Independent evaluation of the OIE Reference Laboratory for Infectious Salmon Anaemia Canada, 31 July – 2 August 2012

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Background

As indicated in the letter of assignment, the OIE Reference Laboratory for Infectious Salmon Anaemia (ISA) in Canada has recently undertaken diagnostic testing of samples and reported results that had not been reproducible in other laboratories. The Government of Canada is working with the laboratory to determine the root cause of the discrepancy in testing results and hopes to implement a series of test panels between this laboratory, the Canadian national aquatic animal health reference laboratory and the OIE Reference Laboratory for ISA in Norway to assess the primers and accuracy of the methods being used. In parallel, the Government of Canada and the OIE agreed that an independent evaluation by scientific experts would be beneficial.

Introduction

The Atlantic Veterinary College (AVC) on Prince Edward Island, Canada, was established in 1986 to support agriculture and fisheries in the eastern seaboard region with a specific remit for aquatic animal diseases. Nevertheless it covers the full range of veterinary disciplines including companion animals. Student numbers are approximately 60 per year, with around 90 graduate students. The first student class graduated in 1990. Research supported by around 15 post-doctoral positions is steadily building up. AVC is fully accredited by the Canadian and American Veterinary Medical Associations, and the Royal College of Veterinary Surgeons (UK).

The College hosts a regional diagnostic laboratory, which was toured by the evaluation panel. This is operated on a commercial basis, and takes samples from veterinarians, the AVC teaching hospital, industry, and the Canadian Government. A full range of services is offered including post-mortem, gross and microscopic pathology, haematology, biochemistry, parasitology, bacteriology, virology and molecular diagnostics. Test capability for ISA is in place, separately from the OIE Reference Laboratory. All data are entered on a LIMS system enabling comprehensive traceability and sample tracking.

AVC also hosts the OIE Collaborating Centre for Epidemiology and Risk Assessment of Aquatic Animal Diseases, directed by Dr Larry Hammell, jointly with the Norwegian Veterinary Institute in Norway. The Collaborating Centre was outwith the remit of the evaluation panel, however it appears to be highly regarded by the aquatic animals industries internationally and within Canada. The panel had the opportunity for discussions with Dr Hammell. Further information about the Collaborating Centre is available on the OIE website, including annual reports from the Centre.

OIE Reference Laboratory for ISA

Organisation

The OIE Reference Laboratory (OIE-RL) for ISA, established in 2004 within the AVC, is organisationally separate from both the diagnostic services unit and the OIE Collaborating Centre for Epidemiology and Risk Assessment of Aquatic Animal Diseases. The OIE-RL is headed by Dr Fred Kibenge who holds the chair of Pathology and Microbiology. His principal responsibilities are as a faculty member, including departmental administration, teaching and research. The work of his laboratory is split approximately 50/50 between OIE-RL activities and research, which includes ISA and other viruses. Dr Kibenge supplied his full CV, including an extensive list of publications. The bench work for the OIE-RL is carried out by Dr Molly Kibenge who has post-doctoral experience in molecular virology. Her CV was also supplied to the panel.

Facilities

Dr Kibenge has five rooms at his disposal for the activities undertaken as the OIE-RL for ISA. The same rooms are in use for research and other diagnostic activities.

- Room 318(S)

  This room houses a fridge and freezer containing the polymerase chain reaction (PCR) reagents and tissue culture media, and a safety cabinet used for the preparation of PCR master mixes for a range of assays and for the subculture of mammalian and fish cell lines.

- Room 319(S)

  Houses electrophoresis equipment used to resolve PCR products, and a basic gel documentation system. This room was used for gel electrophoresis, gel visualisation and for excising the PCR amplificons from gels for sequence analysis.

- Room 326(S)

  This facility is shared with the Canadian Cooperative Wildlife Health Center. Dr Kibenge has access to a laminar flow cabinet, bench top centrifuges and two bench areas. The cabinet is used for extraction of nucleic acids and for adding templates to the PCR-tubes/plates. The benches are used primarily for storage.

- Room329(S)

  This is a general laboratory housing a wide range of laboratory equipment including a safety cabinet used for sample processing/homogenisation, a bench top centrifuge for 96-well plates, a balance used to weight test samples and a thermal cycler used for conventional PCR assays.

  This was the sample reception room used for unpacking, registering sample information and initial sample processing.

  This room was also used for cloning, virus culture and was the main research laboratory for Dr Kibenge's research team.

- Room 330(S)

  Houses freezers that are used for sample storage prior to processing, two Roche light cyclers, real-time thermal cyclers (one no longer used) and some old gel documentation/photographic equipment that we were informed is no longer in use

Funding

The panel was rather surprised to find that no specific budgetary provision is made, either by the AVC or the Canadian Government, to support the work of the OIE-RL. Consequently all services provided in the context of OIE-RL are charged for on a commercial basis. This includes diagnostic testing, provision of reference materials, organisation of proficiency tests, and so on. While an element of cost recovery is recognised by OIE as necessary for many Reference Laboratory activities, this strongly commercial approach appeared to the panel to be going beyond the spirit of international co-operation and support that underpins the philosophy of the OIE.

Scope

The panel also felt that, although it is a somewhat grey area, the inclusion of many commercial diagnostic tests under the banner of OIE-RL was inappropriate. The extensive use of the OIE emblem by the laboratory seemed rather excessive, and the Expert should review this and consider whether it is fully compliant with the "*Guidelines on the use of the OIE Emblem by OIE Reference Centres*" and "*Guidelineson the use of the title of OIE Expert*". These guidelines have been distributed to all reference centres by the OIE.

Specific observations

The following sub-headings relate to the terms of reference assigned to the evaluation panel by the OIE.

Quality Assurance

There is currently no formal quality system in place within the OIE-RL for ISA. However, Dr F. Kibenge informed the panel that he was planning to work towards accreditation for the diagnosis of ISA.

Standard operating procedures (SOP) were available for the nucleic acid extractions, PCR set up (conventional and real-time) and for sample analysis by gel electrophoresis. Although these were adequate prompts for an analyst familiar with the procedures they follow fall well short of the SOPs required for an internationally recognised standard such as ISO-17025. The panel did not have sight of procedures covering sample receipt and registration or information on the PCR product purification and cloning.

Records were maintained for batches of reagents, and the dates of sample processing were transcribed to a summary sheet for each test sample. However, the audit trail for individual samples was generally poor. Completion of individual stages of the procedure was documented and signed off by the analyst, Dr M. Kibenge, but this information was not contained within a single document and was difficult to track. The use of a sample worksheet to record all reagent information, the equipment used and dates of individual procedures would be beneficial.

There are insufficient traceable records for instrument calibration, monitoring and maintenance, or laboratory and equipment cleaning. There were also no records of analyst training and there was only limited evidence of a continual assessment of competency through participation in proficiency panels in 2009.

The panel has serious concerns regarding the current setup in the OIE-RL. The primary concerns were the cramped, untidy conditions of the laboratories, particularly the general laboratory [room 329(S)] where both sample preparation and post-PCR analysis were performed in close proximity to each other. Dr F. Kibenge has clearly recognised the vulnerability of diagnostic PCR methods to contamination and he has made significant attempts to mitigate the problem by separating some pre-PCR sample processes from the post PCR analysis. However, it is a major concern that room 329(S) is used for sample processing, recombinant plasmid preparation, infection of cell cultures and preparation of ISA virus enzyme-linked immunosorbent (ELISA) antigens. The panel believes that there is a serious risk that the integrity of the test samples will be compromised.

Comparison with DSU

The AVC has a separate diagnostic service unit (DSU) (referred to in the introduction above) with ISO-17025 certified accreditation in place for certain serological tests for terrestrial animal diseases carried out on behalf of the Canadian Food Inspection Agency (CFIA). The DSU laboratories are Biosafety Level 2 throughout and there is a molecular diagnostic suite available with sample processing areas, a clean room for PCR reagent set up and a separate area for PCR amplification and post PCR analysis.

The panel met with Karen Smith, the Quality Assurance Coordinator for the AVC diagnostic services and was informed that they plan to extend their accreditation to other disease diagnostic methods including PCR-based detection and identification. The panel were shown some existing standard procedures held by the AVC diagnostic services, which it was accepted currently fall short of the requirements for an accredited standard. However, it was evident from our (albeit superficial) observations of this paperwork and the molecular biology facilities, that the appropriate standards could be reached in DSU without too much difficulty.

Validation of tests

Dr F. Kibenge was clearly familiar with the OIE Validation Standard as published in the *OIE Manuals*. In regard to the antibody ELISA which had been developed in-house, they had completed Stage 1 validation, although there were no reproducibility data with other laboratories. For Stage 2 they had amassed 3000 samples, although definite negatives were difficult to locate, and initial estimates for diagnostic sensitivity and specificity had been made. The panel felt it was imperative to extend the validation of the test in collaboration with other laboratories internationally. There was also concern that Dr Kibenge seemed unaware of similar tests developed elsewhere. It was noted that it is planned to launch the test commercially for use particularly in checking responses to ISA vaccines. It may also have a place in ISA surveillance.

Novel in house primers had been developed for use in a real-time reverse transcriptase (RT)-PCR and were being validated in comparison with the methods described in the *Aquatic Manual*. This validation process was still at Stage 1, and a summary of results to date, with detailed datasheets, was provided to the panel. The eventual aim is to propose the test for inclusion in the *Aquatic Manual* and possibly for adoption in the OIE Register. The approach to validation was in accordance with the OIE Standard, though still at an early stage in the process.

Testing methods in relation to OIE Standards

Based on the information provided, the RT-PCR-based methods employed by the OIE-RL are consistent with those outlined in chapter 2.3.5 of the *OIE Aquatic Manual* (as detailed in the following section of the report). The primary screen is performed using a real-time RT-PCR assay targeting segment 8 (Snow *et al.* 2006) and this is backed up with a conventional RT-PCR assay targeting segment 8 (Devold *et al.* 2000). However, the panel had concerns that Dr F. Kibenge may deviate from these procedures and use alternative non-validated in-house methodologies.

Interpretation of diagnostic test results

RT-PCR detection procedure for ISA virus RNA

There was no written flow-chart to describe the application of different methods used during a diagnostic investigation and the following information was retrieved during interviews with Dr F. Kibenge:

- The primary test method is a real-time one-step RT-PCR based on ISA virus (ISAV) segment 8 using primers described by Snow *et al.* (2006) which is also one of the recommended methods in the *OIE Aquatic Manual*. The extraction and purification of RNA from tissues and cell cultures is done using a combination of trizol extraction and spin columns.

- Positive test results were evaluated according to a pre-set cut-off point, determined for each method used (*i.e.* different sets of primers and probes).

- Positive tests are confirmed by conventional RT-PCR using primers described by Devold *et al.* (2000) also recommended in the *OIE Aquatic Manual*.

- Products from the conventional RT-PCR are excised from the gel, cloned and sequenced. An in-house conventional RT-PCR was used to amplify a 400 bp product covering the ISAV HE HPR region which was cloned, sequenced and the HPR-type was determined. Where possible (either RT-PCR product from tissue or isolated virus) the complete HE gene was sequenced.

- The following procedures are followed if the results are considered dubious, i.e. in cases where ct-values above the cut-off value are detected:

    - The primary test method is used for retesting of the samples three times using the originally extracted RNA, i.e. no new extractions are performed.

    - If these three repeated analyses give consistent results, the sample is regarded positive even if ct-values are above the pre-determined cut-off value.

Panel concerns regarding the sequence of analyses for RT-PCR

- Confirmation of results from the primary real-time RT-PCR should be done using one of the two other real-time assays described in the OIE *Aquatic Manual*.

- When investigating dubious samples the diagnostic procedure should restart from scratch using a fresh extraction of RNA from the original sample.

- To complete the investigation, sequencing should be performed on positive samples, followed by a phylogenetic analysis, comparing them with sequences from previous detections in the laboratory and in the field. Importantly, this would also serve indirectly as a control for possible cross-contamination.

Cell culture and supporting methods

The laboratory had four different cell culture systems for primary isolation of ISAV: SHK-1 cells, CHSE-214 cells, ASKII cells and TO cells. Dr Kibenge informed the panel that he considered TO cells to be the most sensitive for primary isolation of ISAV, and were the first choice for use in his laboratory. Virus isolation and identification procedures (*i.e.* immunofluorescence and RT-PCR) were variations of commonly used procedures and the assessment panel did not have any significant comments on this, except that, as with other methods in the OIE-RL, the SOP for this work was inadequate for a diagnostic or Reference Laboratory.

Laboratory competence

Observing Dr Molly Kibenge perform a dummy run of the real-time RT-PCR set-up, it was clear to the panel that she was competent in the procedures and has a good understanding of the methodologies.

Interpretation and reporting

Final reports are provided with limited interpretation even when the results of different assays were conflicting. In an example provided to the panel, seven wild caught cutthroat trout were tested by PCR. The results showed all samples to be negative by real-time RT-PCR, while all fish were positive by the conventional segment 8 RT-PCR and also with an in-house conventional segment 6 RT-PCR. In the absence of a real-time RT-PCR result to support the conventional RT-PCR results the panel considers these results to be highly dubious, and that the results should be reported as inconclusive pending further investigation. As a general principle, no further testing should be necessary if a primary screening test (in this case real-time RT-PCR) gives a negative result. The rationale for carrying out "confirmatory" testing (conventional RT-PCR) on negative samples is not clear.

We would regard it as a duty of the OIE-RL to seek an explanation for these discrepancies. We consider there could be several explanations, including cross-contamination in the laboratory, but also that the assays were indeed detecting a new genetic variant of ISAV not picked up by the real-time RT-PCR used. An obvious part of such an investigation would be the use of alternative real-time assays as described in the *OIE Aquatic Manual*, but the panel understands that Dr F. Kibenge used an alternative in-house real-time assay to confirm the presence of ISAV and that these results were not shared nor was an explanation provided for the apparent failure of an assay recommended in the OIE manual. Sequencing followed by a phylogenetic analysis would most probably provide essential information, however Dr Kibenge considered this to be a research issue and had simply reported his findings, even though we were told that further investigation had been initiated.

In conclusion, the panel had concerns that Dr F. Kibenge may deviate from his standard procedures that follow recommendations in the *Aquatic Manual*, and use alternative non-validated in-house methodologies. There seemed to be a lack of appreciation of the obligations on a Reference Laboratory for thorough investigation of dubious or illogical results, together with the need for evaluation of the biological significance of results.

Biosafety and biosecurity

Biosafety and biosecurity were not seen to be a problem. An official Canadian Government certificate of compliance with Biosafety Level 2 was presented.

Development and use of reference material

As shown in the annual reports to the OIE, various reference materials can be supplied by the OIE-RL if requested by laboratories in OIE Member Countries. There had been some limited uptake of this facility (see annual reports). All materials would be charged for.

There are no standard reference materials recognised by OIE, and the team encouraged Dr F. Kibenge to consider developing such, in collaboration with other laboratories. He said it was under consideration, but funding would be a limiting factor for such a project.

Proficiency testing

During interviews with Dr F. Kibenge it appeared that the AVC OIE-RL had only participated in ring tests organised by himself. He claimed they had not participated in ring tests organised by the other Reference Laboratory for ISA because of the difficulty importing infectious samples to Canada. The panel felt this was not insuperable and applications for import permits could be submitted.

The OIE-RL had organised three ring tests, mainly including Chilean commercial laboratories. The first two used cDNA samples and we did not receive any information on the details and results from this testing. The third ring test was organised with 12 Chilean laboratories, one Asian and one European. The OIE-RL at AVC also participated through the provision of coded samples to the analyst (Dr M. Kibenge). Samples used in this test were organ homogenate spiked with cell culture grown virus. A description of this ring test including results is described in a published paper by Kibenge *et al.* (2011).

Support and training for national laboratories in OIE Member Countries

No specific training for national laboratories was reported apart from the twinning project (see below).

Twinning project (Chile)

A formal OIE-funded twinning between AVC and the laboratory of the Catholic University of Valparaiso, Chile, had run from June 2010 to May 2012. The final report is not yet complete, but the annual report at August 2011 indicated the project was on track to strengthen the expertise of the partner laboratory through a workshop for technical staff in Chile, and training of two Chilean personnel at the AVC. The partner laboratory was keen to move toward eligibility for OIE Reference Laboratory status in its own right, although Dr Kibenge felt they needed further strengthening before this could be possible.

Interactions with other OIE Reference Laboratories and Collaborating Centres

Contact or interaction with the OIE ISA Reference Laboratory in Oslo, Norway has been almost absent with the exception of exchange of a few ISAV isolates. There has been no proficiency testing, coordination of procedures used by these two laboratories or exchange of information and experiences. During our interview with Dr F. Kibenge there was no explanation for this lack of interaction other than that it was difficult to submit infectious samples between the laboratories. It was disappointing to note that Dr Kibenge declined an invitation to participate in an OIE-sponsored international symposium on ISA in Oslo two years ago, due to teaching commitments.

It was evident that communication and collaboration between the OIE-RL for ISA and the OIE Collaborating Centre for Epidemiology and Risk Assessment of Aquatic Animal Diseases, both located at AVC, were limited or non-existent. Similarly, dialogue with the DSU at AVC was also limited even though they also carry out both RT-PCR and virus cultivation for ISAV. We have noted earlier the possibility of moving the diagnostic testing work from Dr F. Kibenge's laboratory to the DSU facility.

Our general impression is that the OIE-RL at AVC, with the exception of their activities in Chile, has very limited contact/interaction with other laboratories/groups, including other laboratories/groups at AVC, the Canadian national governmental reference laboratory for ISA, and wider Reference Laboratory networks existing within the OIE.

Thus it appears that although the OIE-RL at AVC, operating in conjunction with other groups at AVC, has the capacity and capability to fulfil duties as an OIE Reference laboratory, including support on ISA to other laboratories in OIE member countries, it does not deploy these capabilities to fulfil its mission.

Principal Conclusions

- No evidence for any meaningful Quality System in the OIE-RL
- OIE-RL falls well short of acceptable Quality Standards
- OIE-RL uses the OIE template for validation of tests, but needs to involve other laboratories in the process
- Competent laboratory analyst but lack of comprehensive records of continuing professional training and development
- The OIE expert recognises the importance of laboratory organisation and work flow patterns to avoid contamination, but has not optimised the system. Incompatible pre- and post-PCR procedures were undertaken in the same laboratory.
- The OIE expert is clearly knowledgeable about ISA, with many peer reviewed publications, but has a research focus, rather than that of a diagnostician
- Results of tests reported without contextual interpretation
- No detailed investigation of apparently anomalous results
- No evidence of multidisciplinary engagement for disease investigation
- There is a need for much wider involvement with proficiency test/ring test schemes, either as participant or organiser
- Lack of contact and networking with other laboratories working on ISA
- Lack of understanding of general philosophy of mutual international support within which OIE operates

Recommendations

1. For OIE

1.1. The OIE should consider, in the light of this report, suspension of the Reference Laboratory status of the ISA laboratory at AVC.

1.2. The panel suggests that OIE should consider very carefully before designating Reference Laboratories in university, rather than government laboratories. It is essential in such cases that clear communication routes are established between the laboratory and official veterinary services. Where an OIE-RL is not the National Reference Laboratory for the disease in question, there must be a clear demarcation of responsibilities and a regular dialogue and exchange of materials between the two.

2. For AVC Faculty Management

2.1. If AVC wishes to retain an OIE-RL within the faculty, provision should be made for some level of core funding to support the activities carried out on behalf of the OIE.

3. For the OIE Reference Laboratory for ISA

3.1. The laboratory suite occupied by the OIE-RL has the potential to be better organised in order to provide a quality assured service. However the existence of a well established diagnostic facility in the same building suggests it would be more appropriate to move the ISA testing carried out by the OIE-RL into that facility.

3.2. Such a move would enable a much easier and quicker implementation of an accredited Quality System, under the control of the existing Quality Manager.

3.3. The OIE Expert should arrange to visit other OIE Reference Laboratories, particularly those dealing with virological diseases, to observe how they operate and gain an understanding of the relationship between the OIE, official veterinary services and testing laboratories.

3.4. Much greater clarity is needed on which tests for ISA carried out at AVC are truly in the context of OIE-RL activity. Diagnostic tests provided on a commercial basis should not be processed or reported under the OIE emblem.

3.5. Newly developed tests should be validated according to the OIE Validation Standard in collaboration with other OIE-RLs for ISA and other expert laboratories as appropriate.

3.6. In the interests of transparency, the OIE-RL should actively make available to laboratories in Member Countries information on new and improved tests and their interpretation.

3.7. The OIE-RL should take full advantage of the range of disciplines at AVC, including pathology, epidemiology and risk assessment, when carrying out investigations or test validations under the umbrella of the OIE-RL.

3.8. The OIE-RL should actively work with the other OIE Reference Laboratory for ISA to carry out proficiency tests for national laboratories in Member Countries, and to prepare and make available reference materials.

Post script

The panel was aware that the CFIA had carried out an evaluation of the OIE Reference Laboratory earlier this year (CFIA 2012). We did not have sight of that report prior to our investigation in order to ensure a fully independent evaluation. Following completion of our report we took note of the Canadian report, which raises many of the same issues as identified by the panel.

Acknowledgements

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