

Detection of turkey coronavirus in commercial turkey poults in Brazil

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Poult enteritis complex has been incriminated as a major cause of loss among turkey poults in other countries. We have observed this in Brazil, associated with diarrhoea, loss of weight gain and, commonly, high mortality. In this study, we have used the reverse transcriptase polymerase chain reaction (RT-PCR) to detect turkey coronavirus (TCoV) in sick poults 30 to 120 days of age from a particular producer region in Brazil. The RT-PCR was applied to extracts of intestine tissue suspensions, and the respective intestinal contents, bursa of Fabricius, faecal droppings and cloacal swabs. Primers were used to amplify the conserved 3' untranslated region of the genome, and the nucleocapsid protein gene of TCoV. Histopathological and direct immunohistochemical examinations were performed to detect TCoV antigen in infected intestine and bursa slides. All the results from stained tissues revealed lesions as described previously for TCoV infection. The direct immunohistochemical positive signal was present in all intestine slides. However, all bursa of Fabricius tissues analysed were negative. RT-PCR findings were positive for TCoV in all faecal droppings samples, and in 27% of cloacal swabs. Finally, the best field material for TCoV diagnosis was faecal droppings and/or intestine suspensions.

Introduction

The Brazilian turkey industry ranks second in production in the world, with 187 million carcasses produced between 2005 and 2006, corresponding to 29% of the international market. The farms are localized in the south and partially in the centre of the country, on a high production scale. Despite a conventional vaccination schedule, poult enteritis complex (PEC) has affected turkeys from 6 weeks of age or more, resulting in a substantial economic loss.

According to Cavanagh (2005) the primary disease presentation of PEC is diarrhoea, restlessness, and a general poor condition of the poult. In cases in which morbidity and mortality are high in poults of up to 30 days, the disease maybe classified as poult enteritis and mortality syndrome. Although the aetiologic agent (or agents) that cause PEC has not been definitively identified, several viruses have been associated with PEC-like disease, including turkey coronavirus (TCoV) (Brown *et al.*, 1997; Breslin *et al.*, 2001; Guy, 2003; Ismail *et al.*, 2003). More recently, poult enteritis and mortality syndrome has been described affecting poults in Great Britain, resulting in a first report of this disease there (Culver *et al.*, 2006).

Coronaviruses had often been described as being fastidious. This claim arose from the difficulty that virologists had experienced in finding types of cells in which to grow coronaviruses *in vitro* (Gough *et al.*, 1988,

2006; Brown *et al.*, 1997; Cavanagh, 2005). TCoV cannot be grown in cell culture, so its diagnosis is based upon the indirect immunofluorescence technique, commercial infectious bronchitis virus (IBV) enzyme-linked immunosorbent assay and molecular approaches from clinical and isolated field samples (Breslin *et al.*, 2000). No study has been conducted to compare tissues and samples for the detection of TCoV by reverse transcriptase polymerase chain reaction (RT-PCR).

The aim of this study was to detect TCoV from poults affected by PEC, by analysing cloacal swabs, faeces and tissue suspension by RT-PCRs, comparing the results with histopathological and direct immunohistochemical findings.

Materials and Methods

Origin of clinical samples. In January 2006, clinical samples (intestine, bursa of Fabricius, pool of faeces, individual faeces, cloacal swabs) from affected poults, ranging from 30 to 60 days of age, were received for virological investigation. The poults were from a multi-age farm with more than 20 000 birds on site, divided into five houses. The samples were taken from individual houses containing approximately 4000 birds, of which 4% showed stunting, depression, acute enteritis and mortality of three birds per day. The samples were collected 2 weeks after the start of the signs, and were sent to the laboratory on ice.

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Preparation of clinical samples for pathology. Slices of the ileo-caecal junction (intestinal contents (IC), $n=10$) and bursa of Fabricius (BF, $n=10$) from affected poult were collected at necropsy and fixed in 10% neutral buffered formaldehyde, and, after embedding in paraffin blocks, sectioned at 2 mm. Slides for histopathology were stained with haematoxylin and eosin using routine methods, and were finally examined by light microscope. Slides for immunohistochemistry were stored non-stained in paraffin blocks until use (Gough *et al.*, 1988; Brown *et al.*, 1997).

Preparation of biotin labelled antibody. The IgY against IBV was produced by vaccination of 10 inbred C/O line White Leghorns chickens at 1 day of age by the intra-ocular route with purified M41 strain, as described by Cardoso *et al.* (1999) with some modifications. After 2 weeks the chickens received a second inoculation, this time of commercial vaccine, 1 ml per bird (H120) by the intra-muscular route, and 21 days later they were bled from the wing vein. The γ -globulin fraction was prepared by a salting-out procedure by addition of 35% (v/v) ammonium sulphate (A-2939; Sigma) followed by IgY fraction purification using chromatographic separation on the Sephadex-G200 (Pharmacia). The purified IgY was conjugated to biotin (Biotin disulfide *N*-hydroxysuccinide ester, catalogue number B-4531; Sigma) according to Harlow & Lane (1988) and was used as primary antibody.

Direct immunohistochemical analyses. Unstained sections (4 μ m) were used for direct immunohistochemical examination after deparaffinization, rehydration and washes in buffered saline added by 0.1% Tween 80. The first step was to microwave the sections in citrate buffer (pH 6.1) for 15 min at 700 W to activate the viral antigen, normally damaged by formaldehyde fixation. Just before staining, slides were treated three times with hydrogen peroxide 50% (30 V) for 30 min to inactivate endogenous peroxidase, commonly found in inflammatory reactions. The slides were then washed for 5 x 10 min in buffered saline to remove the residues between each step of the reaction, and the non-specific binding was blocked using dried 15% non-fat milk for 90 min. Viral antigen was demonstrated by the avidin-biotin complex immunoperoxidase method as described by Psalla *et al.* (2006) and Hussain & Nagaraja (1993) with some modifications. The optimum primary antibody dilution determined by previous titration on indirect enzyme-linked immunosorbent assay was 1:200 in phosphate-buffered saline plus 10% non-fat dried milk. Slides were covered by 200 μ l diluted antibody overnight at 4°C in a humidified chamber. After five washes, 100 μ l/slide of streptavidin-peroxidase complex (Sigma product number S-5512) was added and incubated for 1 h at 37°C. In addition, substrate made fresh in the dark, by mixing equal volumes of 0.02% hydrogen peroxide and 0.6 mg 3,3'-diaminobenzidine tetrahydrochloride (catalogue number 15972-011; Gibco BRL®), was added to the slides for 30 min at room temperature. The reaction was stopped by washing with tap water and the specific brown colour was revealed after counterstaining with Meyer's haematoxylin. An intense dark red deposit was considered positive. Negative controls consisted of sections treated with buffered saline instead of biotin-labelled antibody.

Preparation of clinical samples for RT-PCR. Intestinal suspensions (IS) were prepared by cutting slices from the ileo-caecal junction, divided into 100 samples; the respective IC, a total of 100 samples; and tissue suspensions of all individual BF. In addition, faecal droppings (FD) consisted of 1200 g faeces divided into 100 samples, and, finally, individual cloacal swabs (CS), a total of 100, were also prepared. The FD, BF, IS and IC specimens were homogenized in 2 volumes of minimal essential medium (pH 7.4) and clarified by centrifugation at 3000 $\times g$ for 20 min. The supernatant was first filtered once through a 0.75 μ m paper filter (Millipore) and then twice through a 0.25 μ m syringe filter (Corning). These suspensions were heated at 100°C for 30 min in a water bath before total RNA extraction was performed. The individual CS were hydrated by addition of 200 μ l minimal essential medium plus 20 μ g/ml proteinase K (GIBCO-BRL) and heated at 56°C for 30 min. After heating, the swabs were removed, and total RNA was extracted. The infectious bronchitis virus strain M41 and Newcastle disease virus strain La Sota were used as positive and negative control of all reactions, respectively.

RNA extraction. Total RNA was extracted by Trizol® standard protocol, based on guanidinium isothiocyanate and acid-phenol with some modifications (Sellers *et al.*, 2004). Two hundred microlitres of clinical suspension were mixed with 500 μ l Trizol® reagent and incubated for 10 min at room temperature. After addition of 200 μ l chloroform, it was mixed vigorously for 10 s and centrifuged at 13 000 $\times g$ for 10 min. The upper aqueous phase was mixed with an equal volume of cold isopropanol and incubated on ice for 10 min. The total RNA precipitate was pelleted by centrifugation at 13 000 $\times g$ for 10 min and washed with ethanol. The RNA was dissolved in 30 μ l of diethylpyrocarbonate-treated sterile, double-distilled water and stored at -20°C.

Primers. We used primer combination UTR11-/UTR41+, corresponding to the conserved region of the IBV 3'-untranslated region (UTR), which produces a very sensitive RT-PCR (Adhzar *et al.*, 1996; F. Culver & D. Cavanagh, personal communication). Although the TCoV genome has not been completely sequenced, there is sequence information reported for the nucleocapsid (N) gene used by Sellers *et al.* (2004), Spackman *et al.* (2005) and Loa *et al.* (2006) for multiplex RT-PCR. We used the TCoV nucleo forward and reverse primers described by Sellers *et al.* (2004). Before the RT-PCR was performed, RNA was heated for 3 min at 100°C, followed by 2 min at 72°C and, finally, 2 min at 50°C.

Protocol two-step RT-PCR. Samples were analysed with a two-step RT-PCR. cDNA synthesis was performed using the Superscript II RNase H- Reverse transcriptase (Invitrogen) according to the manufacturer's protocol, except that the temperature used for the transcription was 50°C. A sample of 10 pmol each reverse primer (TCoV nucleo reverse and UTR11-) was used and the reaction was performed in a thermocycler (MJ Research) at 50°C for 30 min, followed by an inactivation step at 70°C for 15 min. DNA (2 μ l) was added to a 25 μ l PCR reaction and amplified using TAQ DNA polymerase (Invitrogen) 5 u/ μ l. The primers used were UTR11- and UTR41+ (to give a product of 251 base pairs (bp)) and TCoV nucleo forward and reverse (to give a product of 598 bp). Amplification involved an initial denaturation step at 94°C for 5 min followed by 40 cycles at 94°C for 15 sec, 55°C for 30 sec, and 68°C for 1 min.

Protocol one-step RT-PCR. Optimization of a one-step RT-PCR requires compromises in the concentration of PCR reagents, principally the primers used. The RT-PCR was performed following the instructions of the One-step Superscript III® RT-PCR commercial kit. Briefly, 2 μ l first strand product was used as a template for amplification in a 50 μ l reaction containing 20 pmol primers and 1.6 mM MgCl₂. All PCR conditions were the same as described above. However, the amount of mixture RT-TAQ DNA polymerase *platinum* enzymes was calculated as 2.5 u/50 μ l reaction. A total of 10 μ l PCR products were electrophoresed at 100 V for 1 h in 1.5% agarose gel in 1 \times Tris-borate EDTA buffer and were visualized by ethidium bromide staining and ultraviolet transilluminator. Gel images were captured using Kodak DC290 digital camera and ADOBE 6.0 software. The specificity of both RT-PCRs was tested by addition of other common avian RNA virus (Newcastle disease virus La Sota strain). The sensitivity was calculated by diluting the TCoV and IBV M41 RNA in 10-fold steps up to a dilution of 10⁶.

Statistical analyses. Statistical comparisons of positive results among all the methods were performed with a two-sample *t* test and the *P* value was determined. The sensitivity and specificity were both calculated by standard formulas.

Results

Clinical signs. The poult were described as depressed, anorexic and having watery diarrhoea, with poor performance, mortality of 5%, and the appearance of acute enteritis.

Pathology. Microscopic examination of the intestine (ileo-caecal junction) revealed marked degeneration

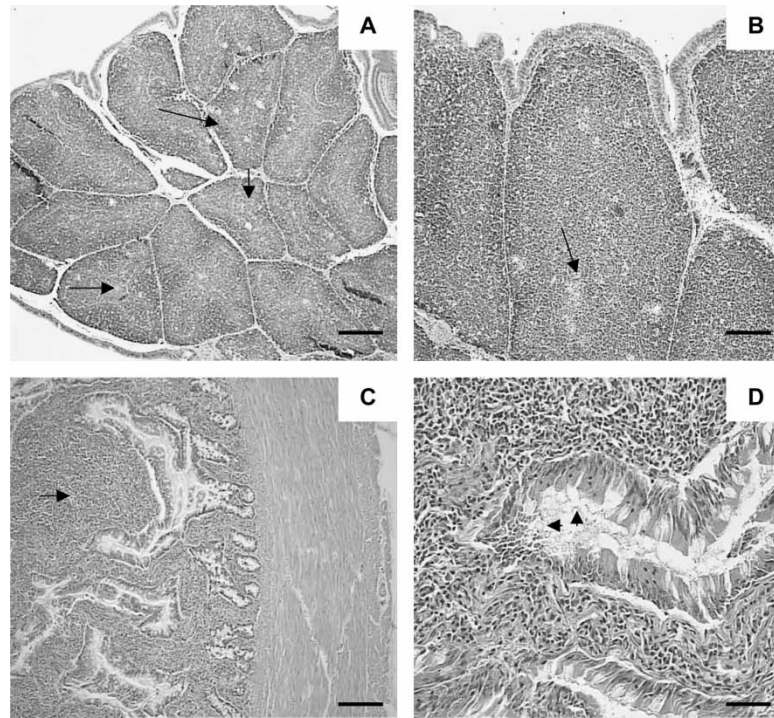


Figure 1. *a:* Low-magnification image of the BF showing mild to moderate lymphocytic destruction and depletion in the medulla. Bar = 30 μ m. *b:* Large numbers of lymphoblast cells with follicular atrophy. Bar = 30 μ m. *c:* Low-magnification image showing extensive monocytic infiltration and destruction of the villous. Bar = 30 μ m. *d:* High-magnification image of the intestine showing the lumen filled with desquamated epithelial cells and mucous. Bar = 30 μ m.

and destruction of the villous epithelium and hyperactivation of the intestinal glands (Figure 1c). The basal lamina was infiltrated with mononuclear cells. Submucosa oedema was also observed (Figure 1d). The intestinal lumen was filled with desquamated epithelial cells in addition to mucous exudates. The BF lymphoid follicles suffered mild to moderate lymphocytic destruction and depletion, especially in the medulla. Many lymphoblast cells associated with follicular atrophy were observed (Figure 1a,b). TCoV antigen was detected in all intestinal sections, being present within enterocytes (Figure 2). In contrast, viral antigen was not found in the BF tissue of any specimens analysed in this work or in any negative controls (data not shown).

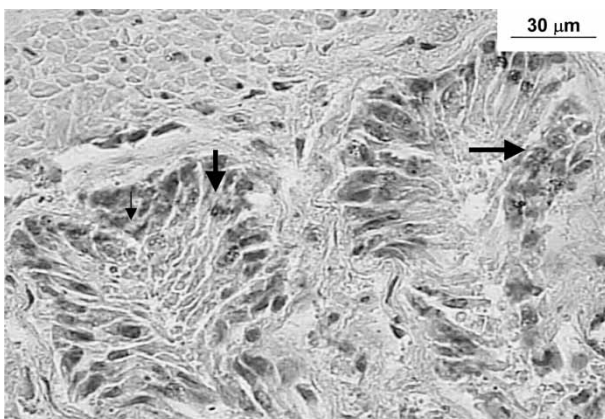


Figure 2. *a:* Low-magnification image of infected intestine submitted to a direct immunohistochemical analysis showing a positive signal. *b:* High-magnification image showing virus-specific brown colour inside enterocytes (arrows). Bar = 30 μ m.

RT-PCR. The RT-PCRs applied here yielded a distinct band of 251 bp for the 3' UTR and 598 bp for the TCoV nucleocapsid protein gene region (Figure 3a,b). Regarding the FD samples, all of them were positive, as well as the IS samples, for the both regions of the genome studied. However, none of the BF samples were positive. Using the 3' UTR primers in the one-step protocol, 27% of the CS samples were positive. All of these samples were also positive using the primers for the N gene region, producing a product of 598 bp, confirming the coronavirus infection. Heating of the RNA was successful in decreasing non-specific bands, especially in faecal samples. The protocol one-step RT-PCR was the most sensitive for the assay, capable of detecting the RNA even after 10^6 -fold dilution. In our analysis, negative results were always negative for both primers used and no amplification products were observed with Newcastle disease virus either for diethylpyrocarbonate water.

Discussion

Due to the complexity and the multifaceted nature of enteric diseases, PEC requires a multidisciplinary approach that can probably only be achieved by combining scientific expertise and resources from different countries. A coronavirus described 30 years ago to be involved with enteritis of turkeys in the USA, where it has been most thoroughly studied, was detected here, causing the same clinical signs and economic losses for poults in Brazil. Moreover, TCoV has been confirmed in Great Britain (Cavanagh *et al.*, 2001), including associated with losses that correspond to poult enteritis and mortality syndrome (Culver *et al.*, 2006).

In spite of high turkey production in Brazil there has been no published report of TCoV detection, or other

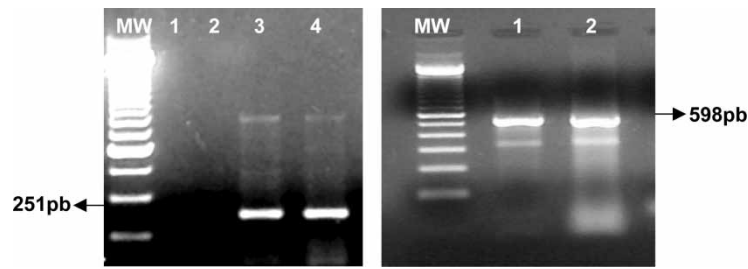


Figure 3. Agarose gel electrophoresis of the one-step RT-PCR products. 3a: Using the primer pair specific to the 3' UTR region. Lane MW, 250 bp size markers; lane 1, water; lane 2, negative control (*Newcastle disease virus*); lane 3, reference positive control (*IBV*); lane 4, positive field sample. 3b: Using the primer pair specific to the nucleocapsid protein gene of *TCoV*. Lane MW, 1 kb size markers; lane 1 reference positive sample (*IBV*); lane 2, field sample.

complicating pathogens, involved in PEC cases, unlike in the USA and Great Britain (Ismail *et al.*, 2001a, b; Ismail *et al.*, 2003; Cavanagh *et al.*, 2001; Cavanagh, 2005).

RT-PCR protocols have been commonly and successfully applied in the USA and the UK (Breslin *et al.*, 1999a,b, 2000). We have used RT-PCRs, histopathological and immunohistochemical examination, and have compared them for detection of *TCoV*.

The birds studied here showed depression, loss of weight, cessation of eating, with wet droppings—signs compatible with those describe in the literature (Cavanagh, 2005). Histopathological examination revealed lesions typically described by other investigators as being associated with *TCoV* infection (Brown *et al.*, 1997; Breslin *et al.*, 2000; Ismail *et al.*, 2003).

In addition, the direct immunohistochemical analysis showed virus antigen in all tissues analysed, except not in the BF samples, using only one primary antibody, which represents an advantage over other conventional protocols. Normally, immunohistochemical reaction protocols involve using two antibodies, the primary antibody and the second, which is a conjugated antibody directed against immunoglobulin of the target animal species. Research in the mid-1990s showed that coronavirus from turkeys were genetically (Breslin *et al.*, 1999a,b) and antigenically (Guy *et al.*, 1997; Breslin *et al.*, 2000; Guy, 2003) closely related to *IBV*, as confirmed elsewhere (Cavanagh *et al.*, 2001). It is true that using polyclonal antibodies increases sensitivity and is less laborious compared with monoclonal antibody protocols (Cardoso *et al.*, 1999). Based on these findings, results from direct immunohistochemical examination using the chicken IgY biotin-labelled primary antibody revealed good results on field samples in a one-step reaction.

Nevertheless, most *TCoV* antigen detection from infected tissues is based on indirect immunofluorescence methodology, which needs frozen tissue sections to be performed and a special microscope to evaluate them. Formalin-fixed tissues present three great advantages over frozen tissue: first, when *in situ* assays are necessary; second, keeping the tissue conservation for further study; and third, long-term storage of paraffin blocks.

The positive RT-PCR reaction of RNA extracted from FD, BF, IS, IC and CS samples using primers to the 3' UTR region provided molecular evidence that coronavirus was present, confirmed using primers specific for the nucleocapsid gene (Sellers *et al.*, 2004). Although it would be desirable, for logistical reasons in the field, to be able to detect *TCoV* by CS samples, in our study they were less suitable (i.e. gave fewer positive results) than FD, IS and IC samples. One possible explanation for this

was the high humidity and temperatures commonly found in the outbreak region. These conditions could have increased the chance of microbes (bacteria and moulds) growing on the CS, which might have damaged the nucleic acid of the *TCoV*, thus reducing the sensitivity of detection.

Considering Brazilian turkey industry needs, further investigations are necessary—firstly, to characterize the *TCoV* by virus isolation; secondly, by gene sequence comparison with other *TCoVs*; and finally, by investigation of the other pathogens (e.g. bacteria) that have been described as being involved in PEC associated with *TCoV*.

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Non-English Abstracts

Detection of turkey coronavirus in commercial turkey poults in Brazil

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Détection du coronavirus de la dinde chez des dindonneaux au Brésil

Le complexe entéritique du dindonneau (PEC) a été incriminé comme étant une cause majeure des pertes observées chez les dindonneaux dans d'autres pays. Nous avons observé ceci au Brésil, associé à une diarrhée, une réduction du gain de poids et généralement une mortalité élevée. Dans cette étude, nous avons utilisé une réaction de transcription inverse et de polymérisation en chaîne (RT-PCR) pour détecter le coronavirus de la dinde (TCoV) chez des dindonneaux malades âgés de 30 à 120 jours dans une région du Brésil où la production est importante. La RT-PCR a été appliquée à des extraits de suspensions de tissu d'intestin (IS), ainsi qu'aux contenus intestinaux (IC), bourse de Fabricius (BF), fientes fécales (FD) et écouvillons cloacaux (CS) issus des mêmes animaux. Des amorces ont été utilisées pour amplifier la région 3' conservée non traduite (3'UTR) du génome, et le gène de la protéine de la nucléocapside du TCoV. Des examens d'histopathologie et d'immunohistochimie directe ont été réalisés pour détecter l'antigène TCoV dans les coupes d'intestins infectés et de bourse. Tous les résultats obtenus à partir des tissus colorés ont révélé des lésions comme celles décrites précédemment dans les cas d'infection par le TCoV. Le signal positif de l'immunohistochimie directe a été présent au niveau de toutes les coupes d'intestin. Cependant, toutes les BF analysées ont été négatives. Les résultats de RT-PCR ont été positifs pour le TCoV dans tous les échantillons de FD, et dans 27% des CS. Finalement, le meilleur matériel pour diagnostiquer le TCoV a été les fientes fécales et/ou les suspensions d'intestin.

Nachweis von Putencoronavirus bei kommerziellen Putenküken in Brasilien

Der Enteritiskomplex bei Küken wird in vielen Ländern als häufigste Ursache für Verluste bei Putenküken angesehen. Wir haben diesen Krankheitskomplex verbunden mit Diarrhoe, Gewichtsverlusten und meistens hohen Mortalitätsraten auch in Brasilien beobachtet. In dieser Studie verwendeten wir die Reverse Transkriptase-Polymerasekettenreaktion (RT-PCR) für den Putencoronavirus(TCoV)-Nachweis bei erkrankten Putenküken im Alter von 30- 120 Tagen aus einer bestimmten Produktionsregion in Brasilien. Die RT-PCR wurde mit Extrakten von Darmgewebeproben (IS), entsprechendem Darminhalt (IC), Bursa Fabricii (BF), Kotproben (FD) und Kloakenabstrichen durchgeführt. Es wurden Primer für die Amplifikation der konservierten, nicht translatierten 3' Region (3'UTR) des Genoms und des Nukleokapsid-Proteingens des TCoV verwendet. Zum Nachweis des TCoV-Antigens in Schnitten von infiziertem Darm- und Bursagewebe wurden histopathologische und direkte immunhistochemische Untersuchungen vorgenommen. Alle beobachteten Veränderungen in den gefärbten Geweben stimmten mit den in der Literatur beschriebenen Veränderungen einer TCoV-Infektion überein. Bei der direkten immunhistochemischen Untersuchung wiesen alle Darmschnitte positive Reaktionen auf, wohingegen alle BF negativ waren. alle FD-Proben sowie 27% der CS-Proben waren in der RT-PCR für TCoV positiv. Daraus ergibt sich, dass sich Kotproben und/oder Darmsuspensionen am besten für die Diagnose von TCoV in Probenmaterial aus dem Feld eignen.

Detección de coronavirus del pavo en pavipollos comerciales en Brasil

El síndrome de enteritis del pavipollo (PEC) ha sido identificado como una de las principales causas de pérdidas económicas en pavipollos en otros países. Nosotros hemos observado este síndrome en Brasil, asociado a diarrea, pérdida de peso y, normalmente, alta mortalidad. En este estudio se ha llevado a cabo la

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transcriptasa reversa y reacción en cadena de la polimerasa (RT-PCR) para la detección del coronavirus del pavo (TCoV) en pavipollos enfermos de entre 30 y 120 días de vida, procedentes de una región concreta de Brasil. La RT-PCR se realizó a partir de extracciones de homogeneizados de tejido intestinal (IS) y los respectivos contenidos intestinales (IC), bolsa de Fabricio (BF), heces (FD) e hisopos cloacales (CS). Para la amplificación de la región no codificante 3' (3'UTR) del genoma y del gen de la nucleocápside del TCoV se utilizaron cebadores. Se llevaron a cabo estudios histopatológicos y la técnica de inmunocitoquímica directa para la detección del antígeno de TCoV en preparaciones de intestinos infectados y de bolsas de Fabricio. Todos los resultados de las tinciones mostraron lesiones previamente descritas en la infección por TCoV. Se observaron tinciones positivas en inmunocitoquímica directa de todas las muestras intestinales. Sin embargo, todas las BF analizadas fueron negativas. Todas las muestras de FD y un 27% de las de CS fueron positivas mediante RT-PCR. Finalmente, se determinó que la mejor muestra para el diagnóstico de TCoV son las heces y/o los homogeneizados intestinales.

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