Annex 4

Indonesia – Netherlands Partnership on Veterinary Control of Highly Pathogenic Avian Influenza

Mission Report Module 6; January – February 2010

Report on the diagnostic training (module 6): 23rd of January till the 3rd of February 2010
Rene Heutink and Gerard Wellenberg, The Netherlands.

Working programme

Date /Time	Activity	Contact
23rd of January	Departure from Schiphol at 20.45 hour	
24th of January	Arrival at Jakarta	Ibu Ratina and ibu Deswarni (Jakarta lab).
25th and 26th of January	Visit the Jakarta lab	Ibu Ratina and ibu Deswarni (Jakarta lab).
27th of January	Visit IPB (Rene) and the BBPMSOH (Gerard)	Ibu Ketut and ibu Yuni (BBPMOSOH); Ibu Sri (IPB).
28h of January	Visit the Cikole lab in Lembang	Ibu Mudji, Pak Umar and colleagues
29th of January	Visit the Cikole lab in Lembang.	Ibu Mudji, ibu Frida and pak Umar
30th of January	Visit the Cikole lab in Lembang	Ibu Mudji, ibu Frida and pak Umar
1st of February	Visit the Cikole lab in Lembang, and visit Medion	Ibu Mudji, ibu Frida and pak Umar
2nd of February	Writing the report and prepare the IPB visit on February 3rd.	
3rd of February	In the morning IPB Bogor. In the afternoon to the airport of Jakarta. February 4, 2010; arrival Amsterdam	Ibu Sri.

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Visit Jakarta Dinas Peternakan Laboratory: Monday 25 and Tuesday 26 January, 2010.

Discussion with ibu Ratina and ibu Deswarni on the real-time PCR results (H5- and M-real-time PCR) and on the progress of the analyses of the collector house study.

Geelong ring trial October 2009
The results of the Geelong ring trial no 7 (October 2009) were reviewed. The M- and the H5 real-time PCR, and the conventional H5-PCR results of all 10 samples were correct (100% score).
The results of the 10 sera, which were tested for H5 antibody titers by HI, were equal or within the range of plus-minus one log2 titer step of the median H5 antibody titer (Geelong). This means that the Jakarta lab scored good results in the H5 antibody test.

Kingfisher training (96-well RNA extraction method)
In the afternoon, a training was given on the Kingfisher RNA/DNA extraction machine. Two kits of the Ambion Viral extraction kit AM1836 were given to Ibu Ratina for training. With the Kingfisher, in combination with the the Ambion kit, a total of 96 RNA extractions can be performed within 50 minutes.

The protocols on the use of the Ambion AM1836 and the AM1840 were given to ibu Ratina and discussed. A standard operating procedure was written to use the Kingfisher96.

The new experimental H5-PCR probe (based on the VLA probe) was used and compared with the Geelong H5-PCR probe. Some of the examined H5-PCR positive samples showed a low plateau with the new H5 probe and not with the Geelong H5 probe. It was decided not do analyze more samples with the new H5-probe.

Neuraminidase (N1) PCR training
A theoretical training was given on the use of two N1-primers (VLA primers; described for the implementation of a conventional N1-PCR). Primer sequences were blasted, and the amplified product was studied (FAO has asked to perform the N1-PCR on H5 positive samples from the wet-marked study).

Action list after visit Jakarta laboratory January 25th, 2010

Description	Responsible	Deadline
HI-tests		
Reference sera were handed over	Gerard	Done
PCR's		
Kingfisher 96 reagents were handed over (AM1836)	Gerard	Done
Implement software for the performances of the AM1836 and the AM1840 extraction kits	Ibu Ratina	February 2, 2010
Implement hard-ware (plastics and the deep-well magnetic block) for the Kingfisher96	Ibu Ratina	February 2, 2010
Quality System: ***Equipment***		
SOP on the use of the Kingfisher	Rene	Done

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Visit BBPMSOH: Wednesday, January 27th, 2010

Visit BBPMSOH for HI training (Gerard).
The AI vaccine testing for potency at BBPMSOH was performed by HI-test. Antibody titers of vaccinated chicken were tested against H5N2 antigen and against H5N1 antigen.
The HI-test was performed as described by the OIE (OIE manual 2008), and the HA-titer was determined using the OIE 2008 method by making more serial dilutions of e.g. 1:2, 1:3, 1:4 and 1:5.
The adapted procedure for the determination of the HA titer was not ready yet.
Also the use of a serum control sheet/book was not implemented, but will be completed for future test runs (ibu Yuni).

Influenza antigens and control sera for HI tests were given to ibu Yuni.

The RNA extraction and the performances of the conventional PCR to detect viral RNA in influenza vaccines were explained. Ibu Yuni will prepare a standard operating procedure and will send it to Gerard (before January 28, 2010; Performed on February 02). She also performed and compared 4 different RNA extractions on influenza vaccines. After extraction, the conventional M-PCR was performed successfully for 3 of the 4 extraction methods. She could continue with performing the conventional PCR on influenza vaccines now.

Action list after visit BBPMSOH laboratory January, 2010
New actions are added to this list.

Description	**Responsible**	**Progress**
Serology tests		
Prepare a serum control book / sheet	Ibu Yuni	
PCRs		
Perform RNA extractions (4 ways) and the PCR for the detection of influenza viral RNA in vaccines.	Ibu Yuni and Ibu Ketut	Done
Quality System: ***Equipment***		
Prepare a standard operating procedure (SOP) on the RNA extraction and the PCR on influenza vaccines	Ibu Yuni	Done
Adapt the standard operating procedure on the HA titration according to the OIE manual 2008.	Ibu Yuni	

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Visit to IPB Veterinary Faculty on 27 January and 3 February, 2010

Visit to IPB for virus isolation (Ivo, René and Patrick (other appointment))

Unfortunately the ordered embryonated SPF eggs were not delivered on time (Wednesday 27, 2010), so the decision was made to discuss the virus isolation protocol again with ibu Sri (prevention of cross-contamination and bio-safety). These topics were already addressed by Guus Koch (August 2009). Decided was to plan a follow-up training on Wednesday February 3, 2010.

The following points were discussed:

- Protocol for virus isolation (SOP)

  According to ibu Sri a virus isolation protocol has been made, but this could not been shown due to computer problems. Technicians still inoculate different amounts of sample (0.1-0.2 ml), and the procedure to avoid cross contamination was not recorded in the protocol. **Action Ibu Sri;** standardization of the amount of inoculated sample (advice 0.2 ml) and implementation of the procedure to prevent cross contamination. Briefly, put only materials in the cabinet which are used for one sample; so use small aliquots of antibiotics, etc. Change gloves after each sample. After a sample had been inoculated, clean and remove all the equipment and let the cabinet run for 1-5 min. Never handle two samples at the same time!

- Lamp to observe embryo's (embryonated eggs)
  A new lamp was delivered.
- Needles
  New blunt needles were delivered to inoculate eggs
- Raw data sheet: Form for recording the mortality of the eggs
  Not prepared. **René** showed ibu Sri the CVI-form on Wednesday February 3, 2010. **Action Ibu Sri;** create a IPB working form for recording raw data such as mortality.
- Checking inoculated eggs for non-specific reactions (embryonic death).
  All the inoculated eggs with embryonic death within 24 hours after inoculation should be checked with HA (or matrix-PCR). When these tests are negative new eggs should be inoculated after adding higher concentrations of antibiotics. Also filtration of the sample through 0.45 um filters could help to reduce bacterial contamination. Filtration will reduce the detection limit and should be applied only when other measures are ineffective.
  Samples should be inoculated onto blood agar plates routinely to check for bacterial contamination. Bacterial culturing plates should be read after 24 hours and 48 hours, against a light source, to check for the presence of bacteria. According to ibu Sri this will be done when eggs die (within 24 hours) and no agglutination was found.
  Action Ibu Sri: Implement this procedure in the protocol.

<u>Bio-safety issues</u>

The lab of IPB is mostly used for AI-virus isolation. According to ibu Sri, no rabies work has been performed in the AI-lab during the last half year. Unfortunately no logbook was created to monitor this (listing should comprise the workers name, date and time of starting and finishing work, pathogen and disinfection method).

Action Ibu Sri: create logbook at the entrance of the lab (this was implemented on February 03, 2010).

The lab is closed, and the owner of the key is ibu Sri. All technicians, working at this lab are aware of the risks of acting with high concentrations of HPAI virus. Technicians are using lab coats which stay in the lab and the coats are washed frequently. At this moment slippers are used in the lab, but the advice is to use shoes which totally cover the feet. In contrast to the masks and gloves which are

being used, no safety glasses (goggles) are used. Advice is to use them. Materials should first be disinfected with EtOH before they can be removed from the safety-cabinet.

There are still problems with the cabinet but progress has been made. Probably a wrong HEPA-filter has been used. A technician was already called to fix this (this problem was not yet fixed on Wednesday 03, 2010).

On **Wednesday 03 February, 2010**, the following progress was recorded at IPB:

- a raw version of the virus isolation protocol was made by ibu Sri, but not finished. She will send the next version to pak Rene for comments.
- in total 18 samples from the Jakarta lab (collector house study samples 2009) were inoculated on embryonated eggs (including a H5N1 positive control (105 EID50/ml) and a negative control)
- Eggs were observed for embryonic abnormalities (and death) on day 1 and 2 pi. All embryo's were death according to the technician and placed at 4C (including the negative control eggs).
- On Wednesday the HA test was performed on allantoic fluids of eggs inoculated with a sample from Jakarta, and the positive and negative control eggs.
- The HA was performed on glass slides using 5% chicken red blood cells. The HA was positive for the H5N1 inoculated eggs (3x), negative for the negative control eggs and one of the three eggs of the Jakarta samples also showed positive haemagglutination (weak).
- Samples were stored at -70C for bacteriological and PCR examinations (M-PCR).
- All other eggs were examined for HA in the afternoon. Samples were stored for performing the second passage (all examinations checked by pak Patrick).

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Visit to Cikole laboratory on 28, 29 and 30 of January and 1 February 2010.

Visit to Cikole lab, Lembang, West-Java

Geelong ring trial October 2009

Conventional H5 PCR

The AAHL ring trial 2010 results of the Cikole conventional PCR were correct except for sample #9 (1: 10,000 dilution of the H5N1 positive sample #8).

Implementation of the real-time PCR

The real-time PCR equipment from Applied Biosystems (7300; ABI) was installed in November 2009. After installation, a training was given by ABI to pak Umar, ibu Frida, pak Nurherman, ibu Dian, and ibu Titin. After this training in December 2009, no real-time PCR test runs have been performed by the Cikole lab.

The M-PCR and H5-PCR primers-probe (Geelong primers and probes) were given to pak Umar. Stock solutions and working solutions were prepared, and a M- and H5 real-time PCR test run was performed, using RNA extraction from Geelong samples and H5N1 dilutions. Practical trainings were given to ibu Dian and ibu Frida.

The Geelong ring trial samples (RNA extracts) were tested by M- and H5 PCR, including RNA extracts of a H5N1 tenfold dilution row (prepared in October 2009). This to determine whether the detection limit was satisfactory compared to the Cikole conventional PCR and the real-time PCR of Jakarta.

Results of the real-time M-PCR on Geelong samples (ring trial samples 2009) were good. The Ct-values of the Cikole real-rime M-PCR were comparable to the median Ct-values of the labs that performed the Geelong ring trial 2009. The results of the real-time H5-PCR on the H5N1 positive Geelong samples were correct. However, weak positive reactions (doubtful reactions; Ct-values of 36 and 38) were recorded for the NDV and PMV7 samples.

Further instructions were given to ibu Frida and pak Umar to get more experience on real-time PCR, and to examine the detection limits of the M- and the H5 real-time PCR on samples from Geelong. The real-time PCR should be performed in the future with reagents similar to the reagents that are used at the DIC's.

The standard operating procedure of the M- and H5 real-time PCR (protocol Geelong AAHL) was written and screened for mistakes. In addition, real-time PCR working sheets (for archiving raw data) were written, protocols to prepare working solutions of primers and probes were written and discussed. These working sheets should be implemented by Cikole for real-time PCR test runs.

AI-serology

The HI-results of the Geelong ring trial 2009 were correct (the same or one log2 titer step higher or lower compared to the median HI titers of the other participating labs).

The three H5N1 antigens from Vaksindo were screened in the HI-test using well defined batch control sera. Results were compared with the results obtained with the Bbalitvet 2003 antigen. The new batch of Bbalivet H5 HI-antigen was tested too (batch control).

New antigens H5 HI test (Cikole)

Table 1: **Bbalitvet 2003 and Vaksindo antigens**

sample code	Antigen 1		Antigen 2		Antigen 3		Bbalitvet	
S1	2	2	0	0	3	3	2	0
S2	1	1	0	0	0	0	2	2
S3	4	4	2	2	4	4	3	3
S4	3	4	2	1	4	4	3	4
S5	4	4	2	3	4	4	3	3
S6	4	3	3	3	4	5	4	4
S7	4	3	3	3	5	4	5	5
S8	nd	nd	nd	Nd	nd	nd	nd	nd
S9	4	4	2	2	4	4	3	4
S10	6	5	5	5	5	6	4	5
S11	5	5	3	3	4	5	5	4
S12	5	5	5	4	5	6	4	4
S13	0	0	0	0	0	0	2	2
S14	3	3	2	2	4	3	0	2
S15	0	0	0	0	0	0	0	0
S16	3	3	0	0	4	3	4	3
Mean	3,2	3,1	1,9	1,9	3,3	3,4	2,9	3,0
Pos H5	5		3		4		5	
Pos H5 new	4		2		3		4	
H7	0		0		0		0	
negative	0		0		0		0	

Next mission. Focus on:
1: Implementation real-time PCR and training DIC protocol

Action list after visit Cikole laboratory October, 2009
New actions are added to this list.

Description	Responsible	Progress
Serology tests		
Clean the serology room thoroughly and throw all expired reagents, kits and plastic bags with sera in the autoclave.	Umar and Sri Mudji	
Select sera from SPF chickens (neg control sera) and positive control sera for Mg testing (increase quality of Cikole lab)	Umar and Nurherman	
Serum control book should always be filled in.	Umar and colleagues	
PCRs		
Clean PCR freezers and fridges in PCR room I and II, and destroy all PCR samples and PCR tubes with amplicons that are not needed anymore	Ibi Frida, pak Umar and Nurherman	
Order reagents for real-time PCR (DIC protocol; contact DIC Waters) and order new storage boxes and labels	Ibu Mudji	

Order at least 4 Qiagen RNA extraction kits AI project	Ibu Mudji	
Order new fridge-freezer combination PCR room I	Ibu Mudji	
Perform test runs of the real-time PCR using the M and H5 primers/probe on Geelong and field samples for practicing	Umar and colleagues	
Use PCR room I for preparing the PCR mixes	Ibu Frida	
Use only filter tips	Ibu Frida	
Quality System: ***Equipment***		
Inform for improvement of the ELISA test system	Gerard	
Remaining subjects		
Visit of ibu Frida to Holland in March-April 2010	Gerard	
Reports of experts should be communicated with lab personnel	Sri Mudji	Always

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Visit to Medion on Monday 1st of February 2010

Visit Medion (ibu Anita and colleague). Discussions on the real-time H5 PCR plots of test runs, that were already performed, the IBDV PCR, IBV PCR and on the Marek virus PCR.

Next mission. Focus on:
1: Training to improve the molecular-biology standard of the Medion lab.

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Wednesday 3rd of February.
In the morning: Improvement of virus isolation in the IPB lab (see above for more information).

Wednesday 3rd of February
Back to Holland.

Gerard Wellenberg and Rene Heutink