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ORIGINAL ARTICLE

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Transmission Kinetics and histopathology induced by European Turkey Coronavirus during experimental infection of specific pathogen free turkeys

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Abstract

Numerous viruses, mostly in mixed infections, have been associated worldwide with poult enteritis complex (PEC). In 2008 a coronavirus (Fr-TCoV 080385d) was isolated in France from turkey poults exhibiting clinical signs compatible with this syndrome. In the present study, the median infectious dose (ID_{50}), transmission kinetics and pathogenicity of Fr-TCoV were investigated in 10-day-old SPF turkeys. Results revealed a titre of $10^{4.88}$ ID_{50}/ml with 1 ID_{50}/ml being beyond the limit of genome detection using a well-characterized qRT-PCR for avian coronaviruses. Horizontal transmission of the virus via the airborne route was not observed however, via the oro-faecal route this proved to be extremely rapid (one infectious individual infecting another every 2.5 hr) and infectious virus was excreted for at least 6 weeks in several birds. Histological examination of different zones of the intestinal tract of the Fr-TCoV-infected turkeys showed that the virus had a preference for the lower part of the intestinal tract with an abundance of viral antigen being present in epithelial cells of the ileum, caecum and bursa of Fabricius. Viral antigen was also detected in dendritic cells, monocytes and macrophages in these areas, which may indicate a potential for Fr-TCoV to replicate in antigen-presenting cells. Together these results highlight the importance of good sanitary practices in turkey farms to avoid introducing minute amounts of virus that could suffice to initiate an outbreak, and the need to consider that infected individuals may still be infectious long after a clinical episode, to avoid virus dissemination through the movements of apparently recovered birds.

KEYWORDS
coronavirus, histopathology, transmission, turkeys

1 | INTRODUCTION

Coronaviruses, order *Nidovirales* family *Coronaviridae* are enveloped viruses with a genome of single stranded positive sense RNA. To date four genera of coronaviruses exist, alpha, beta, delta and gamma, defined on the basis of phylogenetic groups. The genus gamma-coronavirus is mainly composed of viruses isolated from birds (avian coronaviruses, AvCoVs), including infectious bronchitis virus (IBV), Turkey coronavirus (TCoV) and guinea fowl coronavirus (GfCoV) (Ducatez, Liais, Croville, & Guerin, 2015; Fehr & Perlman, 2015; Masters & Perlman, 2013).

Infectious bronchitis virus is a highly contagious virus transmitted very quickly among naive birds in the field. It is responsible worldwide for respiratory diseases, egg drop with poor eggshell quality, reduced hatchability, nephritis and sometimes, in early infection of future breeders, genital atrophy responsible for the syndrome of "false laying" in chicken breeders or layers (Jackwood, & Wit, 2013).

Turkey coronavirus, originally identified in the USA in the 1970s as one of the agents responsible for an acute enteritis named bluecomb (Panigrahy, Naqi, & Hall, 1973; Ritchie, Deshmukh, Larsen, & Pomeroy, 1973) and since with a multifactorial disease known as poult enteritis complex of turkeys (PEC) (Barnes, Guy, & Vaillancourt, 2000), has now been detected in most areas where turkeys are farmed (Breslin, Smith, Barnes, & Guy, 2000; Cavanagh et al., 2001; Dea & Tijssen, 1988; Domańska-Blicharz, Seroka, Lisowska, Tomczyk, & Minta, 2010; Martin, Vinco, Cordioli, & Lavazza, 2002; Maurel et al., 2009; Teixeira et al., 2007), although TCoVs isolated in Europe have been shown to have a different genetic lineage to those isolated in the USA (Brown et al., 2016; Maurel et al., 2011). PEC includes several intestinal disorders that occur in turkeys mostly within the first three weeks of life (Guy, 2013) and its clinical signs often include diarrhea, stunting, anorexia, dehydration, weight loss, and immune dysfunction (atrophy of the thymus and the bursa of Fabricius) that promotes secondary infections. The wide distribution of both IBV and TCoV and their highly contagious nature have considerable economic repercussions.

The contagious nature of a disease can be measured by the "reproduction number" (R0) defined as "the expected number of secondary cases produced by a single (typical) infection in a totally susceptible population" (Masters & Perlman, 2013). The parameters necessary to calculate R0 are (a) the speed of transmission and (b) the shedding duration of the infectious viruses. Generally, a virus with an R0 less than 1 will disappear quickly because an infected individual will have a low ability to infect another. A virus with an R0 greater than one will spread in the susceptible population. For IBV, an R0 of 19.95 has been estimated (de Wit, de Jong, Pijpers, & Verheijden, 1998), which is a figure comparable to the R0 of highly contagious human viruses such as measles virus (R0 12-18) (Masters & Perlman, 2013). For TCoV, R0 has not yet been fully calculated; however, a study with an American TCoV isolate demonstrated that infectious virus particles can be shed up to six weeks post-infection in experimentally infected turkeys (Breslin et al., 2000).

The current study focused on strain Fr-TCoV 080385d that was detected in France in 2008 in turkeys with clinical signs compatible with PEC. Fr-TCoV is the only European TCoV strain isolated to date, although coronaviruses have been detected in turkeys in Poland, Great Britain and Italy (Cavanagh, 2001; Domańska-Blicharz et al., 2010; Martin et al., 2002). The aim of this study was to determine the transmission properties of the virus by evaluating its ID_{50} and reproduction number (R0) under experimental conditions in 10-day-old SPF turkeys, in order to better understand the diffusion of the disease. Histopathological examination and in-situ detection of TCoV antigen at the sites of replication in the intestinal tract were also performed.

2 | METHODS

2.1 | Ethics statement

Three animal experiments (Exp 1, 2 and 3) were performed in agreement with the national regulations of the French Ministry for higher education and research on animal welfare and after approval from the French Agency for Food, Environmental and Occupational Health & Safety's (ANSES) ethical committee.

2.2 | Virus preparation and titration

Virus Fr-TCoV 080385d isolated from duodenal contents of 42-day-old turkeys affected by PEC in November 2008 was propagated by inoculating embryonated SPF turkey eggs (Anses, Ploufragan, France) via the intra-amniotic route, as previously described (Guionie et al., 2013). Because Fr-TCoV 080385d does not induce clinical lesions in the embryo, the intestines of inoculated embryos were screened 4 days post-inoculation by qRT-PCR (Maurel et al., 2011), and the intestines of positive embryos were collected and pooled to prepare a virus stock (22). Five-fold serial dilutions of this stock were inoculated into seven eggs per dilution, and a titre of $10^{4.01}EID_{50}$/ml was calculated according to Reed & Muench (20).

2.3 | RNA extraction and qRT-PCR RT-PCR

One hundred microliters of intestinal or cloacal swab material was lysed with 300 µl of Buffer RLT (Qiagen, France) by mixing and incubating at room temperature for 15 min. RNA was extracted using MagAttract RNA Tissue Mini M48 kit or MagAttract Virus Mini M48 kit for BioRobot M48 (Qiagen, France) and eluted in 100 µl of buffer AVE following the manufacturer's instructions. The presence of TCoV genome was detected using a qRT-PCR specific for Avian Coronaviruses (Maurel et al., 2011). The limit of detection (LoD) and the linear phase of this qRT-PCR were described as 2 log10 and from 3 to 9 log10 copies per microliter of extracted RNA, respectively. In this study, samples were considered positive with a result higher than 2 log10 copies per microliter of extracted RNA. All results are given as copy number (cp)/µl of extracted RNA expressed in log10 together with the SD

2.4 | Exp 1. Titration of Fr-TCoV in 10-day-old SPF Turkeys

Thirty 10-day-old SPF turkeys were separated in 5 groups of 6 birds, and housed for 3 days in negative pressure isolators allowing ad lib feeding and drinking. Each isolator had a cardboard floor with a metal grid platform underneath and a surface area of 1.4 m^2. Groups 1, 2, 3 and 4 were inoculated via the oral route with 0.25 ml of

strain Fr-TCoV 080385d diluted to $10^{-1.5}$, $10^{-3.0}$, $10^{-4.5}$ and $10^{-6.0}$ respectively in MEM Hepes (Gibco, France) supplemented with penicillin (200 μ/ml final concentration) and streptomycin (0.2 mg/ml final concentration). Control group 5 was inoculated with MEMH plus antibiotics alone via the same route. At 1-day post-inoculation (dpi), two SPF turkey contacts were introduced into groups 1–4 as sentinels to demonstrate horizontal transmission of infectious virus. From 1 to 3 dpi, cloacal swabs were collected from all subjects, sampling the contacts first, followed by those that had been inoculated. RNA was extracted from these samples for molecular analysis as described above. The 50% endpoint was calculated using the method of Reed and Muench (Reed & Muench, 1938).

2.5 | Exp 2.: Transmission by contact from a seeder bird

Thirty-two 10-day-old SPF turkeys were separated into groups, one containing 29 subjects and a second containing 3. Each group was housed in a separate negative pressure room at a density of seven birds per m² and floors were covered with wood chippings (reproducing common commercial rearing conditions in France). The group of three subjects was inoculated with 0.25 ml of strain Fr-TCoV 080385d diluted at $10^{-4.5}$ in the same media as used in Exp. 1, via the oral route. At 1 dpi, cloacal swabs were collected to confirm their Fr-TCoV 080385d positive status by qRT-PCR. At 2 dpi, one positive subject was placed as a seeder infected bird among the group of 29 SPF subjects (contacts). Cloacal swabs were collected from all subjects every 2 hr until 16 hr post-contact (hpc), at 24 hpc and 2 days post-contact (dpc) then weekly until 41 dpc.

During the 2-hr-sampling regime, the order in which the subjects were taken was respected throughout. This ensured that each subject was sampled precisely every two hours. Sampling staff wore a new pair of sterile gloves for each sampled bird, so as not to transfer the virus through bird-handling. RNA was extracted from these samples to perform qRT-PCR, to determine infection and the excretion period for each subject.

The transmission characteristics were assessed considering the evolution of individuals through the susceptible, infectious and recovered stages (SIRmodel). Susceptible (S) animals correspond to naïve individuals who are exposed to the virus shed by infectious (I) animals. The individuals then turn to the recovered (R) stage at the end of the shedding period. The transmission rate, denoted as β, reflects the number of new infections generated by one typical infectious individual per time unit. In this study, owing to the high transmissibility of the virus, a two-hourly time scale was selected. With these notations, the probability for a susceptible individual to become infected on a time interval $[t, t + \Delta t]$ is given by $p_t = 1 - \exp(-\beta I_t \Delta t/N)$, where N is the total number of individuals involved in the experiment (here, $N = 30$). Therefore, the number of new cases on each time interval $[t_i, t_{i+1}]$ follows a binomial distribution with parameters S_{t_i}, the number of susceptible individuals at time t_i, and p_{t_i}, the probability of infection. The number of susceptible and infectious animals was updated for each sampling interval, as well as the number of new cases, allowing the estimation of the transmission rate parameter β. The generalized linear model approach was used for the estimation, using the complementary log-log link function and taking $\log\left(\frac{I}{N}\Delta t\right)$ as offset variable (Becker, 1989; Eblé, De Koeijer, Bouma, Stegeman, & Dekker, 2006; Velthuis, De Jong, Kamp, Stockhofe, & Verheijden, 2003).

The duration of excretion of Fr-TCoV 080385d was measured in terms of presence of viral RNA during the course of this experiment, independently from the infective capacity of the detected viral particles. A further experiment (Exp 3.) was therefore conducted in SPF turkeys, to assess the infectivity of the samples confirmed positive by qRT-PCR.

2.6 | Exp 3. – Assessing infectivity of TCoV at different sampling times

Exp 3 objectives were (a) to assess the shedding duration of infectious virus in samples collected at different time points during Exp 2, (b) to evaluate tissue distribution of Fr-TCoV in infected birds, (c) to perform a preliminary assessment of the airborne route of transmission.

One representative positive sample selected at 6 dpc of Exp 2. (codified T6) was diluted (same media as Exp. 1) so as to inoculate via the oral route $10^{5.7}$ RNA copies in three 10-day-old SPF turkeys. They were housed in a negative pressure room, under the same rearing conditions as in Exp 2, with three 11-day-old SPF turkeys introduced as contact-birds at 1 dpi to demonstrate horizontal transmission. Cloacal swabs were collected daily for qRT-PCR analyses from all birds until 3 dpi, when the birds were humanely euthanized and duodenum, jejunum, ileocaecal junction and bursa of Fabricius were collected. These samples were fixed for 24 hr in 4% formaldehyde then transferred to 70% ethanol and finally embedded in paraffin wax for histopathology and anti-TCoV immunohistochemistry (see section Histopathology). This process was repeated using one representative positive sample from 13, 21, 27, 34 and 41 dpc of Exp 2. (codified T13, T21, T27, T34 and T41, respectively) to make a total of six experiments. Airborne transmission was evaluated in each of these experiments by using six 10-day-old SPF turkeys housed in a park in the same containment cell but separated from the other animals, at a distance of 3 meters. The sampling programme was as described above. Housing, circulation of personal, change of boots, clothes and gloves was organized to minimize physical contamination.

2.7 | Histopathology and anti-TCoV immunohistochemistry

Duplicate tissue slides were cut from formalin-fixed, dehydrated, and paraffin-embedded intestinal samples (duodenum, jejunum, ileocaecal junction, bursa of Fabricius), collected from Exp. 3. For routine histopathologic evaluation, one slide was stained with haematoxylin and eosin (HE) according to standard laboratory procedures. The other duplicate slide was deparaffinized with xylene and rehydrated in alcohol series and subsequently subjected to endogenous

peroxidase inactivation in 1% hydrogen peroxide in methanol for 20 min, antigen retrieval via boiling in Tris-ethylenediaminetetraacetic acid (EDTA), pH 9.0 for 10 min and double washing in phosphate buffered Normal Antibody Diluent (NAD, ScyTek Laboratories, Logan, USA) containing 0.1% Tween-20. Tissue sections were then incubated with mouse monoclonal Ab anti IBV M-protein 25.1 (D274, Centraal Veterinair Instituut, Lelystad, the Netherlands) diluted 1:400 in NAD for 60 min at room temperature. Based on pilot experiments (data not shown), this Ab successfully cross-reacted with Fr-TCoV which is likely due to the highly conserved nature of the targeted protein in avian gamma coronaviruses (>90% amino acid identity) (Brown et al., 2014).

Primary antibody binding was detected via subsequent incubation with Dako Envision HRPO labeled polymer goat anti-mouse (Dako, by Agilent Technologies, Santa Clara, USA) diluted 1:1 in NAD (30 min, room temperature), and visualized by administration of 3-Amino-9-ethylcarbazole (AEC, Dako). Fr-TCoV-induced histopathology and Fr-TCoV protein expression were assessed by light microscopy (BX40, Olympus, Tokyo, Japan).

3 | RESULTS

3.1 | Exp 1. Titration of Fr-TCoV in 10-day-old SPF Turkeys

Fr-TCoV was detected with qRT-PCR at 1 dpi in all six inoculated subjects of group 1 (dilution $10^{-1.5}$, mean ± SD 5.19 ± 0.94 $\log_{10}$ cp/µl), in 5 out of 6 subjects of group 2 (10^{-3}, 4.46 ± 1.81 $\log_{10}$ cp/µl) and in 3 out of 6 subjects in group 3 ($10^{-4.5}$, 3.59 ± 1.37 $\log_{10}$ cp/µl). At 2 and 3 dpi, all subjects of these groups, including contact-birds, were positive, demonstrating horizontal transmission. No viral RNA was detected throughout the experiment in groups 4 (10^{-6}) and 5 (MEMH). The result obtained at 1 dpi (before horizontal transmission) gave a virus titre of $10^{4.88}$ ID_{50}/ml.

3.2 | Exp 2. Transmission by contact from a seeder bird

The following data are shown graphically in Figure 1.

An inoculated subject with a viral RNA load of 5.28 $\log_{10}$ cp/µl at 1 dpi that had been placed among 29 contacts, transmitted the virus to one contact between 8 and 10 hpc, though the level of viral RNA detected at 10 hpc in this newly infected bird (2.05 $\log_{10}$ cp/µl) was almost at the LoD. However, between 10 and 12 hpc the level of viral RNA detected in the same bird increased to 3.39 $\log_{10}$ cp/µl and a second contact was positive at 2.19 $\log_{10}$ cp/µl. Between 12 and 14 hpc, seven contacts were positive with values ranging from 2.24 to 4.77 $\log_{10}$ cp/µl. Between 14 and 26 hpc, 23 contacts were positive with a mean ± *SD* of 4.86 ± 0.99 $\log_{10}$ cp/µl. In the subsequent days (2–13 dpc), all contacts were positive with mean ± *SD* values of 6.05 ± 0.54, 5.07 ± 0.76, 5.57 ± 0.38, 4.80 ± 0.76 $\log_{10}$ cp/µl at 2, 3, 6, 13 dpc respectively. The transmission rate β was estimated to be 0.42 $Turkey^{-1}$ h^{-1} (confidence interval [0.27, 0.62]). Otherwise stated, one infected animal had, on average, infected 1 animal every 2.5 hr. For the following 2 weeks (21 and 27 dpc), viral RNA was detected in almost all contacts (N = 25, mean ± SD 3.46 ± 0.79 and N = 27, mean ± *SD* 3.58 ± 0.80 $\log_{10}$ cp/µl respectively). At 34 and 41 dpc, the number of positive contacts was reduced to 16 and 21 respectively with RNA loads near to the LoD (mean ± SD 2.85 ± 0.72 and 2.63 ± 0.54 $\log_{10}$ cp/µl). This result would suggest the shedding period to be longer than 41 dpc. However, this estimation was based on the detection of viral RNA, ignoring the infective potential of the viral particles, which was investigated as Exp. 3.

3.3 | Exp 3: Assessing infectivity of TCoV at different sampling times

The data are shown graphically in Figure 2. In three out of six Exp 2. samples (T6, T27 and T41), the number of positive inoculated birds and the level of viral RNA detection increased over time during the sampling period, culminating at 3 dpi with RNA detected in all birds including contacts (mean ± *SD* = 4.89 ± 0.69, 5.75 ± 0.32 and 4.55 ± 0.70 cp/µl, respectively). No viral RNA was detected throughout the period, neither in inoculated or contact subjects exposed to T13, T21 and T34, nor in subjects assigned to the assessment of airborne transmission.

3.4 | Histopathology and anti-TCoV immunohistochemistry: Widespread antigen distribution in lower gut in the absence of microscopic lesions

Intestinal samples taken from infected subjects at 3 dpi from Exp. 3 showed well-preserved characteristic architectural features. Except for some very mild hyperemia and rare epithelial desquamation, no clear histopathological changes were seen in any of the samples (Figure 3a). Immunohistochemical staining showed an abundance of viral protein expressed in the ileum, caeca and bursa of all inoculated or contact subjects exposed to T6, T27 and T41 (expression in the caecum and bursa is shown for T41 in Figure 3). As shown in Figure 4 histograms, antigen detection in the other regions of the intestine (duodenum or jejunum) was inconsistent in both the inoculated and contact-birds exposed to the same samples, as illustrated by the fact that no viral protein was detected in the duodenum of any contact subject exposed to T6, T27 and T41. No viral protein expression was seen in any of the intestines taken from the inoculated and contact subjects exposed to T13, T21 and T34.

In all positive cases, viral protein prominently presented in both enterocytes and goblet cells and in limited situations in desquamated cells and intraluminal debris. Viral protein expressing enterocytes and goblet cells were mainly situated in the villar region and hardly in the crypt region. The number of cells per tissue section containing viral protein varied from only few to very many. In general, the highest number of cells containing viral protein was found in the caudal intestinal tract (ileum, caecum and bursa). A positive sample (a

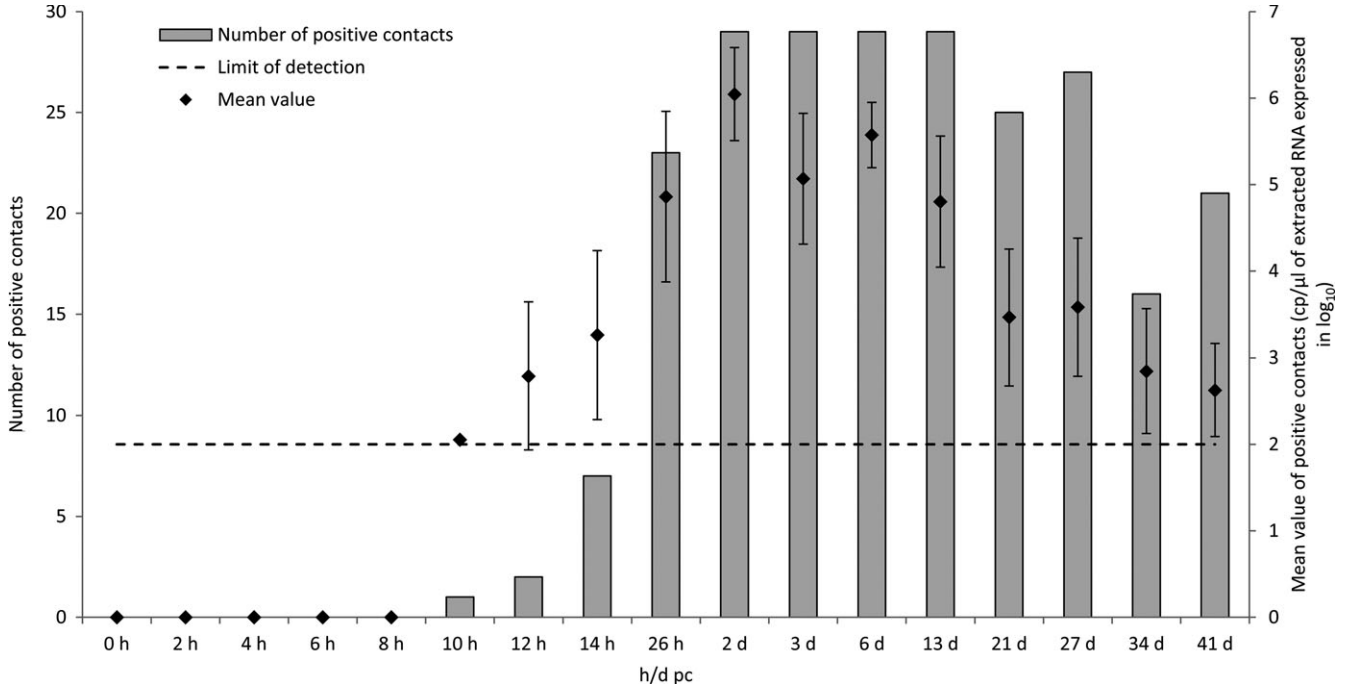

FIGURE 1 Detection of Fr-TCoV080385d viral RNA in 29 SPF turkeys placed in contact with one seeder turkey at 10 days of age. The *x*-axis represents sampling dates (hours (h) or days (d) post-contact). The 2 hr sampling period is underlined. The *y*-axis to the left represents the number of positive contacts. The *y*-axis to the right represents the viral RNA load in positive subjects, expressed as mean $\log_{10}$ copy number per microliter

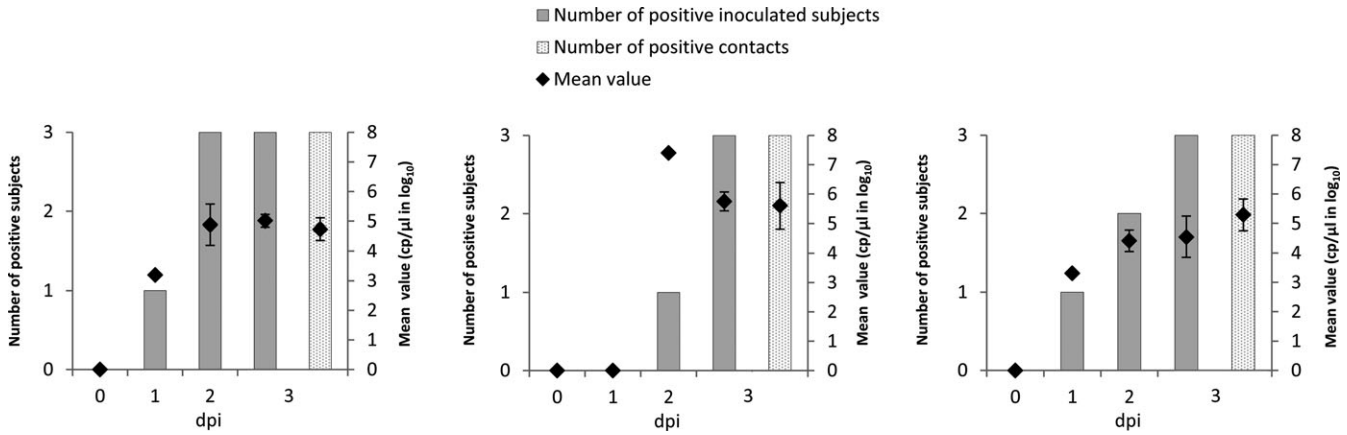

FIGURE 2 Exp 3 = Detection of infectious Fr-TCoVin SPF turkeys inoculated with intestinal samples collected in Exp2 at 6, 27 or 41 days post-exposure (a, b or c, respectively). The x-axis represents sampling dates (days post-inoculation in Exp 3). The y-axis to the left represents the number of positive subjects. The y-axis to the right represents the mean viral RNA copy number in positive subjects

subject inoculated with T41) showing virus protein in caecal enterocytes is shown in Figure 3b. In the bursa of Fabricius and near the caecal tonsil, viral protein expression was mostly restricted to the epithelium covering the lymphoid tissue (Figure 3c,d), but less distinct and fragmented staining was also regularly seen subepithelially in the lymphoid follicles. Incidentally, individual cells in the lymphoid follicles seemingly showed viral protein comparable (in terms of quantity) to the epithelial viral protein expression. One subject (negative control from airborne transmission experiment), which was negative by qRT-PCR, was used as a control for histopathology. This subject neither showed histopathological changes nor viral protein expression in any of the examined intestinal samples.

4 | DISCUSSION

When considering viral infection in animals the infectious dose, the transmission rates, the age of animal infected and environmental conditions are all influencing factors of efficacy. In the current study the infectious dose, transmission rate, duration of excretion and

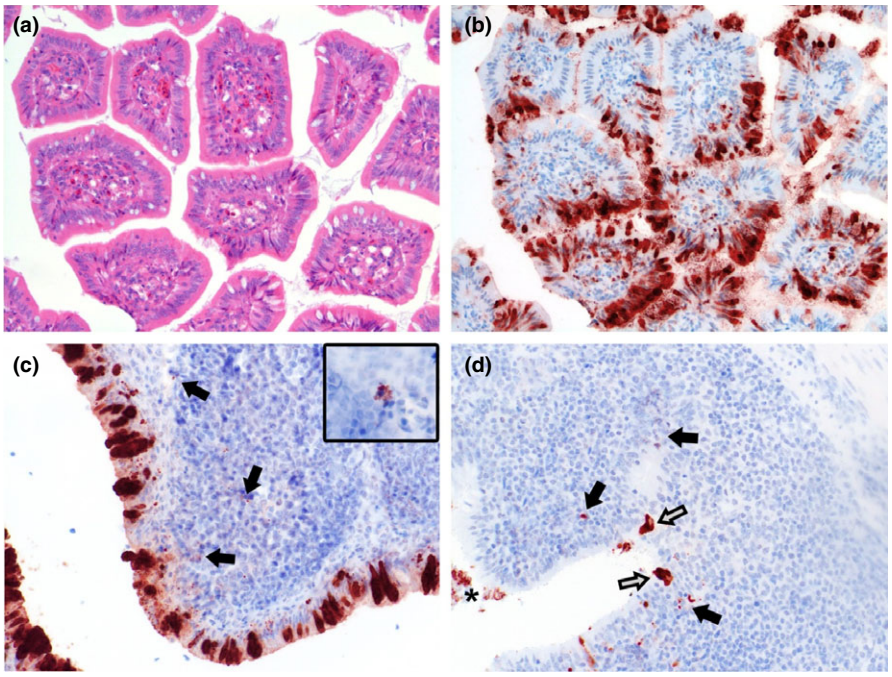

FIGURE 3 TCoV histopathology and protein expression, 3 dpi. Panels a and b: Serial transversal sections of the caecal mucosal folds (villi). Although no clear virus-induced histopathologic epithelial changes are present, besides very mild hyperemia of the lamina propria (a), abundant viral protein expression in the caecal enterocytes is seen (brown staining in b). Panels c and d: Viral protein expression in the epithelium (white arrows) and more fragmented and dotted, finely dispersed immunohistochemical signal subepithelially (black arrows) in the lymphoid follicles of the Bursa of Fabricius (c) and the caecal tonsil (d). Panel c inset: occasional single cells in the bursal lymphoid follicles, demonstrating the intracytoplasmic presence of viral protein. All depicted tissues are taken from the same T41 contact subject. All tissues are visualized by light microscopy at 200× (a, b, d), 400× (c) or 800× (inset c) magnification. A = hematoxylin and eosin staining; B, C + inset, D: immunohistochemistry anti TCoV. Asterisk: desquamated cells in the intestinal lumen [Colour figure can be viewed at wileyonlinelibrary.com]

induced histopathology in ten-day-old SPF turkeys for the European strain of turkey coronavirus Fr-TCoV 080385d were evaluated.

The controlled experimental infections performed in this study demonstrated the Fr-TCoV080385d virus stock to have a titre in 10-day-old SPF turkeys of $10^{4,88}$ ID_{50}/ml. Considering the same virus stock had been titrated previously in embryonated turkey eggs and found to have a virus titre of $10^{4,01}$ EID_{50}/ml, this would suggest that 10-day-old SPF turkeys are more sensitive than embryonated eggs (1 EID50 = 7 ID50) in recovering infectious TCoV, although the repeatability of Reed & Muench titration needs to be taken into consideration. Similarly, at one ID_{50}/ml, viral RNA levels were also beyond the limit of detection of a well characterized qRT-PCR (Maurel et al., 2011), so that 10-day-old turkeys might be also more sensitive than qRT-PCR to detect infectious TCoV. Such a high susceptibility of young hosts was also reported in a recent paper where an enteric coronavirus of pigs (porcine epidemic diarrhea virus, PEDV) was shown to infect more efficiently 5-day-old piglets than tissue culture (minimal infectious dose = 0.056 TCID50) (Thomas et al., 2015). RNA levels at the PEDV MID have also been reported to be beyond the limit of detection by qRT-PCR (Goyal, 2014). The effects of turkey age on the ID_{50} of Fr-TCoVwere not investigated in the current study however, the fact that TCoV associated enteric disorders such as PEC or poult enteritis mortality syndrome (PEMS) are predominantly diseases of younger subjects (Barnes et al., 2000) lends support to more resistance in older birds.

An extremely rapid transmission via the oro-faecal route was observed under the current experimental conditions for 10-day-old SPF turkeys resulting in almost all of the naïve contact subjects being infected from one infectious individual within 24 hr. No airborne transmission over a distance of 2–3 m was observed. Mathematically the results of the oro-faecal transmission gave a calculated rate of one newly infected individual every 2.5 hr and provided a figure for the first parameter in estimating an Fr-TCoV R0. This transmission rate was measured in terms of detection of viral RNA and the passage of each individual from a negative to positive status above the threshold of the qRT-PCR used. Considering what was discussed previously regarding the relative sensitivity of qRT-PCR and 10-day-old turkeys to detect infectious TCoV, the actual rate of transmission could be quicker. The qRT-PCR used in this study is in the authors' experience the most sensitive tool for detecting Fr-TCoV and thus until a more sensitive method becomes available the calculated figure of 2.5 hr cannot be refined. Nevertheless, these results showed that Fr-TCoV can be transmitted extremely rapidly and also draw attention to the fact that when calculating transmission rates, no matter the pathogen, results will relate to the sensitivity of the diagnostic tools applied. In other words, more sensitive tools for detection equals increased transmission rates.

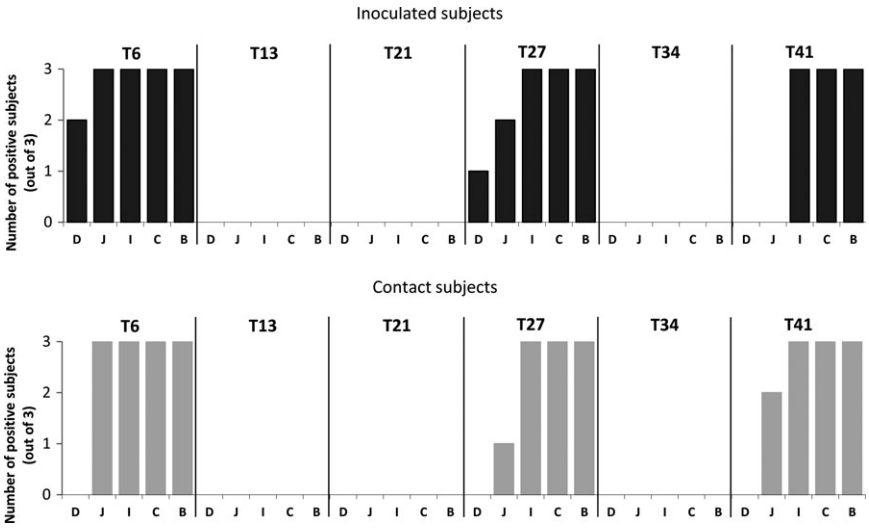

FIGURE 4 Detection of viral antigen in intestinal tissues. Immunohistochemistry of: intestinal tissues D = duodenum, J = jejunum, I = ileum, C = caeca B = bursa of Fabricius, taken 3 dpi from subjects inoculated with samples T6, T13, T21, T27, T34 orT41 of Exp 2 (a) and from their corresponding contacts (b)

The shedding duration of infectious virus, as the second R0 parameter, was more complicated to determine, as detection of viral RNA could not be taken to represent infectious particles. Thus, this parameter could only be solidly determined if individual re-infection of SPF turkeys with each of the samples at every date was performed. This requisite could not be fulfilled in the current study and was also difficult to justify on an ethical level in respect to the principals of the 3 Rs (Russell & Burch, 1959).

Although the "duration of excretion" for every individual could not be obtained, the experiments performed in the current study (in which one sample from each date was re-inoculated) revealed that some subjects continued to shed infectious virus for at least six weeks when others ceased at two. At this time the authors have no data on why some subjects stopped excreting infectious virus four weeks in advance of others or if, in fact, excretion detected at six weeks was representative of subjects with intermittent excretion profiles as has been observed in cats experimentally infected with FECV (Kipar, Meli, Baptiste, Bowker, & Lutz, 2010). In cats, this intermittent excretion has been suggested to be linked with persistent infection in the colon (lower intestine) from which viruses then have the potential to re-infect the small intestine at any time. The presence of virus in both regions of the gut can then result in renewed excretion (Kipar et al., 2010). Concerning Fr-TCoV these questions should be assigned to specifically designed trials and histopathological examination, however, the present study seems to indicate a clear tropism of Fr-TCoV for lower intestine, as described for FECV (Kipar et al., 2010). Indeed, in the current study, Fr-TCoV's ability to infect the turkey intestinal tract was successfully demonstrated with immunohistochemical staining. The virus showed preferred tropism for the lower intestines similar to what has been also observed for US lineage TCoV (NC95) (Guy, Smith, Breslin, Vaillancourt, & Barnes, 2000), which likely reflects distribution of the TCoV receptor (Ambepitiya Wickramasinghe et al., 2015). This distribution pattern could be time-dependent though and since all tissue samples were collected at a single time point (3 dpi in Exp 3), evaluation of intestinal segments taken at different time points after infection would be needed to fully confirm these observations.

Regarding specific cell tropism within the target tissue, gut-associated lymphoid tissue (among which the so-called caecal tonsil) and the lymphoid tissue in the bursa of Fabricius might be of special interest. Although viral protein expression was most distinctively seen in epithelial cells (enterocytes) overlying these subepithelially-located lymphoid tissues, in several birds a delicate, finely dispersed and dotted immunohistochemical staining signal was present subepithelially within these lymphoid tissues. Taking into account both the signal's fragmented subtle aspect compared to the signal at the epithelial level and otherwise the biological function of these lymphoid aggregates, the authors speculate that this might be the result of immunohistochemical demonstration of degraded viral protein within phagocytozing antigen-presenting cells. Occasionally, as shown in the inset picture in Figure 3c, individual cells in the subepithelial lymphoid tissues displayed a 'full-cytoplasmic' immunohistochemical staining signal comparable to the signal seen in the majority of infected enterocytes. Since, upon viral inoculation of an animal, such abundant intracellular signal is usually seen in cells known to be within the virus' tropism (in other words 'presumably correlated with true viral replication'), it is therefore possible that Fr-TCoV can incidentally replicate in antigen-presenting cells (APCs), represented by dendritic cells and monocytes/macrophages. APC tropism was recently demonstrated for subtypes of TCoVs relative IBV, with this ability to infect APCs being a determinant for cell-mediated viremic systemic spread and subsequent infection of the chicken's urinary tract (Reddy et al., 2016). However, although the stellate morphology and the localization of the depicted cell (see inset Figure 3c) suggest that it likely belongs to one of the mentioned APC families, additional assays characterizing these cell types (for example double immunohistochemical staining for both viral protein expression and cell-characterizing protein epitopes) are needed to confirm a APC tropism for TCoV.

Fr-TCoV viral protein expression was not correlated with histopathologic changes in the sampled tissues collected here, contrary to previous observations following inoculation with a TCoV of US lineage that did induce lesions, albeit without associated clinical signs (Guy et al., 2000). This discrepancy could be explained by the

difference in age of the birds inoculated as in the US TCoV study, birds had been inoculated at 6 days of age; alternatively, like the distribution pattern discussed above, this discrepancy might be a time-dependent feature, with 3 dpi in our study being too early for morphologic changes – resulting from epithelial damage, local tissue reactions and influx of immune cells to manifest. Furthermore the specific date post-inoculation when microscopic lesions were observed in the US TCoV study was not given (Guy et al., 2000). It is equally possible that under experimental conditions the European lineage of TCoV simply has a different pathogenic profile to those of the US lineage. Infection studies for EU and US TCoVs in turkeys of the same age and under the same controlled conditions are required for comparative analysis into the pathological profiles of these different lineages.

In conclusion an extremely low dose of European isolate Fr-TCoV strain 080385d is required for infection of 10-day-old turkey poults under experimental conditions. The virus spreads very quickly via the oro-faecal route among susceptible subjects (a new subject at least every 2.5 hr) and infectious virus may continue to be excreted for at least six weeks after the initial infection which may be linked to a preferential tropism for the lower intestines. These results stress the importance of good sanitary practices at the entrance to livestock buildings and the need to consider that infected individuals may still be infectious long after the clinical episode.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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