TURKEY CORONAVIRUS IN POLAND
- PRELIMINARY RESULTS

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Abstract

The presence of turkey coronavirus (TCoV) was monitored in meat-type turkey flocks in Poland between 2008 and 2009. Clinical samples (10 individual faecal swabs/flock) from turkeys, aged from 7 d to 19 weeks, from 136 flocks were collected from different regions of the country. TCoV detection was accomplished by molecular techniques. The prevalence of coronavirus in 7.3% of tested flocks was found. To investigate the virus shedding and its presence in the intestines, liver, kidneys, and bursa of Fabricius of infected SPF turkeys and contact chickens, the quantitative real time reverse transcription-polymerase chain reaction was applied. The presence of virus in turkey swabs from 5 to 14 d post infection and also in intestines and bursa of Fabricius was detected. Surprisingly, contact chickens revealed to be sensitive to TCoV infection.

Key words: turkeys, coronavirus, poult enteritis complex, morbidity, experimental infection, Poland.

Poult enteritis complex (PEC) is characterised by diarrhoea, depression, and a general poor condition of young turkeys. In cases in which morbidity and mortality are high, the disease could be classified as poult enteritis and mortality syndrome (PEMS). These infectious and transmissible conditions could be attributed to multiple factors, and coronavirus (CoV) was shown to be one of the causative agents involved in its aetiology (1, 2, 8, 11, 21). This virus belongs to highly diverse Coronaviridae family, Gammacoronavirus genus, group 3a together with infectious bronchitis virus (IBV). In this genus there are viruses distantly related as CoV from beluga whale (SW1) with the largest genome and CoV from birds of different families with the smallest genome. However, they have unique genomic features such as distinct transcription regulatory sequence and coding potential for small open reading frame (ORF), which distinguish them from other viruses (24). The genome of turkey CoV (TCoV) is a single-strand positive-sense RNA with the following organisation: 5′-UTR-Pol-S-3a-3b-E-M-ORFX-5a-5b-N-UTR-3′ (23). CoV possess four major structural proteins: spike (S), membrane (M), small envelope (E), and the most conserved among coronaviruses nucleocapsid (N).

TCoV has been known since about 60 years. From the beginning of 1950’s, the disease named “mud fever” or “bluecomb disease” caused severe economic losses for the turkey industry in the USA and Canada and TCoV was indicated as the aetiological factor (18, 20). In addition to North America, TCoV was also reported in Australia, Brazil, Italy, UK, and France (5, 6, 17, 21). In recent decade, Polish turkey industry has developed dynamically with 7 million heads raised in 1995 and above 29 million in 2009 (http://www.stat.gov.pl/gus/5840_4127_ENG_HTML.htm). The farms are mainly localised in western and north-eastern part of the country. Despite the high level of hygiene/biosecurity implemented, the clinical signs typical for PEC are observed.

The aim of this study was to detect TCoVs in commercial meat-type turkey flocks in Poland and to examine the virus load in experimentally infected SPF turkeys and contact SPF chickens.

Material and Methods

Sample collection and preparation. During two-years monitoring period (2008-2009), clinical samples from affected and healthy poults, from 7 d to 19 weeks of age, were collected from 136 flocks (10 individual faecal swabs/flock) located in different regions of the country. All samples were stored in -20°C until processing. After slow thawing, each swab was hydrated in PBS with antibiotics, incubated for 1 h at room temperature, and clarified by centrifugation at 1,500 g for 20 min. Two hundred and fifty microlitres of supernatant from five pooled swabs (two pools/flock) were used for total RNA extraction using RNeasy Mini kit (Qiagen, Germany).
The ELISA-IB (IDEXX, USA) was used for serological examination of 570 serum samples from 55 flocks taken between 2006 and 2009.

**Molecular methods.** At the beginning, amplification of the conserved region of 3’ untranslated region (UTR) was carried out by standard reverse transcription-polymerase chain reaction (RT-PCR) using primers UTR11/-UTR41+ according Cavanagh et al. (4). Later, the real time RT-PCR (rRT-PCR) aiming at 5’UTR of IBV genome was used (3).

For absolute quantification of TCoV genome, the standard RNA for the rRT-PCR assay was obtained. A 1432-bp RT-PCR product containing the 5’UTR fragment of reference IBV Beaudette strain was amplified using primer pairs M215F/M1646R (available on request) and this product was cloned into pCR® 2.1-TOPO vector (Invitrogen, USA), linearised at the SpeI promoter according to the manufacturer’s guidelines. After few DNase RQ1 treatments to remove residues of plasmid DNA, the transcripts were purified by phenol/chloroform extraction, resuspended in nuclease-free water, and quantified by fluorescent staining by Quant-iT™ RiboGreen® RNA Kit (Invitrogen, USA). The exact number of RNA molecules was calculated to be about 100 template copies (Ct value = 37).

**Clinical signs and quantification of viral load in experimental turkeys and chickens.** All TCoV infected turkeys developed watery diarrhoea just after 3 dpi and it lasted for 10 d, so no birds were showing such symptoms after that time. At necropsy, the intestines were bloated with thin, pale, and parchment-like walls; the bursa of Fabricius was atrophic on the 21st dpi. Starting from 10 dpi, diarrhoea was also observed in contact chickens; pathological changes in the intestines were also seen on the 21st dpi.

The detection limit of the rRT-PCR was assessed as 4.2 x 10^3 copies µl^{-1}. Ten-fold dilutions of standard RNA were used to construct a standard curve covering a linear range of seven orders of magnitude (from 10^3 to 10^9 copies of standard RNA) and linearity was observed over the entire quantification range (y=-0.2641x+11.046) with R^2=0.9972 (Fig. 1). Using the slope from the linear equation, the overall efficiency of the assay was estimated to be 83.69%. For the elimination of false positive results, the limit of detection and quantification were slightly raised and determined to be about 100 template copies (C, value = 37).

TCoV genome was detected only in swab from one turkey at 5 dpi (below 400 copies), from swabs of four turkeys at 7 dpi (approximately 400 copies), and from all animals at 10 (approximately 4 x 10^6 copies) and 14 (approximately 4 x 10^5 copies) dpi (Fig. 2). No viral load was detected in swabs collected from animals at 21 dpi. Interestingly, all chickens in contact group revealed TCoV genome in their cloacal swabs at 10 (approximately 4 x 10^6 copies), 14 (4.5 x 10^6 copies), and 21 (approximately 400 copies) dpi. As regards to tissues, TCoV RNA was detected in trace quantities (below 400 copies) in the duodenum and jejunum at 7 dpi, in the duodenum, jejunum (above 4 x 10^5 copies), and caecum (approximately 700 copies) at 14 dpi, and in the duodenum (approximately 700 copies) and caecum (4.5 x 10^6 copies) at 21 dpi (Fig. 3). In all tested parts of the intestines of contact chickens TCoV genome was detected (approximately 400 copies). The liver and kidneys of infected and contact birds seem to be free of virus; however, the bursa of Fabricius of one turkey and all chickens contained RNA TCoV (above 4 x 10^5 copies).

The examination of cloacal swabs at six different time points from TCoV infected turkeys revealed that viral load, achieved plateau at 10 dpi, completely disappearing at 21 dpi. Contact chickens infected with the virus achieved virus plateau at 10 dpi and then showed a distinct decreasing at 21 dpi. The virus seems to settle mainly in the caecum and bursa of Fabricius of both bird species; however, some virus load in the liver and kidneys in chickens was found.

**Results**

**Turkey flocks monitoring.** Coronavirus was detected in ten turkey flocks (7.3%) of 136 examined. Five positive flock of 80 analysed (6.2%) were identified in 2008 and five of 56 (8.9%) in 2009. Most of them were in the same province, Wielkopolskie, two in Kujawsko-Pomorskie, and one flock in Malopolskie and Dolnoslaskie. The positive samples were from 5-15 weeks old birds. Most of TCoV positive birds showed clinical signs related to PEC as diarrhoea, thermoregulation troubles, uneven growth, and in one flock increased mortality up to 5%. Three flocks were treated for aspergillosis and in five flocks other enteroviruses (astrovirus, rotavirus, and parvovirus) were also detected. The ELISA revealed TCoV antibodies in birds of nine (16%) flocks but only single serum in each flock was positive (3.7% of all 570 studied sera).
Fig. 1. The rRT-PCR standard curve – the plot of the Ct values vs log10 of ten-fold serial dilutions of standard RNA. The slope (y) and efficiency (R²) are given.

Fig. 2. The average viral load in swabs of experimentally infected turkeys and contact chickens.

Fig. 3. The average viral load in three parts of the intestine of experimentally infected turkeys and contact chickens.
Discussion

In this study, the method applied for detection and quantification of TCoV was based on 5'UTR fragment of the IBV genome (3). The region targeted by this assay was highly conserved and showed 98% identity with sequences of most IBV isolates (7). The assay was shown to be linear over a range of seven magnitudes, from 10^1 to 10^7 RNA copies, ensuring an accurate measurement of TCoV RNA loads in clinical samples.

The presence of turkey coronavirus in 7.3% of the analysed Polish flocks between 2008 and 2009 was found. Such occurrence was markedly lower than that detected in PEC suspected turkey flocks in France (35%) as reported by Maurel et al. (17), and that detected in UK (32%) (12) and in Brazil (82%) (22). On the other hand, no TCoV infection was found in the USA as reported by numerous authors (14, 15, 19). Such low incidence of TCoV in Polish PEC suspected turkey flocks could indicate that other viruses were responsible forenteric conditions of the birds. Other enteric viruses, such as: astroviruses, rotaviruses, reoviruses, adenoviruses, and paroviruses are known to be responsible for conditions, including: diarrhea, depression, growth depression, and impaired feed consumption. The presence of some of these viruses (astro-, rota- and paroviruses) was detected in a number of turkey flocks studied herein (data not shown). The identification of TCoV RNA in low number of the examined turkey flocks goes along with results of serological studies, which detected antibodies only in 3.7% of samples. Using the same kind of ELISA IBV IDEXX test it was found that 50% of field samples from turkeys in Indiana, USA, had TCoV antibodies (16). On the other hand, the sera from turkey flocks from Ontario, Canada, were studied using both commercial IDEXX and recombinant TCoV N-based ELISA and different prevalence was obtained, 16.6% and 70.2%, respectively. The reason for such differences could be the low level of cross-reactivity between the TCoV-specific antibodies and IBV antigens (9).

As estimated, the rRT-PCR used in the studies in the course of TCoV infection in turkey poult's was extremely sensitive as it could detect 100 RNA copies µl^-1. It seems that the virus shedding, measured by this assay, began from 5 dpi when TCoV genome was detected in one turkey. In the next time-point more (7 dpi – 4/9) or all animals (10 and 14 dpi – 7/7) were positive achieving plateau at 10 dpi, when viral load was the highest. Afterwards, gradual disappearing was noted with no virus detection at 21 dpi. The observed duration of shedding, from 5 to 14 dpi, is similar to previous reports of Gomaa et al. (8). The viral load investigation in three parts of the intestine revealed that virus gradually was settled down in the duodenum and ileum at 7 and 14 dpi. At 21 dpi lower virus loads in these parts of the intestine, and the highest in the caecum was found. This might be the result of the presence of the caecum tonsils in the caecal wall and their role in the defence system of birds. In other studies, the observed time of virus presence in intestines was different. The TCoV isolate - ATCC VR-911 up to 2 weeks post inoculation and TCoV-NC95 up to 7 weeks were detected (1, 13). Such differences could result from different pathogenicity represented by these strains, applied dose, and used chicken breed. The presence of virus in the bursa of Fabricius was also detected and this is in accordance with previous studies (11, 17).

Surprisingly, contact 1-d-old chickens revealed to be sensitive to TCoV infection. The virus shedding began at 7 dpi and lasted up to the 21st dpi (end of experiment). The virus load was the highest at 10 dpi. Some clinical signs as mild diarrhea from 10 dpi was also observed. Moreover, TCoV RNA in the intestine, bursa of Fabricius, liver, and kidneys was also detected. It was previously found that TCoV are not pathogenic for chickens in spite their replication in the gut, so their role as carriers of the virus was suggested (13). No clinical signs or microscopic changes, but only TCoV replication in respiratory cells of paranasal sinus and Harderian glands of chicks were observed in studies of Gomes et al. (10). The presence of virus in the intestine and bursa of Fabricius but not in the trachea, lung of chickens was detected in other studies on chicken susceptibility to TCoV infection (11). The finding that TCoV PL/G116/09 caused some clinical signs and pathological lesions is something unexpected and needs to be clarified. In spite the fact that material used for turkey infections was filtered and isolation of bacteria on agar blood plates, as well as molecular methods for rota-, astro- and paroviruses detection, gave negative results, the presence of other bacteria or viruses exaggerating the TCoV infection effect could not be excluded.

In both bird species no seroconversion was observed. However, commercial ELISA IBV IDEXX test was used for the detection of TCoV antibodies and IBV antigens rather poorly cross-reacted with to TCoV specific antibodies (9).

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References

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