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Subclinical avian hepatitis E virus infection in layer flocks in the United States

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Highlights

- Circulation of avian HEV was determined in healthy commercial layer farms in the United States.
- A fluorescent microbead immunoassay (FMIA) was developed for detecting anti-avian HEV IgY antibodies.
- Prevalence of avian HEV infection was high among clinically healthy laying hens.
- Different avian HEV genotype 2 strains were circulating within a farm.

Abstract

The objective of this study was to determine patterns of avian HEV infection in naturally infected chicken farms. A total of 310 serum samples and 62 pooled fecal samples were collected from 62 chicken flocks on seven commercial in-line egg farms in the Midwestern United States and tested for avian HEV circulation. Serum samples were tested for presence of anti-avian HEV IgY antibodies by a fluorescent microbead immunoassay (FMIA) which was developed for this study. The FMIA was validated using archived samples of chickens with known exposure (n = 96) and compared to the result obtained with an enzyme-linked immunosorbent assay (ELISA) based on the same capture antigen. There was an overall substantial agreement between the two assays (κ = 0.63) with earlier detection of positive chickens by the FMIA (P = 0.04). On the seven farms investigated, the overall prevalence of anti-avian HEV IgY antibodies in serum samples from commercial chickens was 44.8% (20-82% per farm). Fecal samples were tested for avian HEV RNA by a nested reverse-transcriptase PCR. The overall detection rate of avian HEV RNA in fecal samples was 62.9% (0-100% per farm). Sequencing analyses of partial helicase and capsid genes showed that different avian HEV genotype 2 strains were circulating within a farm. However,

no correlation was found between avian HEV RNA detection and egg production, egg weight or mortality. In conclusion, avian HEV infection is widespread among clinically healthy laying hens in the United States.

Keywords: Avian hepatitis E virus (avian HEV); Chickens; Fluorescent microsphere immunoassay, RT-PCR, Subclinical infection.

Introduction

Hepatitis E virus (HEV) are non-enveloped, single-stranded RNA viruses with an icosahedral capsid symmetry that belongs to the family *Hepeviridae* (Meng et al., 2012). HEV has been identified in several animal species (Meng, 2013) and, based on host tropism and genetic relatedness, strains genetically characterized thus far can be classified into two genera: genus *Orthohepevirus* (all mammalian and avian hepatitis E virus [avian HEV] isolates) and genus *Piscihepevirus* (cutthroat trout virus) (Smith et al., 2014). Currently, four avian HEV genotypes have been described in chicken flocks worldwide (Johne et al., 2014). Genotype 1 has been identified in Australia and Korea, genotype 2 is present in North America, genotype 3 is present in Europe and China and, more recently, a novel putative genotype 4 has been detected in Hungary and Taiwan (Marek et al., 2010; Banyai et al., 2012; Kwon et al., 2012; Hsu and Tsai, 2014a). The avian HEV genome contains three open reading frames (ORFs): ORF1 encodes non-structural viral proteins, ORF2 encodes the capsid protein and ORF3 encodes a small multifunctional phosphoprotein (Meng et al., 2012).

A HEV-related sequence was first detected in chickens with big liver and spleen disease in Australia (Payne et al., 1999). A similar disease manifestation, designated as hepatitis-splenomegaly syndrome (HSS), was first associated with avian HEV in North America (Haqshenas et al., 2001). In both broiler breeder hens and egg-type chickens ranging from 30 to 72 weeks of ages, HSS is clinically characterized by increased mortality and decreased egg production. On gross examination, blood-tinged fluid can frequently be observed in the coelomic cavity and livers and spleens are typically both enlarged (Meng and Shivaprasad, 2013). A significant proportion of chicken flocks worldwide are seropositive to avian HEV, even though seropositive flocks do not necessarily suffer from HSS (Meng and

Shivaprasad, 2013). Thus far, no clear association between avian HEV strains and pathogenicity has been established (Marek et al., 2010). Avian HEV isolates obtained from healthy chickens were able to induce liver lesions but did not produce clinical HSS in specific-pathogenic-free chickens (Billam et al., 2009). However, apparently healthy chickens with reduced egg production have been shown to be positive for avian HEV RNA in the absence of other pathogens, suggesting that subclinical infection with avian HEV may impair egg production in broiler breeders (Sprygin et al., 2012). Therefore, additional studies are needed to elucidate the significance of avian HEV infections in clinically healthy laying hens.

Current methods to demonstrate avian HEV infection include conventional and real-time reverse transcriptase (RT) PCR assays for detection of avian HEV RNA (Sun et al., 2004; Bilic et al., 2009; Troxler et al., 2011) and commercial and in-house enzyme-linked immunosorbent assays (ELISA) and agar gel immunodiffusion (AGID) tests for detection of anti-avian HEV antibodies (Huang et al., 2002; Morrow et al., 2008; Zhao et al., 2013; Hsu and Tsai, 2014b). ELISAs based on the ORF2 capsid protein have been widely adapted and are commonly used in serological surveys (Peralta et al., 2009; Kwon et al., 2012; Zhao et al., 2013; Liu et al., 2014). Fluorescent microbead immunoassays (FMIA) are increasingly being used in veterinary serology (Wagner et al., 2011; Langenhorst et al., 2012; Gimenez-Lirola et al., 2014). An advantage of this new technology is the ability to screen for antibodies against multiple pathogens simultaneously in one reaction well using a small amount of sample, thus saving time, labor and reagents. In addition, FMIAs may have improved sensitivity compared to conventional serological assays, such as ELISAs (van Gageldonk et al., 2008).

The objective of this study was to evaluate the avian HEV infection dynamics, including fecal avian HEV RNA shedding and prevalence of anti-avian HEV antibodies, in healthy layer hen flocks in the Midwestern United States. For serology purposes, an FMIA to detect anti-avian HEV IgY antibodies was developed, validated and compared with an indirect ELISA.

Material and methods

Farm selection and characterization

Seven commercial egg farms, designated A through G, volunteered to participate in this study. The final number of participating farms was determined based on responses to an email sent to the farms' managers and/or veterinarian-in-charge, and to on-farm availability to provide the specified samples and production data. Participating farms were located in four U.S. Midwestern states: Iowa, Illinois, Michigan and South Dakota. At the time of sample collection Iowa represented the largest U.S. egg producer and Michigan was ranked seventh. The number of sampled farms did not reflect the state's egg production. Each farm had an egg production facility wherein eggs were collected from multiple chicken houses and transported to an egg processing room via a collection of belts and conveyors. These in-line operations produced washed and graded whole shell eggs for retail and/or food service customers. Chickens on each farm were vaccinated against Marek's disease, infectious bronchitis, Newcastle disease, avian influenza, fowl pox, *Mycoplasma gallisepticum*, and *Salmonella* Enteritidis.

Samples were collected between October and December 2013. At the time of sample collection, chickens in flocks on participating farms were healthy and none had clinical signs associated with HSS. A flock consisted of all chickens housed in one building. All flocks on

each farm were sampled (n = 5 to 11, average n = 8). Production data available for each flock included average hen-day egg production (%), weekly mortality (%), and egg weight.

Sample collection

A total of 310 serum samples were collected from 62 flocks divided among seven egg farms. From each flock, blood samples were taken from each of five chickens arbitrarily selected from different locations within a house. Blood samples were acquired by venipuncture of the brachial vein in 5 ml serum tubes (n = 25 to 55/farm; average n = 42). In addition, one pooled fecal sample was obtained from each house by collecting and pooling 2 g of fresh droppings from manure pits at five arbitrarily selected sites.

Blood and fecal samples were collected on the same day from all flocks on a farm, labeled with the house number, placed in insulated boxes with ice packs (4 °C) and shipped on the day of collection to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL), Ames, Iowa. Upon arrival, blood was centrifuged at 1500 ×g for 10 min to harvest serum and aliquotted into 4 mL plastic tubes. Fecal samples were homogenized and an aliquot of 0.4 g was resuspended in 4 ml PBS, vigorously vortexed, and centrifuged at 1500 ×g for 10 min. All samples were stored at −80 °C until testing. Shipping and storage conditions were similar for all samples.

Fluorescent microbead immunoassay (FMIA) development

Experimental serum samples

Ninety-six serum samples from 36 specific-pathogen-free chickens from a previous avian HEV study (Billam et al., 2009) were used as positive and negative controls to develop the FMIA. Briefly, 24 6-week-old chickens were intravenously inoculated with avian HEV

genotype 2. Blood samples were collected before inoculation and weekly thereafter for a total of four weeks. Twelve chickens where sham-inoculated and served as negative controls. Seroconversion started at day post-inoculation (dpi) 14 and at dpi 28 all inoculated chickens had seroconverted to avian HEV as previously determined by an in-house ELISA (Billam et al., 2009). A total of 36 negative control samples (all samples collected on dpi 0) and 60 samples (dpi 7–28) from chickens infected experimentally were tested. Specifically, negative control samples were used for estimating diagnostic specificity and 24 serum samples from experimentally infected chickens collected on dpi 21 (n = 12) or 28 (n = 12) were used for estimating diagnostic sensitivity of the FMIA.

Antigen and conjugation to carboxylated paramagnetic microbeads

A truncated recombinant ORF2 capsid protein of avian HEV expressed in *Escherichia coli* and purified by affinity chromatography as described previously (Haqshenas et al., 2002) was used as antigen for the FMIA. Fluorescent microsphere coupling was performed using a method described previously (Gimenez-Lirola et al., 2012) by addition of 25 µg of the avian HEV recombinant ORF2 capsid protein to 2.5×10^6 carboxylated-fluorescent microbeads (bead region 64, Luminex Corp., Austin, TX, USA).

FMIA

The assay was performed as described previously (Gimenez-Lirola et al., 2012) with exception that a 1:2000 dilution of a biotin-conjugated goat anti-chicken IgY Fc (Gallus Immunotech) was used. Samples were analyzed using a Luminex-100 flow cytometer (Luminex) at default settings set by the manufacturer. Median fluorescence intensity (MFI) of the reporter signal estimated from 50 beads was used for the data analysis. A set of internal standard controls described as high positive control, low negative control and cut off control,

were selected after initial analysis and subsequently included on each plate. Additionally, a blank control (serum diluent) was included on each plate. The MFI data was corrected for background levels by subtracting the blank control MFI from the sample MFI (MFI-Bkg). Results were presented as antibody index (MFI-Bkg sample/MFI-Bkg cut off serum). Samples with an index value below 0.9 were considered negative and those above 1.1 were considered positive. Values from 0.9 to 1.1 were considered inconclusive.

Cut-off value determination

Sensitivity and specificity were evaluated using receiver operator characteristic (ROC) curve analysis. The optimal cut-off points determined to be a sample MFI value ranging between 1008 and 1297 giving an overall diagnostic sensitivity and specificity of 100%. The cumulative area under the ROC curve (AUC) indicated that the aHEV ORF2-based FMIA was 100% accurate. The analysis was performed using GraphPad Prism v. 6.01 (GraphPad Software).

Assay reproducibility

The reproducibility of the FMIA was evaluated by utilizing six different chicken sera. The coefficient of variation (CV) was used to evaluate the intra- and inter-assay variation. The inter-assay CV determined by each sample tested in three different runs on different occasions ranged from 0.57% to 7.83%; while the intra-assay CV determined by three replicates tested within the same run ranged from 0.74% to 7.99%, indicating that the results were reproducible.

Comparison of FMIA and ELISA

In order to evaluate the FMIA, a subset of the experimental (n = 48) and the field (n = 56) serum samples were tested for the presence of specific anti-avian HEV IgY antibodies by an in-house ELISA described elsewhere (Billam et al., 2009) using the same antigen used in this study. For comparison, the results were presented as an antibody index as specified for the FMIA test (sample OD or MFI-Bkg/cut off serum mean OD or MFI-Bkg).

RNA extraction and avian HEV RNA detection

RNA extractions from the fecal samples were performed using the QIAamp Viral RNA Mini kit (Qiagen). Extracts were subsequently used for detection of the partial helicase and capsid genes of avian HEV as described previously (Sun et al., 2004) in nested RT-PCR reactions. Briefly, for the helicase gene detection, external primer set 5′-TGTTATYACACCCACCAARACGYTG-3′, and 5′-CCTCRTGGACCGTWATCGACCC-3′; and internal primer set 5′-GCCACGGCTRTTACACCYCAYGT-3′, and 5′-GACCCRGGRTTCGACTGCTT-3′ were used. For the capsid gene, external primer set 5′-TCGCCYGGTAAYACWAATGC-3′, and 5′-GCGTTSCCSACAGGYCGGCC-3′; and internal primer set 5′- ACWAATGCYAGGGTCACCCG -3′, and 5′-ATGTACTGRCCRCTSGCCGC -3′ were used. PCR products were examined on a 1% agarose gel and amplicons with the expected size (386 bp for the helicase gene and 242 bp for the capsid gene) were excised and purified with the QIAquick Gel Extraction Kit (Qiagen).

Sequencing and phylogenetic analyses

Sequencing of at least two avian HEV capsid and two helicase RT-PCR positive samples from each farm was performed directly on both strands at the Iowa State University DNA Facility, Ames, Iowa, USA (Applied Biosystems 3730xl DNA Analyzer). Sequences

were aligned with published data using BLAST at the national Centre for Biotechnology Information (NCBI)[1]. Sequences were compiled using Lasergene software and the Clustal V alignment algorithm (DNAStar). Identical nucleotide sequences were represented as one sequence and used in phylogenetic analysis. For sequence analysis, the 361 bp sequences of the helicase gene and 232 bp of the capsid gene were compared to each other and to sequences of other avian HEV isolates representing genotypes 1 (GenBank accession numbers AM943647and JN597006), 2 (GenBank accession numbers AY535004, EF206691, and EU919187), 3 (GenBank accession numbers AM943646 and GU954430), and 4 (GenBank accession numbers JN997392 and KF511797). Sequences reported herein were deposited in the GenBank database under the accession numbers KJ495790 to KJ495804 and KJ914879 to KJ914889.

Statistical analysis

Summary statistics including normality were calculated for all the farms. For each flock actual egg production, mortality and egg weights were compared to the breed standards. Breed standard values were defined and provided by the genetic companies for each week of the egg chicken production cycle for the specific genetic line utilized and were valid for the year in which samples were collected. Breed standards are publically shared by all genetic companies and are applicable for any commercial farm located in the U.S. Statistical analysis of the data among farms was performed by one-way analysis of variance (ANOVA). Pearson's correlation test was used to correlate presence of avian HEV RNA in feces with egg production, egg weight, and hen mortality. A kappa index (κ) was performed to determine the agreement of positive and negative results between ELISA and FMIA. The strength of agreement was scored as follows: ≤0, poor; 0.01 to 0.2, slight; 0.21 to 0.4, fair;

[1] http://www.ncbi.nlm.nih.gov

0.41 to 0.60, moderate; 0.61 to 0.80, substantial; and 0.81 to 1, almost perfect. $P < 0.05$ was set as the statistically significant level. The statistical analyses were performed using the SAS v.9.2 (SAS Institute).

Results

Evaluation and validation of the FMIA

The earliest detection of avian HEV-specific IgY antibodies was detected at dpi 14 in 29.2% (7/24) avian HEV infection. On dpi 21 and onwards all 24 chickens were positive for anti-avian HEV IgY antibodies.

To further evaluate the avian HEV FMIA, the same antigen used in the FMIA was coated on 96-wells ELISA plates. Internal FMIA controls, 48 experimental serum samples, and 56 field serum samples were tested by the ELISA. There was a substantial agreement between ELISA and FMIA when using the ORF2 recombinant capsid antigen ($\kappa = 0.63$). Although there was no difference for the overall detection rates between assays (43.4% [53/122] FMIA vs. 36.0% [44/122] ELISA; $P = 0.052$), when considering only the experimental samples subset, FMIA showed a higher detection rate at 14 dpi (7/12 vs. 1/12, $P = 0.04$) (Table 1).

Production data

Egg production, mortality and egg weights were compared to breed standards. Average egg production and average mortality data for participating flocks on each of the seven investigated farms are shown in Fig. 1. A summary of median, maximum and minimum mortality, egg production and egg weights for each farm are shown in Fig. 2.

Overall performance of all farms at the time of sample collection was satisfactory and mortality rates were mostly within an acceptable range when compared to breed standards (average ± SD, 0.13 ± 0.06) with exception of Farm B that presented an average 10% increase in mortality rates ($P < 0.05$) (Fig. 2). Maximum reported weekly mortality was 0.27% at 87 weeks of age on Farm B, 0.27% at 81 weeks of age on Farm E, and 0.27% at 109 weeks of age on Farm G (Fig. 1).

Egg production met or exceeded standard egg production curves in 5 of 7 farms (Figs. 1 and 2). Specifically, egg production on Farms A and B was on average 4 to 7% lower when compared to the breed standards while Farm C presented on average a 3.5% higher egg production ($P < 0.05$) (Fig. 2). On Farm A, egg production in 92 to 97 week old flocks was on average 11% less than expected and on Farm B, egg production decrease was on average 18% from 87 to 94 weeks of age (Fig. 1). On Farm D, there was a 12% drop in egg production in the 89 week old flock; however the overall egg production was on average 5% higher than the breed standard in the other flocks (Fig. 1). Lack of egg production from hens on Farm B at 77 weeks of age and on Farm G at 76 weeks of age was due to induction of molt prior to onset of a second production cycle, a routine procedure used by commercial egg producers in the Midwestern United States.

Egg weights were within an acceptable range when compared to breed standards with exception of a flock in Farm G had a maximum decrease of 10.09% when compared to the standard (Fig. 2). In all other flocks the maximum egg weight decrease was lower than 5.00% (average ± SD, 0.97 ± 2.67) with exception of Farm F that exceed the breed standards (Fig. 2).

298 *Detection of anti-avian HEV IgY antibodies*

299 The detection rates of anti-avian HEV IgY antibodies are shown in Fig. 1 and Table 2.
300 All seven investigated chicken farms had at least one seropositive animal. Considering all
301 farms, the overall detection rate of anti-avian HEV IgY antibodies was 44.8% (145/310). The
302 proportion of positive animals ranged from 20% (5/25, Farm F) to 82.0% (41/50, Farm B) per
303 farm. Considering all the examined animals by rank of age, seropositive animals were
304 detected at any age, but chickens older than 50 weeks were found to have higher
305 seropositivity rates compared to 16-26 and 31-45 weeks old chickens ($P < 0.05$).

306

307 *Detection of avian HEV RNA and correlation with production data*

308 The detection rates of avian HEV RNA are shown in Fig. 1 and Table 2.The overall
309 detection rate of avian HEV RNA in the pooled fecal samples of the seven studied farms was
310 62.9% (39/62) considering the combined results of both RT-PCR assays utilized (capsid
311 gene, 32/62; helicase gene, 38/92). The majority of flocks (31/39) were RT-PCR positive for
312 both capsid and helicase genes. However; on Farm B, the 69 week old flock was only
313 positive for the capsid gene, and the 91week old flock was only positive for the helicase
314 gene. On Farm C, the flocks with layer hens at 16, 23, 23, 43, 94 and 103 weeks of age were
315 positive only for the helicase gene.

316

317 All tested samples from Farms E ($n = 7$) and F ($n = 5$) were negative for avian HEV
318 RNA which coincided with the lowest detection of anti-avian HEV IgY antibodies among all
319 tested farms, 22.9% and 20%, respectively (Table 2). Avian HEV RNA was detected in 40%
320 (4/10) of the samples on Farm G and in 75-100% of the samples on Farms A, B, C, and D
321 (Table 2). No correlation was found between avian HEV RNA detection and hen-day egg
322 production ($P = 0.09$), weekly hen mortality ($P = 0.37$) or egg weight ($P = 0.15$).

Sequence and phylogenetic analyses

At least two avian HEV RNA positive samples were selected for sequencing of both a 361 bp region in the helicase gene and a 232 bp region of capsid gene in each farm. The sequences obtained were compared with the reference strains of each avian HEV genotype. All sequences clustered within avian HEV genotype 2 previously identified in the USA (Fig. 3).

Sequence analysis of the helicase gene from Farms A to G presented 82.3- 99.7% of nucleotide sequence identity. Within a farm, nucleotide sequence identities varied from 85.2-99.7%. Between sequences described here and the USA prototype avian HEV, the nucleotide sequence identify was 84.9-88.6% and it was 84.9-97.2% to the avirulent avian HEV-VA strain. Sequence identities with avian HEV genotype 1, 3 and 4 isolates varied between 76.5-82.5%.

Sequence analysis based on the capsid gene revealed similar results. Among farms, nucleotide sequence identity of avian HEV strains varied between 80.6-97.0%. Within a farm, nucleotide sequence identities varied from 85.2-97.3%. Between sequences described here and the USA prototype avian HEV and avirulent avian HEV-VA strains, there was 85.1-88.9% and 79.7-90.1% nucleotide sequence identity, respectively. Sequence identities with avian HEV genotype 1, 3 and 4 isolates varied between 73.8-77.9%.

Discussion

Serological surveys have shown a worldwide distribution of avian HEV infection, although clinical cases of HSS seem to occur infrequently (Meng and Shivaprasad, 2013).

Often avian HEV is considered as minor causative agent in diagnostic investigations but is frequently regarded as insignificant when another poultry disease has been diagnosed in the same chickens. However, drops in egg production and increased mortality have been reported in clinically healthy chickens infected with avian HEV in the absence of other known pathogens (Sprygin et al., 2012). To further investigate the patterns of avian HEV infection in apparently healthy chickens, fecal samples were tested for HEV RNA by RT-PCR and serum samples were tested for anti-avian HEV IgY antibodies by FMIA. In addition, mortality rates and egg production, parameters usually affected during HSS outbreaks, and egg weight as indirect method to evaluate the liver function (Husbands, 1970), were acquired from the flocks. Egg weight is largely determined by yolk weight (Jaffe, 1964), and yolk is synthesized in the liver hepatocytes.

Although Farm B, which presented the highest detection rates of avian HEV, showed the overall highest increase in mortality and highest decrease in egg production within the farms sampled, no associations between egg production data, mortality rates and detection rates of avian HEV RNA were found when using the cumulative data of all farms. Different management factors and biosecurity measures of each farm, diet, host genetics and presence of other pathogens could partially explain differences between production performances among flocks (Gerber et al., 2014). In fact, a prospective study has found that nearly identical avian HEV nucleotide sequences have been retrieved from broiler breeder flocks over two years, independent of clinical signs (Troxler et al., 2014). In this same study, viral load did not seem to have an effect on pathogenicity (Troxler et al., 2014). Recently, a pilot study using experimentally infected broiler breeder chickens could reproduce more typical macroscopic and microscopic avian HEV lesions than previous studies with young chickens (Park et al., 2015). However, production losses commonly associated with avian HEV

373 outbreaks have not been reproduced experimentally to date. Further systemic prospective
374 studies addressing avian HEV subclinical infection and its impact on production data on a
375 larger scale are needed.
376
377 Testing serum samples from chickens with unknown avian HEV exposure in this
378 study indicated that the seropositive rate of anti-avian HEV IgY antibodies was 44.8%
379 (145/310) in sexually mature chickens at various stages of egg production and that all farms
380 studied were seropositive for anti-avian HEV IgY antibodies with a detection rate ranging
381 from 20% to 82% per farm. This finding is in agreement with previous reports from the U.S.
382 and other countries (Huang et al., 2002; Peralta et al., 2009; Kwon et al., 2012; Zhao et al.,
383 2013). The present study also suggests that the likelihood of being seropositive increases with
384 age, as previously described (Huang et al., 2002; Peralta et al., 2009; Troxler et al., 2014).
385
386 Avian HEV RNA was detected in five of the seven farms tested, including in hens
387 close to the end of the egg production (> 100 weeks of age) and in farms with high numbers
388 of avian HEV seropositive chickens. This suggests that the humoral response may not offer
389 complete protection against avian HEV fecal shedding and/or that chickens might be re-
390 infected at different points of the production cycle with different strains of avian HEV.
391 Indeed, sequencing of avian HEV partial helicase and capsid genes showed that different
392 strains of avian HEV existed on the same farm. Similarly, HEV re-infection in seropositive
393 animals has been reported in adult pigs (de Deus et al., 2008). Possible factors that could
394 explain the co-circulation of different avian HEV sequences on a farm include co-mingling
395 animals from different sources, a common food supplier or shared workers between different
396 farms; however, these factors were not investigated in the present study. Although there is no
397 currently available information regarding the cross-protection among different avian HEV

genotypes, vaccination of pigs with recombinant capsid antigens derived from HEV isolated from swine, rat, and avian induced a strong IgG anti-HEV antibody response but only a partial cross-protection to a HEV genotype 3 challenge (Sanford et al., 2012).

Interestingly, avian HEV RNA was not detected in any of the flocks in Farms E and F although anti-avian HEV antibodies were detected in most of the flocks on all farms investigated. The failure in detecting avian HEV genome could be due to a lower amount of viral shedding into the fecal material of chickens without clinical signs. As pooled samples were used for the analysis, the dilution effect could have decreased the viral copies below the detection limit of the assay used. Alternatively as the avian HEV genome shows a high variability (Sprygin et al., 2012), the primers used for avian HEV RNA detection in the present study may have not amplified the viral genome due to mismatches in the sequences. In addition, lack of detection could be due to poor quality of samples and possible degradation of viral RNA, although samples from all farms were processed and tested similarly. It is also worth noting that Farms E and F contained the lowest number of flocks among the farms sampled in the present study which could have decreased the chances of detecting positive samples if the viral shedding was low. A higher number of fresh fecal samples from each flock should be tested to address these issues.

In conclusion, avian HEV infection was detected directly (RNA) or indirectly (antibodies) in all studied U.S. farms. Different strains of avian HEV genotype 2 were found in different flocks within a farm. There was no clear association between levels of egg production or mortality and detection rates of avian HEV RNA.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig. 1. Actual and standard egg production (%), actual and standard weekly mortality (%), anti-avian HEV IgY antibodies detection rate by FMIA (%) (grey bars) and avian HEV RNA detection by nested RT-PCR (red boxes indicate the age group that was found positive for HEV RNA) for flocks on Farm A (*n*=11 flocks), Farm B (*n*=10 flocks), Farm C (*n*= 11 flocks), Farm D (*n*= 8 flocks), Farm E (*n*= 7 flocks), Farm F (*n*= 5 flocks) and Farm G (*n*= 10 flocks).

Fig. 2. Difference (%) of actual mortality (A), egg production (B), and egg weights (C) in Farms A through G when compared to the breed standards. The box-and-whiskers plots show cumulative results of all flocks within a farm. Different superscripts (a,b) indicate significant ($P < 0.05$) differences between farms for the measured parameter.

Fig. 3. Phylogenetic tree based on 361 bp of the helicase gene (A) and 232 bp of the capsid gene (B) of the avian HEV isolates. Sequences that were obtained in this study are indicated in a black box. The phylogenetic tree was constructed by the NJ method implemented in the Lasergene software (DNASTAR). The nucleotide substitution per 100 residues is given; bootstrap values are indicated for the major nodes. Genotype classification represented as proposed by Marek et al. (2010) and Hsu and Tsai (2014).

Table 1

Comparison of anti-avian HEV antibodies detection rates with ELISA and FMIA on samples obtained from 12 experimentally infected chickens on different days post infection. Data presented as number of positive chickens/total number of chickens tested (mean group antibody index ± standard deviation).

Assay	0	7	14	21
FMIA	0/12 (0.04±0.07)	0/12 (0.03±0.02)	7/12 (2.62±3.08)^{A*}	12/12 (6.87±3.17)
ELISA	0/12 (0.20±0.04)	0/12 (0.23±0.08)	1/12 (0.41±0.32)^{B}	9/12 (1.89±0.93)

* Different superscripts (^{A,B}) within a column indicate significant differences in detection ($P < 0.05$) within groups.

584 **Table 2**

585 Detection rates of anti-avian HEV IgY antibodies in serum samples tested by FMIA and
586 avian HEV RNA in fecal samples tested by RT-PCR from Farms A through G. Data
587 presented as number of positive samples/total number of samples tested (prevalence).

Farm	Anti-avian HEV IgY antibody (%)	Avian HEV RNA (%)
A	29/55 (52.7)^{B*}	10/11 (90.9)^{B}
B	41/50 (82.0)^{C}	10/10 (100)^{B}
C	18/55 (32.7)^{AB}	9/11 (81.8)^{B}
D	24/40 (60.0)^{BC}	6/8 (75.0)^{B}
E	8/35 (22.9)^{A}	0/7^{A}
F	5/25 (20.0)^{A}	0/5^{A}
G	36/50 (72.0)^{BC}	4/10 (40.0)^{B}

588 *Different superscripts (^{A,B,C}) within a column indicate significant differences in detection
589 rates ($P < 0.05$) among groups.

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