FLUAID

Generation of information and tools to support the management of the avian influenza crisis in poultry

STREP

Sustainable management of Europe's natural resources
Sixth Framework Programme
Priority SSP/8.1
Policy-oriented research

Final publishable activity report

Period covered: from 01-01-2006 to 31-10-2009 Date of preparation: January 2010

Start date of project: 01-01-2006 Duration: 46 months

Project coordinator name: Dr Ilaria Capua

Project coordinator organisation name: Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe)

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Section 1 – Project objectives and major achievements for the whole project

Avian influenza (AI) outbreaks have recently caused severe losses to the poultry industry, its stakeholders and, ultimately, to the EU taxpayer. In addition, the ongoing global H5N1 crisis is a serious concern for food security and human health. It is estimated that since 2000, over 200 million birds have died or have been culled following infection with influenza viruses subtypes H5 or H7. Approximately 50 million of these birds were from Europe. Importantly, human infections have also been reported in several of these outbreaks. In Asia, the Middle East and Africa, due to both social conditions and the particular characteristics of the H5N1 virus, the crossing of the species barrier represents a serious potential risk of a new human pandemic virus emerging. The increased relevance of AI in the fields of animal and human health, has highlighted the lack of scientific information on several aspects of the disease, which has hampered the adequate management of some of the recent crises thus resulting in millions of dead animals and concern over loss of human lives and over management of the pandemic potential.

The primary goal of the FLUAID project "*Generation of information and tools to support the management of the avian influenza crisis in poultry*" was the joint development and application of novel technologies to combat AI infections. This goal has been achieved through the interaction of leading European institutes along with the active collaboration of laboratories in Australia, Indonesia, Pakistan, South Africa, Egypt and Vietnam.

Particpant	**Participant name**	**Country**
P1	Istituto Zooprofilattico Sperimentale delle Venezie	Italy
P2	Veterinary Laboratories Agency	UK
P3	Central Institute for Animal Disease Control	Netherlands
P4	Agence Francaise de Securité Sanitaire des Aliments	France
P5	National Agricultural Research Centre	Pakistan
P7	Institute of Virology and Immunoprophylaxis	Switzerland
P8	Australian Animal Health Laboratory	Australia
P10	Innovative Diagnostic Vet	France
P11	University of Stellenbosch	South Africa
P12	Department of Animal Health	Indonesia
P13	National Center for Veterinary Diagnosis	Viet Nam
P14	Forsite diagnostic Limited	UK
P15	Central Laboratory for Veterinary Quality Control on Poultry production	Egypt

The first deliverables of the project were the evaluation and identification of diagnostic tools and vaccines to be used in outbreak management and in the application of control measures based on vaccination. A pilot study was also planned with the aim of identifying prototype strains for a future EU vaccine bank. This study was to allow the identification of suitable strains for conventional vaccine production and the development and validation of companion diagnostic tests which can be used in combination with the DIVA (Differentiating Infected from Vaccinated Animals) strategy. In addition FLUAID has generated data on the presence of virus in the meat of vaccinated versus unvaccinated birds. To complement this work, studies on pathogenesis in reservoir species and on the genetic and antigenic variability of endemic strains were undertaken. These studies shed light on areas such as the emergence of antigenic drift in avian viruses and on the most beneficial gene constellation that supports viral replication and transmissibility.

The results generated by FLUAID, therefore, have identified candidate strains for an EU vaccine bank and validated companion diagnostic tests. Attempts to make novel, rapid and sensitive pen-side tests were unsuccessful although evaluation studies on existing tests particularly in countries were H5N1 is endemic has provided valuable information. This, coupled with improved knowledge on pathogenesis and transmission of the AI virus in ducks, quail and indigenous chickens will contribute to the development of more effective control measures for the disease to be applied globally. In this way European experience, knowledge and scientific achievements will gain visibility and will be used to support decision making and further research.

The work performed and the most significant results achieved by FLUAID are summarised below.

Major Achievements of the project

Objective 1: *Studies on the development of protoype vaccines*

- **Identification of prototype strains :** Using phylogenetic analysis and cross-HI data from a group of selected H5 viruses circulating in birds in Europe, The Middle East and Africa 2005 and H7 isolates from the Italian epidemics (2002-2004) significant data has been obtained that has identified suitable candidates for a European vaccine bank. For the H5 viruses two LPAI isolates obtained from Italian waterfowl e.g A/mallard/Italy/3401/2005(H5N1) and A/duck/Italy/775/2004(H5N3) showed the broadest cross reactivity with the other H5 viruses tested. The study of Italian H7 viruses revealed that one virus isolated in 2003 [i.e. A/turkey/Italy/2987/2003 (H7N3)] is the most suitable vaccine candidate given its high level of cross reactivity with the other H7 viruses tested. **(*Beato, M.S., Monne, I., Mancin, M, Bertoli, E, Capua, I. A proof of principle study to identify suitable vaccine seed candidates to combat introductions of Eurasian lineage H5 and H7 subtype avian influenza viruses. Submitted to Avian Pathology*)**

- **Reassortant viruses for use in DIVA strategies**: To complement these studies experiments were also performed to generate reassortant viruses with rare neuraminidases that could be used as vaccines as part of a DIVA (Differentiating Infected from Vaccinated Animals) strategy. The primary objective was to make available candidate H5 and H7 strains that have neuramminidase antigens different from N1, N2, N3, N7, as the latter appear to be common in nature. Candidate strains with uncommon N antigens, preferably N5 and N8 are less likely to interfere with diagnosis in a "DIVA" strategy based on heterologous neuraminidase testing. An H7N5 virus was obtained by reassortment between a LPAI H7N3 and an H12N5 virus. The protective capacities of this reassortant H7N5 virus when used as a vaccine were also evaluated *in vivo*. SPF chickens were vaccinated with inactivated H7N5 and then challenged with both an HPAI H7N1 and LPAI H7N3 AI virus. The DIVA strategy was successfully used to detect and confirm infection in the vaccinated birds. No clinical signs were observed in any of the vaccinated birds. In addition the vaccine totally suppressed shedding after challenge with H7N1 HPAI and significantly reduced shedding after infection with H7N3 LPAI. Work on the creation of a reassortant H5N5 virus was initiated but due to technical difficulties was not completed within the time frame of the project. Nevertheless four putative H5N5 viruses have been identified by PCR following reassortment between an H12N5 and H5N3 but they still need to be characterised further to confirm the N-type. (***Beato, M.S., Rigoni, M., Milani, A., Capua, I (2007) Generation of avian influenza reassortant viruses of the H7N5 subtype as potential vaccine candidates to be used in the framework of a "DIVA" vaccination strategy. Avian Diseases 51, 479-480.***)

- **Assessment of viral colonization** of muscles following experimental challenge with HP and LP H7N1 viruses in vaccinated and unvaccinated turkeys: It is now generally accepted that in most poultry species, highly pathogenic avian influenza (HPAI) viruses cause viraemia and systemic infection with virus replication in vital organs and in muscle tissue. In contrast, there is little information about the ability of low pathogenicity avian influenza (LPAI) viruses to replicate and persist in meat. The aim of this study was to determine the ability of LPAI and HPAI viruses of the H7 subtype to colonize turkey muscle tissue following experimental infection. In addition the efficacy of vaccination in preventing viraemia and thus meat colonisation in turkeys was investigated. Data obtained confirms the ability of HPAI viruses to cause a generalized infection together with colonization of muscle tissue as already reported in other. studies. The finding of main interest from this study was the ability of an H7N1 LPAI virus to be detected from the blood and muscle of experimentally infected turkeys by means of Real Time PCR. This is in contrast with the general belief that LPAI is a localised infection without viral replication in blood. However, despite the viraemia detected, no virus was isolated from the muscle of the infected birds. Additional data generated from this experiment in vaccinated birds is the absence of detectable virus in the blood and in meat of vaccinated turkeys infected with both LPAI and HPAI viruses and underlines the effectiveness of vaccination in preventing viraemia and meat colonisation. In addition, data obtained show that H7N1 HPAI viruses are not transmitted by swill-feeding ***(Toffan, A., Beato, MS., De Nardi, R., Bertoli, E., Salviato, A., Cattoli, G., Terregino, C., Capua, I. (2008) Conventional inactivated bivalent H5/H7 vaccine prevents viral localisation in muscles of turkeys infected experimentally with LPAI and HPAI H7N1 isolates. Avian Pathology 37:407-12)***

Objective 2. *Development of companion diagnostic tests based on anti-N detection systems*

- Using monoclonal antibodies produced by P3 (e.g. anti-N2 and anti-N3) and P10 (anti-N1) **three companion diagnostic** kits have been produced during this project. All three kits have undergone preliminary evaluation by P1 and have shown to have high specificity and sensitivity. The N1 kit has been successfully commercialised by P10 and has also undergone further evaluation as part of the EPIZONE EU network of excellence **(*Dundon W, Pizzuto, M, Koch, G, Kuehn, K, Harder, T, Mahmood, S, Slomka, M, Schmitz, A, Jestin, V, Jørgensen, P, Stahl, K, Marche, S, Van Den Berg, T, Capua, I, Uttenthal A (2008) Inter-laboratory evaluation of two commercial DIVA kits for Avian influenza. Second Annual EPIZONE meeting June 4-6, Brescia, Italy)***

Objective 3: *studies on antigenic drift and genetic variability of selected H7 isolates*

- Using cross-HI, phylogenetic analysis and principal component analysis (PCA) of the HA gene of almost 50 H7N3 subtype viruses that were isolated at various times during avian influenza epidemics in northern Italy, P1 has shown and determined the extent of antigenic drift. The genetic analysis of the H7N3 shows a high degree of similarity among groups of isolates from 2002, 2003 and 2004. Despite this high homology between the isolates, distinct groups of viruses could be identified according to the year of isolation.The antigenic trajectory analysis used has been able to identify drift variants among the viruses tested. **(*Beato, MS., de Jong, M.C.:, Fusaro, A., Capua, I (2010) The evolutionary trajectory of H7N3 avian influneza in turkeys in Italy. In preparation*)**

Objective 4: *development and validation of antigenic penside diagnostic tests*

- **Lateral Flow Device (LFD) development, optimization and evaluation:** A significant effort was employed by one of the commercial partner of the project to develop a Lateral Flow Device for the rapid diagnosis of AI. Two separate batches and 7,000 device were produced and then evaluated by a number of consortium (P5, P11, P12, P13 and P15) and non-consortium members. Although the sensitivity of the LFD proved high the specificity was low (between 68 – 71%) and so it was decided not to continue a more in depth validation of the LFD. In order to compensate for the poor performance of the LFD, two commercial LFDs were evaluated by one EU partner and two non-EU partners that are experiencing H5N1 outbreaks in their countries. The data obtained was highly informative and will be of use to the diagnostic veterinarian and decision makers. (***Slomka, M.J., Coward, V.J., Pavlidis, T., Kerr, L., Mahmood, S., Banks, J., Brown, I.H. (2009) Problems with experimental approaches for evaluations of commercially available lateral flow devices for rapid penside detection of avian influenza viruses. 7th International Symposium on Avian influenza, Athens, GA, USA; 5-8 April.)***

- **Transfer of laboratory based Real-Time RT-PCR assays to portable instruments:** It was important to confirm that the recommended molecular diagnostic procedures for AI diagnosis could be transferred to portable PCR instruments that could be employed at the penside. This was achieved in collaboration with Italian firebrigade who possess such an instrument. Protocols for the identification of the M, H7 and H5 genes were successfully transferred to a mobile platform.

Objective 5 :*Tropism, pathogenesis and transmission in different avian species*

- Tropism: Analyses investigated the tropism of different influenza A isolates in cells from human, porcine and avian species. Particular attention was placed on their interaction with cells of the porcine innate immune system, to provide information on the potential behaviour of AI virus compared with mammalian influenza viruses in mammalian hosts. Data has been produced that describes the interaction of influenza viruses with porcine endothelial cells. In addition, comparisons have been made between avian and mammalian influenza viruses to determine their efficiency and capacity of inducing type I interferon production by porcine dendritic cells.

- Vertical transmission of H5 viruses in ducks: Experiments carried out on Mule and Muscovy ducks using a series of H5 LPAI virus have clearly concluded that vertical transmission of these viruses do not occur in ducks. In addition, it was shown that there was no persistence of H5 LPAI in embryonating Mule and Muscovy duck eggs inoculated with virus. This work (summarised in the table below) has provided important and reassuring information to the veterinary and the duck production professional community. ***(Picault J-P., Amelot M., Lamandé J., Oger A., Allée C., Le Bras M-O., Ogor K., Guillemoto C., Pierre I., Le Coq L., Bonnion D., Le Coq T., Le Moal L., Mangart J-M., Jestin V. (2008) Virus H5 FP : a priori pas de contamination verticale. Filières Avicoles, 706, 90-92)***

LP H5 viruses	Virus persistence in inoculated Muscovy duck eggs	Virus persistence in inoculated Mule duck eggs	Virus persistence on issues from inoculated Mule duck eggs	Vertical transmission on challenged Muscovy duck breeders	Vertical transmission on challenged Mule duck breeders
A/duck/France/05066b/05 (H5N1)	No	No	No	nd	nd
H5N2 A/chicken/France/03426a/03	No	No	nd	nd	nd
H5N2 A/duck/France/05056a/05	No	No	nd	nd	nd
H5N3 A/duck/France/02166/02	No	No	nd	nd	nd
H5N3 A/duck/France/05054a/05(*)	No	No	No	No	No

Nd: not determined

- **Ascertain the role of quail as a silent reservoir of infection:** Data on viral shedding and seroconversion over an extended period of time following experimental infection with HPAI and LPAI H7 subtype viruses in quails has been generated. In addition lateral spread from experimentally infected quails with H7 LPAI viruses to uninfected quails has been investigated along with the efficacy of vaccination on clinical protection and viral shedding.It has been clearly shown that in quails LPAI and HPAI influenza viruses used in the present experiments replicated primarily in the respiratory tract with high titres. Although no clinical signs were recognized, LPAI infected quails shed virus via the respiratory apparatus in high concentrations (up to $10^{5.2}$ EID_{50}/0.1 ml) and for long periods of time, increasing the possibility of infection spreading. Also, in quails antibody response were not easily detectable and interpretable due to low and inconsistent titres detected by HI (<1:64) (Fig 2) and the majority of animals started sero-conversion only after two-three weeks post infection. There was also an important

lack of agreement between positive birds by molecular diagnostic and virus isolation approaches and serological testing highlighting the need for a reliable serological test for quail. The data generated from the transmission experiments confirmed that LPAI could be readily transmitted between contact birds in a very short time (2 days post inoculation).

The vaccination studies indicated that a two-dose vaccination programme commencing at 4 weeks old, based on an inactivated, conventional vaccine registered for chickens, is not successful in preventing clinical signs and mortality and in suppressing shedding of viable virus among quails. The poor clinical and virological protection of vaccinated and infected quails is most probably due to the low antibody response induced by vaccination suggesting that the perpetuation of the infectious cycle may not be interrupted in quail by vaccination. For this reason vaccines and vaccination schemes must be re-evaluated in quails.

Overall the studies undertaken in quails during this project strongly suggest that quail farms should be strictly monitored both serologically and virologically for the presence of AIVs in order to prevent further virus transmission to other avian hosts. The studies also underline the complexities of managing outbreaks of AI when different species are involved and minor species should always be taken into consideration when designing monitoring programmes aiming at the eradication of AI considering the different responses to AI tests and infection. (***De Nardi, R., Toffan, A, Beato, M.S., Cilloni, F., Bertoldi, E., Savliato, A., Cattoli, G., Capua. I., Terregino, C. (2010) Comparative study on clinical behaviour and shedding levels in commercial Japanese quails and SPF chickens experimentally infected with low pathogenicity avian influenza viruses of the H7 subtype. In preparation.***)

- **Vaccination of native chickens:** In Southeast Asia the majority of local AI outbreaks have occurred in back yard flocks of native chickens. It was therefore considered important to determine whether these birds can be protected against infection by conventional vaccination. With this in mind two transmission experiments were carried out with H5N1 virus in vaccinated and unvaccinated native chickens. The results have shown that transmission of H5N1 virus was rapid and efficient in unvaccinated birds. Vaccination with a heterologous vaccine completely prevented transmission in vaccinated birds.**(*Poetri ON, Bouma, A, Murtini, S, Claassen, I, Koch, G., Soejoedono, RD, Stegmen, JA, vanBoven, M. (2009). An inactivated H5N2 vaccine reduces transmission of highly pathogenic H5N1 avian influenza among native chickens. Vaccine, 11(27), 2864-2869*)**

- **Quantitative transmission in experimental infected ducks and the effect of vaccination:** Domestic ducks play an important role in the epidemiology of H5N1 avian influenza. Although it is known that vaccines that have a high homology with the challenge virus are able to prevent infection in ducks, little is yet known about the ability of genetically more distant vaccines in preventing infection, disease, and transmission. The effect of a widely used H5N2 vaccine (A/Chicken/Mexico/232/94/CPA) on the transmission of H5N1 virus (A/Chicken/China/1204/04) in ducks was studied. The quantitative analyses show that despite the low level of homology between the virus and vaccine strain transmission was significantly reduced two weeks after a single or double vaccination. Mortality and disease rates were reduced markedly already one week after a single vaccination. ***(Van der Goot, JA, Van Boven, M, De Jong, MCM, Koch, G. (2008) Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine. Virology. 382(1):91-7)***

- **Effect of host age on the shedding and tissue dissemination of a HPAI H5N1 virus in infected Pekin ducks:** Pekin ducks in two age-matched groups 8 and 12-weeks-old were each infected with 10^6 EID50/0.1ml of HPAI A/turkey/Turkey/1/05 (H5N1, clade 2.2). Each day for five days, birds were monitored clinically, and cloacal and oropharyngeal swabs collected, before three birds from each group were selected randomly for post mortem examination. Tissue samples were collected for examination by real-time RT-PCR (RRT-PCR), histopathology and immunohistochemistry (IHC). Severe clinical signs, including incoordination and torticollis were observed in the 8-wo group resulting in 100% mortality by 4dpi. Mild clinical signs were observed in the 12-wo group with no mortality. RRT-PCR and IHC results demonstrated the systemic spread of H5N1 virus in birds of both age groups. Higher levels of virus shedding were detected in oropharyngeal swabs than in cloacal swabs, with similar levels of shedding detected in both age groups. Variations in level and temporal dissemination of virus within tissues of older ducks, and the presence of the virus in brain and heart were observed, which coincided with the appearance of clinical signs preceding death in younger birds. These results are consistent with reports of natural infections of wild waterfowl and poultry possibly indicating an age related association with dissemination and clinical outcome in ducks following infection with H5N1 HPAI virus. ***Löndt, B.Z., Nunez, A., Banks, J., Alexander, D.J., Russell, C., Richard- Löndt, A. C., Brown, I. H. (2009)The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin (Anas platyrhynchos) ducks infected experimentally. Influenza and Other Respiratory Viruses. 4, 17-25)***

- **Effect of H7N1 vaccination on highly pathogenic avian influenza H7N7 virus transmission in turkeys:** A study was undertaken to determine transmission of highly pathogenic avian influenza (HPAI) H7N7 virus in 12-week-old turkeys. Cloacal and tracheal swabs as well as serum samples were taken to monitor the infection both in inoculated and in susceptible contact turkeys, which were all either unvaccinated, vaccinated once or vaccinated twice with H7N1. Swabs were tested by real-time RT-PCR and serum samples with haemagglutination inhibition test (HI). Unvaccinated contact birds had a mean infectious period of 6.2 days, and an estimated transmission rate parameter of 1.26 per infectious bird per day. However, no virus shedding was found in inoculated vaccinated turkeys and thus it can be concluded that vaccination with H7N1 protected against challenge with HPAI H7N7 virus. ***(Bos ME, Nielen M, Koch G, Stegeman A, De Jong MC (2008). Effect of H7N1 vaccination on highly pathogenic avian influenza H7N7 virus transmission in turkeys. Vaccine. 26(50):6322-8.)***

- **Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches (*Struthio camelus)*:** In this study 177 serum samples from ostriches (*Struthio camelus*) infected experimentally with A/ostrich/South Africa/Middleton/2004 (H5N2) HPAI virus were collected. These samples were tested using the haemagglutination inhibition (HI) test, the agar-gel immunodiffusion (AGID) test and three ELISA kits. The HI test, with homologous antigen and including pre-treatment of sera with 10% chicken red blood cells, was considered as gold standard. Detectable specific antibodies appeared on day 7 post infection and persisted until the termination of the experiment. The relative sensitivity and specificity of the tests under evaluation and Cohen's K value were calculated. The data obtained will be of assistance to decision makers in drafting guidelines for the definition of the health status of ostriches and for trade purposes. (***Toffan, A, Olivier, A., Mancin, M., Tuttoilmondo, V., Facco, D., Capua, I., Terregino, C. (2010) Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches (Struthio camelus) Avian Pathology 39, 11-15).***

- **Validation of diagnostic tests for detection of avian influenza in vaccinated chickens.** Vaccination is an attractive tool for the prevention of outbreaks of highly pathogenic avian influenza in domestic birds. It is known, however, that under certain circumstances vaccination may fail to prevent infection, and that the detection of infection in vaccinated birds can be problematic. The characteristics of three serological tests (immunofluorescent antibody test (iIFAT), neuraminidase inhibition (NI) assay, and NS1 ELISA) that are able to differentiate infected from vaccinated animals were evaluated. To this end, data of H7N7 infection experiments were analyzed using Bayesian methods of inference. The results show that the N7 iIFAT and the NI assay have good sensitivities for detecting antibodies but substantially lower sensitivities for detecting infection. The NS1 ELISA has a low sensitivity for both detecting antibodies and infection. In addition, the estimated specificities of the N7 iIFAT and the NI assay were higher for the NS1 ELISA. The analysis also indicated a strong association between the duration of virus excretion of infected birds and the probability of developing antibodies. (***Van der Goot, JA, Engel, B., van de Water, S.G.P., Buist, W., De Jong, M.C.M, Koch, G., van Boven, M., Stegeman, A. (2010) Validation of diagnostic tests for detection of avian influenza in vaccinated chickens using Bayesian analysis. Vaccine in press)***

Section 2 – Workpackage detail for the entire project

WORKPAKAGE 1
Coordination

Partners involved: P1

Overall Objectives:

1. Liase with Commission, OIE, FAO
2. Liase with Partners and ensure deadlines are respected
3. Identify risks associated with non-delivery
4. Identify problem-solving options
5. Organise meetings
6. Develop website
7. Manage staff exchange

Deliverables: D1 to D7

Workpackage 1 overview.

The primary goal of **WP1** was the coordination of all the scientific project work and the facilitation of communication between the partners. The workpackage ensured (as far as possible) that all scientific project milestones and deliverables were reached properly and in-time. WP1 managed all of the projects administrative and financial activities, organized the annual meetings, identified sources of funding for staff exchange, developed and maintained the web-site.

Organization of the Annual Meetings

Four consortium meetings were organised during the lifetime of the project. Each meeting was successful in its own right allowing for a forum of discussion and exchange between the partners. Attendance at these meetings was good. The minutes of all the meetings are available in pdf format on the website (www.fluaid.eu)

Date	Location	Period covered	Description
4/4/2006	Cambridge, UK		Kick off
19/3/2007	Legnaro, Italy	01/2006 – 01/2007	First Annual
13/3/2008	Legnaro, Italy	02/2007 – 01/2008	Second Annual
18/03/2009	Legnaro, Italy	02/2008 – 03/2009	Third Annual
22/10/2009	Legnaro, Italy	03/2009 - 10/2009	Final

Amendment Request Process.

This was one of the most labour intensive activities under this work package that required frequent communication with the partners in order to make sure that the necessary information and documentation were correct and complete.

The amendment process involved:

1. removal of P6 (and its replacement with CLQP (P15)
2. removal of CSL (P9) and inclusion of FORSITE (P14).
3. affiliation change of P3 (CIDC to CVI)

The whole amendment process was coordinated under the constant guidance of the legal officer of the EU Commission and involved at least 8 different partners. The process was officially concluded on J**anuary 14, 2008.**

Consortium Agreement

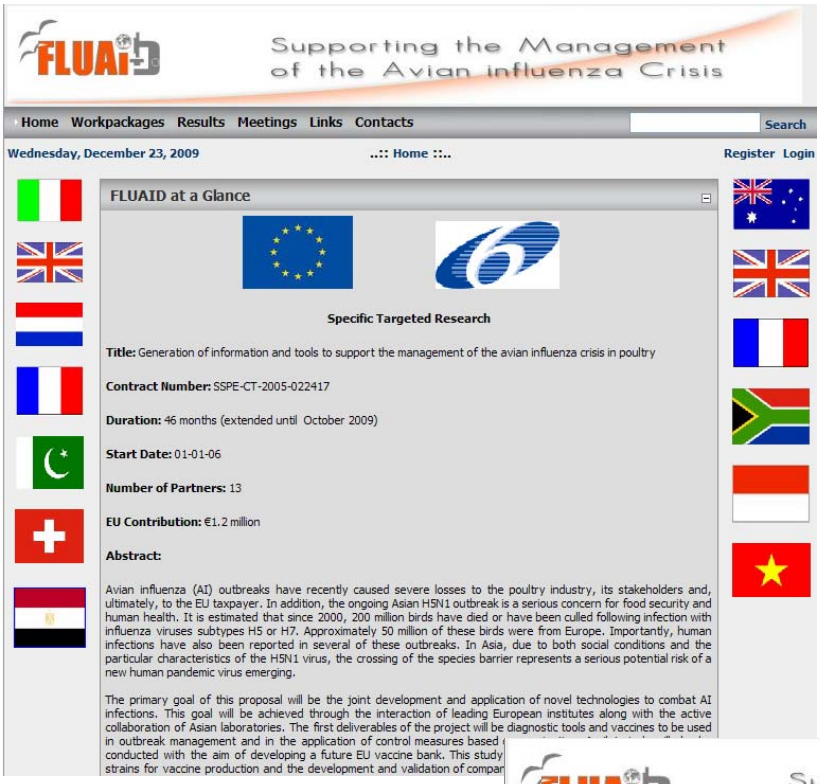
A consortium agreement was prepared under WP1. The final document was signed by all partners

Maintenance of FLUAID website

The website (www.fluaid.eu) for the FLUAID project was maintained and updated throughout the reporting period. The website provides information about the objectives of the project, the members of the consortium and the progress of the project.

Minutes of the annual meeting were uploaded on the website and a PDF version of all the presentations held during the meeting in order to serve for future reference to all the partners.

It was decided during the final FLUAID meeting that the website would be maintained for 18 months from the end of the project.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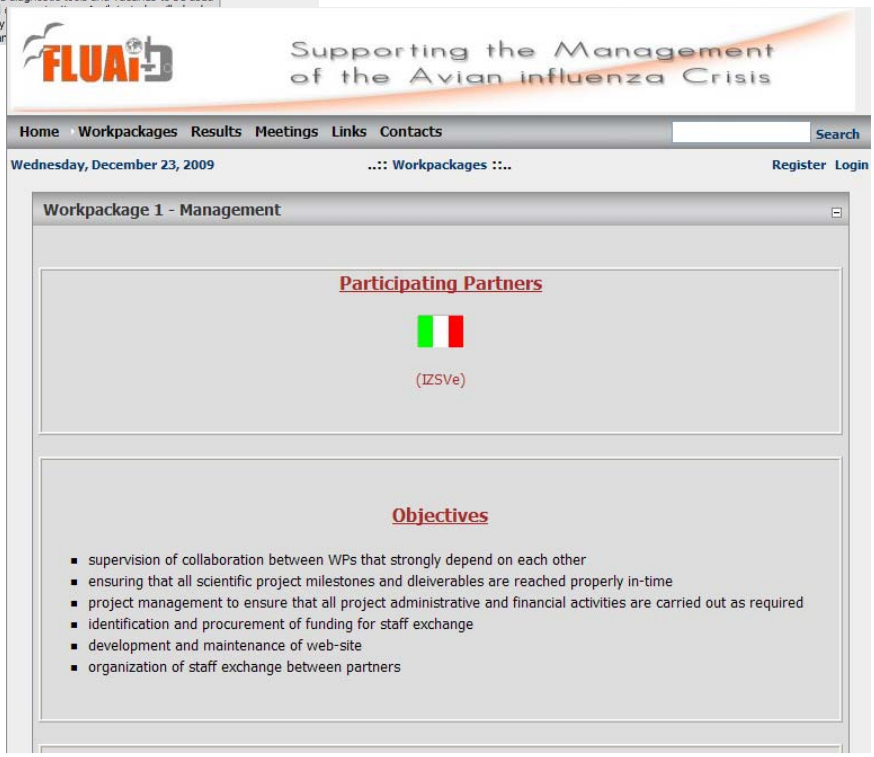
FLUAID at a Glance
Specific Targeted Research
Title: Generation of information and tools to support the management of the avian influenza crisis in poultry
Contract Number: SSPE-CT-2005-022417
Duration: 46 months (extended until October 2009)
Start Date: 01-01-06
Number of Partners: 13
EU Contribution: €1.2 million
Abstract:
Avian influenza (AI) outbreaks have recently caused severe losses to the poultry industry, its stakeholders and, ultimately, to the EU taxpayer. In addition, the ongoing Asian H5N1 outbreak is a serious concern for food security and human health. It is estimated that since 2000, 200 million birds have died or have been culled following infection with influenza viruses subtypes H5 or H7. Approximately 50 million of these birds were from Europe. Importantly, human infections have also been reported in several of these outbreaks. In Asia, due to both social conditions and the particular characteristics of the H5N1 virus, the crossing of the species barrier represents a serious potential risk of a new human pandemic virus emerging.
The primary goal of this proposal will be the joint development and application of novel technologies to combat AI infections. This goal will be achieved through the interaction of leading European institutes along with the active collaboration of Asian laboratories. The first deliverables of the project will be diagnostic tools and vaccines to be used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Workpackage 1 - Management
Participating Partners
(IZSVe)
Objectives
supervision of collaboration between WPs that strongly depend on each other
ensuring that all scientific project milestones and dleiverables are reached properly in-time
project management to ensure that all project administrative and financial activities are carried out as required
identification and procurement of funding for staff exchange
development and maintenance of web-site
organization of staff exchange between partners

Staff exchange:

The facilitation of staff exchange between partners was another remit of Workpackage 1. The project itself had no funds assigned specifically to staff exchange so suitable funds had to be identified from other sources.

In March 2006 a proposal called FLUTRAIN "*Training and technology transfer of Avian Influenza diagnostics and disease management skills*" was submitted to the EU Commission following Call FP6-2005-SSP-5B-INFLUENZA. P1, P2, P3, P13 and P14 are all part of the FLUTRAIN consortium. Funds from this project have been used for staff exchange between the partners of FLUAID.

(1) In December 2007 P1 sent an expert to the Central Laboratory for Veterinary Quality Control on Poultry production, cairo Egypt (P15) in order to assist them in the implementation of routine sequencing of circulating H5N1 viruses. The seven day mission included an assessment of the sequencing instrumentation of P15 and on-site staff training in sequencing procedures (see Annex 2 for detailed mission report).

(2) In 2008 (9/07/08 to 1/08/08) Dr Abdullah Abdulzaher Selim from the Central Laboratory for Veterinary Quality Control on Poultry production (P15) was hosted by P1 to assist in animal trials that were relevant to vaccination strategies in Egypt

(3) Dr Naila Siddique of the National Reference Lab For Poultry Diseases (NARC) Pakistan (P5) attended a training course entitled "Sequencing of Avian influenza genomes" held by P1 as part of the FLUTRAIN project from the 29th Sept to 3rd Oct 2008

(4) In January 2009 (26th-28th), four junior members of the Central Laboratory for Veterinary Quality Control on Poultry production (P15) attended a 3-day training course of Avian influenza given by P1 and sponsored by DG-SANCO. The four participants were then hosted by P1 for an extra 2 days of general laboratory

(5) Dr Zaheer Ahmed of the National Reference Lab For Poultry Diseases (NARC) Pakistan (P5) attended a training course organised by P1 and funded by FAO from 06/07/09 to 17/07/09.

Liason with OIE and FAO

The data from the activities of the project were made available to international organizations via their inclusion as activities carried out by the coordinator.

The ostrich sera ELISA work carried out in WP4 was requested by the EU and OIE and is now being cited as a recommended test for international trade purposes.

WORKPACKAGE 2
Vaccination

Partners involved: P1, P2, P3, P5, P10

Overall objectives:

Identify suitable candidates for an EU vaccine bank
Develop a companion diagnostic test based on anti –N detection systems
Generate data on consumer safety
Evaluate the occurrence of antigenic drift (WP2.3)

Deliverable: D8 to D11

Workpackage 2 Overview and impact

This workpackage generated data and instruments that can play an important role in the implementation of vaccination as a tool to support the eradication of AI.

Important steps toward the identification of prototype strains for an EU vaccine bank have been made. Three companion diagnostic kits, one of which is already commercially available, have been produced and evaluated by consortium partners.

One of the major concerns of the implementation of a vaccination programme for AI is the possibility of viral antigenic drift occurring. Using valuable data and material gathered during the Italian AI epidemics (1999-2000, 2003) the occurrence of antigenic drift in the presences of a DIVA strategy has been evaluated. The data generated by FLUAID should assist EU decision makers in the implementation of future vaccine programmes

Finally, important information on consumer safety has also been generated that will be of primary interest to DG-SANCO and the poultry industry .

Workpackage 2.1

Partners: P1, P2

Deliverable: D8

Generation of preliminary data, mapping the antigenic characteristics of H5 and H7 isolates by cross HI in order to identify prototype strains for an EU vaccine bank.

Experimental design and key results

In order to identify prototype H5 and H7 subtype strains for an EU vaccine bank viruses were selected on the basis of their epidemiological relevance, country and year of isolation.

H5 viruses. Twenty-seven isolates of H5 subtype obtained between 1980 and 2006 were used in this study. Twenty-one Highly Pathogenic AI (HPAI) H5N1 viruses representative of distinct genetic clades, namely 0, 1, 2.1, 2.2, 2.3, 2.5 clades (WHO, 2008), collected in Asia, Europe, Africa and Middle East between 2004 and 2006, were included in the antigenic study. Other LPAI and HPAI H5 viruses genetically unrelated to the 2004-2006 HPAI H5N1 viruses were included in this study as reference strains.

H7 viruses. Twenty-six H7 isolates were used in this study. In particular representative H7N3 strains isolated during the recent Italian epidemics (2002-2004) and H7 strains obtained from the wild bird surveillance programme between 2004 and 2007 in Italy, were selected according to the topology of the HA gene. Other H7 isolates obtained from European and non European countries were included as unrelated viruses.

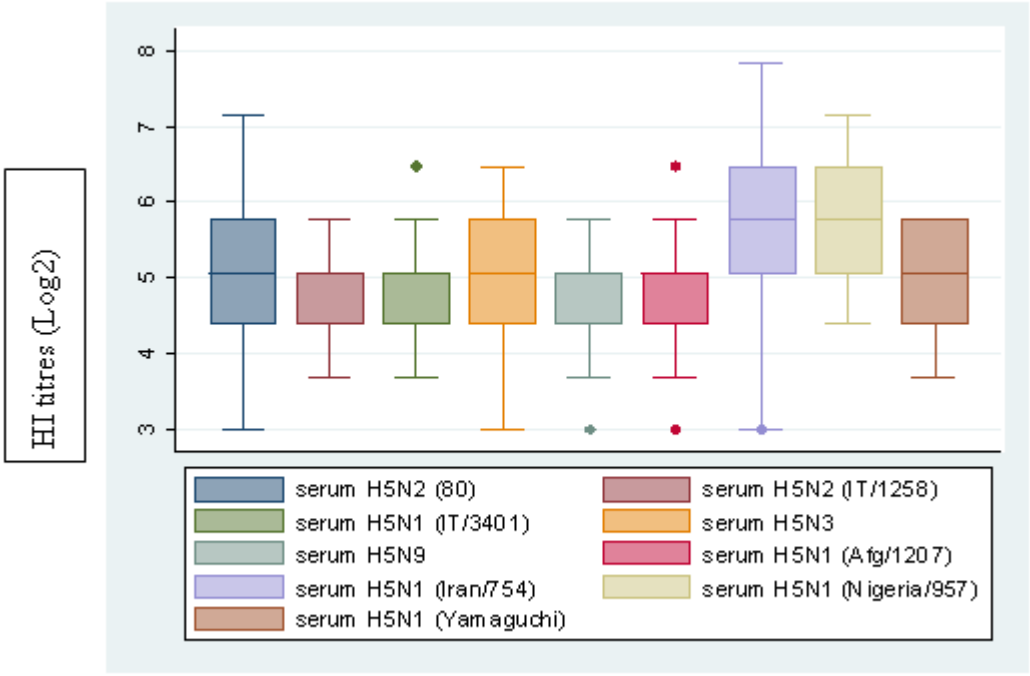
The aminoacid sequences of the HA gene of the H7 and H5 viruses were phylogenetically analysed in order to include in this study strains representative of different genetic clusters. Based on this analysis nine H5 isolates and eleven H7 isolates were selected to produce hyper-immune sera in SPF chickens.. This sera was then used in cross haemagglutination inhibition (HI) tests from which the antigenic relatedness between isolates was evaluated as described using the Archetti and Horsfall formula (Archetti & Horsfall, 1950). All the data generated was analysed statistically

The results indicated that for H5 viruses, two LPAI isolates obtained from waterfowl, A/mallard/Italy/3401/2005(H5N1) and A/duck/Italy/775/2004(H5N3) showed the broadest cross reactivity. In the analyses of one-way HI tests antisera to the H5N1 HPAI strains Iran/754 and Nigeria/957 also showed a high cross reactivity with all strains tested, suggesting that these

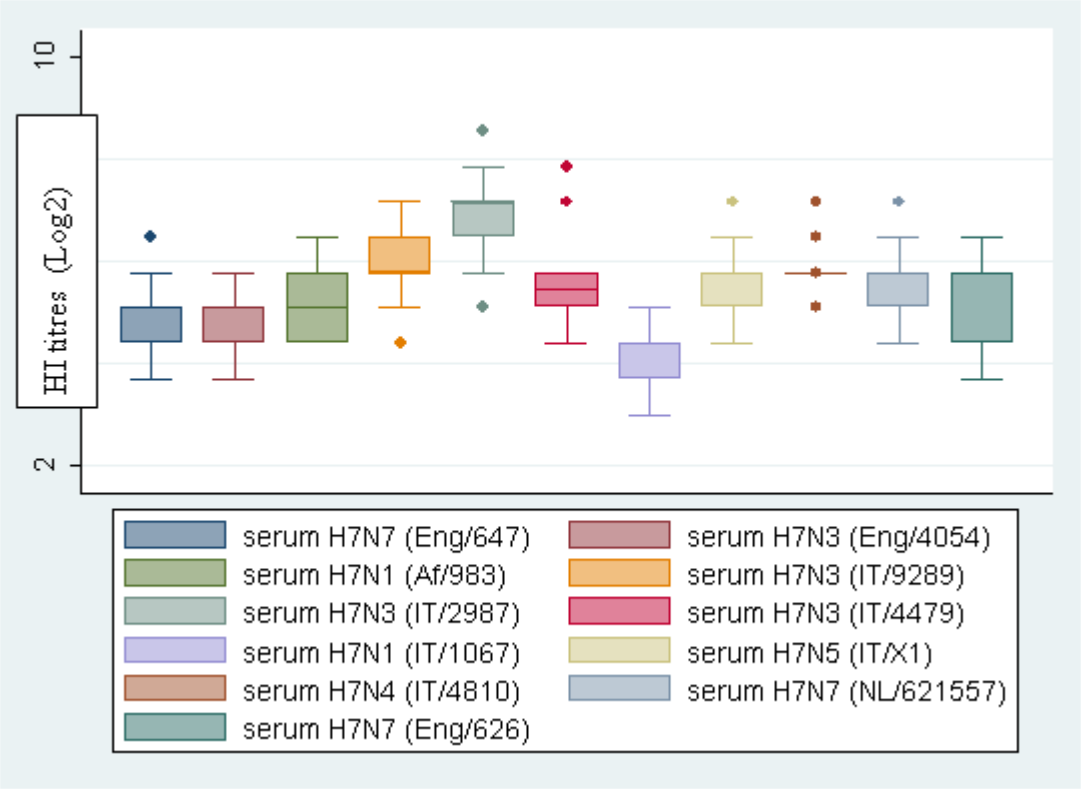
viruses also have HA antigenic characteristics suitable as seed viruses for the generation of engineered vaccines.

The study of the H7 virus subtypes indicates that the H7N3 virus A/Turkey/Italy/2987/2003 shared the highest R (an indicator of antigenic relatedness) values with all viruses tested. The statistical analysis of the one-way HI titres confirmed this finding with a high level of cross reactivity with the antiserum to virus A/Turkey/Italy/2987/2003.

The box plot below shows the distribution of titre values from cross-HI data obtained testing all H5 viruses against all anti H5 sera. Each box indicates the degree of dispersion in the data, the line inside the box is the median, the points above and below the box are the outliers.



This other box plot below shows the distribution of titre values from cross-HI data obtained testing all H7 viruses against all anti H7 sera. Each box indicates the degree of dispersion in the data, the line inside the box is the median, the points above and below the box are the outliers.



Workpackage 2.1

Partner: P1

Deliverable: D8

Generation of reassortant viruses of H5 and H7 subtypes with rare N groups (N5/N8)

Experimental design and key results

In order to generate a reassortant H7 virus with a rare N group to be used as a vaccine as part of a DIVA strategy, two viruses, A/Chicken/Italy/9289/02-H7N3 subtype (LPAI) and A/duck/Alberta/76-H12N5 subtype were selected. The viruses were mixed in proportion of 1:1 and inoculated in the allantoic cavity of 9-11 day old specific pathogen free (SPF) embryonated chicken eggs. The allantoic fluid from the dead embryos, incubated at 37°C, was harvested and incubated for 30 minutes with chicken antiserum against the H12N5 subtype virus in a 1:1 proportion at room temperature prior to being inoculated in eggs. The allantoic fluid was again harvested and tested for the evidence of hemagglutination and then typed using the hemagglutination inhibition (HI) test. Plaque assay was used to screen the viral suspension and identify reassortant viruses.

The full characterization of the two reassortant viruses was obtained by sequencing the eight genes and comparing the sequences with those of the two parental viruses.

Plaques (n=70) were picked and each was diluted in 250 µl of PBS with antibiotics and inoculated in embryonated eggs. From the dead embryos the allantoic fluid was harvested and processed for RT-PCR analysis in order to detect the N3 gene. All positive clones were discarded. This screening yielded two viral clones which were negative to N3 and were subsequently typed by means of HI and neuraminidase inhibition (NI) tests to confirm the identification of H7N5 viruses. The two reassortants were purified by plaque assay.
Two reassortants of the H7N5 subtype (A/Av-R1/Italy/05 and A/Av-R2/Italy/05) were generated by this method. Sequence analysis revealed that the A/Av-R1/Italy/05 acquired the HA and NP gene from the H7N3 virus and the NS, PB1, PB2, M, PA and N genes from the H12N5 virus. The A/Av-R2/Italy/05 acquired the HA, NP and PB2 genes from the H7N3 virus and the NS, PB1, M, PA and N genes from the H12N5 virus.

The method described above was used to generate reassortant viruses of the H5N5 subtype. The parental viruses selected were A/duck/Alberta/76-H12N5 and A/duck/Italy/775/04- H5N3.
The two parental viruses were mixed and inoculated in the allantoic cavity of 9-11 day old specific pathogen free (SPF) embryonated chicken eggs. The allantoic fluid from the dead embryos,

incubated at 37°C, was harvested and incubated for 30 minutes with chicken antiserum against H12N5 virus (A/duck/Alberta/76) in a proportion of 1:1 at room temperature prior to being inoculated in eggs, to select a viral suspension of the H5 subtype.
Viral suspensions were screened using a specific RT- PCR to detect the N5 subtype. Four positive allantoic fluids for N5 were identified and were screened by plaque assay. Plaques were picked and inoculated into 9- to 11 day old SPF chicken eggs. Characterisation of the N subtype is ongoing.

Workpackage: 2.1

Partner: P1

Deliverable: D8

Evaluation of the protective capacities of a reassortant H7N5 vaccine *in vivo* as part of a DIVA strategy

Experimental design and key results

With the aim of evaluating the protective capacities of an H7N5 reassortant vaccine SPF chickens were challenged with an HPAI H7N1 subtype (A/Turkey/Italy/4580/99), and LPAI H7N3 subtype (A/Turkey/Italy/2962/02) viruses at two different doses e.g. 10^7 and 10^5 EID_{50}/0.1 ml oronasally. Vaccine (H7N5 - A/Av-R1/Italy/05) was administered sub-cutaneously at two weeks of age (0.5 ml/bird) and boosted (1ml/bird) three weeks later, at five weeks of age, by the same route. Blood samples were obtained from each bird before each vaccination and before challenge. Chickens were inspected clinically twice a day. Tracheal and cloacal swabs were collected on days 3, 5, 7 and 10 p.i.. On day 7, 14 and 21 blood samples were collected from vaccinated and unvaccinated birds. The efficacy of the vaccine was evaluated by assessing viral shedding both by virus isolation and RRT-PCR. Serological investigation was performed by haemagglutination inhibition (HI) test according to EU Directive 05/96/EU.
To determine whether this vaccine could be used as part of a DIVA strategy, the iFAT test for the detection of N1 and N3 were used as previously described (Cattoli *et al.*, 2003; 2006).

No clinical signs were observed in any of the birds belonging to the vaccinated group infected with HPAI and LPAI viruses at both doses. Mild clinical signs were observed in the unvaccinated birds infected with LPAI strain with doses of 10^5 and 10^7 EID_{50}. They showed depression and mild respiratory signs starting on day 3 pi. All birds challenged with 10^5 and 10^7 EID_{50} of HPAI strain exhibited severe clinical signs and died within the first 48 hours pi. Infection was achieved in the unvaccinated challenged birds with 10^5 and 10^7 EID_{50}/0.1ml with the low path virus. The vaccinated birds challenged with the HPAI isolate at both doses, 10^5 and 10^7 EID_{50}/0.1ml, did not shed detectable levels of virus as virus isolation from tracheal and cloacal swabs collected throughout the period of the experiment was negative. Infection was achieved in birds vaccinated and challenged with LPAI strain at both doses. Analysis of the virological data obtained from tracheal and cloacal swabs of the vaccinated and control group infected with 10^7 EID_{50}/0.1ml of the LPAI strain showed a reduction in the number of animals shedding viable virus. The same pattern was observed when the vaccinated and control groups challenged with the lower dose (10^5) were compared. In addition a reduction of the duration of shedding in this group was alos observed.

Sera from vaccinated and unvaccinated infected birds with LPAI. The iIFA test (IFAT) revealed N3 antibodies in 3 out of ten vaccinated birds receiving the lower dose of LPAI H7N3 on day 14 p.i. and seven out of ten birds on day 21 p.i.. In unvaccinated chickens infected with 10^7 EID_{50} of LPAI H7N3, the iIFAT was positive in two out of ten on day 7 p.i. and nine out of ten on day 14 and 21 p.i..

Sera from vaccinated infected birds with HPAI. The iIFA test (IFAT) revealed N1 antibodies in two out of ten vaccinated birds receiving the higher dose of HPAI H7N1 on day 7 p.i. ,eight out of ten birds on day 14 p.i. and nine out of ten on day 21 p.i.. Two out of ten vaccinated and infected birds, with the lower dose, were positive on day 7 p.i. and 3 out of ten on day 14 and 21 p.i. respectively.
Comparing the vaccinated challenged and control group infected with both doses a greater number of positive animals was observed with 10^7 EID_{50} than 10^5 EID_{50}/0.1 ml infecting dose.

In summary, the H7N5 vaccine totally suppresses shedding after challenge with H7N1 HPAI virus of either high (10^7 EID_{50}/0.1 ml) or low (10^5 EID_{50}/0.1 ml) doses as no detectable levels of virus were isolated from either tracheal and cloacal swabs even though, from the analysis of the serological data it appears that infection was achieved in the vaccinated groups challenged with HPAI. In contrast active infection was achieved in both naïve and vaccinated challenged birds with LPAI isolate.
Virological results obtained from the vaccinated challenged groups with LPAI virus are in agreement with serological data showing the presence of anti-N3 antibodies at each day of sampling. However, a reduction of the number of positive samples in the vaccinated group compared with the unvaccinated control one was detected.
The different results obtained from the vaccination challenge study with HPAI virus and LPAI virus indicate that for the particular HPAI strain used, a higher viral dose is necessary to achieve an active infection.

In conclusion, the H7N5 virus is a strong vaccine candidate because of the cross-protection immunity conferred by the H. In addition it possesses an N-type that is rarely found in nature and so there is a low possibility of interference when using a DIVA strategy from anti-N5 antibodies derived from infection with N5 subtype field isolates.

Workpackage: 2.1

Partner: P1, P3, P10

Deliverable: D9

Development and validation of anti-neuraminidase antibody detection ELISAs for N1-N2-N3 and N7 in different avian species

Experimental design and key results

The aim of this WP was to produce monoclonal antibodies against 4 NA types, N2, N3, N5 and N7. Purified H5N2, H7N3, H6N5 and H7N7 were used for immunization. Mouse monoclonal antibodies were produced by standard protocols. Hybridomas were screened in ELISA using microtitre plates that were coated with purified influenza viruses of different HA and NA subtypes. For this 17 viruses were grown and purified. Hybridomas were selected that bound to viruses with the same NA type as the virus used for immunization but not to viruses with heterologous NA subtypes. All hybridomas that reacted with all viruses or with viruses with different NA type were disregarded as having the wrong specificity. In practice the whole procedure turned out to be more complex and laborious than previously anticipated.

To obtain Mabs against N2, mice were immunised with A/MallardNetherlands/2/05 H5N2. Two fusions were performed. After the first fusion hybridomas were screened in an ELISA using microtitre plates coated with A/Chicken/Netherlands/2/86 (H3N2) and normal allantoic fluid as negative control. Based on a positive ELISA signal with H3N2, 69 hybridomas were selected, grown and frozen down. The supernatants of the 69 hybridomas were tested in ELISA using A/Chicken/Netherlands/2/86 (H3N2), A/Mallard/Netherlands/2/05 (H5N2), A/Turkey/Mass/85 (H6N2) and A/Shearwater/Australia/2576/02 (H15N6) and normal allantoic fluid as antigens. None of the hybridomas reacted with N2 viruses only. However, 2 hybridomas did seem to be specific for H5 subtype viruses or specific for an epitope present only on the N2 of the H5 virus but not of the H6 virus and 3 hybridomas were possibly directed against other viral proteins like the M, NP or conserved epitopes of HA of NA. Hybridomas generated after a second fusion reaction were screened on SF21 insect cells that were infected with N2 recombinant baculovirus obtained from P1. Twenty seven clones reacted weakly in an immunofluorescence test while an additional 4 reacted strongly. In total 6 clones were selected for further subcloning. One of the 6 selected clones had neuraminidase inhibiting activity in a conventional neuraminidase inhibition assay. Using recombinant N2, two monoclonal antibodies inhibited neuraminidase activity in a ELISA based assay. The resulting Mab was provided to P10 for the development of a companion anti-N2 ELISA

Mice immunized with H7N3 were screened on SF 21 cells infected with N3 recombinant baculovirus. From this fusion 6 clones were selected, subcloned and frozen. Theses clones bound to recombinant N3. The resulting Mab was provided to P10 for the development of a companion anti-N3 ELISA

To obtain Mabs against N7, 4 mice were immunised with A/Swan/Netherlands/06003448/06 (H7N7). Unfortunately, although the virus was of low pathogenicity for chickens one of the mice died shortly after immunisation. The lung of the dead mouse were positive in the M gene RT-PCR which suggests that the mice died because of the influenza infection. Another mouse died after the 3rd immunisation. Again lung suspension of reacted positive in the M RT-PCR. The remaining mice were used for a fusion but none of the hybridromas that were selected because of positive ELISA using H7N7 virus as antigen stained insect cells infected with N7 recombinant baculovirus in an indirect immunofluorescence test. Selection for MAbs against N5 was hampered by the lack of N5 recombinant baculovirus.

Since the attempts to produce MAbs were only successful for N2 and N3, it was decided to switch to the use phage libraries of lama VHH genes fragments (Frenken et al, 2000). Lamas were immunized with a mixture of influenza viruses and peripheral blood was used to prepare phage libraries of lama VHH fragments. Purified influenza viruses and transiently expressed NAs of all subtypes were used for panning of phages carrying the appropriated antibody specificity. Currently, we believe to have enriched phages with antibody specificities against all NA subtypes. For N2 lama VHH fragments were cloned and expressed in yeast and E. coli. All lama VHH fragments were sequenced. Based on the VHH sequences we recognize 12 different groups that may reveal recognition of 12 different N2 epitopes.

Ref: G. Frenken, R.H. van der Linden, P.W. Hermans, J.W. Bos, R.C. Ruuls, B. de Geus and C.T. Verrips, Isolation of antigen specific llama VHH antibody fragments and their high level secretion by *Saccharomyces cerevisiae*, *J. Biotechnol.* **78** (2000), p. 11

Validation of companion kits

Anti-N1

The sera tested were from (A) chickens vaccinated with an H7N5 subtype virus and challenged with an H7N1 subtype virus (n=33); (B) turkeys (n=13) and chickens (n=8) negative for type A influenza virus; (C) unvaccinated turkeys infected with H7N1 subtype virus (n=41); (D) unvaccinated turkeys infected with H7N3 subtype virus (n=40). Each sera was tested in duplicate. The results obtained from the ID Screen® ELISA were compared to the indirect immunofluorescence antibody assay (iIFA) which was taken as the gold standard. Cohen's Kappa statistic (K) value was calculated to assess the agreement between the tests.

The overall sensitivity of the ID Screen® ELISA test as compared to the gold standard iIFA was 93.0% (CI 95% 85.0-98.0), 91% for chickens (CI 95% 76.0-98.0) and 95% for turkeys (CI 95% 83.0-99.0). The overall specificity of the ID Screen® ELISA test as compared to the gold standard iIFA was 100% (CI 95% 94.0-100.0), 100% for chickens (CI 95% 63.0-100.0) and 100% for turkeys (CI 95% 93.0-100.0). The K value was calculated as 0.9264 indicating “excellent agreement” between the two tests according to Landis & Koch.

Anti-N2

Sera tested:

A total of 82 sera from chickens were tested.

Analytical sensitivity and specificity were evaluated by testing 43 hyperimmune SPF chicken:
24 sera (16 positive sera for different AI subtypes, namely H1N1, H2N3, H3N8, H4N8, H5N1, H5N2, H5N3, H5N9, H6N2, H7N1, H7N3, H7N4, H8N4, H9N2, H9N7, H10N1, H10N8, H11N6, H11N9, H12N5, H13N6, H14N5, H15N9, H16N3)
2 positive sera for Newcastle Disease (APMV1)
8 sera positive for APMVs subtype 2-9
1 positive serum for Egg Drop Syndrome '76 (EDS 76)
7 positive sera for different serotype of Infectious bronchitis virus (IBV)
1 SPF negative chicken serum

Diagnostic sensitivity and specificity were estimated by testing a total of 42 serum samples from chickens expected to be positive. The sample size was small due to the lack of availability of field sera from birds infected with N2 subtype virus.

HI / ID-Screen	POSITIVE	NEGATIVE	Total
POSITIVE	42	0	42
NEGATIVE	0	0	0
Total	42	0	0

Sensitivity: 100%
Specificity: 100 %

NB: The sample size was very small so the results should be considered as indicative only

Anti-N3

Sera tested:

A total of 277 sera from 2 different avian species (chicken and turkey) were tested.

Analytical sensitivity and specificity were evaluated by testing 43 hyperimmune SPF chicken:
24 sera (16 positive sera for different AI subtypes, namely H1N1, H2N3, H3N8, H4N8, H5N1, H5N2, H5N3, H5N9, H6N2, H7N1, H7N3, H7N4, H8N4, H9N2, H9N7, H10N1, H10N8, H11N6, H11N9, H12N5, H13N6, H14N5, H15N9, H16N3)
2 positive sera for Newcastle Disease (APMV1)
8 sera positive for APMVs subtype 2-9
1 positive serum for Egg Drop Syndrome '76 (EDS 76)
7 positive sera for different serotype of Infectious bronchitis virus (IBV)
1 SPF negative chicken serum

Diagnostic sensitivity and specificity were estimated by testing a total of 239 serum samples. In detail 106 chicken sera (54 expected positive for N3 and 52 expected negative) and 128 turkey sera (104 expected positive for N3 and 24 expected negative) were analyzed.
Sera used belonged to the repository of the OIE/FAO Reference Laboratory for Avian Influenza, Legnaro (PD), Italy.

Interpretation of the results:
According to the 2006/437/CE Commission Decision and OIE manual sera that give HI result lower than 1:16 were considered negative.
All tested sera were divided into two categories: positive samples (positive and doubtful) and non-positive samples. Sensitivity (Se) and specificity (Sp) were calculated using the HI results as gold standard.

Results
A summary of the results are showed in table 1.
The ID Screen® Influenza N3 Antibody competition test results correlated with the HI data with a sensitivity of 100% and a specificity of 93.9%.

Table 1: Correlation between HI test and ID Screen® Influenza N3 Antibody competition

HI / ID-Screen	POSITIVE	NEGATIVE	Total
POSITIVE	161	7	168
NEGATIVE	0	109	109
Total	161	116	277

Conclusion
The ID Screen® Influenza N3 Antibody competition results test obtained a 100% agreement with hyperimmune SPF chicken sera.
The ID Screen® Influenza N3 Antibody competition results correlated very well with the expected results. In particular no false negative were observed giving sensitivity of 100%. Some false positive results (compared to the HI test) were observed (specificity of 93.9%) but this is probably due to the different diagnostic targets of the two tests analyzed (i.e. antibodies against HA vs antibodies against N3). To confirm this data other tests (i..e. immunofluorescence against N3 or ELISA against NP) could be tested and compared with the ID Screen® Influenza N3 Antibody competition test.
In conclusion, due to the results obtained, the ID Screen® Influenza N3 Antibody competition appears to be suitable tool for the detection of chicken and turkey sera that are positive for avian influenza viruses of the N3 subtype.

Workpackage 2.1

Partner: P1

Deliverable: D10

Assessment of viral colonization of muscles following experimental challenge with HP and LP H7N1 viruses in vaccinated and unvaccinated turkeys

Experimental design and key results

It is now generally accepted that in most poultry species, highly pathogenic avian influenza (HPAI) viruses cause viraemia and systemic infection with virus replication in vital organs and in muscle tissue. In contrast, there is little information about the ability of low pathogenicity avian influenza (LPAI) viruses to replicate and persist in meat. The aim of this study was to determine the ability of LPAI and HPAI viruses of the H7 subtype to colonize turkey muscle tissue following experimental infection. In addition the efficacy of vaccination in preventing viraemia and thus meat colonisation in turkeys was investigated.
Four groups of 15 (6-to-7 weeks old) turkeys were used. Two of these groups were immunized subcutaneously (at 19 and 40 days of age) with 0.5 ml of a commercially available inactivated bivalent vaccine (H7N1/H5N9). The other two groups were left unvaccinated. One group of vaccinated birds and one group of unvaccinated birds were infected intranasally with 10^6 EID50/100µL of A/turkey/Italy/4580/99 (HPAI). The remaining groups were infected with the same dose of H7N1 A/turkey/Italy/3675/99 (LPAI). On day 1, 2, 3, 4 and 5 blood (with anticoagulant) was collected from each bird and the presence of viraemia was evaluated by Real Time Reverse Transcriptase PCR (RRT-PCR). Three birds from each group presenting viraemia were sacrificed on the day of testing. When blood samples yielded negative results three turkeys were sacrificed randomly. When dead birds were found, organs were collected on the day of death and breast (deep and superficial) and thigh muscles were collected. Samples were analyzed by RRT-PCR and by virus isolation in 9-11 embryonated specific pathogen free (SPF) chicken eggs according to EU Directive 92/40 (1).
All unvaccinated HPAI infected turkeys showed severe clinical signs starting from day 1 post infection with 100% mortality by day 4 post infection. Virus was detected by RRT-PCR and virus isolation in all of the blood and muscle samples collected from these birds. Vaccinated turkeys challenged with HPAI did not show any clinical signs and none of these birds were found positive for viraemia by RRT-PCR. In addition the muscle samples from these birds yielded negative results both by RRT-PCR and virus isolation.

Unvaccinated turkeys infected with LPAI showed depression and mild conjunctivitis on days 3 and 4 post infection. On day 2 post infection blood samples from two turkeys were positive for viraemia by RRT-PCR. This data was confirmed by virus isolation in only one of the birds. No virus was recovered in muscle samples from any of the birds infected with LPAI. On the basis of

the previous results it was decided not to perform the challenge of vaccinated turkeys with LPAI virus.

Conclusion
Data obtained confirms the ability of HPAI viruses to cause a generalized infection together with colonization of muscle tissue as already reported in other studies.
The finding of main interest from this study was the ability of an H7N1 LPAI virus to be detected from the blood. This is in contrast with the general belief that LPAI is a localised infection without viral replication in blood. However, despite the viraemia detected, no virus was isolated from the muscle of the infected birds. These results are in partial agreement with a previous reports which describe the isolation of an H9N2 from imported chicken meat and the detection of virus in blood following experimental infection. This suggests that the presence of viable LPAI virus in meat may be strain dependant and that, therefore, further investigations are necessary before any general conclusions are drawn.
The absence of detectable virus in the blood and in meat of vaccinated turkeys infected with both LPAI and HPAI viruses underlines the effectiveness of vaccination in preventing viraemia and meat colonisation.

To determine whether poultry meat infected with a HPAI virus could be spread to other birds by swill-feeding.

Experimental design and key results

Two groups of seven-week-old SPF chickens and two groups of turkeys of the same age were used. One group of chickens and one group of turkeys were vaccinated with a commercially available inactivated bivalent (H7N1/H5N9) vaccine. The other groups were left unvaccinated.
At 50 days of age, experimental groups of birds were fed with a meat homogenate prepared as described below and were observed daily for clinical signs. Tracheal and cloacal swabs were collected on days 3, 5 and 7 after administration of the homogenate. Serum samples were also collected on 7, 14 and 21 days post-administration of the homogenate.
The infective meat homogenate was prepared with meat samples that were positive by virus isolation (VI) collected from unvaccinated turkeys infected with the HPAI virus A/turkey/Italy/4580/99. Muscles that yielded the highest viral titres were homogenised together with sterile quartz sand. The meat homogenate was further titrated in embryonated SPF eggs to determine the infectious dose. Two grams of the infective meat homogenate titrating $10^{3.6}$ EID_{50}/0.1g were administered to each animals by the oral route.

Results
No clinical signs were observed in any group fed with infective meat. Neither the vaccinated nor unvaccinated birds showed increased antibody levels on day 21 post infectious meat administration. All tracheal and cloacal swabs collected throughout the experiment yielded negative results.

Conclusion
Feeding meat obtained from experimentally infected turkeys to vaccinated and unvaccinated poults

did not result in infection of either group. Thus H7N1 LP and HPAI viruses cannot be transmitted by swill feeding practices under experimental conditions-.

Workpackage: 2.3

Partner: P1

Deliverable: D11

Evaluation of antigenic drift and genetic variability in H7 isolates of the Eurasian lineage.

Experimental design and key results

Background

During 2002-2004 Italy experienced outbreaks of AI involving an H7N3 subtype influenza A virus of low pathogenicity. The H7N3 circulated in poultry from October to December 2002 in absence of vaccination. From January 2003 the virus continued to spread in unvaccinated and vaccinated farms with the eradication of the infection 8 months later. About one year after, in 2004, the H7N3 subtype re-emerged in vaccinated turkey farms. The vaccination programme, designed was based on a "DIVA" strategy and was carried out using an AI inactivated heterologous vaccine (strain H7N1), started at the end of December 2002.

Forty-one H7N3 isolates were analyzed by cross-HI test: 16 belonging to the 2002 epidemic (no vaccination campaign in place), 14 belonging to the 2003 epidemic, of which 7 isolated from non vaccinated birds, and 11 viruses belonging to the 2004 epidemic all but one from vaccinated farms. In addition, three viruses isolated in different periods were included e.g. H7N3 A/mallard/Italy/33/2001 and two strains isolated in 2007.

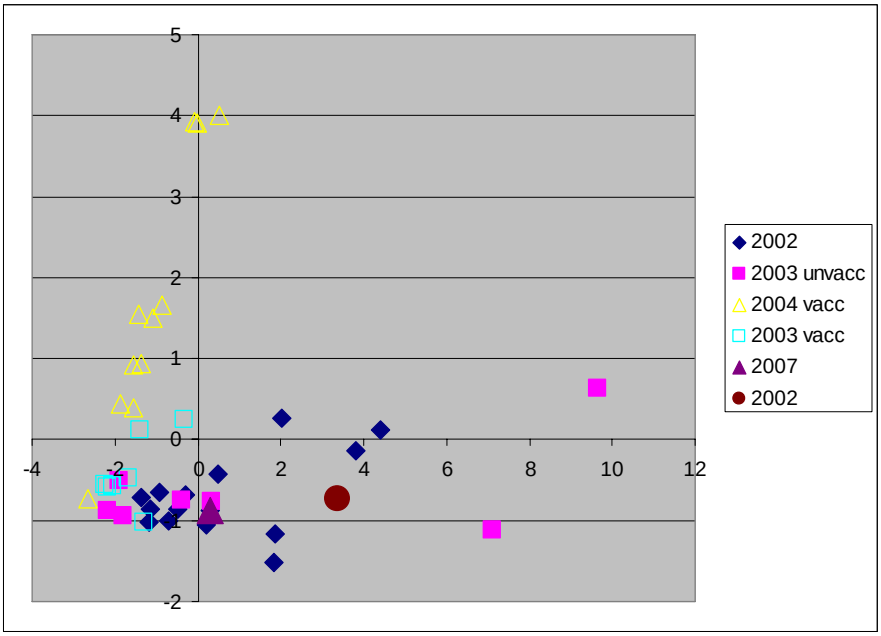
The sequence of HA1 protein of all isolates was generated and analysed phylogenetically.

Antigenic analysis was performed by cross HI and data were analysed statistically using the principal component analysis (PCA).

Comparison were made between isolates from 2002 and 2004 by analysing the HI values obtained by testing the same serum with all viruses isolated in 2002 and all viruses isolated in

2004.The results highlighted the differences in cross reactivity of the same serum against the two groups of viruses (isolated in 2002 and 2004).

PCA analysis of the HI data generated



Looking at the figure above derived form the PCA analysis carried out on HI data it can ba speculate that:

1. From 2002 to 2004 selection has acted on the circulating viruses so that the later viruses have a lower titre against sera made for the ancestral virus and in less variance in the titres against that ancestral virus.

2. 2002 blue diamond viruses indicate a natural evolution of antigenicity in "all directions" since there is no unidirectional push to evolve in one direction over another

3. In 2003 deviant types were found on vaccinated farms (viruses isolated in 2003). Probably they are less fit in terms of their HA-activity. A similar random evolution indicates a similar dispersion pattern a year later 2003 (unvaccinated pink squares)

4. In 2004 this lead to the emergence of new, fitter viruses that still had a titre with the sera against the ancestral virus but are deviant. When vaccination arrives, viruses are pushed in one "small antigenic pool" (light blue squares). Here, from this pool, viruses try to find the antigenic make-up that will allow the viral population to escape the vaccine, thus driving the viral population away from the small antigenic pool into a mono-directional more fit variant (yellow triangles).

WORKPACKAGE 3
Diagnostics and novel technologies

Partners involved: P2, P5, P13, P14, P15

Overall objectives:

Develop rapid antigen detection pen-side tests for H5/H7 AIV
Develop rapid pen-side Real-Time RT-PCR for H5/H7 and generic AI detection

Deliverables: D12 to D16

Workpackage 3 Overview and impact

The primary goal of WP3 was the development and evaluation of a Lateral Flow Devices (LFD) for the penside diagnosis of AI. P14 was the main participant responsible for this task which they performed as required. Unfortunately, following a preliminary evaluation by consortium members, the LFD that was produced by P14 proved to be unsuitable for it purpose due to specificity (false positive) issues. It was impossible to resolve these issues within the time frame of the project so alternative analyses of other commercially available LFDs was undertaken. This analyses produced some valuable data but more importantly it resulted in the setting up of good collaboration between some of the EU and non-EU partners of the project.

The transfer of Real-time RT-PCR protocols for the diagnosis of AI to penside instruments was also the remit of WP3 and this has been obtained.

The data and deliverables of this workpackage will be of interest to poultry veterinarians and professionals involved in AI management and control.

Workpackage: 3.1

Partners: P2, P5, P13, P14, P15

Deliverable: D12 to D16

Development of pen-side tests for antigen detection

Experimental design and key results

A prototype LFD for the detection of all AI strains was designed by P14 and two pilot batches produced for preliminary evaluation by the consortium partners.

Monoclonal anti-influenza A (NP) IgG2a antibody (HYB 340-01, Statens Serum Institut) was selected as the test capture line on a nitrocellulose membrane at concentration of 1.0mg/ml. The selection based upon a preliminary study performed with CSL (previously P9) purified stocks was then re-optimised for commercially obtained batch of antisera prior to pilot batch production. Monoclonal anti influenza A (NP) IgG1 antibody (HYB 340-05) was selected for conjugation to polystyrene microspheres at 1.0mg/ml dilution.

Once manufactured each sub batch of LFDs (50 in small scale production format) were quality control checked at 6% check rate for inactivated antigens of PMV and Avian influenza strain H7N1 in assay buffer.

Batch ‘E’ and batch ‘F’ were produced from two separate batches of each HYB 340-01 and HYB 340-05. 3000 of Batch ‘E’ and 4000 of Batch ‘F’ were produced; however inter-batch variation (HYB 340-01) resulted in a significant difference in performance, batch ‘E’ demonstrating the superior. Batch ‘F’ produced false positive responses under certain conditions and was attributed to too high antibody concentration on the capture line.

Following a successful preliminary assessment by P2 the LFD was selected for further validation and large scale production.

Evaluation of LFDs by consortium partners

P2

A preliminary experimental evaluation of Forsite's first prototype AI LFDs using swabs from experimentally-infected poultry (H7N1 LP and HPAI) at VLA was carried out. Comparison with M gene RRT PCR testing of the same swabs led to the determination of a detection limit of approximately 10,000 EID_{50}/ml for the Forsite protype AI LFD, and a similar sensitivity was observed for established commercial AI LFDs that included products from Quickvue, Anigen and Synbiotics.

Evaluation of P14's "batch E" AI LFD in a separate study involving VLA Biotechnology Dept and VLA Regional Laboratories in the UK involved testing of 841 swabs (oro-pharyngeal and cloacal) collected from wild birds. Although AI LFDs are designated for poultry testing, this exercise with wild bird swabs resulted in only nine reactors by the Forsite AI LFD (false positives, as shown by M gene RRTV PCR testing of the same swabs) that suggested an encouragingly high specificity of 98.9% for this test relative to M gene RRT PCR. P14 then proceeded to manufacture a large-scale lot (batch H) of AI LFDs for distribution to other FLUAID partners for field evaluation with poultry specimens.

A key requirement for AI LFDs is to validate these tests in poultry scenarios, so the involvement of the Egyptian CLQP institute (P15) provided an opportunity to assess the Forsite AI LFD with clinical specimens obtained in the field. The endemic disease status of H5N1 HPAI in Egypt provided an ideal opportunity for kit evaluation. The role of P2 was to design and agree a clear and objective protocol for AI LFD evaluation with P15. Field evaluation of the Forsite prototype AI LFD was conducted in Egypt during June-July 2008 by P15, where it must be emphasised that this is not a "peak season" for H5N1 HPAI in this country. A key element in the study design was to ensure that all swabs (oro-pharyngeal and cloacal) were tested by both the Forsite AI LFD and M gene RRT PCR. Prior to engaging P15 in this study, an AI RRT PCR MiniPanel was organised by P2 for P15 to test blindly, and perfectly good results were obtained. The superior sensitivity and specificity of M gene RRT PCR served as an objective means of determining whether the swabs were AI positive or not.

1. Thirty-six swabs were collected from 18 chickens at a proven H5N1 HPAI outbreak at one backyard farm (June 2008). Sensitivity and specificity of the prototype Forsite AI LFD relative to M gene RRT PCR were 100% and 71% respectively, where the latter was due to five swabs that gave false positive results by the AI LFD.

2. Two hundred and five chicken swabs were collected from 106 chickens, 95 of which were considered sick, at other premises and live bird markets (LBMs), although none of these were proven H5N1 HPAI outbreaks. Sensitivity and specificity of the prototype Forsite AI LFD relative to M gene RRT PCR were 100% and 68% respectively, where the latter was due to 66 swabs that gave false positive results by the AI LFD.

3. Fifty-five swabs were collected from farmed anseriformes (23 ducks and six geese) from locations where there was no clinical suggestion of notifiable disease. Testing of the Forsite AI LFD in comparison to M gene RRT PCR revealed 13 false positives by the former test, resulting in a specificity of 76%.

The above results indicated a false-positive problem with the Forsite AI LFD kit, and this was independently confirmed by testing during summer 2008 by the Irish NRL where swabs were collected from healthy poultry. The same protocol was agreed between P2 and the Irish NRL, namely a comparison of the prototype Forsite AI LFD relative to M gene RRT PCR. There was no AIV infection in the Irish poultry as evidenced by negative M gene RRT PCR results for all collected swabs, but specificity calculations similarly affirmed a false-positive problem for the prototype AI LFD:

1. Thirty-one swabs from 16 healthy farmed turkeys were tested, and 14 swabs gave false positive results that translated into a specificity of 55% for the prototype Forsite AI LFD relative to M gene RRT PCR.

2. One hundred and thirty-two swabs from 67 broiler chickens were tested, and 17 swabs gave false positive results that corresponded to a specificity of 87% for the prototype Forsite AI LFD relative to M gene RRT PCR.

3. Finally, the Irish NRL tested 28 swabs collected from 14 SPF chickens. Ten swabs gave false positive results that translated into a specificity of 64% for the prototype Forsite AI LFD relative to M gene RRT PCR.

These specificity problems that had been observed with swabs collected in the field in Egypt and Ireland were promptly communicated to P14 during summer 2008. There was neither opportunity nor resource for Forsite (P14) to consider a redesign of their AI LFD within the lifetime of the FLUAID project. However, the practicality and logistics of organising rigorous and objective AI LFD evaluation had been proven by P15 in Egypt. Consequently P2 and P15 agreed a programme of work for to similarly assess two established commercial AI LFDs during 2009 in order to complete the FLUAID project (please refer to activity 4 below).

P2 has conducted its own assessment of commercially-available AI LFDs during the FLUAID project. LFDs from Quickvue, Anigen, Synbiotics, Rockeby and Binax were compared to M gene RRT PCR.

Dry swabs (oro-pharyngeal and cloacal) were collected from experimentally-infected SPF chickens and domestic ducks for testing according to the manufacturers' instructions. This included the collection of pre-infection swabs as well as swabs from uninfected controls. Post-infection swabs were collected in a series of *in vivo* studies that included infection of these poultry species with different notifiable AIVs that included H5N1 HPAI (A/turkey/Turkey/05), H5N2 (LP and HPAI; A/ostrich/S Africa/06), H5N8 (HPAI; A/duck/Ireland/83), H7N1 (LP and HPAI; A/turkey/Italy00), H7N3 (HPAI; A/chicken/Chile/02) and H7N7 (HPAI; A/chicken/Germany/34). Overall sensitivity and specificity results for the five commercial AI LFDs relative to M gene RRT PCR were as follows:

Table 1: *Sensitivity and specificity calculations for five commercial AI LFDs relative to M gene RRT PCR. These were determined for all swabs that were collected dry and tested as per the manufacturers' instructions.*

Test	No of swabs tested	Sensitivity	Specificity
A	175	34/67=45.8%	108/108=100%
B	170	29/60=48.3%	110/110=100%
C	149	39/76=51.3%	71/73=97.3%
D	148	18/69=26.1%	79/79=100%
E	145	27/66=40.9%	79/79=100%

The specificity of all five commercial AI LFDs was high. For the sensitivity calculations relative to M gene RRT PCR, this may be influenced by (i) the sensitivity of a given AI LFD test and (ii) the sample population tested. All five tests were tested using approximately 145 swabs (Table 1) sourced from the same birds, while the Quickvue and Anigen AI LFDs were assessed by an additional approximately 30 swabs from other birds. Other than two false-positive reactors by the Synbiotics kit (Table 1), it was noted that all positive AI LFD results were observed in swabs obtained from HPAI infected chickens, and these were confirmed as genuine positives by positive M gene RRT PCR results. Semi-quantitative interpretation of the M gene RRT PCR revealed these AI LFD positives to be swabs where the viral load was >10,000 EID_{50}/ml, in agreement with the sensitivity limit of commercial AI LFDs that had been established previously (activity 1, above). These AI LFD positive chicken swabs were obtained from birds that were either displaying advanced clinical signs or were dead due to HPAI disease. For example, swabs were obtained post-mortem from chickens that had died due to H5N1 HPAI, where 20 swabs (ten oro-pharyngeal and ten cloacal) were tested by each of the five AI LFDs:

Table 2: *Results of five commercial AI LFDs used to test 100 swabs obtained from chickens (carcasses) that had been infected with H5N1 HPAI (A/turkey/Turkey/05).*

Test	No. of AI LFD positives	
	Oro-pharygeal	Cloacal
A	5/10	8/10
B	9/10	8/10
C	7/10	8/10
D	2/10	2/10
E	6/10	7/10

All the above chicken carcass swabs (Table 2) were derived from both oro-pharyngeal and cloacal sites where H5N1 HPAI shedding was approximately 10,000-30,000 EID_{50}/ml. These results indicated that while these AI LFDs lack the superior sensitivity of M gene RRT PCR, they do have sufficient sensitivity to detect AIV shedding from ill or dead chickens that had been shedding sufficiently high titres of H5N1 HPAI.

Negative AI LFD results in ducks infected with HPAI reflected the significantly lower shedding of various HP AIVs observed in experimentally-infected ducks. This included the testing of 10 swabs (five oro-pharyngeal and five cloacal) from ducks infected by H5N1 HPAI by each of the five commercial AI LFDs. Similar negative AI LFD results were obtained from ducks infected with H5N8 HPAI (A/duck/Ireland/83) where 24 swabs (12 oro-pharyngeal and 12 cloacal) were tested by each of the five AI LFDs. The Quickvue and Anigen AI LFDs were used to test swabs

from ducks infected with H7N3 HPAI (A/chicken/Chile/02; 15 oro-pharyngeal and 15 cloacal swabs) and H7N7 HPAI (A/chicken/Germany/34; 15 oro-pharyngeal and 15 cloacal swabs), where all were negative by AI LFD testing. Semi-quantitative interpretation of M gene RRT PCR results obtained from swabs from the same ducks showed that none of the shedding titres was greater than 10,000 EID_{50}/ml for any of these experimentally-infected ducks. This indicated that AI LFDs may be an insufficiently sensitive method for detecting HPAI infection in ducks.

During 2009, P2 agreed with P15 a protocol for the evaluation of Quickvue and Anigen AI LFDs in comparsion to M gene RRT PCR in Egypt. One reason for the choice of these commercial AI LFDs was their proven high specificity when P2 used these to test swabs from uninfected poultry. The 2009 workplan in Egypt was based on the similar approach which P15 previously employed in the field for the assessment of the prototype Forsite AI LFD during 2008. However, it is important to note that the 2009 study was conducted during February-April 2009 when there is a higher probability of encountering H5N1 HPAI outbreaks in Egypt. In addition, it was agreed that P15 would focus on proven or highly-likely instances of H5N1 HPAI outbreaks in order to facilitate an investigation of a greater number of AI-positive swabs which in turn would provide a more accurate measurement of the Quickvue and Anigen AI LFDs' sensitivity. Finally, other LFD data obtained with Quickvue and Anigen kits by P2 with feathers from (i) chickens that had been experimentally-infected with H5N1 HPAI (A/turkey/Turkey/05) and from (ii) H5N1 HPAI turkeys infected in the field (UK November 2007 H5N1 HPAI outbreak) had suggested that feathers may be an appropriate specimen for testing by these commercial AI LFDs. Consequently this field evaluation of Quickvue and Anigen AI LFDs by P15 provided an opportunity to compare testing of feathers to swabs obtained from H5N1 HPAI infected poultry in Egypt.

P15

The LFDs supplied to P15 were evaluated by comparing samples with Real-Time RT-PCR for the M gene following recommended protocols. The samples analysed by P15 are summarised in the table below

Lateral flow device LFD penside testing of AIV antigen

Provinces	No. of swabs (tracheal + cloacal)	Farm samples	LBM samples
Giza	56	56	-
Kaliobia	90	-	90
Menia	50	-	50
Dakhlia	30	-	30
Luxor	40	20	20
Sharkia	20	20	-
Cairo	10	-	10
Gharbia	20	20	-
Total	316	116	200

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LBM= Live bird markets

Analysis of the results revealed that there were 120 positive and 180 negative samples according to the LFD while only 35 positive and 275 negative samples according to the Real-Time RT-PCR. This demonstrated an extremely low specificity of 35% for the LFD

Given the poor results with P14's LFD two commercial LFDs were evaluated. Again, the performance of these LFDs was compared to RRT-PCR results for the same samples.

For the study samples were collected from clinically positive outbreaks of H5N1 primarily form backyard flocks. Three buccal and three cloacal swabs were taken from each bird in addition to 3 calamus feathers. A total of 182 samples were tested.

Kit	Sensitivity	Specifcity
A	81.25	92.85%
B	81.25	85.7%

NB: samples from galliformes only

Sample type*	Sensitivity	Specifcity
Oropharyngeal	78.5%	100%
Cloacal	66.7%	66.8%

* Anseriformes only

P13

In 2008, P13 (NCVD, Vietnam) also received the Forsite prototype AI LFD for field evaluation. In brief, the P13 results similarly revealed false-positive problems as were observed by P15 (CLQP) and the Irish NRL (activity 2, above). In order to maintain contact and collaboration with P13, P2 proposed that for the remaining working period of the project P13 would collect clinical specimens from known H5N1 HPAI outbreaks in Vietnam and send them for laboratory analysis at P2. Clinical specimens were collected from four locations (A, B, C and D), from 25, 25, 21 and 23 birds respectively. Sampling at location A included 15 chickens and 10 Muscovy ducks, location B included 15 ducks and 10 chickens, while samples from locations C and D were from chickens (n=21) and ducks (n=23) respectively. The types of clinical specimens received from Vietnam included the following:

1. Oro-pharyngeal swabs, cloacal swabs and feathers stored in 1ml virus transport medium (brain heart infusion broth) containing antibiotics (BHIB). Collected from all sampled birds at all four locations.

2. BHIB from swabs and feathers, where 100ul of BHIB was spotted onto FTA cards. Ten birds sampled at each location in Vietnam.

3. BHIB from swabs and feathers, where 100ul of BHIB was mixed with an equal volume of "RNA Later" which is a known preservative for RNA-containing clinical specimens. Ten birds sampled at each location in Vietnam.

4. Sera obtained from all live birds at the four locations, namely 15 chickens and eight Muscovy ducks (location A), 13 ducks and ten chickens (location B), 16 chickens (location C) and 18 ducks (location D). The aim was to use these sera in a comparison of H5-specific antibody detection in a comparison of HI testing and commercial H5 antibody-detection ELISAs.

Because the above clinical specimens arrived at P2 at a late stage of the FLUAID project (i.e. July 2009) it was possible to complete thorough testing of all the above clinical specimens obtained from the four poultry locations in Vietnam. Any incomplete work will, however, continue beyond the completion date of the FLUAID project using other resources at P2.

Workpackage: 3.1

Partner: P1, P2

Deliverable: D16

Development of a rapid pen-side Real-Time RT-PCR for H5/H7 and generic AI detection

Experimental design and key results

General Remarks,
Initially this deliverable was entrusted to P2 who attempted, without success to identify a source for a suitable pen-side molecular platform that could be used to evaluated Real-Time RT-PCR protocols for M (generic AI) , H5 and H7. P1 were able to identify a mobile laboratory belonging to the Veneto branch of the Italian firebrigade (Comando dei Vigili del Fuoco, Mestre) that possessed a suitable platform and who were willing to perform evaluation tests in collaboration with P1

Instrument: R.A.P.I.D system (Ruggedized Advanced Pathogen Identification Device), Idaho Technologies, USA

Viruses: Subtype H5N1 (EID_{50} $10^{5.83}$) and H7N1 (EID_{50} $10^{7.375}$)

Sample preparation: Serial dilutions of each virus were made in duplicate and RNA was extracted from each dilution using the NucleoSpin RNA II kit (Macherey- Nagel). The sensitivity of the Real-Time RT-PCR protocol was calculated by testing each sample in triplicate.

Protocols

The protocols were those currently used by the Community AI reference laboratory (VLA, Weybridge, UK), P2 e.g. Matrix (M) gene amplification, N1 gene amplification, H5 gene amplification and H7 gene amplification

Results and conclusions

For comparison purposes each protocol was performed both in the R.A.P.I.D instrument and using the Roche Light Cycler. The R.A.P.I.D instrument performed well for each of the four protocols and proved to be just as sensitive as the laboratory based Real-Time instrument. This suggests that the R.A.P.I.D instrument would function well as a pen-side portable molecular device for the diagnosis of AI if used with the recommended protocols..

WORKPACKAGE 4:
Tropism, pathogenesis and transmission in different avian species

Partners involved: P1, P2, P3, P4, P7

Overall Objectives:

1. Develop in-vitro systems for modelling and identifying host adaptation and tropism of HPAI viruses
2. Evaluate the susceptibility of Anseriformes (ducks, Muscovy, mule ducks) for LPAI and HPAI viruses
3. Study transmission dynamics of selected AI viruses in ducks, quails, indigenous chicken breeds from Asia
4. Evaluate the effect of vaccination on the transmission in ducks and quails

Deliverables: D17 to 30

Workpackage 4 Overview and impact

A significant amount of important data was generated within this workpackage. P7 concentrated specifically on in vitro assays for characterizing both existing and emerging avian influenza virus (AIV) strains with respect to their species tropism and zoonotic potential. The results obtained have provided data on tropism of different influenza virus strains in cells from human, porcine and avian species. Particular attention was placed on the viruses ability to interaction with cells of the porcine innate immune system thus providing information on the potential behaviour of AIV compared with mammalian IV in mammalian hosts. These data will be of particular interest to the influenza research community

The other four partners of the workpackage (P1,P2, P3 and P4) concentrated on the pathogenesis and transmission in different avian species which included chickens (indigenous varieties), turkeys, quail and ducks. Important work on serological response in ostriches was also carried out.

Therefore the key conclusions and observations from this workpackage as regards animal experiments areas follows

1. Vertical transmission of H5 LPAIV in ducks does not occur

2. An age related association with clinical disease and viral dissemination exists in Pekin ducks when infected with H5N1 of the Eurasian lineage.

3. LPAI infected quails shed high concentrations of virus via the respiratory apparatus even though they show no clinical signs. Infected quail can also transmit the virus to contact birds rapidly. This suggests that quail may act as a reservoir of infection play a role in virus transmission.

4. Vaccination of quail using standard vaccination protocols for chickens and turkeys does not provide clinical protection.

5. Quail farms should be carefully monitored and managed given their ability to mask infection and transmit to other avian species

6. Native chickens develop substantial HI antibody titers directed against H5N1 upon vaccination with a heterologous H5N2 vaccine, and that this provides a level of protection that is generally sufficient to prevent a productive H5N1 infection.

7. Vaccination with a heterologous H7N1 vaccine protects 12-week-old turkeys against challenge with H7N7 HPAI virus and no transmission of the virus occurs between the vaccinated turkeys .

8. An H5N2 vaccine strain with a low level of genetic and antigenic homology with the H5N1 challenge virus is able to reduce transmission in ducks significantly.

9. Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches revealed that validated ELISA tests can be considered suitable test for screening purposes and should be preferred to AGID. In addition, the HI test (under specific conditions) remains a suitable test to monitor the circulation of AI in ostriches.

The results obtained in WP4 will be of assistance to EU decision makers and poultry veterinarians in the design and implementation of vaccination and management strategies for AI.

Workpackage: 4.1

Partner: P7

Deliverable: D17, D18, D20

Results from tropism studies

Experimental design and key results

General remarks:

During the course of the project, P7 focused on *in vitro* assays for characterizing both existing and emerging avian influenza virus (AIV) strains with respect to their species tropism. Added to this were a number of swine and human influenza virus (IV) isolates, for comparison of their interaction with cells of different species. Analyses investigated the tropism of the different IV strains in cells from human, porcine and avian species. A particular attention was placed on their interaction with cells of the porcine innate immune system, to provide information on the potential behaviour of AIV compared with mammalian IV in mammalian hosts.

Accordingly, the main goal for P7 in this project was to establish *in vitro* assays (D17, D18, D19) to identify and characterize the various IV strains with respect to species tropism. In addition, the interaction of various strains with the innate immune system was analyzed with respect to the potential for providing information of relevance to the behavior of certain influenza in mammalian hosts (D21). From this work, a list of available IV characterized in terms of their capacity to induce innate responses, would be prepared (D20).

Initiation of the work established the cell lines to be employed for the *in vitro* assays, as well as characterization in terms of their sialic acid receptors. Both cell culture (MDCK cells) and hen egg-passaged stocks of different subtypes of AIV were prepared (reference mammalian influenza viruses were also prepared to be employed as sources of reference). This effort was accompanied by the establishment of SOPs for handling the viruses within the BSL3 laboratory of the institute.

Primary and established Cells

The following cell lines were employed in this project: MDCK (canine epithelial cell line), Vero cells (monkey epithelial cell line), MRC-5 (human epithelial cell line), NBTr (newborn piglet trachea epithelial cell line), CHTr (chicken trachea

epithelial cell line), CEF (chicken embryonic fibroblast cell line), MD11 (chicken macrophage cell line). In addition, primary cultures of blood monocytes and macrophages from pigs, humans and

chickens have been established. Both blood dendritic cells and monocyte-derived dendritic cells of porcine origin are in routine production, using SOPs established in-house.

Influenza virus stocks.
Master and working stocks were prepared for the influenza viruses which have been listed in the deliverable D20. All viruses were grown in SPF 11-day embryonated eggs to produce high titre allantoïc fluids, and certain selected viruses were also prepared using MDCK cells. These preparations were titrated in both SPF 11-day embryonated eggs and on MDCK cells. Certain of the LPAI viruses had to be passaged 2 or 3 times in eggs to obtain usable working titres.

Characterization of sialic acid receptor expression
This work (towards D19) was performed using biotinylated lectins MAA and SNA. The previous reports that MDCK cells express both α2,3 and α2,6-linked sialic acid receptors were confirmed. In additon to this, it was noted that the Vero cells and CEF cells were dominated by of α2,3-linked sialic acid receptors, while the MRC-5 cells expressed mainly α2,6-linked sialic acid receptors. As for the primary cells, it was interesting to note that porcine, human and chicken peripheral blood monocytic cells efficiently bind both lectins, indicating expression of α2,3 and α2,6-linked sialic acid receptors.

Immunofluorescence-based detection of IV after infection of MDCK cells.
This was monitored using a monoclonal antibody (mAb) against the nucleoprotein of IV, which cross-reacts with all the IV studied so far. These analyses will be completed in year 2, and extended to include analyses using a mAb against the matrix protein. For detection of HA of an H5N2 virus, P7 have also successfully used a chicken serum from vaccinated animals.

Infection of porcine PBMC and enriched natural interferon producing cells (NIPC, plasmacytoid dendritic cells) with various strains of AIV, and quantification of virus-induced IFNα by ELISA.
Porcine PBMC or enriched NIPC (plasmacytoid DC or pDC) (D17, D18) were infected at various multiplicities of infection using certain of the above viruses for 1-2 hours. The inoculum was then replaced with normal growth medium, and after 24 hours the supernatants were tested for IFN- γ by ELISA. Initial results are showing that LPAI viruses induce IFN-γ production by PBMC, wherein increased levels are seen when purified NIPC are employed. With all avian viruses tested (H5N2, H7N1, H7N3) the IFN- γ response was relatively independent of the virus dose used. In contrast, human H1N1 induced IFN- γ secretion in a clear virus dose-dependent manner.

This work was extended to other mammalian influenza viruses during years 2 and 3. Furthermore, before these results can be interpreted, the influence of several parameters which could influence the activation of NIPC, including a comparison of egg-grown with MDCK-grown virus, the presence of serum in the cell culture medium and the presence of trypsin, had to be investigated. This was performed during year 2 of the project.

Preparation of H5-pseudotyped lentiviruses.

In order to generate these pseudotyped lentiviruses, the vesicular stomatitis virus (VSV) G sequence in the plasmid encoding the envelop protein of the lentivirus vector system was replaced by an H5 sequence (Swiss and Chinese isolates). This allowed the generation of lentiviruses

bearing the influenza virus H5 protein on their surface. The lentivirus system employed is composed of 3 plasmids encoding for the envelope (pMD2G), the packaging system (pCMV-dR8.91) and the vector (pLVTHM). Therein, the packaging system encodes only *gag* - the virion main structural protein - *pol* - responsible for the retrovirus-specific enzymes - and *rev* - a post-transcriptional regulator necessary for efficient *gag* and *pol* expression. In the original three-plasmid system, the envelope plasmid encodes the VSV-G protein as a structural envelop protein giving a broad cellular tropism to the viral particles. P7 replaced the VSV-G sequence by a full length haemagglutinin sequence from both Swiss and Chinese H5N1 influenza virus isolates. After optimization of the lentivirus production, P7 can currently achieve up to 30% infection rates – measured by expression of the encoded eGFP - in HEK 293 cells and MDCK cells using the H5 pseudotyped particles. One important element is the addition of bacterial neuraminidase in the medium during the production of the HA-pseudotyped lentiviruses. P7 are currently improving the protocol to increase the titres of the lentiviruses. The next step will be to determine whether the tropism of the H5-pseudotyped lentiviruses relates to H5 avian influenza viruses, but this forms part of a parallel project.

In the second year of this project, the work characterized the interaction of IV with various subsets of dendritic cells (DC), including conventional DC as well as plasmacytoid DC (D17, D18). All of the IV strains have been prepared under BSL3 conditions, in eggs, with a certain number also prepared, as deemed necessary for the aims of the work, in the MDCK cell line (D20).

The interaction of IV with natural interferon producing cells (NIPC), also called plasmacytoid DC (pDC) was analyzed with respect to the virus characteristics, and also the cell activities. Based on the lack of knowledge concerning IV interaction with pDC, and the important role of pDC in innate antiviral immune responses, P7 investigated IFN- γ responses of porcine pDC to various IV originating from birds, swine or human in a comparative manner. The IV strains employed are from the list of deliverable D20.

With all viruses, a dose-dependent IFN-γ response was obtained correlating more to haemagglutination units (HAU) than infectious virus titres. This related to the observation that inactivated virus was as efficient in inducing IFN- γ as infectious virus. Surprisingly, all avian IV were more efficient at inducing IFN- γ with lower viruses doses; they also induced higher absolute values if IFN- γ compared to mammalian (human and swine) isolates. Amongst all IV, the H5N1 strain was the most interferonogenic. No difference was found between pairs of avian IV differing in the cleavage site of the haemagglutinin.

P7 have also analyzed the interaction of IV with porcine cDC. These were generated either from purified monocytes stimulated with GM-CSF and IL-4 or from bone marrow haematopoietic cells stimulated with GM-CSF or Flt3L (D17). Both the monocyte-derived DC as well as the bone marrow-derived DC were susceptible to all IV strains used in terms of nucleoprotein expression for up to 48h.

The third year of the project elaborated on the work of the second year, with confirmation of the information obtained, along with extension of the characterization in terms of virus replication in the DC, and the level of interferon being induced. Moreover, these characteristics were defined with respect to the requirement for live and replicating virus.

In view of the results from year 2, P7 investigated in year 3 whether IV uptake in pDC differs dependent on virus origin and haemagglutinin receptor binding, and whether virus uptake in pDC occurs through the classical IV receptor in a sialic acid-dependent manner. Furthermore, the fate of internalized virus within the endosomal system required clarification – an issue of relevance for a TLR7-dependent pDC activation. While a potent activation of pDC could help the host in

controlling avian IV infection, our results could also be of relevance for the pathogenesis of fatal H5N1 infections considering the "cytokine storm" hypothesis. This was also an effort in year 3.

P7 also extended the year 2 effort on the interaction of IV with porcine cDC generated from purified monocytes or from bone marrow haematopoietic cells. Both sources of cDC were shown in year 2 to be susceptible to all IV strains. This was confirmed in year 3, with respect to nucleoprotein expression. However, all viruses were inefficient at producing progeny infectious virus, showing that the replication in the cDC was abortive. Moreover, a similar abortive infection could be registered following infection of the pDC. Nevertheless, this abortive replication was an important event for the induction of cytokine secretion by both the cDC and the pDC. This was particularly notable with the bone marrow-derived DC, and for the release of pro-inflammatory cytokines.

From this, our results indicate that certain avian isolates of IV, in particular the HP H5N1 can more efficiently infect and activate porcine DC. It is considered that the activation is due to the replicative form of the virus RNA (dsRNA) interacting with TLR3 and/or cytosolic helicases). The capacity of inactivated virus to activate the DC is likely to be transmitted via the remnants of the now inactivated RNA genome. There will be adequate RNA remaining to activate via perhaps TLR7, but certainly via cytosolic helicases. Only when the RNA of the virus is removed, as when using virosomes of H5N1 origin, there is no longer an activation of the DC. This would confirm that the activation was dependent on the viral RNA, but not necessarily functional RNA in terms of virus replication. However, replication of the virus would allow for an elaboration of the viral RNA present, and therefore the degree of DC activation and induction of pro-inflammatory cytokines.

Workpackage: 4.2

Partner: P2

Deliverable: D22

Evaluation of the susceptibility of anseriformes to HPAI viruses by experimentally infecting ducks with different doses and through different routes with selected H5 and H7 HPAI strains

Experimental design and key results

Two main experiments were carried out:

1. Time course studies in 4 week old Pekin ducks (n=20) experimentally infected via intranasal and intraocular routes with H5 and H7 HPAI to evaluate shedding and dissemination of virus throughout organs.

Viruses used:

Virus	Type	Dose (EID50/ml)
A/turkey/Turkey/1/05	H5N1	$10^{6.7}$
A/turkey/Ireland/1744/83	H5N8	10^{6}
A/duck/Ireland/113/83	H5N8	$10^{6.5}$
A/chicken/Germany/34	H7N1	10^{6}
A/chicken/Chile/4968/02	H7N3	$10^{5.5}$
A/chicken/England/1158/08	H7N7	$10^{6.5}$

Shedding was detected by collecting buccal and cloacal swabs daily, and tested by M gene Real time RT-PCR (Spackman *et al.*, 2002). Dissemination of virus through the ducks was examined by collection of key tissues for M gene RRT-PCR and neucleoprotein immuno histochemistry (NP-IHC). Final bleeds were taken and serum tested by HI and ELISA.

Calculation of median infectious dose (ID50) and median lethal dose (LD50) of 3 and 4 week old Pekin ducks experimentally infected via intraocular and intranasal routes with H5 and H7 HPAI.

Viruses used:

Virus	Type	Dose (EID50/ml)
A/turkey/Turkey/1/05	H5N1	10^1 10^2 10^3 10^4 10^6
A/ostrich/Italy/984/00	H7N1	10^1 10^2 10^3 10^4 10^6
A/chicken/Germany/34	H7N1	10^2 10^3 10^4 10^5 10^6

Shedding detected by collecting buccal and cloacal swabs, and tested by M gene Real time RT-PCR (Spackman *et al.*, 2002). Tissues were taken for any dead birds and tested for viral infection by M gene RRT-PCR. The median infectious dose (ID_{50}) and median lethal dose (LD_{50}) was calculated.

Time course study in ducks revealed 3 distinct profiles

I. Shedding observed predominantly through the buccal cavity throughout the time course. There was progressive accumulation and systemic spread of virus in key organs including the heart, brain, liver and lung. Virus was detected by NP-IHC and was localised in neurons and glial cells, indicative of targeted neurological infection. Ducks infected with the HPAI viruses A/turkey/Turkey/1/05 (H5N1) and A/chicken/Germany/34 (H7N1) displayed this infection profile and many ducks showed clinical signs and died.

II. Shedding observed through buccal and cloacal cavities but only at low levels. Virus detected by NP-IHC in scattered enterocytes and underlying lymphoid tissue targeting the intestinal tissues and indicative of an epithelial infection. Ducks infected with the HPAI viruses A/turkey/Ireland/1744/83 (H5N8) and A/duck/Ireland/113/83 (H5N8) displayed this infection profile and no clinical signs or mortality was observed during the time course. When sera were tested by HI, seroconversion was detected for A/turkey/Ireland/1744/83 and A/duck/Ireland/113/83 at 232 and 28 respectively. Ducks from both groups gave a positive result by generic AI ELISA.

III. Minimal or no shedding detected from swabs or tissues and no clinical signs observed. No seroconversion detected by HI or ELISA. Ducks infected with the HPAI viruses A/chicken/Chile/4968/02 (H7N3) and A/chicken/England/1158/08 (H7N7) displayed this infection profile.

2- Dose study to calculate median infectious dose (ID_{50}) and median lethal dose (LD_{50})

Virus	LD50 (EID50/ml)	ID50 (EID50/ml)
A/turkey/Turkey/1/05	10^3	$\leq 10^1$
A/chicken/Germany/34	$10^{5.7}$	10^3
A/ostrich/Italy/984/00	$>10^6$	$10^{4.2}$

Studied carried out with A/turkey/Turkey/1/05 produced the greatest amount of shedding and mortality from all dose groups. Ducks infected with A/chicken/Germany/34 also showed shedding from all dose groups with mortality observed from the three highest doses. Shedding was only detected from ducks infected with A/Ostrich/Italy/984/00 for the highest two dose groups and no mortality was observed.

A higher dose of H7N1 A/ostrich/Italy/984/00 was required to cause mortality and infection than H7N1 A/chicken/Germany/34 or H5N1 A/turkey/Turkey/1/05.

The effect of host age on the shedding and tissue dissemination of a HPAI H5N1 virus in infected Pekin ducks.

Experimental design and key results

Infections of 8- and 12-wo Pekin ducks with 10^6 EID_{50} of A/turkey/Turkey/1/05 H5N1 HPAI were undertaken. The virus, A/turkey/Turkey/1/05 HPAI H5N1, was selected for use as a contemporary 'Eurasian lineage' H5N1 virus, representing the westward movement of these viruses from their origins in South East Asia into Europe.

Samples of tissues (brain, heart, spleen, lung, liver, kidney, intestine, caecal tonsil, trachea, breast muscle, thigh muscle and wing feathers) were collected for RRT-PCR. Tissue samples collected for histopathology and immunohistochemistry [using an anti-nucleoprotein monoclonal antibody (NP)] examination included those selected for RRT-PCR as well as nasal turbinates, air sacs, gizzard, proventriculus, pancreas, duodenum, thymus, bursa, adrenals, thyroid, ovary/testes and sciatic nerves. The presence of virus was determined by matrix gene real-time RT-PCR and HI

Severe clinical signs, including depression, reluctance to feed, tremors, loss of balance and torticollis were observed in the 8-wo group resulting in 100% mortality by 4dpi. However, only mild clinical signs, mainly conjunctivitis, were observed in the 12-wo group with no appearance of nervous signs or mortality over an observation period of 7dpi. Gross pathological observations of the 8-wo ducks, included lesions and haemorrhages in the pancreas. At post mortem examination, inoculated 8-wo ducks showed mild rhinitis from 1 dpi, with increased severity from 3 dpi. Multifocal necrotizing pancreatitis was observed from 2 dpi, with severity and extension of lesions progressing with disease advancement, and occasional multifocal haemorrhages. Air sacculitis was evident from 2 dpi onwards. Myocarditis was observed at 3 dpi in one of three birds.

In 12-wo ducks, a mild rhinitis was observed from 1 dpi, with a similar presentation through the course of the experiment. Mild pancreatitis and airsacculitis was observed from 3 dpi. The appearance of gross lesions occurred later than in 8-wo ducks with the severity of the lesions milder, more limited in extent, and with a slower progression.

The presence of histopathological lesions were observed in the 8-wo challenged ducks from 2 dpi. Lesions were milder and less frequent in the 12-wo ducks than in ducks in the 8-wo group. The detection of viral antigen by immunohistochemistry in 8-wo infected ducks occurred in a higher number of cells than in 12-wo ducks for almost any particular tissue. An increase in the number of immunolabelled cells in conjunction with disease progression was more marked in 8-wo birds.

Higher levels of viral shedding were detected in oropharyngeal swabs than in cloacal swabs in both 8- and 12-wo age groups, with similar levels of shedding detected in both age groups. In keeping with clinical signs, virus detection in the various tissues tested, including trachea, caecal tonsil, thigh muscle, heart and brain, showed much higher levels in 8-wo ducks, compared to older ducks, in which virus detection was usually minimal.

In contrast, substantially higher levels of virus were detected in the liver, kidney, intestine and spleen tissue of 12wo ducks, compared to 8wo ducks . Interestingly, the only organ to show similar dissemination of virus in both age groups was the lung. In the skeletal muscle tissues, similarly high levels of virus were detected in the breast muscle of 8-wo birds with minimal detection in tissues of 12-wo ducks. An age dependency for the temporal dissemination of virus within these skeletal muscle tissues was also observed with the delayed infiltration into tissues in older ducks. In the heart samples from birds in the 12-wo group, little virus was detected, but higher levels of virus was detected in 8-wo ducks peaking at 3 dpi.

Generally, the highest levels of viral RNA was detected in the brain samples from 8-wo ducks on days 3 and 4 pi, with an increase in levels in the brain samples from 12-wo ducks only on day 5 pi). Although, the mean viral titres in the brain samples of 12-wo ducks increased sharply at 5 dpi, this was due to a single individual bird that showed substantial amounts of virus present without the presentation of clinical signs. A similar age dependency for temporal viral dissemination, as seen for skeletal muscles, was also observed in brain and heart tissues which coincided with the appearance of clinical signs preceding death in younger birds.

Although ducks and some other aquatic birds had been previously thought to be “resistant” to most HPAI viruses, which caused only asymptomatic infections, they have recently been shown to be highly susceptible to certain HPAI H5N1 isolates . In the study performed an age related association with viral loads excreted and the tissue distribution of virus (as detected by IHC and real-time RT-PCR) over time post infection was identified. This association also coincided with the appearance of clinical signs and mortality. No clinical or pathological evidence of debilitating disease was observed in 12-wo Pekin ducks but “abundant excretion” levels were not observed.

Although the nature and location of histological lesions observed in both age groups of ducks were similar, the severity and prevalence of these lesions were lower in older ducks which is consistent with the reduced clinical disease at 12-weeks-old.

Interestingly, virus was detected in wing feathers of both age groups is suggested as a possible sampling method for HPAI surveillance in wild bird populations. However, in older ducks in the present study, detection of virus was shown to be related to the level of calcification of the feather shaft and therefore the age of the bird sampled may affect the sensitivity of feather sampling as a method of surveillance.

Since wild waterfowl (represented by Pekin ducks as a model in this study) of migratory age, infected with H5N1, appear healthy without the presentation of clinical signs, they could act as

long-distance vectors. Although 12-wo Pekin were able to shed virus without showing clinical signs or succumbing to disease, the viral load shed was low and therefore it remains to be seen whether this shedding dose would be sufficient to allow transmission to susceptible hosts.

Although a more mature immune system in older ducks is an obvious candidate for this phenomenon, the fact that viral loads in organs such as liver, spleen and intestine were higher in older ducks, does indicate that a more successful immune response may not be the sole contributing factor.

Workpackage: 4.2

Partner: P4

Deliverable: D23

Data on potential persistence of H5 LPAIV in embryonating duck eggs and their Issues.

Experimental design and key results

An evaluation of the persistence of low pathogenic H5 influenza viruses in duck eggs was carried out using a model where both SPF Muscovy duck eggs, and Mule (AI antibody and virus free) duck eggs were inoculated with ten-fold dilutions of the virus at tires over and under the fifty per cent infectious dose (EID 50 %). About 20 embryonating eggs per dilution were inoculated via the yolk sac at 6 to 7 days old with H5 LP avian influenza strains previously isolated in France (Cherbonnel M. et al., Av. Dis., 2007, 51, 408-413).

Eggs were candled daily to detect embryo mortality. The incubation was stopped after 29 to 30 days of incubation in order to avoid hatching and possible inter-contamination. In contrast, in two of the experiments Mule duck eggs were allowed to hatch and the ducklings were then tested up to 4 weeks of age. In order to check for the presence of the AI M gene, allantoïc fluid (AF) was collected from dead embryos. For embryos that died late in the experiment or survived the experiment, trachea and intestine were sampled. All these samples were tested using the standard AI gene M-based real-time RT-PCR (EU Diagnostic Manual).

In the two experiments where ducklings were allowed to hatch, all birds were sampled as follows :

- at one week of age : oro-pharyngeal and cloacal swabs were tested by AI gene M-based real-time RT-PCR .

- at four weeks of age : blood samples to test sera for HI antibodies against two H5 antigens (the homologous challenge virus and LP H5N2 A/duck/France/05057b/2005) and in competitive influenza NP-ELISA (in-house test).

Results

Embryos inoculated with the highest virus doses consequently died early on in the experiment , i.e between 3 and 10 days post-inoculation (dpi). They were nearly all highly positive by AI gene M-based real-time RT-PCR.

AF from all of the embryos that died late in the experiment (possibly due to incubator problems), in addition to the respiratory and digestive tracts from all embryos still surviving at 22 to 24 dpi (end of the experiment), were found negative by molecular tests.

All ducklings hatched from inoculated eggs were found negative by molecular and serological tests.

Study of vertical transmission of LPAIV H5N3/05054a in Muscovy ducks breeders

Protocol

The SPF Muscovy duck breeders (26 females and 5 males), established in a P3 security level protected housing were challenged oculo-nasally at 33 weeks of age (just after point of lay) with 10^7 EID_{50} of the H5N3 LP AIV strain A/duck/France/05054a/2005 selected due to its high virus-shedding charactersitics.

Eggs laid after inoculation were collected during two successive periods and were treated differently before being incubated separately in order to assess true vertical transmission as opposed to pseudo-vertical transmission:

- period 1 (group 1) = from 3 to 9 dpi : egg shells were carefully disinfected before incubation ;
- period 2 (group 2) = from 10 to 17 dpi : egg shells were not disinfected before incubation and dirty eggs were preferably selected.

Progeny of the two groups hatched separately. Ducklings were individually checked over a 7 week period for the presence of the H5 virus (RNA) and antibodies, as follows :

- at 4, 7, 10, 14, 17 and 21 days-of-age : oro-pharyngal and cloacal swabs of all birds were tested by AI gene M-based real-time RT-PCR ;
- at 3, 4 and 7 weeks-of-age : blood sampling of all birds to test sera using :

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- HI to the challenge-virus homologous antigen and an LP H5N3 virus (A/duck/France/02166/2002) ;
- AI NP-based competitive ELISA.

Results

The infection of the duck breeders was confirmed by the positive seroconversion of all of them against H5 (HI antibodies) at 4 weeks post-inoculation.

The first group of ducklings consisted of only 25 birds (due to an incubation problem) versus 60 for the second group. None of these 85 ducklings were found positive by molecular tests (AI gene M-based rRT-PCR) regardless of the type of swab used and the time of sampling. Similarly, none of these birds showed either H5 HI antibodies or NP-ELISA antibodies during the whole experiment period (7 weeks of age).

Study of vertical transmission of LPAIV H5N3/05054a in Mule breeders

Protocol

The protocol differed slightly from the previous one in that shells from eggs collected were not disinfected and that breeders were tested molecularly after challenge (in addition to the serological testing).
Details are the following : the AI antibody and virus free Mule breeders (23 females and 6 males) were challenged oculo-nasally at 40 weeks of age with 10^6 EID_{50} of strain H5N3/05054a. The breeders were sampled at day 3, 5, 7 and 10 post challenge (cloacal swabs) to check for the presence of the challenge virus by real-time RT-PCR (gene M). Serum was tested at day 17 post-challenge for :

1) HI antibodies against the challenge virus and another H5 with a different neuraminidase (N2) ;
2) AI NP-based competitive ELISA antibodies.

Eggs from challenged breeders were collected from day 3 to day 12 post-challenge. They were incubated in two steps (at day 7 and day 12 post-challenge) to limit embryo mortality during storage. The 40 duckling issues from the second incubated group (B) were mixed at one day old with the 26 ones from the first incubated group (A) to favour inter-group contamination.

Ducklings were swabbed (cloaca) for real-time RT-PCR AIV gene M at :

- 3, 7 and 10 days of age for group A
- 3, 6 and 10 days of age for group B.

Blood was collected from ducklings for serological testing by using the same antigens and tests as for their parents at :

- 21 and 30 days of age for group A
- 16 and 25 days of age for group B.

Results

Parents

All parents (but one, found positive later) were found positive by AI gene M-based real-time RT-PCR at day 3 post-challenge. Using this molecular test, positive breeders decreased regularly until day 10 post-challenge (2 birds positive out of 29).

At day 17 post-challenge, all parents were found strongly positive serologically with both tests used (HI with both H5 antigens and AIV NP-ELISA).

Ducklings

Progeny from infected Mule breeders were constantly found negative in AI gene M-based real-time RT-PCR as well as for HI antibodies to both H5 antigens. Only ducklings from group B showed transitory AI NP-ELISA antibodies at 16 days of age which correlated well with post-challenge seroconvertion of their parents ; they return negative at 25 days of age and birds from group A in close contact with them remained negative all through the experiment, confirming a non-infectious process in ducklings. This is strongly indicative of maternally derived antibodies being present in birds hatched from eggs collected from infected breeders.

Conclusions

In our experiments, infection of duck breeders from two species (Muscovy and Mule) with a low pathogenic H5 influenzavirus did not lead to infection of their progeny. Moreover, when inoculated early into embryonated duck eggs (Muscovy and Mule ones), this virus and four other LP H5 viruses did not permit embryos inoculated with high doses to survive and to hatch. Ducklings that hatched from inoculated eggs never showed the presence of virus RNA or antibodies to the challenge virus.
These results therefore invalidate the hypothesis of vertical transmission of H5 LPAIV in ducks. This is important and reassuring information for professionals involved in duck production including national veterinary authorities in charge of disease management.

Workpackage: 4.2

Partner: P1

Deliverable: D22 and D24

Data on viral shedding and seroconversion over an extended period of time following experimental infection with HPAI and LPAI H7 subtype viruses in quails.

Experimental design and key results

Four groups of fifteen SPF chickens each and four groups of fifteen quails each were formed. Two groups of quails were infected with two HPAI H7N1 viruses (A/quail/Italy/1764/00 and A/turkey/Italy/4580/99) at the dose of 10^4 EID_{50} /0.1ml respectively. The other two groups of quails were infected with two LPAI viruses A/turkey/Italy/8000/2002 (H7N3) and A/quail/Italy/4610/2003 (H7N2) at the dose of 10^6 EID_{50}/0.1ml. All animals were infected via the ocular and oronasal route. The same experimental design was applied for SPF chickens. Clinical signs were recorded for all animals twice a day. Tracheal and cloacal swabs were collected every day from day 2 to 11 post infection (p.i.) and then on day 19 and 25 p.i.. On days 7, 13, 19, 25, 33, 44, 58, 73, 93, 125, 146 and 171 p.i. blood was collected from each bird. From sick and dead birds internal organs were collected for histopatological examination.

In quails and chickens, LPAI and HPAI influenza viruses used in the present experiments replicated primarily in the respiratory tract with high titres. Clinical behaviour and duration of viral shedding of both groups of chickens were different compared to quails. All A/quail/Italy/1764/00 infected chickens died within 24 hours p.i. while the group infected with A/turkey/Italy/4580/99 shed viruses for prolonged period compared to quails (9 days p.i.).
Although no clinical signs were recognized, LPAI infected quails shed virus via the respiratory apparatus in high concentrations (up to $10^{5.2}$ EID_{50}/0.1 ml) and for long periods of time, increasing the possibility of infection spreading.
On day 3-4 p.i. the number of shedding quails was higher with HPAI viruses (11 quails infected with A/quail/Italy/1764/00 and 14 with A/turkey/Italy/4580/99) than LPAI viruses (7 quails infected with A/turkey/Italy/8000/2002 and 9 with A/quail/Italy/4610/2003). LPAI viruses replicated in respiratory tract with higher titre than HPAI viruses ($10^{4.4}$ EID_{50}/0.1ml for A/turkey/Italy/8000/2002, $10^{4.9}$ EID_{50}/0.1ml for A/quail/Italy/4610/2003and $10^{3.6}$ EID_{50}/0.1ml ml for A/quail/Italy/1764/00, $10^{2.9}$ EID_{50}/0.1ml for A/turkey/Italy/4580/99).

In quails antibody response is not easily detectable and interpretable due to: low and inconsistent titres detected by HI (<1:64), the lack of agreement between the total number of RRT-PCR and

virus isolation positive animals and the number of sera testing positive highlights the need to identify a reliable and suitable serological test for quail.

We believe that validated commercial ELISA tests for the serological diagnosis of AIV infections in quails sera can be considered suitable test for screening purposes and are preferred to AGID.

Findings reported suggest that Japanese quails are readily infected with LPAI H7 viruses and that they may represent a source of infection for other poultry species during outbreaks. Therefore, it is reasonable to postulate that, during the 2002-2004 Italian H7 epidemic, quails might have played a role as reservoir of infection.

Evaluation of lateral spread from experimentally infected quails with H7 LPAI viruses to uninfected quails

Experimental design and key results

To determine the lateral spread characteristics of LPAI H7 viruses in a susceptibility host population, 4 groups of 5 quails each were used. Two groups were challenged with A/turkey/Italy/8000/2002 (H7N3) and A/quail/Italy/4610/2003 (H7N2) LPAI viruses respectively via the ocular and oronasal route with 100 µl of a viral suspension containing 10^6 EID_{50}/0.1ml. On day two p.i. 5 uninfected quails were placed in contact with each infected group.
Tracheal and cloacal swabs were collected daily from all birds (infected and in contact) of both groups until 11 day p.i. and 9 respectively for viral shedding. Clinical signs were recorded for all animals twice a day. The same experiment was carried out twice to asses its repeatability.

No severe clinical signs were observed in infected and in contact quails. All inoculated and contact quails were found positives by virus isolation in tracheal and/or cloacal swabs throughout the experiment. Virus was detected in all inoculated quails for 5 - 9 days p.i. All in contact quails became infected from the 2^{nd} to the 3^{rd} day post contact (p.c.) until day 7-8 p.c.

In conclusion, no severe clinical signs were observed in the sentinels put in contact with challenged quails throughout the study. On the other hand the high viral replication in quails allowed efficient viral transmission to other quails. In fact, both LPAI viruses were transmitted to all contact birds in a short period (2 days post inoculation). This data suggests that both H7 LPAI viruses have the capacity to replicate efficiently and can be rapidly transmitted in quail.

Evaluation of efficacy of vaccination on clinical protection and viral shedding

Experimental design and key results

Three groups of ten quails each were vaccinated with three different commercial available vaccines. One vaccine contained H7N1 LPAI (A/CK/Italy/473/99) (vaccine A), one vaccine contained the virus A/CK/Mexico/232/94/CPA H5N2 subtype (vaccine B) and third vaccine contained the A/CK/Mexico/232/94/CPA H5N2 and NDV (LaSota strain) (vaccine C). A two shot vaccination (0.5 ml/bird) 21 days apart, was administered subcutaneously in the back of the neck to all quails at four weeks of age.
Sera were collected from all vaccinated birds 21 days post vaccination, every week for thirty five days after second vaccination and on day 14, 21 and 30 p.i.. AGID, commercial c-ELISA and HI were used for the detection of antibodies against AI.

Four weeks after the second vaccination 100 µl of a suspension containing 10^6 EID_{50} of LPAI H7N2 virus (A/quail/Italy/4610/2003) was used to challenge quails vaccinated with vaccine A via the ocular and oronasal route. The antigen used in the HI test was inactivated A/African Starling/983/79 (H7N1). With the same method quails vaccinated with vaccine B and C were infected with HPAI H5N1 (A/turkey/Turkey/2005). Tracheal and cloacal swabs were collected on day 4 post-challenge from each bird. Serum samples were collected every week for thirty days after challenge.

The results of the study indicate that a two-dose vaccination programme commencing at 4 weeks old, based on an inactivated, conventional vaccine registered for chickens, is not successful in preventing clinical signs and mortality and in suppressing shedding of viable virus. All vaccine titres were below the value considered protective for animals (1:32). The poor clinical and virological protection of vaccinated and infected quails is most probably due to the low antibody response induced by vaccination. This evidence suggests that the perpetuation of the infectious cycle may not be interrupted in quail by vaccination. For this reason vaccines and vaccination scheme must be re-evaluated in quails. This study highlights the necessity for the pharmaceutical companies to improve the quality of vaccines for avian species different from chicken. Currently, standard serological tests for AI do not permit to draw final conclusions on infection in quails. The results of the study indicate that low HI titres should not be underestimated and virological and serological tests must be associated.

Workpackage: 4.3

Partner: P3

Deliverable: D25 to D30

Data on vaccination of native chickens

Experimental design and key results

A commercially available oil-emulsion adjuvanted vaccine (Vaksiflu N2®, PT. Vaksindo) was used containing an inactivated low pathogenic H5N2 strain isolated from turkeys [A/turkey/England/N28/73(H5N2)]. The vaccine dosage was 0.5 ml per bird, containing 256 HAU per dose, and was administered intramuscularly in the breast muscle. Chickens were vaccinated at the age of 4 weeks and received a booster vaccination 3 weeks later.

The challenge virus H5N1 strain (A/chicken/Legok/2003), was used at 10^6 EID_{50} per ml. The protein homology of the haemagglutinin (HA1) of the vaccine strain and the challenge strain was 92%. Each inoculated bird received 0.1 ml inoculum which was administered intratracheally.

Two transmission experiments were carried out with groups of native chickens:

In Experiment 1, one group consisted of 20 unvaccinated birds, and the other group contained 20 vaccinated chickens. The aim of Experiment 1 was to quantify virus transmission in homogenous groups of chickens, homogenous with respect to vaccination status. Five unvaccinated sentinel layer type chickens were housed in the same room between the two experimental groups (physically separated from the vaccinated and unvaccinated groups) to detect whether virus transmission between the two groups of native chickens occurred.

Three weeks after the second vaccination, 10 unvaccinated and 10 vaccinated chickens were removed from their group and placed in two cages in a separate room; the vaccinated ones in one and the unvaccinated ones in another cage. These 20 birds were inoculated with H5N1 AI virus strain. After 24 h, these inoculated birds were re-united with the birds from their original group. The non-inoculated birds were subsequently exposed to the inoculated birds. Thus each group, vaccinated and unvaccinated, consisted of 10 inoculated birds and 10 contact-exposed birds, thus homogeneous with respect to vaccination status.

Experiment 2 was carried out in which 10 vaccinated birds were housed together with 10 unvaccinated birds. The unvaccinated birds were inoculated as before according to the method described in the previous section, and the vaccinated birds were contact-exposed to these inoculated ones. The aim of this experiment was to determine whether vaccinated birds would be

protected against virus transmission when exposed to a high virus load, excreted by unvaccinated pen mates.After inoculation, birds were kept and observed for 4 weeks.

The main results of this study indicate that native chickens develop substantial HI antibody titers directed against H5N1 upon vaccination with a heterologous H5N2 vaccine, and that this provides a level of protection that is generally sufficient to prevent a productive H5N1 infection. Moreover, the results provide a proof-of-principle that vaccination with a heterologous vaccine can reduce transmission levels of H5N1 influenza virus to the extent that no epidemics can occur. In addition, the unvaccinated native chickens were not resistant to infection, and showed signs of infection that are comparable to layer chickens infected with H5N1 virus.

Data from transmission experiments with Pekin Ducks after a single vaccination.

Experimental design and key results

Pekin ducks were vaccinated with an inactivated oil emulsion vaccine based on the strain A/Chicken/Mexico/232/94/CPA H5N2 (Intervet Schering-Plough Animal Health, The Netherlands). They were then challenged with A/Chicken/China/1204/04 (H5N1) according to the table below.

Treatment groups [a]	Age at vaccination	Age at challenge
Unvaccinated	na	8 weeks
Single vaccination, one week [b]	7 weeks	8 weeks
Single vaccination, two weeks [c]	6 weeks	8 weeks
Double vaccination, two weeks [d]	6 and 9 weeks	11 weeks

na, vaccination was not applied.

[a] Every treatment group consisted of two groups of 10 ducks.
[b] Challenge one week after vaccination.
[c] Challenge two weeks after vaccination.
[d] Challenge two weeks after the second vaccination.

Virus isolation, Real-time PCR, HI and ELISA were performed on samples and sera collected during the experiments in order to understand the infection dynamics. Contact ducks were included in the experiment in order to determine the reproduction ratio (ability to transmit) between birds.

In the unvaccinated groups several birds showed clinical symptoms. Two inoculated ducks died, and eight other birds showed depression and/or conjunctivitis. In all vaccinated groups there was a marked reduction in the number of birds that showed symptoms. In the groups that were challenged one week after a single vaccination only two ducks showed conjunctivitis. In the groups that were challenged two weeks after a single vaccination three ducks showed symptoms: one duck showed conjunctivitis, one duck had a swollen oropharynx (possibly due to the swabbing), and one duck showed conjunctivitis and a swollen oropharynx. In the groups that received a double vaccination two ducks showed conjunctivitis.

Virus was isolated from ducks that were challenged one week after a single vaccination as well as all of the contact birds. Challenging after two weeks from a single vaccination reduced the transmission dynamics (reproduction ratio) significantly. The best reduction in transmission of the virus was seen when ducks were doubly vaccinated and then challenged two weeks later.

Overall, the results demonstrate that a widely used H5N2 vaccine strain that has a HA1 protein homology of 84% with the H5N1 challenge virus not only prevented severe morbidity and mortality but also significantly reduced virus excretion and transmission of H5N1 in ducks two weeks after vaccination.

Effect of H7N1 vaccination on highly pathogenic avian influenza H7N7 virus transmission in turkeys

Experimental design and key results

Sixty four-week-old female turkeys were challenged by inoculation with highly pathogenic avian influenza H7N7 virus (A/Chicken/Netherlands/621557/03). The turkeys were challenged both intranasally (0.1 ml) and intratracheally (0.1 ml) with diluted allantoic fluid containing 10^6 median egg infectious dose (EID_{50})/ml.

Turkeys were vaccinated with an inactivated oil-emulsion vaccine, which was commercially available and contained H7N1 antigen (A/Chicken/Italy/99).

The birds were randomly divided into three parallel groups of 20 turkeys based on their wing clip identification number (group 0: 1–20, group 1: 21–40, group 2: 41–60). The control group did not receive vaccination. Group 1 was vaccinated once at 10 weeks of age and the third group was vaccinated twice; at 6 weeks and at 10 weeks of age. The three treatment groups were then each

divided into two groups of 10 animals, to form two replicates per treatment. Each subgroup was housed in a separate room. From each subgroup five contact animals were housed separately before the challenge. The other five birds were inoculated at 12 weeks of age. The contact birds were placed back with the inoculated birds in the same room the day after the challenge. At day 28 post challenge all remaining live birds were euthanized and the experiment was terminated.

Immunofluorescence using an N7-recombinant baculovirus, Real-time PCR, HI and ELISA were performed on samples and sera collected during the experiments in order to understand the infection dynamics. Contact turkeys were included in the experiment in order to determine the reproduction ratio (ability to transmit) between birds.

The data generated indicate that vaccination with H7N1 in turkeys was highly efficacious, Turkeys vaccinated once showed almost no virus shedding after challenge with H7N7 virus, and no transmission of the virus to vaccinated contact turkeys was observed. When the birds were vaccinated twice, no virus shedding was demonstrated at all after challenge, and hence transmission could not be quantified. Unvaccinated turkeys however, showed symptoms of illness, shed virus and were fully able to transmit the virus to susceptible contact turkeys. These results indicate that vaccination of turkeys could be useful as additional control measure to control an epidemic of HPAI as it was able to induce clinical protection and reduce virus shedding and virus transmission.

Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches (*Struthio camelus)*.

Experimental design and key results

Despite the inclusion of ostriches (*Struthio camelus*) in the World Organisation for Animal Health (OIE) and European Union (EU) definitions of "poultry" (Terrestrial Animal Health Code, 2008 and 1990/539/EEC), they are phylogenetically and thus anatomically and physiologically very different from conventional poultry. Scientific literature dealing with ostrich serology for the detection of avian influenza antibodies is limited and most of it does not report data on the sensitivity or specificity of the tests used, due to the lack of availability of appropriate samples. In this study 177 serum samples from ostriches (*Struthio camelus*) infected experimentally with A/ostrich/South Africa/Middleton/2004 (H5N2) HPAI virus were collected. Following challenge, almost all birds exhibited respiratory signs, such as conjunctivitis, ocular discharge, nasal discharge, tracheal foam, pharyngitis and coughing. A few birds showed diarrhoea. The average duration of clinical signs was 5 days with a range from 1 to 11 days. Eight birds showed clinical signs for a single day only. In general the birds recovered rapidly and despite the high dose of infection clinical signs were mild.

Test/method	Se and [CI]	Sp and [CI]	Cohen's K and p-value	p-value of McNemar test
HI with homologous ag without pre-treatment	81.39 [71.55; 88.98]	98.84 [93.69; 99.97]	K=80.23 p<0.05	p<0.05
HI with hetelogous ag without pre-treatment	27.91 [18.77; 38.62]	100 [95.80; 100*]	K= 27.91 p<0.05	p<0.05
HI with hetelogous ag and pre-treatment with 10% RBCs solution	50 [23.04; 76.96]	93.75 [69.77; 99.84]	K=44.95 p<0.05	p<0.10
Com. ELISA 1	91.86 [83.95; 96.66]	89.41 [80.85; 95.04]	K=82.28 p<0.05	p=NS
IZSVe ELISA	98.84 [93.69; 99.97]	86.05 [76.89; 92.58]	K=84.88 p<0.05	p<0.05
Com. ELISA 2	97.64 [91.76; 99.71]	93.02 [85.43; 97.40]	K=90.65 p<0.05	p=NS
AGID	91.86 [86.95; 96.66]	97.67 [91.85; 99.71]	K=89.53 p<0.05	p<0.10

Samples from these birds were tested using the haemagglutination inhibition (HI) test, the agar-gel immunodiffusion (AGID) test and three ELISA kits. The HI test, with homologous antigen and including pre-treatment of sera with 10% chicken red blood cells was considered as the gold standard. Detectable specific antibodies appeared on day 7 post infection and persisted until the termination of the experiment. The relative sensitivity and specificity of the tests under evaluation and Cohen's K value were calculated.

The results of this study give indications as to which tests can be used for serological diagnosis of AIV infections in ostriches. Validated ELISA tests for the detection of AI antibodies in ostriches sera can be considered suitable test for screening purposes and should be preferred to AGID. The HI test with pre-treatment with 10% chicken RBC suspension, and using the virus to which the birds have been exposed (or genetically and antigenically closely related with it) as the HA antigen remains a suitable test to monitor the circulation of AI virus of known subtype.

The results obtained will also be of assistance to decision makers in drafting guidelines for the definition of the health status of ostriches and for trade purposes.

Validation of diagnostic tests for detection of avian influenza in vaccinated chickens using Bayesian analysis.

Experimental design and key results

Vaccination against highly pathogenic avian influenza (HPAI) is one of the tools that can be used to prevent and control epidemics of HPAI. However, it is known that under certain circumstances vaccinated birds may still be infected and shed virus without showing clinical symptoms. This implies that there is a risk of silent spread of virus in vaccinated populations. Therefore, it is increasingly believed that vaccination programs should always be accompanied by active surveillance.

Virus detection in vaccinated birds has the disadvantage that it is only successful in the acute phase of the infection. Therefore, serological DIVA tests are promising because birds can be randomly selected from a vaccinated flock and antibodies might persist for a long time.

The characteristics of three serological tests (immunofluorescent antibody test (iIFAT), neuraminidase inhibition (NI) assay, and NS1 ELISA) that are able to differentiate infected from vaccinated animals were investigated. Data of H7N7 infection experiments wereanalyzed using Bayesian methods of inference. These Bayesian methods enable validation of the tests in the absence of a gold standard, and allow one to take into account that infected birds do not always develop antibodies after infection. The results showed that the N7 iIFAT and the NI assay have sensitivities for detecting antibodies of 0.95 (95% CI: 0.89–0.98) and 0.93 (95% CI: 0.78–0.99), but substantially lower sensitivities for detecting infection: 0.64 (95% CI: 0.52–0.75) and 0.63 (95% CI: 0.49–0.75). The NS1 ELISA has a low sensitivity for both detecting antibodies 0.55 (95% CI: 0.34–0.74) and infection 0.42 (95% CI: 0.28–0.56). The estimated specificities of the N7 iIFAT and the NI assay are 0.92 (95% CI: 0.87–0.95) and 0.91 (95% CI: 0.85–0.95), and 0.82 (95% CI: 0.74–0.87) for the NS1 ELISA.

According to this analysis, not all infected animals in the population produce antibodies, which implies that improving the tests in detecting antibodies will only have a limited effect on the sensitivity in detecting infection. However, because the likelihood of producing antibodies is associated with the duration of virus excretion, the birds missed by the test are probably not the birds contributing most to the spread.

It is concluded that the N7 iIFAT and the N7 NI assay are useful tests in detecting silent transmission, because the birds that are most significant in spreading virus will be detected. On the other hand, due to the lower specificity, the N7 iIFAT and the N7 NI are less suitable for declaring freedom of disease after vaccination.

Deliverables List for whole project

Del. no.	Deliverable name	WP no.	Status	Dissemination level	Lead contractor
D1	Active and updated web-site	1	√	PU	P1
D2	Annual Report	1	√	CO	P1
D3	Annual Report	1	√	CO	P1
D4	Identification and procurement of funding for staff exchange	1	√	PU	P1
D5	Staff exchange between partners	1	√	PU	P1
D6	Final scientific report to the EU	1	√	PU	P1
D7	Final administrative report to the EU	1	√	CO	P1
D8	Identification of prototype strains for an EU vaccine bank	2	√	PU	P1
D9	Development and validation of anti-neuraminidase antibody detection ELISAs for N1-N2-N3 and N7 in different avian species	2	√ **Not for H7**	PU	P1, P3, P10
D10	Data on the presence of AI in meat of vaccinated versus unvaccinated animals	2	√	PU	P1
D11	Data on the occurrence of antigenic drift in H7 isolates of the Eurasian lineage	2	√	PU	P1
D12	Evaluate prototype LFD for AI antigens in trials and define performance criteria	3	√	PP	P9
D13	Completed LFD optimisation and antibody evaluation and final prototype selected	3	√	PP	P2 + P14
D14	Supply LFD and guidance notes to all laboratories for final evaluation	3	√	PP	P2, P14
D15	Rapid penside tests for H5/H7 AIVantigens	3	**X**	PP	P2
D16	Rapid penside Real Time RT-PCR for H5/H7 and generic AI detection	3	√	PP	P2

D17	Protocol for testing targeting of influenza virus to dendritic cells and\or macrophages	4	√	PU	P7
D18	Protocol for determining the capacity of influenza virus and/or vaccine to induce type I interferon and TNF-α production *in vitro*	4	√	PU	P7
D19	Test protocol for analysing influenza virus isolates in terms of altered tropism for NeuAcα 2,3-gal (mainly avian viruses) and NeuAcα 2,6-gal (mainly human/porcine isolates)	4	√	PU	P7
D20	Available influenza viruses of current interest analysed for their capacityto induce type I interferon and TNF-α production *in vitro*	4	√	PU	P7
D21	Genetic factors that determine cell tropism	4	√	PU	P7
D22	Data on the incubation time of selected AI virus infection in ducks and quail	4	√	PU	P1 + P3
D23	Data on the occurrence of vertical transmission by infecting muscovy ducks	4	√	PU	P4
D24	Data on the pathogenesis of AI in quails	4	√	PU	P1
D25	Parameters that describe transmission dynamics in groups of ducks	4	√	PU	P3
D26	Parameters that describe transmission dynamics in groups of indigenous chicken breeds	4	√	PU	P3
D27	Improved AI surveillance methodology in waterfowl and quail	4	√	PU	P1, P3, P4
D28	Efficacy of vaccination on virus transmission within a group of ducks	4	√	PU	P3
D29	Data that can be used for modelling the efficacy of vaccination in the field	4	√	PU	P1, P2, P3, P4
D30	Reagents for evaluation of DIVA tests and ready to use, on-site tests and real time RT-PCRs	4	√	PP	P1, P2, P3, P4

Section 4 – Using and disseminating the knowledge

Dissemination of the knowledge arising from the project

Workpackage 1

Title	FLUAID website
WP	1
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientist, Stakeholders, Decision makers, Public)
Note	Project website www.fluaid.eu was established in June 2006 and is updated regularly. It will be maintained for 18 moths beyond the conclusion of the project.

Title	The EU project FLUAID
Authors	WG Dundon I Capua
Citation	Third Annual EPIZONE meeting, Antalya, Turkey, 12-15 May (2009)
Citation type	Oral presentation
WP	1
Partner(s)	P1
Type of Audience	Medium (EU Veterinarians, Scientist, Decision makers)

Title	Season's Greetings Card
Description	A greeting card highlighting the deliverables of FLUAID and inviting colleagues to visit the website – December 2009
WP	1
Partner(s)	All Partners
Type of Audience	Large (International Veterinarians, Scientist, Stakeholders and Decision makers)

Supporting the Management of the Avian influenza Crisis

The FLUAID project "*Generation of information and tools to support the management of the avian influenza crisis in poultry*" SSPE-CT-2005-022417 has concluded as of the 31st of October 2009.

The main achievements of this 13 - partner international consortium are:

- Assessment of the risk of transmission of highly pathogenic avian influenza from infected meat.
- Quantification of viral transmission in ducks and turkeys infected experimentally and the effect of vaccination thereon.
- Development of companion diagnostic tests based on anti-N1, N2 and N3 detection systems.
- Evaluation of antigenic drift and genetic variability of Eurasian lineage H7 isolates.
- Evaluation of rapid antigen detection pen-side tests for avian influenza.
- Interaction of avian influenza with host interferon producing cell types.
- Identification of suitable H5 and H7 subtype viruses as candidates for an EU vaccine bank.
- Studies on the occurrence of vertical transmission of H5 viruses in ducks.
- Determination of the role of quail as a silent reservoir of infection.
- Evaluation of various serological tests for the detection of antibodies against highly pathogenic avian influenza in ostriches infected experimentally.

We invite you to visit the Project website at www.fluaid.eu for further information.

Workpackage 2

Title	Generation of avian influenza reassortant viruses of the H7N5 subtype as potential vaccine candidate to be used in the framework of a "DIVA" vaccination strategy.
Authors	Beato, MS, Rigoni, M., Milani, A., Capua I.
Citation	Avian Diseases Special issue. 51; 479-480 (2007)
Citation type	Peer-reviewed publication
WP	2
Partner(s)	P1
Type of Audience	Large International (Veterinarians and Scientist)

Title	Conventional inactivated bivalent H5/H7 vaccine prevents viral localisation in muscles of turkeys infected experimentally with LPAI and HPAI H7N1 isolates.
Authors	Toffan, A., Beato, MS., De Nardi, R., Bertoli, E., Salviato, A., Cattoli, G., Terregino, C., Capua, I.
Citation	Avian Pathology Aug;37(4):407-12 (2008)
Citation type	Peer-reviewed publication
WP	2
Partner(s)	P1
Type of Audience	Large International (Veterinarians and Scientist)

Title	Avian influenza viruses in poultry products: a review.
Authors	Beato, M.S., Alexander, D.J., Capua, I.
Citation	Avian Pathology, 28, 193-200. (2009)
Citation type	Peer-reviewed publication
WP	2
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientist, Decision makers)

Title	Vaccination prevents viral colonization of muscles in experimentally infected turkey challenged with highly pathogenic and low pathogenicity avian influenza viruses of the H7N1 subtype.
Authors	Toffan, A., Beato, M.S, De Nardi, R., Bertoli, E., Cattoli, G., Terregino, C., Capua, I.
Citation	Vaccination – a tool for the control of avian influenza, Verona, Italy 20-22 March 2007
Citation type	Poster presentation
WP	2
Partner(s)	P1
Type of Audience	Medium International (Veterinarians, Scientists, Decision makers)

Title	Evaluation of an avian influenza natural reassortant (H7N5) as a candidate vaccine strain to be used in the framework of a "DIVA" strategy.
Authors	Beato, M.S., Toffan, A., De Nardi, R. Masiero, E. Bertoli E. Capua I.
Citation	Vaccination – a tool for the control of avian influenza, Verona, Italy 20-22 March 2007
Citation type	Poster presentation
WP	1
Partner(s)	P1
Type of Audience	Medium International (Veterinarians, Scientists, Decision makers)

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Title	Assessment of viral colonization of muscles following experimental challenge with HP and LP viruses in vaccinated and unvaccinated turkeys
Authors	Toffan, A., Beato, M.S., De Nardi, R., Terregino, C., Salviato, A., Ormelli, S., Cattoli, G., Capua, I.
Citation	56th Western Poultry Conference, Las Vegas 17-29 March 2007
Citation type	Poster presentation
WP	2
Partner(s)	P1
Type of Audience	Medium International (Veterinarians, Scientists, Decision makers)

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Title	The control of avian influenza in poultry.
Authors	Toffan, A.
Citation	XVIII European Symposium on the Quality of Poultry Meat and XII European Symposium on the Quality of Eggs and Egg products of WPSA, Prague (CH) 3-4/09/2007
Citation type	Oral presentation
WP	2
Partner(s)	P1
Type of Audience	Medium (EU Poultry breeders and veterinarians)

Title	A proof of principle study to identify suitable vaccine seed candidates to combat introductions of Eurasian lineage H5 and H7 subtype avian influenza viruses
Authors	Beato, M.S., Monne, I, Mancin, M, Bertoli, E., Capua I.
Citation	Submitted to Avian Pathology
Citation type	Peer-reviewed publication
WP	2
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientists, Decision makers)

Title	The evolutionary trajectory of H7N3 influenza in turkeys
Authors	Beato, M.S., de Jong, M. C. M., Fusaro, A., Capua I.
Citation	in preparation
Citation type	Peer-reviewed publication
WP	2
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientists, Decision makers)

Workpackage 3

Title	Diagnosis of Avian influenza poultry outbreaks: considerations for RRT-PCRS and rapid penside testing with lateral flow devices.
Authors	Slomka, M.J., Coward, V.J., Irvine, R., Pavlidis, T., Aly, M.M., Hassan, M.K.A., Flynn, O., Raleigh, P.J., Kerr, L. Banks, J., Brown, I.H.
Citation	7th International Symposium on Avian influenza, Athens, GA, USA; 5-8 April (2009)
Citation type	Oral Presentation
WP	3
Partner(s)	P2, P15
Type of Audience	Medium International (Veterinarians, Scientist, Decision makers)

Title	Problems with experimental approaches for evaluations of commercially available lateral flow devices for rapid penside detection of avian influenza viruses.
Authors	Slomka, M.J., Coward, V.J., Pavlidis, T., Kerr, L., Mahmood, S., Banks, J., Brown, I.H.
Citation	7th International Symposium on Avian influenza, Athens, GA, USA; 5-8 April. (2009)
Citation type	Poster presentation
WP	3
Partner(s)	P2
Type of Audience	Medium International (Veterinarians, Scientist, Decision makers)

Title	Evaluation of lateral flow devices (LFDs) for penside detection of avian influenza. Initiative from FLUAID."
Authors	Slomka M,
Citation	First Annual Epizone Meeting in Lublin 30/5/07.
Citation type	Oral presentation
WP	3
Partner(s)	P2
Type of Audience	Medium (EU Veterinarians, Scientist, Decision makers)

Workpackage 4

Title	An inactivated H5N2 vaccine reduces transmission of highly pathogenic H5N1 avian infleunza among native chickens.
Authors	Poetri, ON, Bouma, A, Murtini, S, Claassen, I, Koch, G., Soejoedono, RD, Stegmen, JA, vanBoven, M.
Citation	Vaccine, 11(27), 2864-2869 (2009)
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P2
Type of Audience	Large International (Veterinarians and Scientist)

Title	The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin (Anas platyrhynchos) ducks infected experimentally.
Authors	Löndt, B.Z., Nunez, A., Banks, J., Alexander, D.J., Russell, C., Richard- Löndt, A. C., Brown, I. H.
Citation	Influenza and Other Respiratory Viruses. 4, 17-25 (2009)
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P2
Type of Audience	Large International (Veterinarians and Scientist)

Title	Pathogenesis of Highly Pathogenic Avian Influenza (HPAI) A/turkey/Turkey/1/2005 H5N1 in Pekin ducks (Anas platyrhynchos) infected experimentally.
Authors	Löndt, BZ, Nunez, A, Banks, J., Nili, H, Johnson, LK, Alexander DJ
Citation	Avian Pathology. 37(6), 619-627 (2008)
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P2
Type of Audience	Large International (Veterinarians and Scientist)

Title	Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine.
Authors	van der Goot JA, van Boven M, Stegeman A, van de Water SG, de Jong MC, Koch G.
Citation	Virology. 382(1):91-7 (2008)
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P3
Type of Audience	Large International (Veterinarians and Scientist)

Title	Effect of H7N1 vaccination on highly pathogenic avian influenza H7N7 virus transmission in turkeys.
Authors	Bos ME, Nielen M, Koch G, Stegeman A, De Jong MC
Citation	Vaccine. 25;26:6322-8. (2008)
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P3
Type of Audience	Large International (Veterinarians and Scientist)

Title	Effect of vaccination on transmission of HPAI H5N1: The effect of a single vaccination dose on transmission of Highly Pathogenic Avian Influenza H5N1 in Peking Ducks.
Authors	Van der Goot, JA, Van Boven, M, De Jong, MCM, Koch, G.
Citation	Avian Diseases Special issue. 51; 323-324 (2007)
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P3
Type of Audience	Large International (Veterinarians and Scientist)

Title	Virus H5 FP : a priori pas de contamination verticale
Authors	Picault J-P., Amelot M., Lamandé J., Oger A., Allée C., Le Bras M-O., Ogor K., Guillemoto C., Pierre I., Le Coq L., Bonnion D., Le Coq T., Le Moal L., Mangart J-M., Jestin V.
Citation	Filières Avicoles, 706, 90-92 (2008)
Citation type	Poultry sector publication
WP	4
Partner(s)	4
Type of Audience	Small (French Poultry breeders and veterinarians)

Title	Comparative study on clinical behaviour and shedding levels in commercial Japanese quails (*Coturnix coturnix japonica*) and SPF chickens experimentally infected with low pathogenicity avian influenza viruses of the H7 subtype.
Authors	De Nardi, R, Toffan, A., Beato, M.S., Cilloni, F., Bertoli, E., Salviato, A., Cattoli, G., Capua, I., Terregino, C. (2008)
Citation	BirdFlu2008: Avian influenza and human health, St. Hilda's College, Oxford, UK. 10-11th Sept.
Citation type	Poster presentation
WP	4
Partner(s)	P1
Type of Audience	Medium International (Veterinarians, Scientists)

Title	Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches (Struthio camelus).
Authors	Toffan, A, Olivier, A, Mancin, M, Tuttoilmondo, V, Facco, D, Capua, I, Terregino, C..
Citation	Avian pathology (2010) 39, 11-15.
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P4
Type of Audience	Large International (Veterinarians and Scientist)

Title	Validation of diagnostic tests for detection of avian influenza in vaccinated chickens using Bayesian analysis.
Authors	Van der Goot, JA, Engel, B., van de Water, S.G.P., Buist, W., De Jong, M.C.M, Koch, G., van Boven, M., Stegeman, A. (2010)
Citation	Vaccine (2010) in press
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P4
Type of Audience	Large International (Veterinarians and Scientist)

Title	Comparative study on clinical behaviour and shedding levels in commercial Japanese quails (*Coturnix coturnix japonica*) and SPF chickens experimentally infected with low pathogenicity avian influenza viruses of the H7 subtype.
Authors	De Nardi, R, Toffan, A., Beato, M.S., Cilloni, F., Bertoli, E., Salviato, A., Cattoli, G., Capua, I., Terregino, C. (2010)
Citation	in preparation
Citation type	Peer-reviewed publication
WP	4
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientists, Decision makers)

NB: There are a number of publications in preparation by the various partners. Once published they will be cited on the FLUAID website.

Additional Publications citing FLAUID

Title	A closer look at the NS1 of influenza virus (Review).
Authors	Dundon, W.G., Capua, I
Citation	Viruses 1, 1057-1072 (2009)
Citation type	Peer-reviewed publication
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientists)

Title	Reassortant Avian Influenza virus (H5N1) in poultry, Nigeria, 2007.
Authors	Monne, I., Joannis, T. M., Fusaro, A., De Benedictis, P., Lombin, L. H., Ularamu, H., Egbuji, A., Solomon, P., Cattoli, G., Capua, I
Citation	Emerging Infectious Diseases 14 (4) (2008)
Citation type	Peer-reviewed publication
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientists)

Title	Genome analysis links recent European and African H5N1 influenza viruses.
Authors	Salzberg, S.L., Kingsford, C, Cattoli, G, Spiro D.J., Janies D.A., Aly M.M. , Brown I.H., Emmanuel Couacy-Hymann, E., De Mia G.M., Dung D.H., Guercio, A, Joannis, A., Maken Ali, A.S., Osmani, A, Padalino, I, Saad, M.D., Savić,V., Sengamalay, N.A, Yingst, S., Zaborsky, J, Zorman-Rojs, O., Elodie Ghedin and Capua, I
Citation	Emerging Infectious Diseases 13:713-8 (2008)
Citation type	Peer-reviewed publication
Partner(s)	P1
Type of Audience	Large International (Veterinarians, Scientists)

Photos of clinical signs and post-mortem lesions of Avian influenza. collected during the experiments funded by FLUAID were used for:

1) Atlas of transboundary animal diseases. This replaced an older publication by the OIE known as the "Illustrated Manual for the Recognition and Diagnosis of Certain Animal Diseases", volumes 1 and 2.

2) EPIZONE. A database with pictures, slides, videos or other material. which show clinically diseased animals, pathological or pathomor-phological changes or electronmicrographical pictures of virus particles. This material shall be made accessible in a web-accessible library via the EPIZONE homepage. http://www.epizone-eu.net/cps/databases/Disease%20images/Forms/AllItems.aspx

3) E-LEARNING COURSE for the continuing education of veterinarians at the Institute of Virology, University of Veterinary Medicine Hannover, Germany. The Institute was involved in a project. funded by the European Union, concerning the control of epidemics in livestock.

4) Avian Influenza and Newcastle Disease: A Field and Laboratory Manual. Capua I. & Dennis J. A. Springer Verlag. 2008.

5) E-LEARNING COURSE "Gestione di una emergenza epidemica di influenza aviaria" for the continuing education of Italian veterinarians. The aim of this course was to assist veterinarians in managing outbreaks and surveillance programmes for avian influenza.

Coordinator's acknowledgements

I would like to thank Roberta Bassan, Sergio Bazan, Alessandro Bertoldi and Giovanni Meneghetti for their support and advice on financial issues. I would also like to thank William Dundon for his management skills and efforts.

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