

ORIGINAL ARTICLE

An experimental study of the survival of turkey coronavirus at room temperature and +4°C

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Turkey coronavirus (TCoV) is a gammacoronavirus (*Coronaviridae*, *Nidovirales*) responsible for digestive disorders in young turkeys. TCoV has been associated with poult enteritis complex, a syndrome that severely affects turkey production. No medical prophylaxis exists to control TCoV, therefore sanitary measures such as cleaning and disinfection are essential. It is thus important to evaluate temperatures that allow persistence of TCoV in the environment. Two series of aliquots of a suspension of a French isolate of TCoV (Fr TCoV) were stored at room temperature or +4°C for 0 to 40 days. As TCoV does not grow in cell culture, the presence of residual infectious TCoV in the stored samples was tested by inoculating embryonated specific pathogen free turkey eggs. As TCoV does not induce lesions in the embryo, virus replication in the jejunum and ileum of the embryos was detected 4 days post inoculation, using RNA extraction and a real-time reverse transcriptase-polymerase chain reaction based on the nucleocapsid gene. No surviving virus was detected after 10 days storage at +21.6 ± 1.4°C or after 40 days storage at +4.1 ± 1.6°C, these temperatures being representative of the mean summer and winter temperatures, respectively, in the major French poultry-producing region. The relatively short survival of the virus at room temperature should contribute to limited virus survival during summer months. However, infectious virus was still detected after 20 days storage at the cooler temperatures, a finding that suggests prolonged survival of Fr TCoV and easier transmission between poultry farms in a cool environment are possible.

Introduction

Turkey coronavirus (TCoV) belongs to the genus *Gammacoronavirus* in the subfamily *Coronavirinae*, part of the family *Coronaviridae* in the order *Nidovirales* (De Groot *et al.*, 2008). TCoV is an enveloped virus with a 28-kb-long single-stranded positive-sense RNA genome (Cao *et al.*, 2008; Gomaa *et al.*, 2008; Jackwood *et al.*, 2010). Together with infectious bronchitis virus (IBV), TCoV is one of the two most economically significant avian coronaviruses. Unlike IBV, however, TCoV has not been adapted to grow in cell cultures and can only be propagated *in ovo* or *in vivo*.

TCoV was shown in the 1970s to be one of the causative agents of an enteric disease known as bluecomb, transmissible enteritis or coronaviral enteritis of turkeys (Guy, 2008). More recently, TCoV and several other agents (viruses, bacteria, protozoa, fungi) have been associated with poult enteritis complex (PEC) (Barnes & Guy, 2003). PEC is a general term for a group of multifactorial, transmissible infectious diseases of young turkey poults up to 7 weeks of age, with signs including enteritis, retarded development, impaired feed utilization and frequent immune dysfunction (Barnes *et al.*, 2000). PEC severely affects turkey production (Barnes *et al.*, 2000).

In Europe, TCoV has been detected in the UK (Cavanagh *et al.*, 2001), and more recently in France (Maurel *et al.*, 2009, 2010, 2011) and Poland (Domanska-Blicharz *et al.*, 2010). A coronavirus was also detected in turkeys with enteritis in Italy (Moreno Martin *et al.*, 2002). In France, since 2003, the number of turkey flocks exhibiting clinical signs compatible with PEC has increased and PEC has become an important concern (Germain & Rousseau, 2005). A survey performed from 2007 to 2009 and based on a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay specific for the nucleocapsid gene of IBV and TCoV (Maurel *et al.*, 2009) revealed that 37% of turkey flocks suspected of PEC were positive for TCoV, whereas only 17% were positive among flocks without enteritis or with an enteritis not evocative of Poult Enteritis (PEC)/Mortality Syndrome (PEMS). The virus was found predominantly in the digestive contents (41% in the jejunum, 39% in the caecum) (Maurel *et al.*, 2011). The genetic characterization of French strains of TCoV (Fr TCoV) demonstrated that although they are related to their North American (NA TCoV) counterparts, they form an independent sublineage and might have been generated by recombination events involving different

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IBV strains than those involved in the emergence of NA TCoV (Jackwood *et al.*, 2010; Maurel *et al.*, 2011). Interestingly, the IBVs most related to Fr TCoV appeared to be 4/91 and QX (ITA/90254/2005), respectively, which represent some of the more recently emerged IBV serotypes in Europe (Worthington *et al.*, 2008).

To date, no medical prophylaxis exists to control TCoV infection. It is therefore essential to control this virus by sanitary measures, such as cleaning and disinfection. In this respect, evaluating the survival of Fr TCoV at room temperature or lower is an important step.

Materials and Methods

Intra-amniotic route of inoculation for embryonated turkey eggs.

Eighteen-day-old to 21-day-old embryonated specific pathogen free (SPF) turkey eggs were candled to ensure embryo viability. They were placed vertically with the air chamber uppermost. The top of the shell was disinfected with iodine and alcohol, and a small hole was drilled at the centre of the air cell. A 21-gauge (0.8 mm), 1.5-inch (40 mm) needle mounted onto a 1 ml syringe held vertically was slowly but completely inserted into the hole and 0.1 ml inoculum was injected. The needle was removed slowly along the same vertical axis and the hole was sealed with melted wax. The eggs were returned to the incubator at +37°C, where they were kept vertically with the air chamber uppermost until 4 days post inoculation.

Virus stock. The 080385d virus used in this study was isolated in November 2008, from the duodenal contents of 42-day-old turkeys affected by PEC. A search for astrovirus, reovirus, adenovirus, and serial passage in chicken hepatocyte cultures failed to detect any virus other than Fr TCoV. The virus was propagated serially five times in embryonated SPF turkey eggs (Anses, Ploufragan, France), inoculated as described above. As Fr TCoV does not induce embryo lesions, the jejunum and ileum of the inoculated embryos were harvested 3 to 6 days post inoculation (depending on the age of the inoculated embryos). The collected material was diluted w/v in phosphate-buffered saline at +4°C, then ground with a Thurrax blender (IKA, Staufen, Germany). The suspension was centrifuged at 3000 ×g for 10 min and the supernatant centrifuged a second time at 3000 ×g for 5 min. The collected supernatant was considered the virus stock.

Virus titration in specific pathogen free turkey eggs. The virus was serially diluted in a stabilizing solution (phosphate buffer + sucrose 20 g/l) supplemented with 0.2% of a penicillin (100,000 u/ml)–streptomycin (100 mg/ml) antibiotic solution and 0.4% of fungizon preparation (0.5 mg/ml), from 10⁻¹ to 10⁻⁵ or from 4⁻¹ to 4⁻⁵ when high or low virus titres were expected, respectively. Each dilution was inoculated in embryonated SPF turkey eggs as described above (four to eight eggs per dilution). Replication of the virus in each inoculated embryo was assessed by harvesting the jejunum and ileum followed by the molecular detection of the TCoV genome (see below). Titres were calculated using the Reed and Muench method for the determination of 50% endpoints (Reed & Muench, 1938) and were expressed as median infectious dose per millilitre of virus (EID₅₀/ml).

Molecular detection of the TCoV genome. Three freeze–thaw cycles were performed to increase the lysis of the tissues and possibly maximize the extraction of TCoV genetic material. Then 140 µl phosphate-buffer saline solution was added to the tissues. Seventy microlitres of this mixture were mixed with 330 µl RLT lysis buffer (Qiagen, Courtaboeuf, France) and incubated at room temperature for 15 min to inactivate the virus. RNA was extracted from the resulting 400 µl using the MagAttract RNA Tissue Mini M48 kit for automated extraction and the M48 Biorobot (Qiagen).

The presence of TCoV genome was assessed using a real-time RT-PCR based on a conserved part of the nucleocapsid gene of avian coronaviruses (primers and protocol according to Maurel *et al.*, 2011).

In this real-time RT-PCR, a cycle threshold (Ct) higher than 35 was considered non-specific (Maurel *et al.*, 2011). With such a protocol, embryos containing infectious Fr TCoV typically produced values ranging from 23 to 35 Ct.

Virus incubation at room temperature and +4°C. Fr TCoV was diluted to obtain an initial titre of 3.2 log₁₀ EID₅₀/ml. To mimic the presence of organic material and possibly promote virus stability, the dilution medium (MEMH) was supplemented with 5% foetal calf serum. Aliquots of the initial virus suspension (400 µl/tube) were placed in an isothermal box containing a thermometer at room temperature. Duplicate tubes were removed from the box and stored at -70°C after 0, 2, 5, 7, 10, 14, 21, 28, 35 and 42 days storage at room temperature. Another series of aliquots was placed in a cold room at +4°C, also containing a thermometer. Duplicate tubes were removed from this second series and were frozen at -70°C after 5, 10, 15, 20 and 40 days of exposure. The temperatures of both rooms were registered daily.

Assessing the stability of Fr TCoV RNA at room temperature. The first duplicates, harvested after 0 and 42 days of storage at room temperature, and 40 days of storage at +4°C, were directly submitted to real-time RT-PCR as described above, prior to egg inoculation.

Assessing Fr TCoV persistence in samples stored for different durations.

The first duplicates harvested after different times of storage at the different temperatures were used to test for the presence of residual infectious virus in the sample by inoculating SPF embryonated turkey eggs as described above. Whenever the first duplicate was shown to still contain live Fr TCoV, the second duplicate was used to titrate the virus as described above.

Results

Optimization of the intra-amniotic route of inoculation for embryonated turkey eggs. To achieve reliable virus delivery into the desired embryonic cavity, some efforts were made to optimize inoculation via the intra-amniotic route into embryonated SPF turkey eggs. Using this methodology, the proper targeting of the inoculum was checked by inoculating 1% methylene blue dye into 20-day-old embryonated SPF turkey eggs, then opening the eggs to check the location of the dye deposit. Out of 40 eggs inoculated by four people, 38 were suitable for analysis of the inoculation point. Inoculation proved to have been performed into the amniotic sac, yolk sac or allantoic fluid in 34 (89%), three (8%) and one (3%) of the inoculated eggs, respectively. The comparison of the results by the different manipulators showed that the percentage success ranged from 6/9 (66%) for one person to 10/10 (100%) for two others. Only those who succeeded best in the intra-amniotic inoculation test inoculated eggs in the subsequent experiments.

Validation of the method for the detection of residual live virus after storage.

The TCoV RNA signal from the initial virus suspension kept for 0 or 42 days at room temperature was 27 ± 4 Ct. This demonstrated that the amount of viral RNA in the inoculum was not affected by the duration of storage. However, when the same samples were inoculated into embryonated SPF turkey eggs, viral RNA was detected in the digestive tracts of the inoculated embryos after 0 days of storage (27.5 Ct), but not after 42 or 40 days (>40 Ct) at room temperature or +4°C, respectively. The results observed at 42 and 40 days strongly suggested that the starting inoculum was so diluted when inoculated into the embryonated eggs that the viral genome could not

Table 1. Detection of infectious Fr TCoV in embryonated SPF turkey eggs by real-time RT-PCR, according to the number of days of storage at room temperature or at +4°C.

Days at room temperature or +4°C	Number of eggs with infectious virus/number of eggs inoculated		Mean (standard deviation) Ct ^a	
	Room temperature	+4°C	Room temperature	+4°C
0	7/7	ND	27.5 (3.0)	
2	4/4	ND	26.6 (0.6)	
5	3/3	4/4	26.1 (2.4)	29.0 (2.9)
7	1/4	ND	29.1	
10	0/4	3/4	Not detected	30.0 (0.6)
14	0/3	ND	Not detected	
15	ND	3/4		30.8 (3.3)
20	ND	2/3		30.2 (2.7)
40	ND	0/4		Not detected
42	0/3	ND	Not detected	

ND, not done. ^aCycle threshold determined using a real-time RT-PCR specific for avian coronaviruses (Maurel *et al.*, 2011).

be detected, unless TCoV replication occurred in the inoculated eggs, as was observed after 0 days of storage. Consequently, in the subsequent experiments, a positive real-time RT-PCR signal from the digestive tract of inoculated embryos was interpreted as the presence of infectious virus, not as residual non-infectious RNA derived from the inoculum.

Detection of residual infectious virus after different storage times. The mean room temperature was +21.6°C (minimum: +19.7°C; maximum: +25.3°C; standard deviation: 1.4°C). After 2 and 5 days of storage, all inoculated eggs were positive (Ct = 26.6). After 7 days, only one out of four amplified the virus (Ct = 29.1). From 10 days of storage onwards, none of the inoculated eggs allowed detection of any virus replication (Table 1).

The mean refrigeration temperature was +4.1°C (minimum: -0.2°C; maximum: +9.9°C; standard deviation: 1.6°C). After 5 days of exposure, all inoculated eggs were positive (Ct = 29.0). After 10 or 15 days, three out of four had supported virus replication (Ct = 30.4). After 20 days, two out of three inoculated eggs were still positive (Ct = 30.8), whereas no virus was detected after 40 days of storage (Table 1).

Quantitative decrease in virus titre during storage. The virus titre measured after 0 days of storage was 2.8 log₁₀ EID₅₀/ml, which was consistent with the initial titre of the virus stock (3.2 log₁₀ EID₅₀/ml), considering the possible effect of one freeze – thaw cycle (titres were calculated after the aliquots had been stored at -70°C).

After storage at room temperature, the titres of the residual virus at days 2 and 5 were similar, and lower than or equal to 1.5 log₁₀ EID₅₀/ml (decrease of at least 1.3 log₁₀ EID₅₀/ml). The decrease continued at 7 days, with a titre lower than or equal to 0.7 EID₅₀/ml (decrease of at least 2.1 log₁₀ EID₅₀/ml). Finally, no virus titre could be detected at 10 days (Figure 1).

After refrigerated storage, the titre of the residual virus proved stable from 0 to 5 days (2.8 log₁₀ EID₅₀/ml) and a virus titre of 2.3 log₁₀ EID₅₀/ml (decrease of 0.5 log₁₀ EID₅₀/ml) was still detected after 20 days of storage. No surviving virus was detected after 40 days of storage at +4°C (Figure 1).

Discussion

The protocol used here was developed to try to simulate the survival of Fr TCoV in the environment at +20°C and +4°C. These temperatures were selected as representative of the mean summer (+16.6 to +19.2°C) and winter (+5.9 to +6.2°C) temperatures in Brittany, the largest poultry-producing area in France, over the 1981 to 2010 period (<http://www.meteo-bretagne.fr/normales-climatiques-Rennes>). Indeed, in this region and during this period, the mean number of days above +25°C or below 0°C did not exceed 39.6 and 33.5 days, respectively. In addition, the +4°C temperature is likely to be comparable with temperature during refrigerated shipment of unfrozen biological samples. However, our study does not mimic the survival of virus in litter or dried faeces, and so forth, where relative humidity is different. The use of a virus suspension, instead of dried viruses on surfaces, decreased the risk of titre variation, simplified the study, and gave consistent data. This simplified protocol was all the more interesting as this virus does not grow in cell culture, requiring a multi-step procedure for titration of the virus, comprising embryonated SPF turkey eggs, embryo dissection and real-time RT-PCR. Another significant improvement would have been to start with a virus titre initially higher, in order to obtain values of decrease in virus titre on a larger scale. However, this was not possible because titres obtained when propagating Fr TCoV in turkey eggs currently did not exceed 4 log₁₀ EID₅₀/ml, a titre that could not be increased either by inoculating the embryonated SPF

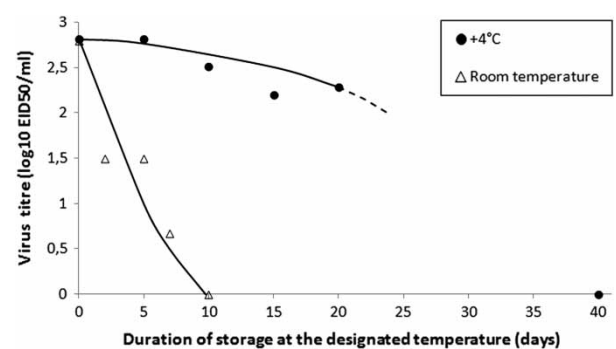


Figure 1. Reduction in Fr TCoV titre with storage time at different temperatures.

turkey eggs at a younger age or by increasing the passage level of Fr TCoV in eggs (data not shown).

Our data suggest that Fr TCoV in suspension survives for less than 10 days at room temperature and for 20 to 40 days at +4°C. The virus titre loss was only 0.5 log₁₀ EID₅₀/ml after 20 days at +4°C, which strongly suggests that the virus might survive much longer than 20 days. However, survival between 20 and 40 days at +4°C was not evaluated.

Coronaviruses, being enveloped viruses, are expected to be less stable in the environment than non-enveloped viruses. Data available for the severe acute respiratory syndrome (SARS) coronavirus infecting humans are in the same range as reported here for Fr TCoV. SARS-CoV dried on plastic retained its infectivity for as long as 6 days (loss of 3 log₁₀ TCID₅₀/ml) at room temperature (+21 to +25°C), the total loss of the initial 7 log₁₀ TCID₅₀/ml being obtained after 9 days (Rabenau *et al.*, 2004). This stability is much higher than previously described for human coronavirus HCoV-229E, which completely lost its infectivity within 72 h under the same conditions. Another study using a suspension of SARS-CoV showed a loss of 3.7 log₁₀ TCID₅₀/ml after 7 days and 5.7 log₁₀ TCID₅₀/ml after 13 days, at +22/ +25°C (Chan *et al.*, 2011). The Fr TCoV titre reduction was less in the first days (2.1 log₁₀ EID₅₀/ml after 7 days) than that previously described for a suspension of SARS-CoV (Chan *et al.*, 2011) but Fr TCoV could not be detected at 10 days.

The effect of temperature was recently studied in two animal coronaviruses: transmissible gastroenteritis virus (TGEV), an enteric pathogen of swine; and mouse hepatitis virus (MHV), a respiratory and enteric pathogen of laboratory mice (Casanova *et al.*, 2010). This study investigated viral inocula placed on stainless steel surfaces, then in a controlled relative humidity environment (20% and 80% of relative humidity). Our data, being obtained from viruses in suspension, most closely compare with data obtained under 80% relative humidity. At +20°C, after 7 days, the titre decrease was 1.7 log₁₀ for TGEV and 4 log₁₀ for MHV. After 10 days the titre decrease was 2 log₁₀ for TGEV and at least 5 log₁₀ for MHV, indicating that infectious MHV was no longer detected. Compared with our data, the titre decrease was higher with MHV than Fr TCoV, but similar between TGEV and Fr TCoV, an interesting finding considering that both viruses are enteric pathogens. At +4°C MHV and TGEV persistence was longer, showing a titre decline after 28 days of 3.2 and 2.5 log₁₀ for TGEV and MHV, respectively. The Fr TCoV titre in suspension at +4°C was more stable (decrease of only 0.5 log₁₀ after 20 days).

As far as avian viruses are concerned, it is difficult to compare the temperature sensitivity of Fr TCoV with that of IBV, because very limited published information is available regarding the latter. An early study of IBV survival in lyophilized preparations (Hofstad & Yoder, 1963) can hardly be compared with the present study due to the presentation of the samples being so different. Lyophilized IBV strains 33 and 97 both exhibited a similar decay in titre (about 2.3 log₁₀ EID₅₀/ml) when stored for 30 days at +37°C, but no loss in virus titre was measured when the viruses were stored at +4°C for the same time. This is consistent with a greater thermal stability of avian coronaviruses at lower temperatures. In another study dating back to

the same period, the presence of salts in solution was shown to stabilize the titre of the IBV-42 (Beaudette) strain at +25°C. Indeed, the virus titre strongly decreased by 3.5 log₁₀ EID₅₀/ml when incubated for only 15 minutes at +25°C, whereas it was stable in salt solution (Hopkins, 1967). Clearly, more recent studies on the stability of IBV in the environment are required if comparisons are to be made between TCoV and IBV. In general, the data available regarding animal coronaviruses are in the same range. Virus survival ranges from 1 to 2 weeks at +20°C, whereas survival is longer than 20 days at +4°C.

The longer survival of TCoV at cooler temperatures could be a factor allowing an increased persistence or transmission of the virus during the cooler seasons of the year. Indeed, the strain used in this study (080385d) was isolated from a diseased flock in November, usually a rather cold and humid period in western France. However, this may not reflect the seasonality of enteric disorders in France. In their survey of 81 French turkey flocks, performed during the period 2007 to 2009, Maurel *et al.* (2009) detected 24 flocks positive for Fr TCoV. Fifteen of these flocks (63%) were detected during warm periods (spring–summer), as compared with only nine flocks (37%) in autumn and winter. Although this survey was not designed to precisely assess seasonality of Fr TCoV infections, the annual distribution of PEC reports to the French Nationwide Network for Epidemiological Observations in Poultry (RNOEA) (Souillard *et al.*, 2007) confirms that the proportion of PEC cases declared from 2007 to 2011 was higher during the warmer periods (77% of the total declared PEC cases) than during the colder ones (23% of the total reported cases) (Toux & Souillard, personal communication, 2011). The period of highest PEC incidence hence appears not to be the most favourable for Fr TCoV survival in the environment. However, it should be remembered that PEC is a multifactorial syndrome, so that the reported PEC cases may be caused by a variety of agents (Fr TCoV, astroviruses, reoviruses, adenoviruses, bacteria, etc.), some of which may account for additional summer cases. Furthermore, the survival of Fr TCoV is only one possible factor that might contribute to the seasonality of the disease, and other possibly more significant interfering factors, such as possible vectors or seasonality of turkey production, might also play a role, so that Fr TCoV virus survival according to the outdoor temperature might not be the most important parameter for its transmission between turkey flocks.

Based on the data of the present study, it can be concluded that a Fr TCoV strain survives for less than 10 days in a liquid culture medium kept at room temperature, but for more than 20 days at +4°C. Interesting future work would be to assess virus survival: at the higher temperatures that are reached during steam cleaning on the surfaces of farm equipment; at +10°C (the temperature for pre-licensing activity testing of agricultural disinfectants); and at –5°C, the usual temperature during shipment of frozen samples to diagnostic laboratories. The results of such studies might help understanding of TCoV epidemiology and ensure efficient shipment of samples for virus isolation.

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