Turkey arthritis reovirus diagnostic submission analysis and development of a serological test

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Dedication

I dedicate this thesis to my husband Fabian, who never failed to support and love me; David and Alina who are the reason of my happy life; my Mon Enma Vaca, whose memory keeps me going; my father Belisario Barrera and my sister Nancy Barrera who always have been supporting and helping me.

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General Abstract

Turkey reovirus (TRV) has been associated with several clinical syndromes including lameness, enteritis, myocarditis and immunosuppression in turkey flocks. Lameness and enteritis are the current field problems and an increase in cases of lameness has been reported, possibly due to emergence of novel genotypes of the virus. It is, therefore, important to understand factors responsible for this increase and to devise methods for the rapid detection of the virus and its antibodies. Currently, TRV is detected through RT-PCR and virus isolation but no specific serological test is available to detect anti-TRV antibodies in turkeys. In this thesis, I report retrospective analysis of lameness submissions to the University of Minnesota Veterinary Diagnostic Laboratory (UMN-VDL) in the last eight years and confirm that submission of TRV positive cases have indeed increased. There were some months during which the probability of positive cases was numerically high. In addition, the submission of reovirus-positive lameness cases came from many different states indicating that the problem is widespread. There was a positive correlation between age of the turkey and TRV-related lameness; the number of TRV positive cases increased as the flocks aged.

In addition, I developed a whole-virus ELISA (enzyme-linked immunosorbent assay) to detect anti-TRV antibodies. The TRV isolate used for this ELISA was a representative of several TRV genotypes based on Sigma C and B conserved epitopes. The test was validated using serum samples from experimental and clinical cases of TRV. At 0.4 cut-off, the diagnostic sensitivity and specificity of this ELISA were 0.92. In summary, the development of an ELISA with high sensitivity and specificity should provide an opportunity for early diagnosis of reovirus exposure and to follow the dynamics of reovirus immune response over time.

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List of abbreviations and glossary

TRV = Turkey reovirus virus

CRV = Chicken reovirus

CARV = Chicken arthritis reovirus

TARV = Turkey arthritis reovirus

TERV = Turkey enteritis reovirus

UMN = University of Minnesota

VDL = Veterinary Diagnostic Laboratory

ARIMA = Auto regressive integrated analysis

ELISA = Enzyme-linked immunosorbent assay

ROC = Receiver operating characteristic curve

RT-PCR = reverse transcriptase polymerase chain reaction

rRT-PCR = real time reverse transcriptase polymerase chain reaction

QT-35 = Japanese quail fibrosarcoma cell line (Quail tumor 35)

General Introduction

General Introduction

Avian reovirus (ARV) belongs to the genus Orthoreovirus under the family *Reoviridae*. The ARVs can infect both domestic poultry and wild birds. The viruses that infect domestic chicken and turkeys are called CRV (chicken reovirus) and TRV (turkey reovirus), respectively. These viruses have been recognized for more than 50 years; the CRVs are the causative agents of runting-stunting syndrome and arthritis in chickens while TRVs are reported to cause enteritis, myocarditis, and arthritis in turkeys. The TRVs causing enteritis and arthritis are tentatively divided into TERV (turkey enteric reovirus) and TARV (turkey arthritis reovirus), respectively. In some instances, TRVs can also be isolated from apparently healthy turkeys. The designation of TERV or TARV is based entirely on the tissue (intestine or tendon) from which the virus was isolated. Both TERV and TARV can infect the gastrointestinal tracts of turkeys and they currently cannot be differentiated with molecular tests (PCR) or whole genome sequencing.

Although TERVs have been known for more than 50 years, the TARVs were first reported in the 1980s and were isolated from cases of lameness in turkeys. After these reports, there were no documented reports of TARV for nearly 30 years. In 2010-2011, lame turkeys were submitted to the University of Minnesota Veterinary Diagnostic Laboratory. From gastrocnemius tendons of these cases, my research group isolated a reovirus and named it TARV to differentiate it from TERV. Subsequently, they fulfilled Koch's postulates by orally inoculating turkeys with TARV, producing histological evidence of gastrocnemius tenosynovitis, and then re-isolating the virus from gastrocnemius tendons. In that study, neither CRV nor TERV was able to produce arthritis/tenosynovitis in inoculated turkeys (Sharafeldin et al. 2014)

Turkey arthritis reovirus (TARV) is an economically important pathogen because it results in performance losses due to lameness/tenosynovitis in turkeys. Since 2011,

hundreds of reovirus-induced lameness diagnostic cases have been submitted to the UM-VDL. Furthermore, recent studies have suggested that there are several TARV genotypes and that an increase in lameness cases could be associated with the emergence of new genotypes.

There is currently limited data on TARV epidemiology, distribution and prevalence in the US turkey flocks as well as limited diagnostic tools to detect exposure to TARV. With this study, I aimed to retrospectively analyze data on lameness cases submitted to the UMN-VDL in the last eight years to evaluate trends, seasonality, distribution and correlation with age of the flock. Understanding the dynamics of lameness diagnostic cases may help inform turkey producers and veterinarians about the potential patterns of lameness clinical cases and promote more research to further increase the knowledge about TRV epidemiology and transmission within and between flocks. Additionally, TARV is currently detected using RT-PCR and virus isolation; however, no serological specific- tests are available to detect anti-reovirus antibodies in infected turkey flocks. For that reason, my second aim was to develop a turkey- specific ELISA to detect turkey reovirus antibodies. This assay could allow for detection of TRV exposure at earlier ages before clinical signs occur and would allow us to understand the dynamics of TRV infection in turkey flocks. I describe a highly sensitive and specific indirect enzyme-linked immunosorbent assay (ELISA) to detect several TRV isolates circulating in commercial flocks. Overall, my thesis should increase the understanding of TRV lameness dynamics over time and provide a new serological diagnostic tool that turkey producers and veterinarians may use to enhance detection of TARV and to characterize immune responses in the field.

Chapter 1: Literature Review

History and significance of turkey reovirus

Turkey reovirus (TRV) can cause considerable economic losses due to mortality and decreases in growth and feed efficiency of turkey flocks (Simmons et al. 1972, Tang, Lin, et al. 2015, Spackman, Day, and Pantin-Jackwood 2010). It has been reported that TRV causes enteritis (Simmons et al. 1972, Carver et al. 2001), myocarditis (Franca et al. 2010, Shivaprasad et al. 2009) and arthritis/tenosynovitis (Mor et al. 2012, Tang, Lin, et al. 2015). For these reasons, TRV-associated disease is considered one of the most important diseases of commercial turkey flocks in the U.S and worldwide (Rosa et al. 2014, Alafaleq et al. 1989, Al Afaleq and Jones 1989, Adair, Burns, and McKillop 1987).More studies are needed to better quantify the economic impact of TRV infections.

TRV has been associated with different syndromes in US turkey flocks. Initially, TRV was isolated from poults with enteritis and high mortality (Gershowitz and Wooley 1973, Simmons et al. 1972, Heggen-Peay et al. 2002, Simmons et al. 2000). The effects of enteritis were worse when poults were infected with multiple enteric viruses (King 1976, Kaleta, Siegman, and Mikami 1975, Lozano et al. 1989). TRV has also been associated with arthritis/tenosynovitis and it has been confirmed experimentally that TRV is the causal agent (Al Afaleq and Jones 1989, Alafaleq et al. 1989, Sharafeldin, Mor, Bekele, et al. 2015). TRV has been associated with clinical cases of myocarditis in poults but this causality has not been confirmed experimentally (Franca et al. 2010, Shivaprasad et al. 2009). Finally, it has been reported that TRV causes immunosuppression due to bursal atrophy (Rosa et al. 2014, Day, Spackman, and Pantin-Jackwood 2008).

TRV has been described in US turkey flocks since 1969 (King, Kawamura, and Anderson 1969). For 40 years, TRV has generally been regarded to be an enteric pathogen of poults. TRV was initially identified as the cause of poult enteritis with high mortality (Simmons et al. 1972, Gershowitz and Wooley 1973). By oral inoculation TRV

was shown to cause enteritis in poults (Nersessian et al. 1986) and in 1989, TRV was associated with a field case of lameness in turkeys (Al Afaleq and Jones 1989). TRV was later reported as the cause of myocarditis in poults (Franca et al. 2010, Shivaprasad et al. 2009). Finally, 25 years after the first report of TRV-associated lameness in turkeys, TRV was isolated from field cases of lameness characterized by arthritis and tenosynovitis (Mor et al. 2012). Our research group, in order to categorize turkey reoviruses, classifies TRV into two different groups based on tissue from which the virus was isolated: turkey enteric reovirus (TERV) and turkey arthritis reovirus (TARV). Since our research at the UM-VDL demonstrated that reovirus can cause lameness in turkeys, the laboratory has become a center for reoviral lameness testing and receives many samples from throughout the US. This provides us with data on eight years (2010-2018) of lameness case submissions for epidemiological analysis.

Turkey reovirus prevalence and distribution in US

TRV has been circulating in US turkey flocks at least for the last five decades. Since it was first reported in 1969 in Wisconsin (King, Kawamura, and Anderson 1969), there are numerous reports of the syndromes associated with TRV in US commercial flocks. Although the data is limited, it seems that TRV can be found in healthy and diseased flocks worldwide (Jones 2013). Clearly, there is a need to determine TRV distribution and prevalence in US turkey commercial flocks as well as to characterize the predominant TRV genotypes. Finally, limited data are available on the dynamics of TRV-associated arthritis, enteritis and myocarditis cases submitted to the veterinary diagnostic laboratories (Franca et al. 2010, Jindal et al. 2009). It is important to characterize the features and trends of TARV-associated lameness cases submitted to the diagnostic laboratories to help understand the distribution, flocks of origin and potential seasonality patterns of TARV infections.

Turkey reovirus characteristics

TRV in an avian reovirus within the *Orthoreovirus* genus and the *Reoviridae* family. TRV diameter is about 70-80nm. TRV is non-enveloped, double protein capsid shell, double-stranded RNA (dsRNA) and segmented genome with 10 segments: Large (L1, L2 and L3), Medium (M1, M2 and M3) and Small (S1, S2, S3 and S4) (Teng et al. 2014, Tang, Lu, et al. 2015). These segments encode for eight structural proteins (λA, λB, λC, μA, μB, σA, σB and σC) and four non-structural proteins (μNS, P10, P17 and σNS) (Varela et al. 1996). The σC protein encoded by the S1 segment is the most abundant, immunogenic and responsible of cell attachment (Martinez-Costas et al. 1997).

Turkey reovirus origin, evolution and genetic diversity

It is believed that TRV has evolved to infect turkey populations after potential introductions of avian reoviruses from chickens (Day, Pantin-Jackwood, and Spackman 2007, Sellers et al. 2004). Genetic diversity in TRV has been described as high in all genome segments between species. Recent studies have shown that TRV and CRV can re-assort M segments and that M segments from TRV and CRV isolates can be grouped within the same genotypes or gene constellations (Mor, Marthaler, et al. 2015). Additionally, TRV L segments had clustered with CRV L segments, which further support re-assortment and potentially the chicken origin of TRV (Mor, Sharafeldin, et al. 2014). Moreover, the S segments genetic analysis further support the findings in L and M segments although there was more differentiation between avian species and potential coevolution of different genotypes. Finally, there was not enough evidence to suggest that TARV and TERV were different viruses and it was suggested that TARV may have evolved from TERV and/or CARV (Mor, Verma, Sharafeldin, et al. 2014).

Pathogenesis, clinical signs and lesions of TRV-associated syndromes

TRV is a virus present in the virosome of healthy turkeys. TRV-associated enteritis is produced when enterocytes are infected by TRV (also called TERV). The produced enteritis is characterized by mild diarrhea and depression about 7 days post inoculation in commercial poults (Spackman et al. 2005, Nersessian et al. 1986). In addition, there is a mild increase of crypt depth due to crypt hyperplasia. There is also granulocyte and lymphocyte infiltration in the lamina propria and submucosa 2 weeks after inoculation (Spackman et al. 2005, Heggen-Peay et al. 2002). In contrast to the intestinal findings, TRV was consistently detected in the bursa and lesions were characterized by lymphoid depletion that caused bursal atrophy and fibroplasia (Pantin-Jackwood, Spackman, and Day 2007, Heggen-Peay et al. 2002, Day, Spackman, and Pantin-Jackwood 2008). Additionally, TRV has been associated with clinical cases of myocarditis. TRV was isolated from enlarged hearts of turkeys with mild to severe ventricular dilation, whitish discoloration, hydropericardium, pale areas in the liver, congestion, edema in the lungs and enteritis with pale serosa and watery contents. Microscopically, TRV-associated myocarditis was characterized by moderate to severe degeneration and necrosis of myocytes as well as lymphocytic infiltration in myocardium and epicardium (Franca et al. 2010, Shivaprasad et al. 2009). Even though many features of the TRV-associated syndromes have been described, a better understanding of target cells where TRV replicates is still needed.

TARV causes lameness in turkeys due to arthritis/tenosynovitis of the tibiotarsal-tarsometatarsal (hock) joint often characterized by swelling and rupture of the gastrocnemius and/or digital flexor tendons due to fibrosis (Al Afaleq and Jones 1989, Sharafeldin et al. 2016). Microscopically, TARV lesions are characterized by lymphocytic inflammation in the subsynovium of tendons along with synoviocyte hypertrophy. As tendon lesions progress, there is fibrosis of the tendon and tendon sheath that promotes stiffness (reduced tendon elasticity) and ultimately leads to gastrocnemius

tendon rupture in some birds. Turkeys orally inoculated with TARV demonstrate reduced weight gain (Sharafeldin, Mor, Bekele, et al. 2015, Sharafeldin et al. 2016). Finally, it has been shown that TARV grows well in intestine and this may explain why TARV is capable of crossing the intestinal barrier to reach the blood and then the gastrocnemius tendon to produce lameness (Sharafeldin, Mor, Sobhy, et al. 2015).

TRV epidemiology and transmission

TRV is ubiquitous and rather hardy virus in the environment. TARV survives in litter and drinking water for about a week (Mor, Verma, et al. 2015), but is inactivated in 10 minutes or less when exposed to most commercial disinfectants (Mor, Bekele, et al. 2014). It is believed that TRV are distributed worldwide and it is part of the virosome of healthy turkeys (Jones 2013, Awe 2014). There are no studies reported on the prevalence of TRV in US commercial flocks, especially the prevalence and distribution of the different genotypes associated with clinical disease. Enteric reoviruses as well as other viruses, have been reported in the feces of turkeys of various ages in field cases throughout the U.S. (King 1976, King, Kawamura, and Anderson 1969).

Reports often document a co-infection of TRV with other enteric viruses. (Awe 2014, Jindal, Mor, and Goyal 2014). It has been demonstrated that CRV can be transmitted vertically through eggs and horizontally through fecal and contaminated litter (Al-Muffarej, Savage, and Jones 1996, Jones 2013). It is unclear whether resident TRV produces disease or new TRV genotypes are transmitted from other populations to the affected flocks. It is critical to advance the understanding of TRV origin in clinical cases as well as to determine the routes of introduction. Strain-specific CRV vaccines can reduce vertical transmission of CRV and therefore reduce clinical signs in growing chickens (Awandkar et al. 2017, Jones 2013). Similar studies have not been conducted on

TERV or TARV and vertical transmission of TARV in turkeys has not yet been documented.

TRV diagnostics

TRV-associated syndromes can be diagnosed based on clinical history and signs, histological lesions, virus isolation in eggs and/or QT-35 cells and/or detection by RT-PCR in the affected tissues (Jones 2013). Recently, a TARV-specific RT-PCR was developed to aid in the diagnosis of lameness produced by TARV (Mor, Verma, Bekele, et al. 2014). TRV can also detected using molecular sequencing techniques such as Sanger and Next Generation Sequencing (NGS) (Tang, Lu, et al. 2015, Mor et al. 2012).

Furthermore, response humoral immune response to TRV is detectable about 1-week post inoculation using virus neutralization tests (Adair, Burns, and McKillop 1987, Nersessian et al. 1985). High throughput turkey-specific serological tests such as ELISA are not available for TRV. A CRV ELISA test for chickens is available but it does not reliably detect TRV antibodies in turkeys due to low cross-reactivity between CRV and TRV and the need to use turkey-specific antibodies in the assay (Spackman et al. 2005, Al Afaleq and Jones 1989). A TRV ELISA is urgently needed to characterize TRV infection dynamics in turkey flocks and assist in the diagnosis of TRV-associated syndromes.

TRV control

TRV vaccination has been anecdotally described as the main control measure based on the successful experiences reported in the control of CRV (Rosenberger et al. 2013). However, the increasing genetic diversity of TRV isolates makes the efficacy of the available vaccines relative and strain specific (Troxler et al. 2013, Vasserman et al.

2004). In chickens, CRV-associated syndromes have been controlled by vaccinating breeders *in ovo* and coarse-spray in chicks with inactivated and/or live attenuated vaccines (Awandkar et al. 2017, Lublin et al. 2011, Guo et al. 2004, van Loon et al. 2003, Guo et al. 2003, Giambrone and Hathcock 1991). There is an urgent need for new TRV vaccines and new vaccine platforms that identify new vaccine antigens or even better, a vaccine that has wide cross-protection among different reovirus genotypes regardless of species.

Concluding remarks

TRV-associated infections have been reported for several decades in the US turkey flocks but only TARV-associated lameness, TERV-associated enteritis and TRV-associated immunosuppression have been reproduced experimentally. TRV-associated myocarditis has not been reproduced experimentally yet. Currently, TRV lameness cases have been associated with new TRV genotypes and TRV lameness in turkeys remains a major issue in turkey production. Currently, there is limited information on distribution and dynamics of the TRV lameness cases submitted to the diagnostic laboratories. This information may be useful to understand trends and patterns of lameness cases over time. Finally, there is an urgent need for a high throughput serological test such as an ELISA test to assist in the diagnosis, research and control of TRV infections in turkey flocks.

Chapter 2: Retrospective analysis of turkey arthritis reovirus diagnostic submissions in Minnesota

ABSTRACT

Turkey arthritis reovirus (TARV) causes tenosynovitis in turkeys resulting in decreased profits for the producers due to increases in morbidity, mortality, and feed conversion ratio. There is limited information on TARV epidemiology including the dynamics of diagnostic submissions to the veterinary diagnostic laboratories. In this study, we retrospectively analyzed 719 cases of lameness in turkeys submitted to the Minnesota Veterinary Diagnostic Laboratory from March 2010 to May 2018. Almost all submissions were pools of tendons from several lame turkeys, which were tested by virus isolation and/or real-time RT-PCR. Most of the submissions were from Minnesota. We found 52% of the submitted cases to be positive for TARV. The TARV positive submissions increased considerably in the last few years. There was no statistical evidence that lameness submissions were seasonal, although TARV positive submissions were higher in January, April, July, and December. TARV positive submissions also increased as flocks aged. In summary, we found that lameness submissions have increased in the last few years, have varied over time and are correlated with age of the bird. This information is important in understanding and informing the risk of TARV as well as its epidemiology, transmission, and control.

Introduction

Turkey reoviruses (TRV) are widespread in turkey flocks (Pantin-Jackwood et al. 2008) and have been arbitrarily divided into TERV (turkey enteric reovirus) and TARV (turkey arthritis reovirus) depending upon clinical signs and tissue from which these viruses are isolated (Sharafeldin et al. 2014, Sharafeldin, Mor, Bekele, et al. 2015). TARV causes lameness/tenosynovitis, which is characterized by swelling of intratarsal (hock) joints, fibrosis in chronic cases and rupture of gastrocnemius and/or digital flexor tendons (Mor et al. 2012). Histologically, TARV lesions are characterized by synovial hyperplasia and lymphocytic infiltration (Mor et al. 2012, Sharafeldin et al. 2014, Sharafeldin, Mor, Verma, et al. 2015). Infection with TARV results in economic losses to turkey producers due to decreased growth, increased feed conversion ratio, culling of lame birds, increased morbidity and occasional mortality (Sharafeldin et al. 2014, Lu et al. 2015). In addition, there are animal welfare concerns due to lameness-associated pain and inability of lame birds to reach feed and water.

Experimentally, TARV has been shown to produce clinical disease in susceptible turkeys (Sharafeldin, Mor, Bekele, et al. 2015). Genetic characterization of TARVs indicates that they are different from chicken arthritis reovirus (CARV) and that there is low or no cross protection between these viruses (Tang, Lu, et al. 2015). Vertical transmission of chicken reovirus from hens to chicks has been documented (Al-Muffarej, Savage, and Jones 1996) but it is not known if TARV are capable of vertical transmission. Studies indicate that TARVs can survive 7-10 days in turkey litter and drinking water (Mor, Verma, et al. 2015) and that oxidizing disinfectants can kill TARVs in 10 minutes or less (Mor, Bekele, et al. 2014).

Recently, there has been a perceived increase in the number of arthritis/tenosynovitis cases associated with TARV in US turkey flocks (Tang, Lin, et al. 2015, Dandar et al. 2015). The purpose of this study was to analyze diagnostic submissions of turkey lameness to the University of Minnesota Veterinary Diagnostic Laboratory (UMN-VDL) retrospectively, to evaluate trends of TARV infection including correlation of seasonality and age on TARV infection.

Materials and Methods

Data cleaning, consolidation and validation

Database from the UMN-VDL from March 2010 to May 2018 was searched for submissions of turkey cases that were tested for reovirus infections. It was common practice during this period to test all cases of lameness for reovirus. Incomplete and duplicate cases and/or data entry results were deleted. After data cleaning, aggregation and selection, we included only lameness submissions that consisted of tendons and joints to reflect cases associated with arthritis/tenosynovitis in turkeys. Data on other specimens such as feces, semen, heart and spleen were not included. Additionally, we included only the submissions that had results of TARV detection by real-time RT-PCR (rRT-PCR) and/or virus isolation. A sample consisted of a pool of tendons from 2 to 6 legs (gastrocnemius and/or digital flexor tendons) within the same submission.

Data tabulation

After data validation and aggregation, monthly submissions were tabulated to plot the number of TARV positive and negative submissions over time. Submissions were tabulated by specimen type, submitter, number of sample pools per submission, and type of test conducted on each sample. Additionally, geographic distribution of submissions

by state and distributions by month of the year and age of the flock were plotted using the maps (Original S code by Richard A. Becker 2018), ggmap (Kahle and Wickham 2013), ggplot2 (Wickham 2016) and ggpubr (Kassambara 2018) packages in R statistical software (R Core Team 2017).

Seasonality and age of the bird

After tabulation and plotting, seasonality of TARV positive submissions was assessed using classical time series regression analysis (ARIMA and Fourier regressions with different time periods in the trigonometric functions) and logistic regression models using the forecast (Hyndman 2016), astsa (Stoffer 2016) and lme4 (Bates et al. 2014) packages. In the logistic regression, month of the year was used as a fixed predictor of seasonality and the age was categorized to simplify the model. Age category was used as a covariate in the logistic regression. Finally, the predicted probabilities from the bivariable logistic regression were plotted to show variations by month and age over time.

Results

The 719 submissions from March 2010 to May 2018 consisted of a total of 926 samples of tendon and/or joint fluid (Table 1). A total of 54 clients from 14 different states submitted these cases for diagnosis. A large majority of submissions were from Minnesota and Wisconsin (Table 1). The samples had been tested by virus isolation in both QT-35 cells and SPF eggs. The majority of samples tested TARV positive in the first two passages in cell cultures but one passage was enough in SPF eggs (Table 2). A small subset of TARV were isolated only in SPF eggs and some required additional passages. A detailed geographic distribution of submissions from each state is shown in Fig. 1.

There was an increase over time in the number of monthly submissions and the number of TARV positive submissions in the last two years (Fig. 2). Overall, there were 98 months of data and 52% (374/719) of the submissions tested TARV positive. The ARIMA and Fourier time series regression models tested with 3, 4, 6 and 12 months as

Table 1 Details of submissions included in the study

Description	Category	No. submissions	No. (%) TARV[a] positives
Specimen type	Tendon	683	357 (52)
	Joint fluids	15	6 (40)
	Both	21	10 (48)
Number of pooled samples	1	605	302 (50)
	2	86	55 (64)
	3	28	17 (61)
State	Minnesota	353	187 (53)
	Wisconsin	151	87 (58)
	Other 12 states	215	100 (47)
Total		719	374 (52)

[a] = Turkey arthritis reovirus (TARV)

Table 2 Detection of TARV[a] in the 926 samples included in the study

Description	Category	No. Tested	No. (%) TARV[a] positives
Specimen	Tendon	880	414 (47)
	Joint fluids	46	13 (28)
Test	Virus isolation	570	260 (46)
	RT-PCR[b]	89	12 (14)
	Both	267	155 (58)
Virus isolation	QT-35[c] cells only	125	111 (89)
	Eggs only	6	5 (83)
	Both cell and eggs	706	299 (45)
QT-35 cells	1 passage	125	123 (98)
	2 passages	177	175 (99)
	3 passages	171	14 (8)
	4 and 5 passages	324	1 (0)
Eggs	1 passage	255	244 (96)
	2 passages	153	32 (21)
	3 passages	270	4 (2)

[a] = Turkey arthritis reovirus (TARV)
[b] = real-time reverse-transcriptase polymerase chain reaction (RT-PCR)
[c] = Japanese Quail fibrosarcoma cell line (QT-35)

Fig. 1. Geographic distribution of TARV submissions to UM-VDL from 14 states 2010-2018

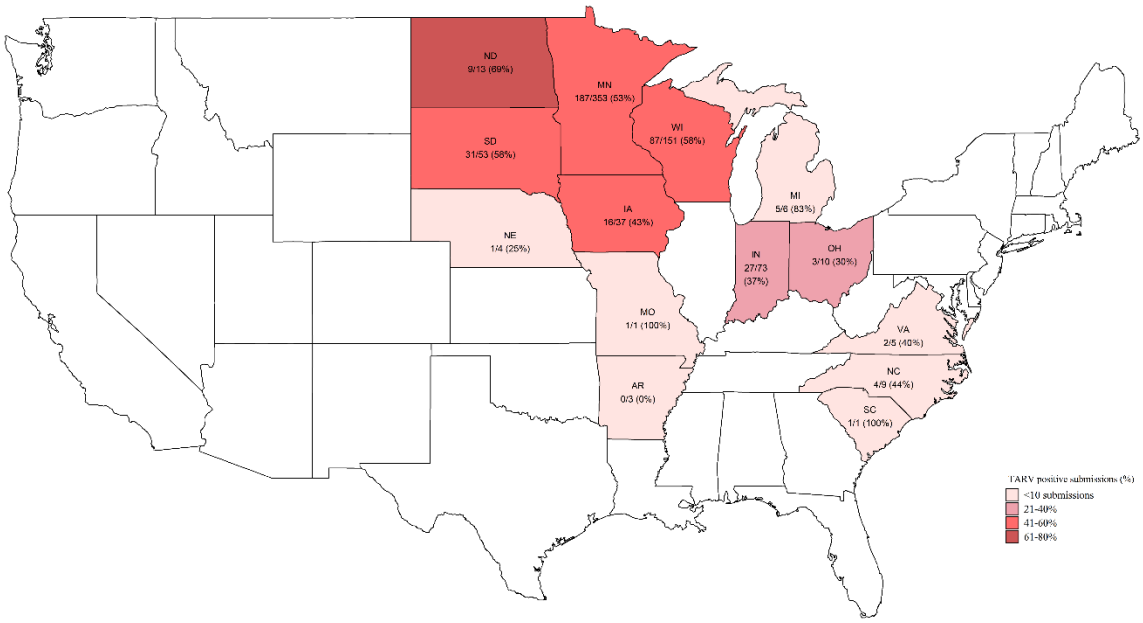
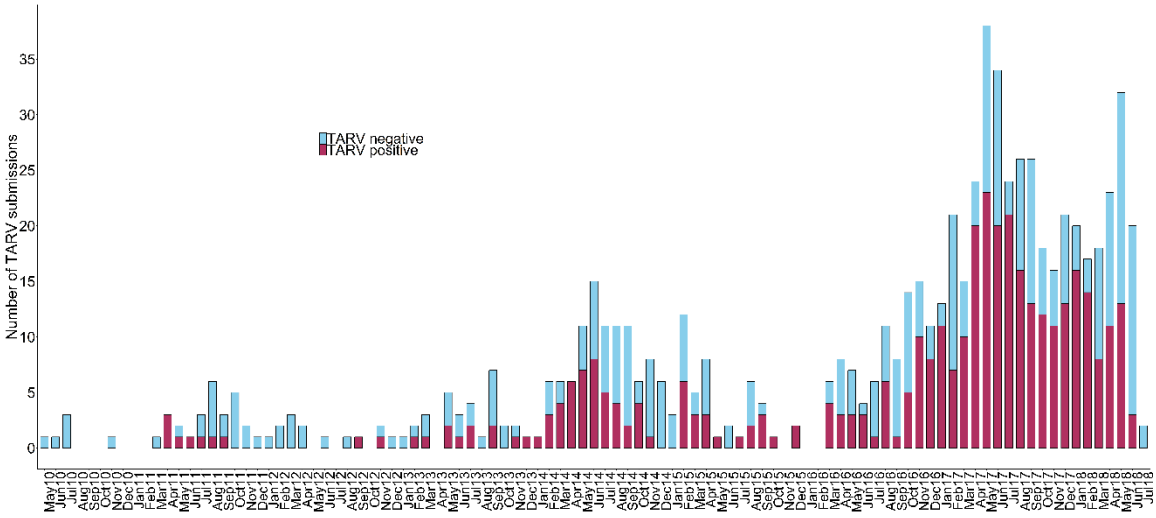


Fig. 2. Bar graphic showing TARV positive and negative samples on a month basis 2010-2018



time periods of the trigonometric functions were not significant in this study. Month of the year (p-value=0.30) and age category (p-value<0.01) were both included in the final logistic regression model to obtain probabilities of TARV positives over time. Even though TARV positive submissions were not seasonal in this study, there were certain months that had higher probabilities of TARV positive cases (Fig. 3). Overall, the probability of TARV positive varied by month and was higher in January, April, July and December. Most of the submissions were from 11 to 18 weeks old turkeys (Fig. 4). Overall, the number of TARV positive submissions and the probability of TARV positive cases increased as flocks aged as shown in Table 3.

Table 3 TARV[a] positive submissions, month and flock age logistic regression results

Variable	Category	Probability of TARV[a] positive submission	p-value
Flock age	0-2 weeks	0.21	Ref.
	3-5 weeks	0.48	0.05
	6-10 weeks	0.52	0.01
	11-15 weeks	0.59	< 0.01
	16-18 weeks	0.70	< 0.01
	19-80 weeks	0.50	0.01
	Unknown	0.42	0.04
Month	12 months	-	0.30

[a] = Turkey arthritis reovirus (TARV)

Discussion

Turkey arthritis reovirus (TARV) is an important pathogen of turkeys in the U.S. In this study, we determined TARV detection dynamics in diagnostic submissions as well as its spread and potential association with certain months and flock ages. We documented that TARV diagnostic submissions have increased in the last few years. We also reported that TARV was widespread in U.S. flocks and that TARV positive submissions varied over time, were higher in certain months and increased as flocks

aged. Veterinarians and producers can use this information to apply control methods when the risk of TARV infection is high.

Our data clearly indicate that TARVs are widespread in the Midwestern US and that more data on how TARVs persist and transmit between flocks are urgently needed to formulate biosecurity and management strategies to control TARV infections. Our results also raise concern about the recent increases in the disease produced by this newly re-emerging virus. Even though the increased awareness among veterinarians likely impacted the increasing number of submissions, not only was there a real increase in the number of submissions, but also in the number of TARV positive submissions.

Fig. 3. TARV submissions by month (A) and probability of TARV positive by month (B), cumulative 2010-2018

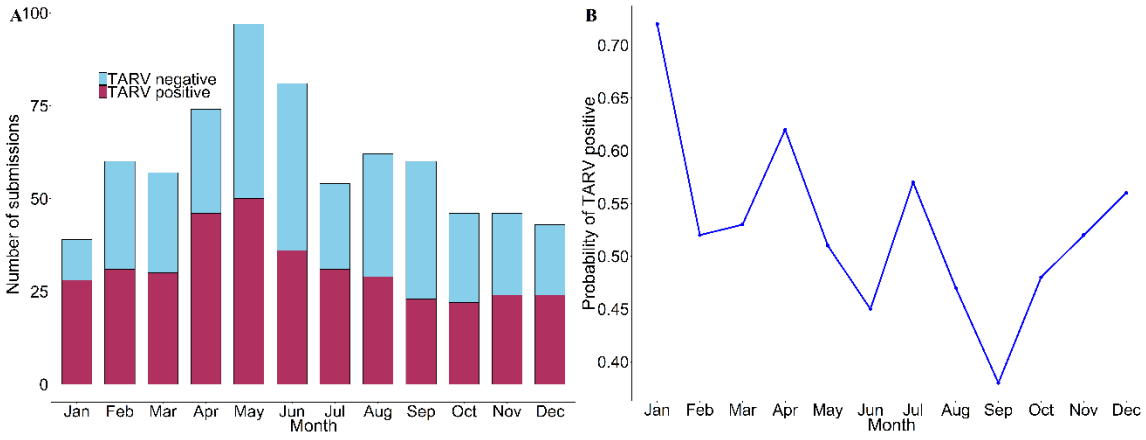
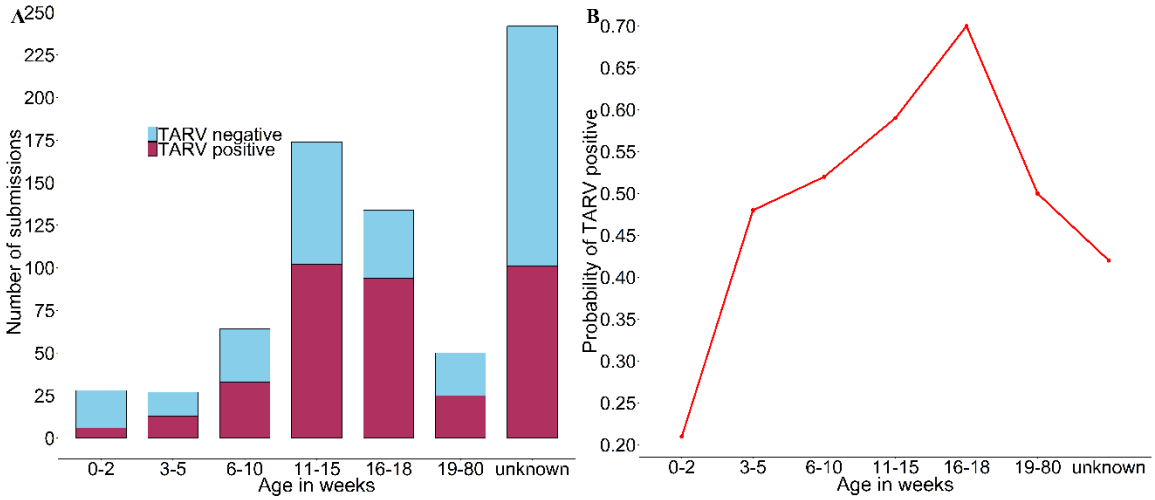


Fig. 4. TARV submissions by age categories (A) and probability of TARV positive by age category (B)



We did not find statistical evidence that TARV diagnostic submissions were seasonal in nature. However, there were certain months that had higher probabilities of TARV positive submissions. This indicates the need for additional studies to determine if TARV is indeed seasonal so turkey producers and veterinarians can focus control efforts in higher risk periods. Additional research will also highlight the underlying factors responsible for these trends, if any. There are very limited data on TARV dynamics within flocks and how climate and other management practices can impact TARV infections in US turkey flocks. Our study is the first one exploring TARV seasonality.

Our age correlation results support previous studies in which TARV was experimentally demonstrated to produce clinical disease in older turkeys although they were infected at one week of age (Sharafeldin, Mor, Bekele, et al. 2015). This is believed to be partially due to increased body weight of older turkeys putting more pressure on the joints. Our findings agree with these experimental studies and with the field reports in which more clinical cases are observed in turkeys 13 weeks of age and older. There is still lack of information on the age at which turkeys become susceptible to TARV infection and the factors that lead TRV to become TARV or TERV.

The gastrocnemius tendons alone or in combination with digital flexor tendons are commonly tested to confirm TARV etiology. The majority of the samples were tested by virus isolation in both QT-35 cells and eggs. Tendon maceration is currently challenging and TARV rRT-PCR is still not as sensitive as virus isolation to detect TARV in tendons. The methods to recover the virus from tendons are still not fully optimized and validated. Considering that a majority of submissions consisted of one tendon pool, we speculate that diagnostic sensitivity may have impacted our results through lower detection rates. There is a need for additional studies on the effect of tendon collection, processing and pooling on diagnostic sensitivity. The extent of this

impact is difficult to quantify given the current and limited diagnostic tools for TARV detection.

Conclusions

We have documented that TARV diagnostic submissions in Minnesota have increased in the last few years. Our results also suggest that TARV is widespread in the Midwest and that there are higher number of submissions in certain months of the year. Finally, we corroborated previous findings in which TARV cases were higher as turkeys aged. Our results suggest the need for future studies to further understand TARV dynamics, transmission and epidemiology within and between flocks. This information may prove to be critical for veterinarians and producers engaged in improving the health of turkeys.

Chapter 3: Development of an enzyme-linked immunosorbent assay to detect antibodies against turkey reovirus

ABSTRACT

Turkey reovirus (TRV) causes significant economic losses to the US turkey industry due to enteritis, pericarditis and severe lameness/tenosynovitis. In recent years, there is an increase in diagnostic cases submitted for lameness and associated with TRV in turkeys 12 weeks of age and older. A turkey-specific serological test for detection of anti-reovirus antibodies has not been available. We developed and optimized a whole-virus ELISA test based on a TRV isolate from a case of lameness in turkeys. ELISA optimization was done with checkerboard titration varying antigen, serum and conjugate dilutions. Optimum reagent concentrations were 1:160, 1:200 and 1:6000 for antigen, serum and conjugate, respectively. The ELISA was validated with serum samples (n=159) collected from turkeys experimentally inoculated with 17 TARV strains (n=107), 2 TERV strains (n=11), non-TRV inoculated turkeys (n=13) and TARV-associated lameness field cases (n=28). Sample to positive (S/P) ratios and TRV status were used in a receiving operating curve (ROC) analysis to determine optimum S/P ratio cut-off. For the estimate S/P ratio cut-off value of 0.4 the diagnostic sensitivity and specificity was 0.92 for both. Our ELISA was capable of detecting antibodies from different TRV strains currently circulating in US turkey flocks and had good diagnostic sensitivity and specificity. This is the first TRV ELISA test available for producers and veterinarians at the University of Minnesota Veterinary Diagnostic Laboratory and promised to be a useful adjunct for early detection of reovirus exposure in turkey flocks and to characterize the immune response dynamics in turkey flocks infected with reovirus.

Introduction

Avian reoviruses are double-stranded RNA viruses belonging to Orthoreovirus genus under the family *Reoviridae.* These viruses can infect a variety of avian species including chickens and turkeys. Chicken reovirus (CRV) causes runting-stunting syndrome and arthritis in chickens while turkey reovirus (TRV) is responsible for enteritis and arthritis/tenosynovitis in turkeys. The TRVs are arbitrarily divided into turkey enteric reovirus (TERV) and turkey arthritis reovirus (TARV) depending upon the clinical signs produced and the tissues from which they are isolated. Infection with TARV is economically important for turkey producers because it causes lameness, swelling of the intratarsal (hock) and joints, and rupture of gastrocnemius and/or digital flexor tendons in turkeys (Sharaf Eldin 2015, Sharafeldin et al. 2014, Sharafeldin, Mor, Bekele, et al. 2015). Histological lesions associated with TARV infection include infiltration of inflammatory cells in the gastrocnemius tendon sheath synovium along with hyperplasia and hypertrophy of synoviocytes (Sharafeldin, Mor, Verma, et al. 2015, Sharafeldin, Mor, Bekele, et al. 2015, Sharaf Eldin 2015). In chronic stages of TARV infection there is fibrosis of the gastrocnemius and digital flexor tendons (Sharafeldin, Mor, Verma, et al. 2015, Sharafeldin, Mor, Bekele, et al. 2015, Sharaf Eldin 2015).

In the U.S. there has been an increase in number of cases of TARV-associated lameness in turkeys 12 weeks of age and older (Tang, Lin, et al. 2015, Sharafeldin, Mor, Verma, et al. 2015, Sharafeldin, Mor, Bekele, et al. 2015, Lu et al. 2015). Infection of turkeys with reoviruses can be detected by RT-PCR and virus isolation (Mor, Verma, Bekele, et al. 2014, Jindal et al. 2012) but a turkey-specific serological test for the detection of antibodies to turkey reovirus is not available. The commercial enzyme-linked immunosorbent assay (ELISA) to detect antibodies against chicken reovirus (CRV) does not reliably detect anti-TRV antibodies because of low cross-reactivity between CRV and

TRV and the lack of turkey-specific immunoglobulins in the assay (Spackman et al. 2005, Al Afaleq and Jones 1989). In this study, we report the development of a whole virus ELISA test to detect antibodies against turkey reoviruses. The development of a reliable serological test should be useful promised to be a useful adjunct for early detection of reovirus exposure in turkey flocks and to characterize the immune response dynamics in turkey flocks infected with reovirus, and ultimately help with controlling reovirus infections.

Methods

Source of the virus

A single TARV was chosen as representative strain for the whole virus ELISA by analyzing whole genome sequences of 32 field isolates of TARV and 14 reference sequences from GenBank after confirming that Sigma C and B protein epitopes were conserved among the majority of all isolate sequences (Yin, Qin, Sun, Gao, Qi, et al. 2013, Yin, Qin, Sun, Gao, Gao, et al. 2013, Clark et al. 2016).

Source of sera and conjugate

Positive control turkey serum was obtained from Dr. Jack Rosenberger (Aviserve, Newark, DE). This serum was prepared against the O'Neil strain of TARV, which has been used in many experimental studies (Sharafeldin et al., 2014, 2015). The conjugate was a horseradish peroxidase (HPR)-conjugated, anti-turkey IgY antibody prepared in goats (Abcam, Cambridge, MA). The substrate for the conjugate was hydroxide peroxide, and the color indicator was TMB (tetramethylbenzidine). A serum sample originating from SPF poults and known to be negative for TRV antibodies was used as a negative control.

ELISA development and optimization

The selected isolate was propagated in QT-35 cells with DMEM (Dulbecco's Modified Eagle Medium) containing 2% fetal bovine serum. After 80-90% of the monolayer showed cytopathic effect (CPE), it was washed with PBS followed by the addition of 0.5 mL of a non-ionic detergent (0.5% IGEPAL solution in PBS) in each of the 75 cm^2 flask. The flask was placed on a rocking platform for 1 hour at 4°C. The cell culture fluid was then centrifuged at 800 g for 15 min at 4°C to remove cellular debris. The supernatant was labeled as positive antigen and stored at -80°C. The same procedure was applied to non-infected cells for the preparation of negative control antigen.

The optimum concentrations of antigens, serum and conjugate were determined by classical checkerboard titration. First, the antigen was diluted two-fold (1:10 to 1:20480) and serum and conjugate dilutions were kept constant. Second, antigen and conjugate dilutions were kept constant, and serum (positive and negative sera) was diluted two-fold diluted (from 1:50 to 1:800). Finally, antigen and serum dilutions were kept constant and conjugate was diluted two-fold (from 1:1500 to 1:12000). Briefly, 96-well microtiter plates were coated with positive and negative antigens by diluting in coating buffer (1.8 g sodium carbonate and 2.9 g sodium bicarbonate in 1 liter of distilled water) and placing in alternate rows of the plate. The plates were incubated at 37°C overnight and then washed three times with a washing solution (53.6 g sodium phosphate dibasic, 169.6 g sodium chloride and 10 mL Tween-20 in 20 L of distilled water). The wash solution was aspirated and the plates sealed and stored at -20°C.

On the day of use, the antigen-coated plates were placed at room temperature for 15 minutes. They were then washed three times (with soaking for one minute between each wash step). The solution was then aspirated followed by the addition of 300uL/well of blocking buffer (1% of nonfat dry milk, pH 9.6). The plates were incubated at 37°C for 2 hours and then washed three times with the wash solution. Serum samples were diluted in dilution buffer (1% nonfat dry milk, pH 7.4) followed by the addition of 100uL/well of this dilution in both positive and negative antigen-coated wells, plates were incubated at 37°C for 1 hour and then washed three times again. Conjugate was diluted in dilution buffer and 100uL were added to each well, incubated for 1 hour and washed. Then, 100ul of TMB was added to each well and kept at room temperature in darkness for 15 min. The reaction was stopped by adding 100uL of stop solution (1 M phosphoric acid) to each well. The optical density (OD) was read at 450 nm in an ELISA reader (Vmax kinetic microplate reader, Molecular Devices, Sunnyvale, CA). The OD difference (ODD) was obtained by subtracting the negative antigen OD from the positive antigen OD for each serum sample or dilution. Finally, the sample to positive (S/P) ratio was calculated as the ratio of the sample ODD and the average ODD of positive serum control.

Test validation

The ELISA was validated using hyperimmune sera from poults inoculated with 17 different TARV isolates, 2 TERV isolates, and non-inoculated poults. Serum samples from field cases of turkey reoviral arthritis were also used.

Statistical analysis

A Receive operator characteristic (ROC) analysis curve was used to determine the optimum S/P cut-off value to maximize diagnostic sensitivity and specificity for the

ELISA. S/P ratios and the TRV status of each sample was used in the ROC analysis that was calculated assuming a 50% prevalence by the method described by Greiner et al. (Greiner, Pfeiffer, and Smith 2000) in the EpiTools epidemiological calculators tool (Sergeant 2019).

Results

The optimum dilutions of antigen, serum and conjugate were 1:160, 1:200 and 1:6000, respectively. A total of 159 serum samples were tested from which 107 samples were from poults inoculated with seventeen different TARV clinical isolates, 11 from poults inoculated with two different TERV isolates, 13 from non-inoculated poults, and 28 from field cases of turkey reoviral arthritis. The distribution of S/P ratios obtained from the tested samples is shown in Fig. 5. A total of 13 samples (8%) were considered truly negative because they were obtained from un-inoculated poults that were free from TRV infection. The remaining 146 (92%) samples were considered TRV-infected.

Diagnostic sensitivity (Se) and specificity (Sp) curves calculated with the obtained S/P ratio and status of each sample using the ROC analysis are shown in Fig. 6. As expected, diagnostic Se decreased as the S/P ratio cut-off value increases. In contrast, diagnostic Sp increased as the S/P ratio cut-off value increased. Considering a TRV prevalence of 50% and maximizing diagnostic Se and Sp, the optimum S/P ratio cut-off value was 0.28 for a diagnostic Se and Sp of 0.99 (0.95-1.00) and 0.92 (0.67-0.99), respectively. For a S/P cut-off value of 0.4, diagnostic sensitivity and specificity was estimated as 0.92 for both Se (0.87-0.96) and Sp (0.67-0.99). Therefore, we set an S/P ratio of 0.4 as the cut-off value to differentiate between positive and negative samples. Fig. 7 shows the distribution of the tested samples based on an S/P ratio cut-off of 0.4.

Using this value, 85% (139/159) of the tested samples were classified as positive and 15% (24/159) as negative.

Discussion

We have developed, optimized and validated a whole-virus ELISA test to detect TRV antibodies. Using a cut-off of 0.4 S/P ratio, this ELISA showed diagnostic sensitivity and specificity of 0.92 and was able to detect TRV antibodies in poults infected with both TARV and TERV. Several types of ELISA have been developed for

Fig. 5. Sample to Positive (S/P) ratios of 159 serum samples

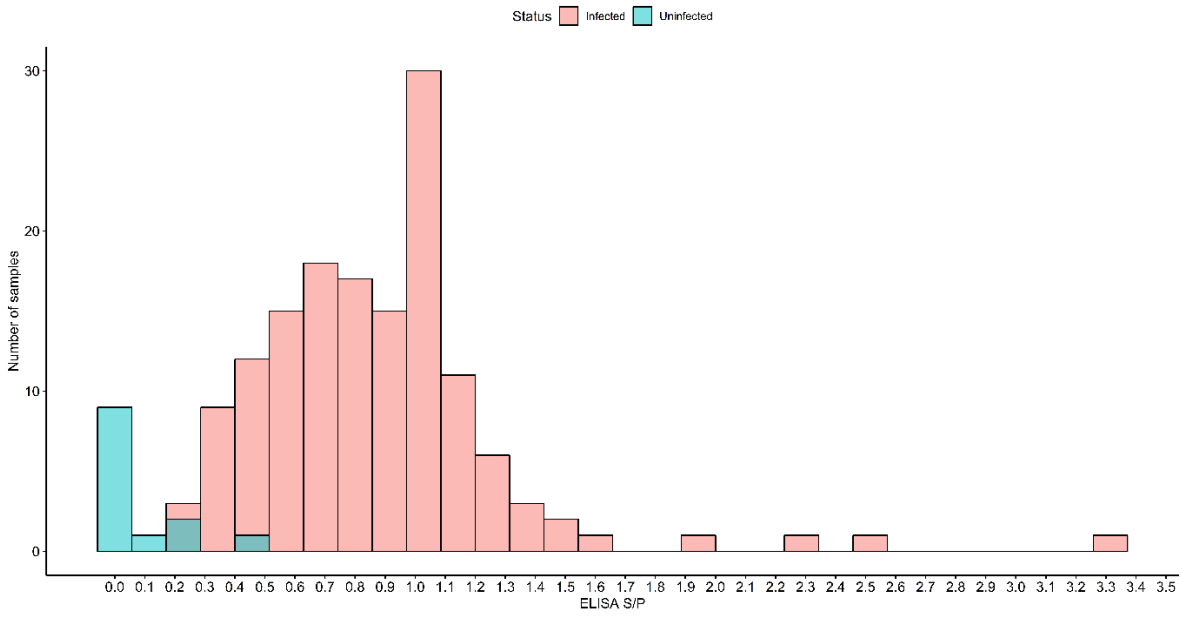


Fig. 6. Diagnostic sensitivity and specificity curves after the ROC analysis

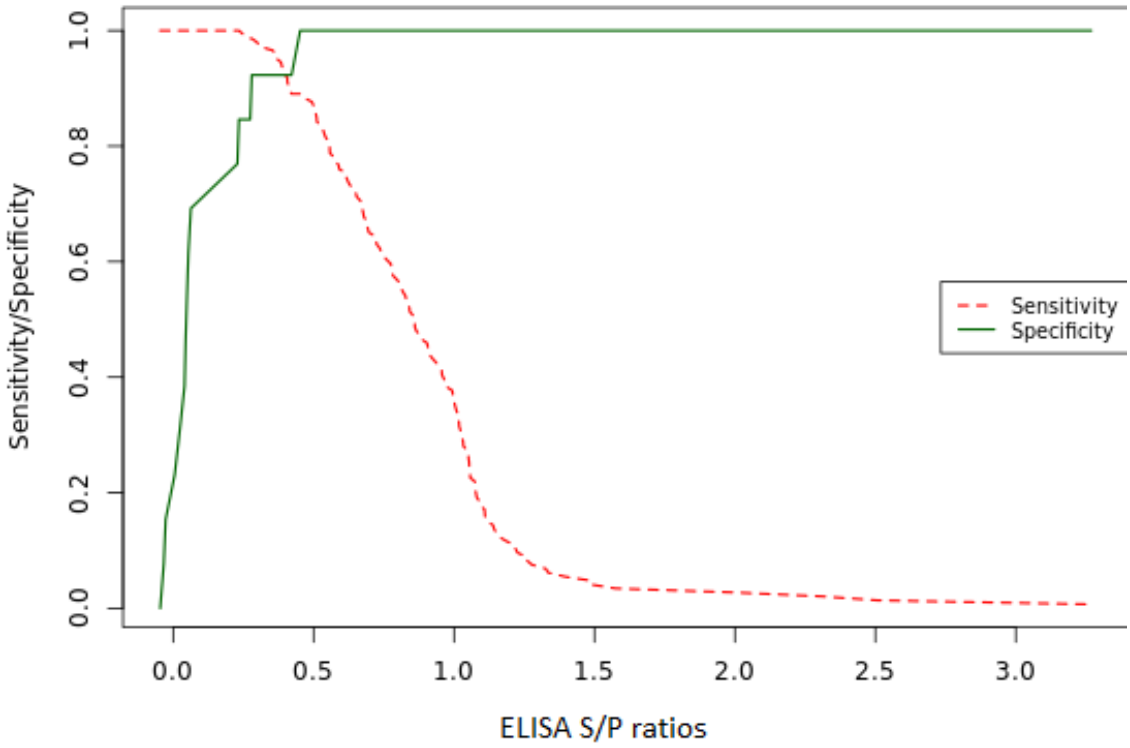
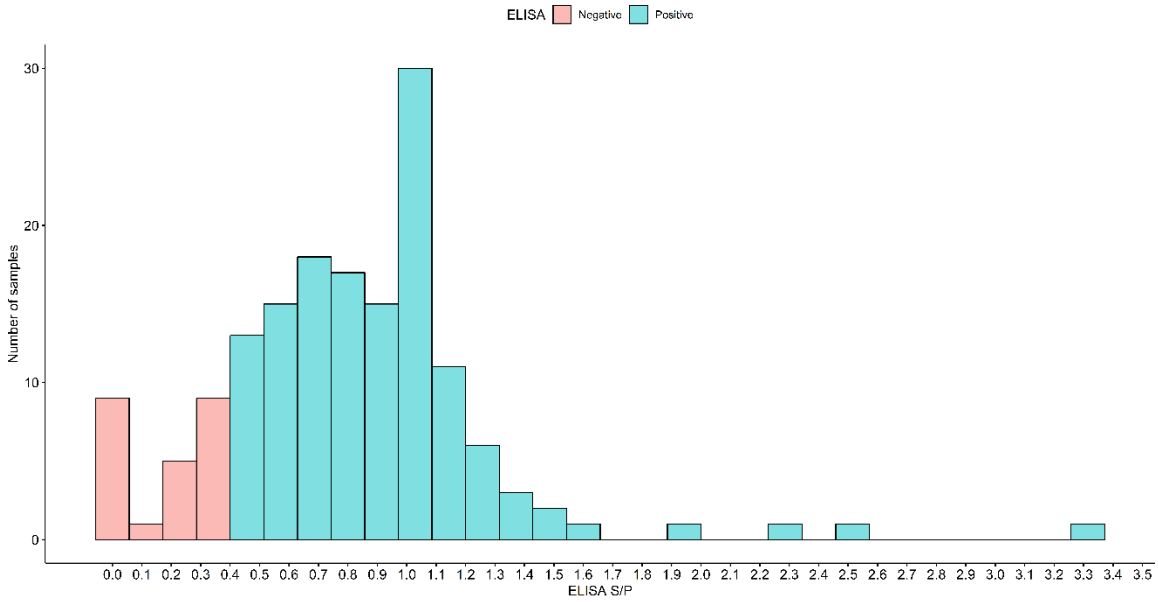


Fig. 7. Distribution of anti-reovirus antibody based on a S/P cut-off value of 0.4, n=159



detecting antibodies against avian reoviruses in chickens and ducks (Majumder et al. 2018, Yun et al. 2015, Yang et al. 2010); however, no turkey-specific assays have been described. The chicken ELISA is based on expressed and purified CRV subunit proteins. We did not compare our assay to those in the reports or against other commercial ELISA tests for anti-reovirus antibody because the other assays do not use anti-turkey IgY conjugate as the determinant antibody.

The TRV isolate used in this ELISA was chosen based on the sequence similarity among conserved epitopes in Sigma B and C proteins (Yin, Qin, Sun, Gao, Qi, et al. 2013, Yin, Qin, Sun, Gao, Gao, et al. 2013). Even though we selected this isolate from a group of 32 field isolates, there is still a high possibility that the selected isolate was not representative of the current TRV strains circulating in the US turkey flocks given that we did not select for differences in all reoviral segments that are reported as immunogenic. Additionally, some studies have reported that particular reoviral segments used for ELISA tests can background signals when a whole virus ELISA is used (Lin, Shen, and Lee 2012, Chen et al. 2004).

To validate our ELISA, we used a set of samples from TRV inoculated and non-inoculated poults as wells as from field samples of turkey reoviral arthritis in which TARV was isolated. Although the total number of samples used is acceptable, only 13 true negative samples were available. Because of this, there is a possibility that the ROC statistical model used in this study could have some limitations since the model requires large numbers of samples within a binormal distribution (López-Ratón et al. 2017, Fawcett 2006, Greiner, Pfeiffer, and Smith 2000). Whether or not this could have affected our ability to accurately estimate S/P cut-off points and diagnostic sensitivity and specificity, remains to be determined. Nonetheless, this ELISA should be of help to producers and veterinarians in diagnosing TRV infections and designing appropriate control strategies in commercial turkey flocks.

Chapter 4: General discussion and conclusions

General discussion and conclusions

I retrospectively analyzed submission data from lameness cases submitted to the University of Minnesota Veterinary Diagnostics Laboratory (UMN-VDL) from 2010-2018 and found that lameness cases associated with TRV have increased in the last few years. TRV-positive lameness cases were found to be widely distributed in the Midwestern US, were not seasonal although there were certain months with higher rates of positive cases, and were correlated positively with flock age.

Even though the majority of lameness cases were from several companies in Minnesota and Wisconsin, this data confirmed the field perception of an increase in the TRV lameness cases in the last few years. This increase is likely a result of increased numbers of field cases, increased producer and veterinarian awareness about TRV lameness, and the industry submitting cases to the UMN-VDL because the lab is one of the few diagnostic labs that has a PCR assay for avian reovirus. More studies are needed to fully determine if TRV transmission or associated clinical signs are seasonal in nature. Our analyses of lab submissions support general clinical findings and previous experiments in lameness associated with TRV is most recognizable as the turkeys age and gain weight.

Moreover, I developed, optimized and validated a whole-virus enzyme-linked immunosorbent assay (ELISA) to provide a high throughput, turkey-specific serological test for TRV antibodies. This assay could assist US turkey producers and veterinarians in early diagnosis of TRV exposure as well as following the dynamics of the immune response in turkeys during reovirus infection\. The assay could play a role in regular control or detection of reovirus infection in at-risk turkey flocks. The ELISA was capable of detecting TRV antibodies in many experimental and clinical samples of turkeys infected with a number of TRV isolates currently circulating in US turkey flocks.

Diagnostic sensitivity (Se) and specificity (Sp) were good and the test is currently available at the UMN-VDL customers.

The TRV ELISA uses a reovirus isolated from a clinical case of lameness, and the isolate is representative of a group of clinical isolates based on the conserved epitopes in Sigma C and B proteins. Whether or not the selected isolate is representative of all TRV genotypes currently circulating in US turkey flocks needs to be tested. In addition, using a whole-virus as antigen provided a broad epitope to detect turkey antibodies against all TRV proteins, but at the same time this likely increased the background noise in the obtained optical density values. This is why we needed to test the same sample with and without antigen to account for that. Finally, the ratio of TRV-negative to positive samples was not ideal and more negative samples need to be tested to more accurately estimate the optimum S/P cut-off value and the respective diagnostic Se and Sp.

Overall, my thesis addressed two critical aspects of TRV infections in the US turkey flocks. I have analyzed submission data for turkey lameness cases from 2010-2018 and developed a turkey-specific ELISA to detect TRV antibodies. Both findings are critically important for US turkey producers and veterinarians given the increase in TRV lameness cases and genetic diversity in the last few years. US turkey producers, veterinarians and researchers may use these findings to answer more questions about TRV epidemiology, transmission and control.

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