



Evaluation and validation of selected loop mediated isothermal amplification (LAMP) and other rapid tests with emphasis on avian influenza and testing its suitability under field conditions

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1 Executive Summary

The diagnosis of Influenza A, in particular, of highly pathogenic avian influenza (HPAI) H5N1 viruses, currently creates challenges for laboratories in resource constrained settings:

- I. The OIE-required gold-standard (virus isolation by propagation in embryonated chicken eggs) takes long time (up to seven days) and requires high biosafety and bio-containment standards.
- II. Usually, molecular technology requires constant -20°C freezing conditions which are rarely fulfilled as power cuts often occur.
- III. Material and equipment for molecular biological techniques (e.g. PCR, sequencing) are relatively expensive and not affordable for labs which have a limited budget. They also require RNA-extraction step in advance, which- by use of commercial kits – lead to further high costs.
- IV. Therefore, rapid tests (immunochromatography lateral flow devices) are frequently used as the only means of detection. The lateral flow devices were distributed widely by different stakeholders. However, they lack in sensitivity and are often expired when used.

Assuming this scenario for many countries in the world under-detection of Influenza A is likely and thus contributes to the spread and/or endemicity of certain virus strains, e.g. HPAI H5N1. Limitations in the diagnosis of animal diseases further reduces reporting activities to the OIE through the World Animal Health Information System (WAHIS) and may result in a lack of information related to a specific disease situation in a country.

Often, limited access to updates on modern molecular diagnostic methods and reagents leaves laboratory staff unaware of novel technology that may suit their laboratory capabilities. Capacity building and laboratory networking, also to encourage sending of samples to Reference laboratories, is therefore essential to fill this gap.

To support laboratory diagnostics in developing countries it would be necessary to have diagnostic technology that fulfils the following:

- High sensitivity, high specificity,
- robust,

- cheap and easy to obtain independently (procurement without the support of donors),
- easy to handle and interpret results,
- equipment-free,
- no requirement for long-term (1-2 years) freezing conditions for storage.

In this study three major goals were targeted:

(i) Scientific validation of a sample of modern diagnostic tools (“robust tests”) for sensitive and specific detection of influenza A viruses, in particular a promising technology, called loop mediated isothermal amplification (LAMP).

(ii) Assess cost of molecular diagnosis of influenza viruses and infection. The main aim of this part was the evaluation of possible ways to reduce costs of avian influenza surveillance in developing countries.

(iii) Enhancing international laboratory networking and capacity building of lower resourced laboratories. Furthermore, the application and training capacities of techniques for test validation should be enhanced.

We evaluated kits of three different modern diagnostic approaches (LAMP, real time PCR, immunochromatography lateral flow devices) and various methods for saving resources.

Scientific validation of novel rapid diagnostic kits

LAMP assays: We validated 4 different LAMP kits, one from IAEA (H5 specific) and 3 from EIKEN Chemical Co., Ltd. Company (Flu A Influenza Virus Detection Kit, Influenza Virus subtype H5 Detection Kit, H1pdm2009 Influenza Virus Detection Kit; <http://loopamp.eiken.co.jp/e/lamp/>; Japan):

(i) IAEA-H5-specific RT-LAMP assay: The evaluated H5-specific RT-LAMP assay produces results in a short time. It has sensitivity comparable to RT-PCR while producing results more rapidly. The IAEA-H5-specific RT-LAMP assay has the advantage of being delivered in lyophilized format, which facilitates transport and storage for several months independent of a cold chain. In general, this H5-specific RT-LAMP assay was found to be efficient. We could successfully apply the kit in Kazakhstan and Japan to identify several H5N1 isolates belonging to different clades. This finding emphasizes the scope of application of the kit.

However, the following challenges arose during our evaluation:

- The cut-off time discriminating positive and negative results is critical: By choosing different assay running times either sensitivity or specificity decreases. Therefore, interpretation of the results is critical. Although a solution may exist, the investigators were unable to resolve these issues.- Our attempts to modify the RT-LAMP assay for result interpretation by the naked eye failed due to the composition of enzymes and buffers required for the lyophilisation process. Use of the current kit is restricted to laboratories which have access to sophisticated optical reading equipment (qPCR cycler or for example a Qiagen ESEQuant Tube Scanner). Although many labs in developing countries have a qPCR cycler, the equipment may not be well maintained (and thus not usable) or lab-staff maybe overwhelmed using the complicated and difficult-to-use software if they have not been trained sufficiently. The easier-to-operate Qiagen ESEQuant Tube Scanner, on the other

hand, is an expensive piece of equipment which can only be used for LAMP-assays and not for any other purpose. During joint meeting with IAEA kit developers, this issue also was considered.

To sum up, the IAEA-H5-specific RT-LAMP assay has the advantage of high sensitivity, but disadvantage of lower specificity. The kit would be useful for analysis of highly positive samples (which usually need less than 25-30 minutes to reveal positive results), but harbors the risk of false positive results. The latter should be considered when increasing the cut-off to 35-40 minutes or higher (recommended cut-off by IAEA is 60 minutes). Reduction of the cut-off to 30-35 minutes (same cut-off as all Eiken kits) can minimize the chance of false positive results, but weak positive samples may be considered as false-negative. Resulting lower sensitivity would not be a big issue for highly positive samples (for example samples which usually are collected during HPAI outbreaks). Equipment-free assay visualization and improving specificity still need further consideration if the kits are to be suitable for use in low resource laboratories.

As a final conclusion, the IAEA-H5-specific RT-LAMP assay is a robust and fast-working tool which can be used for screening during an outbreak scenario (testing samples of dead/sick birds) in a low resource/remote laboratory (provided it has a qPCR cycler) but it should not be considered for active surveillance activities in a non-outbreak setting. In any case, positive results need to be confirmed by any other of the OIE recommended assays (PCR or virus isolation).

(ii) Eiken Influenza Virus subtype H5 Detection RT LAMP assay: The Eiken-H5-specific RT LAMP assay has the advantage of easy handling, naked-eye visualization with rapid results. The kit has a cut-off value of 35 minutes (similar to other Eiken kits). The sensitivity of the kit is not high, but it has a high specificity. Sensitivity of the kit is lower than IAEA-H5-specific RT-LAMP assay when we consider 35 minutes as cut-off for both kits. Possibly, the lower sensitivity of the kit in *our* experiments is related to using H5N1 isolates which are more prevalent in Europe, while the kit was developed to detect South-East Asian H5N1 isolates.

The benefit of this kit is its naked-eye visualization (no need for result-reading instruments), but it needs cold storage with a limited expiry date. Therefore, the Eiken-H5-specific RT LAMP assay is a robust tool; but dependence on cold storage and its low sensitivity require further modification. This kit could be an option for analysis of highly positive samples in countries with sufficient resources.

(iii) Eiken-H1pdm 2009 Influenza Virus Detection RT LAMP assay: The H1N1 pandemic (pdm)-specific RT-LAMP assay from Eiken has the same advantages as the H5-specific kit with the same cut-off value of 35 minutes and naked-eye visualization. Although the kit was developed for identification of human H1N1pdm influenza viruses, we could successfully apply it for identification of H1N1pdm viruses in pig samples and also for re-assorted H1N1pdm and swine influenza viruses. Since this reagent kit comes in a dried form like IAEA kit, it does not need to be stored at -20°C (storage at 2 to 8°C) and can be easily handled (no need of reagent preparation, no need of complicated RNA extraction procedure).

(iv) Eiken generic AI LAMP kit: The Eiken *Flu A Influenza Virus Detection RT-LAMP Kit* has been developed based on detection of matrix gene of influenza viruses of all subtypes and has the advantage of easy handling, naked-eye visualization, short run-time and high sensitivity. This kit has also a cut-off value of 35 minutes, but compared to the other two subtype-specific Eiken kits, it has a higher sensitivity. Yet, the high sensitivity of the kit is related to a lower specificity (similar to IAEA-H5-specific RT-LAMP assay). Eiken

recommends the use of their specific turbidimeter for the amplification. Therefore, using real-time PCR cyclers may be a cause of lower specificity.

Combined usage of this kit and H5 specific kit could solve the problem of sensitivity and specificity when used during highly pathogenic avian influenza outbreaks or during passive surveillance activities. Anyhow, the price of the kit as well as export limitations and customs are limiting factors for many countries, and a short expiry date adds to it.

Immunochromatography lateral flow devices:

(v) *Pan AI (Synbiotics) and H5 specific (Quicking Biotech Company, China) lateral flow devices:* Our results of the evaluation of the two rapid test kits based on immunochromatography confirm the poor sensitivity of those kits. Sensitivity and specificity of the kits tested are not at all sufficient for reliable diagnosis of AI infections and may only be applied on flock-basis upon HPAI-suspicion (see Marek et al., 2012).

Tetracore PAN-AI RTqPCR kit:

(vi) *The commercial Tetracore PAN-AI RTqPCR kit* in a lyophilized format is highly sensitive and specific, easy to handle and can be stored at room temperature for one year, but is expensive and needs a qPCR cyclers.

Cost-benefit molecular diagnosis of influenza viruses

Demands for monitoring wild birds and domestic poultry for avian influenza virus (AIV) have increased enormously worldwide in the recent years due to a panzootic spread of highly pathogenic avian influenza (HPAI) H5N1. Active surveillance activities rely mainly on collection of oropharyngeal, cloacal and faecal swab samples. These swab samples are then transported to diagnostic laboratories where they are processed for molecular analysis and virus isolation. The whole process of sample collection, transport and analysis is time-, labour- and money-consuming. In the case of larger outbreaks, the burden on diagnostic capacity is likely to be excessive. Therefore, strategies to economize both surveillance activities and outbreak analyses are sought. Real-time RT-PCRs (RT-qPCR) have been recommended as rapid and highly sensitive methods to speed up high quality virological diagnosis. Yet, RT-qPCR is a cost-intensive assay. The whole process of nucleic acid extraction and subsequent amplification requires expensive consumables and reagents (including extraction kits), equipment and skilled staff. Several advanced laboratories in Africa and Asia are attempting to use RT-qPCR, but are often confronted with constraints including procurement cost, maintenance costs, technical challenges with maintenance, and availability of skilled personnel to use the equipment. Reducing hands-on time and economizing sample processing and analyses can translate into substantial cost savings, thus increasing surveillance activities and sample investigations.

(vii) We studied and compared the results of using a sample pooling policy for processing high numbers of samples. We found high efficiency when analysing sample pools of five samples by RT-qPCR. This pooling policy is beneficial with respect to saving hands-on time, consumables and costs during wild bird monitoring studies or poultry surveillance programs especially when negative AI results are expected for the majority of

samples.

(viii) Reducing the reaction volume of influenza A virus generic as well as of subtype-specific RT-qPCRs to 12.5 µl (2.5 µl template) instead of 25 µl did not adversely affect the limit of detection for RT-qPCR assays. A significant economic benefit without impeding detection efficacy can be achieved when sample pools of five samples are analyzed by RT-qPCR using a reduction of reaction mix to the half the original volume.

(ix) For decades, rayon swabs have been used for collecting oropharyngeal, cloacal, or fecal samples from wild birds in AIV monitoring studies. A new generation of nylon swabs has been made available for influenza sample collection. Nylon flocked swabs are designed to absorb and release a greater volume of sampling materials than do rayon swabs and, therefore, should improve sampling efficacy.

We have studied the effect of using two commercial swab products of the rayon-tipped and flocked nylon type on the quantity of detection of AIV RNA in swab samples in different storage conditions. In summary, there did not appear to be any any significant association between swab type (rayon vs. nylon) and the sensitivity of AIV RNA detection. Although the superior efficiency of nylon swabs has been stated by several independent studies, our studies showed that rayon swabs allowed an at least similar rate of RNA recovery. Considering the lower costs, rayon swabs seem to be a preferred choice for sample collection, especially in laboratories with limited resources.

(x) Viral loads and sample matrix as well as time and storage conditions between sampling and processing are known to affect the recovery rate of infectious/live virus. It is possible that improper handling and prolonged storage of swabs in inappropriate conditions may adversely affect the quality of samples, thereby leading to inactivation of viral infectivity and accelerated RNA degradation of viral RNA.

In this regard, we have selectively examined the effect of different storage conditions on the quantity of AIV RNA detection. If swabs need to be stored for prolonged time periods, a continuous temperature of 4°C provides the best results for RNA recovery rates.

Degradation of viral RNA in virus suspensions in viral transport media over a period of 2 weeks was negligible using constant storage conditions at 4°C or 20°C. Temperature shifts (4°C, -70°C, and 4°C), however, had a detrimental effect. The influence of swab storage conditions on infectivity has not been investigated in this study, but temperature fluctuations and prolonged storage time at temperatures above 4°C are expected to have a much greater influence. Therefore, storage of swab samples at a constant temperature of 4°C is recommended when virus isolation is to be attempted.

International Laboratory Networking and Capacity Building

Another aim of the project was to enhance international laboratory networking and capacity building of lower resource laboratories in Asia and Africa for a possible future application of the targeted diagnostic tool/method/kit.

Therefore, hands-on workshop on "Training on modern diagnostic tools for the diagnosis of Influenza A and enhancing networking between laboratories" was carried out at the FLI. Three veterinary researchers from

Animal Health or Research Institutes in Nigeria, Iran and Kazakhstan participated in the workshop. Besides the program comprising novel technologies including LAMP, the participants had ample opportunity to establish contacts with researchers at the FLI and were able to visit various OIE Reference Laboratories. During a field trip to Kazakhstan in summer 2013, the LAMP methodology as well as RT-qPCR methods were successfully applied in the laboratory by using Asian H5N1 viruses. Lab staff was trained on the spot.

For the scientific evaluation of different LAMP assays (EIKEN, IAEA), a trip to Japan deemed necessary to meet the developers of the LAMP methodology. This meeting served in first line for testing the IAEA-H5-specific RT-LAMP assay by using H5N1 viruses of different clades and secondly, in exploring the possibilities for modification of LAMP components to finally meet the required optimum conditions of robustness, highest sensitivity and specificity, long storage without freezing chain, cost-effectiveness and independence of equipment.

2 Conclusions

Molecular assays targeted to nucleic acid identification are becoming increasingly important in laboratory diagnosis world-wide. The advantages of molecular assays are rapidity, sensitivity, specificity, increased biosafety and accuracy. Disadvantages are the need for sophisticated equipment, skilled personnel, expensive reagents and consumables, and the cost of sample preparation (RNA extraction). Those disadvantages are bearable for laboratories in industrialized countries, but not for countries with limited resources (developing countries).

There is a clear need for robust and safe molecular assays which are affordable and applicable in low resource laboratories. LAMP (potentially equipment-free), rapid immunochromatography lateral flow devices, realtime PCR kits are some promising molecular or immunological assays available on the market.

In frame of this OFFLU project we have evaluated several tests targeting avian influenza for their suitability in laboratories in low resource settings. Furthermore, we have explored modifications to existing (and internationally recommended) methods for AI diagnosis which can save resources.

The following table summarizes the required practicability for each assay or method tested (NT= not tested; green field: good performance, yellow: improvable)

Assay or method	Requirement for a perfect diagnostic assay to be applied in resource-constrained labs								
	sensitive	specific	No cold chain	Equipment-free	RNA-extraction-free	Robust	rapid	Commerc. available	Easy handling
IAEA H5-specific RT-LAMP									
EIKEN generic RT-LAMP									
EIKEN H5-RT-LAMP									
EIKEN H1-RT-LAMP									
Quicking H5-lateral flow									
Synbiotics AI lateral flow									
Tetracore generic RTqPCR									
Tetracore direct RTqPCR	NT	NT				NT			NT
RT-qPCR (reduced volume)									

2.1 Sensitivity and Specificity

With regards to sensitivity and specificity RT-qPCR is superior to all other assays and methods evaluated in

this study. Reducing the reaction volume by half confirms the high sensitivity and specificity. Compared to the EIKEN RT-LAMP H5 specific assay, the IAEA-H5-specific RT-LAMP assay is more sensitive, but lacks in specificity. There is a clear need for further modification and optimization. The rapid immunochromatographic lateral flow devices, particularly the H5-specific test failed in good performance of sensitivity and specificity which disqualifies the test for its application. Under certain conditions the Synbiotics rapid test may be applicable (in outbreak situation as a pre-screening of organs or body fluids of carcasses). Any result need to be confirmed by one of the OIE recommended tests.

2.2 Cost-effectiveness and availability

With rare exceptions, most countries in Asia and Africa face limits in their laboratory budget. This is more pronounced in the field of veterinary medicine. Furthermore, procurement of diagnostic reagents and consumables from Europe or USA is often difficult and costly (shipment costs, taxes, customs, mode of payment) and only works with the support of development aid organizations. Even if a perfect tool is commercially available, problems with procurement are limiting factors which require future consideration and attention. This becomes more important when considering that the least suitable assay (the Quicking Biotech H5-specific immunochromatography lateral flow device) is one of the cheapest and easiest to obtain. Underinvestment in laboratory budgets can drive laboratories towards using less sensitive and specific diagnostic tests.

2.3 Independence of the cold chain

A major limiting factor in the application of molecular diagnostic tools in resource-constrained settings is the need for permanent electricity supply to maintain the cold chain. Most molecular reagents (particularly enzymes) require stable freezing conditions. Thawing and freezing in case of electrical power outage quickly damages the reagents. A generator is not always a solution when fuel is expensive or hard to come by and when it needs to be manually switched on (e.g. if there is a power cut at night the operator may be asleep). It is for these reasons that ideally the kit needs to be stored at room temperature rather than under cool or freezing conditions. The Tetracore PAN-AI RTqPCR kit is an example of a kit which can be stored at room temperature

2.4 Independence of equipment

Ideally, a molecular assay should function without expensive or sophisticated equipment. Usually, gene amplification requires a PCR cycler automated by a computer. Often, the cycler or computer programs are not easy to handle and need regular maintenance. The EIKEN-LAMP assay does not require any sophisticated equipment: Amplification happens under isothermal conditions and results can be read by naked eye. None of the other tests (apart of the immunochromatography lateral flow devices) meet this requirement. Unfortunately, the IAEA-H5-specific RT-LAMP assay cannot be easily modified in a way to read results by naked eye. If so, the assay would come as non-lyophilized assay losing its capability of long term storage at 4°C.

2.5 Independence of Nucleic acid extraction

Gene amplification requires nucleic acids (NA) extracted from the sample. There are various techniques for manual RNA extraction. While the one method based on Guanidinium thiocyanate-phenol-chloroform

(TRIzol®) is laborious and has a serious health hazard when directly exposed to the user, the other more rapid silica-membrane based method is safe but expensive (50 samples = 150-260€). Recognizing a clear advantage in omitting the either laborious or expensive method, the Tetracore Direct RT-qPCR kit and the IAEA-H5-specific RT-LAMP assay simplify the molecular diagnosis by using the sample without prior extraction of RNA. For the latter a simple heating of the sample at >90°C is the only preparative step needed prior to amplification.

In summary, the IAEA-H5 RT-LAMP kit seems to be most suitable for its application in lower resource laboratories. However, it is not the perfect tool and still requires improvement.

While awaiting optimization, we validated and recommend the following modification to real time PCR as the gold standard for molecular diagnosis in order to reduce costs and save resources:

- Using rayon swabs (e.g. ear cleaning cotton buds!) for sampling birds and shaking the sample for at least 10 minutes in viral transport media has the same sensitivity as using expensive nylon flocked swabs.
- Samples can be stored at moderate room temperature or +4°C for prolonged period of time without serious loss in RNA yield. Although there appears to be no loss in RNA yield this may negatively impact on the ability to isolate live virus from the sample.
- Pooling five samples together for RT-qPCR does not have an impact on sensitivity.
- Reducing the reaction volume of RT-qPCR components by half reduces the costs but does not reduce sensitivity.
- 1% Hypochlorite should be used for decontamination of work areas and pipettes. It is effective for viral and nucleic acid decontamination.

The development of a robust, sensitive, specific and cost-effective rapid (molecular) test is a target for evolving diagnostic capacity in veterinary laboratories which should one day be achieved. However another target should be to focus on improving the procurement system world-wide. There are huge efforts to ameliorate molecular diagnostic capacities and to train scientists and laboratory staff in developing countries through twinnings, projects and various international and national programs, but these efforts are compromised when latterly, these countries are not able to freely order, pay for and receive diagnostic material and equipment. A user-friendly laboratory procurement system would surely depend on the support of the country's government and veterinary service, but international (developing aid) organizations together with industrial companies should consider a possible pathway towards more flexible and cheaper distribution and maintenance of diagnostic material and equipment. Supporting reference laboratories in a way to take over responsibility for material distribution for the laboratories in their range may pose one starting point for finding a solution.

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Our results on methods to save resources and using different swab types and storage conditions were published in two publications:

- Fereidouni SR, Harder TC, Gaidet N, Ziller M, Hoffmann B, Hammoumi S, Globig A, Starick E. Saving resources: avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR. *J Virol Methods*. 2012 Dec;186(1-2):119-25. doi: 10.1016/j.jviromet.2012.08.002.
- Fereidouni S, Globig A, Starick E, Hoffmann B, Harder T. Effect of swab matrix, storage time and temperature on detection of avian influenza virus RNA in swab samples. *Avian Dis*. 2012;56(4):955-8.