea water, and PBS as control, in accordance with their main characteristics: pH, salinity, protein content, oxygen, nitrite, ammonium, phosphate content, at three temperatures: 4-8 ^oC; 22 ^oC and 35 ^oC. Viability of the influenza virus was tested periodically by microELISA technique on MDCK cells. Our results show that the viability of the virus is maintained at least 20 days at low temperature and 12 days postinfection at higher temperature.

Deviations from the project work programme, and corrective actions taken

As stated in the first interim report, part of the work (tasks 5 and 6) was anticipated. As planned, the other tasks started at M19 but underwent some delay when the A(H1N1)2009 pandemic broke out.

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP N.	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 4-1	Approvable and standardised protocols for testing the effect of chemical and physical treatment of different types of water on influenza virus survival	4	M19-M33	M33	10	3	2
D 4-2	Data on the efficacy of chemical and physical treatments of water for the reduction of virus load and virus survival	4	M19-M33	M33	10	2.5	2
D 4-3	Guidelines for treatments of different types of water aiming to reduce virus survival	4	M30-M33	M42	10	2.5	2

D 4-4	Scientific publications in peer-review journal	4	M30-M33	M42	2	0,5	2

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP N	Date due	Actual/Forecast delivery date	Lead contractor
M 4-1	Choice of types of water treatment to be tested	4	M20	M33	2
M 4-2	Choice of an experimental model for testing water treatment on virus survival; expected result: standardised testing protocol	4	M26	M33	2
M 4-3	Implementation of the experimental model for water treatment testing and choice of the best combinations of physical and chemical factors.	4	M33	M33	2

WORK PROGRAMME II

"*Survival of AIVs 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.

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, as rough and porous surfaces. Moreover, we obtained from a major worldwide airline company 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 non porous and smooth surfaces, assays were carried out on watch glass which enabled us to choose the strategy for virus recovery (see protocol soon on web site).

For porous and rough surfaces, assays were carried out without prior sterilisation of weaved tissue handkerchiefs and fabrics used in passenger cabins in civil aeronautics (see protocol soon on web site).

Both kinds of surfaces were treated using the mechanical effect of beads [(Balls-1/4 Ceramic Sphere (MPBiomedicals, LLC ref: 6540-412)] with the Ball mill FastPrep–24 apparatus (MPBiomedicals ref: 6004500), on 15 mL plastic tubes holder for FastPrep–24 apparatus (12x15ml Adapter MPBiomedicals ref: 6002526)

Assays were preformed in triplicate and with several conditions of time and speed, in order to validate the best method for virus recovery, were tested.

The tested conditions were the following:

30 seconds at 6M/S, 30 seconds at 4M/S, 40 seconds at 4M/S and 60 seconds 4M/S plus twice 40 seconds at 4M/S.

The ratios of virus recovery on weaved tissue handkerchiefs were estimated by $TCID_{50}$/mL and for fabrics used in passenger cabins in civil aeronautics by $TCID_{50}$/mL and qRT-PCR (M gene).

For viral recovery on weaved tissue handkerchiefs we choose 30 seconds at 4M/S conditions. In fact these conditions allowed recovering of virus with a loss of $10^{0.59}$.

For fabrics used in passenger cabins we choose 30 seconds at 6M/S conditions. In fact these conditions allowed recovering virus with a loss of $10^{3.79}$.

Detection and titration of virus infectivity in the course of time (from 5 min. to three days in) at given temperatures (4℃, 25℃ and 35℃) (Task 4) were car ried out after using the strategy previously chosen for virus recovery on watch glasses.

The assays were carried out on washed, degreased (acetone alcohol) and sterilised by autoclave (20 minutes at 121℃) watch glass. Watch glasses were p ut in a hermetic box with desiccant to facilitate the drying of the suspension.

Fig. WP5-1: Picture of watch glasses in the hermetic box

We used the following viral strains:

The Assay with Influenza A/Duck/Cambodia/D4(KC)/2006 (H5N1) was performed at 3 temperatures (4℃, 25 and 35℃) and survival evalua tion was performed by titration by $TCID_{50}$/mL and compared with qRT-PCR for M gene after 1 day, 2 days and 8 days. Each watch glass was contaminated with 50µL containing a A/Duck/Cambodia/D4(KC)/2006 (H5N1) virus stock suspension with a titre of $10^{8.5}$ $TCID_{50}$/mL. The calculated titre for each watch glass was $10^{8.5}$ $TCID_{50}$/mL. For each condition, assays were preformed in triplicate. After each time point, virus was collected with 500 µL of culture medium.

Table WP5-1 : Results:

Temperature	Time	Average titre $TCID_{50}$/mL (Standard deviation)	Average loss $TCID_{50}$/mL	Gene M detection copies number/ 5 µL (Standard deviation)
4℃	Immediately after contamination	$10^{6.70}$ (0.18)	$10^{0.80}$	7.77×10^{9} (2.45×10^{9})
	Day 1	$10^{6.44}$ (0.10)	$10^{1.06}$	2.61×10^{9} (1.30×10^{8})
	Day 2	$10^{6.52}$ (0.25)	$10^{0.98}$	3.10×10^{9} (6.50×10^{8})
	Day 8	$< 10^{6.70**}$ (NA*)	$10^{5.83}$	1.25×10^{9} (2.70×10^{8})
25℃	Day 1	$10^{3.94}$ (0.34)	$10^{3.65}$	1.22×10^{9} (3.50×10^{8})
	Day 2	$10^{2.11}$ (0.54)	$10^{5.39}$	4.28×10^{8} (3.50×10^{7})
	Day 8	$< 10^{6.70**}$ (NA*)	$> 10^{5.83}$	2.06×10^{7} (1.57×10^{7})
35℃	Day 1	$< 10^{2.22}$ (0.51)	$> 10^{5.83}$	2.33×10^{8} (4.00×10^{7})
	Day 2	$< 10^{6.70**}$ (NA*)	$> 10^{5.83}$	1.28×10^{7} (4.20×10^{6})
	Day 8	$< 10^{6.70**}$ (NA*)	$> 10^{5.83}$	1.02×10^{6} (7.13×10^{6})

**= Calculated detection threshold

*NA= not applicable: Do not result from a measure but it is obtained from the calculated detection threshold

As already explained in Work Package 0 progress, we decided to study the survival of the new A(H1N1) pandemic virus in the environment, because Influenza A virus survival in different environmental conditions is dependent on virus isolates even within the same subtype. Our results showed that this virus has the ability to persist on glass surface for extended periods of time. On smooth nonporous surface, the H1N1pdm virus retained its infectivity for at least 4 days at 35°C and up to 56 days at 4°C.

Additional experiments suggest that external viral structures in direct contact with the environment must be involved in virus loss of infectivity.

All the data generated by this study are described in a full research paper entitled "Long-lasting Persistence of the Novel Pandemic Influenza A(H1N1) Virus in the Environment" which will be soon submitted for publication.

To proceed with task 7, a BSL3 climatic chamber with controlled temperature and humidity has already been designed (see previous report), and its making will be starting as soon as we have the funding (thanks to the sponsorship of Groupe Accor

(http://www.accor.com/fileadmin/user_upload/Contenus_Accor/Presse/Pressreleases/2010/FR/100118_CP_Partenariat_Accor_Institut_Pasteur_FR.pdf)

and BNP Paris CBI

(http://cercle-actionnaires.bnpparibas.com/focus.php)).

Meanwhile, the use of a BSL3 half-suite isolator allowed us to start the experiments with fewer controlled parameters. The aerosol nebulizer (Palas PLG2000) and the air biocollectors (Sartorius MD8 Airport and MD8 Airscan) have been ordered, installed and tested in this environment (see pictures below).

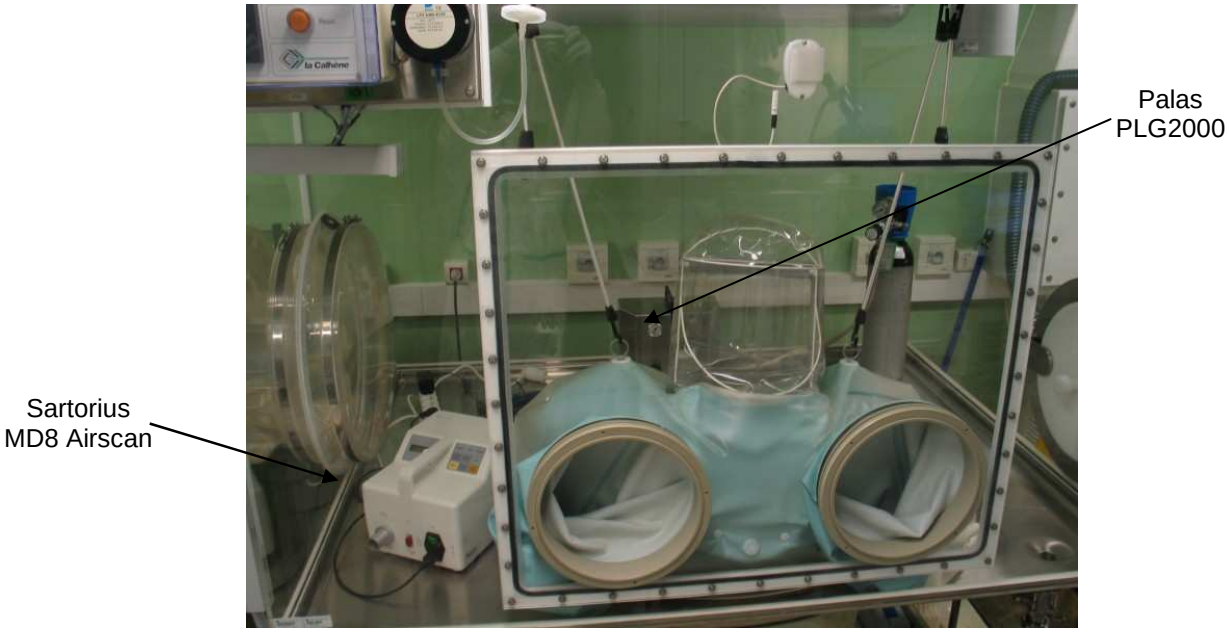


Fig.WP5-2: Picture of the half-suite isolator with the aerosol nebulizer and air sampler installed

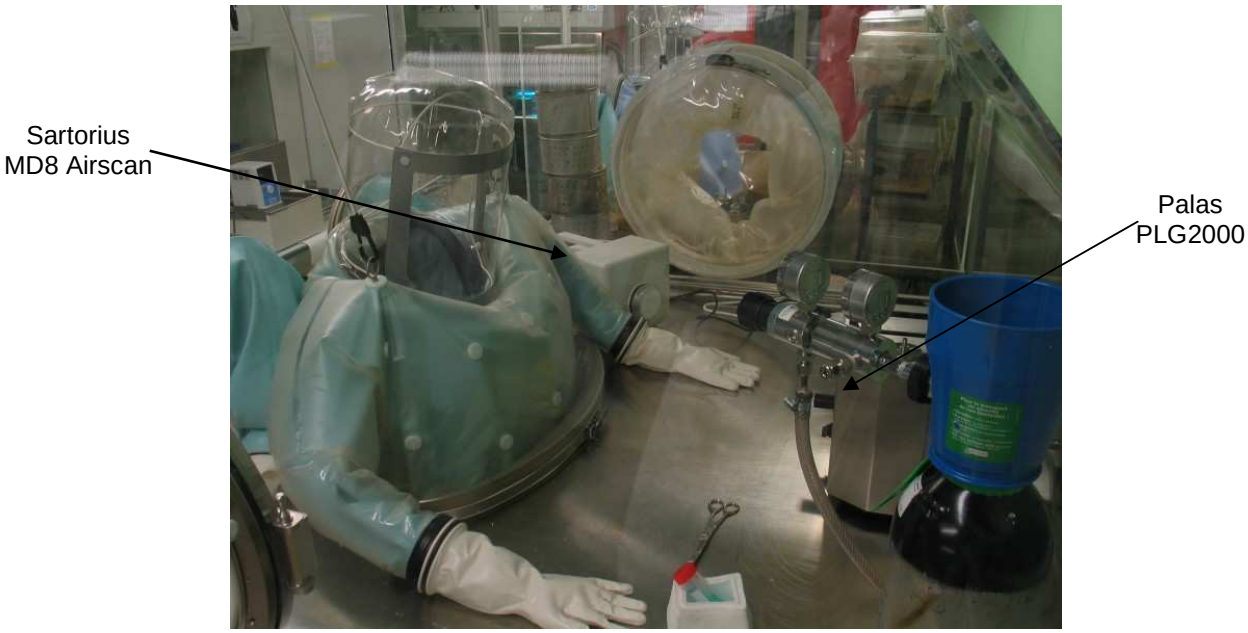


Fig. WP5-3: Another view of the half-suite isolator with the aerosol nebulizer and air sampler installed

The whole experimental procedure has been elaborated and submitted to the biosafety committee of the IPP, which just approved it (M36). The protocols for the bioaerosol generation (WP5-task 5) and the air sampling and virus collection (WP5-task 6) have been written and are under testing (available soon on the RIVERS website). The detection of virus in bioaerosols and its infectivity in the course of time will be starting soon.

Currently, the scientific community has still a limited knowledge on many aspects of the ecology and environmental properties of influenza viruses. Studies on the survival of influenza viruses in the atmospheric environment and on inert surfaces are rather limited and difficult to interpret because of the methodological difficulties. Further studies are needed in order to develop suitable control strategies to prevent risk of infection. Consequently, conducting environmental studies on the resistance of influenza viruses has been an important focus of WP5.

In this context, the underground public transportation in Paris appeared as an essential sampling site because of its representativeness of the urban environment and its convenience for sampling (confined space, dry air and high density of population). The aim of the present study was to determine whether respiratory viruses, particularly human influenza viruses or respiratory syncytial virus, could be detected in the air and on inert surfaces of subway trains at the peak of the influenza virus epidemic in metropolitan France. Samples were taken twice a week throughout the active circulation of influenza viruses in the northern hemisphere, during rides within the busiest lines of the Parisian subway network. A standalone portable device, the Airport MD 8 (Sartorius), was used to collect air samples (500L in 10 min) on gelatin membranes. Surface smears were taken on door handles and bars of the subway cars using nylon swabs.

A total of 72 air samples and 150 surface samples were collected. Searches for influenza viruses or respiratory syncytial viruses were performed by standardized quantitative and classical RT-PCR methods. Analysis revealed that one air sample was positive for influenza A virus. Unfortunately, virus culture using MDCK cells did not lead to virus isolation. Presently, focus has been placed on the search for other viruses in the collected samples, particularly rhino-, entero- and noroviruses.

Deviations from the project work programme, and corrective actions taken

Delays are the main deviation from the initial and revised programme especially due to the A(H1N1) pandemic which broke out in the middle of the RIVERS project.

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP N.	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 5-1	Approvable and standardised protocols for influenza virus recovery from various surfaces	5	M25	M38	3	2	1
D 5-2	Integrated air system for respiratory virus survival in aerosols	5	M33	M42	3	1	1
D 5-3	Data (database) about IV survival in the air and on various kind of surfaces and in various conditions	5	M33	M42	3	2	1
D 5-4	Guidelines for recommendations	5	M33	M42	3	0	1
D 5-5	Peer-review scientific publications in international	5	M23	M42	0.5	0.67	1

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP N	Date due	Actual/Forecast delivery date	Lead contractor
M 5-1	Choice of surfaces: sterilisation methods, preparation of experiment friendly surfaces	5	Choice: M12 Sterilisation: M20	M42	1
M 5-2	Availability of strategies for virus recovery from various surfaces	5	M38	M42	1
M 5-3	Availability of aerosol generator and choice of biocollector	5	M38	M42	1
M 5-4	Implementation of an air system to study virus survival	5	M38-42	M42	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

In Asia (Cambodia)

As explained for WP 2, in Cambodia, In response to the notification of a confirmed case of influenza subtype H5N1 infection in humans or poultry, the environmental contamination by the H5N1 virus was studied. The investigation was conducted in households surroundings in the vicinity of the index household. Environmental specimens such as mud, pond water, water plants, soil, insects, aquatic animals and swabs were collected and processed as indicated in WP2.

Each year since the beginning of the RIVERS project and until now, the environmental investigations were conducted in the location with influenza virus (H5N1)–associated outbreaks.

H5N1 outbreak in 2009 and 2010

At the beginning of 2010 in Takeo province, which is located in the south next to the Vietnamese border, an outbreak of H5N1 AIV was reported in poultry. This outbreak caused thousands of death for the poultry in a single village. The sampling process was conducted in the surroundings of the households and in the field of villagers as detaild in WP2.

In December 2009, one confirmed human case of H5N1 was reported from the province of Kampong Cham, in a village where a previous H5N1 outbreak had already been reported in 2007. As reportd for WP2, a total of 82 specimens were collected in the location surrounding the index household with 37 out of these are now in process of analysing to investigate the possible environmental contamination of the H5N1 virus. The 11 positive samples are currently proc

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

The main activities carried out during the second 18-months period were related to WP6 and as well as 2 and 9. Two directions were followed (1) improvement and standardisation of the molecular detection methods for AIV and (2) contribution to the development of models for virus survival in its natural environments.

Within the framework of this project, the following activities were undertaken in Montpellier and in Cambodia in coordination between the Pasteur Institute of Phnom Penh and the UR AGIRs of CIRAD:

- Publication of an article in the International Journal of Applied Earth Observation and Geoinformation, Tran, A., et al., Remote sensing and avian influenza: A review of image processing methods for extracting key variables affecting AIV survival in water from Earth Observation satellites. Int. J. Appl. Earth Observ. Geoinform. (2009), doi:10.1016/j.jag.2009.09.014 Recent studies have highlighted the potential role of water in the transmission of avian influenza (AI) viruses and the existence of often interacting variables that determine the survival rate of these viruses in water; the two main variables are temperature and salinity. Remote sensing has been used to map and monitor water bodies for several decades. In this paper, we review satellite image analysis methods used for water detection and characterization, focusing on the main variables that influence AI virus survival in water. Optical and radar imagery are useful for detecting water bodies at different spatial and temporal scales. Methods to monitor the temperature of large water surfaces are also available. Current methods for estimating other relevant water variables such as salinity, pH, turbidity and water depth are not presently considered to be effective.

- Our work in 2008 allowed us to identify environmental indicators in connection with floods and landuse, from MODIS data. These indicators are linked to epidemiological data in order to identify risk factors for the occurrence of avian influenza cases. A model to detect water surfaces and periods of floods has been created.
- Field data, in Cambodia in several spots known for previous H5N1 outbreaks, have been collected between March and August 2009 (before and at the end of rainy season), in order to validate the model.
- June 2009: a mission of modeler war organized in Cambodia in order to work on the H5N1 data available in IPC.
- October 2009: starts of the analysis of correlation between environmental conditions linked to water and the number of H5N1 cases in Cambodia. This analysis should be finished end June 2010.

In Europe (Bulgaria)

Prevalence of AIV in Bulgaria

1. Regionally

Samples studied were collected from fresh foeces and cloacal swabs from mule ducks and fecal materials from *Anser albifrons* and *Branta ruficollis* along *Via Pontica.*

Samples from mule ducks from the following regions in Bulgaria were collected in a specific schedule and were sent for laboratory diagnostics:

- Regions of Stara Zagora, Haskovo, Dobrich and Yambol– samples collected and sent five times monthly in the period November 2008 – March 2009.
- Regions of Plovdiv and Pazardjik– samples collected and sent two times monthly in the period March 2009 – April 2009.

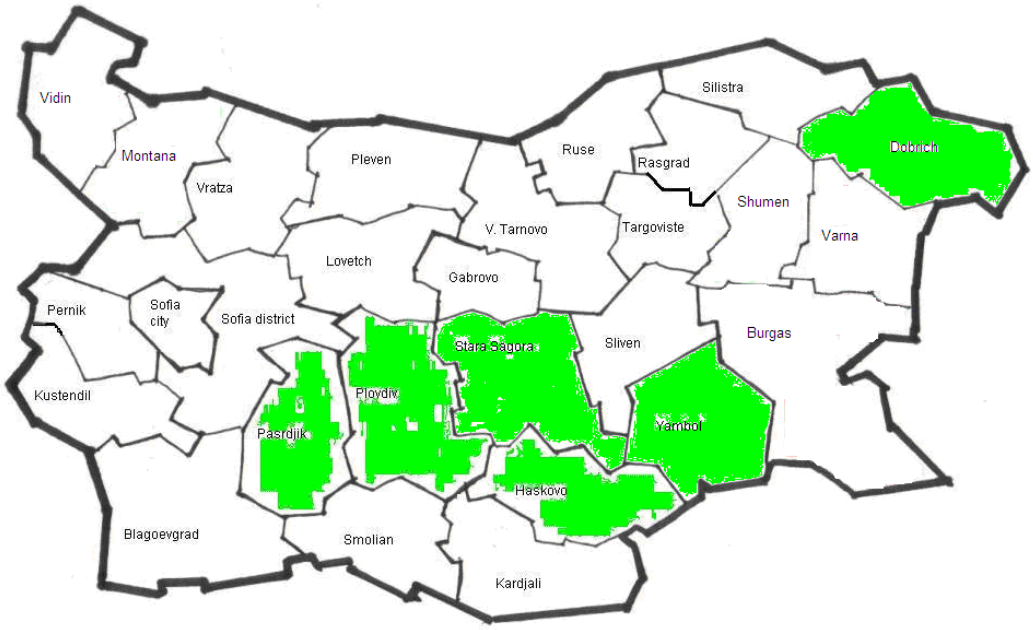


Fig. WP6-1: Map of Bulgaria with regions with positive LPAI

Samples were collected from farms are listed below:

Stara Zagora region:

- *"Mebida" Ltd, Bratya Daskalovi village*
- *"Камtrick" Ltd, Suedinenie village*
- *"Jessica Pan" Ltd, Oryzovo village*
- *"Heron 77 – Slav Yanchev" Oryzovo village*
- *"Yankovi Brothers – Yuri Yankov", Granit village*
- *"Beximpo – B.Georgiev", Cherna Gora village*

- *"Detelina – V. Lesov", Malak Dol village*
- *"Vety" Ltd –B. Tabakov, Golyam Dol village*
- *"Irony - 89", Mirovo village*
- *"Avita", Kolarovo village*
- *"P.S.V. Group" Ltd, Bozduganovo village*
- *"Ani Max", Yagoda village*
- *"Todor Peltekov", Gita village*

Haskovo region:

- Farm for adolescent mule ducks – G. Vanchev, *Knijovnik village*
- Farm for adolescent mule ducks – Z. Vancheva, *Knijovnik village*
- Farm for adolescent mule ducks – Z. Kolev, *Knijovnik village*
- Farm for adolescent mule ducks - T. Tenev, *Knijovnik village*
- Farm for adolescent mule ducks - J. Taneva, *Knijovnik village*
- Farm for adolescent mule ducks - K. Koleva, *Knijovnik village*
- Farm for adolescent mule ducks - S. Stamov, *Knijovnik village*
- H. Uzunov", Knijovnik village
- Farm for adolescent mule ducks - K. Dimitrov, *Malevo village*
- Farm for adolescent mule ducks K. Dimitrov, *Manastir village*
- Farm for adolescent mule ducks, *Dolno Voyvodino village*
- "Gray", *Tunkovo village*
- "Pro Agro 2000" Ltd, Poultry Farm - *Haskovo town*
- "Tedymex", Poultry Farm - *Haskovo town*
- "Vipera"-D. Kiryakov, *Konstantinovo village*
- "Agrofix", *Simeonovgrad town*
- "Helix" Ltd, *Krepost village*
- "SIG" Ltd, *Dobrich village*
- "Gray"-G.Antonov, *Haskovo town*
- "Matev" Ltd, *Chernokonevo village*
- "Radina"-R. Marchev, *Uzundjovo village*

Dobrich region:

- "Chance 61" Ltd- V. Vassilev
- "BMW"- S. Zankov, *Lomnica village*
- "Poultry farm V. Dimitrov, *Balchik town*

Yambol region:

- Aliance Agricol" Ltd, *General Inzovo village*
- Aliance Agricol" Ltd, *Pobeda village*

Plovdiv region:

- "Sifiana 2006", *Begovo village*
- "Des-93"D. Genov, *Ceretelevo village*
- "Kotov"Ltd A. Kotov, *Carimir village*
- "Mini 2008"N. Panishev, *Pudarsko village*
- "Venelin-Nedyalko Tabakov", *Pudarsko village*
- "Venelin-Nedyalko Tabakov", *Brezovo town*
- "MIZ" Ltd, *Brezovo town*
-"DINO" Ltd- I. Bodoshki*, Belozem village*
- "GIDIK" Ltd – Y. Gidishki, *Rakovski town*
- "Gendov" Ltd – Y. Gendov, *Rakovski town*
- "LIMP" Ltd , *Shishmanci village*
- "Yankovi Brothers", *Gradina village*

Pazardjik region:

- "KWELE", *Chernogorovo village*
- "Tonyvet-eco-27" Ltd *, Pishtigovo village*
- "Lulchev -55" Ltd , *Topli Dol village*
- "Deltastroy Gemishev & Co" Ltd, *Malo Konare village*

Table WP6-1. Detection of Influenza A virus strains in birds - Regional Diagnostics Laboratory of Influenza A and NDV in birds, Aksakovo, Varna region, Bulgaria, 2009

	Number of samples studied	PCR and virological	Serology	Positive	Negative
IAV	3383	661	2722	5	3378

2. Overall situations in Bulgaria

Table WP6-2 : Confirmation of cases, detected in regional laboratory in Aksakovo and detection of Influenza A virus strains collected from birds on all the territory of the country - National Reference Laboratory, Sofia, Bulgaria, 2009.

Method	Number of samples studied	Results
Serology	9367	-
Virological (embryonated chicken eggs)	2289	22 low-pathogenicity strains isolated from mule ducks imported from France - **H4N6, H3N2 and H4N2**
Molecular detection (qRT-PCR)	1119	-
Total	12775	22 low-pathogenicity strains isolated from mule ducks imported from France - **H4N6, H3N2 and H4N2**

In the period November 2008 -2009 22 low-pathogenicity strains of Influenza A viruses were isolated and laboratory confirmed in Bulgaria. All the strains H4N6, H3N2 and H4N2 were isolated from samples from mule ducks, imported from France.

Table WP6-3: Low-pathogenicity strains isolated and laboratory confirmed in Bulgaria

Numl	Strain	Subtyp	Date of isolation	Site of Isolation	Type of bird
1	**9/WP/MD/28-34**	**H6N2**	26.11.2008	"Yankovi Brothers – Yuri Yankov", Gr village	mule ducks
2	**11/WP/MD/36-45v**	**H6N2**	26.11.2008	Beximpo – B.Georgiev", Cherna Gora villag	mule ducks
3	**61/WP/MD/244**	**H3N2**	09.12.2008	"Heron 77 – Slav Yanchev" Oryzovo village	mule ducks
4	**68/WP/MD/270-275**	**H6N2**	09.12.2008	Detelina – V. Lesov", Malak Dol village	mule ducks
5	**76-77/WP/MD/297-30**	**H6N2**	09.12.2008	"P.S.V. Group" Ltd, Bozduganovo village	mule ducks
6	**78/WP/MD/307-313**	**H6N2**	09.12.2008	"Todor Peltekov", Gita village	mule ducks
7	**121-122/WP/MD/478 482**	**H6N2**	16.12.2008	farm for adolescent duck-mulards, Knijov village	mule ducks
8	**127/WP/MD/493-496**	**H4N2**	16.12.2008	farm for adolescent duck-mulards, Knijov village	mule ducks
9	**105/WP/MD/428-437**	**H4N2**	16.12.2008	"Gray"-G.Antonov, Haskovo town	mule ducks
10	**173/WP/MD/647-651**	**H6N2**	12.01.2009	"Tedymex", Poultry Farm - Haskovo town	mule ducks
11	**174/WP/MD/652**	**H6N2**	12.01.2009	"Tedymex", Poultry Farm - Haskovo town	mule ducks
12	**175/WP/MD/653-662**	**H6N2**	12.01.2009	"Pro Agro 2000" Ltd, Poultry Farm - Hask town	mule ducks
13	**191-192/WP/MD/718 721**	**H4N2**	15.01.2009	farm for adolescent duck-mulards, Mana village	mule ducks

14	**247/WP/MD/871-875**	**H3N2**	09.02.2009	"Jessica Pan" Ltd, Oryzovo village	mule ducks
15	**248/WP/MD/876**	**H3N2**	09.02.2009	"Jessica Pan" Ltd, Oryzovo village	mule ducks
16	**263/WP/MD/933-938**	**H6N2**	09.02.2009	"Todor Peltekov", Gita village	mule ducks
17	**285/WP/MD/1036-10**	**H6N2**	10.02.2009	"Gray"-G.Antonov, Haskovo town	mule ducks
18	**286/WP/MD/1046**	**H6N2**	10.02.2009	"Gray"-G.Antonov, Haskovo town	mule ducks
19	**369/WP/MD/1295-13**	**H4N2**	16.03.2009	"Gray"-G.Antonov, Haskovo town	mule ducks
20	**396/WP/MD/1385**	**H4N2**	16.03.2009	farm for adolescent duck-mulards –Knijov village	mule ducks
21	**402/WP/MD/1403**	**H4N2**	15.03.2009	farm for adolescent duck-mulards –Knijov village	mule ducks
22	**524/WP/MD/1699-17**	**H6**	07.04.2009	"Venelin-Nedyalko Tabakov", Pudarsko villa	mule ducks

Persistence of AIV in cloacal samples

Materials and Methods

Virus and innoculum preparation

The low-pathogenic avian influenza A virus (LPAIV) of the H6N2 subtype obtained from a mallard duck (*Anas plathyrynchos*) was used at a titre of 10^5 ELD50 /0.1 mL (ELD_{50} mean embryo lethal doses causing a 50% death rate in inoculated CEs) (Zarkov et al., 2006). Allantoic fluid was collected after inoculation of LPAIV (H6N2 subtype) into the allantoic sac (100 μL) of 5 to 9-day old CEs. Embryos were observed daily for 120 hours (when all were dead). Allantoic fluid derived from them was explored by haemagglutination assay (HA) (Anonymous, 2005). Samples with haemagglutin titres of 1:128 were stored at -84℃ until used in the experiment.

Birds and protocol design

9 mallard ducklings (*Anas plathyrynchos*) 30–day-old and were used in this experiment. 7 ducklings were intravenously infected with 100 μL allantoic fluid from infected CEs (CE) while 100 μL allantoic fluid from intact CE was intravenously injected to the other birds (uninfected control group, n = 2). The 2 groups of infected and uninfected birds were kept separately in 4 x 4 m rooms at 1.8 m feeding and watering front, 20℃ and 70% humidity. No vaccine and antibiotic were administered to the birds.

Fecal swabs from all infected and uninfected birds were collected on day 5 post infection (P.I.) and were stored in riginal form without any further processing. In intervals of two days (2, 4, 6, 8, 10, 12, 14, 16, 18, 20 ден) parts of fecal samples were taken and infectious vitus titers were examined by cultivation in CEs.

Virus isolation method

A 20% suspension of the fecal samples (w/v) was prepared in MEM (pH: 7.2-7.4) supplemented with penicillin G (2.10^6U/L), streptomycin (200 mg/L), polymyxin B (2.10^6 U/L), gentamicin sulfate (250 ml/L), nystatin dehydrate ($0.5.10^6$ U/L), sulphamethoxazole (0.2 g/L) and foetal bovine serum (0.5%). After homogenization and centrifugation (800 g, 4℃ for 10 min), the supernatant (200 μL) was inoculated into the allantoic sac of three 9-day old CE. The infected embryos were incubated at 36℃ for 120 hours, then the dead and living CE were cooled at 4℃ for another 2 hours and the allantoic fluid was collected. The presence of the haemagglutinating virus were determined by the haemagglutination assay (HA) and the viral haemagglutinins by the haemagglutination inhibition (HI) test. Serial dilutions (50 μL) of the allantoic fluids (1:2 – 1:4096) were prepared in a micro plaque with buffered saline and 50 μL of 1% hen erythrocyte suspension were added. HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The highest dilution of the allantoic fluid preventing the spot-like agglutination of erythrocytes corresponded to the haemagglutinating viral titre. The haemagglutinins from the H6 isolates were identified by the HI test using a chicken monospecific hyperimmune serum diluted to 1:256. The micro plaque remained at room temperature for 30 min before the results were read. Positive HI (presence of agglutination) evidenced the subtype of the viral haemagglutinins.

Virus titers were calculated by the method of REED & MUENCH (1938). For titration sample were taken from cloaca of bird №2 on day 2-, 4-, 6-, 8. The results were recorded and are summarized in table 1.

Results

A. Time of persistence of virus in fecal samples

Control birds feces gave always negative results for H6N2 subtype virus for the whole experimental period.

The period of avian influenza A virus H6N2 subtype tenacity varied from all infected feces between the 2 to the 8 P.I. days (Table 14).

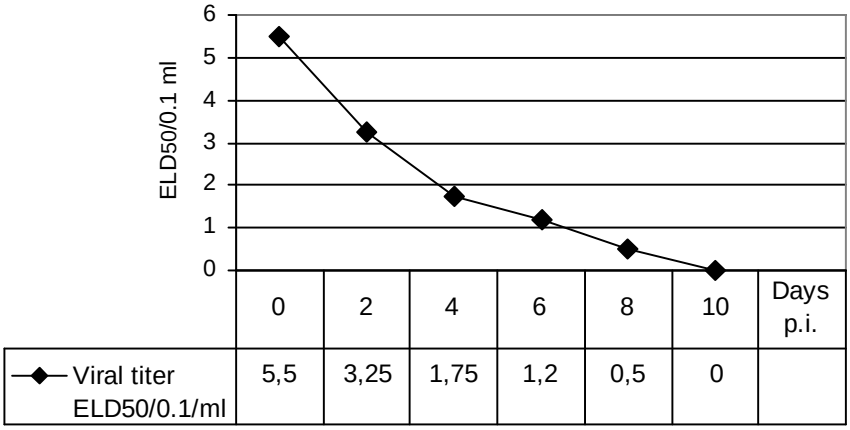
Table WP6-4:. Isolation of H6N2 subtype virus from feces of infected ducklings

	Presence of virus									
Days	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
Duck №1	+	+	+	+	-	-	-	-	-	-
Duck №2	+	+	+	-	-	-	-	-	-	-
Duck №3	+	+	+	+	-	-	-	-	-	-
Duck №4	+	+	+	+	-	-	-	-	-	-
Duck №5	+	+	+	+	-	-	-	-	-	-
Duck №6	+	+	+	-	-	-	-	-	-	-
Duck №7	+	+	+	+	-	-	-	-	-	-

B. Viral titer in fecal samples

During the period of observation viral titers were reduced from 10 $^{5.5}$ ELD_{50}/0.1 ml (day 0) to 10 $^{0.5}$ ELD_{50}/0.1 ml (day 8) as seen in Fig 2. the sharpest was decrease between day 2 and day 4 - 10 $^{1.5}$ ELD_{50}/0.1 ml /from10 $^{3.25}$ ELD_{50}/0.1 ml to10 $^{1.75}$ ELD_{50}/0.1 ml

Fig. WP6-2 **Titration in embryonated chicken eggs of A/duck/Bulgaria/05 H6N2 from fecal sample of experimentally infected with IAV (H6N2) duck (day 0-10)**



Discussion

In the present study, the LPAIV H6N2 subtype virus was successfully isolated from feces from previously intravenously infected *Anas plathyrynchos* ducklings for period to 8 days. Experiments were preformed in specific conditions of storage of fecal swabs (20℃ and 70% humidity) and normal bacterial microflora. These parameters of humidity and temperature are considered as optimal for housing of poultry (closed farms according BDS standart). The period of storage is similar to data published by Lu et al. (2003) – 7 days but these studies were performed keeping temperature without monitoring of humidity. The infectious virus titer decreased sharply after day 4. The transmittion of infection during housing of domestic ducks in closed poultry farms is influenced not only by the period of persistence of virus in feces but by the contact of susceptible birds with feces infected with virus (Sturm-Ramirez et al., 2004).

AIV might be isolated from feces of wild birds (Webster et al., 1978) and the method is widely used by investigators including for isolation from *Anas plathyrynchos* which are the main reservoir of the virus. Nevertheless, there are too little data for persistence of AIV in open areas (ground, marsh-water, plants) (Vong et al., 2006). PCR studies determined 35 % positive for viral RNA samples but it is remained unclear if the virus is infectious for birds. The time of persistence of IAV in feces in open areas is an important topic due to the variety of abiotic (physical and chemical components) and biotic factors (other microorganisms).

Deviations from the project work programme, and corrective actions taken

During the M19-M36 period, delays for this WP were due to the overload of work linked to the A(H1N1) pandemic.

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP N	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 6-1	Data on the prevalence of AIVs in the surroundings of farms with present and past outbreaks of HPAIV H5N1	6	M33	M36	7	7	3
D 6-2	Data on variations in prevalence and in virus load in the surroundings of farms with present and past outbreaks or in areas with wild brid H5N1 mortality, in the course of time throughout the year in tropical and non tropical regions of the world	6	M33	M36	7	6.5	3
D 6-3	Data on virus survival in bird guano	6	M20	M42	7	6.5	3
D 6-4	Publications in peer reviewed international journals	6	M36	M42	0.7	1.,1	3

List of Milestones

Milestone no.	Milestone name	WP N.	Date due	Actual/Forecast delivery date	Lead contractor
M 6-1	Earth sampling and collection from commodity surfaces (input from WP5)	6	M33	M42	3
M 6-2	Detection and quantification of viable viruses in solid samples with standardised protocols	6	M33	M42	3
M 6-3	Detection and quantification of viable viruses in solid samples (biota, mud)	6	M33	M42	3
M 6-4	Data analysis and transfer to WP9	6	M33	M42	3

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 which was conforted by the results and conclusions from WP0 between M19 and M36. 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, during the M1-M18 period, we had 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 (see first interim report). This tool is now also used by WP0.

Deviations from the project work programme, and corrective actions taken

During the M19-M36 period, delays for this WP were due to the overload of work linked to the A(H1N1) pandemic.

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP N.	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 7-1	General information on avian flu virus survival in natural condition when dried	7	M27	M42	9	4	5
D 7-2	Scientifically tested information on dried AIV stability in parameterised conditions	7	M27	M42	10	4	5
D 7-3	Standard Operating Procedures for virus disinfection/inactivation in different settlements	7	M33	M42	5	4	5
D 7-4	Training of Chinese and Asian scientists on Biosafety and virus inactivation	7	M19	M42	5	0	5
D 7-5	Publications in peer reviewed international journals	7	M36	M42	1,1	0.7	5

List of Milestones

Milestone no.	Milestone name	WPN	Date due	Actual/Forecast delivery date	Lead contractor
M 7-1	Virus stocks and titration. RNA quantification. Preparation of surface/supports. Preparation of instrumentation. Prove of concept (dehydratation, elution, reproduction of results)	7	M22	M42	5
M 7-2	Experiments of dehydrated virus inactivation on different surfaces under different conditions for different viruses	7	M27	M42	5
M 7-3	Comparative results with other scientists from different workpackages. Publications	7	M33	M42	5

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

WP Leader: Partner 7 – Institut Pasteur de Lille (Jean-Marie DELATTRE)

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.

Animal materials involved in this project include birds 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 poultry meat were evaluated. With a suitable method, quantitative studies of the impact of food processing conditions on AIV was 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 not impossible 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.

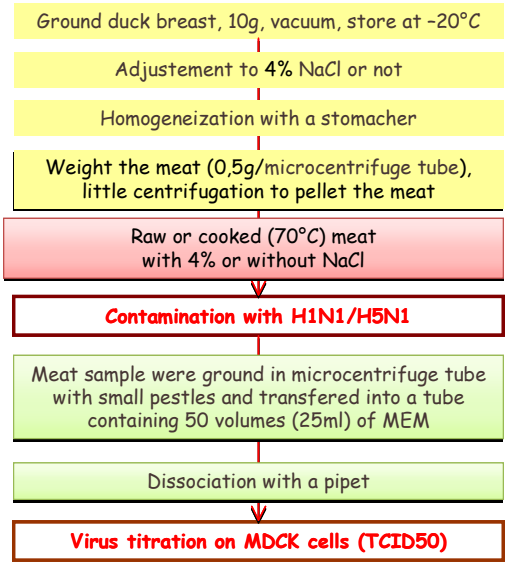
Progress towards objectives

Poultry meats could be contaminated by differents contamination routes. Data concerning chicken and duck thigh and breast naturally infected by H5N1 were available. Some experiments were carried out to put in evidence infection of thigh and breast by intravenous (Suarez et al., 1998 ; Tumpey et al., 2002), intra-tracheal (Suarez, Perdue et al., 1998) and intranasal route (Suarez, Perdue et al., 1998 ; Swayne et al., 2005 ; Thomas et al., 2007 ; Brown et al., 2008 ; Das et al., 2008 ; Thomas et al., 2008) and by ingestion of infected chicken meat (Swayne et al., 2004 ; Brown, Stallknecht et al., 2008). Domestics cats were experimentally infected after feeding on infected chicken carcasses (Rimmelzaan, 2006). And during outbreaks, various carnivores, including domestics cats, tigers, leopards, stone martens and dogs, were infected possibly by consuming infected bird carcasses. Moreover meat could possibly be contaminated during slaughter and evisceration of infected poultry (Harder et al., 2009).

<u>Extraction and detection of AIV in poultry meat (Task 3)</u>

To study survival/inactivation of AIV in poultry products, a protocol for extraction and detection of AIV from poultry meat was based on previously described methods (Swayne, 2006 ; Thomas and Swayne, 2007 ; Thomas et al., 2008). Recovery of two Influenza strains, H5N1 (A/HK/156/97) and H1N1 (A/PR/8/34), was validated.

Experimental protocol is illustrated below:



Briefly, duck or chicken breasts were ground and stored frozen. After defrost of 10 g ground meat, salt concentration was adjusted or not to 4 % (w/w) by addition of 2 ml of a salt suspension containing 0.4 g NaCl and homogenisation with a stomacher. pH and aW were checked before and after addition of salt. 0,5 g of meat was dispensed in 1,5 ml polypropylene microcentrifuge tubes and centrifuged to pack the meat in the bottom of the tube. 10 µl of a viral suspension (10^8 $TCID_{50}$/ml) were added in each tube, to be sure of homogene repartition of virus, and homogenised with 0.5-ml pestles. Samples were kept 1 hour at room temperature. Meat was transfered into a tube containing 50 volumes (25 ml) of culture medium (MEM). Ground meat was dissociated with a pipet. Mixture was serially 10-diluted in MEM and immediately titrated on cells.

Preliminary tests were carried out in order to determine conditions to remove cytotoxicity and titration interference, when meat was 50-diluted before titration on cells, and to obtain a good virus recovery from meat. No significant decreases of infectious titres was immediately observed when virus was added in raw or cooked meat with or without NaCl. About 2 10^5 $TCID_{50}$ / ml were recovered when 8 10^5 was inoculated. No cytotoxicity and no interference were observed in the previously described conditions.

Evaluation of individual effect of main parameters, involved in cooking of poultry meats, and their interaction on survival of H5N1 in poultry matrices (Task 1 and 2)

This workpackage consists in evaluating the impact of parameters involved in cooking of poultry meats. Based on results obtained with enteric viruses, chemical and physical factors, such as pH (Salo et al., 1976 ; Siegl et al., 1984), divalent cations (Wallis et al., 1961 ; Wallis et al., 1965 ; Fujioka et al., 1975 ; Salo and Cliver, 1976) and other environmental solid particles (Ward, 1976) have been reported to substantially decrease sensitivity of viruses to heat inactivation treatment (Siegl, Weitz et al., 1984 ; Scholz et al., 1989). Influence of pH, salinity and temperature on Influenza virus infectivity in water has been investigated (Brown et al., 2009). Influenza virus infectivity decreased in water with low pH from 8,6 to 5,8, increased temperature from 4 to 37°C and high salinity (3%). Time required to reduce infectivity decreased with low pH, increased temperature, and high salinity.

The experimental domain would be defined according to the chemical characteristics of poultry meat. Poultry meats (thigh and breast) were characterised by pH ranges from 7 for chicken meat or 6,3 for duck meat (just after slaughtering) to approximately 5,5 (24h post-mortem) (Baeza, 1995 ; Debut et al., 2003). Based on available data (“Report on Salt: Evaluation and recommendations” from Agence Française de Sécurité Sanitaire des Aliments - 2002), salt concentration added for taste before meat cooking represents about 0,2 to 0,4% (w/w) and were explored (Salt can be added in high concentration in food conservation processing, use of solution containing up to 26% (w/v) for pickling brine or approximately 20% (w/w) for salting process). Chicken meat are cooking according to (i) guidance documents from WHO (OMS, 2005 ; OMS, 2007): INFOSAN notes, disseminated through the International Food Safety Authorities Network, gives recommandations on proper food handling and cooking and on published data (Swayne and Beck, 2005); (ii) Food Safety and Inspection Service (FSIS) guidelines, from current US Department of Agriculture (USDA), based on performance standards for the production of meat and poultry products requiring relative reduction (6 $\log_{10}$ lethality) of Salmonella of beef meat (cooked, roast and corned). The compliance guidelines provided temperature for cooking uncured poultry (30 sec 71°C) and for cooking cured poultry (1 min 68°C) and provided a time-temperature for fat level range from 1 to 12% and species (chicken and turkey) needed to obtain 7 $\log_{10}$ lethality of Salmonella.

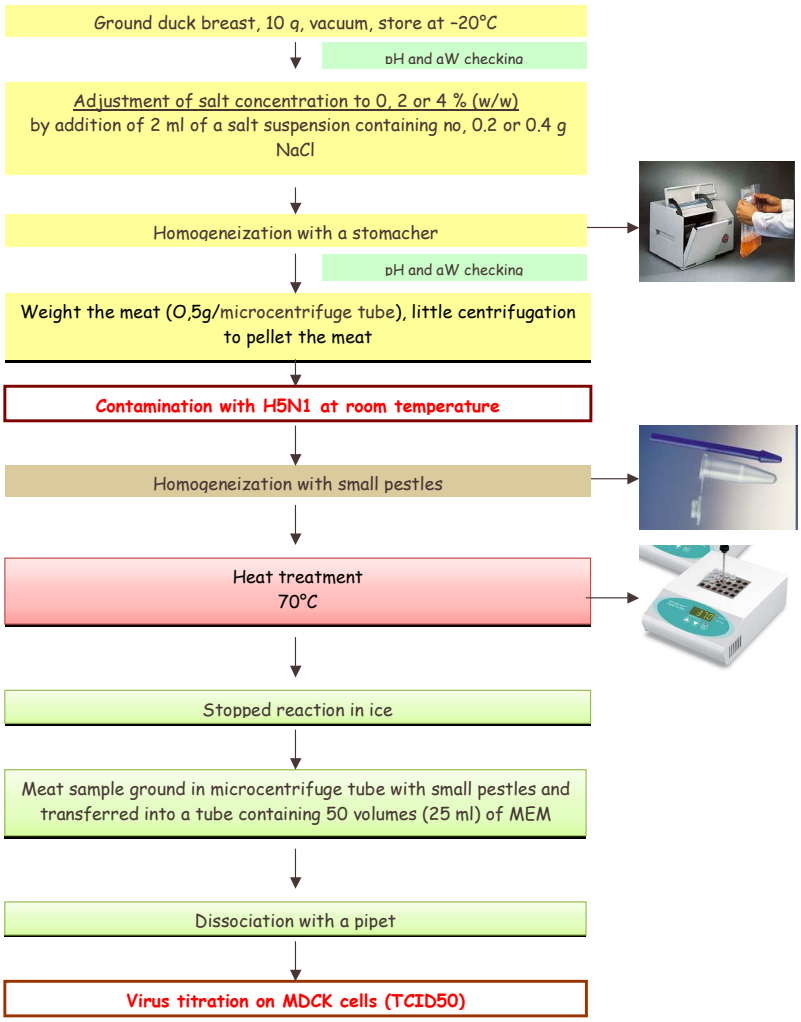
Inactivation of Influenza viruses in food has been poorly investigated (Doyle et al., 2007). Heat is known to affect the stability of viruses and thus been effective means of virus inactivation. However, a reproducibible microassay using small pieces of meat and a thermocycleur heating block, was developed to measure thermal inactivation of H5N1 in naturally infected poultry. No infective virus isolated from meat after it changed from pinked-tan to white color and it was exposed to 70°C for 1 sec with reaching temperature in 40 seconds (Swayne, 2006). Using this assay, survival curves were constructed for H5N1 virus in chicken meat exposed temperature range from 57 to 61°C and a linear mode l provided a good fit for all curves. D values (time required to reduce virus titers by one log) were calculating at different temperatures by linear regression. Similar D-values, regression plots of log D-values and line equations were observed in both meat types, thigh and breast, for H5N1 (Thomas and Swayne, 2007), and for both virus strains, H5N1 and H5N2, in breast (Thomas, King et al., 2008). A regression plot of log D-value versus temperature yields an equation that can be used to calculate Z-value (temperature increase to reduce D-value by 1 log) and to predict D values for additional temperatures. Z-value describes the temperature dependance of a thermal inactivation. Combined model line equation are available to calculate D-values for H5N1 in chicken meat versus temperature. Times predicted at given internal temperature needed to obtain 11-log reductions of virus titer and achieved in chicken meat cooked were

compared to current USDA FSIS time-temperature guidelines for 7-log reduction of Salmonella. Thermal Influenza inactivation was faster than Salmonella.

Based on the previous data, the impact of salt concentrations, ranging from 0 to 4% (w/w), on cooking of artificially infected poultry breast at pH 5.5 was evaluated.

With the suitable method for extraction and detection of AIV described above, quantitative studies of the impact of food processing conditions on AIV was possible. The thermal inactivation procedure was based on available data (Swayne, 2006 ; Thomas and Swayne, 2007 ; Thomas, King et al., 2008). Experiments were performed with H5N1 virus (A/HK/156/97). Since thermal inactivation of Influenza viruses have been mainly investigated on chicken meat, four repetitions of experiments were performed in ground duck meat to obtain survival curves. One experiment was however carried out on ground chicken breast, to fit predictive model for poultry matrices.

Experimental protocol is illustrated below:



Briefly, meat was prepared and contaminated as previously described. The tube-holding heating block was preheated at 26℃. The tubes containing the sam ples were placed in the test instrument until temperature of 26℃ was reached. The targeted temperature was set to 70℃ for testing kinetics of thermal inactivatio n. Single samples were removed during the ramp-up period of the heating. The temperature was reached in approximately 12 minutes. Upon removal from the block, samples were immediately chilled in ice. Meat samples were ground in microcentrifuge tube with 0.5-ml pestles and transfered into a tube containing 50 volumes of culture medium. Ground meat was dissociated with a pipet and titrated on cells.

Viral inactivation curves were constructed on viral residual titres measured during the ramp-up period of the heating from 26℃ up to the targeted temperatur e of 70℃.

Development of inactivation predictive models (Task 3)

The experimental data obtained for all survival kinetics will be processed using the statistical software S-PLUS 2000. A modelling approach will be implemented, using available models to describe viral inactivation kinetics. (in progress)

Validation of model predictive data (Task 5)

No model-predictions can be used in confidence unless it was validated on independent data on foods (Delignette-Muller, 1997). Model predictions will be then confronted to experimental results obtained on poultry products with different salt concentration values. Heat treatments, range from 26 to 70℃, of H5N1 and H1N1 in poultry products (duck and chicken) supplemented with salt concentrations (0 to 4%) will be performed.

Deviations from the project work programme, and corrective actions taken

Thermoresistance studies, performed with enteric viruses, have shown several factors, as fat, sugar and protein, could increase heat stability of HAV in shellfish (Millard et al., 1987 ; Croci et al., 1999), in fruit based products (Deboosere et al., 2004) and in dairy products (Parry et al., 1984 ; Bidawid et al., 2000). Evaluation of parameters on survival/inactivation was conducted in meat rather than in liquid model medium (Task 1).

Moreover, duck meat was characterised by a 24h post-mortem pH 5.5. $NaHCO_3$ (1N) is classically used to neutralize lactic acid. Meat, with added salt 4%, could be adjusted with $NaHCO_3$ (1N) to pH 6.5, but significant changes were observed in consistence of meat, which became more elastic and gelatinous. Structural modifications have been shown to change viral thermoresistance. Evaluation of effect of salt content and temperature on survival/inactivation was conducted in meat at natural post-mortem pH (5.5) (Task 1).

List of Deliverables (M19-M36)

Del. No.	Deliverable name	WP N°	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 8-1	A report will be produced every 6 months	8	M18	M42	6	3.17	7
D 8-2	A final report describing the influence of studied parameters on AIV in food products will be prepared at the end of the project	8	M36	M42	7	5	7
D 8-3	Scientific publications in peer-review journal	8	M36	M42	1	0.5	7

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP N	Date due	Actual/Forecast delivery date	Lead contractor
M 8-1	End of WP8 task 1[Evaluation of pH level, salt content and temperature on various survival/inactivation, in laboratory medium]	8	M42	M42	7
M 8-2	Report	8	M18	M42	7
M 8-3	End of WP8 task 2 [Study of the interaction of factors (temperature/pH, temperature/Aw, etc) on the behaviour of various viral strains]	8	M20	M42	7
M 8-4	End of WP8 tasks 3 [Development of inactivation predictive models]& 4 [Extraction of H5N1 virus from various food products (chicken, turkey,…)]+ report	8	M27	M42	7
M 8-5	End of WP8 task 5 [Validationwith contaminated food products, of the predictive data obtained from laboratory media] + final report	8	M33	M42	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 objective of the WPG9 was to implement a conceptual model of influenza virus spread, acting within computationally suitable environment, and starting from many classes of simple, data driven, descriptive models concerned with the individual studies of the stability of viral strains in water reservoirs, as well as the stability of influenza viruses on dried surfaces – again under laboratory-controlled, and natural conditions. 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 influenza virus' perpetuation, as well as it's possible methods of deactivation. And the second, as means to achieve the main goal, to built a series of partial models, each simulating partial aspect of influenza virus behavior, stability and perpetuation in specific environments.

Progress towards objectives

It was already estabilished during the first stage of the project (D9-1) that no commercial software was available to even approximately fulfil the necessary requirements for an agent-based model of IV survival in various environments. Therefore it was decided to construct a modular system of various tools – to act on experimental data as soon as they will became available form other consortium partners.

Two simulatory models of the influenza viral spread, infecting guinea pigs in laboratory-controlled conditions, at various temperature and humidity regimes (partial fulfilment of D9-4, and M9-4), have been built and validated. (*CBAC* ***33*** *(2009) 176-180****,*** and *CBAC* ***33*** *(2009) 339-343*). The most important shortcoming of the first model was the fact that the simulations carried were not able to cover scenarios similar to the experimentally observed for temperature of 20 C, and 35% humidity. This problem has been solved in the second model, which is much better optimized for dealing with small data sets than preceding simulations. The results obtained are disturbed to much lesser degree by the input sparseness – the situation rather common in many studies involving infectivity data, esp. for pathogens of high biohazard risk, and we believe might be of value for other situations. It would be interesting and highly beneficial to calibrate the model with larger data sets, should such data be available. Nevertheless, the results presented are sufficiently accurate to be incorporated as a module into a large-scale modeling effort. It can be expected, that due to a high similarity of the AIV H5N1 serotype, it would be fairly straightforward to repeat the simulations and modelling, as soon as data on H5N1 stability in various environments will be available.

Two main modules of the final simulation platform were designed, implemented and tested (D9-6, and M9-5). First module realized a country-wide model of social contacts, quintessential for a simulation of influenza spread has been implemented, Particular emphasis was put on contact patterns arising from daily commuting to schools, offices and other workplaces. In order to reproduce the map of contacts, we were using geo-referenced, individual-based models (IBMs). Within this framework, the set of different stochastic algorithms was employed, utilizing available aggregated data. Based on such model system, selected statistical analyses were performed, in particular the accessibility of schools, or location of emergency rescue service units. The results (*Journal of Artificial Societies and Social Simulation,* ***13*** *(2010) 13*) show a good agreement with available census data, reproducing well local population densities, average times in transit, and other indicators decisive for assessment of individual contacts and daily mobility patterns.

This platform then served as a base for the next stage – providing social context for a large-scale epidemiological simulations. The second main module involved a construction of an agent-based model for studying the effects of influenza epidemic in large-scale (38 million individuals) stochastic simulations, together

with the resulting various scenarios of disease spread. Simple transportation rules were employed to mimic individuals' travels in dynamic route-changing schemes, allowing for variance of contacts, and their possiblr effects on infection spread. Parameter space was checked for stable behavior, especially towards the effective transmission rate variability. Although the model was based on quite simple assumptions, it allowed observe two different types of epidemic scenarios: characteristic for urban and rural areas. This differentiates it from the results obtained in the analogous studies for the UK or US, where settlement and daily commuting patterns are both substantially different and more diverse. The resulting epidemic scenarios from ABM-simulations were compared with simple, differential equations-based, SIR models – both types of our results have shown strong similarities and correlations (submitted to *Physica A*, positive reviews). The software platforms developed in both stages are currently used in the next step of the project, employed to study various epidemic mitigation strategies. As the human infectivity data of the H5N1 serotype are as yet unknown, we were 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. The simulations are scaleable, thus it will be not particularly difficult to transform the model, from it's current capacity, towards countries of different sizes – provided that census data will be available at sufficiently detailed granulation.

The next setp of simulatory effort will be a software module allowing development and testing of scenarios for influenza spread involving more than one serotype. The recent emergence in 2009 of the novel, swine-like variant of A/H1N1 serotype indicates that many phenomena of epidemic spread within context of several competing virus types might be of rather high interest. In order 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 was developed The method is of general use, and is potentially applicable to very many areas of genomic research (*Comptes Rendus Biologies, **332** (2009) 336-350*).

Deviations from the project work programme, and corrective actions taken

Up to date only very limited amounts of experimental data usable by this WP were released by the other WPs of the project. Which is 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 inactivation. As soon as respective usable data should become available, the modelling efforts will continue.

List of Deliverables (M19-M36)

Deliverable D9-1 was already fulfilled; deliverables D9-2, D9-3, D9-4, D9-5, D9-6 and D9-7 will be prepared and delivered in the next 6 months.

Del. No.	Deliverable name	WP N.	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 9-1	Assessment guidelines for appropriate analytical, algorithmic, and software tools optimally suited for modelling AIVs at various descriptive details levels, different temporal and spatial scales	9	M24	M42	4	2.2	8
D 9-2	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in various water environments, laboratory-controlled and natural	9	M20-M33	M42	4	2.3	8
D 9-3	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in a dry state at various surfaces, laboratory-controlled and natural	9	M24-M33	M42	4	2.2	8
D 9-4	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and	9	M24-M33	M42	4	2.4	8

	deactivation in air at laboratory-controlled environements						
D 9-5	Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in avian feces and farm manure	9	M24-M33	M42	2	0	8
D 9-6	Multi-scale agent-based simulation model of possible determinants for AIVs stability, perpetuation and deactivation	9	M33	M42	16	9,17	8
D 9-7	Peer-review scientific publications in international journals	9	M36	M42	2	3.6	8

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP N.	Date due	Actual/Forecast delivery date	Lead contractor
M 9-1	Choice of software tools for modelling of virus viability and perpetuation.	9	M20	M42	8
M 9-2	Implementation of simulation models of AIVs perpetuation, viability and deactivation in various water environments.	9	M20-M33	M42	8
M 9-3	Implementation of simulation models of AIVs perpetuation, viability and deactivation in a dry state.	9	M24-M33	M42	8
M 9-4	Implementation of simulation models of AIVs perpetuation, viability and deactivation in air at various	9	M24-M33	M42	8
M 9-5	Implementation of hierarchical, multi-scale agent-based simulation model of AIVs perpetuation and possible deactivation	9	M33	M42	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 except postponing the phase between M37 and M42.

List of Deliverables

Deliverable D10-1 will be prepared and delivered in the next 6 months.

Del. No.	Deliverable name	WP N.	Date due	Actual/Forecast delivery date	Estimated indicative person-months	Used indicative person-months	Lead contractor
D 10-1	Final report and recommendations for specific policies to prevent and control avian influenza	10	M36	M42	8	0	1

List of Milestones (M1-M18)

N/A – Not Applicable

Milestone no.	Milestone name	WP N..	Date due	Actual/Forecast delivery date	Lead contractor
M 10-1	Expected results: Integration of proposed recommendations into relevant policies and guidelines for prevention and control measures to present and possibly future outbreaks of avian influenza A(H5N1) in Europe.	10	M36	M42	1

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 second 18 months of activity have been related to maintaining a minimum momentum of the project among partners in spite of their heavy mobilisation against the A(H1N1) pandemic.

a/ Meetings

Very few meetings took place during this period because of the A(H1N1) pandemic which forced us to cancel the big mid-term meeting planned in Cambodia at the beginning og September 2009. Instead a number of "bilateral" meetings took place especially between Partner 8 and 7 and 1 and between partners 4 and 6 (see Collaborations, visits, training of people, exchange of materials in Annex).

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 has been maintained (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.

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.

The project is required to set up a scientific advisory board. A list of potential candidates has been identified but they have been too busy because of theA(H1N1) pandemic itself and now the aftermath and the reviews and inquiries.

e./ Summary

In summary, the RIVERS project which was successfully initiated did not progress as much as planned during the second 18 month period due to the A(H1N1) pandemic and in spite of each partner making its utmost to work on RIVERS as shown by the person-month used during this period.

Deviations from the project work programme, and corrective actions taken

Due to the pandemic caused by the novel influenza A(H1N1)v virus, all project-widemeetings were cancelled during the period.

List of Deliverables (M19-M36)

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-M42	M1-M42	39	19	1

List of Milestones (M19-M36)

Milestone no.	Milestone name	WP no.	Date due	Actual/ Forecast delivery date	Lead contractor
M11-1	Activation of Consortium internet site	11	M1-M36	M2	1
M11-2	Reportings and organisation of meetings	11	M1-M36	M1-M42	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 even through difficult times due to the A(H1N1) pandemic in 2009/2010.:

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 specific efforts of the consortium are outlined as detailed in WP11 progress report.

To accommodate the research, a rich IT infrastructure had been established and was mai maintained during the second period within the consortium, including a public and members only www facility with information about the project and its activities, email lists.

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 but they have been overwhelmed by their workload during the A(H1N1) pandemic and even after because of lessons being drawned everywhere and at every level.

In summary, the RIVERS project which was successfully initiated did not progress as much as planned during the second 18 month period due to the A(H1N1) pandemic and in spite of each partner making its utmost to work on RIVERS as shown by the person-month used during this period.

Contractors

(Comments regarding contributions, changes in responsibilities and changes to consortium itself, if any)

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) as reported at the end of the first 1_ month period.

Table Sec3-1: 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 the second interim reporting, RIVERS has resumed a momentum compatible with the achievement of the objectives of each of the WPs within the time frame of the project extended by 6 month and this inspite of the delay in delivering some WPs. 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 M42.

Person-Months Status Table

WP0

Name	Position (professional category)	Person-Months spent on RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.3
Ana M. BURGUIERE	Deputy head of laboratory	0.1
Jean-Claude MANUGUERRA	Senior scientist, Head of laboratory	0.1
Christophe BATEJAT	Head technician	0.1
Gilberte CORALIE	Senior technician	0.1
Frederic FICHENICK	Technician	0.1
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	10.4
Sayuri SHEGEMATSU	PhD Student (Japan)	2.3

WP1

Name	Position (professional category)	Person-Months spent on RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.1
Ana M. BURGUIERE	Deputy head of CIBU	0.1
Jean-Claude MANUGUERRA	Head of laboratory	0.1
Christophe BATEJAT	Head technician	0.1
Gilberte CORALIE	Senior technician	0.1
Frederic FICHENICK	Technician	0.1
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	1
Sayuri SHEGEMATSU	PhD Student (Japan)	0.1
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent personnel:		
Philippe BUCHY	Head of Virology Unit	3
Horm Srey Viseth	Research Assistant	1
Personnel funded by the project:		
-	-	-

Participant 7 – Institut Pasteur de Lille (IPL)		
Permanent staff:		
DEBOOSERE Nathalie	Project Leader / Senior Engineer	4
GACHET Jessica	Technician	2
PINON Anthony	Bio-statistician / Senior Engineer	1
Personnel funded by the project:		
GACHET Jessica	Technician	2

WP2

Name	Position (professional category)	Person-Months spent on RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.1
Jean-Claude MANUGUERRA	Head of laboratory	0.1
Christophe BATEJAT	Head technician	0.1
Gilberte CORALIE	Senior technician	0.1
Frederic FICHENICK	Technician	0.1
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	1
Sayuri SHEGEMATSU	PhD Student (Japan)	0.1
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
Mihai Maria Elena	Participant (researcher, biochemist)	0.50
Tecu Cristina	Participant (senior physician, PhD	0.50
Ustea Luiza	Technician	0.50
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 of Virology Unit	3
Horm Srey Viseth	Research Assistant	5
Ramona Gutierrez	DVM, PhD student	9

Y Bun Thin	Lab technician	6
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent Personnel funded by the project:		
DONG CHANGGUI	Researcher	2
CAI CHEGUO	Researcher	1
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
E. ALBINA	Scientist	0.62
P. GIL	Laboratory technician	5.50
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
Hongbo Zhang	Researcher assistant	18

WP3

Name	Position (professional category)	Person-Months spent on 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	3.5
Horm Srey VISETH	Research Assistant	3.5
Ramona GUTIERREZ	Vet	14
Personnel funded by the project:		
-	-	-

WP4

Name	Position (professional category)	Person-Months spent on RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana M. BURGUIERE	Deputy head of CIBU	1
Jean-Claude MANUGUERRA	Head of laboratory	1
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
Onu Adrian	Participant (researcher, PhD)	0.50
Lupulescu Emilia	Leader of the WP4 (senior physician, researcher)	1.00
Mihai Maria Elena	Participant (researcher, biochemist)	1.00
Tecu Cristina	Participant (senior physician, PhD	1.00
Ustea Luiza	Technician	0.75
Ene Mirela	Technician	0.75
Enache Nuti	Technician	0.75
Dobre Emilia	Technician	0.75
Personnel funded by the project:		
-	-	-

WP5

Name	Position (professional category)	Person-Months spent on RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.17
Ana M. BURGUIERE	Deputy head of laboratory	0.5
Jean-Claude MANUGUERRA	Senior scientist, Head of laboratory	0.1
Christophe BATEJAT	Head technician	0.3
Gilberte CORALIE	Senior technician	0.3
Frederic FICHENICK	Technician	0.3
Jessica VANHOMEWEGEN	EUPHEM ECDC Fellow	0.5
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	3
Sayuri SHEGEMATSU	PhD Student (Japan)	0.5

Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent personnel funded by the project:		
DONG CHANGGUI	Researcher	3
CAI CHEGUO	Researcher	1

WP6

Name	Position (professional category)	Person-Months spent on RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.1
Ana M. BURGUIERE	Deputy head of laboratory	0.1
Jean-Claude MANUGUERRA	Senior scientist, Head of laboratory	0.1
Christophe BATEJAT	Head technician	0.23
Gilberte CORALIE	Senior technician	0.1
Frederic FICHENICK	Technician	0.1
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	0.1
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
Mihai Maria Elena	Participant (researcher, biochemist)	0.50
Tecu Cristina	Participant (senior physician, PhD)	0.50
Ene Mirela	Technician	0.50
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	2.5
Georgi GEORGIEV	Professor, DMV, DSc, Dept. Exotic and Dangerous Viral Infections, Mational Diagnostic Resarch Vetreinary Institute, Sofia	2.5
Ivan ZARKOV	Professor, DMV, DSc, Department of Virology, Faculty of Veterinary Medicine, Thracian University, Stara Zagora	4
Lora SIMEONOVA	Research scientist,MS	1.5

	Department of Virology, MICB	
Gabriela GUJGULOVA	Research scientist, DMV Dept. Exotic and Dangerous Viral Infections, Mational Diagnostic Resarch Vetreinary Institute, Sofia	1.5
Lucia MUKOVA	Research assisant,MS Department of Virology, MICB	1
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
Philippe BUCHY	Head of Virology Unit	0.5
Horm Srey Viseth	Research Assistant	0.5
Ramona Gutierrez	DVM, PhD student	1
Ngin Yous	Lab technician	1
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent personnel funded by the project:		
DONG CHANGGUI	Researcher	2
CAI CHEGUO	Researcher	1
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
Patricia GIL	Laboratory Technician	0.85
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 RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
Ana M. BURGUIERE	Deputy head of laboratory	0.1
Jean-Claude MANUGUERRA	Senior scientist, Head of laboratory	0.1
Christophe BATEJAT	Head technician	0.1
Gilberte CORALIE	Senior technician	0.23
Frederic FICHENICK	Technician	0.1
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	0.1
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		

Permanent personnel funded by the project:		
DEUBEL VINCENT	Principal Investigator	1.5
ZHOU PAUL	Principal Investigator	1
TOYODA TETSUYA	Principal Investigator	1
LU PENG	Technician	1
CAI CHEGUO	Researcher	3
WANG WEI	Researcher	2
REN PEIJUN	Technician	2
DONG CHANGGUI	Researcher	11
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 RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.1
Ana M. BURGUIERE	Deputy head of laboratory	0.1
Jean-Claude MANUGUERRA	Senior scientist, Head of laboratory	0.1
Christophe BATEJAT	Head technician	0.1
Gilberte CORALIE	Senior technician	0.1
Frederic FICHENICK	Technician	0.1
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	0.07
Participant 7 – Institut Pasteur de Lille (IPL)		
Permanent staff:		
DEBOOSERE Nathalie	Project Leader / Senior Engineer	3
GACHET Jessica	Technician	4
ALEXANDRE Virginie	Technician	2
PINON Anthony	Bio-statistician	1
Personnel funded by the project:		
-	-	-

WP9

Name	Position (professional category)	Person-Months spent on RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.2
Ana M. BURGUIERE	Deputy head of laboratory	0.1
Jean-Claude MANUGUERRA	Senior scientist, Head of laboratory	0.1
Personnel funded by the project:		
Amélie DUBLINEAU	PhD Student (France)	0.33
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
Flavie GOUTARD	Scientist	3.5
Participant 8 – Interdisciplinary Centre for Mathematical and Computational Modelling (ICM)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
Jan RADOMSKI	Senior researcher	5
Tomasz BADOWSKI	Student trainee	1
Szymon JARANOWSKI	Student trainee	1
Piotr PŁOŃSKI	Student traniee	3
Tomasz ŻUK	Research assistant	4
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 RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		

Alexandrescu Ion Viorel	Participant (senior physician, PhD)	0.50
Personnel funded by the project:		
-	-	-
Participant 4 – Institut Pasteur du Cambodge (IPC)		
Permanent staff:		
-	-	-
Personnel funded by the project:		
-	-	-
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
Flavie GOUTARD	Scientist	0.3
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 RIVERS Project
Participant 1 – Institut Pasteur Paris (IPP)		
Permanent personnel:		
India LECLERCQ	Associate professor Paris University Denis Diderot P7	0.5
Ana M. BURGUIERE	Deputy head of laboratory	1
Jean-Claude MANUGUERRA	Senior scientist, Head of laboratory	2
Sophie ABLOTT	Contract manager	5
Personnel funded by the project:		
-	-	-
Participant 2 – Institutul Cantacuzino (IC)		
Permanent personnel:		
Alexandrescu Ion Viorel	Participant (senior physician, PhD)	0.50

LUPULESCU Emilia	Participant (researcher, senior physician)	0.50
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 of Virology Unit	1.5
Personnel funded by the project:		
-	-	-
Participant 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)		
Permanent personnel funded by the project:		
DEUBEL VINCENT	Director	0.5
SUN BING	Co-Director	0.5
Participant 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)		
Permanent + non-permanent staff:		
Dominique MARTINEZ	Scientist	0.7
Cam-Tu HO	Financial Officer	0.92
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

During this reporting period, coordination activities have been mainly focused on administrative management (collection of report and forms from all partners for the reporting period M1-M18. From spring 2009, due to the emergence of the pandemic prone influenza A(H1N1)v, an effort was made to liaise with all partners to seek their position and approval about asking the EU for an additional no cost 6 months. The consortium was relieved that the EU accepted their request.

The main mid term meeting originally planned in September 2009 in Cambodia was called offbecause most important collaborators could not or even were not allowed by their institute to leave their working place for long distance travels. Consequently, the communication between members was entirely or so through electronic means.

Each head of partner team made their utmost to push the project forward in spite of unexpected contogencies linked to the H1N1 pandemic. They stayed in contact with the global coordinator.

Section 4 – Other issues

NOT RELEVANT

Annex – Plan for using and disseminating the knowledge

Section 1 – Exploitable knowledge and its use

Not applicable during the second 18 month period because of the A(H1N1) pandemic in 2009/2010.

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

Section 2 – Dissemination of knowledge

Not applicable during the second 18 month period because of the A(H1N1) pandemic in 2009/2010.

The following table shows the main dissemination activity undertaken by all RIVERS partners during the first reporting period (01/08/2008 to 31/01/2010).

Date	Type[1]	Type of audience[2]	Countries addressed	Size of audience	Partner responsible/ involved
01/08/2008 – 31/01/2010	RIVERS Website	General public and scientists	Worldwide	Unknown	Partner 1 (IPP)/ All

[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.*

Section 3 – Publishable results

List of RIVERS-related publications during the period 01/08/2008- 31/01/2010

PARTNER 1 – Institut Pasteur Paris (IPP)
1. Berthet N, Leclercq I, Dublineau A, Shigematsu S, Burguière AM, Filippone C, Gessain A, Manuguerra JC. High-density resequencing DNA microarrays in public health emergencies., Nat Biotechnol. 2010 Jan;28(1):25-7. 2. Dublineau A., Leclercq I., Batéjat C., Shigematsu S., Burguière A. Manuguerra J.-C. Long-lasting Persistence of the Novel Pandemic Influenza A(H1N1) Virus in the Environment, submitted for publication
PARTNER 2 – Institutul Cantacuzino (IC)
PARTNER 3 – The Stephan Angeloff Institute of Microbiology (MICB)
PARTNER 4 – Institut Pasteur du Cambodge (IPC)
PARTNER 5 – Chinese Academy of Sciences, Pasteur Institute of Shanghaï (IPS)
PARTNER 6 – Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD)
1. Tran, A., et al., Remote sensing and avian influenza: A review of image processing methods for extracting key variables affecting AIV survival in water from Earth Observation satellites. Int. J. Appl. Earth Observ. Geoinform. (2009), doi:10.1016/j.jag.2009.09.014
PARTNER 7 – Institut Pasteur de Lille (IPL)
1. Développement d'une technique d'adsorption - élution pour la concentration de virus *Influenza A* dans les eaux de surface » was presented in Journées Francophones de Virologie, April 2009. (Poster) 2. Optimisation et validation d'une méthode de concentration et de détection de virus Influenza A dans l'eau de surface, basée sur les méthodes utilisées pour la détection des virus entériques » was submitted to Journées Francophones de Virologie, March 2010 (Oral communication) 3. A publication intented for "Applied and Environmental Microbiology") is in progress.
PARTNER 8 – Interdisciplinary Centre for Mathematical and Computational Modelling (ICM)
1. T.Zuk, F.Rakowski, and J.P.Radomski, "A *model of influenza virus spread as a function of temperature*

and humidity", Computational Biology and Chemistry, **33** (2009) 176-180,

2. F.Rakowski, M.Gruziel, M.Krych, and J.P.Radomski, *"Large scale daily contacts and mobility model – an individual based countrywide simulation study for Poland"*, Journal of Artificial Societies and Social Simulation, 13 (2010) 13,

3. T.Zuk, F.Rakowski, and J.P.Radomski, *"Probabilistic model of influenza virus spread as a function of temperature and humidity"*, Computational Biology and Chemistry, **33** (2009) 339-343,

4. J.P.Radomski, and P.P.Slonimski, *"ISSCOR: Intragenic, Stochastic Synonymous Codon Occurrence Replacement – a new method for an alignment free genome sequence analysis"*, Comptes Rendus Biologies, **332** (2009) 336-350,

5. F.Rakowski, M.Gruziel, L.Bieniasz-Krzywiec, and J.P.Radomski, *"Influenza epidemic spread simulation for Poland - a large scale, individual based model study",* submitted to Physica A, positive reviews,

6. RIVERS internet site at Intedisciplinary Center for Modelling, Warsaw Uniwersity: http://rivers.icm.edu.pl/

PARTNER 9 – Wuhan Institute of Virology (WIV)

1. Chen, J., Fang, F., Yang, Z., Liu, X., Zhang, H., Zhang, Z., Zhang, X., and Chen, Z. (2009). Characterization of highly pathogenic H5N1 AIVs isolated from poultry markets in central China. Virus Res 146(1-2), 19-28.

2. Chen J, Yang Z, Chen Q, Liu X, Fang F, Chang H, Chen Z et al. Characterization of H5N1 influenza A viruses isolated from domestic green-winged teal. Virus Genes 2009 Feb;38(1):66-73.

3. Zhang Hongbo, Chen Quanjiao, Xu Bin, Li Yan, Chen Ze. Characterization of H9N2 influenza viruses isolated from Dongting Lake wetland in 2007 (Submitted)

4. Zhang, H, Xu, B, Chen, Q, Chen, J, Chen, Z. Characterization of An H10N8 Influenza A Virus Isolated from The Water of Dongting Lake Wetland.(Submitted)

Collaborations, visits, training of people, exchange of materials

J.P. Radomski visited several consortium partners:

Type of travel	Date
Meeting in Inst. Pasteur, Paris	8.09.08
Meeting in Inst. Pasteur, Paris	3.12.08
Meeting in Inst. Pasteur, Paris	12.03.09
Meeting in Inst. Pasteur, Lille	26.05.09
Partial meeting of RIVERS in Inst. Pasteur, Paris	2.07.09
Meeting in Inst. Pasteur, Paris	16.09.09