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Final report

Project

Control and characterisation of highly pathogenic avian influenza (HPAI) strains in poultry in Indonesia

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2 Executive summary

The project was developed in 2006 at the time when H5N1 HPAI had spread throughout Indonesia, control was limited and outbreaks were common in a wide number of avian species. The project objectives aimed to address some of the most pressing issues predominant at the time these being: efficacy of inactivated AI vaccines in commercial chickens; characterisation of AI isolates from vaccinated commercial poultry; development of new reagents for DIVA testing; development of a simple state-transition model of HPAI infection and provide specialised training in molecular approaches for characterisation of viruses and for generation of diagnostic reagents.

The project was a collaboration between the University of Melbourne School of Veterinary Science and the Indonesian Research Center for Veterinary Science with participation of the Australian Animal Health Laboratory and the AusVet Pty Ltd. The project commenced in late 2007 and objectives were revised in mid 2008 due to commencement of other projects in Indonesia, in particular the FAO/OFFLU project on characterisation of AI isolates from vaccinated commercial poultry.

Following the incursion of H5N1 into Indonesia in 2003, the Indonesian Government opted for vaccination as a control measure and, in line with the FAO/OIE global strategy for HPAI control, allowed the use of heterologous AI vaccines only. At that time 21 different AI vaccines were registered for use, however limited information was available on their effectiveness. To assess the efficacy of some of these vaccines we developed surveillance questionnaire and initially conducted asurveillance study on broiler and layer farms from commercial Sector 3 in an area with a high concentration of poultry. Results were entered and managed using a newly developed web based database tool, and showed that in this sector none of the farms surveyed had suffered HPAI outbreaks since 2007 in spite of outbreaks reported in these districts. Only one of the broiler farms practiced AI vaccination, the remainder relying entirely on biosecurity for protection. All layer farms used vaccination with no uniform regime; on 44 farms, 19 different AI vaccines were used, most of H5N2 subtype with some using recently introduced H5N1. In a longitudinal study that involved 15 layer farms from the same sector, the level of protective immunity afforded by H5N2 vaccines used was not adequate against the majority of H5N1 strains. The results further suggested that vaccines based on the local H5N1 strains were superior and provided protective immunity against most, but not all, Indonesian H5N1 antigenic variants. Hence the data from this study do not support the use of heterologous H5N2 vaccines in Indonesia.

Following introduction of vaccination in Indonesia the use of a DIVA strategy to monitor vaccination programmes was also recommended basd on the principle that the test can ***D***ifferentiate Infected from ***V***accinated ***A***nimals. This mirrors the use of the test in effective eradication programs elsewhere. Different approaches were taken in DIVA test development including assessment of the neuraminidase, NS1 and M2e proteins as detecting reagents. These proteins were produced either by purification from the virus or recombinant means and the antigenicity of expressed proteins was found to be adequate. The specificity of various DIVA tests was evaluated on reference and field sera and a test based on either the recombinant or peptide M2e was selected as a test of choice. Limited evaluation of this test in commercial flocks indicated AI challenge in the absence of overt outbreaks.

A model to describe the spread of HPAI in the poultry sector and predict the impact of vaccination programs on the incidence of disease was conducted and found to be functionally sound and as the basis of future modelling scenarios. Transfer of technology was successfully achieved in the area of recombinant antigen expression, antigenic and genetic characterisation of AI viruses and antigens, and biosecurity principles. Project outputs have immediate and also long-term applications in Indonesia, as well as globally,

and are expected to contribute to the framing of national strategies for improvements of AI control measures.

3 Background

In Indonesia outbreaks of HPAI in poultry were first notified in August 2003. Initial cases were not contained and HPAI H5N1 virus has since spread throughout the country, with 31 out of 33 provinces now affected. Significant mortalities that occurred early following introduction have been replaced by endemic circle with H5N1 infections reported in chickens, ducks and quails. H5N1 virus has also infected humans with Indonesia having the highest number of cases, 166 from 500 total reported worldwide, with fatality rate of 82% (137/166). These infections are of particular concern because of the severity of the disease, the high mortality rates and the possibility of further virus evolution and a human pandemic. The source of H5N1 influenza virus infections for the majority of human cases in Indonesia has been linked to direct contact with poultry. The total population of poultry is 1.4 billion, consisting of 320 million native (village) chickens, 95 million layers, 970 million broilers and 34 million ducks. The majority of these birds are in Java and Sumatra (62% and 18% respectively). The poultry are kept in either modern commercial systems (Sectors 1 and 2) or traditional village and backyard systems (Sectors 3 and 4).

Vaccination has been adopted as the principal control strategy in Indonesia and is anticipated to become a long-term solution for the Indonesian poultry industry. While this approach is in line with the FAO Global Strategy for Control of HPAI, it has been recognised that there are a number of local factors that differ from those in developed countries influencing, to a large degree, the success of H5N1 control. Of the countries in Asia that have been affected by HPAI, only Indonesia, China and Vietnam have adopted vaccination to control the H5N1 virus.

Following initial outbreaks of H5N1 in Indonesia in 2003, inactivated vaccines based on H5N1, H5N2, H5N7 or H5N9 subtypes were imported for use largely in Sectors 1 and 2 comprising large scale production units. In late 2005 a plan for the local production of 300 million doses of heterologous H5N2 inactivated vaccines was released. For that purpose two H5N2 strains (from Mexico and the UK) were imported for two vaccine manufacturers. In 2007 the Central Government approved the manufacture of H5N1 vaccine based on a strain isolated in 2003 and in 2009 new H5N1 vaccine strains based on WJ/PWT-Wij/06) were also approved for use. At present 24 imported or locally manufactured AI vaccines are registered for use in Indonesia. To date there are no field data to support the effectiveness of any of these vaccines. In some instances, inappropriate vaccines have been used, leading to disenchantment with vaccination and refusal to vaccinate, particularly in Sector 4, the smallholder sector. In 2008 a Laboratory trail conducted in the USA using several Indonesian H5N1 isolates showed that one of the H5N2 vaccines did not protect against strain WJ/PWT-Wij/06 isolated in 2006.

Influenza viruses are segmented RNA viruses that are divided into types A, B and C on the basis of their internal nucleocapsid (NP) and matrix (M1) proteins. Only type A influenza viruses cause natural infections of birds. The virus genome consists of 8 RNA segments that code for ten viral proteins (Steinhauer & Skehel, 2002). On the virion surface are haemagglutinin (H), neuraminidase (N), and matrix (M2) proteins. The internal proteins are three polymerase proteins, PA, PB1 and PB2, the nucleoprotein (NP), the matrix protein (M1) and the non-structural protein NS1 and NS2. The non-structural protein NS1 is produced during infection, but it is not packaged within the virus particle. Antibodies to H are the main determinant of protection, with at least 5 antigenic sites that are capable of producing neutralising antibodies. Variation in any of these antigenic sites enables the virus to escape vaccinal immunity. Antibodies to the N are thought to be of lesser importance, but greater protection is achieved in animals immunised with both H and N. Antibodies are also made to the internal NP and M1 proteins and because these proteins are conserved within all type A influenza viruses, they are used as target antigens in serological assays.

Influenza viruses undergo frequent changes. Antigenic drift, which results from the accumulation of mutations in the antigenic domains of H, is the most common mechanism. It is believed that this type of change is selected within immune populations. In poultry, immunity against AI did not exist until recently as all incursions of LPAI and HPAI into poultry were eradicated. Mutations due to immunological pressures have been documented in countries where vaccination has been practised (Mexico and Pakistan) (Lee *at al* 2004; Swayne & Kapczynski 2008).

Phylogenetic analysis, based on sequences of H and N genes, can indicate relationships between different AI virus strains. For example the origin of the H5N1 virus could be traced to a virus prevalent in aquatic birds in South China however following its spread, H5N1 has evolved in a separate direction in Indonesia from that of the H5N1 viruses in other Asian countries (Chen *et al* 2006). This reflects different adaptations of H5N1 viruses to the ecological niches in which they replicate. Sequencing of six other influenza virus genes enables can detect if a virus is a mixture of two influenza strains and if re-assortment has taken place, an event that may facilitate movement of influenza viruses across a species barrier. Of the three pandemic viruses that emerged in the 1918 (H1N1), 1957 (H2N2) and 1968 (H3N2), the latter two arose by re-assortment of two influenza viruses. The concern at present is that there is an immense reservoir of H5N1 virus in poultry that mutate rapidly but can also recombine and acquire genes from other influenza viruses such as H1N1 currently circulating in pigs in Indonesia. Therefore the consequences of the long-term persistence of H5N1 in poultry are difficult to predict, particularly its potential as a threat to public health.

During project implementation the following projects were ongoing in Indonesia: (i) An ACIAR funded project on the epidemiology of AI in ducks (AH/2004/040); (ii) an Indonesian - Dutch Government bilateral programme on technical assistance to national and regional institutions dealing with AI control, including support for production of AI vaccines: (iii) FAO-Indonesian Government surveillance project to monitor H5N1 in Sector 4; (iv) Indonesian Government funded AI surveillance in Sector 4 that included H5N1 strain characterisation. Hence the aims of this project did not overlap or duplicate any other major research being undertaken on HPAI in Indonesia.

Significance for Australia

Australia is free from HPAI and incursion and spread of HPAI strains into local poultry would represent a significant threat to the profitability and sustainability of the local poultry industry. The Australian Government has invested significant resources in upgrading Australian readiness to respond to HPAI incursion into local poultry and in the case of human pandemic. Since the presence of H5N1 influenza virus in Indonesia is a potential source for transmission to Australia, the Australian Government, public and poultry sectors have been vitally interested in the achievement of effective control of H5N1 influenza virus in Indonesia. To that end, the Australian Government has provided support for Indonesia's activities in both human and animal sectors through its various agencies, aiming to strengthen its technical and response capability and learn from the strategies and outcome put in place.

4 Objectives

The overall aims of the project were to determine efficacy of AI control in Indonesia in the semi-intensive poultry sector and develop a simple test for discrimination of infected and vaccinated poultry.

The specific objectives were to:

1. Assess the efficacy of various inactivated AI vaccines and vaccination regimes in preventing shedding and transmission of H5N1 infections in commercial chickens.

2. Develop reagents for DIVA testing.

3. Develop sustainable protocols for investigating vaccine efficacy for HPAI in Indonesia.

4. Characterise at genetic and antigenic levels AI isolates from vaccinated commercial poultry.

5. Provide specialised training and capacity building for Indonesian scientists in molecular approaches for characterisation of viruses and for generation of diagnostic reagents.

6. Develop a simple state-transition model of HPAI infection in commercial and/or village flocks and validate the model against real outbreaks.

7. Communicate and disseminate results to the relevant stakeholders.

5 Methodology

Objective 1. *To assess the efficacy of various inactivated AI vaccines and vaccination regimes in preventing shedding and transmission of H5N1 infections in commercial chickens*

In Indonesia at least 24 different vaccines are available for control of AI. However a limited amount of information exists on the effectiveness of most of these vaccines against challenge with Indonesian H5N1 (Swayne *et al* 2006). Consequently poultry producers have been facing a difficulty choice of which vaccine to use and are guided by factors other than vaccine efficacy. The prevailing field situation in all poultry sectors has been signalling that the efficacy of some, if not most, vaccines is not as good as indicated by laboratory studies carried out under controlled conditions.

Many factors influence AI vaccine efficacy including how closely vaccines match the antigenic type of the prevailing field virus, if sufficient antigenic mass is present in a given dose, timing and frequency of vaccination, how well other pathogens are controlled, and not least, the manner in which vaccine has been handled before and during vaccination. Laboratory studies with two H5N2 vaccines of Mexican and European origin, used frequently for vaccination in Indonesia, showed that they protected chicks against mortality and clinical disease, however significant proportion of the vaccinated birds did shed virus following challenge with an Indonesian H5N1 virus (Swayne *et al* 2006). Shedding of AI virus, particularly if in large amounts, almost always leads to genetic and antigenic changes in circulating field viruses, necessitating frequent vaccine changes. (van den Berg *et al* 2008).

Our hypothesis was that in intensive commercial Sector 1 and 2, where vaccination is frequent, the likelihood of vaccine failures and the emergence of new AI variants is the highest. Furthermore that in Sector 1 companies using different AI vaccines either homologous or heterologous, there will be different changes detected in circulating H5N1, eventually leading to establishment of H5N1 of distinct lineages.

This objective was realised through collaboration with three commercial companies from Sectors 1 and 2, and research conducted at Bbalivet through the following activities:

Activity 1. *Source inactivated vaccines, reagents and test kits.* For laboratory vaccination trails vaccines based on H5N2, H5N9 and H5N1 strains were to be of the same batch as used for vaccination of commercial flocks and were to be sourced directly from collaborating companies insuring data from field and Laboratory vaccinations could be compared. Vaccination response was to be measured using the HI and SN tests and H5N1 strains from 2003 (BL/03) and 2006 (WJ/PWT/06) that differ genetically and antigenically. Monoclonal antibodies that are able to antigenically differentiate H5N1 isolates were to be sourced from a Laboratory in Hong Kong and used to determine variation in Indonesian H5N1 strains.

Activity 2. *Establish collaboration with the Dutch group conducting trials with the H5N1 vaccine*. At the time of the project inception, an Indonesian - Dutch Government bilateral programme on HPAI control (2005 – 2009) had commenced. Part of this project also anticipated laboratory and field trials with a locally produced H5N1 vaccine to determine its effectiveness. The intention was to ascertain that this project uses the same test procedure, method of vaccination and sampling to enable comparison of results and to tailor field and laboratory trials so that they do not overlap in their aims and outcomes.

Activity 3. *Undertake baseline trials in laboratory*. The aim was to obtain baseline data when vaccination was conducted in the laboratory, under controlled conditions, and without challenge with other field pathogens, such as vvNDV and vvIBDV that are common and significant disease burden in Indonesia. Day-old layer chicks were to be sourced from collaborating companies, transferred to experimental isolation rooms at

Bbalitvet and vaccinated with the same vaccine, and at the same intervals, as used by collaborating commercial companies. For each vaccine there were to be 7 groups with 30 chicks, 6 groups to be vaccinated either 1, 2, 3, 4, 5 or 6 times, with one group non-vaccinated. Chicks were to be bleed 4 weeks post vaccination and just prior to each vaccination and antibody titres determined using HI and SN tests with at least two antigenically different H5N1 strains and prevalent in the region; sera were also to be tested in DIVA test. At the end of vaccination, 20 chicks in each group were to be challenged with two different H5N1 strains to determine level of protection afforded by each vaccines when given between 1 and 6 times.

Activity 4. Undertake baseline collection of data on passive and active antibody status as well as infectious status in vaccinated commercial poultry at different locations. Intention was to follow at least one layer flock from each of three different commercial companies, located in different parts of Java, which use different AI vaccines. Flocks were to be vaccinated as per company vaccination protocols, with husbandry and biosecurity as practiced by the company. Flocks were to be sampled just prior to each vaccination, and 4 weeks after vaccination and cloacal swabs collected at the time. Sera were to be tested for AI antibodies by HI and SN tests using two H5N1 strains, and also in DIVA test, and NDV and IBDV antibody titres also determined. Swabs were to be tested by RT-PCR for the presence of H5N1 virus followed by virus isolation from RT-PCR positive samples.

Activity 5. *Undertake laboratory challenge trails in vaccinated layers*. At the end of vaccination trails each group of chicks was to be challenged with BL/03 and WJ/PWT/06 strains to determine level of protection afforded by each vaccine when given between 1 and 6 times. Challenge experiments were to be conducted in Bbalivet BSL3 facility planned to be operational in 2008. In case a variant H5N1 was isolated during the field vaccination trails, such virus was to be used preferentially for challenge.

Objective 2. To *develop reagents for DIVA testing*

Initial outbreaks of N5N1 in Indonesia were not controlled allowing for rapid spread of virus throughout bird populations and becoming endemic in domestic and commercial poultry. In 2004 the Indonesian Government adopted vaccination as a strategy for control of disease that was in line with the FAO Global Strategy for Control of HPAI. Essence of this strategy is based on the use of heterologous inactivated AI vaccines that allow identification of vaccinated chicks that are infected following field challenge. Accordingly Government of Indonesia approved the use of vaccines developed form heterologous subtypes such as H5N2, H5N7 or H5N9 anticipating the use of DIVA test to monitor the freedom of flocks from H5N1 and leading to eventual eradication.

The DIVA strategy is applicable in a number of disease control scenarios, however each requires an appropriate DIVA test; hence there could be a number of DIVA tests each targeting different part of the virus. In the case of Indonesia where initially only heterologous inactivated vaccines where approved, choice of DIVA tests was restricted to detecting either the N1, NS-1 or M2e antibodies.

The project aimed to use all of the available DIVA tests and approaches known and considered feasible at the time; aims where to be realised through the following activities:

Activity 1. Sourcing reagents and available DIVA test kits. It was anticipated at the time of project inception that a commercial DIVA tests might become available due to high interest in the test and its potential to contribute to effective AI control. A number of laboratories have described various DIVA tests in Italy, Belgium, Korea and the USA (Capua *et al* 2002; Tumpey et al 2005; Lambrech *et a*l 2007; Avellaneda et al. 2010) and release of at least some of these tests was anticipated. Since all four tests used different formats, the intention was to source all tests available and evaluate their comparative performance in Indonesia using reference and field sera.

Activity 2. *Support the test development underway in Balitvet*. In 2004 the Indonesian Government approved the use in poultry of heterologous H5N2 vaccines only This meant that the most appropriate DIVA test could be based on detection of the N1-specific antibodies originating from field challenge with H5N1. In 2006 Bbalitvet commenced a project on development of an ELISA-based DIVA test for N1- specific antibodies. In 2007 this development was incorporated into this project, as it was complementary with other approaches planned in the project and to be undertaken at the University of Melbourne. The approach was to grow H5N1 virus in embryonated chicken eggs, purify the N1 protein from the virus and use the purified N1 protein as a coating antigen in ELISA. Further, in order to increase test specificity N1-specific antibodies were generated in rabbits and used to develop a blocking ELISA. All the work on the N1-DIVA test development and evaluation was to be carried out in Bbalitvet.

Activity 3. Generate peptides, peptide sera and develop an ELISA. In addition to the N1-specific DIVA tests, alternative DIVA strategies have emerged such as using two other AI proteins, the M2e and NS1, to differentiate field infections from vaccine responses (Suarez 2005). An ELISA for detection of M2e antibodies was evaluated in Belgium using a synthetic peptide specific for the M2e as a detecting antigen (Lambrecht et al 2007). The same approach was also evaluated in this project and involved the following; available M2e sequences of Indonesian H5N1 strains were aligned and a consensus sequence for the M2e protein deduces from this comparison. Two peptides, 24 and 18 amino acids long, were then synthetised and used as coating/detecting antigens in ELISA. The specificity of this ELISA was determined using reference sera to a number of AI subtypes as well as using sera from chicks vaccinated and challenged with different local H5N1 isolates. The antisera to the M2e specific peptides were also produced in rabbits and in chickens in order to determine if these mono-specific M2e sera could be used in devising different format of DIVA tests, such as competitive instead of direct ELISA.

Activity 4. *Express NS1 and M2e in vitro using different expression systems*. The NS-1 and M2e proteins of AI virus have been considered as possible DIVA antigens because they are only found in cells infected with live AI virus, whereas an inactivated AI virus, or vaccine, do not contain significant amounts of either the NS-1 or the M2e proteins. Therefore if post-vaccination sera were to contain antibodies to the NS-1 or M2e, it meant that vaccinated chickens have been effectively challenged by a field strain of AI. Since the NS-1 or M2e are not present in the AI virus, the only way of getting the purified NS-1 or M2e for ELISA was to express these proteins *in vitro* using expression vectors. The approach was to use the consensus sequences of NS-1 and the M2e from Indonesian H5N1 strains, make synthetic genes for both, and evaluate expression of these in *E. coli* using different expression vectors. Further to purify these recombinant antigens, use these either directly in ELISA or use antisera to these as capture antibodies, and compare specificity of all tests using initially reference sera to live and inactivated AI viruses of different subtypes. From these comparison the most appropriate DIVA test was to be selected.

Activity 5. Generate AI antisera in a challenge experiment using Bbalitvet AI isolates. Aim was to produce H5N1 antisera with known history for evaluation of specificity and sensitivity of M2e DIVA test. Such sera can only be produced in experiments conducted in the laboratory and in case of H5N1 only in the BSL3 facility that exists in Bbalitvet. Also since M2e antibody responses can only be measured in vaccinated and surviving chicks it means that AI vaccination has to be suboptimal to allow for chicks to be challenged and for multiplication of HPAI virus to take place without mortalities. Therefore vaccines that provide only partial protection need to be selected for this purpose. Chicks, SPF 2 weeks of age were to be vaccinated in BSL2 facilities at Bbalitvet with one of H5N2 type vaccines and sera collected 4 weeks after vaccination. At 4 weeks after vaccination chicks were to be challenged with one antigenically different Indonesian H5N1 isolate, swabs collected for virus isolation at 2, 5, 7 and 14 days after challenge and chicks also bleed at 7, 14, 21 and 28 days after challenge. Sera to be tested for HI antibodies and in M2e DIVA test and results correlated with virus isolation.

Activity 6. Generate chicken and rabbit sera against M2e and develop a competitive ELISA. The aim was to attempt to develop a competitive ELISA for detection of M2e antibodies. The approach was introduced in the concluding stage of the project as a possible alternative to direct M2e ELISA, in which some field sera induced non-specific binding and affected test specificity and reliability. Rabbits and chickens were to be immunised with the M2e peptides, immune sera collected, the IgY isolated and coupled to the horseradish peroxidase and used in a competition ELISA as detecting antibodies. The work was to be conducted in Bbalitvet.

Activity 7. Undertake isolation of rabbit anti-M2e recombinant antibodies. Since monoclonal antibodies have been used successfully in ELISA as competitive antibodies aim was to attempt to develop rabbit monoclonal antibodies specific for the M2e from rabbits immunised with the M2e peptide in activity 2.6. For this spleen cells were to be collected from rabbits with high titre to the M2e peptide. From spleen lymphocytes immunoglobulin genes specific for the M2e were to be isolated, assembled *in vitro* and expressed in *E coli*. Phage expressing antibodies with specificity for the M2e were to be selected and characterised for binding to the M2e protein. If M2e specific antibodies were isolated they were to be assessed for usefulness in a competitive ELISA. Work on isolation of recombinant antibodies were to be conducted in Australia, the University of Adelaide with participation of one staff member from Bbalitvet.

Objective 3. To develop sustainable protocols for investigating effectiveness of vaccination for HPAI in Indonesia

Despite the increased understanding of various facets of AI vaccination, H5N1 infections and disease have been observed in vaccinated poultry in Indonesia. At the most general level, these infections may be due to either “failure of the vaccine” or “failure to vaccinate”. In the commercial sector it is the former which is more likely, and specifically a mismatch between the vaccine antigen and the challenge circulating strain. The primary purpose of this strategy was therefore to develop robust and appropriate methodologies to assist the small-scale commercial sector to be able to assess the effectiveness of their vaccination regimes.

Activity 1. *Meetings with poultry representative bodies*. The aim was to engage as wide participation of Sector 3 farms as possible. Originally this was to be achieved through local poultry representative bodies, but this needed to be adapted and more emphasis was placed upon building a strong engagement with the district departments of livestock (“Dinas Peternakan”).

Activity 2. Training course on investigations into vaccine efficacy. The intention was to explain to the staff involved in this objective the concept of “investigations into vaccine efficacy”; Aims to be achieved and the strategy and methodology to be used. Primarily trained were to be staff from Bbalitvet participating in this objective and also staff from DINAS who would facilitate with selection of farms, engagement off and visits to farms during the survey period.

Activity 3. Protocols for undertaking investigations into effectiveness of vaccination.
The original intention was to develop documentation on how to approach and undertake on-farm investigations of vaccine failures, but this needed to be modified when the problem of delayed notification was fully appreciated. Accordingly, the focus shifted to a proactive assessment of vaccine efficacy, and this involved an initial survey to understand vaccine practices on the farms (*Activity 4*), and then a follow on longitudinal field study of vaccine responses (*Activity 5*).

Activity 4. Collection of data using developed Survey Questionnaires. An enrolment and engagement survey were to be developed aiming to gain an understanding of vaccine usage on both broiler and layer farms, within the broader context of farm-level biosecurity

and production. This survey form was to be translated and administered to about 60 Sector-3 broiler and layer farms in the surrounding Districts of Cianjur, Sukabumi and Tangerang. All visits was to be made with staff of the local Dinas Peternakan, and results entered into a project specific database.

Activity 5. Collection of samples and testing of sera from the longitudinal study on vaccination efficiency. Following on from a trail sero-survey with Dinas Peternakan, a more rigorous longitudinal study was planned in broiler and layer farms in Sukabumi and Cianjur to collect serum and data to assess vaccine responses. A detailed sampling protocol to be developed, and sera tested against a range of antigens, including that which was closest (homologous to) the vaccine used on the farm.

Activity 6. Overall analysis and communication of the results. All data from the longitudinal survey to be entered into the web-based *SurvIT* data management system; Hi titres to be compared and conclusions derived on the vaccine efficacy and likely level of protection. All data from this survey to be relayed back to the participating farms with the interpretation of the data obtained. Data to be also disseminated to the DGLS and CMU as well as other poultry industry personal and regulatory authorities.

Objective 4. *To characterise at genetic and antigenic levels AI isolates from vaccinated commercial poultry*

The aim of this objective was to analyse H5N1 strains from intensive poultry Sectors 1 and 2 in order to determine influence of various inactivated vaccines in emergence of H5N1 variant. The hypothesis was that heterologous H5N2 and H5N7 vaccines provide variable and insufficient protection allowing virus circulation in vaccinated flocks over prolonged period and leading eventually to genetic changes. These changes are more frequent in intensive commercial Sectors 1 and 2 and hence more likely source of new antigenic variants than other less intensive Sectors 3 and 4. Additionally that in flocks vaccinated with different vaccines, genetic changes will be different and directed by existing vaccinal immunity. The work on this Objective was aligned with the Objective 1, from where the majority of isolates were expected to come from. The aims were to be realised through the following activities:

Activity 1. *Compile existing sequence data for Indonesian H5N1 strains for all 8 segments.* In order to provide full assessment of genetic changes in isolated H5N1 variants the complete genomic analysis was to be undertaken. Since an AI virus consists of 8 segments that code for ten AI proteins the intention was to compile pre-existing sequence data for all Indonesian H5N1 strains that are available in GeneBank. From this comparison of sequences, primers were to be selected for sequencing of gene segments that code for the H, N, M, NP, NS1, PA, PB1 and PB2 proteins.

Activity 2. *Obtain isolates from layers and broiler breeders in Sectors 1 and 2.* Swab samples were to be collected in Activity 1.4 (Objective 1) from at least one layer flock from three different commercial companies, located in different parts of Java, which use different AI vaccines. Cloacal swabs were to be collected prior to each vaccination from 20 chicks in each flock and at regular intervals throughout the rearing period and during lay. Tissue and swab samples were also to be collected from other flocks if there was an outbreak within the Company, regardless of location. Samples were also to be collected from other poultry companies in Sector 1, 2 and 3 that were not participating in longitudinal trails but where willing to submit samples for analysis to this project.

Activity 3. Perform PCR or Real-time PCR and limited gene sequencing. Cloacal swabs were to be screened if contained H5N1 using PCR, followed by limited HA gene sequencing at Bbalitvet to establish degree of genetic variation in virus positive samples. Following confirmation that H5N1 is present, virus isolation from PCR positive samples would be undertaken in embryonated chicken eggs at Bbalitvet.

Activity 4. Sequence the entire genome. Those H5N1 isolates identified to differ by preliminary HA sequencing were to be fully sequenced. For this cDNA would be made at Bbalitvet and transferred to the University of Melbourne where all 8 segments would be sequenced using primers designed in the Activity 4.1. Before cDNA is transferred to the University of Melbourne approval was to be obtained from AQIS for importation and work with this cDNA, as well as a Material of Transfer Agreement was to be signed between Bbalitvet and the University of Melbourne to cover the use of this cDNA in Australia. Obtained sequences were to be aligned with sequences for other Indonesian H5N1 strains available in Gene Bank, amino acid changes identified and compared with the critical changes identified previously in H5N1 from Indonesia. Phylogenetic analysis was also to be performed to identify relationship between these and H5N1 isolated from poultry in Sectors 3 and 4.

Activity 5. Propagation of prototype N5N1 and other field isolates in tissue culture. Those H5N1 that differ significantly in their HA gene were to be propagated in embryonated chicken eggs and also cloned in tissue culture to be used for antigenic analysis of H5N1 strains. Some clones were to be tested in eggs for pathogenicity index, ones that differ selected and sequenced and sequences compared for changes in all 8 genes.

Activity 6. Test selected strains for pathogenicity in chicks, as vaccines or as challenge viruses (Contingent on additional funding). Those H5N1 isolates that differ significantly in their HA gene sequence and also antigenically by HA and SN tests to be tested for pathogenicity in chickens in experiments conducted in BSL3 laboratory at Bbalitvet. Also to be tested in challenge experiments using existing AI vaccines, particularly the vaccine used for vaccination of flocks from where they have been isolated.

Objective 5. *To provide specialised training and capacity building for Indonesian scientists in molecular approaches for characterisation of viruses and for generation of diagnostic reagents*

Bbalitvet has been actively involved in the national efforts to effectively respond to and control HPAI. While staff involved in this project have had the majority of the essential skills required, the aim was to support strengthening of the already existing skill base and enable development of some specialized skills which were identified in the course of the project as needed. The particular area of skill strengthening was in the area of molecular biology and protein expression and purification. The enhancement of skills was to be achieved either by training, working on the project activities in research laboratories in Australia, direct transfer of technology from Australia to Bbalitvet and facilitating particular research activities to be carried out in Bbalitvet rather than in Australia.

Activity 1. *Familiarization with software for data analysis, design of primers and phylogenetic analysis.* While these skills were considered to exist in Bbalitvet, at the time their use was limited both in extent and scope. Intention was to undertake complex analysis of the entire AIV genome, not undertaken at Bbalitvet, with proficient application of software not commonly used or available for analysis of molecular events in virus evolution.

Activity 2. Training in molecular techniques for protein expression and purification. Aim was to make Bbalitvet self sufficient in producing reagents for DIVA test that were developed at the University of Melbourne. In particular, this would involve development of skills in areas of recombinant protein expression, purification and standardisation. Further to utilise new technologies for antigen and antibody production to increase DIVA test specificity. This would also enable exposure to alternative techniques used in developing functional diagnostic tests.

Activity 3. *Training to estimate genetic relatedness, antigenic drift and re-assortment*. Aim was to increase capacity at Bbalitvet in using advanced molecular techniques for analysis of re-assortment, recombination and antigenic drift, events all anticipated to be detected in H5N1 isolated from commercial poultry. Also to provide facilities for analysis of antigenic relatedness used for antigenic and serological mapping of H5N1 isolates.

Objective 6. *To develop a simple state-transition model of HPAI infection in commercial and/or village flocks and validate the model against real outbreaks*

Activity 1. Develop the model & confirm model structure. Most of the modelling activities were, at the time of inception of this project, focused on regional/country levels rather than individual flocks and have been developed in relative isolation and used for theoretical analysis and application rather than to support disease control within a country. The Australian Department of Agriculture Fisheries & Forestry (DAFF) and the Australian Biosecurity CRC for Emerging Infectious Diseases were jointly supporting modelling for AI preparedness in Australia. This project aimed to simulate outbreaks of HPAI in the Australian poultry industry and spread between enterprises in various sectors (broilers, layers, ducks, turkey and game birds). In support of this aim, a simple state transition model was to be developed and tested to describe the expected behaviour of avian influenza in an infected flock.

Activity 2. Develop a bio-economic model for Sector 3 poultry flocks in West Java. For this revised objective, gross-margin financial models were to be developed for both layer and broiler flocks. Survey questionnaires were to be developed to support these models and coded into the SurvIT database to be developed as part of Objective 3. The questionnaires were to be piloted in Sector 3 flocks (Objective 3). The available data were to be analysed in the model.

Activity 3. Use model to analyse data. The questionnaires were to be trialed on broiler and layer farms from Sector 3. Data were to be analysed to evaluate financial situation of surveyed farms. Some refinement of economic model were to be undertaken using data collected and development of models in R statistical software undertaken

Objective 7. *Communication and Collaboration*

Activity 7.1. Renew Contacts with DGLS: to strengthen better linkages between the project and DGLS so that DGLS is familiar with the scientific output of the project in particular in respect of the DIVA test and assessment of AI vaccines performance in Sector 3 farms. Bbalitvet has a scientist Dr Tatty Syafriati working in DGLS and this contact was to be utilised to arrange briefing meetings with the Director of DGLS and HPAI Campaign Management Unit (CMU).

Activity 7.2. Renew contacts with FAO: to maintain linkages between the project and the FAO/OFFLU so that scientific outputs of the project and their possible application are made known to FAO. *Both* FAO and OFFLU have developed good network and communication pathways in Indonesia so that the results of the project may be shared to leaders of Indonesian poultry Industry and other major stakeholders through this network.

Activity 7.3. Communicate results in Indonesia and elsewhere: to disseminate project results to a wider scientific and poultry industry communities.

6 Achievements against activities and outputs/milestones

Objective 1: To assess the efficacy of various inactivated AI vaccines and vaccination regimes in preventing shedding and transmission of H5N1 infections in commercial chickens

no.	Activity	outputs/ milestones	completion date	comments
1.1	Source vaccines, reagents and test kits	Standardized procedure & reagents in place	30 June 2007	One vaccine of N5N2 subtype sourced from collaborating company 1; Others were to be sourced from collaborating commercial companies once collaboration was established. HA & HI test were those used by Bbalitvet and using two H5N1 strains from 2003 (BL/03) and 2006 (WJ/PWT/06). Serum neutralisation was developed using BL/03 & WJ/PWT/06 strains. Monoclonal antibodies requested from Dr Peris laboratory in HK however no reply was obtained; Lack of Mabs did not impact on the results obtained since SN test also revealed significant antigenic differences between H5N1 strains tested. (PC, A)
1.2	Collaboration with the Dutch group	Comparable approach and test procedure used/ Complementarity between trails	31 May 2009	Methodology sourced. Laboratory trail with H5N1 vaccine in Medion completed. Field trails using H5N1 vaccine discontinued. (A, PC)
1.3	Baseline trials in laboratory	Establish levels and duration of Ab responses in controlled conditions	31 Jan 2008	First lab trail commenced in Jan 2008 with six vaccinations done by Aug 2008. Just before challenge the trail was terminated. HA and SN titres determined in some sera only. (PC)
1.4	Undertake baseline collection of data on passive and active antibody status as well as infectious status in vaccinated commercial poultry at different locations	Assess degree of correlation between Ab levels in field and Lab vaccination and duration of Ab levels following different vaccination regimes. Testing of methods for AI virus recovery from field samples	31 May 2008	Serum and swabs collected from three broiler and layer flocks from the collaborating company as in 1.3 with the first sampling of broilers at 1 week old and layers at 4 week old just prior the first vaccination. HI and SN titres determined in some sera only. (PC)
1.3 to 1.6	All activities	Comparison of immunity in field and Lab vaccination	Feb 2010	All activities on this Objective terminated June 2008.

PC = partner country, A = Australia

Objective 2: *To* develop reagents for DIVA testing

no.	Activity	outputs/ milestones	completion date	comments
2.1	Source reagents and available DIVA test kit	To be used as standard	October 2007	Reagents for three tests described in literature requested from the laboratories in the USA, Belgium and Italy. None were available. The NS1 AIV Ab ELISA test from Animal Genetics (South Korea) requested however the test is no longer available. Reference antisera to a number of AIV subtypes imported from the UK. (A)
2.2	Support test development underway in Balitvet	Specificity of NA based DIVA test determined	January 2008	NA protein purified from H5N1 virus and used as coating Ag in ELISA. N1 antibodies detected in chicks vaccinated with inactivated H5N1; however cross-reactive NA antibodies detected also in chicks vaccinated with H5N2. Thus NA1 could not be used for DIVA, although it could be useful for measuring vaccination response. The IgG isolated from rabbit sera produced against the N1 protein, labelled with horseradish peroxidase and used in a competitive ELISA. (PC)
2.3	Generate peptides, peptide sera and develop an ELISA	Specificity of peptide based DIVA test determined	May 2010	Two M2e peptides synthetised and used in an ELISA as coating antigens. Both peptides reacted with reference sera from chicks infected with live H5N1 but not with those from chickens immunised with inactivated H5N1 and H5N2 vaccines. Thus feasibility of using Me2 peptides for DIVA confirmed. (A)
2.4	Express NS1 and M2e *in vitro* using different expression systems	Specificity of NS1 & M2e based DIVA test determined	31 May 2009	NS1 gene expressed in two systems *in vitro*. Evaluation using reference and field sera showed significant non-specific reaction with some field sera. Additional purification as recommended by others did not improve test specificity. Therefore approach abandoned in favour of Me2. (A) Synthetic M2e gene made, incorporated into expression vector, recombinant Mbp-M2e protein expressed, purified and evaluated in direct ELISA. Good results obtained with reference sera however non-specific reaction occurred with some field sera. Mbp-M2e ELISA optimised to reduce non-specific reactions and evaluated using field sera; test discriminated between infected and vaccinated birds, but the background noise was rather high and about 5% of sera reacted non-specifically with the Mbp protein. (A & PC)

2.5	Generate AI antisera in a challenge experiment using Bbalitvet AI isolates (PC)	For evaluation of sensitivity of DIVA an	30 September 2010	Sera from three challenge experiments were collected and tested in M2e ELISA and correlated with virus isolation from swabs. M2e antibodies were detected for the first time at 14 days and also at 21 days after challenge. Detection of M2e antibodies correlated well with virus isolation in all chicks, whereas all virus negative chicks were also M2e antibody negative. (PC)
2.6	Generate chicken and rabbit sera against M2e and develop a competitive ELISA (PC)	Competitive ELISA based on the M2e	30 September 2010	Rabbits and chickens immunised with M2e peptides produced sera of high titres that reacted with both M2e peptide and Mbp-M2e protein. IgG/IgY was purified from both rabbit and chicken sera, coupled to the horseradish peroxidase and used in a competition ELISA as detecting antibodies. Chicken sera known to be positive for M2e antibodies was not able to out-compete anti-peptide M2e antibodies-HRP. (PC)
2.7	Undertake isolation of rabbit anti-M2e recombinant antibodies (A)	Competitive ELISA based on rabbit anti-M2e recombinant antibody	*30 September 2010*	Spleen lymphocytes from one M2e immune rabbit were purified, cDNA generated and immunoglobulin heavy and light chains isolated, assembled *in vitro* and expressed in *E coli* using phage display technology. Phage expressing antibodies with specificity for the M2e were rescued by process of panning. Ten clones were selected of which only one was stable and able to be propagated and purify further. The ability of this M2e specific phage as competing antibody is being evaluated. (A & PC)

PC = partner country, A = Australia

Objective 3: To develop sustainable protocols for investigating effectiveness of vaccination for HPAI in Indonesia

no.	**activity**	**outputs/ milestones**	**completion date**	**comments**
3.1	Meetings with poultry representative bodies	Obtain formal agreement of collaboration between industry bodies and Balitvet	30 November 2007	A meeting was held with the Chairman of PINSAR, the Indonesian Poultry Information Centre, in January 2008. The Chairman was interested in the project, but nevertheless expressed a general reluctance to advise members of PINSAR to actively collaborate with the project. Accordingly, the project proceeded to develop relationships with the poultry farms via the district Dinas Peternakan offices.

3.2	Training course on investigations into vaccine efficacy	Participants adopt the concept	31 March 2008	Training was given to Bbalitvet staff on epidemiological approaches to vaccine breakdown investigations, and discussions were held at length of how to apply these to the specifics of the Indonesian small-scale commercial sector. From this was identified the need for better field data capture, and practical training in survey methodologies was given, and applied to two field surveys. (PC & A)
3.3	Protocols for undertaking investigations into effectiveness of vaccination	Documentation on how to approach and undertake investigations	30 November 2009	As initially conceived, the project would develop reactive protocols to investigate apparent vaccine breakdowns. This relied upon rapid notification of breakdowns by the producers, but the project was advised that such notifications were generally delayed. Therefore, the project needed to adapt and develop a more proactive approach, of direct engagement with producers. This led to new objects, viz. 3.4, 3.5 and 3.6 – see below. (PC & A)
3.4	Collection of data using Survey Questionnaires	Estimation of success of current AI vaccine in preventing HPAI outbreaks	Nov 2009	Two surveys were undertaken. The first (in early 2008) was an enrolment and engagement survey, aiming to gain an understanding of vaccine usage on both broiler and layer farms, within the broader context of farm-level biosecurity and production. A Survey Questionnaire developed, adjusted following phase 1 enrolment visits and translated into Bahasa Indonesia; visits made to 61 farms with assistance of DINAS and Survey Questionnaire completed for all 61 farms. Data was entered into specially designed and developed web-based epidemiological survey management tool ("*SurvIT*") *(PC & A)*
3.5	Collection of samples and testing of sera from the longitudinal study on vaccination efficiency	Data on likely protective efficacy of the currently used vaccines against newly emerged H5N1 variants	Feb 2010	Following on from the analysis of the initial survey (Objective 3.4) a more targeted survey was undertaken to investigate immunological responses in both broiler and layers. Sampling protocols were developed and sera collected from 32 farms with testing against a range of antigens corresponding to vaccines currently used in Indonesia. This allowed for a comparative assessment of the efficacy of vaccination on the sampled farms. (PC & A)

3.6	Overall analysis and communication of the results	Report / scientific paper of the overall sub-project	Feb 2010	Web based data base SurvIT developed to collate and analyse the data. All data from the Survey Questionnaires and from sera HI testing entered into SurvIT and analysis performed; Output results generated and prepared for dissemination and publication. (PC & A)

PC = partner country, A = Australia

Objective 4: To characterise at genetic and antigenic levels AI isolates from vaccinated commercial poultry

no.	**activity**	**outputs/ milestones**	**completion date**	**comments**
4.1	Compile sequence data for Indonesian H5N1 strains for all 8 segments	Foundation data for Indonesian H5N1 strain to which all other sequences will be compared; consensus sequences and primers for sequencing of all 8 genes	30 November 2008	Gene sequences for all 8 gene segments from Indonesian and South East Asian H5N1 strains deposited in GeneBank were aligned and primers selected to be used for sequencing of H5N1 isolated in this project. (A)

4.2	Obtain isolates from layers and broiler breeders in Sectors 1 and 2	For assessment of type of AI circulating in vaccinated flocks	30 November 2009	AI samples for this analysis were to come from Objective 1. Agreements were negotiated with the commercial sector regarding the confidentiality of origin of the samples and the results to be obtained. Contract signed with one Sector 1 Company in November 2007. From December 2007 until February 2008 five samples obtained from five commercial companies in Sector 1 & 2 vaccinated with different heterlogous AI vaccines. H5N1 viruses Isolated from all five samples and two cloned in tissue culture. No further work carried out on these isolates as the Objective 1 and the work on characterisation of these isolates were suspended in June 2008. Bbalitvet granted access to five H5N1 strains isolated by Bbalitvet during surveillance between 2003 and 2006 being: Indo/BL/2003, WJ/TasikSol/06, WJ/TasikSob/06, WJ/Hamd/06 and WJ/SMI-US/06; Bbalitvet also grated access to three clones from tissue culture adapted Indo/BL/2003 obtained in the Objective 1. Swab samples collected from layers pullets from the Collaborating Company 1 (see objective 1) at 4 weeks prior the 1st and 2nd vaccination. Swab samples collected from 20 multi-age farms in the area of Sukabumi and Bandung. (PC)
4.2a	Collect samples from layers and broilers in Sectors 3 currently surveyed in Objective 3	Estimated sequence variation among AI isolates from vaccinated flock in Sector 3	September 2010	There were no outbreaks reported on 15 farms in Sector 3 that were surveyed from March to September 2010 and hence no samples were collected. (PC)

4.3	Perform Real time PCR and limited gene sequencing	Obtained sequence information indicates that AI isolates differ (or do not differ) from original H5N1 strains	31 January 2010	cDNA made from eight H5N1 strains to which Bbaitvet has granted access to the project and cDNA transferred to the UoM for full genome sequencing. Swab samples collected from layer pullets from the Collaborating Company 1 (see objective 1) at 4 weeks prior the 1st and 2nd vaccination were tested in PCR and all were negative. Swab samples, collected from 20 multi-age farms in the area of Sukabumi and Bandung that were vaccinated with four different vaccines, were tested in PCR. Samples from 13 farms were positive for H5N1 in PCR however no AI virus was isolated from any samples. (PC)
4.4	Sequence the entire genome	Confirmation and identification, or other wise, of changes in viruses recovered from vaccinated flocks	*Nov 2009*	Sequencing completed using cDNA at UoM for five H5N1 samples isolated by Bbalitvet between 2003 and 2007: Indo/BL/2003, WJ/TasikSol/06, WJ/TasikSob/06, WJ/Hamd/06 and WJ/SMI-US/06. Analysis showed that in strains with variation in HA gene, NS1 and M2 genes also varied. *Three tissue culture adapted clones of Indo/BL/2003 sequenced (all 8 gene segments).* Five H5N1 from Objective 1 that originated from Sector 1 and 2 layers not sequenced, as Bbalitvet did not approve their use in the project. (A)
4.5	Propagation of prototype N5N1 and other field isolates in tissue culture	Cloned candidate strain for homologous vaccine development	*Aug 2009*	Reference strain Indo/BL/2003 and another two field isolates from Sector 2 cloned and propagated in tissue culture for SN test. Three clones of Indo/BL/2003 selected for identity testing and estimation of sequence variation during adaptation in tissue culture. No significant changes identified. Not completed as the Objective 1, and all related work related, terminated in June 2008. *(PC)*
4.6	Test selected strains for pathogenicity in chicks, as vaccines or as challenge viruses (Contingent on additional funding)	Determine if isolated strains differ in virulence from other H5N1 & if existing vaccine(s) are protective	*February 2010*	Not completed as the Objective 1, and all related work terminated in June 2008

PC = partner country, A = Australia

Objective 5. *To provide specialised training and capacity building for Indonesian scientists in molecular approaches for characterisation of viruses and for generation of diagnostic reagents*

no.	activity	outputs/ milestones	completion date	comments
5.1	Familiarization with software for data analysis, design of primers and phylogenetic analysis	Staff able to analyse the data on AI strains collected in Indonesia and at Balitvet and other data deposited in GeneBank	31 May 2008	Dr Indi Drhamanyanti, responsible for molecular work at Bbalitvet, spent one week at UoM, and attended 2 days Angis course on gene sequence analysis and genomic data base software. (PC, A)
5.2	Training in molecular techniques for protein expression and purification	Training in molecular techniques for protein expression and purification	30 September 2010	Dr Simson Tarigan, visited and worked at the UoM on three occasions learning molecular techniques for AI gene cloning, *in vitro* expression and protein purification as well as large scale production of expressed NS1 and Mbp-M2e for DIVA test. Dr Tarigan also spent 4 weeks at the University of Adelaide in July 2010 and worked on isolation of rabbit anti M2e recombinant antibodies. Drs Sumaningsi granted JAF in 2007; undertook advanced English language course in Melbourne. Commenced work for Master degree at UoM in June 08; Converted to PhD in July 2010 and expected to complete PhD in July 2011. (PC, A)
5.3	Training to estimate genetic relatedness, antigenic drift and re-assortment	Capability at Balitvet of using generated sequence data for analysis and predictions	September 2010	Ms Atik Ratnawati spent 4 weeks in Australia learning tissue culture techniques for AI isolation and antigenic typing as well as principles of sequencing; also received training at the Australian Animal Health Laboratory in laboratory biosafety Ms Risa Indriani spent 4 weeks at AAHL testing sera collected from Sector 3 for HI titres using antigenically different HA antigens. (A)

PC = partner country, A = Australia

Objective 6: *To* develop *a bio-economic model of Sector 3 poultry production in West Java and use this model to investigate enterprise profitability and potential impact of HPAI outbreaks and control measures (revised)*

no.	activity	outputs/ milestones	completion date	comments
6.1	Develop the model & confirm model structure (original objective)	Specified model parameters & structure adopted by the project team	May 2008	A simple state transition model was developed and tested using standard parameters. This model appears functionally sound and shows promise as the basis of future modelling scenarios if suitable parameters for the Indonesian situation can be developed. (A)
6.2	Develop a bio-economic model for Sector 3 poultry flocks in west Java (Revised)	Completed, functional bio-economic model	May 2009	Biological flock model developed and tested; Farm financial model developed taking inputs from biological model; Models developed in Excel; Models tested and appear realistic; Data requirements for survey design for Objective 3 completed. *(A)*
6.3	Use model to analyse data from Obj. 3 farms:	Analyse and report data from Objective 3 farms	Feb 2010	Survey trialed on 10 broiler and 18 layer farms in late 2009; Data analysed to evaluate financial situation of surveyed farms. However, significant problems and inconsistencies identified in data collection prevented detailed analysis of results; Some refinement of economic model undertaken using data collected; Development of models in R statistical software undertaken. (PC, A)

PC = partner country, A = Australia

Objective 7: Communication and Collaboration

no.	activity	outputs/ milestones	completion date	comments
7.1	Renew Contacts with DGLS	Better linkage between the project and DGLS	**September 2010**	The scientific output of the project in particular the results of DIVA test development and serological surveillance in Sector 3 farms reported to the DGLS and CMU in Sept 2010**.**
7.2	Renew contacts with FAO	Better linkage between the project and FAO	September 2010	The scientific output of the project presented to FAO in September 2010 and at DGLS-OFFLU meeting held in October 2010 in Jakarta.
7.3	Communicate results in Indonesia and elsewhere	Disseminate project results	September 2010	There was an opportunity to present the major outcomes of the DIVA test development and serological surveillance in Sector 3 farms and their implication to the Indonesian poultry industry personnel at the OFFLU meeting held in October, in Jakarta.

7 Key results and discussion

Objective 1: To assess the efficacy of various inactivated AI vaccines and vaccination regimes in preventing shedding and transmission of H5N1 infections in commercial chickens

Analysis of efficacy and potency of three commercial vaccines

Several important outcomes were expected from this objective: (i) comparison of three different AI vaccine most frequently used in Indonesia for potency and efficacy, that is level of antibodies they induced and cross-protection they afford; (ii) capacity of each vaccine to prevent shedding following field challenge and (iii) assessment of variables that may influence efficacy of AI vaccination in field. Work was to be undertaken in collaboration with three commercial Sector 1 and 2 companies using layer flocks. The hypothesis was that in this commercial sector, where the use of AI vaccines is the most intensive, the likelihood of vaccine failures and the emergence of new AI variants is the greatest. H5N1 viruses, isolated from cloacal swabs of vaccinated layers were to be analysed for antigenic changes using HA and SN tests and also for genetic changes in Objective 4. Therefore a comprehensive analysis of efficacy and potency of three commercial vaccines was to be achieved aiming to provide scientific rational for their use. Laboratory vaccination of layers chicks with the H5N2 vaccine commenced in mid September 2007., however the objective was terminated in June 2008 due to some internal issues in Bbalitvet which were not resolved in time to resume the work and enable completion. The results showed that at least two to three vaccinations were needed to reach HI antibodies titres considered as protective. It was also of interest that some companies commenced vaccination as early as 4 weeks of age with 6 vaccinations prior to the point of lay.

Comparison of SN and HI tests for antigenic differentiation of H5N1 strains

Two methods, HI and SN test, were established to analyse serological responses following vaccination using a reference H5N1 antisera. For SN test three Indonesian H5N1 isolates were cloned three times and then propagated in chicken embryo fibroblast to obtain high titre virus stock. Identity of clones with the original, allantoic fluid propagated H5N1 isolate, was confirmed for one strain only using complete genome sequencing. Propagation and cloning of H5N1 strain BL/03 in CEF cells did not cause any significant change in the passaged virus allowing for direct comparison of SN and HI titres. The SN test has been rarely used previously to analyse serological responses in AI vaccination. Comparison of SN test with commonly used HI test in this study however showed that different results and conclusions may be obtained using the SN test. While the HI test indicated that vaccinated chicks had protective antibody titres, particularly against heterologous H5N1 virus, the SN antibody titres were more strains specific and below values that could be considered protective. In particular lack of SN antibody titres were detected in layers vaccinated with H5N2 in the Laboratory and in field against an H5N1 variant isolated from an outbreak in flocks vaccinated with the same vaccine suggesting that the newly isolated H5N1 was an antigenic variant. Further SN testing revealed greater antigenic differences between H5N1 isolates than did the HI test. Analysis of antibody titres showed that it is difficult to maintain high antibody titres using the H5N2 vaccine in question, which are needed to achieve protection against H5N1 challenge. However these findings needed further work using antigenically different H5N1 to establish true value of SN in comparison to the HI test, currently the test of choice

Collaborations with Sector 1 companies for vaccine efficacy trials

A further outcome of this objective was establishment of effective collaborations with Sector 1 companies. In its first year of operation, the project signed a collaborative agreement and commenced field and laboratory trails with one Sector 1 company and

was negotiating to commence trails with two companies from Sector 1 and 2, when this objective was terminated. The willingness of Sector 1 and 2 companies to collaborate with the project reflected relevance of the work to be undertaken and was in contrast with some other initiatives in Indonesia that were unable to establish collaboration with these Sectors.

Overall, the achievements in the first six months of this objective indicated that significant new data on vaccine efficacy would have been obtained with immediate practical application as well as for the future of AI control in Indonesia. This type of data is still lacking, not only in Indonesia but also elsewhere.

Objective 2: To *develop reagents for DIVA testing*

DIVA test strategies – Options and Considerations

Different strategies can be used for differentiation of infected and vaccinated animals (DIVA). All DIVA strategies, except for use of sentinel chicks and monitoring individual birds for infectious status, are based on serological detection of infection by detecting antibodies to either Neuraminidase (N) or non-structural proteins the NS1 and M2e. Choice of antibody to target for detection in DIVA is determined foremost by the type of AI vaccine used. Developments of most AI vaccines to date, and in particular new generation of recombinant vaccines, have taken into account the need for DIVA test application, resulting in so cold “DIVA vaccines”.

Target antigens for DIVA

The most commonly used AI vaccines in poultry have been inactivated vaccines derived from heterologous strains that allow detection of N-specific antibodies. In analogy, in Indonesia if N1-specific antibodies are detected in either H5N2 or H5N9 vaccinated chicks, it would mean that vaccination did not protect chicks against field challenge, since N1 antibodies could have been induced only by a field challenge with H5N1. Since heterologous AI vaccines are now considered inadequate for protection against H5N1, new types of AI vaccine are being developed such as reverse genetic, subunit, DNA and viral vector vaccines (van den Berg *et al*, 2008). In application of many of these new generation vaccines the N1-specific antibodies are targets for DIVA test; however in others the NS1 or M2e antibodies are targeted to detect H5N1 infection. In Indonesia there has been a gradual change in type of vaccines used; initially only heterologous vaccines such as H5N2 and H5N9 were registered for use, however because of their inadequate efficacy, homologous H5N1 vaccines have been also introduced over the last two years. Further there is now also a prospect of using recombinant vaccines, such as reverse genetic derived vaccines, that would further influence type of DIVA test that can be applied. To allow for all of these eventualities the project approach was to evaluate feasibility of using DIVA based on detection of antibodies to all possible target antigens, that is the N1, NS1 and M2e proteins.

The type of DIVA test – ELISA

In addition to choosing the target antigens for DIVA, the choice had also to be made on the type of DIVA test. Although most DIVA are serological test, the formulation of the test may vary and furthermore there are significant limitations due to the use of HPAI strain H5N1, particularly outside of BSL3 facilities. The first DIVA test developed and used in poultry for AI control was a serological test in which post-vaccination sera were tested for the presence of N-specific antibodies in an immunofluorescence (IF) test (Capua *et al* 2002). An immunofluorescence based DIVA test is not user friendly and also requires BSL3 facilities, and therefore is not feasible for routine mass screening as required in Indonesia. A simpler DIVA test such as enzyme linked immunosorbent assay (ELISA) has obvious advantage since it is a robust, cheep test that allows high throughput and can be used in most diagnostic laboratories without the need for specialised equipment.

Commercial DIVA tests

Although a number of laboratories have described various DIVA tests, none are commercially available. Reasons for their absence on the market are not entirely clear. An ELISA for detection of NS1 was available in early 2008, however test was withdrawn from the market due to its lack of specificity. Hence no commercial DIVA test could be sourced for evaluation and as a standard for validation of DIVA tests developed in this project.

Evaluation of DIVA test based on the detection of antibodies to Neuraminidase subtype 1 (N1) protein

Since the detection of N1-specific antibodies is the most frequently suggested strategy for DIVA and furthermore only heterologous AI vaccines were approved for use in Indonesia at the time of inception of this project, we undertook to develop an N1-based DIVA test. The N1 protein was purified from H5N1 virus and used in indirect ELISA for detection of antibodies in vaccinated chicks. High titres of N1 antibodies were detected in chicks vaccinated with inactivated H5N1 however equally high titres were also detected in chicks vaccinated with H5N2. This meant that this type of assay could not be used for DIVA due to existence of cross-reactive antibodies between N proteins of different AI subtypes. There are published papers that used similar approach that is the N protein derived form expression *in vitro*, and were able to differentiate between the N proteins of different subtypes using ELISA (Kwon et al. 2009; Liu et al. 2010). It is not obvious why there is such a discrepancy between ours and other published N1 based ELISA, unless the N1 protein expressed *in vitro* is antigenically modified and is no longer recognised by cross-reactive antibodies. Cross-reactive N antibodies are known to exist and are detected in ELISA, unlike N - subtype antibodies that are only detected in specific tests such as neuraminidase inhibition test and by monoclonal antibodies. Thus N1 ELISA developed could not be used as DIVA test. However this proved to be of no importance since Indonesia has moved towards use of H5N1 inactivated vaccines, instead of heterologous H5N2 vaccines, meaning that, at least at present, the N1- based DIVA test is no longer possible to use in Indonesia.

The developed N1-ELISA however could have other uses; one is to measure N-specific antibody responses in vaccinated chicks that could be a useful adjunct in evaluating efficacy of recombinant vaccines that have heterologous N proteins. Also the N1-ELISA could be used for detection of H5N1 virus. Methods currently used for detection of H5N1 are either expensive or complicated and require sophisticated laboratory set up. A need for a simple, specific, sensitive and cheep test for detection of H5N1 have been long recognised. To evaluate the possibility of using an ELISA for detection of N1, the purified N1 protein was used to immunise rabbits and high titre of anti-N1 sera was generated. From this sera IgG/IgY antibodies were purified and coupled to horse-radish peroxidase (HRP). When rabbit anti-N1 antibodies and rabbit anti N1 IgG HRP were used together in a sandwich ELISA they were able to detect the H5N1 virus. The possible usefulness of this test for detection of H5N1 is current being evaluated.

Evaluation of DIVA test based on the detection of antibodies to the non-structural NS1 protein

The DIVA test based on the detection of antibodies to the non-structural NS1 protein of AIV has been proposed by a number of researchers as useful test for differentiation of infected and vaccinated chicks as its application is independent from the type of vaccine used (Suarez 2005;Tumpey et al 2005; van den Berg *et al* 2008). As NS1-ELISA was developed for use in research laboratories only, we also embarked on the development of this test using similar approach, albeit with some modifications. Recombinant GST-NS1 protein obtained by expression in *E. coli* reacted only with sera from live AIV infection and not with sera obtained following vaccination and therefore this recombinant protein was

able to act as differentiating antigen. However significant non-specific reactions were obtained with some field sera. Additional purification of the recombinant antigen as recommended by others, ELISA test optimisation and pre-treatment of sera did not improve test specificity (Zao et al 2005). Significant proportions of non-specific reactions were due to the GST present in the recombinant protein and hence the GST-NS1 recombinant protein was not feasible to use with commercial sera. Also at the same time other researchers published results showing that the NS1 might not be antigenic enough to be used in DIVA. For that reason, and for interest of time, the work shifted to evaluating the usefulness of the M2e protein.

Evaluation of DIVA test based on the detection of antibodies to the M2e protein

For the evaluation of usefulness of the M2e as detecting antigen in DIVA, two approaches were taken: the M2e was made as a recombinant protein and as a synthetic peptide. This was for the reasons: (a) it is cheaper to produce recombinant protein then M2e synthetic peptide, and this could be relevant if test was to be used on a large scale in Indonesia; and (b) antigenicity of the M2e may be altered by either approach, hence they were to be compared.

DIVA based on the recombinant Mbp-M2e protein

Since the M2e is a small protein of only 24 amino acids, to ease purification of recombinant M2e protein it is necessary to couple it to another carrier protein. Since work with recombinant NS1 showed that GST is not suitable as carrier protein because of reaction with non-infected chick sera, the maltose binding protein (Mbp) was used as carrier protein as preliminary evaluation showed that it had low or no cross-reaction with sera obtained from commercial layers in Australia know to be AIV antibody free. The recombinant Mbp-M2e protein was expressed in good amounts and sufficient to make test economical. In ELISA the Mbp-M2e did not react with SPF sera of chicks vaccinated with killed H5N1 and H5N2, whereas it reacted well with reference sera to H5N1 strains obtained by vaccination with homologous inactivated H5N1 followed by live virus challenge. Live challenge sera from three different H5N1 strains differed in affinity of binding, however all had antibody levels detectable at sera dilution on 1/100. Further, in ELISA the Mbp-M2e reacted with reference sera obtained following infection of chicks with low pathogenic AI strains of different subtypes. Non-specific binding with some sera was observed at low dilution of sera however these were below background level of mean + 2 standard deviation (STD) of negative sera. The M2e specificity of all reactions in the Mbp-M2e ELISA was confirmed using western blotting. Using sera from experimentally vaccinated and challenged chicks Mbp-M2e ELISA was able to discriminate well between infected and vaccinated birds, however the background noise with some sera was higher then expected. If the cutoff point of mean + 2 STD was applied however the positive reactions were still high and cleary differentiated.

With field sera from vaccinated layers in Mbp-M2e ELISA the background noise was also higher then expected and about 5% of sera reacted non-specifically with the Mbp protein. The resons for the higher background level are being investigated and additonaly the expression of Mbp-M2e recombinant protein is being optimised to remove excess of the Mbp protein found to be present in some preparations of recombinant Mbp-M2e. Overal the Mbp-M2e ELISA is able to discriminate well between infected and vaccinated birds, the test is senitive and specific on the flock level, however some negative sera have a backgrund noise which is not desirable. The recombinant Mbp-M2e protein proved to be however a valuable reagent. It enabled us to use western blotting as a validation assay for all experiments that involved detection and characterisation of the M2e responses in AIV infected chickens.

DIVA test based on the M2e peptide

The M2e peptide ELISA in which an 18 amino acids long M2e peptide was used as coating antigens proved to be specific and sensitive for DIVA application. Only reference sera obtained by challenge with three live H5N1 strains and seven low pathogenic AIV of various subtype were positive in M2e peptide ELISA. For all parameters, except for the background noise, the M2e peptide and Mbp-M2e ELISA gave the same results with reference sera. Using sera from experimentally vaccinated and challenged chicks, M2e peptide ELISA discriminated between infected and vaccinated chicks and results corelated well with virus isolation from individual birds. M2e antibodies were detected for the first time at 14 days after challenge. There were however differences in aborbance values (OD) for different sera and OD could be separated into those with high, medium and low OD. With field sera obtained from vaccinated layers from Sector 3, M2e peptide ELISA separated flocks into two groups. The first group consisted of flocks wit a high propotion (≥40%) of chickens positive for M2e. Information from the farms indicated that flocks were in area with low biosecurity and poor health or where AI had been rampant. Flocks in the second groups were all M2e negative and consistent with field reports of flocks in good health and good bisecurity. Specificity of this response, particularly with sera that have low OD is being determined.

Evaluation of M2e based competitive DIVA test?

Because of higher then desirable background noise and some non-specific reaction in Mbp-M2e ELISA attempts were made to increase test specificity by changing test format from direct to competitive ELISA. Competitive ELISAs have been used with other antigens (N antigens from AIV) and other diseases (FMDV) to increase test specificity. For this format of ELISA, monospecific high titre anti M2e sera was produced in rabbits and chickens as well as homologous anti M2e HRP conjugate to enable assay of chicken M2e antibodies in test sera. In this format of ELISA plates were coated with Mbp-M2e recombinant antigen, and test sera containing M2e antibodies are expected to block binding of anti M2e IgG HRP. However none of reference sera, known to be positive for M2e antibodies were able to block binding of anti M2e IgG HRP to coated M2e antigen. This was disappointing and possibly could be explained by rabbit and chicken anti M2e peptide antibodies having higher affinities and displacing the week chicken anti M2e antibodies generated by challenge and infection with live virus. This aspect of the M2e ELISA is being investigated further.

An effective competitive ELISA for detection of antibodies to Neuraminidase N1, N2 and N3 has been described recently using mouse monoclonal antibodies (Moreno *et a*l. 2009). We used recombinant antibody approach instead of conventional mouse monoclonal antibody approach. Rabbit recombinant anti M2e peptide antibodies were first attempted to obtain since they were available and was an easier approach to develop reagents and methods necessary, than chicken anti M2e recombinant antibodies. Phage expressing antibodies with specificity for the M2e were selected and the ability of this M2e specific phage as competing antibody is being currently evaluated Although these recombinant antibodies may not be able to be used in a competitive ELISA they are expected to be of value in understanding the antigenicity of the M2e proteins and be useful in improving M2e based ELISA and its use as DIVA reagents

In summary To develop new reagents that can be used in a DIVA test applicable in Indonesia we generated three types of reagents and evaluated usefulness as DIVA tests of three ELISAs for detection of antibodies, to the N1, NS-1, and M2e proteins. From these evaluations a DIVA test based on either recombinant Mbp-M2e protein or M2e peptides were found to be the most specific for differentiation of infected from vaccinated chicks. The specificity of M2e-DIVA test was confirmed in experimentally vaccinated and H5N1 challenged chicks. In vaccinated commercial chicks the M2e-DIVA test was able to differentiate flocks that were positive for M2e antibodies. The final proof for specificity of this M2e-DIVA test needs however to be obtained in longitudinal studies on vaccinated

layers where infected chickens are detected by the ultimate DIVA test, which is virus isolation.

Objective 3. To develop sustainable protocols for investigating effectiveness of vaccination for HPAI in Indonesia

The primary purpose of this objective was to gain a better understanding of the field use of AI vaccine, and their effectiveness in semi intensive commercial Sector 3. The effectiveness of registered AI vaccines in Indonesia, although critical for successful AI control, has been largely unknown. The outcomes of this strategy aimed to assist national veterinary authorities in devising future scenarios for more effective AI control**.** The conduct and outcomes were dependent on several critical components including: the availability of advanced epidemiological skills in Bbalitvet for data collection and analysis; willingness of a sufficient number of farms to participate in the surveys; involvement of DINAS Peternakan in all farm visits; plausible survey design that took into account farm workflows and also the need to manage the data collected effectively. The effort from all parties involved was significant and important outcomes were achieved.

Enrolment of farms from Sector 3

There has been a prevailing perception in Indonesia that it is difficult to involve commercial poultry sectors in studies on AI. This project, with the help of three DINAS Peternakan, was successful in enrolling a large number of farms from Sector 3 in the study. From 61 farms visited in three districts in West Java in the first survey (in early 2008), all were enrolled in the study testifying to the willingness and interest of this sector to participate in well-structured surveys that also benefited farms as well. Feedback to participating farms was part of the survey and this was important for continuing collaboration. Important aspect was also the design of the survey and confidentiality of information obtained. Detailed data on farm management, husbandry, biosecurity, economics and vaccination were collected in the survey and coded, contributing to farm anonymity.

Profile of farms enrolled and vaccination practiced

In total 17 broiler and 44 layer farms, evenly spread among three districts in West Java, were surveyed and serum samples collected at the time of the survey. The subsequent serological surveys of broiler farms (in 2009-10) showed that at 3 weeks of age maternal AI immunity was not present in any of the broiler flocks. Since only one broiler farm was vaccinating against HPAI, it means that most broiler farm must rely entirely on biosecurity measure for protection.

In contrast, all 44 layer farms surveyed practiced AI vaccination with most of the birds, all of the cycles. However no common vaccination protocol was used; the 1st vaccination was given at different ages, and the last before the point of lay. It was of interest that 44 farms used 19 different AI vaccines. At the time 21 different AI vaccines were registered for use in Indonesia of which only two were based on an Indonesian H5N1 subtype from 2003. All farms, but one, used the single H5N2 vaccine. Preliminary serological analysis of nine different layer farms sampled at the point of lay showed however that flocks were not fully protected against all circulating H5N1 strains.

Level of protection in vaccinated layers

In order to assess the level of protection in layer flocks vaccinated with different AI vaccines more structured serological survey was done on 15 layer farms, of which 10 used H5N2 subtype vaccines and 5 used H5N1 vaccine. Serological responses were measured using H5N2 strain and four H5N1 strains consider by antigenic mapping conducted by the OFFLU project to be the most prevalent and relevant H5N1 strains in Indonesia.

Overall the results indicate that there is little support for the use of H5N2 vaccines in Indonesia in the current situation. Although not all H5N2 vaccine responses were measured, it was apparent that H5N2 vaccines based on either Mexico or UK H5N2 strains were not as efficacious, and that inferior manufacturing was not the reason, but instead was due to antigenic differences between H5N2 strains used and challenge strains prevailing in Indonesia. Also that vaccines made in Indonesia from local strains provide higher HI titres than any of H5N2 vaccines. Hence our results indicate that H5N2 vaccines should be discontinued and replaced by the H5N1 vaccines based on the more recent strains.

Further important outcome was that none of the 61 participating farms reported an AI outbreak during the observation period of two years. Hence the results suggest that the surveyed Sector 3 farms have effective biosecurity procedures in place and that in spite of the suboptimal vaccines of H5N2 subtype farms in the Sector 3 have a satisfactory level of AI protection.

The SurvIT web-based data management tool

Another positive outcome was the use of the *SurvIT* web-based data management tool that assisted in the design and implementation of the specific surveys used on the enrolled farms. The development of *SurvIT* was undertaken to fulfil the specific project objective of managing the survey data, but will have wider application as it is not AI specific. With further enhancements this tool should be able to provide comprehensive analysis and to be shared with other projects working on animal diseases in Indonesia and beyond.

Objective 4: *To* characterise at genetic and antigenic levels AI isolates from vaccinated commercial poultry

The key expected outcome for this Objective was identification of changes occurring in vaccinated commercial flocks intensively reared. The results were to complement data obtained by other projects in Indonesia that focused on less intensively reared poultry in Sector 4; and also to highlight the weaknesses of using multiple vaccines of heterologous type that do not provide adequate protection allowing for further diversification of circulating H5N1. The approach taken stipulated isolation of variants from poultry companies in Sector 1 and 2 using different AI vaccines and was linked with the conduct of the Objective 1.

As outlined previously the Objective 1 was terminated by Bbalitvet nine months after the project commenced; field and laboratory trails were discontinued and H5N1 virus samples obtained form Sector 1 and 2 rendered invalid. Hence the planned outcomes could not have been achieved for this Objective either.

Source of H5N1 isolates used

In Indonesia commercial Sectors 1, 2 and 3 operate independently from the Government. Since compensation for AI positive flocks has not been available and the Sector feared repercussion of declaring flocks as H5N1 positive, the AIV status in these Sectors has remain largely unknown. While the Government and FAO in partnership have obtained comprehensive information on virus changes that have taken place in Sector 4, situation in Sector 1 and 2 has remained speculative. Only very recently, in October 2010, have the first data on extensive virus mutation in one company from Sector 1 come to light. It indicated existence of multiple antigenic variants of H5N1, influenced by the type of vaccine used and which have diverged from antigenic groups found in the Sector 4. It is the scenario that was predicted to happen and was to be researched and demonstrated in this Objective.

This project was successful in establishing contacts with several Commercial companies in Sector 2 that were willing to participate in longitudinal studies for monitoring serological

and infectious status of some of their layer flocks. The project guaranteed confidentially of all information as this was of the major concern and of paramount importance. At the time the project commenced, outbreaks in Sector 1 and 2 have occurred and the project obtained samples from five flocks vaccinated with different H5N2 and H5N7 vaccines. Two samples were from an AI outbreak in layers of the Company with which agreement on collaboration has been signed just prior to the outbreak. H5N1 was recovered from all five flocks and all were different from the reference strain BL/03 and from each other by HI test. Further work on characterisation of these samples did not proceed however as Bbalitvet did not allow access to the samples by the project.

H5N1 strains used

Since approval from Bbalitvet has not been obtained for use of H5N1 isolated by the project and the Objective 2 needed information on the sequence variation in the NS1 and the M2e proteins for DIVA test development, five H5N1 samples isolated by Bbalitvet were used. Strains Indo/BL/2003, WJ/TasikSol/06, WJ/TasikSob/06, WJ/Hamd/06 and WJ/SMI-US/06 were fully sequenced since only HA and the N genes from these isolates were available in the GeneBank. No significant variations were found in the PB1, PB2, NP and PA genes whereas strains that had variation in HA gene had also variations in the NS1 and M2 genes. These results were not unexpected since all of the isolates originate from non vaccinated village poultry and were isolates from early years (2006) where a small degree of amino acid changes has occurred in the HA gene. The full genome sequences for these strains will be deposited in Gene Bank once the approval from Bbalitvet has been obtained.

Complete genome sequencing of three tissue culture obtained clones from Indo/BL/2003 strains was undertaken to determine if propagation in tissue culture of H5N1 strains results in genetic changes. There were no significant changes in any of the eight segments from three clones indicating that tissue culture grown virus was antigenically identical to the egg propagated H5N1. Therefore data obtained in the SN tests can be directly compared with HI test results for the same virus.

Following termination of field trails in Objective 1, we attempted to isolate H5N1 form Sector 3 farms. Swab samples were collected from 20 multi-age farms in the area of Sukabumi and Bandung, vaccinated with four different vaccines. Samples from 13 farms were positive for PCR however no AI virus was isolated from any PCR positive samples. This was of concern however no further work was carried out to resolve these “false positive” samples.

Objective 5: *To* provide specialised training and capacity building for Indonesian scientists in molecular approaches for characterisation of viruses and for generation of diagnostic reagents

The key output of this objective was strengthening of expertise in molecular biology techniques relevant to characterisation of AIV strains and generation of regents for diagnostic tests. There was a good participation in this activity from the project staff from Bbalitvet and also from three collaborating institutions in Australia where training, or transfer of knowledge took place. In the course of the project four staff members from Bbalitvet worked in laboratories in Australia and one attended course in Bioinformatics in Australia.

The leader of DIVA test development team at Bbalitvet Dr Simson Tarigan transferred and implement successfully technology for recombinant protein expression and purification from the University of Melbourne to his laboratory. The technology was used to produce and evaluate DIVA reagents in Bbalitvet and for future use. There was further benefit from experience with techniques used in protein chemistry and immunochemistry. This acquired knowledge and skills enabled successful project outcomes for DIVA test and

furthermore it ensures sustainable application of this test in Indonesia. This enhanced knowledge has already been applied in other related areas currently underway in Bbalitvet and also enabled for further scientific research, in collaboration with the University of Adelaide, related to DIVA strategies.

A staff member from Zoonoses team in Bbalitvet Ms Atik Ratnawati has been trained in PCR technique and the use of the SN test for antigenic analysis of AIV isolates at the University of Melbourne. Since then she has taken part in Bbalitvet activities on genetic characterisation of AIV isolates but has not been able to apply the SN test in this project. She also received training at the Australian Animal Health Laboratory in laboratory biosafety, since she is anticipated to work with other zoonotic agents in the newly commissioned Zoonosis Laboratory at Bbalitvet. Her training in this project is a contribution to building the long-term capacity of Bbalitvet for work with zoonotic agents such as Nipah, Rabis and Anthrax.

A number of senior staff from Bbalitvet Dr Abdul Adjid, Ms Risa Indriani, and Dr Tatty Syafriati have been involved in the development of the questionnaire and survey for vaccine practices in commercial Sector 3 farms. Also the use of web based data system for questionnaire development, data entry and results analysis. This is a new activity previously not undertaken in Bbalitvet and enabled exposure to basic aspects of epidemiology and how complex surveillance data can be effectively managed.

Intended training in approaches for advanced molecular analysis was only partially accomplished; Dr Indi Dharmayanti attended a bioinformatic course however further training did not take place as the activities in the area of AIV gene analysis at Bbalitvet was curtailed. Also there was no interest by molecular diagnostic section at Bbalitvet to be involved in furthering their expertise and skills in this area through this project.

Ms Risa Indriani worked at Australian Animal Health Laboratory with sera obtained from Sector 3 farms, as Bbalitvet did not have the panel of Indonesian reference strains for mapping of serological responses to different antigens. This enabled exposure to standards used in such typing as well as access to new reagents that have now been transferred and used in Bbalitvet. This is particularly beneficial to Bbalitvet that is now part of the network of Laboratories for antigenic mapping of Indonesian H5N1 isolates.

The John Allwright Fellow Ms Sumaningsi has been working towards here PhD degree on antigenic characterisation of the M2e protein since August 2008. She has acquired a range of skills in molecular biology techniques, including PCR, sequencing, generation of recombinant constructs, site directed mutagenesis, protein expression and antigenic analysis of proteins and peptides. Those skills are applicable and will contribute to the ongoing and future activities of Bbalitvet in the area of molecular diagnosis of animal diseases.

The project has had some capacity building outcomes for the University of Melbourne and the University of Adelaide and for the Australian Animal Health Laboratory. The work with recombinant protein expression and use of gene fragments and peptides has resulted in further improvement of capacity in these areas in the School of Veterinary Science the University of Melbourne and benefited other projects currently on the way in the School by utilising the technical expertise gained through this project. The work with expression of recombinant rabbit and chicken antibodies at the University of Adelaide has resulted in development of a new area of expertise and activities at the School of Veterinary Science as Dr Farhid Hematzadeh who worked on this project initially at the University of Melbourne has taken a position of a Senior Lecturer and has continued his activities aligned to this project. The development of the Web based data base has involved Dr Peter Durr from AAHL in some innovation that is likely to have applications to other areas of work but it has also resulted in the effective collection of data that will have benefits at the level of antibody analysis. The scientific outputs from the project will result in publications that will add to the scientific record of the School of Veterinary Sciences at the University of Melbourne and Adelaide and the Australian Animal Health Laboratory.

Objective 6: *To* develop *a bio-economic model of Sector 3 poultry production in West Java and use this model to investigate enterprise profitability and potential impact of HPAI outbreaks and control measures (revised)*

Development of a simple state-transition model of HPAI infection

The original aim of this Objective was "To develop a simple state-transition model of HPAI infection in commercial and/or village flocks and validate the model against real outbreaks". In support of this aim, a simple state transition model was developed and tested to describe the expected behaviour of avian influenza in an infected flock. The model was functionally sound and showed promise as the basis of future modelling scenarios if suitable parameters for the Indonesian situation can be developed.

However, it was not possible to estimate model parameters under Indonesian conditions due to the difficulty experienced in getting outbreak data to validate model and the problems experienced in Objective 1. At the first annual project review meeting it was decided that this approach was no longer relevant, as AI was by then endemic in much of Indonesia and conditions had changed significantly since the project was first developed.

Economic modelling of disease impacts

As a result it was considered that economic modelling of disease impacts and control options/costs would be more useful and relevant to the Indonesian poultry industry, and the objective was modified accordingly to: "To develop a bio-economic model of Sector 3 poultry production in West Java and use this model to investigate enterprise profitability and potential impact of HPAI outbreaks and control measures".

For the revised objective, gross-margin financial models were developed for both layer and broiler flocks. Survey questionnaires were developed to support these models and coded into the SurvIT database developed as part of Objective 3. The questionnaires were piloted in 28 flocks from Objective 3 and significant issues were identified with data quality and interpretation of questions. Unfortunately there was no opportunity before the project finished to re-develop the questionnaire talking into account the problems experienced. The available data was analysed in the model but the problems in the data prevented any real conclusions being drawn.

The financial models developed were internally sound and potentially valuable for the Indonesian poultry industry. However, problems experienced with data collection prevented revision of the models to optimise them for local conditions and to analyse quality data collected from local farmers.

Objective 7. Communication and Collaboration

Aims were to strengthened linkages with and promote scientific output of the project within DGLS and FAO/OFFLU so that the project results may be of use in policies development and future control of H5N1. From meeting that took place with both Organisations two project outputs, the DIVA test and surveillance in Sector 3 farms, where identified as contributing to the current knowledge and future control of AI in Indonesia. In particular the DGLS might use the information obtained form vaccine assessment in Sector 3 in framing its policy on future vaccine use in Indonesia and use DIVA test as adjunct in improving the AI control with possible immediate application of DIVA in its program of targeted vaccination planned for Sector 4.

8 Impacts

8.1 Scientific impacts – now and in 5 years

Indonesia is one of the few countries that have opted for widespread, long-term vaccination to control HPAI. Previous knowledge on AI vaccination obtained in other countries is limited and it has become apparent over the last few years that the AI strategies applied in developed countries and directly implemented in Indonesia had limited success. There are many variables uniquely Indonesian that need to be taken into account and new approaches for AI control devised. During the conduct of this project new results were generated in two areas, development of DIVA test and assessment of efficacy of vaccination in small-scale commercial flocks, both expected to have immediate scientific impact and contribute to the overall increased knowledge and improvement in management of HPAI control.

Assessment on efficacy of vaccination

Experiences with AI vaccination in two countries, Mexico and Italy, have been used as guiding principles in implementing vaccination in Indonesia; in particular the exclusive use of heterologous vaccines, most notably of H5N2 subtype for control of H5N1 infections. While some in poultry industry and scientific community in Indonesia, including this project, have argued that this type of vaccines will lead to increased antigenic variation, regardless the H5N2 and other heterologous vaccines have dominated the vaccine market in Indonesia. Only recently has Indonesian Government, following laboratory challenge studies, allowed manufacture and registration of two vaccines based on local H5N1 strain(s). Use of appropriate vaccines is a cornerstone of AI control, however, in spite of this there have been no data available to support use of any of AI vaccines registered in Indonesia. Our assessment of vaccination in Sector 3 farms for the first time demonstrated the widespread use and dominance of H5N2 vaccines. Also that there is a limitted scientific evidence for use of such vaccines for control of H5N1 in Indonesia; antibody titres induced by these vaccines are less effective against antigenic variants predominating in Indonesia and moreover the locally made vaccines were better inducer of AI immunity then any of imported vaccine made from heterologous strains. Hence the project provided scientific rational and impetus for discontinuation of use of heterologous AI vaccines in Indonesia. This would be a major policy change in AI control in Indonesia, and globally, that would however have a long term consequences for improved AI control.

Development of DIVA test for large scale screening

DIVA strategy used successfully in Italy was also advocated for implementation in Indonesia however the method used in Italy is not applicable on a large scale and in laboratory set up of Indonesia. The DIVA tests evaluated in this project took into the account local needs and identified the M2e antigen as the most feasible antigen for AI DIVA application in Indonesia. Since very little work has been previously carried out on the AIV M2e antigen, scientific studies were also undertaken on the expression and characterisation of antigenic properties and immune responses to the M2e antigen. This work provided the results that scientifically supported the use of this antigen as DIVA marker and also provided confidence in the test ability to differentiate between AI infected and vaccinated chicks.

The use of the *SurvIT* web-based survey data management tools was a new and useful scientific innovation that could be used by other projects on AI in Indonesia and elsewhere.

8.2 Capacity impacts – now and in 5 years

The project made considerable impact on the scientific expertise and capability of Bbalitvet in a number of areas:

The development of M2e as recombinant protein and its use as serological test has involved new technology for Bbalitvet and Indonesia. This technology is likely to be increasingly used as a method for antigen production in the future and some aspect of this technology have already been applied in another project currently undertaken at Bbalitvet.

There has been an extensive training and transfer of technology associated with this project and four staff had an opportunity to further develop their skills in additional molecular techniques, tissue culture and biosecurity, all relevant to the current research in Bbalitvet. Some skills have already been used in other zoonotic diseases research in Bbalitvet.

Bbalitvet does not currently have an epidemiological unit for field data collection and analysis, and therefore this is an area for potential future capacity building. This project began this process by demonstrating and utilising practical epidemiological skills, including questionnaire design, development of sampling protocols, data management and data analysis.

Further benefit to Bbalivet is the increased profile in Indonesian scientific community as the organisation where competent and outcome oriented research can be undertaken. The John Allwright Fellow has develop extensive range of skills that will contribute to the ongoing activities of Bbalitvet in the area of molecular diagnosis of animal diseases.

8.3 Community impacts – now and in 5 years

8.3.1 Economic impact

In Indonesia chicken meat, together with fish, is the primary protein source particularly in rural areas. Typically women and children care for native chicks, whereas chickens reared in semi intensive Sector 3 are often cared for by families. Occurrence of HPAI H5N1 have had significant economic impacts on all poultry due to mortalities, culling, high prices for day-old chicks and sale of birds before reaching marketable age, conservatively estimated to be about $100 million. There are also significant indirect losses, associated with cost of increased biosecurity, vaccination and disease management, estimated to be in the order of $540 million.

The perception that poultry are not safe for human consumption also has had an impact on of all types of poultry, and sales of chicken meat and eggs have been reduced. Further, pressure from the public health sector has been directed particularly towards village and Sector 3 poultry that do not use high levels of biosecurity and for such production to be curtailed. As a result various initiatives are being considered and costly re-structuring of the poultry marketing chains, that had the greatest impact on small poultry producers in terms of limited market opportunities, has commenced.

Considering the current options available in Indonesia, the most immediate and feasible improvement will come from the use of more effective vaccines. In this context, the direct contribution of this project was to identify that differences exist among AI vaccines used and that significant number of vaccines are providing suboptimal protection. Hence these vaccines, rather than being of benefit are the risk factors for an effective control and do not provide optimum return on the investment. The economic gains from an effective control would be immediate particularly in Sector 3, and further gains would be achieved over the period of 2 – 5 years as transmission rate between flocks is reduced, and overall load of virus in the environment is reduced. The application of DIVA technology

developed during this project would provide support for vaccination by determining if there is significant residual exposure to H5N1 field challenge and if there are regional differences.

8.3.2 Social impacts

Human infections and deaths due to H5N1 in Indonesia have been linked with direct contact with infected poultry. International view is still prevailing that the H5N1 in the poultry sector in Indonesia continues to be a serious risk factor for the emergence of human pandemic. The social and economic repercussion of such a pandemic would be considerable in all countries. Finding of this project that the most effective vaccines, which also reduce the load of H5N1 in poultry, are not used, make a contribution to the reduction of H5N1 as a pandemic threat. In general outbreaks of H5N1 in poultry also have social impacts with market reactions and an impact on family income especially of small producers who raise household poultry for supplementary income. Disease outbreaks or disease control culling operations also affect poor families who raise household poultry as a supplementary food source. The outputs of this project when realised have potential to have incremental impacts on efforts to control HPAI in Indonesia and mitigate against the social impacts of the disease

8.3.3 Environmental impacts

There should be a reduction in waste disposal resulting from bird mortalities following application of more effective vaccines. Large quantities of disinfectant have been used on all infected premises, and effective vaccination should reduce usage of such chemicals.

8.4 Communication and dissemination activities

There have been a number of meetings between Project staff and various poultry sectors representatives usually at the higher management level. At the outset the project leaders promoted the project aims within Sector 1 and 2 poultry companies and secured collaboration with two companies. The feedback was part of the collaboration and the results obtained in Laboratory and field trails in Objective 1 were communicated in full to the participating companies. This type of collaboration aligned with the prompt feed back, that included all to understand results interpretation and their implication, was a good mechanism in obtaining access to the relevant poultry Sectors. However not all companies wished to collaborate and share information, likely due to degree of distrust and fear of undesirable exposure. It was far simpler to communicate and collaborate with semi-intensive poultry Sector 3, in particular this was helped by DINAS staff who together with the project staff visited twice 61 broiler and layer farms during the enrolment phase and collection of data on effectiveness of vaccination. Similarly farmers were willing to collaborate and share information looking for some informative results in return that would help with the disease management. This however does not appear to happen regularly in Indonesia, and the success of this project was likely due to an effective feedback on the comparative standing of farms in regards to practices used elsewhere.

The project has also made contact and linkages with FAO project activities in Indonesia relevant to AI. In the inception stage the project participated in a meeting organised by FAO and all major poultry companies, vaccine manufacturers and other poultry veterinary organisation representatives with the aim of involving all poultry Sectors in the FAO antigenic cartography project. This contact continued and scientific outputs of the project were presented to the DGLS and FAO/OFFLU so that the project results may be used in policies development for future control of H5N1. The DIVA test and surveillance in Sector 3 farms were identified as contributing to the current knowledge and future control of AI in Indonesia by both DGLS and FAO. In particular the DGLS might use the information obtained form vaccine assessment in Sector 3 in framing its policy on future vaccine use

in Indonesia and use DIVA test as adjunct in improving the AI control with possible immediate application of DIVA in its program of targeted vaccination planned for Sector 4.

DIVA test developed was distributed to a local private diagnostic laboratory, on a trail basis, to promote the test in a more practical environment and for an independent evaluation of its usefulness. It was trailed on poultry samples from farms around Bogor area and some farms were identified as positive for virus challenge. The feedback was positive and there was a farm that requested regular DIVA testing of their samples, being willing to pay for the cost of testing. This free of charge pilot testing of DIVA could be increased as a part of the market placement.

9 Conclusions and recommendations

9.1 Conclusions

The project aims and expected outputs were highly ambitious and covered many of the key areas of AI control in Indonesia. All planned objectives were based on scientifically sound hypothesis and demonstrated to be true, either in this or other AI projects undertaken in Indonesia. All planned objectives remained highly relevant even today four years after the inception of this project and outputs realised are expected to have scientific, economic and social impacts.

The project was highly successful in establishing effective contacts and collaboration with both intensive and semi intensive poultry Sectors that went beyond simple surveillance. While the assessment of vaccination in Sector 1 and 2 did not conclude, we did obtaining significant, albeit incomplete results in a short time, that demonstrated the uniqueness of this sector as the major ground for emergence and evolution of H5N1 viruses in Indonesia. Although the project did not complete assessment of vaccine efficacy in Sector 2, it obtained some valuable results by doing the similar assessment in Sector 3. The Sector 3 proved to be markedly different and some unexpected results were obtained, particularly the demonstration that a relatively good control of AI is achieved in this Sector in spite of adverse locations of farms in the major outbreak regions in Indonesia. Further studies however are needed to define additional parameters that contribute to effective vaccination in this Sector such as for example level and degree of shedding. There is also a prospect of new alternative vaccines being used in Indonesia in addition to H5N1 vaccines based on multiple strains. The effectiveness of such complex vaccination practices on AI control is unknown and difficult to predict.

Although it is well known in Indonesia that myriad of AI vaccines are registered and available for use, this is the first study to provide comparative field data; it came as a surprise that on 44 farms surveyed in early 2008, 19 different vaccines were used. Even more surprising was that most are heterologous vaccines that do not afford protection even against old strains. Hence an impetus exists now for the major shift in policy on AI immunisation since it is documented that heterologous vaccines have not worked well in Indonesia. Our project has developed the tools to enable the Government of Indonesia to collect data from Sector 3 poultry farms about current vaccination and biosecurity practices, and thus better inform policy in these areas.

DIVA tests, although beset by a number of restrictive factors, did emerged as a plausible technology that if applied in vaccinated flocks could give a simple indication, over time, of effectiveness of vaccination in preventing AI shedding or challenge.

9.2 Recommendations

Infections with H5N1 continue to be a significant veterinary and public health concern in Indonesia, imposing significant economic burden on many public and private sectors and institutions, and the country is still considered as one with the highest potential to generate human pandemic.

The Government of Indonesia is committed to the gradual reduction of H5N1 incidence and is looking for additional and new strategies to achieve this including introduction of disease free control zones, targeted vaccination and alternative vaccines.

To aid in the implementation of any of these strategies additional support tools will be required to monitor the effectiveness of measures adopted. Outputs of this project, the DIVA technology and the survey questionnaire, linked with the *SurvIT* database, could contribute significantly in this next phase of the AI control measures.

The DIVA technology would be applicable in two scenarios; to confirm the efficacy of vaccines in preventing virus shedding and also in confirming the absence of challenge in regions where discontinued vaccination could be implemented. Longitudinal studies that monitor the effectiveness of new vaccines would have an important role in confirming that approach adopted is working. Improved vaccination strategies will result in a significant reduction in the cost of production and improve the health status and the security of the food supply from the poultry sector.

In the previous five years there has been a significant investment by the international donor organisations into improving control of HPAI in Indonesia. Many of these projects are coming to an end and there will be, both internally and externally, shift from HPAI into veterinary services in general. In addition to HPAI, beef production has also become a priority area, which means that resources will need to be shared, and those allocated for HPAI control will decrease. Hence there will be less research activity within the country, and from external sources, that will be reflected onto the effectiveness of measures taken.

10 References

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10.2 List of publications produced by project

Formal publication in Conference Proceedings or Scientific Journal (published and planned)	Outputs described in the publication and their significance to project outcomes.
S Tarigan, R Indryani, Darminto & J Ignjatovic. 2009. *Purification of Neuraminidase from subtype H5N1 Influenza Virus.* Jurnal Ilmu Trernak dan Veteriner 14(1), 75-82	The neuraminidase (N1) protein from an Indonesian H5N1 strain was purified and tested in ELISA as possible DIVA reagent. A range of DIVA options tested aiming to develop the most effective DIVA test.
F Hemmatzadeh, S Tarigan, Sumarningsih, R Indriani, NLPI Dharmayanti & J Igniatovic. *Recombinant M2e ELISA as a tool for differentiating chickens Infected from those Vaccinated with avian influenza virus*. Submitted for publication.	Expression of recombinant M2e protein *in vitro* and its use for differenation of infected from vaccinated chickens was evaluated in laboratory conditions. The developed regent was suitable to be used in a DIVA tests and as an additional tool in improving AI control.
P Durr, R Indriani, RM Adjid, T Syafriaty. Hardiman & J Igniatovic. *Vaccine usage to prevent HPAI-H5N1 in small to medium sized ("Sector 3) poultry farms in western Java, Indonesia*. To be submitted for publication in Transboundary and emerging Diseases.	Results of the survey of vaccination practices in the area are described. It documents for the first time vaccination practices used in this sector in Indonesia over period of 2 years providing concrete evidence for legislator in Indonesia.
P Durr, R Indriani, RM Adjid, & J Igniatovic. SurvIT: A *web-based tool for designing and managing complex epidemiological surveillance data.* To be submitted for publication in Tropical Animal Health and Production..	Development of a web-based application "SurvIT" for managing complex epidemiological questionnaires is described. As the SuvIT was in two languages, Its use in the project enabled effective data entry and analysis in both Indonesia and Australia, at any time, enabling better remote management of the surveillance undertaken in Sector 3 flocks.
P Durr, R Indriani, RM Adjid, T Syafriaty. Hardiman & J Igniatovic. *AI immunity in layers in semi-intensive commercial sector in Indonesia.* To be submitted for publication in Avian Diseases.	Results of surveys on effectiveness of AI vaccines used in Sector 3 layers to elicit cross-protective H5N1 immunity is described. Data provide evidence for legislator in Indonesia that heterologous vaccines are not effective against H5N1 strains circulating at the time in Indonesia.