# Resistance of Influenza Viruses in Environmental Reservoirs and Systems

The surge of the global avian Influenza epizootic caused by the genotype Z high pathogenic avian Influenza virus (HPAIV) has posed numerous questions, in particular to risk managers and policy makers. Scientific knowledge is thin on many aspects of the ecology and environmental properties of HPAIVs, in particular H5N1. At the start of this project, virus survival, a key element in control strategies, was an illustration of this paucity of knowledge. Data from the literature on AIV survival were 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 could not be extrapolated to the current A(H5N1) viruses before a careful consideration. Further, few information was provided regarding the survival of IVs in the air and surfaces. Meantime, no standardised protocols existed to concentrate and detect AIVs in waters, in the air or in/on solid matrices. Ideally, the virus detection techniques 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) joined forces in order to investigate the prevention and control of Influenza outbreaks in animal population at present and at time of restocking.

The objective of our project, whose website URL is www.rivers-project.eu, was 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. Indeed during this project, avian or/and human cases of HPAI occurred in China, Cambodia and Romania and investigations were conducted as planned within infected areas. Policy recommendations might be drawn from the provision of a corpus of data, by gathering data on the survival of avian IVs, in natural environments and in experimental setting.

More specific objectives were: 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 was to elaborate models about the survival of avian Influenza virus in natural environments to demonstrate, in connection with other project and very recent published data, their perpetuation in nature both in biological and environmental reservoirs.

Altough the 2009/2010 influenza pandemic due to the H1N1pdm virus had a negative impact on the project, the hard and dedicated work of all partners allowed us to reach about two thirds of completion rate as far as deliverables were concerned. This rate will probably be higher once final data are available and more paper published. Guidelines and recommendations might also arise from the wealth of data that RIVERS has been generating in three and a half years. It is probable that the impact of RIVERS will be bigger than we can anticipate now not only because of the data generated but also for the connections between teams that it has tighten. For some partners, RIVERS helped kick off some additionnal funding from private sponsors to widen and carry on research on influenza in the environment. RIVERS website has been a great tool and will continue to be operational in the months and years to come to publicise all new data and scientific papers that will be generated after the official end of the program.

***RIVERS Consortium*** (led by Partner 1, Institut Pasteur)

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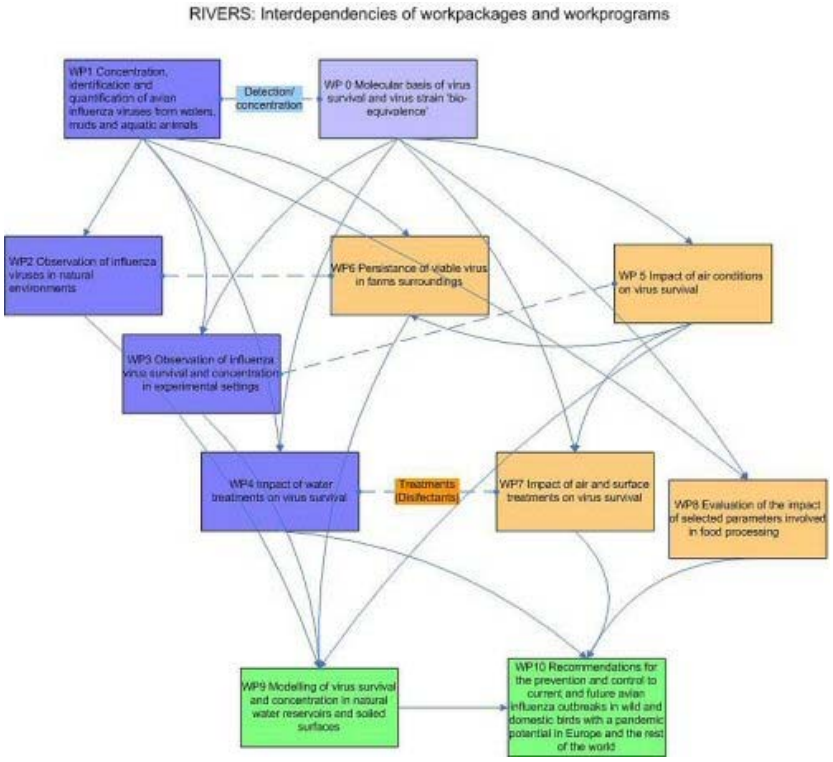
The RIVERS project was built on three workprograms: one focusing on virus in water, one focusing on virus in the air and on the surfaces and one workprogram dedicated to modeling and drawing guide lines. In the first two programs, two paired approaches were followed in parallel: observations in natural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



## 0. Molecular basis of virus survival and strain bio-equivalence

Upstream of all work programs, RIVERS first focused on the concept of virus strain bioequivalence. The aim was to select a non High Pathogenic Avian Influenza (HPAI) virus strain, possibly not H5, giving the same survival profile as the tested model virus HPAIV H5N1 genotype Z strain. To this end, four strains of Influenza Virus (IV) were compared in relation to survival. Effects of temperature and salinity on the persistence of the four strains of IV in water were investigated. Data generated by RIVERS strongly suggested that IVs have the ability to persist in water for extended periods of time, even at 35°C. Viruses can theoretically persist in distilled water for more than 200 days at 4°C. At 25°C, IV has the ability to remain infectious until 40 to 50 days, depending on the strain. Results for salinity trials showed that salt impact on virus survival was less important than that of temperature (at low temperature). Virus infectivity also persisted in saline waters for extended periods of time, and significantly decreased only for a salt concentration of 270 $g.L^{-1}$. However, even then, IVs were still infectious after 37 days.

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

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-. Theybwere 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.

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. 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 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 accepted for publication.

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

Results obtained with H1N1pdm virus and described in the last report (M19-M36) suggested that external viral structures were involved in virus survival. We thus decided to focus on the role of HA glycosylation and composition of the lipid bilayer.

**Role of N-glycosylation in virus survival:**

During the last 6 months, we studied the role of N-glycosylation of the HA protein in virus survival by mutating it at the surface of lentiviral pseudotypes. Generation of pseudoparticles was obtained by combining home-made plasmids from IPS and commercial plasmids from Invitrogen. Transfection of 293T cells by all of these plasmids allowed the generation of lentiviral pseudoparticles expressing the H5 and N1 influenza proteins

at their surface.

In order to assess the impact of glycosylation on the virus survival in water, we generated mutated pseudoparticules lacking one potential glycosylation site. Predictions for N-glycosylation sites on H5 sequence were determined with NetNGlyc 1.0 Server which predicts N-Glycosylation sites in proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons.

We found 9 potential glycosylation sites on the H5 protein. Four were not accurate so we decided to generate 5 mutants. For each site, amino acid Asparagine (N) was replaced by a Glutamine (Q). Mutants were generated from wild-type plasmid pCMVR-HA using the Quick-Change II Site-Directed Mutagenesis kit from Qiagen. The mutated pseudoparticles were generated after transfection of 293 T cells and tested by hemagglutination. Titers obtained were comparable to that of wild-type (WT) for XX/5 mutants. Preliminary results and statistical analysis suggest that N170Q mutant is less stable in water than wild-type and N39Q mutant.

**Role of composition of the lipid bilayer in virus survival**

Two cell lines, one derived from a mammalian species (Dog: MDCK) and one from an avian species (Quail: QT6) were used to grow two strains of influenza A viruses, one A(H1N1) and one A(H5N1). The various viral preparations, differing by their passage history, were compared for their ability to retain infectivity in the course of time in various conditions in water. The virus preparations were left at 25C° in water, the samples were adjusted to the same titre on day0. At each time point, the samples were titrated on MDCK by the TCID50 endpoint method.

The kinetics of virus (H5N1) derived from QT6 reached higher titres on MDCK cells, compared to the same virus derived from MDCK during the whole experimental period (day 0 until day 20). The biggest difference in titres was observed on day 6. The infectivity reached its detection limit of 1,58 log TCID50/ ml on day20. To confirm our first observation according which the virus derived from QT6 retain higher infectivity compared to the virus derived from MDCK when inoculated on MDCK cell line, we attempted the same assay with a different strain, a A(H1N1) strain of a different subtype, which had been derived either from QT6 or MDCK. Here, we observed the H1N1 derived from QT6 showed higher titres compared to the H1N1 derived from MDCK throughout the whole experimental period (the biggest difference of infectivity was recorded on day7). The detection limit was reached on day7 for the virus last passaged on MDCK cells, whereas that of the one passaged last on QT6 reached this limit on day13. Besides, when the viruses were titrated on QT6, the results were opposite. The biggest difference was observed on day 7**.**

Whatever the passage history and the nature of the viral strain, the quantity of RNA recovered at the studied time points remained stable unlike viral ability to infect cells. The results of classical endpoint RT-PCR of the whole RNA fragment strongly suggest that viral genomic RNA is, at least in part, not degraded.

Thus, we studied the phenotype of our viral preparations by plaque assay on MDCK cells. The number of plaques on MDCK cells was higher for viruses assayed with QT6 than for the viruses assayed with MDCK cells. There was no significant difference in the average size of plaques between these 2 virus preparations, no matter what the type of cells. However for QT6, there seems to be 2 groups of plaques: small plaques and big plaques.The analysis indicated that there was no difference in colony size between both virus strains when inoculated on MDCK. On the contrary, the analysis revealed that there was a significant difference in the proportion of the number of small and big colonies for both viruses when inoculated on QT6.

Since the size of plaques is strongly linked to the properties of the NA, we sequenced the NA segment of the virus with different passage history by NA segment, we analysed the composition of mutation after one passage in each corresponding cell line. Thus each virus was passed once on egg and next 4 series of passage on MDCK and the 5th passage either on MDCK or QT6. The average sequence showed no mutation (compared to sequences in NCBI).

To investigate the quasispecies composition of are viral preparation with different passage histories, each vRNA was cloned, after RT-PCR, using TOPO vectorTA cloning kit. There was no mutation conserved throughout the different clones. No significant polymorphism was observed.

**In conclusion, s**urvival kinetics of both strains of viruses showed the viruses derived from QT6, avian cell line resisted more in the water environment. We inoculated on two different cell lines, MDCK and QT6 and the viruses derived from mammlian cells showed smaller plaques on avian cell line, whereas both viruses derived either from MDCK or QT6 cells showed no significant plaque size difference when plaque assays were done on a mammalian cell line.

Taken altogether our results suggest that the loss of viral infectivity was not due to the degradation of viral genome but to the damage to external structures. Since the last report, mutants on putative glycosylation sites have been generated and survival data are now accumulating.

## 1. Survival of Influenza A(H5N1) viruses in waters

### 1.1. Concentration, identification and quantification of avian IVs from waters, muds and aquatic animals

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

Optimised protocols for the concentration, identification and quantification of avian IVs from waters, muds and aquatic animals were developed in a multistep fashion. First quantitative detection methods by RT-PCR and cell culture have been developed to detect H5N1 as current detection techniques are often not sensitive enough, not only for direct analysis of heavily contaminated samples (such as duck ponds) and of solid matrices (sediments, biota, food), but also for analysis of lakes and especially for drinking water after a concentration step. An inventory of the conditions of real-time RT-PCR techniques used by the different partners within RIVERS was made. Kits providing the fastest and easiest way to purify viral RNA have been identified. A qRT-PCR protocol has been adopted as the consortium protocol and was disseminated within it (http://www.rivers-project.eu/internal/Protocoles_and_Articles/P001RIVERS Procedure RT-PCR detection of H5N1 Version 1.pdf ) and soon beyond.

A particular emphasis was then put on the development of viral concentration procedures from surface waters**.** An inventory of methods used for concentrating infectious viral particles was first carried out. The aim was to select the most efficient method useable for large volumes of clean or dirty water. Rapidity and simplicity were also evaluated, since the method has to be applied by each RIVERS partner and if possible in the field. In RIVERS, two methods were evaluated: adsorption/elution on glass wool, as described in the French national standard method (AFNOR XP T90-451) usually used for the detection of enteroviruses, and viral adsorption/elution on a commercial electropositive filter. These methods could be used for primary concentrations, allowing concentration of large volumes of water. The use of a second round of concentration steps with chicken erythrocytes or with poly-ethylene glycol (PEG), in combination or not with filter concentration, was subsequently evaluated. http://www.rivers-project.eu/internal/Protocoles_and_Articles/P002RIVERS Procedure for concentration of avian influenza viruses from water Version 2.pdf.

### 1.2. Technique of concentration, detection and isolation of H5N1 influenza virus in water using red blood cells (by Khalenkov et al. 2008)

This technique of concentration of H5N1 virus from contaminated water was applied to the analysis of water samples collected from natural environments in the H5N1 outbreak-associated areas and water specimens from experimental settings. The volume of water of 50 ml was used with Khalenkov technique to concentrate the virus before the detection by qRT-PCR and viral isolation.

Out of 12 samples collected in 2009, one was positive for both RT-PCR detection and viral isolation. One out 5 samples collected in 2010 was positive and only for Rt-PCr detection.

In various experimental settings, the protocols used allowed the detection by RT-PCR of H5N1 genomic RNA and virus isolation.

### 1.3. Technique of concentration of avian influenza A H5N1 virus from water using filtration on glass wool

This concentration technique was used to analyse the water samples collected from natural environments in the H5N1 outbreak-associated area in order to investigate the environmental contamination of H5N1 virus. The volume of water of 10 litres was used with this RIVERS technique to concentrate the virus before the detection by qRT-PCR and virus isolation.

There was neither positive genomic RNA detection nor virus detection.

### 1.4. Concentration, identification and quantification of avian influenza viruses from mud

Another task was to deal with the analysis of solid matrices (sediments, biota, and food). In this case, concentration steps were not necessarily the limiting factor. Strong matrix effects may occur with two main difficulties. Firstly, mud can trap infectious particles and secondly they can contain a lot of pollutants, potentially acting as polymerase inhibitors, thus lowering PCR performances. Such effects were studied and partially overcome.

As part of this programme, a direct extraction protocol for Influenza A viruses from mud was optimised for small quantities of mud and proved to be efficacious, provided the virus quantity was high enough.

A total of five techniques were used to extract the nucleic acid from mud. Here we tested natural mud collected from a natural pond (in an H5N1-free area).

Recovery of H5N1 viruses from mud with method P003RIVERS

This procedure for concentration of avian influenza viruses from mud has been elaborated by a RIVERS partners (IP de Lille) (P003 RIVERS). This method was tested and validated at IPC for the analysis of field mud samples. The detection threshold and the recovery rates of H5N1 virus were determined. The technique was then used to analyse the mud samples collected from outbreak sites of HPAI H5N1 in Cambodia in 2007 to 2010 in order to investigate the contamination of the environment.

The method is based on an elution step with a beef extract solution (10%) at alkaline pH, in combination with a second concentration step with poly-ethylene glycol (PEG6000). After these 2 following steps of concentration, a total of 1 or 2 ml of concentrated solution containing the virus were obtained from 5 or 10 grams of mud. Then, viral isolation and molecular analysis were done on this final solution in order to detect and quantify the virus obtained..

Among the two methods chosen for a nucleic acid extraction, the MagNa Pure Nucleic Acid Isolation Kit was the best one, giving a recovery rate of vRNA from mud eluate of about 0,5% - 3,8% and 0,2% - 6,1% in the first and second series of testing, respectively. The detection threshold of viral RNA in mud eluate in both testing was 2 $10^4$ RNA copies ($10^2$ EID50) per gram of mud. In mud eluate the infectious virus particles was detected in both testing with the detection threshold of 2 $10^5$ RNA copies per gram of mud.

After mud concentration, the recovery rate of viral RNA was about 0,8% - 3,8% and 0,8% - 9,3% in the first and second rounds of tests, respectively. The detection threshold of viral RNA in mud concentrate in both series of tests was 2 $10^4$ RNA copies ($10^2$ EID50) per gram of mud. In mud, the infectious virus particles were detected in both series with a detection threshold of 2 $10^4$ RNA copies per gram of mud in the first series and of 2 $10^3$ RNA copies ($10^1$ EID50) per gram of mud in second series.

This technique of recovery of avian influenza virus from mud was applied to the analysis of mud or soil samples collected from natural environments in the H5N1 outbreak associated area and from experimental settings. The volume of 5 or 10 grams of mud or soil was used with this method (P003RIVERS) to recover the virus in mud eluate and to concentrate the virus before the detection by qRT-PCR and viral isolation.

Out of 8 samples collected in 2010, 3 were positive for genomic RNA RT-PCR detection. None of the other samples (2007-2009) were positive. There was no virus isolation.

This method P003RIVERS was used to analyse mud specimens collected from our experimental settings. For these specimens the volume of mud was about 1 gram and we adjusted the volume of solution used accordingly,

## 2. Observation of IVs in natural environments

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

### 2.1. Data from China

Water samples were collected in two large lakes in China: Dongting Lake on the Yangtze (Chang Jiang) River downstream of Wuhan (Hubei Province) and Qinghai Lake on the Yellow River (Huang He) where a severe epizootic occurred in 2005 and was an important point for the subsequent geographical spread of HPAI H5N1 viruses. Two methods associated with Influenza virus isolation from water samples have been established, with which many strains of Influenza A virus of subtypes H5N1 and H9N2 have been isolated from the

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

The research 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 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. 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 (GenBank). 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 $TCID_{50}$ 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, the some parameters 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 temperature. 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°C and 16°C, 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°C, H5N1 and H9N2 viruses could retain their infectivity for at least 30 days in the Milli-$QH_2O$ 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 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 stll 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°C it was also able to survive 7 days or so, which strongly suggested the AIVs could be transmitted in the migratory birds through the faecal-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.

**2.2. Data from Cambodia**

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

In response to the notification of a confirmed case of influenza subtype H5N1 infection in humans or poultry, the IPC team 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°C within 36 hours to Institut Pasteur in Cambodia 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. From April 2007 to February 2010, 4 environmental investigations were conducted in the locations with influenza virus (H5N1)–associated outbreaks.

**H5N1 outbreak in 2007**

In 2007, a fatal human case of avian influenza A H5N1 was reported in Kampong Cham Province of Cambodia. A total of 45 environmental samples were taken in this outbreak location. Out of 45 samples, 5 were H5 gene positive and 2 out of these were also M gene positive. However, none of these viruses were subsequently isolated from the positive environmental specimens.

**H5N1 outbreak in 2008**

In November 2008, one human case (non fatal) of avian H5N1 was confirmed in Kandal Province. A total of 66 samples were collected from the environment surrounding the epidemic site. Real-time quantitative RT-PCR performed with all the samples allowed to detect 16 samples positive in gene H5. All positive samples with qRT-PCR were inoculated into embryonated chicken eggs to isolate the virus. All attempts of viral isolation using these samples were negative suggesting that no infectious particles were present.**2.2. 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 84 specimens were collected in the location surrounding the index household. Out of these 84 samples analysed, 8 were H5 tested positive ($10^1$ -$10^3$ RNA copies per ml and $10^3$-$10^4$ RNA copies per gram). The viral isolation detected two positive samples.

**H5N1 outbreak in 2010**

A total of 50 environmental specimens were collected and analysed by qRT-PCR. Of these specimens, 15 were positive with a quantity of virus varying from $10^4$ to $10^5$ RNA copies per ml and $10^2$ to $10^5$ RNA copies per gram. These positive samples were processed for viral isolation and none contained infectious viral particles.

To summarize, from April 2007 to February 2010, the IPC team collected a total of about 250 environmental samples in half a dozen households in 3 provinces where the different outbreaks occurred. From the total of samples, 18% tested positive for H5N1 by qRT-PCR. Viral RNA was frequently detected in farm soil, mud, pond and puddle water, feathers of recently dead poultry, aquatic animal, water plants in households' ponds, poultry faeces, poultry swab, and from straw in poultry cage. The number of RNA copies were the highest in the contaminated mud and straw collected during an outbreak in Takeo province in 2010. Of the 44 specimens positive by qRT-PCR which were then inoculated in eggs, viable H5N1 could only be amplified from 2 samples (soil and water). The longest persistence of viral RNA observed in the environmental specimens was 7 days following the last poultry death.

The investigation in Cambodia demonstrates that H5N1 RNA was frequently present on various environmental surfaces in the households of H5N1-infected patients and in the surrounding environment. We successfully detected viral RNA from mud and dry soil. However, the presence of RNA does not necessarily imply that the virus is still infectious or that human or animal contamination could occur. We were only able to isolate viable virus from 2 out of 44 samples, and there was no correlation between these samples and those in which the highest quantity of RNA was detected. Nevertheless, the results underscore the potential role of the environment in H5N1 human and animal contamination as well as the importance for regular surveillance and disinfection of the surrounding environment following avian influenza outbreaks.

**3. Observation of Influenza virus survival and concentration in experimental settings"**

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

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

Two sets of series of experiments were performed.

**3.1. A first series of 3 experiments were conducted in 1/ aquaria and 2/ in jars with fish.**

**3.1.1. 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 located where there was reported outbreaks of A(H5N1) HPAI.

The first experimental setting in aquaria showed the 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.

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 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 though 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.

In the third series of experiments, aquaria containing rain water and molluscs but no mud were tested at a temperature of 25°C. The organs taken from molluscs 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.

**3.1.2. 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 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 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.

### 3.2. Second set of four experimental settings

#### 3.2.1. Fourth experimental setting in aquarium

Water specimens collected from all aquaria (Aquarium 1- 8) from day1 to day 8 post viral inoculation were processed by the technique of Khalenkov to concentrate the virus in order to detect by qRT-PCR or viral isolation. We observed that the viral RNA was present mostly from day 1 to day 3 after virus inoculation and more frequently in aquarium where the fauna and flora was not introduced (aquarium 1, 5, 7). The viral isolation performed on water specimens collected from day 1 to day 5 post viral inoculation could not detect any infectious virus particles even though in some of these specimens virus RNA was detected by qRT-PCR.

### 3.2. Fifth experimental setting in aquarium

A total of 6 aquaria were used for this 14-day experiment. Two aquaria contained only water and mud while another 4 aquaria contained water, mud, plants, and aquatic animals. The water and mud used in these aquaria was collected from a lake in a site where H5N1 outbreak had occurred in 2006.

For this experimental setting, 4 aquaria 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 and mud were collected daily and the specimens of plant and aquatic animals were taken every 3 days. 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 plant and animal has not been detected.

### 3.3. Sixth experimental setting in aquarium

In this experiment lasting 14 days, three aquaria containing 10 litres of rain water were tested at a temperature of 25°C. No mud was introduced in these aquaria.

The first aquarium was infected with A/CAM/408008/2005(H5N1). A total of 25 molluscs were introduced in this infected aquarium at the same time as of viral inoculation. 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 post-inoculation, 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). 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 aquaria.

We collected a total number of 56 specimens: from the first aquarium (infected): infected molluscs; from the second aquarium (non-infected): non-infected molluscs; from the third aquarium (exposure experiment): infected mollusc placed into the aquaria on days 3 and 6, and "contact" molluscs (originally non-infected) on days 3 and 6.

The organs taken from molluscs of the infected aquarium (IM) on days 2, 3, 4 and 6 were all H5 gene positive. Furthermore, infectious viral particles were successfully isolated from these specimens. Howeve, no viral detection could be done later than 6 days post-inoculation.

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 post-inoculation, with an amount of viral RNA detected in a range of 102-103 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 high concentration of H5N1 virus, 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.

### 3.4. Seventh experimental setting in aquarium

In this experiment lasting 10 days, six aquaria (A1- A6) containing 10 or 5 litres of rain water were tested at a temperature of 25°C. No mud was introduced in these aquaria. The aquaria A1, A3 and A4 were infected with A/Ck/CAM/LC1AL/07(H5N1). A total of 40 molluscs were introduced in each of infected aquarium A3 and A4 at the same time as virus inoculation.

We observed that the molluscs survive the same way in infected and non infected water (A2, A3, A4). A total of 200 specimens were collected:

The analysis of the water sampled from the infected aquarium A1 and A3 showed that infectious virus particles could be isolated on the first 4 days of viral inoculation and the presence of viral RNA was remained until day 8 post-inoculation. The detection of viral RNA and the infectious virus particles is not different in infected water in the presence or absence of molluscs. The amount of viral RNA detected in these aquarium was in a similar range.

Molluscs placed in infected water (A3) were contaminated by the virus. The infectious virus particles were successfully isolated from different organs of molluscs at day 1 and 2 post-inoculation. Whereas the viral RNA was detected at least for 8 days after virus inoculation.

Viral RNA was detected in the infected molluscs' organs (IM-Exp) until 3 days after their being placed in non-infected water (A6). But no infectious virus was successfully isolated from these specimens. As for the non-infected molluscs exposed to infected ones (NIM-Exp) in aquarium A6, viral RNA could be detected in their organs on days 1 and 2 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 aquarium A5 and A6 after it was exposed to infected molluscs.

In this experimental setting with similar conditions as those of the third experimental setting (temperature of 25°C, rain water, no mud, H5N1 virus) the survival of H5N1 virus in water was observed during 4 days with the persistence of viral RNA at least 8 days after viral inoculation. These results were in agreement with the previous ones in the third experimental setting in aquarium with molluscs.

In this infected water the molluscs were contaminated by the virus and carried infectious virus particles for 2 days after inoculation of the water. We observed that in this experiment the infected molluscs carried the infectious virus particles for a short duration (2 days vs 6 days compared to the previous result). Furthermore the

contaminated molluscs may transmit the virus to other molluscs. Here, we observed the presence of viral RNA 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.

**4. Impact of water treatments on virus survival**

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

***Virus.*** Avian influenza virus (AIV) vaccine strain, H5N1 – NIBRG – 14 (originating from NIBSC, UK), with haemagglutinin - HA and neuraminidase – NA genes derived from A/Viet Nam/1194/2004 – clade 1 was propagated in SPF embryonnated eggs in order to obtain the stock virus, which was stored in aliquot, in liquid nitrogen. Virus concentrations used in the experiments were calculated by 50% tissue culture infectious doses (TCID50) having virus titre of $10^{5.25}TCID_{50}$/0.1 ml. High, medium or low concentrations of H5N1 were diluted in PBS and in standing water (lake), running water (river) and sea water.

***Samples:*** Water samples, collected from the typical waterfowl habitats such as river Neajlov, Siutghiol lake and Black Sea, were characterized in terms of chemical parameters using a commercial kit and were stored at -80 $^0$C aliquots using it throughout the experiment. Protein content, total number of alive aerobic particles, heavy metals (lead) and pH of the two types of water were measured.

Substrates to monitor the retention infectivity of the influenza virus after different chemical treatment and virus infectivity testing

***Phenotypic methods:*** Embryonated chicken eggs (4 eggs/each sample, (two or three passages) or certified MDCK cell line.

The first method has the advantage of a negligible effect of disinfectant action on the substrate. Disadvantages consist of embryonated eggs need and consumption of time.

***Molecular method:*** qRT-PCR method quantifying the HA/M gene, on duplicates, avoiding time consuming of the classical method.

***Controls***: virus, disinfectant.

***Disinfectants:*** concentration: recommended by the producer or higher; time of interaction chemicals – virus: recommended by the producer or higher

***UV light:*** For inactivation of virus by UV light, AIV at initial concentration of $10^3$ $TCID_{50}$/0.1 ml in river, lake water sample and in sterile PBS was exposed to ultraviolet light at 500 $\mu W/cm^2$ (at 15 cm distance)(UV-Lampe NU-4 KL, 4W), at room temperature. The total volume and depth of the viral suspension in the Petri dishes were 4 ml and 0.6 cm. Samples have been taken every 10 min after exposure, from each suspension and were inoculated on MDCK monolayer cells. After 24, 48, 72h the supernatant of cells culture was harvested and were stored at -80 $^0$C until tested..

The effectiveness of disinfectant or UV light has been assessed by a *Real-time quantitative RT-PCR* in supernatants collected at different time.

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

To be closer to field situations, the impact of glutaraldehyde on AIV in association with organic materials was studied by infecting river water and standing water (lake or pond water) with H5N1 AIV. The preliminary data showed that a 2% glutaraldehyde preparation efficiently desinfected all the tested liquids at 20 $^o$C or more, except in the case of the highest virus titres. As expected, the presence of organic materials decreased the inactivation efficacy of glutaraldehyde at 37 $^o$C for 40 hours. In these conditions, viable virus was still detectable in the tested water collected from a lake. Globally, our first results, in experimental conditions, had showed that H5N1 AIV were sensitive to inactivation by glutaraldehyde and oxidizing agents.

On another hand, ultraviolet radiation light has been proven to inactivate AIVs in 10 - 20 min in experimental condition if the viral loading was $10^3$ $TCID_{50}$/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.

The team involved in this WP optimized a part of new laboratories techniques during the experiments development of a faster and more sensitive method to assess viral inactivation.

Two groups of disinfectants (Aldehydes and Oxidizing agents) proved to be most effective in inactivation of NIBRG 14 reasortant, in all types of water, at all titers.

Alcohol disinfectants were efficient for the inactivation of a moderate virus titre $10^{3.5}$ EID50/ml , at a minimum concentration 60-70%.

There was no significative diference in disinfection effectiviness of all three water types, probablly explained by the low biochemical differences, except salinity for sea water. The same can be said about the physical factors (temperature and UV radiation.

## 5. Survival of Influenza A(H5N1) viruses in air, surfaces and food products

### 5.1 Impact of air conditions on virus survival

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

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

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

### 5.2 Impact of surface nature and conditions on virus survival

#### 5.2.1 Design of protocols for a maximum recovery of IAVs

Because one of the goals of WP5 was 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).

We developed strategies for virus recovery from various surfaces in order to avoid or minimize virus loss during this operation. Assays were preformed in triplicate and with several conditions of time and speed.

**On porous surfaces:** The ratios of virus recovery from weaved tissue handkerchiefs using a Fastprep apparatus and various beads 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 huge 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 in ) at given temperatures (4°C, 25°C and 35°C) were carried out after using the strategy previously chosen for virus recovery on watch glasses.

The Assay with Influenza H5N1 was performed at 3 temperatures (4°C, 25 and 35°C) 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.

**5.2.2 Evaluation of virus survival on surfaces in laboratory settings**

**5.2.2.1. For smooth non porous surfaces**

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. 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.

**5.2.2.2. For porous and rough surfaces**

We tested the survival of four influenza viral strains on weaved tissue handkerchiefs. Assays were carried out without prior sterilisation of weaved tissue (see protocol on web site).

Once the protocols became available, we used the following viral strains: A/Duck/Cambodia/D4(KC)/2006 (H5N1), A/Hong-Kong/156/97 (H5N1), A/Paris/2590/2009 (H1N1)v and A/New Caledonia/20/99 (H1N1) at 3 temperatures (4°C, 25 and 35°C) and survival evaluation was performed by titration by $TCID_{50}$/mL and compared with qRT-PCR for M gene after 30 minutes (D0 wet), after complete drying of contaminated weaved tissue handkerchiefs (24 hours at 4°C and 6 hours for 25 and 35 °C° (D0 dry)), 1 day after , 2 days after and 3 or 5 days after. Each weaved tissue handkerchief was contaminated with 50 µL of the viral stock strains.

For each condition, assays were performed in triplicate. After each incubation time point, virus was collected with 2000 µL of culture medium and submitted to the recovery method using the mechanical effect of beads (30 seconds at 4M/S conditions).

For each temperature condition, a viral titre control (Liquid) was performed with 2000 µL of culture medium contaminated with 50 µL of viral stock strain, maintained 30 minutes at the temperature condition, and then it was titrated.

For each temperature condition, the comparison of the viral titre control (Liquid) and the D0 wet titres allowed us to evaluate the retention of viral particles in the weaved tissue handkerchiefs, the comparison between the titre of the viral titre control (Liquid) and D0 dry titre allowed us to evaluate the retention of the viral particles in the weaved tissue handkerchiefs plus the loss of infectivity due to the combined effects of temperature and drying.

At 4°C, the loss due to retention in weaved tissue handkerchiefs was approximately of 2 to 3 log excepted for the A/Duck/Cambodia/D4(KC)/2006 (H5N1) strain. At 25 and 35 °C the loss due to retention and loss of infectivity was approximately of 1 log, except for the A/New Caledonia/20/99 (H1N1) strain at 25°C. For this strain at this temperature, there was no loss due to retention. This result is also observed after RNA genome concentration evaluation.

RNA genome concentration were determined by Gene M detection by qRT-PCR (Light Cycler 480) at different days (designed as Dx, x being the number of days) and expressed in log (copy number/mL). All experiments were done in triplicate.

Our results showed that, at all temperature conditions, the loss due to retention in weaved tissue handkerchiefs was approximately of 1 log copy number/mL, the RNA genome concentrations were quite stable from the beginning to the end of the experiments (D3 or D5 dry).

For the A/Duck/Cambodia/D4(KC)/2006 (H5N1) strain, as for titration by $TCID_{50}$ $TCID_{50}$/mL, virus retention was not observed at 25°C.

**5.3. Fields collection of air and surface samples**

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 was 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.

As planned, the Sartorius BioCollector 'Airport MD8' was evaluated in the real environment of passenger cabins of commercial aircrafts. The analysis by qRT-PCR of these air samples for Influenza A and B viruses and RSV showed that one was positive for Influenza B virus and one for RSV A. Virus culture using MDCK, Vero and /or MRC5 did not lead to virus isolation. These results illustrate that, in our real environment observational setting, the MD80 allows virus capture when present. The inability to recover viable virus might be due to the conditions in an environment very hostile to IVs (IVs) with high air turn-over, effcicient HEPA filters and very low humidity.

**6. Persistence of viable virus in farms surroundings**

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

**In Asia (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 2010, the environmental investigations were conducted in the location with influenza virus (H5N1)–associated outbreaks.

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 the paper written by members of the RIVERS consortium, satellite image analysis methods used for water detection and characterization were reviewed, 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 were also available. Current methods for estimating other relevant water variables such as salinity, pH, turbidity and water depth were not considered to be effective.

The work done in 2008 allowed identifying 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, were collected between March and August 2009 (before and at the end of rainy season), in order to validate the model. In June 2009: a mission of modeler took place in Cambodia in order to work on the H5N1 data available in IPC. In October 2009, the analysis of correlation between environmental conditions linked to water and the number of H5N1 cases in Cambodia started. This analysis was finished by the end of the RIVERS project.

**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). 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°C and 70% humidity.

Fecal swabs from all infected and uninfected birds were collected on day 5 post infection (P.I.) and were stored in original form without any further processing. In intervals of two days (2-20) parts of fecal samples were taken and infectious virus titers were evaluated by cultivation in CE. 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 'resistance' varied from all infected feces between the 2 to the 8 P.I. days.

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 titre decreased sharply after day 4. The transmission of infection during housing of domestic ducks in closed poultry farms was influenced not only by the period of persistence of virus in feces but by the contact of susceptible birds with faeces infected with virus.

**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. Also, if any inactivation process would affect the genome integrity, it would be extremely difficult to differentiate this impact from that directed to the viral envelope. Our second postulate was that inactivation of virus would be graduated and dependant on time of exposure to the inactivating agent. Therefore, we have developed tools which would reproduce viral envelopes and which would allow easy quantification of virus replication. In order to avoid the use of infectious particles, we have engineered pseudoparticles containing H1 and N1 envelope proteins and a lentivirus defective RNA containing the reporter gene of luciferase to quantify the efficiency of peudovirus penetration and replication. This tool will also be used by for molecular studies in other components of RIVERS. To quantify the efficiency of virus infection, real-time RT-PCR systems for M1, H1 and H5 genes were developed. Finally, protocols were established and equipment was bought to treat virus particles with an array of physical and chemical agents.

**8. Evaluation of the impact of selected parameters involved in food processing**

Poultry meats can be contaminated by different 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.

**8.1 Extraction and detection of AIV in poultry meat**

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.

**8.2 Evaluation of individual effect of main parameters, involved in cooking of poultry meats and their interaction on survival of H5N1 in poultry matrices**

The impact of parameters involved in cooking of poultry meats was evaluated. 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°C and high salinity (3%). Time required to reduce infectivity decreased with low pH, increased temperature, and high salinity.

Based on the data available in the literature, 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 our second period 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 strain. Since thermal inactivation of Influenza viruses have been mainly investigated on chicken meat, four repetitions of experiments were performed with ground duck meat to obtain survival curves. One experiment was however carried out with 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°C up to the targeted temperature of 70°C.

The experimental domain was 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. Chicken meat were cooked according to (i) guidance documents from WHO (OMS, 2005 ; OMS, 2007): INFOSAN notes, disseminated through the International Food Safety Authorities Network, gives recommendations 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 log10 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 log10 lethality of Salmonella.

A first experimental setting was conducted to study viral inactivation observed at 60, 65 and 70°C. The targeted temperature was reached in 2 minutes and survival curves showed viral inactivation about 2 log units when targeted temperature was reached.

So a second experimental setting was chosen to obtain a slow increase of temperature from 26°C to 70°C, in order to observe one viral residual titre associated to one temperature, previously described for heat inactivation of bacteria. Briefly, meat was prepared and contaminated as previously described. The tube-holding heating block was preheated at 26°C. The tubes containing the samples were placed in the test instrument until temperature of 26°C was reached. The targeted temperature was set to 70°C for testing kinetics of thermal inactivation. In these conditions, the temperature was reached in approximately 12 minutes. Single samples were removed during the ramp-up period of the heating. Upon removal from the block, samples were immediately chilled onto 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°C up to the targeted temperature of 70°C.

### 8.3. Development of inactivation predictive models

The experimental data obtained for all survival kinetics were processed using the statistical software SPLUS 2000. A modelling approach was implemented, using available models to describe viral inactivation taking into account population and temperature variations as a function of time. Unfortunately the modelling has been for the moment unsuccessful. The use of other software, adapted to the data explanation, was still in progress at the official end of the project.

However trends could be observed. No infective virus was isolated from duck and chicken meat after 10 to 11 minutes corresponding to a progressive heating from 26 to 60°C, The range of added salt content studied had no impact on the viral inactivation. Viral inactivation seems slower in chicken meat than in duck meat.

### 8.4. Validation of model predictive data: See the following paragraph

The results obtained are disturbed to a 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 modelling 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.

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

It was assessed during the first part of the project that no commercial software was available, which could even approximately fulfil the necessary requirements to establish an agent-based model of AIV survival in various environments. Therefore, it was decided to construct a modular system of various tools, acting on experimental data as soon as they become available form various component of RIVERS. In order to test and verify this conclusion, a simulatory model of the Influenza viral spread, (strain A/Panama/2007/99(H3N2)) infecting guinea pigs in laboratory-controlled conditions, was built. The experimental findings were reproduced by this model fairy well. It is expected, that due to a high similarity of the AIV H5N1 serotype to the one used by authors of the experimental study on guinea pigs, it would be fairly straightforward to repeat our simulations and modelling, as soon as data on H5N1 stability in various environments will be available from other component of RIVERS.

Two main modules of the final simulation platform were designed, implemented and tested. 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 possible effects on infection spread. Parameter space was checked for stable behaviour, 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 last 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 scalable, 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 was 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*). Based on the ISSCOR method analyses and visualisations of influenza virus hemagglutinin genes of three different A-subtypes revealed some rather striking temporal (for A/H3N2), and spatial relationships (for A/H5N1) between groups of individual gene subsets. The application to the A/H1N1 set revealed also relationships between the seasonal H1, and the swine-like novel 2009 H1v variants in a quick and unambiguous manner. The examples considered so far demonstrated that application of the ISSCOR method for analysis of large sets of homologous genes is a worthwhile addition to a toolbox of genomics – it allows a rapid diagnostics of trends, and perhaps can even aid an early warning of newly emerging epidemiological threats (the work have been just submitted to the Comptes Rendus Biologies). Ever increasing amounts of genetic

information stored in sequential databases require efficient methods allowing for their automatic and routine *in silico* analyses to reveal their phylogenetic relationships. Towards this end we have created several software tools for unambiguous labelling of translated tree nodes, with no constraints on nodes' degree, and for the subsequent finding of evolutionary pathways. Main features of these methods are**:** small demands on computational time, and the ability to analyze phylogenies obtained by any traditional tree-building technique (*QPF: Quick algorithmic solution for unambiguous labeling of phylogenetic tree nodes* CBAC (2010) in press, doi:10.1016/j.compbiolchem.2010.10.002; *DAC – The Use of Neighbor-Joining for Inferring Very Large Phylogenies – Heuristic Method Improvements*, J. Bioinformatics and Compututational Biolology (2010), submitted; *Phylogenetic Topology, Structure, and Other Features of the Sequences Set – Their Influence on Trees' Reconstruction*, CBAC (2010), submitted). Currently the work is in progress to combine these methods with the ISSCOR technique to create an analytical tool aiding multi-strain modelling within a framework of very large scale epidemiological simulations described above.

Up to date, despite the production of a wealth of experimental data released by the other WPs of the project, the amounts of data remained too limited to be used by modellers. This was a crucial impediment to achieve some deliverables, as sufficient database of experimental results is essential to characterize both possible parameterisations, and the intended modelling studies leading to a hierarchy of modules simulating. and maybe ways of it's deactivation.

**RIVERS:** LEVEL OF COMPLETION (in green)

by deliverables

| **Deliverable No**[1] | **Deliverable title** | **% completion** [2] | **Nature** [3] | **Dissemination level** [4] |
|---|---|---|---|---|
| 1 - D 0-1 | Establishment of 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 |  | P | PU |
| 2 -D 0-2 | Experimental tools eg model virus not requiring BSL3 conditions to be used in other WPs |  | P | PU |
| 3 -D 0-3 | Method of virus viability other than virus titration of cell culture (TCID50 titre determination) |  | R | PU |
| 4- D 0-4 | Knowledge on some determinants of virus stability |  | R | PU |
| 5- D 0-5 | Peer-review scientific publications in international journals |  | R | PU |
| 6 - D 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 |  | R | PU |
| 7 - D 1-2 | Standard Operating Procedures (SOPs) available for other partners of the project |  | R | PU |

[1] Deliverable numbers in order of delivery dates: D1 – Dn

[2] Month in which the deliverables will be available. Month 0 marking the start of the project, and all delivery dates being relative to this start date.

[3] Please indicate the nature of the deliverable using one of the following codes:

**R** = Report
**P** = Prototype
**D** = Demonstrator
**O** = Other

[4] Please indicate the dissemination level using one of the following codes:

**PU** = Public
**PP** = Restricted to other programme participants (including the Commission Services).
**RE** = Restricted to a group specified by the consortium (including the Commission Services).
**CO** = Confidential, only for members of the consortium (including the Commission Services).

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|---|---|---|---|---|
| 8 - D 1-3 | Publications in peer review international journals |  | R | PU |
| 9 - D 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) |  | O | PU |
| 10 - D 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 |  | R | PU |
| 11 - D 2-3 | Data on variations in prevalence and in virus load along the stream of rivers (eg Yangtze River) |  | R | PU |
| 12 - D 2-4 | Data on gastropods and bivalves Molluscs regarding their potential role a sconcentrators and conservatories of AIVs in aquatic biotopes |  | R | PU |
| 13 - D 2-5 | Publications in peer reviewed international journals |  | R | PU |
| 14 - D 3-1 | Inventory of all the elements naturally existing in the aquatic environment in the countries where H5N1 virus is circulating |  | R | PU |
| 15 - D 3-2 | Data and analysis |  | R | PU |
| 16 - D 3-3 | Peer-review scientific publications in international journals |  | R | PU |
| 17 - 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 |  | R | PU |
| 18 - D 4-2 | Data on the efficacy of chemical and physical treatments of water for the reduction of virus load and virus survival |  | R | PU |
| 19 - D 4-3 | Guidelines for treatments of different types of water aiming to reduce virus survival |  | R | PU |
| 20 - D 4-4 | Scientific publications in peer-review journal |  | R | PU |

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|---|---|---|---|---|
| 21 - D 5-1 | Approvable and standardised protocols for influenza virus recovery from various surfaces |  | R | PU |
| 22 - D 5-2 | Integrated air system for respiratory virus survival in aerosols |  | R | PU |
| 23 - D 5-3 | Data (database) about IV survival in the air and on various kind of surfaces and in various conditions |  | R | PU |
| 24 - D - 5-4 | Guidelines for recommendations |  In progress | R | PU |
| 25 - D 5-5 | Peer-review scientific publications in international |  | R | PU |
| 26 - D 6-1 | Data on the prevalence of AIVs in the surroundings of farms with present and past outbreaks of HPAIV H5N1 |  | R | PU |
| 27 - 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 bird H5N1 mortality, in the course of time throughout the year in tropical and non tropical regions of the world |  | R | PU |
| 28 - D 6-3 | Data on virus survival in bird guano |  | R | PU |
| 29 - D 6-4 | Publications in peer reviewed international journals |  | R | PU |
| 30 - D 7-1 | General information on avian flu virus survival in natural condition when dried |  | R | PU |
| 31 - D 7-2 | Scientifically tested information on dried avian influenza virus stability in parameterised conditions |  | R | PU |
| 32 - D 7-3 | Standard Operating Procedures for virus disinfection/inactivation in different settlements |  | R | PU |

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|---|---|---|---|---|
| 33 - D 7-4 | Training of Chinese and Asian scientists on Biosafety and virus inactivation |  | O | PU |
| 34 - D 7-5 | Publications in peer reviewed international journals |  | R | PU |
| 35 - D 8-1 | A report will be produced every 6 months |  | R | PU |
| 36 - 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 |  | R | PU |
| 37 - D 8-3 | Scientific publications in peer-review journal |  | R | PU |
| 38 - 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 |  | R | PU |
| 39 - D 9-2 | Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in various water environments, laboratory-controlled and natural |  | R | PU |
| 40 - 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 |  | R | PU |
| 41 - D 9-4 | Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in air at laboratory-controlled environements |  | R | PU |
| 42 - D 9-5 | Descriptive, data driven, low-level simulation models of AIVs perpetuation, viability and deactivation in avian feces and farm manure |  | R | PU |
| 43 - D 9-6 | Multi-scale agent-based simulation model of possible determinants for AIVs stability, perpetuation and deactivation. |  | R | PU |
| 44 - D 9-7 | Peer-review scientific publications in international journals |  | R | PU |
| 45 - D 10-1 | Final report and recommendations for specific policies to prevent and control avian influenza |  In progress | R | PU |

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|---|---|---|---|---|
| **All deliverables** | Total project (including publications) |  | | |