Lithium chloride inhibits the coronavirus infectious bronchitis virus in cell culture.
Sally Harrison, Ian Tarpey, Lisa Rothwell, Pete Kasier, Julian Hiscox

To cite this version:
Sally Harrison, Ian Tarpey, Lisa Rothwell, Pete Kasier, Julian Hiscox. Lithium chloride inhibits the coronavirus infectious bronchitis virus in cell culture.. Avian Pathology, Taylor Francis, 2007, 36 (02), pp.109-114. 10.1080/03079450601156083 . hal-00540068
Lithium chloride inhibits the coronavirus infectious bronchitis virus in cell culture.

| Journal: | Avian Pathology |
|---------|-----------------|
| Manuscript ID: | CAVP-2006-0180 |
| Manuscript Type: | Original Research Paper |
| Date Submitted by the Author: | 01-Dec-2006 |
| Complete List of Authors: | Harrison, Sally Tarpey, Ian; Intervet UK, R&D Rothwell, Lisa Kasier, Pete Hiscox, Julian |
| Keywords: | IBV, LiCl, RNA, protein |
Lithium chloride inhibits the coronavirus infectious bronchitis virus in cell culture.

Sally M. Harrison\textsuperscript{1*}, Ian Tarpey\textsuperscript{2}, Lisa Rothwell\textsuperscript{3}, Pete Kaiser\textsuperscript{3} and Julian A. Hiscox\textsuperscript{1,4}.

\textsuperscript{1}Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, UK, \textsuperscript{2}Intervet UK Ltd, Milton Keynes, UK, \textsuperscript{3}Institute for Animal Health, Compton, UK, \textsuperscript{4}Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, UK.

**Running title** Inhibition of IBV by lithium chloride.

To whom correspondence should be addressed. Tel. 44 (0)113 343 5582. Fax. 44 (0)113 343 3167. E-mail j.a.hiscox@leeds.ac.uk

*Received 9 May 2006*
Lithium chloride inhibits the coronavirus infectious bronchitis virus in cell culture.

Abstract

The avian coronavirus infectious bronchitis virus (IBV) is a major economic pathogen of domestic poultry which, despite vaccination, causes mortality and significant losses in production. During replication of the RNA genome there is a high frequency of mutation and recombination which has given rise to many strains of IBV and results in the potential for new and emerging strains. Currently the live-attenuated vaccine gives poor cross-strain immunity. Effective antiviral agents may therefore be advantageous in the treatment of IBV. Lithium chloride (LiCl) is a potent inhibitor of the DNA virus herpes simplex virus but not RNA viruses. The effect of LiCl on the replication of IBV was examined in cell culture using two model cell types; Vero cells, an African Green monkey kidney-derived epithelial cell line, and DF-1 cells, an immortalised chicken embryo fibroblast cell line. When treated with a range of LiCl concentrations, IBV RNA and protein levels and viral progeny production were reduced in a dose-dependent manner in both cell types, and the data indicated that inhibition was a cellular rather than a virucidal effect. Host cell protein synthesis still took place in LiCl-treated cells and the level of a standard cellular housekeeping protein remained unchanged, indicating that the effect of LiCl was specifically against IBV.
Introduction

Coronaviruses are a family of positive-sense, single-stranded RNA viruses that replicate in the cytoplasm of infected cells. Infectious bronchitis virus (IBV) is a highly pathogenic respiratory pathogen of chickens that can also affect the kidneys and reproductive systems (Cavanagh, 2005; Raj & Jones, 1996), therefore resulting in both bird mortality and decreased reproductivity (Ignjatovic & Sapats, 2000). As with all coronaviruses the IBV input genomic RNA is translated by host cell ribosomes to generate an RNA dependent RNA polymerase (Rep1a, Rep1ab) (Lai & Cavanagh, 1997). This complex is responsible for the transcription of viral sub-genomic mRNAs via a discontinuous mechanism (Pasternak et al., 2006) and the generation of new genomic RNA. Both the genomic and sub-genomic mRNA are 3’ co-terminal and share a common 3’ un-translated region (UTR), whereas the 5’ end of the genomic mRNA has a unique 5’ UTR.

Whilst live attenuated vaccines are used to prevent infection with IBV, these give little cross-strain immunity (Cavanagh, 2005; Gelb et al., 2005; Liu et al., 2006). The problem of vaccination efficiency against IBV is compounded due to the extensive antigenic variation between different strains (Jackwood et al., 2005; Bochkov et al., 2006), caused by the high frequency of mutations due to error-prone replication and also recombination (Wang et al., 1993; Kottier et al., 1995; Lee and Jackwood, 2000). Other important steps that are taken to control IBV infection are serological monitoring to determine virus exposure, reverse transcriptase-polymerase chain reaction (RT-PCR) to detect viral RNA and enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition to detect IBV antibodies (Adzhar et al., 1996; Chen et al., 2003). Due to the high probability of new and emerging strains of IBV and other
coronaviruses in general, such as severe acute respiratory syndrome coronavirus (SARS-CoV) (Peiris et al., 2004), the need to develop alternative strategies to vaccination is paramount (Cavanagh, 2003; Cavanagh, 2005; Weiss & Navas-Martin, 2005).

Our understanding of the action of lithium chloride (LiCl) on the replication of a range of DNA and RNA viruses is limited. LiCl inhibits the replication of the DNA virus herpes simplex (HSV) (Skinner et al., 1980), whereas with the RNA viruses, encephalomyocarditis virus (EMCV), and influenza virus, there was no apparent effect on virus biology (Skinner et al., 1980). Previous studies have also determined that inhibition of virus is specific to the presence of lithium ions, as no reduction in virus replication was seen in cells treated with potassium or sodium chloride (Skinner et al., 1980).

Following the potential application of LiCl to treat DNA virus infection, we investigated the antiviral effects of LiCl on IBV in two cell systems; Vero cells, an African Green monkey kidney derived epithelial cell line, and DF-1 cells, an immortalised chicken embryo fibroblast cell line. Although Vero cells have been used extensively as a permissive cell line to study virus replication (Britton et al., 2005; Casais et al., 2003), cell biology (Dove et al., 2006a) and protein targeting (Dove et al., 2006b; Reed et al., 2006), recent data suggests potential discrepancies in virus biology, in terms of protein trafficking, between avian and mammalian cell lines (Pendleton & Machamer, 2006), therefore both Vero and chicken cells were used in this study.

Materials and Methods
Cell culture and virus production. Vero cells (an African green monkey kidney-derived epithelial cell line) and DF-1 cells (Doug Foster, a chicken embryo fibroblast-derived epithelial cell line) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37°C in the presence of 5% CO₂, as described previously (Dove et al., 2006a). IBV Beaudette US, a strain adapted for growth in Vero cells (Alonso-Caplen et al., 1984), was propagated in Vero and DF-1 cells and the virus harvested at 24 h post-infection (p.i.). Virus titre was calculated by plaque assay titration in either Vero or DF-1 cells (Dove et al., 2006a). All cell culture experiments were conducted in the absence of antibiotic or anti-fungal agents.

Treatment of cells with LiCl. Vero and DF-1 cells were seeded at 2x10⁵ in 7 cm² tissue culture dishes and grown to 70% confluency prior to mock or infection with IBV at 2x10⁶ pfu/ml. At 8 h post-infection the cells were treated with 0, 5, 10, 25 or 50 mM LiCl and at 24 h post-infection mock and infected cells were lysed or prepared for subsequent plaque assays.

Preparation of total cellular protein. Mock and IBV infected Vero and DF-1 cells were harvested 24 h p.i. and lysed using RIPA buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, Complete Protease Inhibitor at a dilution of 1/25 (Roche)). Total protein was quantified by BCA assay (Promega) and western blot analysis performed.

Western blot analysis. Ten µg of total protein were denatured in Invitrogen NuPage LDS sample reducing buffer containing NuPage reducing agent and separated on a
NuPage Bis-Tris 10% pre-cast gel in 1 X MOPS running buffer. Proteins were then electro-transferred onto a polyvinylidene fluoride (PVDF) membrane with transfer buffer that contained 25 mM Bicine, 25 mM Bis-Tris and 10% (v/v) methanol. Western blotting was performed using ECL (luminol 3-aminophthalydrazide) (Sigma). IBV proteins were detected using a chicken anti-IBV polyclonal antibody (diluted 1:20,000) (Charles Rivers). Mouse anti-GAPDH (6C5) antibody (diluted 1:40,000) (AbCam) was used to detect GAPDH. Horseradish peroxidase-conjugated rabbit anti-chicken and goat anti-mouse secondary antibodies (1:1000 dilution) (Sigma) used as appropriate.

**Plaque assay analysis of virus.** 7 cm² tissue culture dishes were seeded at 2x10⁵ with either Vero or DF-1 cells and grown until confluent. A serial dilution of progeny virus was performed in DMEM with 10% FBS ranging from 10⁻¹ to 10⁻³ for IBV grown in Vero cells, and from 10⁻¹ to 10⁻⁶ for IBV grown in DF-1 cells. Individual wells were then infected in duplicate with the range of virus dilutions and incubated at 37°C in the presence of 5% CO₂ for 1 h before being over-laid with 1% low melting point agarose (lmp agarose) in DMEM with 10% FBS. The cells were then incubated at 37°C for 72 h before being stained with gentian violet (1% crystal violet, 10% formaldehyde (40%) and 5% EtOH in phosphate buffered saline (PBS)). Virus titre was determined by counting the number of plaques formed at a specific dilution (Dove *et al.*, 2006a).

**Preparation of total cellular RNA.** Total cellular RNA was extracted at 0 and 24 h p.i. by the RNeasy method according to the manufacturer’s instructions (Qiagen).

**Taqman analysis of IBV genomic and subgenomic mRNA.** IBV genomic and subgenomic RNA levels in mock and virus infected Vero and DF-1 cells treated with 0,
5, 10, 25, 50 mM LiCl were quantified by TaqMan real-time reverse transcriptase polymerase chain reaction (RT-PCR) (for other examples see (Bicknell et al., 2005, Kaiser et al., 2003)). Primers and probes for the IBV 5’ UTR to detect genomic RNA and the cellular 28S rRNA, were designed using the Primer Express software program (Applied Biosystems). A primer and probe set to detect the IBV 3’ UTR was designed manually as the software did not detect any optimum sequences. The primer and probe sets used in this study are detailed in Table 1 and are based upon the Beaudette US strain (accession number AAA46214).

The TaqMan FAST universal PCR Master Mix (Multiscribe) and RNase inhibitor mix (Applied Biosystems) was used to perform real-time quantitative RT-PCR. Detection and amplification of RNA levels using the 28S, 5’ UTR and 3’ UTR probes were carried out using the 7500FAST TaqMan machine (Applied Biosystems). The following cycle profile was used: one cycle of 48°C for 30 min (RT step) and 95°C for 20 sec (Taq activation), then 40 cycles of 95°C for 3 sec (melting step) and 60°C for 30 sec (anneal and extension step). Quantification was based on increased fluorescence detected due the 5’ exonuclease activity of the Taq DNA polymerase during PCR amplification hydrolysing the target specific probes. The reporter signal was normalised by the reference dye 6-carboxy-c-rhodamine, which was not actually involved in amplification. Results were expressed in terms of Ct values (threshold cycle value); the cycle at which the change in reporter dye passes a significance threshold (ΔRn).

Variation in sampling and RNA preparation was accounted for by standardising the Ct values for the IBV 5’ UTR and 3’ UTR-specific products for each sample to the Ct value of 28S rRNA product for the same sample. RNA levels between samples in the same experiment were normalised by pooling values from all samples in that experiment and calculating the mean Ct value for 28S rRNA-specific gene product.
Variations in each individual 28S rRNA sample compared to the mean were then calculated. Differences in input of total RNA were calculated by determining the slope of the 28S rRNA log$_{10}$ dilution series regression line. Using the slopes of the respective IBV 5’ UTR, IBV 3’ UTR or 28S rRNA log$_{10}$ dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust the IBV 5’ UTR or IBV 3’ UTR specific Ct values. This was done as follows:

Corrected Ct value = Ct + (Nt-Ct’)*S/S’ where:

Ct = mean sample Ct, Nt = experimental 28S mean, Ct = mean 28S of sample, S = IBV 5’ UTR/IBV 3’ UTR slope, and S’ = 28S slope.

Results were then expressed as 40-Ct values.

**Results and Discussion**

Previously it has been shown that LiCl has an antiviral effect on HSV (Cernescu *et al.*, 1988; Ziaie *et al.*, 1994; Ziaie & Kefalides, 1989) when used at concentrations ranging from 1-10 mM. The same trend in reduced viral yield is seen in the DNA viruses pseudorabies and vaccinia. However, inhibition was not observed in the RNA viruses influenza and encephalomyocarditis (EMC) (Skinner *et al.*, 1980). This study investigated the effects of LiCl on IBV replication in cell culture.

**Lithium chloride treatment reduces progeny virus production in both IBV infected Vero and DF-1 cells.** To determine whether LiCl had an inhibitory effect on IBV growth *in vitro*, Vero and DF-1 cells were infected with IBV and then left untreated or treated with increased concentrations of LiCl ranging from 5 to 50 mM. Virus progeny
production was quantified by plaque assay at 24 h.p.i. The data indicated that with the lowest concentration of LiCl tested (5 mM) there was an approximately 50% reduction in virus titre compared to untreated IBV-infected cells, and at the highest concentration (50 mM) virus progeny production was abrogated, as determined by plaque assay (Figures 1 and 2). Although the data indicated that IBV grew better in DF-1 cells compared to Vero cells, as evidenced by plaque formation at $10^{-6}$ dilution of progeny virus from DF-1 cells compared to $10^{-3}$ dilution in Vero cells, the equivalent reduction in virus titre with LiCl treatment was observed in both Vero and DF-1 cells. There was no apparent difference in the plaque morphology of IBV between Vero and DF-1 cells and likewise at any concentration of treatment with LiCl.

Lithium chloride does not have a direct virucidal effect on IBV. In the above experiments LiCl may have exerted its inhibitory effect either via interfering with viral or cellular processes or through a direct virucidal effect on progeny virus which is present in the supernatant, both of which would result in a drop in progeny virus production. Therefore to distinguish between these possibilities, i.e. to determine whether LiCl has a direct virucidal effect on IBV, a 0.5 ml preparation of IBV (~2x10^6 pfu/ml) was treated with 5 to 50 mM LiCl. As controls, this concentration of virus was also incubated for 1 and 16 h at either 4°C or 37°C to assess the effects of temperature in the presence of the different concentrations of LiCl. After these treatments the amount of virus was determined by plaque assay. The data indicated that there was no significant variation in virus titre of IBV treated with the range of LiCl concentrations, and therefore LiCl did not have a direct virucidal effect on IBV (Figure 3). However, temperature was shown to influence IBV titre, as when the virus was incubated at 4°C for 1 h, the titre was approximately ten-fold higher than when the virus was incubated at
4°C for 16 h. When the virus was incubated at 37°C for 16 h, the virus was rendered non-infectious (data not shown).

**Lithium chloride treatment causes a dose-dependent decrease in the synthesis of IBV protein in infected Vero and DF-1 cells.** To determine the effect of LiCl on virus protein production, the amount of IBV nucleocapsid (N) protein was assayed by western blot. N protein binds viral RNA with high affinity (Chen et al., 2005; Spencer & Hiscox, 2006) and is one of the most abundantly expressed viral proteins in an infected cell (Laude & Masters, 1995), and can thus be used as a sensitive marker for viral protein production. The potential effect of LiCl on cellular protein expression was determined by examining the amount of GAPDH, a cellular housekeeping protein, compared to total protein present. Cell lysates were prepared from either mock-infected or IBV-infected Vero and DF-1 cells either untreated or treated with 5 to 50 mM LiCl. The yield of total protein was determined using the BCA assay and equivalent amounts of protein extract from each experimental treatment used for western blot analysis. The data indicated that the amount of N protein decreased in proportion to the amount of LiCl treatment with apparent abrogation in the amount of N protein when either Vero or DF-1 cells are treated with 50 mM LiCl. Western blot analysis indicated that the amount of GAPDH did not vary between LiCl-treated or untreated Vero or DF-1 cells. Thus the effect of LiCl on the amount of protein was specific to IBV (Figure 4).

The reduction in progeny virus production could therefore be due to the decreased amount of virus proteins observed in infected cells treated with LiCl. This may be a result of either a reduction in the translation of viral sub-genomic mRNAs or a decrease in the amount of sub-genomic mRNAs themselves. With regard to the former possibility, as the translation of both viral and cellular mRNAs is cap-dependent, if LiCl
affected this then we would predict cellular translation would also be decreased. However, analysis of the amount of GAPDH suggested that this protein was unaffected by LiCl.

Therefore, LiCl may act as an inhibitor at the level of genomic RNA and subgenomic mRNA synthesis (with a corresponding effect on translation). Previous studies on HSV have shown that LiCl inhibits DNA synthesis (Skinner et al., 1980), and therefore it is possible to tentatively hypothesise that LiCl may inhibit RNA dependent RNA polymerases (RdRp), which are characteristic of positive and negative stranded RNA viruses. One possibility is that the activity of components of the IBV-encoded RdRp may be affected by LiCl. As the activity of the SARS-CoV helicase is Mg-dependent (Tan et al., 2004), and metal ions can also inhibit the activity of the SARS-CoV 3CL protease (Hsu et al., 2004), the same may also be true for IBV. Another precedence for this is the inhibition by LiCl of the activity of certain cellular proteins. For example, LiCl can inhibit glycogen synthase kinase 3 beta by several different mechanisms (Doble & Woodgett, 2003; Jope, 2003), including competition for Mg ions (Ryves et al., 2002). These hypotheses were tested by comparing the levels of viral RNA between infected cells treated and untreated with LiCl.

**Lithium chloride treatment causes a reduction in IBV genomic and subgenomic RNA levels in infected Vero and DF-1 cells.** To determine the effect of LiCl on virus genomic and subgenomic RNA levels, TaqMan RT-PCR analysis was performed on RNA extracted from mock and infected LiCl-treated cells at 16 h pi, using primer and probe sets designed against the IBV 5’ and 3’ UTRs respectively. These would detect the genomic RNA (5’ UTR sets) and both the genomic RNA and sub-genomic mRNAs (3’ UTR sets). The data indicated that in both Vero and DF-1 cells treated with LiCl,
there was an overall reduction in viral RNA levels as the concentration of LiCl was increased (Figures 5A and 5B, respectively). For example, there was an approximately 20 fold decrease in subgenomic mRNA levels between untreated cells and those treated with 5 mM LiCl in Vero and DF-1 cells (each 40-Ct value represents a 2 fold difference). The RNA levels indicated that the amount of genomic RNA decreased and was not significantly different between 5 and 50 mM LiCl treatment. However, the total positive-sense RNA (subgenomic and genomic) in DF-1 cells generally decreased in a dose dependent manner (except not between 5 and 10 mM LiCl) as the concentration of LiCl was increased. This may account for the general reduction in the amount of N protein observed in infected cells treated with increasing concentrations of LiCl.

These data suggest the potential use of LiCl as an antiviral agent against IBV and by inference, in terms of having common genome and replication strategies, other coronaviruses. Whether or not LiCl could be used in the field against IBV remains to be determined, but certainly it would have application in the laboratory for studying the molecular biology of IBV.

Acknowledgements

This work was funded by the award of a BBSRC DTA/CASE studentship with Intervet UK, Ltd to JAH.

References
Adzhar, A., Shaw, K., Britton, P. & Cavanagh, D. (1996). Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. *Avian Pathology, 25*, 817-836.

Alonso-Caplen, F. V., Matsuoka, Y., Wilcox, G. E. & Compans, R. W. (1984). Replication and morphogenesis of avian coronavirus in Vero cells and their inhibition by monensin. *Virus Research, 1*, 153-167.

Bicknell, K. A., Brooks, G., Kaiser, P., Chen, H., Dove, B. K. & Hiscox, J. A. (2005). Nucleolin is regulated both at the level of transcription and translation. *Biochemical and Biophysical Research Communications, 332*, 817-822.

Bochkov, Y.A., Batchenko, G.V., Shcherbakova, L.O., Borisov, A.V.L & Drygin, V.V. (2006). Molecular epizootiology of avian infectious bronchitis in Russia. *Avian Pathology, 35*, 379-393.

Britton, P., Evans, S., Dove, B., Davies, M., Casais, R. & Cavanagh, D. (2005). Generation of a recombinant avian coronavirus infectious bronchitis virus using transient dominant selection. *Journal of Virological Methods, 123*, 203-211.

Casais, R., Dove, B., Cavanagh, D. & Britton, P. (2003). Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *Journal of Virology, 77*, 9084-9089.

Cavanagh, D. (2003). Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. *Avian Pathology, 32*, 567-582.

Cavanagh, D. (2005). Coronaviruses in poultry and other birds. *Avian Pathology, 34*, 439-448.

Cernescu, C., Popescu, L., Constantinescu, S. & Cernescu, S. (1988). Antiviral effect of lithium chloride. *Virologie, 39*, 93-101.
Chen, H., Coote, B., Attree, S. & Hiscox, J. A. (2003). Evaluation of a nucleoprotein-based enzyme-linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus. *Avian Pathology, 32*, 519-526.

Chen, H., Gill, A., Dove, B. K., Emmett, S. R., Kemp, F. C., Ritchie, M. A., Dee, M. & Hiscox, J. A. (2005). Mass spectroscopic characterisation of the coronavirus infectious bronchitis virus nucleoprotein and elucidation of the role of phosphorylation in RNA binding using surface plasmon resonance. *Journal of Virology, 79*, 1164-1179.

Doble, B. W. & Woodgett, J. R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *Journal of Cell Science, 116*, 1175-1186.

Dove, B. K., Brooks, G., Bicknell, K. A., Wurm, T. & Hiscox, J. A. (2006a). Cell cycle perturbations induced by infection with the coronavirus infectious bronchitis virus and their effect on virus replication. *Journal of Virology, 80*, 4147-4156.

Dove, B. K., You, J.-H., Reed, M. L., Emmett, S. R., Brooks, G. & Hiscox, J. A. (2006b). Changes in nucleolar architecture and protein profile during coronavirus infection. *Cellular Microbiology, 8*, 1147-1157.

Gelb, J. Jr., Weisman, Y., Ladman, B.S. & Meir, R. (2005). S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). *Avian Pathology, 34*, 194-203.

Hsu, J. T., Kuo, C. J., Hsieh, H. P., Wang, Y. C., Huang, K. K., Lin, C. P., Huang, P. F., Chen, X. & Liang, P. H. (2004). Evaluation of metal-conjugated compounds as inhibitors of 3CL protease of SARS-CoV. *FEBS Letters, 574*, 116-120.

Ignjatovic, J. & Sapats, S. (2000). Avian infectious bronchitis virus. *Review of Science and Technology, 19*, 493-508.
Jackwood, M. W., Hilt, D. A., Lee, C. W., Kwon, H. M., Callison, S. A., Moore, K. M., Moscoso, H., Sellers, H. & Thayer, S. (2005). Data from 11 years of molecular typing infectious bronchitis virus field isolates. *Avian Diseases, 49*, 614-618.

Jope, R. S. (2003). Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes. *Trends in Pharmacological Sciences, 24*, 441-443.

Kaiser, P., Underwood, G. & Davison, F. (2003). Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. *Journal of Virology, 77*, 762-768.

Kottier, S. A., Cavanagh, D. & Britton, P. (1995). Experimental evidence of recombination in coronavirus infectious bronchitis virus. *Virology, 213*, 569-580.

Lai, M. M. C. & Cavanagh, D. (1997). The molecular biology of coronaviruses. *Advances in Virus Research, 48*, 1-100.

Laude, H. & Masters, P. S. (1995). The coronavirus nucleocapsid protein. In *The Coronaviridae*, pp. 141-163. Edited by S. G. Siddell. New York: Plenum Press.

Lee, C. W. & Jackwood, M. W. (2000). Evidence of genetic diversity generated by recombination among avian coronavirus IBV. *Archives of Virology, 145*, 2135-2148.

Liu, S.W., Chen, J.F, Han, Z.X., Zhang, Q.X., Shao, Y.H., Kong, X.G. & Tong, G.Z. (2006). Infectious bronchitis virus: S1 gene characteristics of vaccines used in China and efficacy of vaccination against heterologous strains from China. *Avian Pathology, 35*, 394-399.

Pasternak, A. O., Spaan, W. J. & Snijder, E. J. (2006). Nidovirus transcription: how to make sense...? *Journal of General Virology, 87*, 1403-1421.

Peiris, J. S., Guan, Y. & Yuen, K. Y. (2004). Severe acute respiratory syndrome. *Nature Medicine, 10*, S88-97.
Pendleton, A. R. & Machamer, C. E. (2006). Differential localization and turnover of infectious bronchitis virus 3b protein in mammalian versus avian cells. *Virology, 345*, 337-345.

Raj, G. D. & Jones, R. C. (1996). Immunopathogenesis of infection in SFP chicks and commercial broiler chickens of a variant infectious bronchitis virus of economic importance. *Avian Pathology, 25*, 481-502.

Reed, M., Dove, B. K., Jackson, R. M., Collins, R., Brooks, G. & Hiscox, J. A. (2006). Delineation and modelling of a novel nucleolar retention signal in the coronavirus nucleocapsid protein. *Traffic, 7*, 833-849.

Ryves, W. J., Dajani, R., Pearl, L. & Harwood, A. J. (2002). Glycogen synthase kinase-3 inhibition by lithium and beryllium suggests the presence of two magnesium binding sites. *Biochemical and Biophysical Research Communications, 290*, 967-972.

Skinner, G. R., Hartley, C., Buchan, A., Harper, L. & Gallimore, P. (1980). The effect of lithium chloride on the replication of herpes simplex virus. *Medical Microbiology Immunology, 168*, 139-148.

Spencer, K.-A. & Hiscox, J. A. (2006). Characterisation of the RNA binding properties of the coronavirus infectious bronchitis virus nucleocapsid protein amino-terminal region. *FEBS Letters*.

Tan, K.-P., Shih, K.-N. & Lo, S. J. (2004). Ser-123 of the large antigen of hepatitis delta virus modulates its cellular localization to the nucleolus, SC-35 speckles or the cytoplasm. *Journal General Virology, 85*, 1685-1694.

Wang, L., Junker, D. & Collisson, E. W. (1993). Evidence of natural recombination within the S1 gene of infectious bronchitis virus. *Virology, 192*, 710-716.
Weiss, S. R. & Navas-Martin, S. (2005). Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiology Molecular Biology Review, 69*, 635-664.

Ziaie, Z., Brinker, J. M. & Kefalides, N. A. (1994). Lithium chloride suppresses the synthesis of messenger RNA for infected cell protein-4 and viral deoxyribonucleic acid polymerase in herpes simplex virus-1 infected endothelial cells. *Laboratory Investigation, 70*, 29-38.

Ziaie, Z. & Kefalides, N. A. (1989). Lithium chloride restores host protein synthesis in herpes simplex virus-infected endothelial cells. *Biochemical Biophysical Research Communications, 160*, 1073-1078.
Table 1. Primer and probe sets used for Taqman RT-PCR in this study.

| Target          | Forward Primer | Reverse Primer | Probe $^a$          |
|-----------------|----------------|----------------|--------------------|
| Vero 28S        | GGCGAAAGACTA   | CGAGAGCGCCAG   | TAGTAGCTGGTT       |
|                 | ATCGAACCAT     | CTATCCT       | CCCTCCGAAGTT       |
|                 |                |               | TCCCT             |
| Chicken 28S     | GGCGAAGCC      | GACGACCGATT   | AGGACCGCTACG       |
|                 | AGAGGAAACT     | GCACGTC       | GACCTCCACCA        |
| IBV 5' UTR      | CGTACCGGTTCCT  | GCCCAACGCTAG  | TCACCTCCCCCC       |
|                 | GTTGTGTGTA     | GCTCAA        | ACATACCTCTAA       |
| IBV 3' UTR      | ACGAACGGTAGA   | TGGCGTCCTAG   | TACTCAGCGTG        |
|                 | CCCTTAGATTTT   | TGCTGTACTAA   | CCCGGCA           |
|                 | AATT           |               |                   |

$^a$At the 5’ end of each probe was a fluorescent reporter dye 5-carboxyfluorescein (FAM) and at the 3’ end a quencher N, N, N, N’-tetramethyl-6-carboxyrhodamine (TAMRA).
Figure 1. Plaque assay showing plaque formation in (A) Vero and (B) DF-1 cells infected with progeny IBV from cells treated with the concentrations of LiCl indicated to the left. Due to IBV replicating less efficiently in Vero cells compared to DF-1 cells, only three-ten fold serial dilutions of progeny IBV from Vero cells were required compared to six ten-fold serial dilutions of progeny IBV from DF-1 cells. The experiment was repeated three times and one representative set of data are presented, the average pfu/ml is indicated to the right.
Figure 2. Histogram showing the relative virus titre of progeny virus from Vero cells (grey) and DF-1 cells (dark grey) treated with the concentrations of LiCl indicated on the X-axis as compared to those cells untreated (=100%).
Figure 3. Histogram showing the relative average virus titre of IBV treated directly with the concentrations of LiCl shown at 4°C (light grey) or 37°C (dark grey) for 1 h or at 4°C for 16 h (white), as assayed in DF-1 cells.
Figure 4. Western blot analysis of the amount of IBV N protein in mock and IBV-infected (A) Vero and (B) DF-1 cells treated with the concentrations of LiCl indicated above each blot. GAPDH was used as a marker for cellular protein levels. The migration of molecular weight markers is indicated to the left.
Figure 5. Real-time RT-PCR analysis of the levels of IBV genomic RNA as well as genomic and subgenomic mRNAs, as determined by analysis of the IBV 5’ UTR (light grey) and 3’ UTR (dark grey), respectively, in infected Vero (A) and DF-1 cells (B).