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**Development of a new real-time colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the rapid detection of highly pathogenic H5 clade 2.3.4.4b avian influenza viruses.**

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Since 2016, Europe and France have faced four major highly pathogenic avian influenza viruses (HPAIV) of clade 2.3.4.4b. HPAIV epizootics are characterized by high bird-to-bird transmission, and high mortality rates in wild and domestic birds, causing important losses for the poultry industry. Additionally, HPAIV spread is threatening public health due to their zoonotic potential and more and more frequent spillovers in wild animals. Despite strong biosecurity measures, each year the viruses are spread rapidly and massively causing more and more outbreaks and an increasing number of infected birds.
Early detection is essential to better control and prevent the spread of the virus in the environment. Currently, HPAIV official diagnosis method is based on tracheal swabs analyzed by rRT-qPCR. Even though rRT-qPCR is highly sensitive and specific, this technique is expensive, requires trained staff and sophisticated lab equipment. More importantly, the sample-to-result time can be long and delay the start of the official response for disease control. Therefore, we aimed to develop an on-site molecular-based method which could be performed by farm staff. We developed a real-time colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the specific detection of clade 2.3.4.4b HPAIV.
RT-LAMP primers were designed from the in silico analysis of more than 620 hemagglutinin sequences available online. Primers specificity was tested on 8 avian influenza viruses from different subtypes, clades, and pathogenicity. Absolute quantification was used to calculate the HPAIV H5N8 2020/2021 and H5N1 2021/2022 detection threshold. Additionally, a clinical validation was performed on 198 tracheal swab pools sampled in the last two years on French outbreaks. Pools were simultaneously analyzed by RT-LAMP assay and rRT-qPCR to investigate the RT-LAMP assay sensitivity and specificity. Finally, as a proof-of-concept, we tested this RT-LAMP assay in two duck and one chicken farms officially reported as suspicious for HPAIV less than 24 hours before sampling. The RT-LAMP assay was realized directly on 10 individual tracheal swabs performed on randomly selected birds, without RNA extraction.
In silico and in vitro primers specificity investigation revealed a 100% specificity for clade 2.3.4.4b viruses. The detection threshold, obtained by absolute quantification was found below 10 and 20 copy/µL for H5N1 and H5N8 viruses, respectively. In addition, clinical validation analyses revealed a 100% specificity detection and an overall sensitivity of 86.11%. Interestingly, for samples with Ct value below 25, the sensitivity reached 100%. Finally, the on-site proof-of-concept detection analysis performed in the three suspicious HPAIV farms successfully detected the virus in each farm.
Therefore, we have developed a novel colorimetric RT-LAMP for the specific and sensitive detection of clade 2.3.4.4.b. This assay showed high specificity and sensitivity detection on clinical swabs. 100% sensitivity for samples with Ct<25 allows us to consider this strategy applicable in field as, most of the time, suspicious outbreak animals present low Ct values largely below 25. The proof-of-concept study confirmed our hypothesis. Moreover, this RT-LAMP assay is simple, fast (30min), cheap (~1.5euros/reaction), and requires limited equipment. This could be a valuable tool to improve the urgently needed sample-to-results time.