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Article

Reoviral Hepatitis in Young Turkey Poults—An Emerging Problem

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Abstract

From January 2019 to April 2020, the Minnesota Veterinary Diagnostic Laboratory (MVDL) received cases of hepatitis and spiking mortality in young turkey poults (average age 15.5 days) from multiple turkey-producing states. Meat-type turkeys were mainly affected with few cases from breeders. Of 188 cases, 88 (47.5%) tested positive for reovirus by virus isolation with most of the positive cases from 7–14 days old birds (n=42). Gross lesions consisted of hepatosplenomegaly with acute, multifocal necrosis in both liver and spleen. Microscopically, liver sections showed congestion of hepatic sinusoids and necrotizing hepatitis with infiltration of lymphocytes, plasma cells, and macrophages. Reovirus was detected in liver samples by electron microscopy and *in situ* hybridization (ISH). Sections of spleen showed areas of necrosis with infiltration of the mixed population of inflammatory cells and depletion of lymphocytes. We consistently isolated reoviruses from livers and tentatively named “Turkey Hepatitis Reovirus” (THRv). Phylogenetic analysis of the newly emerged THRvs revealed their clustering into four distinct groups. This study also highlighted the close antigenic relation between TARV and THRv compared to turkey enteritis reoviruses (TERVs), which shed light on the probable origin of this newly emerged pathotype. In summary, further molecular and pathogenicity studies are recommended on THRvs to help diagnose and control this serious variant.

Keywords: turkey hepatitis reovirus; poult; mortality; liver; lameness; turkey reoviruses; hepatitis

1. Introduction

Reovirus belongs to the genus Orthoreovirus in the family *Reoviridae* and infects several avian and mammalian hosts. It is a 70–80 nm non-enveloped icosahedral virus having a segmented, double-stranded RNA genome [1]. Avian reoviruses (ARVs) are ubiquitous in domestic poultry with 80% non-pathogenic [2]. Clinical disease associated with ARVs mostly depends on the virus pathotype, route of exposure, age, and immune status of the affected bird [2]; younger birds are more susceptible to infection with this virus [3]. ARV infects most avian species and has been associated with a wide range of diseases e.g., tenosynovitis, respiratory disease, enteric disease, hydropericardium, hepatitis, splenitis, and runting/malabsorption syndrome in chicken [2,4], duck [5–7], goose [8–10], turkey [11–13] quail [14] and wild birds [15,16].

The avian reovirus genome has 10 segments, which are classified according to their size into: Large (L1, L2, and L3), Medium (M1, M2, and M3), and Small (S1, S2, S3, and S4). All segments are monocistronic except for S1 which is tricistronic. S1 contains 3 viral proteins, two nonstructural proteins (p10 and p17), and one structural one Sigma C (σ C) [17]. The σ C protein is a minor outer-

capsid structural protein translated by the third largest open reading frame (ORF) of the S1 segment [18]. It is made up of 326 amino acids which are divided into two variable regions, 1 to 122 amino acids and 196 to 326 amino acids). It forms a homotrimer responsible for cell attachment [19] and antigenicity as it is the main site of the type- and broad-specific neutralizing epitopes [20]. Sigma C is commonly used for genotypic classification and characterization of ARVs [21,22].

In turkeys, reovirus is mainly associated with enteritis and arthritis. The viruses associated with these disease conditions are tentatively known as turkey enteric reoviruses (TERVs) and turkey arthritis reoviruses (TARVs), respectively. The TERVs have been isolated for years from feces/intestines of apparently healthy poult and from cases of poult enteritis [13,23,24]. In the 1980s, reovirus was detected from cases of arthritis in turkeys [25–27]. No further reports of reovirus-associated arthritis in turkeys were available until the disease reappeared in 2011 [28]. Currently, TARV is one of the top ten diseases, which has a detrimental impact on one of the most promising industries in the US with highly divergent strains circulating in the country [29]. A third type of turkey reovirus causing hepatitis and mortality in poult emerged in 2019 [30–32]. This disease is characterized by spiking mortality, hepatitis, and occasional splenitis. An experimental study from our lab using reovirus isolates from cases of hepatitis and splenitis orally inoculated in one-week-old turkey poult from reovirus infection and vaccination-free breeder flock established Koch's postulates for this disease [33]. This virus has tentatively been named as "Turkey Hepatitis Reovirus" (THRv). The present study reports the isolation of THRv, evaluation of trends in geographical distribution, seasonality, age susceptibility, and diagnostic testing. Additionally, this study represents the first molecular characterization of these novel emergent variants of avian reoviruses.

2. Materials and Methods

2.1. Case Definition

Reovirus was considered the confirmed cause of death if the dead bird had hepatosplenomegaly with histopathologic evidence of hepatic and/or splenic necrosis with the detection of reovirus by one or more of the three tests, e.g., real-time reverse transcription-PCR (rRT-PCR) [33], virus isolation (VI) in cell cultures and/or specific-pathogen-free (SPF) embryonated chicken eggs, and *in situ* hybridization (ISH) on liver sections.

2.2. Retrospective Data Review

The electronic database of the Minnesota Veterinary Diagnostic Laboratory (MVDL) was queried and cases of turkeys with liver and/or spleen lesions submitted from January 2019 to April 2020 were selected. The information included several different clients, geographical location, age of birds, clinical history, and laboratory test results. Complete diagnostic work of 185 cases consisted of necropsy, gross and microscopic examinations, virology, and bacteriology. In some cases, electron microscopy and ISH were also done.

2.3. Pathology

At necropsy, the liver, spleen, lungs, intestine, and other visceral organs showing gross lesions were collected for routine histopathology from cases mentioned in the electronic database from January 2019 to April 2020. All tissues were fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin, sectioned at 4 μ m, mounted on glass slides, and stained with hematoxylin and eosin (H&E). Sections of livers from some cases were tested for the presence of viral mRNA by ISH as described below.

2.4. In Situ Hybridization

The ISH was performed using RNAscope [34], our universal rRT-PCR primers for the S2 gene [33], and RNAscope 2.5 HD detection red kit (Advanced Cell Diagnostics, Newark, CA, USA). Liver

samples negative for reovirus by rRT-PCR and virus isolation were used as negative controls. Briefly, FFPE sections were deparaffinized at 60°C for 90 min, and then transferred to xylene and absolute ethanol twice, incubating for 5 min each time. Endogenous peroxidase was inactivated by 3% hydrogen peroxide for 10 min at room temperature. The sections were then boiled for 15 min in RNAscope target retrieval buffer, followed by 15 min incubation at 40°C with RNAscope protease reagent. Hybridization with S2 gene probe was done at 40°C for 2 hrs. Slides were incubated with amp 1 and amp 6 amplifiers for a time duration recommended by the manufacturer, and the signals were detected with Fast red. Slides were counterstained with 50% Gill's hematoxylin I for 30 seconds and mounted by VectaMount. Sections were then examined under a bright field microscope.

2.5. Bacterial Isolation

Swabs from liver and spleen were cultured on sheep blood agar and MacConkey agar (Becton, Dickinson, USA). Some of the swabs were tested for the presence of Mycoplasma by incubating Frey's broth at 37°C in a 5% CO₂ atmosphere (Becton, Dickinson, USA) followed by plating on agar plates and incubation at 37°C for 2 weeks [35]. Real-time PCR was used for confirmation of Mycoplasma (<https://www.vdl.umn.edu/services-fees/molecular-diagnostics/bacterial-pcr>).

2.6. Virus Isolation

Homogenates of liver were inoculated in SPF embryonated chicken eggs (ECE) as described previously [23]. Briefly, a 10% suspension of pooled liver samples in Hanks' balanced salt solution (HBSS) was homogenized in a stomacher. The homogenates were centrifuged at 1,500 xg for 15 min and the supernatants were inoculated in 6-day-old ECE via the yolk sac route followed by incubation at 37°C. After four days of incubation, the yolk sac and embryo homogenates were processed for blind passage. A total of 1-2 blind passages were given for each sample. Additionally, supernatants were inoculated on QT-35. Inoculated cells were incubated at 37°C and examined daily under an inverted microscope for the appearance of reovirus-specific cytopathic effects (CPE) [24]. Up to three blind passages were given in cell cultures.

2.7. Electron Microscopy (EM)

Purified virus suspension from liver samples was added on parafilm- and formvar-coated copper grids. The samples were post-fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The tissue samples were then dehydrated in graded alcohol and embedded in resin. Following trimming, thin sections (60–70 nm) were obtained with an ultramicrotome (Leica Microsystems, Vienna, Austria). Samples were then negatively stained followed by examination under a 1200 EX II transmission electron microscope (JEOL Ltd., Tokyo, Japan). Images were obtained by using a Veleta 2K 3 2K camera with iTEM software [36].

2.8. Phylogenetic Analysis

The THRV isolates (n=25) from US and Canada (Table 1) were sequenced for genotyping characterization using previously described classification [21,22]. Phylogenetic analysis was conducted by comparing the σ C gene of THRVs with representative turkey and avian reovirus sequences downloaded from the GenBank. Multiple Sequence Alignment was carried out using ClustalW in Clustal Omega software [37], and the identity percentage was determined among various sequences. Furthermore, multiple sequence alignment with distance analysis was also performed on all sequences in this study using the Muscle algorithm within the Geneious Prime software [38]. Finally, a maximum likelihood tree was generated, and its robustness was validated statistically by 1,000 bootstrap replicates with the best fit substitutional models, which was implemented by MEGA11 software [39].

Table 1. The details of turkey hepatitis reovirus isolates analyzed in in this study.

Sequence Name	GenBank Accession Numbers	Country	State	Age (Days)
MVDL-THRV-1/USA/AR/2019	OR525778	United States	Arkansas	14
MVDL-THRV-2/USA/AR/2019	OR525777	United States	Arkansas	22
MVDL-THRV-3/USA/AR/2019	OR525788	United States	Arkansas	-
MVDL-THRV-4/USA/AR/2019	OR525787	United States	Arkansas	21
MVDL-THRV-5/Canada/2019	OR525775	Canada	-	-
MVDL-THRV-6/USA/MN/2019	OR525782	United States	Minnesota	14
MVDL-THRV-7/USA/MN/2019	OR525786	United States	Minnesota	-
MVDL-THRV-8/USA/MN/2019	OR525789	United States	Minnesota	14
MVDL-THRV-9/USA/MN/2019	OR525783	United States	Minnesota	8
MVDL-THRV-10/USA/MO/2020	OR525790	United States	Missouri	14
MVDL-THRV-11/USA/MO/2020	OR525784	United States	Missouri	18
MVDL-THRV-12/USA/AR/2020	OR525785	United States	Arkansas	12
MVDL-THRV-13/USA/MO/2020	OR525776	United States	Missouri	-
MVDL-THRV-14/USA/KY/2020	OR525794	United States	Kentucky	13
MVDL-THRV-15/USA/VA/2020	OR525795	United States	Virginia	10
MVDL-THRV-16/USA/VA/2020	OR525796	United States	Virginia	10
MVDL-THRV-17/USA/IN/2020	OR525793	United States	Indiana	15
MVDL-THRV-18/USA/MN/2021	OR525791	United States	Minnesota	10
MVDL-THRV-19/USA/IN/2021	OR525797	United States	Indiana	11
MVDL-THRV-20/Canada/2021	OR525792	Canada	-	10
MVDL-THRV-21/Canada/2021	OR525774	Canada	-	4
MVDL-THRV-22/Canada/2021	OR525773	Canada	-	13
MVDL-THRV-23/USA/IA/2021	OR525780	United States	Iowa	-
MVDL-THRV-24/USA/IA/2021	OR525781	United States	Iowa	23
MVDL-THRV-25/USA/VA/2021	OR525779	United States	Virginia	-

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. Retrospective Data Summary

The geographic distribution of submissions from the US and Canada is shown in (Table 2) Of the 185 cases, 176 were from 10 states in the US and 9 were from Canada. Of the 176 cases from the US, 122 (69%) were from Minnesota (Figure 2). A large majority of cases (164 of 185 or 89%) were from birds less than three weeks of age while only 21 (11%) were from older birds (Table 3). A total of 88 (47.5%) cases tested positive for reovirus by virus isolation. Most of the virus-positive cases were from 7-21-day-old birds (n=69 or 78.4%) with an average age of 15.5 days. Reovirus was isolated after one (n=74) or two passages (n=10) in QT-35 cells. Four cases were only positive in ECE after two passages. Month-wise submission data and geographical distribution of submissions are shown in Figures 1 and 2, respectively. There appears to be no seasonal effect of this condition, but there was an increase in total submissions and positive cases from Aug-Dec 2019 (Figure 1).

Table 2. Submissions of cases from different geographical locations.

Country	State (Codes)	Submitter	No. of submissions	No. (%) positive for reovirus
	Arkansas (AR)	Client 1	8	8 (100)

US	California (CA)	Client 2	1	0
	Indiana (IN)	Client 3	3	2 (67)
	Iowa (IA)	Client 4	9	0
		Client 5	2	2 (100)
	Michigan (MI)	Client 6	1	0
		Client 7	1	1 (100)
		Client 8	1	1 (100)
		Client 9	1	0
		Client 10	8	3 (37.5)
	Minnesota (MN)	Client 11	54	36 (67)
		Client 12	15	12 (80)
		Client 13	3	0
		Client 14	3	1 (33)
		Client 15	36	6 (17)
	Missouri (MO)	Client 16	7	6 (86)
		Client 17	1	0
	Nebraska (NE)	Client 18	1	0
	North Carolina (NC)	Client 19	3	1 (33)
	Wisconsin (WI)	Client 20	18	9 (50)
Canada	NA	Client 2	8	0
	NA	Client 9	1	0
Total			185	88 (47.5)

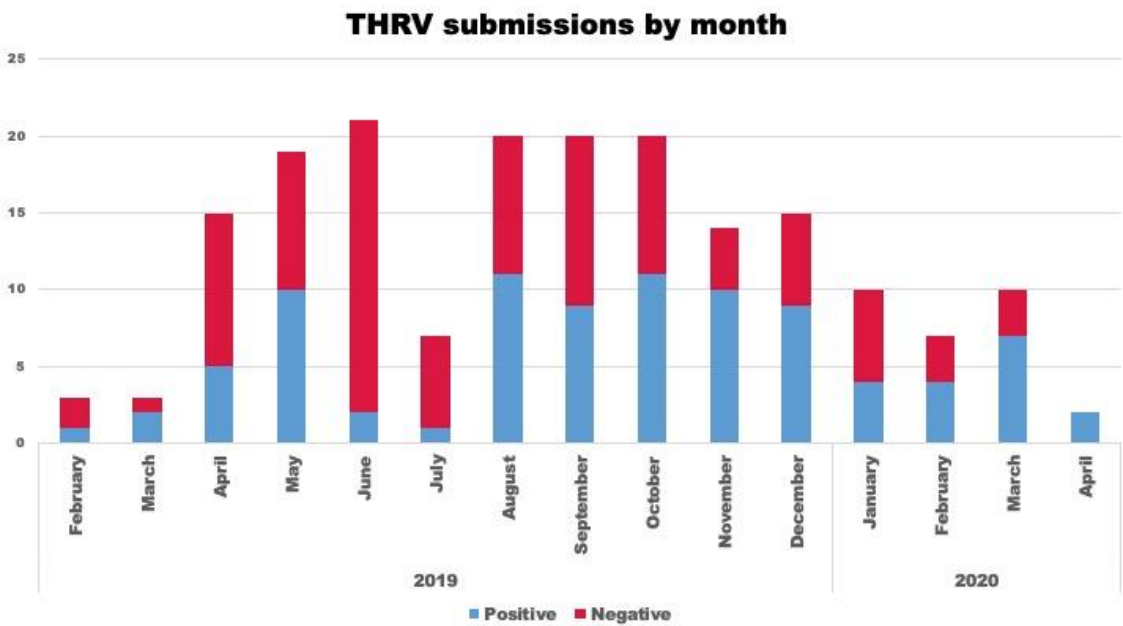


Figure 1. Stacked bar graph representing a month-wise number of Turkey Hepatitis Reovirus (THR​V) positive (red bar) and negative (blue bar) cases out of total number (red + Blue) of cases submitted.

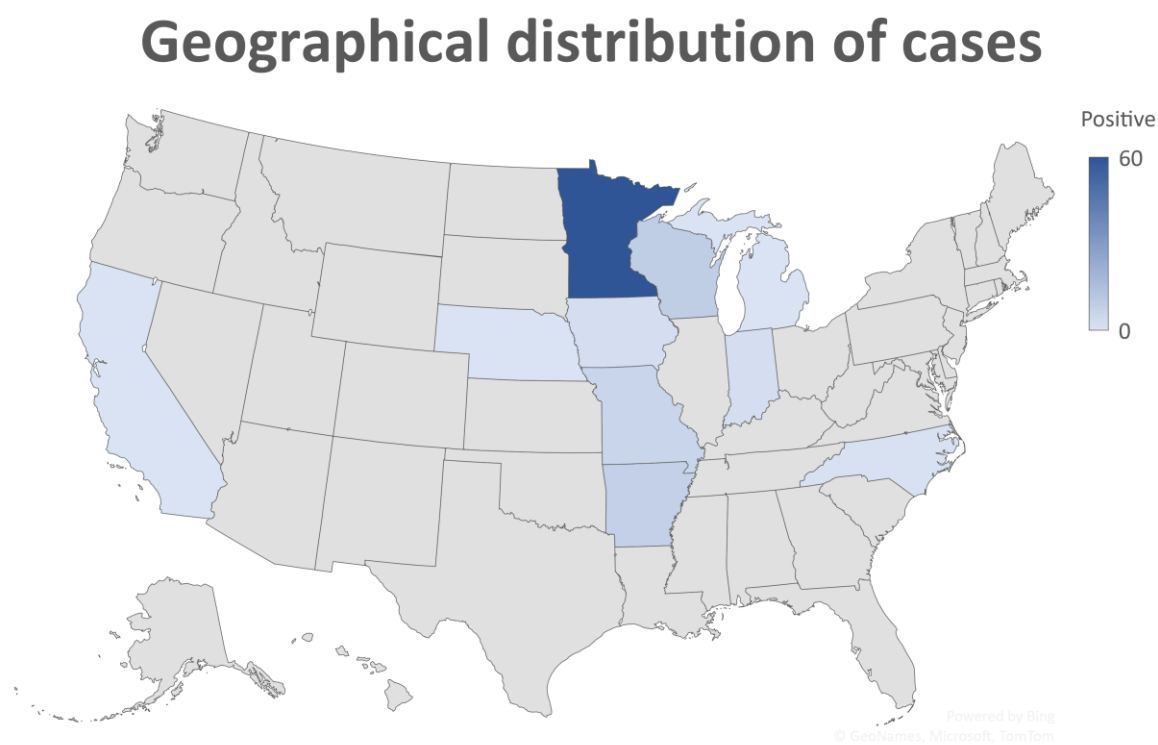


Figure 2. Figure showing the geographical and state-wise distribution of THRV-positive cases received from different states in the US. A darker shade of blue means a higher number of THRV-positive cases received at MVDL.

Table 3. Age-wise distribution of cases .

Age (days)	No. of submissions	No. (%) positive for reovirus
0-6	69	7
7 to 14	60	42
15 to 21	35	27
22 to 28	9	7
29 to 35	7	2
36 to 42	1	1
43 to 49	2	1
>50	2	1
Total	185	88

3.2. Gross Lesions and Histopathology

At necropsy, hepatosplenomegaly was evident with widespread focal areas of necrosis in the hepatic and splenic parenchyma (Figure 3). Microscopically, liver sections showed necrotizing hepatitis with infiltration of lymphocytes, plasma cells, and macrophages and congestion of hepatic sinusoids (Figure 4). Fibrin accumulation was observed in necrotic areas with occasional bile duct hyperplasia in some sections. Sections of spleen showed areas of necrosis with infiltration of a mixed population of inflammatory cells and depletion of lymphocytes (Figure 5). There was prominent reticular cell hyperplasia and proliferation with marked erythrophagocytosis and numerous siderophages in some sections. Sections of the lungs showed interstitial pneumonia characterized by distension of blood capillaries with erythrocytes, cell debris, and mononuclear cells.



Figure 3. Turkey liver and Spleen: Showing white necrotic foci in a turkey poult's hepatic (arrow) and splenic parenchyma (arrow).

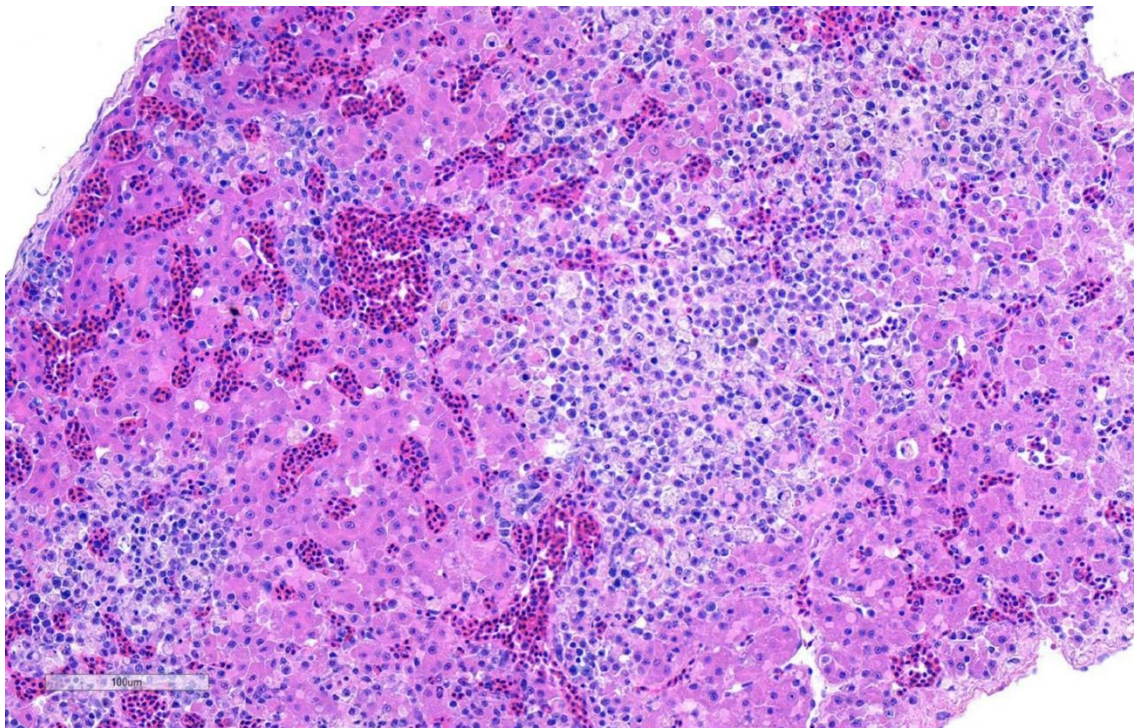


Figure 4. Section of liver showing plum islands of hepatic necrosis having lymphoplasmacytic cell infiltration and congestion of hepatic sinusoids. **H&E. 10X.** .

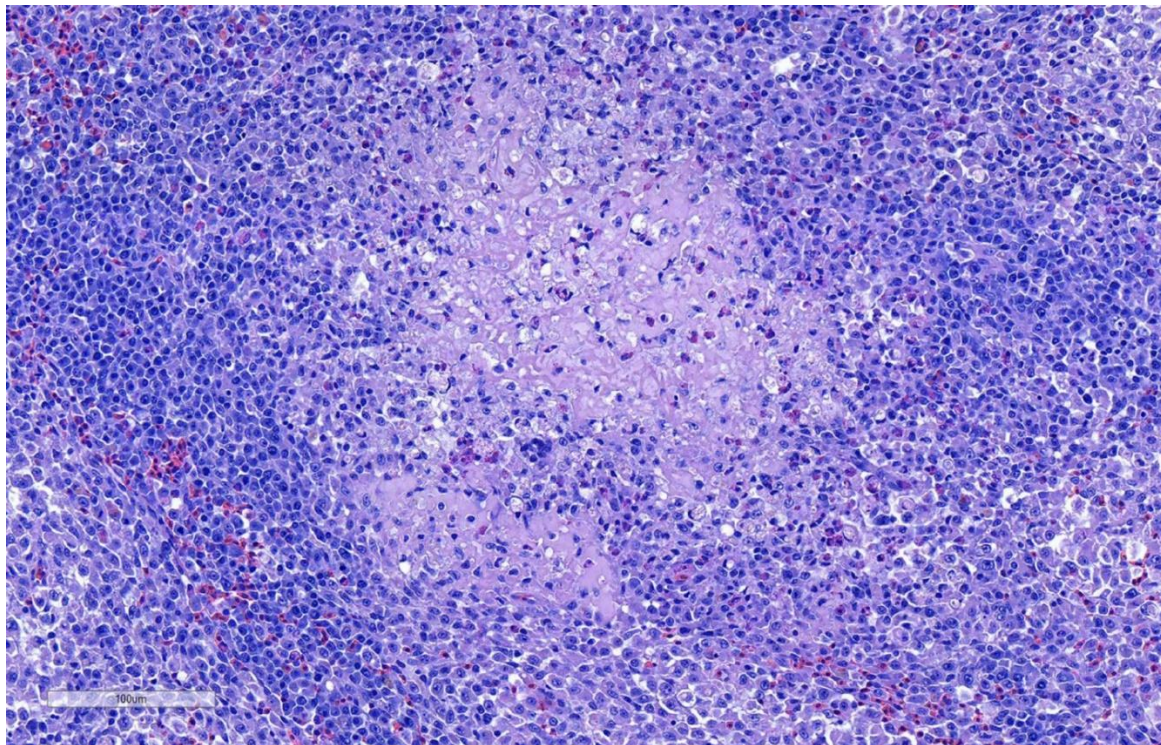


Figure 5. Spleen section showing necrotizing splenitis with infiltration of mixed population of inflammatory cells and depletion of lymphocytes. **H&E. 10X.** .

3.3. *In Situ Hybridization*

The ISH probe specifically designed to target reovirus showed a positive *in-situ* hybridization reaction (Figure 6) surrounding areas of necrosis and cellular infiltration. The positive ISH reaction demonstrating the presence of reoviral mRNA in the hepatic parenchyma suggests that reovirus was directly associated with characteristic hepatic lesions.

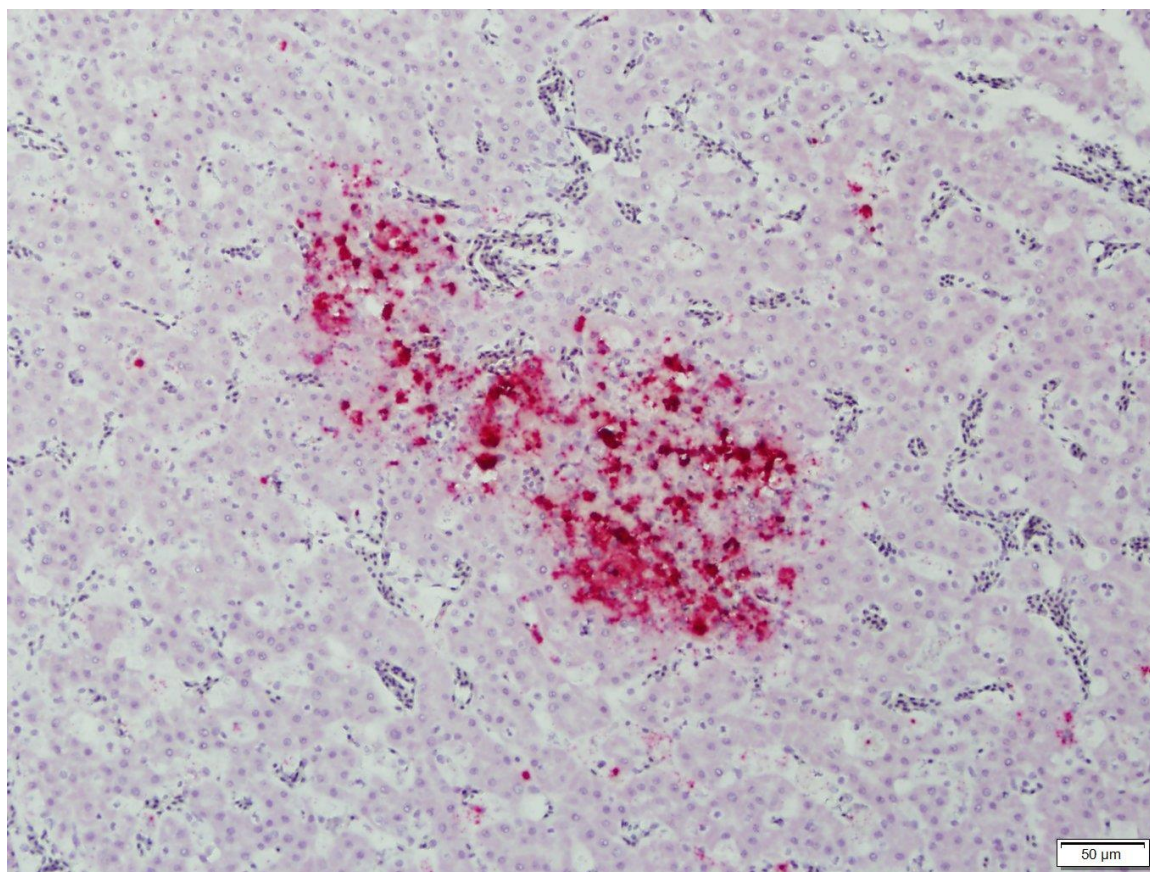


Figure 6. Section of liver demonstrating intense red color as positive ISH reaction showing reoviral mRNA by in-situ hybridization in intralesional cells (arrowheads) in liver. **ISH. 400x.**

3.4. Bacterial Isolation

There was no significant bacterial growth from the liver, spleen, intestine, or lungs except that low numbers of *Enterococcus* sp. and *Klebsiella pneumoniae* were isolated from liver, lung, and trachea of one case. No *Salmonella* sp was isolated.

3.5. Virus Isolation

The embryos inoculated with tissue homogenates showed stunting, hemorrhages, and death of the embryo. The allantoic fluid and stunted embryos were processed for the next passage. Most of the reovirus-positive samples caused death of the embryo in the first two passages. Allantoic fluid and stunted/dead embryos were tested for reovirus by rRT-PCR. Some of these fluids were also subjected to negative contrast electron microscopy. In reovirus-positive cases, characteristic syncytia were seen in QT-35. No adenovirus or picornavirus was isolated from liver samples.

3.6. Electron Microscopy

Ultrastructural examination of pulverized liver samples revealed acute swelling and necrosis of hepatocytes with fibrin deposits surrounding the necrotic hepatocytes. Numerous virus particles were observed in the necrotic debris and within the necrotizing hepatocytes (Figure 7). The virions were distributed in a moderate electron-dense material and arranged in small groups (Figure 8). The mean diameter of the virus particles was ~ 70- 80 nm. The virions were non-enveloped with icosahedral symmetry, consistent with members of the family *Reoviridae*.

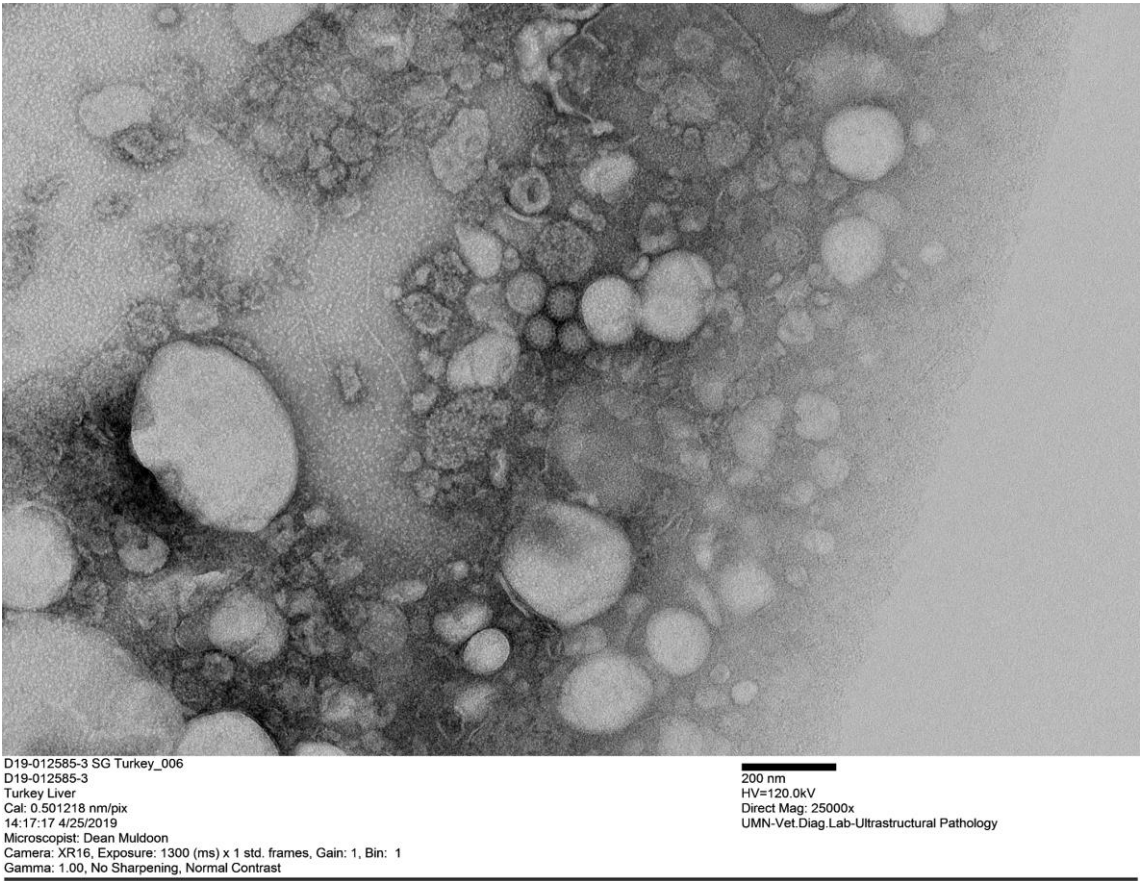


Figure 7. Transmission electron microscopy: liver showing focal area of hepatocellular death where hepatocytes are surrounded by fibrin (F) and one hepatocyte contains large number of viral particles (box). **EM. 4000X.**

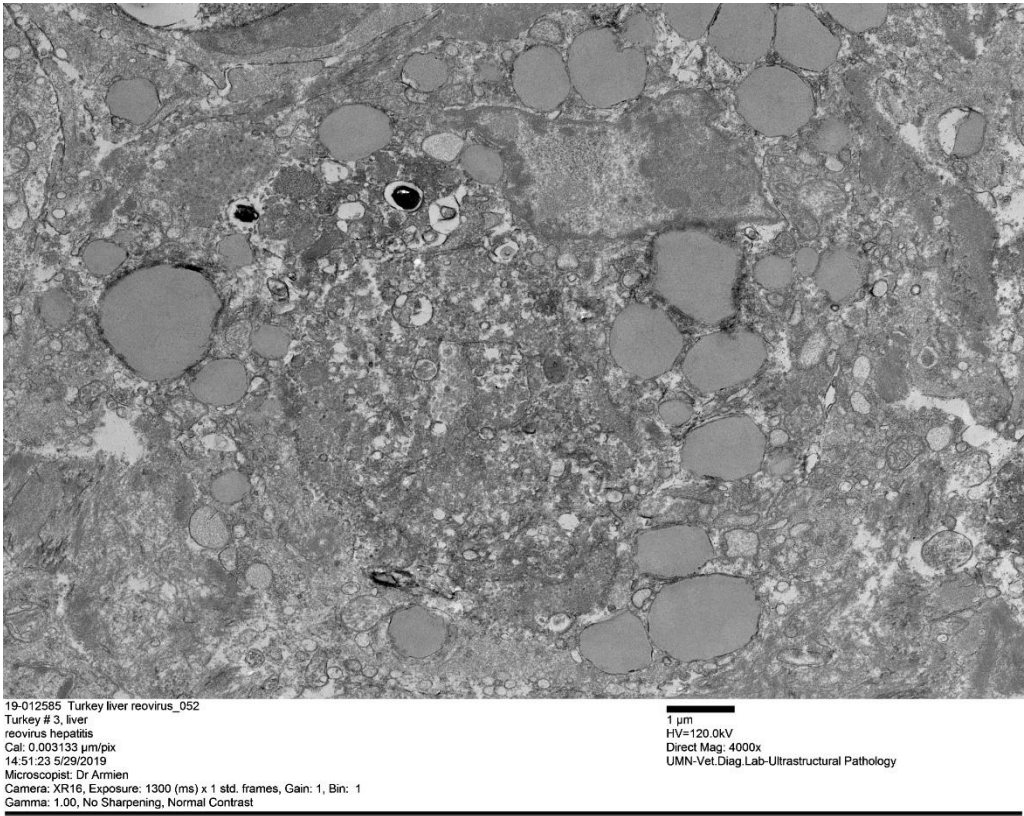


Figure 8. Transmission electron microscopy: electron microphotograph of a pulverized liver showing non-enveloped, spherical, and ~ 70- 80 nm virions. EM. 25000X.

3.7. Phylogenetic Analysis

The phylogenetic analysis based on the complete σ C gene was performed using the maximum likelihood method with high bootstrap values support. All sequences (THRVs and reference sequences) were divided into two main genetic clusters (GCs) (Figure 9) The GC1 contained the classical reoviruses including wild birds and ducks and attenuated vaccines (S1133 and 1733) while GC2 included all TRV (TERV, TARVs and THRVs) and some CARVs. The GC2 was further divided into three subclusters I, II, and III. The 25 THRv sequences were grouped into subcluster I and further subdivided into four groups based on 3% or more nucleotide (nt) and amino acid (aa) identity differences (Figure 9). The intra-group average identity percentages were more than 99% and inter-group average identity percentages were around 96 to 97% nucleotide (na) and amino acids (aa) (Table 4). The group I included six strains from, MN, AR, and VA. Group II had four strains (three from Canada, and one from MO). Nine sequences from MN, AR, and MO were clustered in group III and group IV included six strains from Canada, KY, VA, and IN. In addition to THRVs, subcluster I had different chicken and turkey arthritis and enteritis reovirus sequences. Some of these reference strains were identical to the THRVs while the others showed relative divergence. Interestingly, TARV sequence from SD (Tk_SD_121541_Td_2016) shared 98.7% nt and 99.0% aa identity to group 1 THRVs, hence the only closely related sequence reported so far in the GenBank. The two TARVs from PA (Reo/Turkey/PA/01769/14, and Reo/Turkey/PA/27011/13) were sharing the same node with group II THRVs with 98.3 nt and 98.4% aa identities (Table 4). Three out of total four THRVs from Canada were clustered in group II. Similarly, turkey and chicken reovirus strains from Pennsylvania (Reo/PA/Turkey/27399/12, and Reo/PA/Broiler/27541/12) were also included the same nodes of group III and IV with ~98.0% identity (Table 4). The reference TARV O'Neil and related TARVs were also closely to THRVs mainly with groups 11-1V with ~98.0% identities (Figure 9) The identity percentages between the THRv groups and the subclusters II and III avian and turkey reoviruses were around 90-91% and 86-87%, respectively, dropping sharply to 59% na and 54% aa with the ARVs in GC 2 (Table 4).

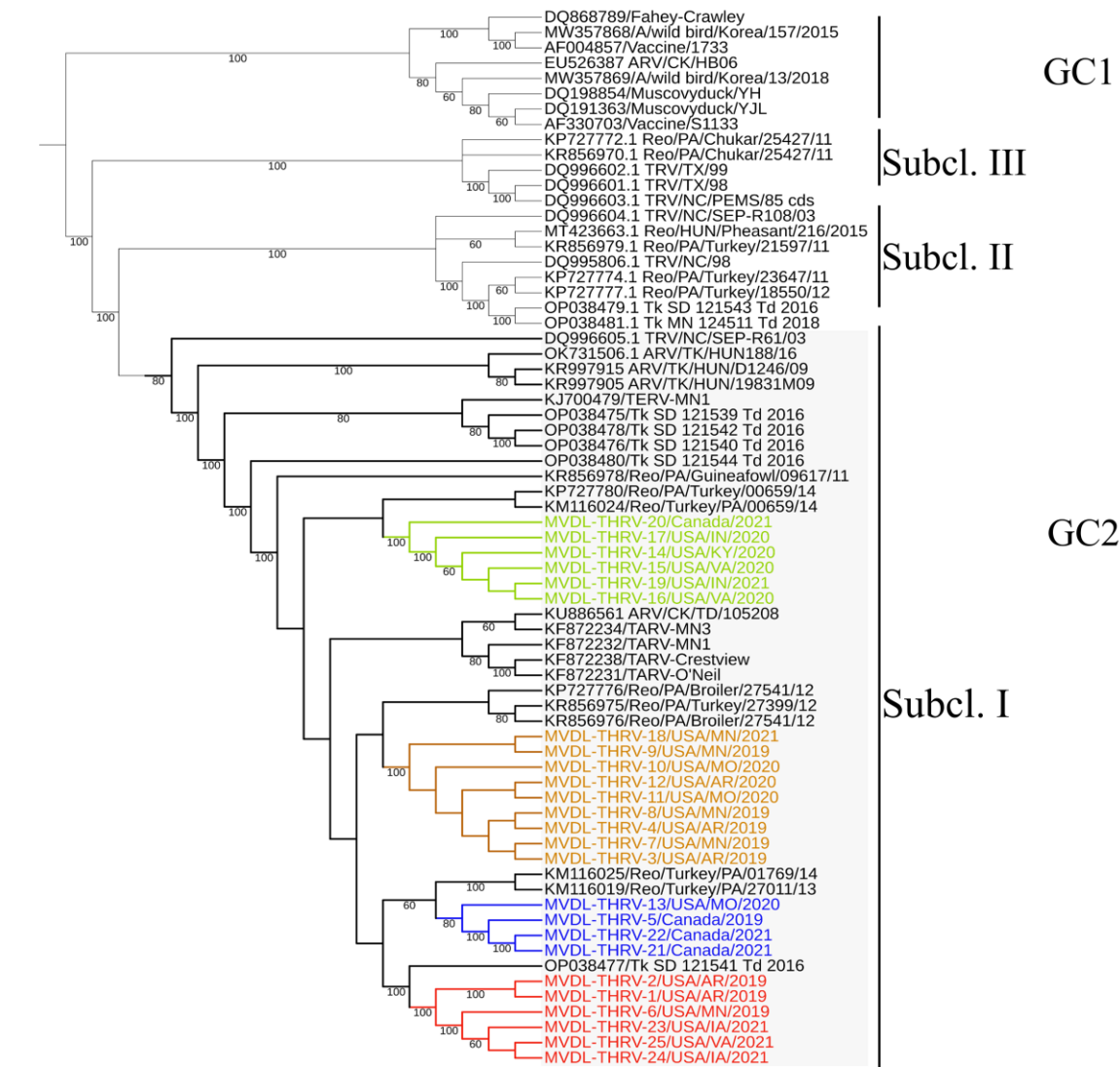


Figure 9. A Maximum likelihood tree was generated based on the nucleotide sequences of the complete σ C gene of 25 THR sequences and representative turkey and avian reovirus sequences from GenBank. The best-fit substitutional model implemented the tree topology and validated by 1000 bootstrap replicates. Genetic cluster 1 (GC1) and Genetic cluster 2 (GC2). Subcluster I (Subcl. I), Subcluster II (Subcl. II), and Subcluster III (Subcl. III). Four distinct groups of THRVs were highlighted with different colors. Group I is marked in red, Group II in blue, Group III in yellow, and Group IV in green.

Table 4. The identity percentage between the four groups of turkey hepatitis reovirus (THR) isolates and turkey arthritis reoviruses (TARVs), turkey enteric reoviruses (TERVs) and chicken reoviruses based on sigma C (σ C) gene.

		Group 1		Group 2		Group 3		Group 4	
		NA	AA	NA	AA	NA	AA	NA	AA
Genocluster 1	THR Group I	99.6	99.5	96.5	95.3	96.4	95.9	96.3	96.0
	THR Group II	96.5	95.3	99.0	99.0	97.0	97.4	96.7	94.0
	THR Group III	96.4	95.9	97.7	97.4	99.6	99.6	97.1	96.0
	THR Group IV	96.3	95.2	96.7	96.2	97.1	96.7	99.4	100
	TARV-O' Neil-like sequences	97.0	97.4	98.0	98.1	98.7	98.2	98.3	97.5
	Reo/Turkey/PA/00659/14	97.2	95.6	97.8	97.3	98.2	98.0	98.3	98.1
	Reo/Turkey/PA/01769/14	97.0	96.0	98.3	98.4	97.8	98.2	96.8	97.4
	Reo/Turkey/PA/27011/13								

Reo/PA/Turkey/27399/12	96.9							
Reo/PA/Broiler/27541/12		95.9	98.0	97.6	98.4	98.4	97.7	97.0
Tk_SD_121541_Td_2016	98.7	99.0	96.3	95.3	96.4	96.1	96.2	95.1
Tk_SD_121539_Td_2016								
Tk_SD_121540_Td_2016								
Tk_SD_121542_Td_2016	94.8	93.6	95.1	94.8	95.2	95.4	95.5	94.7
Tk_SD_121544_Td_2016								
ARV/TK/HUN/19831M09								
ARV/TK/HUN/D1246/09	94.2	93.8	95.4	95.1	95.4	95.8	95.6	95.0
ARV/TK/HUN188/16								
TERV-MN1	95.2	93.9	95.1	95.8	96.1	96.4	96.3	95.5
TRV/NC/SEP-R61/03	93.2	92.6	93.8	94.4	93.9	95.0	94.4	94.8
Subcluster II	90.0	91.0	91.6	91.6	91.6	91.9	92.1	91.6
Subcluster III	86.7	85.7	87.3	87.3	87.0	87.3	87.1	86.1
Genocluster 2	59.4	53.7	59.6	54.9	59.6	54.2	59.6	54.2

The deduced aa alignment of σ C protein between the THRVs and the representative TRVs and ARVs also confirmed the maximum likelihood tree topology and average identity percentage (Figure 10). The unique aa substitutions were detected in each THR/V group. The group I THR/Vs possessed nine aa substitutions (22S, 52N, 79V, 81G, 116T, 118H, 147E, 176A, 245S) and group II had two aa (239V and 249S) in all sequences, but two sequences had three extra substitutions 57E, 69S, and 75I. The characteristic aa substitutions in group III were 44A, and 286A while group IV had seven aa substitutions (38A, 48N, 105E, 129N, 137A, 161Q, and 284N). These unique substitutions were exclusive for THR/V groups and were absent in all other TRVs and ARVs, except those sharing the same nodes with the groups that had one or two amino acids common with these groups. Exceptionally, the Tk_SD_121541_Td_2016 strain, which is highly identical to group I, has all the characteristic amino acids of group I except only Q118H plus it also has L217F. Within subcluster I, the number of mutations between THR/V groups and other AVRs and TRVs showed a gradual increase in the divergence reoviruses, leading up to TRV/NC/SEP-R61/03 which displayed the highest number of mutations. Interestingly, the TERV-MN1 has unique aa mutations D24N, N69D, Q122R, V125A, L230M, V214I, and N236S, hence differentiating it from TARVs, and THR/Vs.

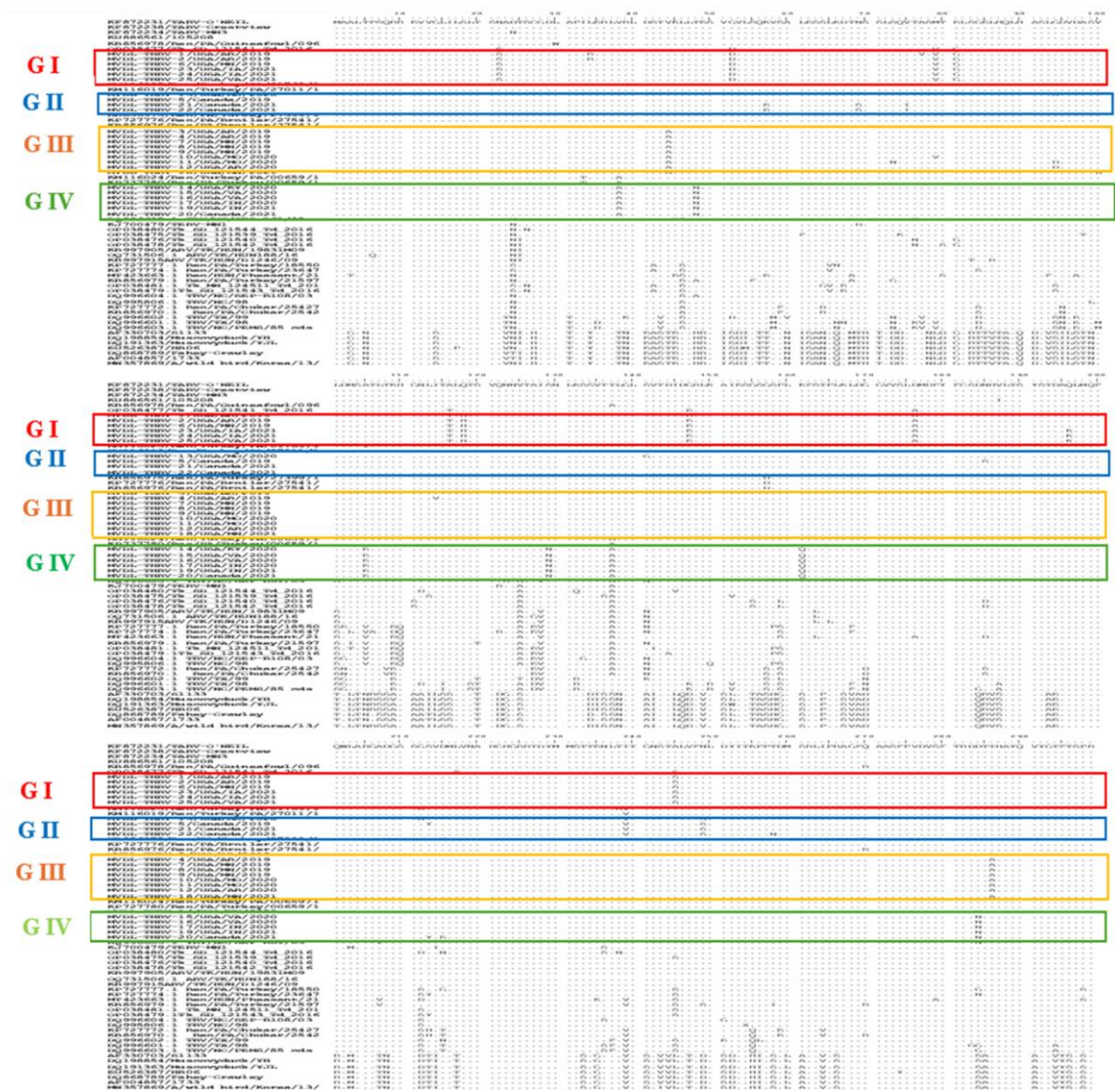


Figure 10. Deduced amino acids alignment of the σ C protein of 25 THRVs and representative sequences of turkey and avian reoviruses. Specific color frames are representative of different groups of THRVs.

4. Discussion

The results of the present study, together with those of an earlier experimental study fulfilling Koch’s postulates [33], establish that turkey reovirus can cause not only enteritis and arthritis but also hepatitis and splenitis leading to spiking mortality in young turkey poults. Since the early 1990s, turkey reovirus seems to have been evolving in terms of its pathogenicity [25,26]. TERV was a cause of concern for years [13,23,24,40]. Subsequently, lameness caused by TARV re-emerged a decade ago resulting in huge economic losses due to non-uniformity of the flock, mortality, and condemnation of carcasses in the grow-out phase [28]. This study reports another condition associated with turkey reovirus i.e., hepatitis in <3-week-old poults characterized by spiking mortality and necrotizing hepatitis and splenitis. Recently, considering the notable rise in reported cases of hepatitis among turkey farms, the USDAH considered THRVs as one of the top ten challenges affecting the turkey industry, ranking them as the 9th most serious concern [41].

A review of data from January 2019 to April 2020 indicates that the cases of THRv are increasing in North America and that the disease occurs mainly in <3-week-old poults (average 15.5 days). In a similar retrospective study on turkey viral hepatitis, turkeys at younger ages were most susceptible with an average age of 29 days [42]. However, this was based on 76 cases reported from California only. Turkey reovirus was isolated from two cases but it was not confirmed as a causative agent of

viral hepatitis. No such cases were reported from Midwest and Eastern states until 2019 when TRV-associated viral hepatitis cases were reported in turkey poult from several states [30–32]. The retrospective analysis study of diagnostic submissions at MVDL from March 2010 to May 2018 reported TARV-associated lameness cases only but no THRv [43]. This also indicates the emergence of THRvs in 2019.

The association of reovirus with turkey hepatitis is not surprising. Previously, reovirus-associated hepatitis has been reported in chicken [3,44–46], psittacine species [47], geese [48], Pekin ducks (*Anas platyrhynchos*) [49], magpies (*Pica pica*) [50], and American crows [51,52]. Occasional investigations into the role of reovirus in turkey hepatitis have been documented. For instance, a surveillance study conducted in Poland, which encompassed laying hens, broilers, and turkeys, identified cases of reovirus-related hepatosplenomegaly, accompanied by the presence of white necrotic foci within the tissue parenchyma [53]. Shivaprasad et al. [54] observed acute centrilobular necrosis with vacuolation of hepatocytes without inflammation in reovirus-infected turkeys; one of the birds had multifocal necrosis of hepatocytes with infiltration of lymphocytes, plasma cells, and macrophages. The present study, however, is more comprehensive, continuous submissions of these cases to our laboratory indicate that THRv can cause hepatitis in addition to arthritis and enteritis. Also, evaluating the economic significance of this newly emerging disease is necessary.

We observed hepatosplenomegaly associated with THRv in turkey poult, primarily from August to December as well as in March and May. (Figure 1). The TARV retrospective study did not find any seasonality pattern and reported that the overall probability of TARV positive cases varied by month and was higher in January, April, July, and December [43]. While our study does not delve into the seasonal aspects of this disease, the seasonality of infection will be important to study in the future to drive efforts for infection control in high-risk periods. Additional research is also needed to understand the effect of climate and management of turkey flocks on turkey reoviruses.

Interestingly, the emergence of the THRvs was consistent with the new duck reovirus (NDRV) detected in ducks and geese which was also associated with hemorrhage and necrosis in the livers and spleens of 5-to-25-day-old ducklings [55]. Additionally, the NDRV infection occurred mainly at young ages, the younger the age of infected ducks, the higher the mortality and morbidity in ducklings as seen with THRv infection in this study. Malkinson et al. [56] also reported a reovirus-associated disease in young Muscovy ducklings, which appeared as early as 10 days of age. The THRv-affected turkey flocks had a history of 2-8% mortality within a span of one week. The morbidity and mortality due to THRvs can be highly variable [29], depending on the strain of THRv as indicated in the pathogenicity study of two THRv isolates [33]. However, the morbidities and mortalities associated with THRvs were significantly lower than NDRV in ducks and geese, causing high morbidity (10-30%) and mortality (40-60%) [6,55].

We developed and used an ISH probe specifically designed to target avian reovirus. Positive *in situ* hybridization reaction demonstrated that reovirus was directly associated with characteristic hepatic lesions, thus confirming it as the etiologic agent. The ISH test developed in this study should be useful for veterinary diagnostic laboratories to detect and confirm reovirus infections. Ancillary tests such as virus isolation from these cases, virus detection by rRT-PCR and electron microscopy, and our previous study pathogenesis [33] further confirm the reovirus etiology of turkey hepatitis. All these ancillary diagnostic observations agree with the data reported in the published literature on duck, chicken, and turkey reovirus w.r.t molecular detection, virus isolation in embryonated eggs and cell culture, ISH, and EM [2,57]. In this study, we also did nucleotide and amino acid sequence analysis of the complete σ C protein of the THRvs with representative sequences of chicken and turkey reoviruses based on the common σ C classification of ARVs conducted by Kant et al. [21]. Initially, GC 2 is more likely to contain the majority of TRVs [22], and THRvs isolated in this study, like other TRVs, were positioned in the same GC 2, with high divergence to GC 1 containing classical avian reoviruses and vaccinal strains (S1133 and 1733) [58–61]. Notably, THRvs were more identical to TARVs like reference TARV-O'Neil and other TARVs with a maximum of 98-99% identity. THRvs were divided into four groups based on distinct monophyletic groups, intra, and inter-groups

average identity percentage, and characteristic aa substitutions for each group. Specifically, these aa substitutions were variable among the groups; as high as nine and seven in the more divergent THRV groups (group I and group IV) versus only two unique substitutions in group II and group III. These consistent aa substitutions were exclusive for the THRV groups (absent in all TARVs and TERVs); therefore, they could act as genetic markers distinguishing THRVs from TARVs and TERVs. These unique aa substitutions may be related to the tissue tropism and virulence of THRVs.

The molecular characterization of all THRVs isolated in this study indicates their close relation to TARVs with maximum identity to the TARV O'Neil strain, suggesting that THRVs are variants of TARV with increased pathogenicity. This is also supported by the pathogenicity study of Kumar et al. [33], who stated that THRVs can cause tenosynovitis in addition to hepatitis. Similarly, chicken arthritis strains like 176, UM 1-203 were reported to cause necrotizing hepatitis and splenitis with high mortality after 48 to 72 hrs post-infection [62,63] The σ C exhibited the highest rate of sequence divergence and rapid evolution and possessed the type- and broad-specific neutralizing epitopes; therefore, the σ C gene could be used as a genetic marker for rapid differentiation between novel THRV and TARV. In a comparative pathogenesis study, Kumar et al. [33] reported that not all THRVs are highly pathogenic which may be correlated with the difference in aa changes in different groups of THRVs. Hence there is a need to correlate genetic mutations in these groups with pathogenicity for better understanding which groups are highly pathogenic.

5. Conclusions

In conclusion, the results of the present study suggest that mortality in young poult was due to multifocal necrotizing hepatitis caused by reovirus infection. This appears to be an emerging disease caused by a previously recognized pathogen. To the best of our knowledge, this study represents the first phylogenetic analysis of the novel THRVs that emerged recently in commercial turkey farms. It revealed that based on σ C, the THRVs isolated from NA commercial turkey farms can be classified into four distinct groups, exhibiting characteristic amino acid changes distinguishing them from CRVs, TARVs, and TERVs. THRVs are more related to most TARVs, with some TARVs displaying a significant degree of identity to certain THRV groups. Further comparative pathogenicity studies are crucial, particularly between representatives from the different groups and closely identical TARVs, to confirm the genetic marker behind the liver tropism of THRVs and understand evolutionary patterns.

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Abbreviations

The following abbreviations are used in this manuscript:

ARVs Avian reoviruses
CPE Cytopathic effects

EM	Electron microscopy
FFPE	Formalin fixed paraffin embedded
H&E	Hematoxylin and Eosin
HBSS	Hanks' balanced salt solution
ISH	<i>In situ</i> hybridization
MVDL	Minnesota Veterinary Diagnostic Laboratory
rRT-PCR	Real-time reverse transcription-PCR
TARV	Turkey arthritis reovirus
TERV	Turkey enteric reovirus
THRV	Turkey hepatitis reovirus

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