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Initial validation of multiplexing a high pathogenicity H5 detection ('HP H5') real-time reverse transcription polymerase chain reaction (rRT-PCR) with an N1-specific rRT-PCR for rapid detection of HPAIV H5N1

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Introduction
The high pathogenicity H5 ('HP H5') reverse transcription polymerase chain reaction (rRT-PCR) reported by James et al. (2022) has proved its value along with an N1-specific rRT-PCR (Payungporn et al., 2006) in statutory notifiable avian disease (NAD) response during the ongoing avian influenza (AI) epizootic in the United Kingdom (UK); the largest ever AI outbreak recorded to date in this country. Both assays have facilitated detection of HP avian influenza virus (HPAIV) H5N1 strain responsible for the epizootic as well as defining the virus found within the wild bird populations, through testing of samples submitted through the UK wild bird surveillance scheme. Multiplexing of both rRT-PCR assays into a single reaction well would provide substantial benefits reducing all the following: reagent costs, sample consumption, hands on processing time, the number of test runs performed and pipetting inaccuracies. Accumulatively these factors will add value to the customer, with an improved timeline and the potential for improved test sensitivity. The aim of this study was therefore to validate the potential to multiplex the HP H5 and N1-specific rRT-PCR assays using clinical samples to compare the diagnostic sensitivity against the respective single-plex assays.
Methods
The same thermal amplification profile is used for both the HP H5 detection and N1-specific rRT-PCR single-plex rRT-PCR assays. Therefore, for the initial validation of a multiplexed assay, the N1-specific primers and probes (Payungporn et al., 2006) were directly added to the HP H5 detection rRT-PCR master mix (James et al., 2022), reducing the equivalent volume of DEPC-water, up to a final master mix volume of 20 µl, adding 5 µl of sample RNA. The resulting multiplex assay was then used to amplify RNA extracted by an automated process from oropharyngeal and cloacal swabs submitted for AI virus surveillance screening from "found-dead" wild birds and from two NAD (i.e., AI and Newcastle disease) investigations on chicken premises. All extracted RNA samples (>340) were simultaneously screened by the single-plex HP H5 detection and N1-specific rRT-PCR assays and by the HP H5/N1 multiplex assay.
Results
Preliminary pilot data using HPAIV H5N1-positive clinical samples collected from wild bird surveillance provided a very promising outcome, where the results from the multiplex system correlated well with the corresponding results obtained from the HP H5 detection and N1-specific single-plex assays.
Conclusions
Optimisation of the thermal amplification and master mix reagent concentrations may improve the performance of the multiplex assays in comparison with both single-plex assays. Successful validation of the HP H5 detection/N1 multiplex rRT-PCR will significantly reduce costs associated with the UK wild bird surveillance scheme by reducing (by up to 50%) the numbers of rRT-PCR test sampling wells required for confirmation of HPAIV H5N1 infection in wild birds and will reduce the numbers of rRT-PCR tests required for statutory confirmation of HPAIV H5N1 infection from official samples submitted during the current AI epizootic in the UK.