Avian Influenza National Reference Laboratory (NRL)

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Afssa’s Laboratory for studies and research on poultry, pig and fish farming in Ploufragan has been France’s NRL for avian influenza (AI) and Newcastle’s disease (ND) since 1992, the year the first European directives on the monitoring of these diseases were issued. This national reference activity, along with an OIE (World Organization for Animal Health) reference activity in infectious bursal disease and avian metapneumoviruses, is currently carried out, in conjunction with research into major and emergent avian and rabbit viroses, on the site dedicated to the Avian and Rabbit Virology, Immunology and Parasitology unit (1,100 m² sq of Level 2 laboratories and 80 m² of Level 3 laboratories). On this same site (Ploufragan), the NRL also has access to the Laboratory for studies and research on poultry, pig and fish farming’s experimental facilities for its chicken pathogenicity tests.

The AI national reference activity began to truly take off in the late 90s with efforts to anticipate the introduction of highly pathogenic (HP) AI viruses in France and to ascertain the situation of French poultry producers with regard to low pathogenic (LP) AI virus infections (H5/H7 sub-types), because of their potential risk to animal health as well as to human public health. This brought about the rapid development of molecular diagnostic tools, while the AI NRL became a driving force in the development, implementation and monitoring of AI surveillance of poultry farms and wild bird fauna in France.

The AI NRL was also set up to provide the necessary responsiveness and to adjust the human resources deployed to the epizootic risk. In normal periods, to date, six full-time equivalent positions have been devoted to the AI/ND reference activity; however, 14 people (13 full-time equivalent positions), also assigned to research positions, are certified in the skills required for the entire AI/ND field of accreditation. These individuals are mobilised in alternation according to the variable needs over the year and depending on epizootic risk. Technical stand-by duty for weekends and holidays can be implemented when necessary. In crisis periods, such as in the winter of 2006, all the unit’s virology personnel were redistributed and mobilised into three teams ensuring a seven-days-a-week relay with extended hours. In addition to this workforce, the laboratory’s Avian pathology animal experimentation and relaying needs (some of which were developed in the NRL in the 1980s), and haemagglutinin typing of haemagglutinating AI and ND viruses with the aid of reference sera and monoclonal antibodies (some of which were developed in the NRL in the 1980s), and haemagglutinating AI and ND viruses. An NRL researcher who had participated in their EU-level standardisation wrote the French reference texts and the corresponding standards thanks to his regular participation in AFNOR working groups on animal immunoserology and virology. The reverse transcription polymerase chain reaction (RT-PCR) sequencing procedures developed ten years earlier in the pigeon ND/paramyxovirus laboratory were applied to the AI diagnosis in the late 1990s. They were then brought up to date with the arrival of automatic sequencing and real-time RT-PCR techniques, then validated in collaboration with the CRL and several other European pilot NRLs. This brought about the creation of the European Manual on AI Diagnosis which Council Directive 2005/94/EC draws on and which represents the regulatory foundation for EU measures in the fight against AI.

This article illustrates the way in which the AI NRL fulfils its NRL missions as set down in the decree dated January 5, 2006, namely the development and methodological validation, including standardisation, accredited laboratory network coordination, performance of official analyses (confirmation in particular), scientific monitoring and technology watch, and answering to all scientific and technical expert assessment requests from the Ministry of Agriculture and Fisheries and, if necessary, from other ministries. This article also situates the AI NRL in the global, and more specifically EU-level, network of AI reference laboratories and attempts to highlight the respective contributions of each to both research and expert assessment work on this topic.

Methodological development, validation, standardisation and accreditation

The AI/ND NRL has been accredited since 1997 for the COFRAC’s programmes 109 (animal serology) and 112 (animal virology) with minor extensions in 2002 and 2003, which cover the following official techniques: ND and AI H5, H6, H7 haemagglutination inhibition (HI) assay, AI agar gel immunodiffusion (AGID) tests, ovoculture isolation and haemagglutinin typing of haemagglutinating AI and ND viruses with the aid of reference sera and monoclonal antibodies (some of which were developed in the NRL in the 1980s), and haemagglutinating AI and ND viruses. An NRL researcher who had participated in their EU-level standardisation wrote the French reference texts and the corresponding standards thanks to his regular participation in AFNOR working groups on animal immunoserology and virology. The reverse transcription polymerase chain reaction (RT-PCR) sequencing procedures developed ten years earlier in the pigeon ND/paramyxovirus laboratory were applied to the AI diagnosis in the late 1990s. They were then brought up to date with the arrival of automatic sequencing and real-time RT-PCR techniques, then validated in collaboration with the CRL and several other European pilot NRLs. This brought about the creation of the European Manual on AI Diagnosis which Council Directive 2005/94/EC draws on and which represents the regulatory foundation for EU measures in the fight against AI.

The AI NRL then improved on its previous achievements and obtained the first COFRAC Animal Health PCR accreditation for four real-time RT-PCR techniques; it continues to improve on these techniques with the construction of an armoured RNA internal control that makes it possible to validate all the stages of the process.

Using real-time RT-PCR techniques and/or RT-PCR sequencing, the AI NRL is able to perform the detection and full characterisation of all eight genes of all the influenzavirus subtypes. The laboratory’s analytical capacity is also expressed in particular, scientific monitoring and technology watch, and answering to all scientific and technical expert assessment requests from the Ministry of Agriculture and Fisheries and, if necessary, from other ministries. This article also situates the AI NRL in the global, and more specifically EU-level, network of AI reference laboratories and attempts to highlight the respective contributions of each to both research and expert assessment work on this topic.

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in its full mastery of bioinformatics tools which enable it to identify specific mutations and monitor the genetic evolution of viruses, LP (low pathogenic) AI H5 in particular, as well as to study their antigenic evolution, within the limits of access to the relevant samples.

The NRL has also developed and/or validated ELISA influenza NP, N1, NS and M2 tests and within the framework of the EU programmes, is pursuing its work on the development of DIVA programmes, is pursuing its work on the development of DIVA tests (in order to distinguish infected fowl from vaccinated fowl) and the evaluation of serological test kits.

**Certified laboratory network coordination and adherence to the European and global laboratory network**

Previously, the NRL offered regular training sessions for diagnostic laboratories specialising in poultry breeding, essentially in basic AI AGID (and Newcastle HI) techniques, and upon request, more specialised workshops (ovoculture in particular). In this way, a network of public and private laboratories specialising in poultry breeding was established. Now that the laboratories are well versed in these techniques, training sessions are only provided upon request (except for the specialised training sessions set up for the transfer of new techniques - NP-ELISA, H5/H7 HI, M and H5 gene real-time RT-PCR).

The NRL has organised yearly inter-laboratory proficiency tests (ILPTs) in accordance with programme 109 (in alternation with influenza AGID or HI - only Newcastle HI prior to 2002, then Newcastle HI and screening for H5/H7) since 1993 and since 2005 on the real-time AI M and H5 RT-PCR tests. Approximately fifteen laboratories participate. The NRL is also preparing for ILPT organiser accreditation.

The NRL participates in all the yearly European ILPTs organised by the CRL (VLA Weybridge, UK) in the domains of activity involving the detection and identification of viruses by RT-PCR / sequencing techniques and HI as well as the detection, quantification and typing of antibodies using AGID and HI techniques respectively.

Every year, in accordance with its obligations, the NRL provides a statement of identified viruses and observed serologically positive cases and also regularly presents scientific papers. In addition to its participation, with the CRL, in four European research programmes, the NRL also transmits strains of particular interest or that pose a problem in terms of identification to the CRL. The NRL is also a member of the FLU.LAB.NET network, that includes all the European NRLs and the major international reference laboratories and facilitates information exchanges. It also participates in working groups for data, expertise and biological material pooling within the framework of the EPIZONE network of excellence.

The AI NRL also contributes to the training of laboratory technicians and managers from other countries.

**Official analysis performance (confirmation)**

By implementing the above-cited techniques, the AI NRL performs all the confirmation analyses requested by the Directorate General for Food (DGAL) in contexts such as clinical suspicion, surveillance and export control. It also performs all the self-monitoring tasks necessary for surveillance of the Laboratory for studies and research on poultry, pig and fish farming's SPF and conventional livestock. Moreover, in the early 2000s, during roll-out of the AI surveillance surveys, the AI NRL performed first-line analyses in minor poultry species sectors for which serological test validation was poorly documented; the NRL then progressively transferred these analyses to the accredited laboratories.

The NRL processed nearly 10,000 tests in 2008, versus close to 18,000 in 2006 (the year of the crisis). The AI NRL therefore performed the identification and virulence characterisation of all the HP (highly pathogenic) H5N1 viruses detected in the winter of 2006 and the summer of 2007 and demonstrated that the second outbreak was a new introduction of the virus, in addition to performing systematic monitoring of the genetic and antigenic evolution of the identified AI viruses (LP H5 virus in particular) and the emerging subtypes, in addition to regular requests to confirm positive H5 serological results.

A diagramme summarising the relationships between the certified laboratories and the AI NRL is shown in Figure 1. The certified laboratories perform first-line real-time RT-PCR tests: 1- to detect the presence of any subtype of influenza virus, and 2- if found to be positive, to detect the presence or absence of the H5 haemagglutinin subtype of the virus (green box, upper left-hand side). In the event of a positive result, the NRL performs the identification and virulence characterisation of the H5 virus through sequencing of the haemagglutinin cleavage motif (red box, lower left-hand side);

- either (if an H5 virus is detected) to immediately confirm or rule out the presence of highly pathogenic H5N1 clade 2.2 viruses (present in Europe in 2006-2008) and in all cases to confirm or determine the virulence characteristics of the H5 virus through sequencing of the haemagglutinin cleavage motif (red box, lower left-hand side);

- or, if the H5 virus has not been detected, to immediately rule out or confirm the presence of H7 haemagglutinin virus subtypes, and if a positive result is found, determine the virulence characteristics of the H7 virus through sequencing of the haemagglutinin cleavage motif (box in the middle left-hand area).

The typing of previously identified H5/H7 viruses continues: the neuraminidase subtype is determined using RT-PCR methods; the strain is isolated in order to push investigations further in the event of a successful outcome: determination of antigenic characteristics, and if relevant, partial or complete genome
sequencing, possible in vivo testing, including an intravenous pathogenicity index (IVPI) test.

If H5/H7 viruses are absent, the most common (H6, H1, H3) and/or emergent (H9) subtypes are sought using RT-PCR methods, and if results are positive, the investigations continue as before.

If the aforementioned subtypes are absent, isolation is attempted in order to identify the virus using reference antisera and then confirmation and possible characterisation is performed using RT-PCR sequencing methods since the NRL is capable of identifying the H1-H15 and N1-N9 subtypes of avian influenza virus.

Phylogenetic studies and reassortment research are then performed. The most interesting strains are added to the collection to be used in pathogenesis research work and vaccinology as well as in methodological development to update existing tests, validate new ones, etc.

**References**


**Conclusion**

Thanks to its efficient utilization of powerful and reliable tools, its constant investment in methodological development, its structure and responsiveness and the coordinated involvement of its personnel in research and/or expert reporting activities, the AI NRL effectively fulfills all its official obligations. In doing so it is able to provide all the scientific and technical support needed for monitoring avian influenza in France and to share its expertise with laboratories in requesting third-party countries.

**Scientific and technical monitoring and support**

Through the contribution of these same researchers to international-level reference and research activities, the NRL fulfills its scientific and technical monitoring mission on a regular basis. As for expert opinion reporting, thanks to its senior researchers, the NRL has always been there to provide responses to the solicitations of its governing bodies – and the Ministry of Agriculture and Fisheries in particular. In fact, these requests were quite intense during the establishment of the initial AI monitoring plans and then during their updating and extension due to the increased risk of HP H5N1 virus introduction in France, as well as during the development of an associated vaccination and monitoring plan and the discovery of the health situation on domesticated water fowl farms. In addition, two senior NRL researchers have been participating in the collective expert evaluation process at Afssa (chairmanship of the emergency collective expert working group, avian influenza ECEAG), Afset (French Agency for Environmental and Occupational Health Safety) and EFSA (European Food Safety Authority). One of them has also participated in expert reporting missions for the FAO (Food and Agriculture Organization) and the OIE (World Organization for Animal Health).

Figure 1. Avian influenza virus detection and identification process.