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Molecular characterisation of avian hepatitis E virus in Australia and development of a rapid genotyping technique

Molecular characterisation of avian hepatitis E virus in Australia and development of a rapid genotyping technique

Researcher Contact Details

Name: Kinza Asif, Martins Olaogun and Amir H. Noormohammadi*

Organisation: The University of Melbourne

Phone: +61 (3) 9731 2275

Email: Amirh@unimelb.edu.au

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Poultry Hub Australia Contact Details

Poultry Hub Australia

CJ Hawkins Homestead, Ring Road

University of New England

Armidale NSW 2350

02 6773 1855

poultryhub@une.edu.au

www.poultryhub.org

Project Summary

Project Title	Molecular characterisation of avian hepatitis E virus in Australia and development of a rapid genotyping technique
Project No.	2020-221
Date	Start: 01 Jan 2021 End: 30th Oct, 2023
Project Leader(s)	Martins Olaogun, Margaret Sexton, Amir H. Noormohamamdi
Organisation	Asia-Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, University of Melbourne
Email	Amirh@unimelb.edu.au
Project Aim	The main objective of this project was to develop a safe, rapid, reliable, robust, and relatively inexpensive WGS platform for accurate strain identification of viral pathogens of poultry directly from clinical specimens and make the new technique available to the Australian poultry to improve her capacity to act immediately and appropriately during viral outbreaks.
Background	Big Liver and Spleen (BLS) Disease was first reported in Australian broiler breeders in 1980 and was later found to be caused by Avian Hepatitis E virus (AHEv). Over the last three years, there has been a significant increase in the number of BLS submissions made to our laboratory from Australian broiler breeder and layer chickens. However, the genotypes of the circulating aHEVs had not been investigated due to various discrepancies between results from the current available genotyping tools, consistent with overseas reports. Thus, in this project, we attempted to develop a cost-effective high-yield protocol to determine the complete genome sequence of Australian aHEV strains directly from clinical specimens and generate accurate data on the viral population structure and distribution. This enabled us to investigate the genetic make-up of the strains responsible for recent outbreaks, facilitate epidemiological investigations, and provide insight onto the diversity and evolution of the virus in Australia and globally.
Research Outcome	A new tool for the detection and discrimination of the Australian aHEVs was developed and proved to be effective in discriminating between different field cases, including those submitted from Australian layer birds.
Impacts and Outcomes	The main outcome of this project is an improved capacity of the Australian poultry industry to: • Determined the exact genotype of aHEVs in Australian meat and layer chicken flocks. • Identified new markers and developed an improved diagnostic assay.
Publications	Presentation at the Poultry Hub idea exchange meeting in Adelaide, October 2023. Publication in a scientific paper is highly desired and feasible. However some additional laboratory sequencing will be required, hinging on the availability of funds and a Masters/PhD student to compile sufficient information for an international journal. Please see section recommendations.

Executive Summary

Avian hepatitis E virus (aHEV) causes BLS disease, a transmissible viral disease of poultry, with significant economic losses and severe welfare concerns to the global poultry industry. At present, there is no effective commercial vaccines nor drugs to prevent and/or treat the disease in birds. During BLS outbreaks, immediate and unambiguous aHEV strain differentiation is vital for epidemiological investigations and choice of control strategy. Whole genome sequencing is used as the gold standard for strain identification of poultry pathogens especially given that the technology has become more cost-effective than ever before. However, its requirement of viable virus for laboratory isolation and propagation from which sufficient high-quality viral DNA/RNA can be extracted has limited its adoption as a routine diagnostic tool for strain identification of poultry and veterinary pathogens. aHEV cannot be efficiently propagated in cell culture except within living organisms or animals and so, isolating and culturing the virus to generate high quality viral RNA for WGS is impaired which prevents routine WGS of aHEV to generate accurate data on viral population structure and distribution during outbreaks. In this project, a recently developed method in our laboratory was used to determine WGS of DNA and RNA viral pathogens directly from clinical liver materials without having to isolate and culture the virus. Using this technique, aHEV RNA was successfully extracted from 4 out of 5 samples. Whole genome sequencing analysis of the two sequenced isolates showed high similarity of 98.9-099.1% with each other and 98.9-99.1% with recently reported Australian strains (GenBank accession # MW589651, MW589652). However, these isolates showed considerably less identity (82%) to the historical Australian strain (AM943647). Moreover, based on whole genome analysis of Australian isolates, several primer pairs were designed targeting variable regions flanked by conserved regions of the genome. Discriminatory powers of these primers were evaluated in PCR-High Resolution Melt (HRM) curve analysis using different field submissions. Results showed that the primer pair F1R1 provided the best discrimination between historical and contemporary isolates with high consistency and greater confidence percentage gap. Further analysis revealed two and possibly three new melt curve patterns, one of the new patterns were generated from specimens obtained from commercial layers. In conclusion, the new rapid genotyping assay has shown potential for routine diagnosis and detection of aHEV isolates in the Australian diagnostic laboratories. Also, this project has detected three new genotypes of aHEV in the Australian poultry industry including one in commercial layers. Future studies should investigate detailed sequences of the new aHEVs detected.

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Introduction

The expanding field of whole-genome sequencing (WGS) has revolutionized our understanding of microbial diversity, biology, and phylogeny and as such, has the potential to improve our understanding of the epidemiology, diversity, evolution, and emergence of new virulent strains of viruses and bacteria of significance in poultry and evolution. With the rapidly falling cost, it is logical that WGS should be used as a standard tool for strain identification of poultry pathogens. However, its requirement of viable virus for laboratory isolation and propagation from which sufficient amounts of high-quality viral DNA/RNA can be extracted has limited its adoption as a routine diagnostic tool for strain identification of poultry and veterinary pathogens. Recently in our laboratory, we have developed rapid and cost-effective methods to determine WGS of DNA viruses directly from clinical materials without having to isolate and culture the virus. The techniques included WGS of different serotypes of fowl adenoviruses (FAdV) directly from liver samples (Asif *et al.*, 2020), Infectious Laryngotracheitis virus from tracheal tissues (Asif *et al.*, 2022) and Fowlpox virus from skin samples (Asif *et al.*, 2021a). A modified version of the techniques reported for FAdV was also used to determine the WGS of an Avian Hepatitis E Virus (aHEV) from infected liver (Asif *et al.*, 2021b). Avian Hepatitis E virus is an RNA virus (Bilic *et al.*, 2009) and causes a transmissible disease in chickens characterized by sudden drop in egg production, enlargement of the liver and spleen, and increased mortality, with significant economic losses and severe welfare concerns to the global poultry industry (Sun *et al.*, 2019; Syed *et al.*, 2017). The virus is nonenveloped and has a 6.6 kb single-stranded RNA. It belongs to the genus Orthohepevirus, of the family Hepeviridae (Bilic*, et al.*, 2009; Sun*, et al.*, 2019) and is genetically and antigenically related to the mammalian hepatitis E virus including the swine and human HEV (Haqshenas *et al.*, 2002) which raises concern of a potential emergence of a zoonotic strain (Meng, 2010, 2016). To date, four major genotypes of aHEV have been identified based on genetic characterisations of segments of the viral genome with varied distribution across Europe, North and South Americas, and Asia(Matczuk *et al.*, 2019; Sun*, et al.*, 2019). While aHEV isolates from different geographic regions are genetically heterogenic, each genotype of aHEV belongs to a single serotype (Marek *et al.*, 2010; Meng, 2010; Zhao *et al.*, 2015), and so, effective strain differentiation is limited to nucleic acid amplification techniques. Since the clinical signs and pathologic lesions of BLS are atypical, diagnosis of clinical BLS is based on the detection of specific aHEV viral RNA in specimens using RT-PCR assays (Sun*, et al.*, 2019), as well as detection of viral antigen or specific antibody using serological assays including Agar gel precipitin (AGP) test, enzyme-linked immunosorbent assay (ELISA) (Clarke *et al.*, 1990; Sun*, et al.*, 2019; Syed*, et al.*, 2017; Wang *et al.*, 2014). However, given that aHEV genotypes tend to be geographically different, the specificity and consistency of the serologic assays remains unknown (Wang*, et al.*, 2014; Zhao*, et al.*, 2015) as evidenced by variations in the results from various serological investigations (Matczuk*, et al.*, 2019; Sun*, et al.*, 2019). Hence, determining the complete genetic make-up of the aHEV isolates during outbreaks would facilitate the development of rapid and effective detection and genotyping assays.

At present, there is no effective commercial vaccines nor drugs to prevent and/or treat the disease in birds. During BLS outbreaks, immediate and unambiguous aHEV strain

differentiation is vital for epidemiological investigations and choice of control strategy (Bilic, *et al.*, 2009; Kwon *et al.*, 2012). Avian hepatitis E virus cannot be efficiently propagated in cell culture except within living organisms or animals (Meng, 2010; Syed, *et al.*, 2017) and therefore, isolating and culturing the virus to generate high quality viral RNA for WGS is impaired which prevents routine WGS of aHEV to generate accurate data on viral population structure and distribution during outbreaks (Lecuit & Eloit, 2015).

Over the last five years, there has been a steady increase in outbreaks in Australian flocks and more interestingly, in the commercial layer industry in at least two Australian states consistent with overseas reports (Crespo *et al.*, 2015). However, the genotypes of the circulating aHEVs had not been investigated due to various discrepancies between results from the current available genotyping tools, consistent with overseas reports (Crespo, *et al.*, 2015; Kwon, *et al.*, 2012; Wang, *et al.*, 2014; Zhao, *et al.*, 2015). In some of these cases, discrepancies between results from serological assays (commercial and in-house ELISAs, and AGP) were observed. Recent investigations in our laboratory have suggested that results generated from serological assays (commercial ELISA and AGP) do not necessarily correlate with those generated using RT-PCR. Notably, some clinical specimens (sera and liver tissues) tested negative in AGP while positive in ELISA and/or PCR. These suggest that there may be genetic and antigenic differences between strains prevalent in Australia. It is notable that that the antigen currently used in Australia for the AGP serological assay is from a historical (broiler breeder) aHEV which may be distinct from the strains responsible for the recent outbreaks, and this may possibly impact the capacity of the assay to detect antibodies to heterologous aHEVs (Kwon, *et al.*, 2012; Wang, *et al.*, 2014; Zhao, *et al.*, 2015). Hence, characterising the genotypes of circulating aHEV strains in Australia using whole genome sequencing would be vital for investigations on epidemiology of the disease, development of an effective genotyping tool, and subsequently, implementation of effective control and prevention strategies.

Objectives

The main objectives of this project were to:

- determine the genotype of aHEVs in Australian meat and layer chicken flocks.
- monitor the presence & spread of aHEV strains in Australia to facilitate the development of new molecular tools to identify new genetic markers for grouping related strains.
- identify new markers for development of improved diagnostic assays and nomination of potential vaccine candidates for the control of the disease.

Methodology

Sample Preparation

The details of samples used in this study are described in Table 1. Samples were obtained from Avian-hepatitis-E virus (aHEV) positive clinical specimens submitted to our laboratory as part of routine diagnostic examination from outbreaks of Big Liver and Spleen (BLS) disease. As such, no formal Animal Ethics approval required. Samples were prepared for RNA extraction as described below. Briefly, approximately 1.5g of aHEV-positive liver and spleen tissues were homogenized in mortar and pestle using sterile sand, and mixed with Medium 199 (M-199, Gibco) containing 1% newborn calf serum (Invitrogen) supplemented with 2 mM L-glutamine, 50 µg/mL of penicillin, 50 µg/mL of streptomycin and 5 µg/mL of amphotericin B. The homogenates were centrifuged at 3900 ×g for 30 min at 4 °C. The supernatants were treated with kaolin hydrated aluminium silicate powder (MP Biomedicals LLC) at the ratio of 5 % of its total volume (25 mL) and the mixture was agitated on a shaker for 30 min at room temperature (~22 °C). This was followed by centrifugation at 1750 ×g for 5 min at 4 °C (Beckman Coulter), and supernatants were collected and processed for viral RNA purification.

Table 1. Details of samples used for the study.

S/N	Sample ID	Farm/location	Layer/Broiler	Age
1	101454 (1)	Victoria	Breeders	48 weeks
2	101454 (2)	Victoria	Broiler Breeder	38 weeks
3	101572 (4)	South Australia	Broiler Breeder	36 weeks
4	W184-21	Victoria	n/a	n/a
5	102378	Western Australia	Broiler Breeder	Multi age

Viral RNA purification

The supernatants were processed for RNA extraction by ultracentrifugation. Briefly, the supernatant was layered onto a 30 % w/v ice-cold sucrose cushion in an open top tube and virions were pelleted at 98,000 ×g for 90 min at 4 °C. The pellets were resuspended in a 2× lysis buffer (0.01 M Tris-HCl [pH 7.5], 0.01 M EDTA, 1% SDS, 1 mg/mL proteinase K in 0.01 M Tris-HCl [Ph 8]) and incubated overnight at 37°C. The aHEV RNA was extracted using QIAzol method doubling the volume of QIAzol and chloroform used. The aHEV RNA quality and quantity were measured using the Quantus Fluorometer (Promega) and micro spectrophotometry (NanoDrop, ND-1000, NanoDrop Technologies, Wilmington, DE) respectively. A NanoDrop 260/280 ratio was used to determine the purity of samples. RNA samples were stored at −80 °C until used for reverse transcription polymerase chain reaction (RT-PCR) and genome sequencing.

RT-PCR

Extracted RNA were subjected to two sets of reverse-transcriptase polymerase chain reactions (RT-PCR). The first RT-PCR targeting Helicase gene and to confirm the presence of aHEV

RNA in the extracted RNA samples, was performed using the 1-Step QIAGEN RT-PCR kit and primer sets and protocols as previously described (Huang *et al.*, 2002).

The second set of RT-PCR was to amplify the complete genome of aHEV from the 5′ ends of CDS 1 to the 3′end of the CDS 2. RT-PCR was performed using the SuperScript IV one step RT-PCR system (Invitrogen) following the manufacturer's instructions and the primer pair BLS-F1 (5′GCATGACCCCATGCCAGGGTAAGAATG 3′) and BLS-R6 (5′ CTATGCCCGAGATGGGAGGATTTC '3′) (Kinza et al 2021). The RT-PCR products (complete genome) were separated by gel electrophoresis on a 1.5 % agarose gel containing gel red stain (Biotium) and visualised on a transilluminator. Amplicons were purified directly using FavorPrep GEL/PCR Purification kit (Favorgen Biotech Corp.) following the manufacturer's instructions (Figure 1).

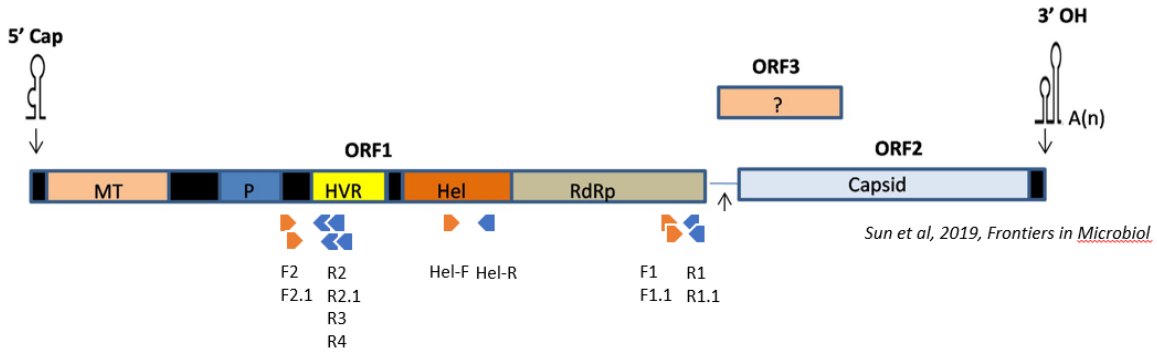

Figure 1. Schematic presentation of primers designed.

Nanopore sequencing

RT-PCR amplicons of complete aHEV genome were subjected to Nanopore sequencing. Sequencing libraries of PolyA+ RNAs and/or RT-PCR amplicons were prepared using the direct cDNA sequencing kit (SQK-DCS109) with native barcoding (EXP-NBD114) according to manufacturer's instructions. Sequencing was performed on a MinION MK-1 device fitted with a FLO-MIN106 (R9.4 chemistry) flow cell at the Asia-Pacific centre for Animal Health, Melbourne Veterinary School, The University of Melbourne, Werribee 3030.

Sequence assembly, annotation, and phylogenetic analysis

Sequence assembly and comparison were performed as described previously (Asif*, et al.*, 2021b). In brief, quality filtered reads from Nanopore sequencing for RT-PCR amplicons of the submissions 101454 and W184-21 were used to generate assembly using Unicycler with the default parameters. ORFs were found in the final assembly using the NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) and annotated according to the already published aHEV genomes. Multiple alignment of the assembled sequences with previously published

sequences was performed in Geneious version 9.1.8. Phylogenetic relationship between aHEV sequences was performed using Neighbour-joining method with Kimura 2-parameter model and 1000 bootstrap iterations in MEGA-X version 10.0.5. Sequences will be submitted to the GenBank prior to the submission of a manuscript for publication.

Genotyping with High-Resolution Melt (HRM) curve analysis

A series of primer sets were designed based on the sequence assembly of the previously characterised Avian Hepatitis E viruses (aHEV) (Asif*, et al.*, 2021b) and those characterised in this study. The primers were designed to target the most variable regions of the genome whilst flanked by conserved regions for primer design. The primers (5'-3') were as follows:

F1: GGCACCCTTCTGTGGAACACTGTCTGG

R1: CCTTCATTTTTAGCCCGCAGTCTGC

F1.1: AACACUGUGUGGAACAUGACGG

R1.1: CAGCAAGTTGGAAAAGGCGCCACATGGACC,

F2: TGCATATTATGGACCGCCGACCGGTAG

R2: CGACGGCAATACAGCAAGAACCTCC

F2.1: CAACCGTCTGCTCCACCATCACTGC

R2.1: CCTCCTCTTGACCAGGTTGTGGC

R4: GCGGCAACATCCAACAAATTCC

R3: CATCCAACAAATTCCCCACGAT

Schematic image represents the location and direction of the primers used according to the aHEV genome (Fig. 1).

Several PCR runs were conducted to determine the optimal PCR conditions with different primer pairs. Finally, a PCR protocol was established with primer set F1 and R1under the following conditions: 1 cycle each of reverse transcription at 50ºC for 30min, enzyme inactivation at 95ºC for 15min, followed by 35 cycles of denaturation at 94ºC for 30sec, annealing at 60ºC for 30sec, extension at 72ºC for 30 sec and final extension at 72ºC for 10min.

Preliminary evaluation of the PCR assays for HRM curve analysis

HRM analysis was performed after PCR amplification using a historical and two variants of a contemporary aHEV from broiler breeders at the temperature ramping from 75-90 ºC, rising by 0.2 ºC/2sec, at three different ramps of 0.05, 0.2 and 0.5 using Rotor Gene Q-Series software version 2.3.1 (Build 49). Normalised melt curves were analysed using normalisation ranges of 78.1-78.6 °C and 84.5-85.1 °C. To verify the accuracy of the method, PCR-HRM with primer pair F1R1 was evaluated on field submissions received for aHEV (previously tested positive by RT-PCR in our laboratory).

Results

Complete genome of aHEV was sequenced using Nanopore sequencing directly from RT-PCR amplicons.

High quality aHEV viral RNA yield was obtained directly from the liver tissues for four of five samples (Fig. 2).

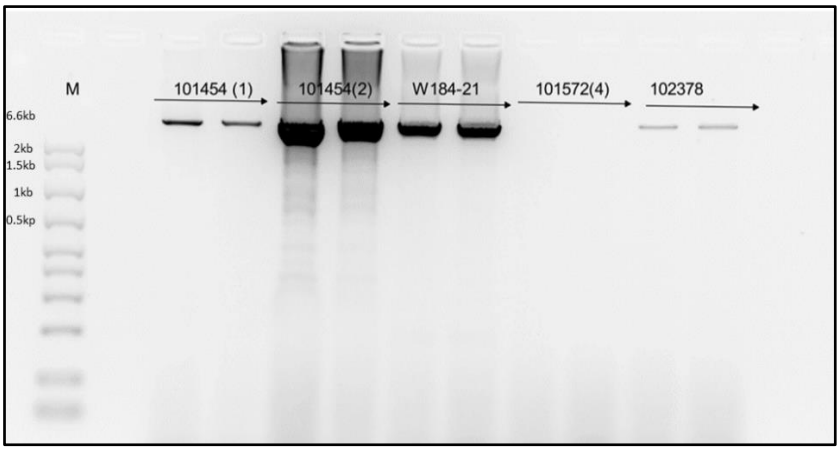

Figure 2. Agarose gel electrophoresis (1.5% agarose) of 6.6kb PCR amplified products using BLS F1-R6 primer sets. Lane M, 2 kb DNA size marker

RT-PCR generated expected amplicons of approximately 6.6kb for four of five samples (Fig. 2). Two of four samples (W184-21 and 101454) were subjected to Nanopore sequencing. Yields and purity of the nucleic acid preparations were deemed suitable for nanopore sequencing and generated a large number of reads of sufficient length for WGS and genome assembly (Fig. 3).

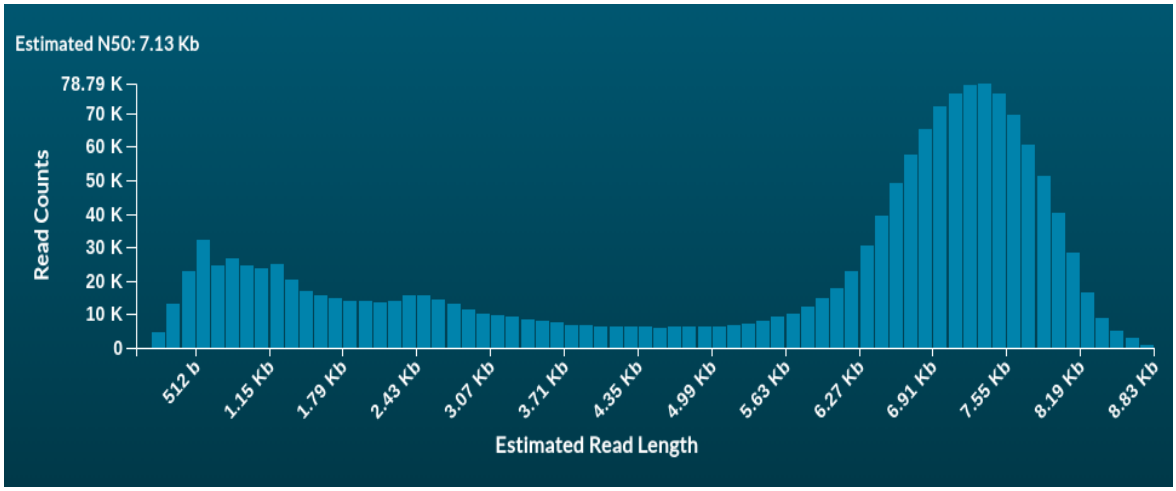

Figure 3. Nanopore read length histogram showing the length of reads generated samples 101454(2) and W184-21

Subsequently, the complete genome of two strains of aHEV were generated (Figure 4).

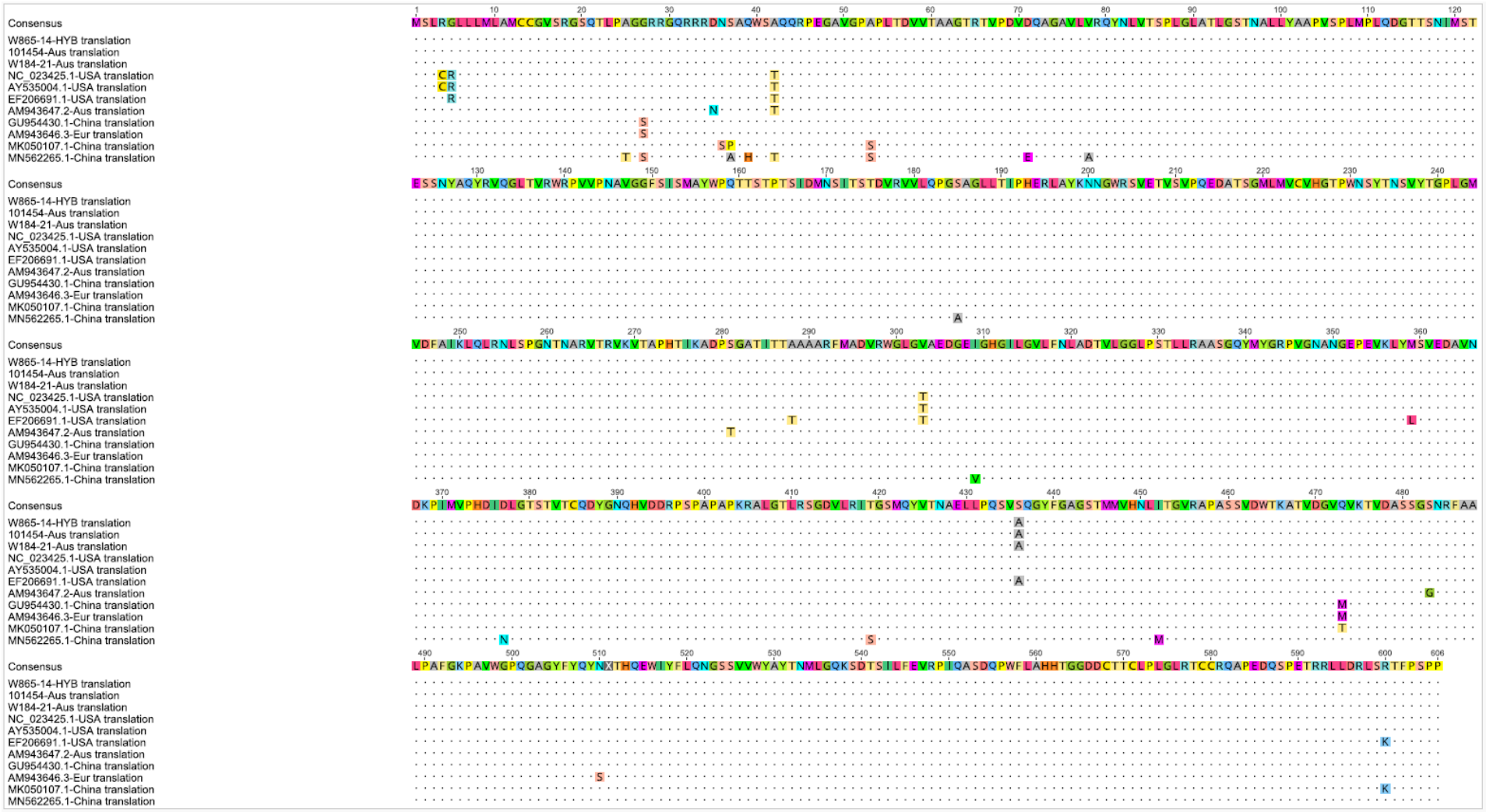

Figure 4. Sequence alignment of the capsid protein aa sequences from 101454 and W184-21 isolates and those of the previously published aHEV sequences.

The newly characterised full genomes were highly similar to the contemporary aHEVs.

The complete assembled genomes were compared against all previously published sequences (Table 2). Comparison of the assembled sequences showed that 101454 and W184-21 were highly similar to each other (98.3% identity) and to the recently reported (Asif*, et al.*, 2021b) aHEV sequence (99.0-99.2%) (Table 2). However, there was considerably less sequence identity to that of the historical Australian aHEV (GenBank accession #AM9436347) sequence (approximately 82% identity).

Nucleotide variations were detected all through the genome although most variations were found to occur in the 5' end of the genome (between bases 1-200 which codes for the amino-terminal domain of the non-structural protein), and early the capsid gene (bases 4725-4940) (results not shown). Comparison between the aa sequences of the capsid protein revealed relatively low level of variation between the aHEV isolates. Most variations were found between aa 1-50 of the capsid protein (Fig.4).

Phylogenetic tree based on complete genome sequences classified 101454 and W184-21 aHEV isolates in a single clade together with the recently reported Australian isolate (Asif*, et al.*, 2021b) and the USA strains AY535004 and EF206691, whereas genotype 1 historical Australian strain AM943647 clustered close to the South Korean strains (Fig. 5).

PCR-HRM based on F1R1 primer set reveals new melt curve patterns.

Preliminary analysis of a combination of primer sets designed in this study in PCR-HRM analysis using a historical and two variants of a contemporary aHEV from broiler breeders revealed that a melt curve at the ramp of 0.2 provided the best discrimination power between different aHEVs and minimum "noise". Based on conventional melt curves, the primer set F1 and R1 appeared to give a better discrimination between specimens tested (Fig. 6A). The helicase gene primers also provided a reasonable discrimination between the melt curves although the temperature differences were less than the F1 & R1 primer set.

Table 2. Sequence identity of the 101454 and W184-21 assembled sequences versus previously published sequences.

	W184-21-Aus	**101454-Aus**	W865-14-HYB	AM943647.2-Aus	AY535004.1-USA	EF206691.1-USA	NC_023425.1-USA	AM943646.3-Eur	GU954430.1-China	MK050107.1-China	MN562265.1-China
W184-21-Aus		98.3	98.9	82.4	87.9	87	87.9	82.1	82.2	81.7	80.6
101454-Aus	98.3		99.1	82.3	88	87.3	88	82.2	82.2	81.6	80.6
W865-14-Recent Aus	98.9	99.1		82.4	88.2	87.2	88.2	82.2	82.2	81.7	80.5
AM943647.2-Hist-Aus	82.4	82.3	82.4		82.4	82.7	82.4	82.3	82.3	82.2	81.2
AY535004.1-USA	87.9	88	88.2	82.4		90	100	82.1	82	82.3	81.2
EF206691.1-USA	87	87.3	87.2	82.7	90		90	82.5	82.5	82.5	81.6
NC_023425.1-USA	87.9	88	88.2	82.4	100	90		82.1	82	82.3	81.2
AM943646.3-Eur	82.1	82.2	82.2	82.3	82.1	82.5	82.1		98.3	86.1	81.3
GU954430.1-China	82.2	82.2	82.2	82.3	82	82.5	82	98.3		86.1	81.4
MK050107.1-China	81.7	81.6	81.7	82.2	82.3	82.5	82.3	86.1	86.1		80.9
MN562265.1-China	80.6	80.6	80.5	81.2	81.2	81.6	81.2	81.3	81.4	80.9	

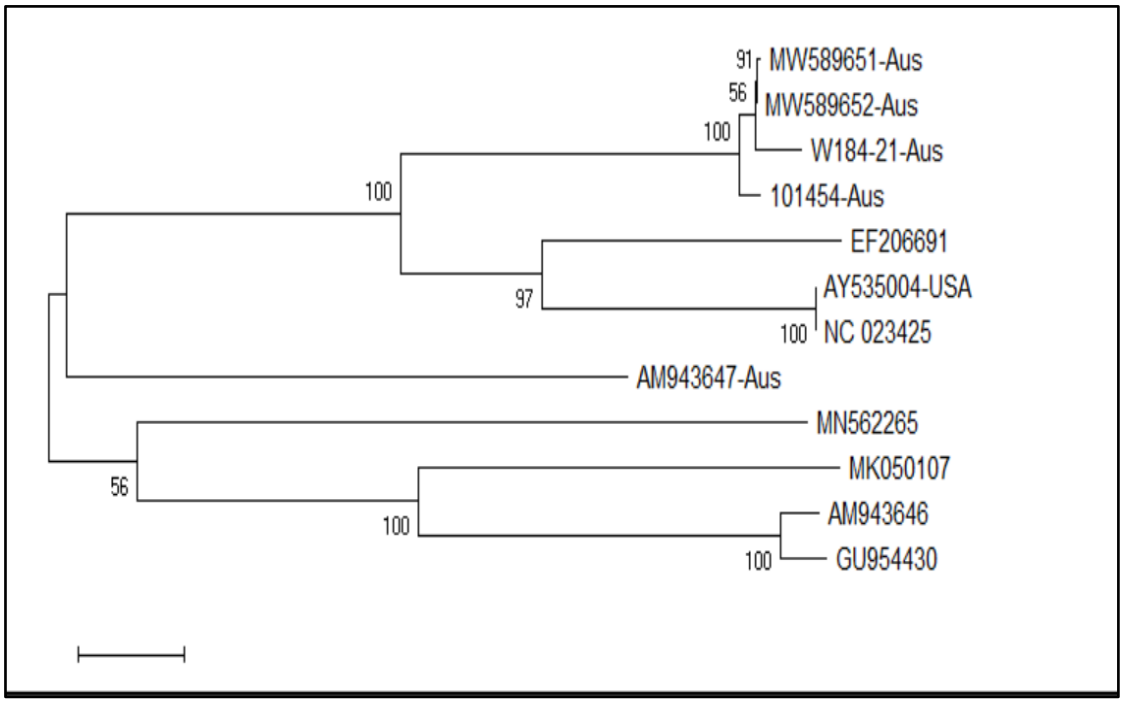

Figure 5. Phylogenetic tree showing the relationship between the 101454 and W184-21 assembled sequences versus previously published sequences. Scale represents 0.020 nucleotide substitutions per site.

Evaluation of the F1 & R1 PCR primer set on field submissions and analysis of the melt curves against historical and contemporary aHEV isolates (Asif, *et al.*, 2021b) revealed two, and possibly three, new melt curve patterns (Fig. 6B). One of the new patterns was generated from specimens obtained from layer birds.

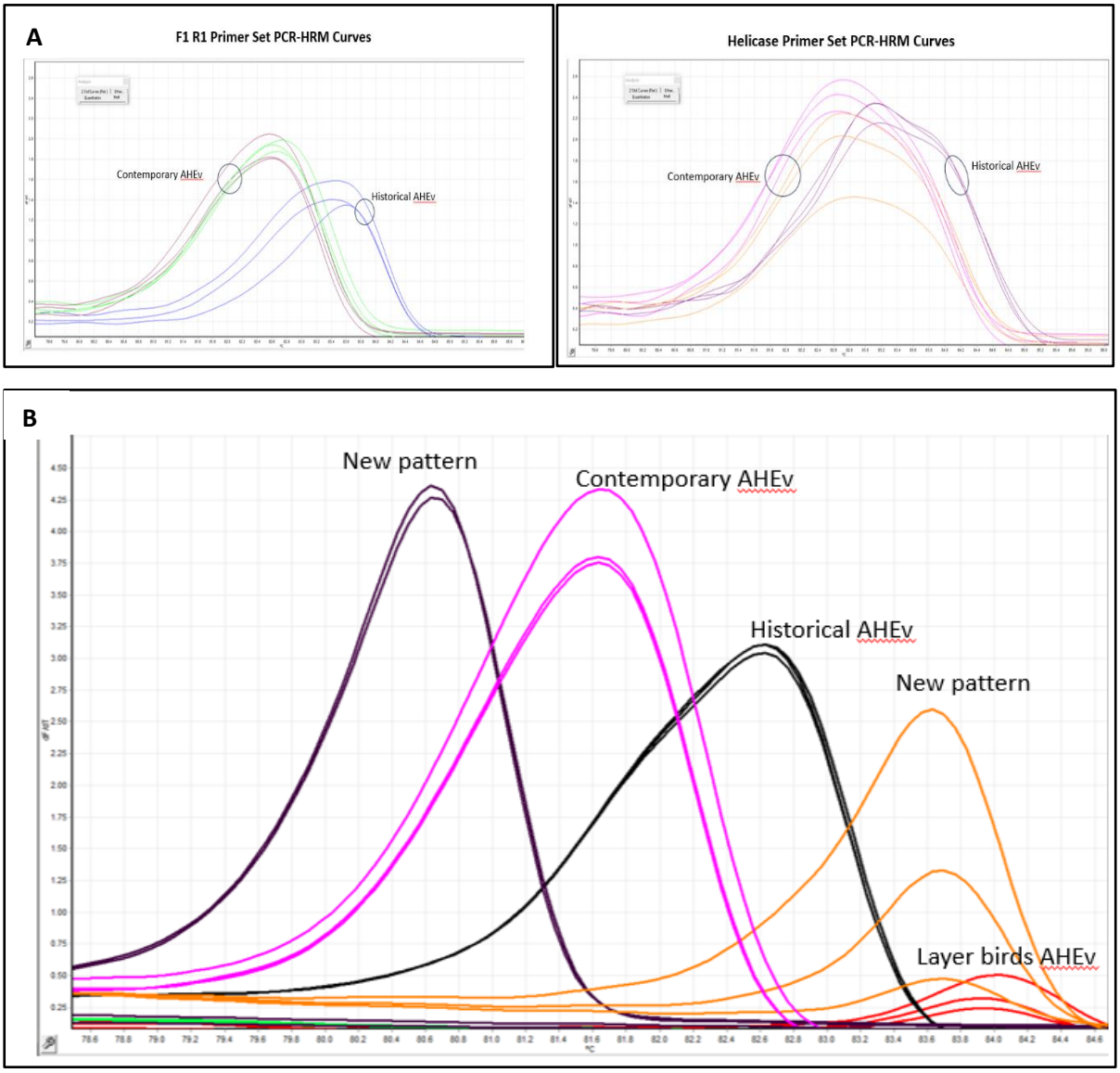

Figure 6. Evaluation of the F1R1 PCR-HRM curve analysis for differentiation of field strains

Analysis of the normalised melt curves using normalisation ranges of 78.1-78.6 °C and 84.5-85.1 °C, showed high consistency between replicates and a high discriminatory power between the new patterns and the historical and contemporary aHEVs (results not shown). This was evident by a wide confidence percentage gap between the patterns (Table 3).

Table 3. Confidence percentage between different isolates

Genotype (replicates)	Confidence %
Variation	0
Variation	0
Variation	0
Historical	100
Historical	100
Historical	100
Variation	0
Variation	0
Variation	0
Variation	0
Variation	0
Contemporary	100
Contemporary	100
Contemporary	99
Variation	0
Variation	0

Discussion

The most significant challenge with aHEV is that it cannot be propagated easily in cell culture and therefore WGS directly from clinical samples is the most suitable option for characterisation of viral isolates. Also, WGS directly from clinical specimens does not run the risk of sequence modifications that could occur after passage *in vitro* (Asif*, et al.*, 2020; Cottone *et al.*, 1998; Hautaniemi *et al.*, 2010).

In this project, the WGS of two Australian aHEV isolates from Australia were characterised directly from clinical liver tissues using kaolin hydrated aluminium silicate. The aHEV isolates used in the study were associated with mortality and loss of egg production in broiler breeders. Sequency analysis and comparison of both isolates with previously sequenced aHEVs revealed that both isolates showed ~98.3% nucleotide identity with each other and 98.9-99.1% with recently reported Australian strain (Asif*, et al.*, 2021b) and 87.9-88% with strains from the USA. However, these Australian isolates showed considerably less nucleotide identity (~82%) with historical Australian strain. Nucleotide variations were detected all through the genome although most variations were found to occur in the 5' end of the genome (between bases 1-200 which codes for the amino-terminal domain of the non-structural protein), and early the

capsid gene (bases 4725-4940) (results not shown). Comparison between the aa sequences of the capsid protein revealed relatively low level of variation between the aHEV isolates. Most variations were found between aa 1-50 of the capsid protein. Complete genome of two isolates of aHEV were assembled and compared to the previously characterised sequences. Nucleotide variations were seen across the whole genome albeit distributed unevenly through the genome. The capsid protein gene showed some variations in the 5' end but generally amino acid sequences showed less variation.

Based on phylogenetic analysis, the newly characterised Australian isolates form a clade with the recently reported Australian strain and aHEV strains from the USA (genotype 2), which is distant from genotype 1 Australian strain, and strains from South Korea, Europe, and China.

A PCR-HRM was developed based on sequence information obtained from WGS obtained in this study and proven useful for rapid genotyping of aHEVs in Australia. The combination between PCR and HRM curve analysis is routinely used in our laboratory for the diagnosis and strain identification of a number of veterinary pathogens including *Mycoplasma synoviae*, *M. gallisepticum*, *Chlamydia spp*, Infectious Bronchitis virus, Avian Nephritis virus, Infectious Bursal Disease virus and Fowl Adenoviruses (Chamings *et al.*, 2015; Ghorashi *et al.*, 2013; Ghorashi *et al.*, 2015; Ghorashi *et al.*, 2011; K. Hewson *et al.*, 2009; Robertson *et al.*, 2009; Steer *et al.*, 2009). Theses assays have been instrumental in defining strains involved in outbreaks of these pathogens in Australia as well as differentiation between vaccine and field strains (Ghorashi*, et al.*, 2013; K. A. Hewson *et al.*, 2010; Jeffery *et al.*, 2007; Robertson *et al.*, 2010; Steer *et al.*, 2011). Following assessment of the AHEv PCR-HRM curve analysis assay on specimens collected during this study and other clinical materials submitted to our laboratory as part of diagnostic investigations, three new viral genotypes, in addition to the Australian historical and contemporary viruses were detected. One of these new genotypes belonged to a commercial layer farm with a history of mild increase in mortality and reduced performance. This particular submission was part of a diagnostic specimen submitted to our laboratory towards completion of this project and therefore it fell outside timeframe of the current project. Therefore, further studies are needed to investigate the detailed sequences of the newly detected aHEVs and their impact on the health of infected birds.

In conclusion, a new tool for the detection and discrimination of the Australian aHEVs was developed and has proven to be effective in discriminating between different field cases, including those submitted from Australian layer birds.

Implications

The main outcome of this project is an improved capacity of the Australian poultry industry to determine the exact genotype of aHEVs in Australian meat and layer chicken flocks, monitor the presence and spread of aHEV strains in Australia and therefore facilitate the development of new molecular tools to identify new genetic markers for grouping related strains and identify new markers for development of improved diagnostic assays and nomination of potential vaccine candidates for the control of the disease.

The immediate impact of the genotyping technique developed here for the Australian poultry industry is the capacity to 1) identify the infected flocks and the exact genotype involved, so that 2) the source of spread can be identified and 3) further spread of the virus and subsequent economical losses can be ceased immediately. This is especially important as the virus is suspected to spread both horizontally and vertically.

Recommendations

Further whole genome sequencing studies are recommended to investigate the detailed sequences of the newly detected aHEVs and their impact (risk) on the health of infected birds. Such studies will need to calculate the correlation between the presence of individual genotypes of aHEV and clinical disease and/or mortality in the affected farms.

Once genotypes with high risk are identified, additional studies should attempt to isolate the live virus from clinical materials (stored in our laboratory) to allow for challenge experiments towards potential vaccine development. Currently, there is no commercial vaccine available for this disease globally. Given the uncertainty of isolation and propagation of the aHEV under laboratory conditions at the time being, inactivated vaccines (as opposed to live vaccines) or non-traditional vaccines such as DNA-, RNA- and recombinant protein vaccines are likely to be more achievable within a short time frame.

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The project leader Dr. Olaogun prepared and submitted the proposal, and was awarded the grant to conduct this project. Professor Noormohammadi, Dr Sexton and Dr Marenda provided direction for this project. Dr Asif conducted WGS of the viral strains and related analysis, developed a new PCR-HRM analysis for an improved detection and strain identification of the viral strains, and prepared the final report for this project. Dr. Songhua Shan (the Universoty of Melbourne) provided assistance in laboratory work and data analysis and specimens for inclusion in this project.

Media and Publications

A presentation on this project was delivered by Dr Kinza Asif at the Poultry Hub Australia Ideas Exchange 2023 in Adelaide, South Australia. A copy of this presentation has been attached.

Intellectual Property or Confidential Information Arising

Confidential information related to farm or company from which clinical specimens collected have been excluded from this report.

Techniques for extraction of viral DNA/RNA using 'Kaolin hydrated aluminium silicate' treatment had been developed independent of this project for whole genome sequencing of Fowl adenoviruses and aHEV directly from liver samples (Asif, *et al.*, 2020; Asif, *et al.*, 2021b). Also, a complete whole genome sequence of a 2014 aHEV from VIC had been determined using PCR based techniques, also independent of this project (Asif *et al*, unpublished data).

The sequences determined as part of this project are highly similar to the ones reported earlier (Asif, *et al.*, 2021b). The information related to development of the new PCR-HRM assay, and the discovery of new genotypes have not been published and will need to be kept confidential until completed (conditioned on availability of additional research funds) and published.

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Appendices

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