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Effects of cold storage on detection of avian infectious bronchitis virus in chicken carcasses and local antibodies in tracheal washes

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Abstract

In order to test the survivability of infectious bronchitis virus (IBV) in dead chicken carcasses during 24 h of cold storage, 7 week-old specific-pathogen-free chickens were infected with virulent IBV Massachusetts strain M41, and were killed humanely 10 days later. Carcasses were stored in a cold room at 4 °C. After 1, 3, 6, 9, 12 or 24 h of storage, necropsies were carried out. Trachea, lung, kidney and rectum were collected for virus isolation by tracheal organ culture (TOC) or embryonated chicken eggs (ECE), and detection by nested reverse-transcriptase polymerase chain reaction (RT-PCR). IBV was detected by RT-PCR at all sampling times, except for 1 and 6 h of storage in kidney and 9 h of storage in kidney and rectum. For ECE, isolation was obtained at all sampling points, except at 1 and 24 h of storage in lungs. Isolation by tracheal organ cultures was less successful, except from rectum. In addition to sampling for virus, tracheal washes were collected from each carcass to measure the ability to detect local antibodies after storage. Levels of IgA in tracheal washes remained high for up to 9 h of storage, suggesting that accurate sampling for research purposes when required must be carried out within this time.

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Keywords: Infectious bronchitis; Cold storage; Virus detection; Local antibodies

1. Introduction

Infectious bronchitis caused by a coronavirus is an important disease in chickens, and it mainly affects respiratory and urogenital systems (Cavanagh and Naqi, 2003; Dhinakar Raj and Jones, 1997). Diagnosis of infectious bronchitis virus (IBV) is confirmed by isolation of the virus using either chicken embryonated eggs (ECE) or tracheal organ culture (TOC) and detection by reverse-transcriptase polymerase chain reaction (RT-PCR) (Cavanagh and Naqi, 2003; Gelb and Jackwood, 1998). Tracheal swabs, oropharyngeal swabs and tissues such as trachea, lungs, kidney, oviduct and caecal tonsils are normally used for isolation (Cavanagh and Naqi, 2003; Gelb and Jackwood, 1998). It is recommended that carcasses should be submitted to the laboratory as soon as possible but no reports are available to indicate an appropriate time limit, beyond which virus detection is impossible. This paper provides information on the probability of IBV recovery from target tissues in carcasses stored at 4 °C for up to 24 h post-killing. Three different methods of demonstrating the presence of IBV, namely isolation by TOC or ECE; and detection by nested RT-PCR were used.

The trachea is recognised as a main target organ for IBV infection, hence an important site for research into study local immune responses (Dhinakar Raj and Jones, 1997; Gillette, 1981; Gomez and Raggi, 1974). In such investigation, tracheal washes are collected for detection of local antibodies (Dhinakar Raj and Jones, 1996; Hawkes et al., 1983) and this is normally done soon after killing. However, no details on the optimal time intervals between killing and collection of tracheal washes have been established. This experiment therefore provided the opportunity to measure the levels of...
IgA and IgG in tracheal washes of chicken carcasses stored at 4 °C and sampled at the same intervals.

2. Materials and methods

2.1. Eggs and chicks

White Leghorn specific-pathogen-free chicken eggs (Lohmann Animal Health, Cuxhaven, Germany) were incubated and hatched at our laboratory. Chicks were housed in isolation rooms in an experimental house. Food and water were provided ad libitum.

2.2. Infectious bronchitis virus

The Massachusetts strain M41 was used after numerous passages in ECE. The titre was $6.9 \log_{10}$ median egg infective doses per ml. Prior to this, the virus had undergone 10 passages in TOC and 2 passages in ECE.

2.3. Experimental design

Chickens were inoculated when seven weeks old, with 100 μl of IBV by the oculo-nasal route. The birds were monitored for clinical signs and were humanely killed at 10 days post-infection. Carcasses were stored at 4 °C. At 1, 3, 6, 9, 12 and 24 h of storage, four carcasses were randomly chosen for tracheal wash collection and virus detection.

2.3.1. Tracheal washes

Tracheal washes were collected as described by Dhinakar Raj and Jones (1996) and stored at −70 °C until further use. They were assayed for IBV-specific IgA and IgG by indirect ELISA (below).

2.3.2. Tissues

Pieces of trachea, lung, kidney and rectum were aseptically collected for isolation or RT-PCR.

A similar group of uninfected chickens kept in a separate isolation pen were used as a control.

2.4. Virus isolation and detection

2.4.1. Sample processing

Each trachea was scraped with a sterile surgical blade and the mucus and epithelium were vortexed in 0.9 ml of virus isolation medium [Eagles serum-free MEM with glutamine, streptomycin (50 μg/ml) and penicillin (50 IU/ml)]. Pieces of lung, kidney or rectum (after squeezing out faecal contents) were homogenised using a sterile pestle and mortar with sterile sand and 0.2 ml of the medium. Subsequently, more medium was added to make a final 1:10 (w/v) dilution of the sample. Prior to centrifugation, a sterile cotton swab was dipped into each of the tissue homogenates for RT-PCR detection of IBV. Swabs were left to dry at room temperature then kept in a cupboard at room temperature until used. The tissue homogenates were centrifuged at 1500 × g for 5 min and the supernatants were collected and stored at −70 °C until processed for virus isolation.

Three different methods were used to detect presence of IBV in the homogenised tissues.

2.4.2. TOC

Isolation of the virus was carried out in chicken TOCs as described by Cook et al. (1976). Three replicates of each homogenate underwent three passages and those TOCs with complete ciliostasis at the third passage were subjected to RT-PCR, to confirm presence of the virus.

2.4.3. ECE

Virus isolation was carried as described by Gelb and Jackwood (1998) using specific-pathogen free chicken eggs of 9–11 days of incubation. This was done for up to three passages. After the third passage, eggs were incubated for 7 days and chilled overnight. Chorioallantoic fluid was collected aseptically and stored at −70 °C for until used. The embryos were examined for typical lesions of IBV. The chorioallantoic fluid from embryos with lesions consistent with IBV after up to three passages was considered positive for virus. Those where embryo lesions were inconclusive after the third passage was subjected to RT-PCR to confirm the presence of IBV.

2.4.4. RT-PCR

The swabs were pooled according to tissues and sampling intervals. RNA was extracted as described previously (Cavanagh et al., 1999). Briefly, each was dipped into 1 ml of guanidine isothiocyanate (solution D) containing 2-mercaptoethanol and being left at −20 °C for several hours. Then, 500 μl of the sample was transferred to a new microcentrifuge tube and 50 μl of sodium acetate (pH 4.1) and 650 μl phenol chloroform were added. The mixture was vortex mixed and centrifuged for 5 min. The top layer was transferred to 500 μl isopropanol, mixed and precipitated overnight at −20 °C. RNA was precipitated using 100% ethanol and the resulting precipitate was suspended in a solution containing sterile tissue culture water, dithiothreitol and ribonuclease inhibitor. RT-PCR was conducted according to method of Cavanagh et al. (1999).

2.5. Detection of antibodies in tracheal washes

IBV-specific IgA and IgG in tracheal washes were assayed using an indirect ELISA (Dhinakar Raj and Jones, 1996) except that the plates were coated with partially purified IBV M41 antigen. This was done by ultracentrifugation of previously clarified allantoic fluid at 20,000 × g for 90 min and the resulting pellet was washed and resuspended in phosphate buffered saline (pH 7.2). The virus suspension was overlaid onto 25% sucrose and ultracentrifuged at 20,000 × g.
Table 1
Detection of IBV by RT-PCR or isolation in TOC or ECE

| Site     | Method | 3  | 6  | 9  | 12 | 24 | Total/24 |
|----------|--------|----|----|----|----|----|----------|
| Trachea  | RT-PCR | +  | +  | +  | +  |    |         |
| TOC      |        | 0  | 1  | 2  | 0  | 0  | 3        |
| ECE      |        | 3  | 4  | 2  | 3  | 1  | 15       |
| Lungs    | RT-PCR | +  | +  | +  | +  |    |         |
| TOC      |        | 0  | 2  | 1  | 1  | 0  | 5        |
| ECE      |        | 0  | 4  | 4  | 3  | 0  | 14       |
| Kidney   | RT-PCR | −  | +  | −  | −  | +  |         |
| TOC      |        | 1  | 2  | 0  | 2  | 0  | 5        |
| ECE      |        | 1  | 2  | 3  | 4  | 2  | 15       |
| Rectum   | RT-PCR | +  | +  | −  | +  |    |         |
| TOC      |        | 3  | 1  | 1  | 1  | 3  | 11       |
| ECE      |        | 4  | 4  | 4  | 4  | 4  | 24       |

* Positive for PCR (pool of four swabs of the respective homogenised tissues).
* Number of birds with positive isolation by TOC (out of four).
* Number of birds with positive isolation by ECE (out of four).

for 3 h. The pellet was washed and resuspended in phosphate buffered saline, aliquoted and stored at −70 °C until used.

Monoclonal antibodies to chicken IgG and IgA were kindly provided by Dr. T.F. Davison (Institute for Animal Health, Compton, Newbury, Berks, UK). Corrected optical density values were calculated by deducting the optical density values of non-antigen coated wells from those of the test wells (Fourrier-Caruana et al., 2003). The mean antibody titres were compared using a Student’s t-test.

3. Results

3.1. Clinical signs and post-mortem lesions

Signs of sneezing, head-shaking and watery eyes were observed from day 2 until day 7 post-infection. At necropsy on 10 days post-infection, no gross lesions were found.

3.2. Detection of virus (isolation and RT-PCR)

3.2.1. TOC

For trachea, virus was isolated at 3 and 9 h of storage only and in lungs between 2 and 12 h but from never more than two out of four chickens (Table 1). At 1, 3 and 12 h of storage virus was isolated in the kidney, and was consistently detected in rectum.

3.2.2. ECE

Virus was consistently detected at all sampling points except at 1 and 24 h of storage in lungs. In total, rectum provided 100% recovery, followed by trachea and kidney (63%), and lungs (50%).

3.2.3. RT-PCR

IBV was detected at all sampling intervals in all four organs, except at 1, 6 and 9 h of storage for kidney and at 9 h of storage for rectum (Table 1).

All control tissues remained negative for virus by isolation and RT-PCR.

3.3. Detection of antibodies

Fig. 1 shows levels of IgA and IgG in TW at different sampling intervals. Levels of IgA at 1, 3, 6 and 9 h of storage were similar and were significantly higher than 12 and 24 h of storage. However, no significant differences were found between the sampling intervals, except levels of IgA at 9 h of storage were significantly higher than 12 h of storage. For IgG, only trace amounts were detected.

4. Discussion

The main aim of this paper was to provide some information on the survivability of IBV in chicken carcasses during 24 h of cold storage. This, in turn, should influence the ability to confirm a diagnosis on materials that is not fresh. The chickens were deliberately killed at 10 days post-infection, as it is well recognised that it is more difficult to recover virus at this stage than at less than 7 days post-infection (Cavanagh and Naqi, 2003; Cook, 2001). Isolation was attempted in TOC or ECE, which have been reported to be equally sensitive for IBV recovery (Cook et al., 1976; Darbyshire et al., 1975; De Wit, 2000) and also detected by nested RT-PCR (Cavanagh et al., 1999; Cavanagh and Naqi, 2003; Elhafi et al., 2004).

In this study, it appears that irrespective of the IBV detection method, the virus was found in all tissues examined for up to 24 h of storage. This suggests that in difficult circumstances, necropsy examination and tissue collection of
carcasses kept at 4 °C could be safely delayed up to 24 h post-killing. Interestingly, irrespective of the method of isolation, consistently higher numbers of recoveries were obtained from the rectum. It is known that clearance of IBV infection first occurs from the respiratory tissues followed by other tissues, with later clearance from kidney and digestive tract (Lucio and Fabricant, 1990). In addition, Ambali and Jones (1990) reported IBV isolation from rectal tissue and replication in the epithelium, suggesting that apart from excretion of the virus via this route, the tissue may play an important role in generating new virus when respiratory replication has declined. Thus, the rectum could be an important but additional tissue for diagnosis of IBV. As for the kidneys, since we have used a non-nephrotropic virus, the frequency of virus detection was low and to be expected. The host, agent and environmental factors may have influenced the results of this experiment (Ambali and Jones, 1997) and a change in these factors, particularly the strain of virus used may have produced a different outcome.

The nested RT-PCR employed in this study, is regularly used in our laboratory for detection of IBV in pooled swabs. For diagnosis of the disease, use of pooled swabs is a routine practice as it is convenient, and represents a flock rather than individual birds. In our study, we also used pooled swabs taken from homogenised tissues where RNA was extracted for RT-PCR. This appears to be the first report where such an extraction has been performed. Other workers have extracted RNA from allantoic fluid (Cavanagh and Naqi, 2003; Gelb and Jackwood, 1998), directly from tracheal or oropharyngeal swabs (Capua et al., 1999; Cavanagh et al., 1999; Jackwood et al., 1997; Elhafi et al., 2004) or directly from non-processed tissues (Falcombe et al., 1997). In this study, positive PCR reactions were found on all occasions, except after 1, 6 and 9 h of storage for lungs and 9 h of storage for rectum. This could have been due to low amount of viral genomes in the tissues or other non-specific inhibitory factors (Kwon et al., 1993), as positive isolations at these times were obtained by ECE. Generally, it appears that RT-PCR has detected positive samples at similar rate as ECE, and was better than TOC. This may demonstrate that direct use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch. Virol. 30, 109–118. Durbuyshere, J.H., Cook, J.K.A., Peters, R.W., 1975. Comparative growth kinetic studies on avian infectious bronchitis virus in different systems. J. Comp. Pathol. 83, 623–630. De Wit, J.J., 2000. Detection of infectious bronchitis. Avian Pathol. 29, 71–93. Dhimakat Raj, G., Jones, R.C., 1996. Local antibody production in the ovulect and gut of hen infected with a variant strain of infectious bronchitis virus. Vet. Immunol. Immunopathol. 53, 147–161. Dhimakat Raj, G., Jones, R.C., 1997. Infectious bronchitis virus: immunopathogenesis of infection in chicken. Avian Pathol. 26, 667–706. Elhafi, G., Nayler, C.J., Sarage, C.E., Jones, R.C., 2004. Micronucleus or autolacive treatments destroy the infectivity of infectious bronchitis virus and avian pneumovirus but allow detection by reverse transcription-polymerase chain reaction. Avian Pathol. 33, 303–306. Falcone, E., D’Amore, E., Di Teodori, L., Silvi, A., Tolle, M., 1997. Rapid diagnosis of avian infectious bronchitis virus by the polymerase chain reaction. J. Virol. Methods 64, 125–136. Fleuret-Caravas, J., Porter, B., Haud, G., Julliot, C., Fudo, P., Tordo, N., Perrin, P., 2003. Inactivated rabies vaccine control and release: use of an ELISA method. Biologics 31, 9–16. Gelb Jr., J., Jackwood, M.W., 1998. Infectious bronchitis. In: Swayne, D.E., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. (Eds.), A Laboratory Manual for the Isolation and Identification of Pathogens. Kennett Square, PA, pp. 169–174. Gillette, K.G., 1981. Local antibody response in avian infectious bronchitis virus-neutralizing antibody in tracheobronchial secretions. Avian Dis. 25, 431–443. Gomez, L., Rapp, L.G., 1974. Local immunity to avian infectious bronchitis in tracheal organ culture. Avian Dis. 18, 346–360. Haukos, R.A., Darbyshire, J.H., Peters, R.W., Mockett, A.F., Cavanagh, D., 1983. Presence of viral antigens and antibody in the trachea of chickens infected with avian infectious bronchitis virus. Avian Pathol. 12, 331–340. Jackwood, M.W., Yousif, N.M., Bitt, D.A., 1997. Further development and use of a molecular serotype identification test for infectious bronchitis virus. Avian Dis. 41, 105–100. Kwon, H.M., Jackwood, M.W., Brooks, P.B., Gelb, J., 1993. Polymerase chain reaction a biotin-labelled DNA probe for detection of infectious bronchitis virus in chickens. Avian Pathol. 33, 149–156. Lucio, B., Fabricant, J., 1990. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. Avian Dis. 26, 506–519.

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