Received: 14 April 2021 | Revised: 11 May 2021 | Accepted: 17 May 2021

DOI: 10.1002/pep2.24234

ARTICLE

PeptideScience WILEY

Antiviral activity of selected cathelicidins against infectious bronchitis virus

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Funding information
Ministry of Economic Affairs, Grant/Award Number: ASIA2

Abstract

Avian infectious bronchitis (IB) is a highly contagious disease caused by infectious bronchitis virus (IBV), a coronavirus of domestic fowl. IB is a major concern in the poultry industry, causing worldwide economic losses through decreased egg production and quality and by increasing the chicken's susceptibility for secondary bacterial infections, particularly *Escherichia coli*. In this study, the anti-IBV activity of cathelicidins, small antimicrobial peptides of the innate immune system was investigated. The cell culture adapted (nonvirulent) IBV strain Beaudette was effectively inhibited by the human cathelicidin LL-37 in bovine hamster kidney-21 cells at non-toxic concentrations. The peptide needed to be present during virus inoculation to effectively inhibit the infection of IBV-Beaudette, indicating that LL-37 likely bound viral particles. However, no clear morphological changes in the IBV virion upon binding were observed by electron microscopy. In this cell culture model, chicken cathelicidins (CATH1-3) were inactive against IBV-Beaudette. In contrast, in multicellular infection models using the virulent IBV-M41 strain the activities of human and chicken cathelicidins were different. In particular, upon inoculation of 10-day-old embryonic eggs with IBV-M41, CATH-2 reduced the viral load to a higher extend than LL-37. Similarly, viral infection of chicken tracheal organ cultures with IBV-M41 was significantly reduced in the presence of CATH-2 but not LL-37. These results indicate a potential antiviral role for CATH-2 upon IBV infection *in vivo*.

KEYWORDS
coronavirus, host defense peptides, innate immunity

1 | INTRODUCTION

Host defense peptides (HDPs) are small cationic peptides that play an important role in the innate host defense against pathogens.[1] These peptides are produced as prepropeptides by epithelial and innate immune cells.[2] Upon release of the HDPs the prepro sequence is cleaved releasing the active peptides locally where they exert their antimicrobial and immunomodulatory activities. While HDPs of the

family of defensins are structurally very similar, characterized by three intramolecular disulfide bonds in the mature peptide, the mature cathelicidin peptides are structurally very diverse.[3–5]

Cathelicidins were originally described as strong antibacterial compounds with activity against a broad range of Gram-positive and Gram-negative bacteria.[6] However, currently their spectrum has been extended toward their antimicrobial activity against fungi and viruses.[7–9] Besides that, these peptides show strong immunomodulatory activity through either direct interaction with immune cells or interaction with microbial products leading to modulation of immune responses through, for example, Toll-like receptors.[10,11]

With respect to antiviral activity the best-described cathelicidin is LL-37, the sole human cathelicidin. For this peptide, antiviral activity has been observed against both DNA and RNA viruses, including influenza, HIV, Vaccinia, PRRSV and Dengue virus.[9,12–15] Similarly, several other cathelicidins from, for example, porcine and bovine origin have also shown antiviral activity, although mainly *in vitro*.[16,17] Information on the role of avian cathelicidins in viral infections is scarce, but a recent report described that among other innate effector molecules, avian defensins and two chicken cathelicidins were upregulated upon infectious bronchitis virus (IBV) infection.[18] While this indicated a potential role for these peptides in antiviral defense, direct antiviral activity of cathelicidins against IBV has not been described yet.

IBV is a positive strand RNA virus belonging to the order *Nidovirales*, family *Coronaviridae*. The virus can cause major health problems with subsequent economic losses in the poultry industry. Its global economic impact has been estimated as the second most damaging poultry disease after highly pathogenic avian influenza (TAFS-Forum 2011).[19] It initially infects the epithelium of the upper respiratory tract of predominantly chickens (*Gallus gallus*), and depending on the virus strain, it can show tropism for other epithelia, including the renal tubuli, the oviduct and parts of the gastrointestinal tract.[20–22] Many different virus strains circulate worldwide (reviewed in refs. [20,23,24]), but studying these field viruses is complicated by the fact that their growth is only supported in complex multicellular systems like embryonated chicken eggs and trachea organ cultures. In contrast, IBV-Beaudette, a highly adapted IBV strain that is nonpathogenic for chickens[25] can infect cell culture cells,[26] and is therefore often used as model for IBV.

In this article, the activity of human LL-37 and chicken cathelicidin 2 (CATH-2) against highly attenuated IBV-Beaudette and virulent IBV-M41 was investigated. Although LL-37 showed strong antiviral activity against IBV-Beaudette, CATH-2 was most effective in inhibiting the virulent IBV-M41 strain in more relevant multicellular models.

2 | MATERIALS AND METHODS

2.1 | Cells, eggs, and viruses

Baby hamster kidney (BHK) 21 cells were obtained from the American type culture collection (ATCC-CCL-10) and used within 20 passages after thawing, BHK-21 cells, the only IBV—susceptible cell line currently available, were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% fetal calf serum (FCS) (Bodinco BV, Alkmaar, The Netherlands), penicillin (100 units/mL), and streptomycin (100 μg/mL) (Thermo Fisher Scientific). All cells were grown at 37 °C in 5% CO_2. Virus stocks of IBV-Beaudette were grown and titrated in BHK-21 cells; virus stocks of IBV-M41 (Animal Health Service, Deventer, The Netherlands) were grown in 10-day-old specific pathogen free (SPF) embryonated chicken eggs (ECEs) (Animal Health Service). Virus titration on BHK-21 (Beaudette) and *in ovo* (M41) was based on 50% infectious dose (ID_{50}) per mL, as determined at day 7 post-infection (pi) according to Reed and Muench.[27]

2.2 | Peptides

Peptides were synthesized using Floc solid-phase synthesis (CPC Scientific, Sunnycale, California). All peptides were purified to a minimum purity of 95% by reverse phase high-performance liquid chromatography prior to biological testing. The sequences of the peptides used in this study are shown in Table 1.

TABLE 1 Host defense peptides

	Amino acid sequence	AA	Charge
CATH-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	26	+8
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF	26	+9
CATH-3	RVKRFWPLVPVAINTVAAGINLYKAIRRK	29	+7
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	+6
CRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	34	+5
HYB1	*RFGRFLRKIRRFRPK*FKRIVQRIKDFLRNLV	31	+12
HYB2	*RFGRFLRKIRRFRPK*RIVQRIKDFLRNLV	29	+11
HYB3	*RFGRFLRKIRRFRFK*RIVQRIKDFLRNLV	29	+11
HYB4	LLGDFFRKSKEKIGKEF*RPK*VQRIKDFLRNLVPRTES	37	+6

Note: Sequences and characteristics of the HDPs used in this study. The hybrid (HYB) peptides are based on LL-37, with CATH-2-based partial sequences indicated in italics.

2.3 | Inhibition of the infectivity of IBV-Beaudette by cathelicidins

BHK-21 cells were seeded in 24-well plates at 200 000 cells per well and inoculated the next day with IBV-Beaudette at a multiplicity of infection (MOI) of 0.1 in DMEM. This MOI provides a good opportunity to measure inhibition of infection without overwhelming the cells with virus particles. The indicated amounts of HDPs were added to the cells before (pre), during (co) or after (post) virus inoculation. After 2 hours of virus inoculation, the cells were washed and fresh culture medium was added. At 8 hours pi, the cells were lysed for RNA isolation.

2.4 | Cytotoxicity

Cytotoxicity of the peptides on BHK-21 cells was determined by WST-1 assay according to the manufacturer (Roche, Basel, Switzerland). BHK-21 cells were seeded in 24-well plates at 200 000 cells per well and incubated with 0-10 μM HDPs (twofold higher concentration than required for antiviral activity) for 10 hours. Subsequently, the cell proliferation reagent WST-1 was added and after 30 minutes absorbance was measured at 450 nm with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and was corrected for absorbance at 630 nm. Nontreated control cells were defined as 100% mitochondrial activity.

2.5 | Real-time PCR for quantification of viral RNA synthesis

From IBV-infected cells, total RNA was extracted using the High Pure RNA Tissue kit (Roche, Mannheim, Germany.) For isolation of RNA from culture supernatant and allantoic fluid, the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. Subsequently, cDNA was generated using the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, California). Reverse transcription quantitative-PCR using SYBR-green was performed as previously described using GAPDH for relative quantitation of IBV gene expression.[28]

2.6 | Electron microscopy

IBV-Beaudette was incubated with various concentrations up to 40 μM (eightfold higher than the maximum activity to ensure possible morphological changes) of LL-37 or CATH-2 for 1 hour at room temperature before inoculation with 4% glutaraldehyde (Polysciences Inc, Warrington, Pennsylvania) at 4 °C overnight. Fixed samples were adhered to carbon coated grids and stained with 1% ammonium molybdate (pH = 6), washed and dried for 1 hour. Imaging was done using a FEI Tecnai 12 electron microscope at 80 kV.

2.7 | *In ovo* inoculation

Ten-day-old embryonated chicken eggs (ECEs) were inoculated into the allantoic cavity with 100 EID_{50} IBV-Beaudette or IBV-M41 premixed with HDPs at 5 μM (max *in vitro* activity). At 12 hours pi, the allantoic fluid was collected and stored at −80 °C before RNA isolation and RT-qPCR. For each condition, five ECEs were inoculated and the experiment was performed in triplicate.

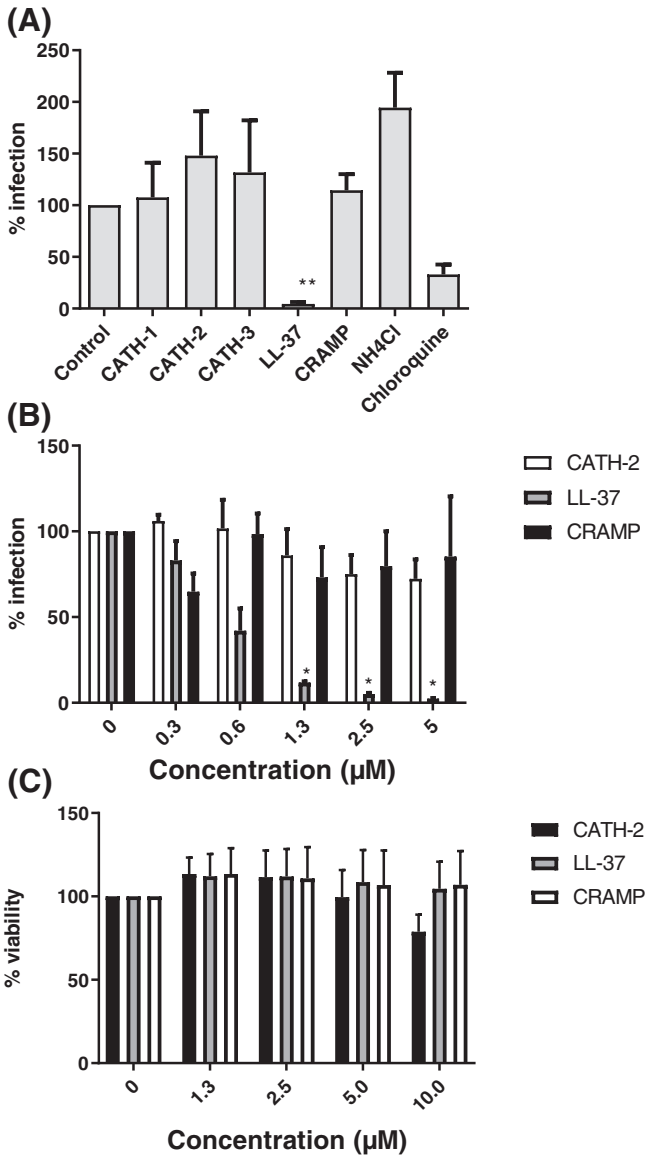

FIGURE 1 LL-37 inhibits viral infection of BHK-21 cells. A, BHK-21 cells were infected with IBV-Beaudette in the presence of different HDPs at a concentration of 5 μM. B, Concentration dependency of antiviral activity of LL-37, CATH-2, and CRAMP. Viral replication was measured after 8 hours using qPCR. C, Effect of HDPs on metabolic activity of BHK-21 cells measured by WST-1 reduction assay. Data are shown as mean ± SEM of at least three independent experiments for each group (in triplicate). * indicates significant difference ($P \leq .05$) compared to no-peptide controls

2.8 | Trachea organ cultures

All experiments involving animals were conducted in accordance with a protocol approved by the Dutch Animal Experimental Licensing Committee (DEC, license AVD108002016642-1), trachea were collected from adult chickens, sacrificed for teaching purposes and flushed four times with phosphate buffered saline (PBS) before being sliced into rings of 2-4 mm thickness. The generated TOCs were incubated in DMEM containing penicillin (100 units/mL) and streptomycin (100 μg/mL) (Thermo Fisher Scientific) for 24 hours while rotating. After inspection for ciliary movement five TOCs per condition were inoculated with different amounts of IBV-M41 premixed with 5 μM LL-37 or CATH-2 in DMEM. At 16 hours pi, supernatant was collected from individual TOCs and stored at −80 °C before RNA isolation and RT-qPCR. TOCs were fixed in neutral buffered 10% formalin in PBS for 24 hours, stored in 70% ethanol and paraffin embedded. The 4 μm sections of TOCs were mounted on glass slides and subsequently deparaffinized and rehydrated in alcohol series.

2.9 | Anti-IBV immunohistochemistry

Immunohistochemistry was performed as previously described[28] using MAb Ch/IBV 26.1 directed against the S2 protein of IBV (Wageninigen Bioveterinary research-Lelystad, The Netherlands) diluted 1:100 in phosphate buffered normal antibody diluent (NAD, ScyTek Laboratories, Logan) + 0.1% Tween-20. Staining was visualized by 3-amino-9-ethylcarbazole (AEC; Agilent, Santa Clara, California).

2.10 | Statistics

Data were analyzed using SPSS version 16.0 software (IBM, Armonk, New York). Gaussian distribution of the data was checked with the Shapiro-Wilk test for normality and data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's post hoc tests. Differences were considered significant if $P < .05$.

3 | RESULTS

3.1 | Human, but not mouse or chicken cathelicidin inhibits IBV infection of BHK-1 cells

To study whether cathelicidins can inhibit the infection of IBV, BHK-21 cells were inoculated with the cell culture-adapted IBV-Beaudette strain at an MOI of 0.1 in the presence of 5 μM of chicken CATH-1, -2, -3, human LL-37, or mouse CRAMP. Intracellular viral RNA levels were quantified at 8 hpi by RT-qPCR. Chloroquine (25 μM) and ammonium chloride (10 mM), both blockers of endocytosis were used as controls. Interesting, while chloroquine showed a strong tendency to block the infection ($P = .055$), ammonium chloride unexpectedly, and for yet unknown reason, rather increased the infection. From the cathelicidins tested, only the human cathelicidin LL-37 reduced the amount of IBV RNA in the cells significantly ($P = .0011$) (Figure 1A).

In order to extend the observations on antiviral activity, BHK-21 cells were incubated with IBV-Beaudette in the presence of different concentrations (0-5 μM) LL-37, CATH-2, and CRAMP. There was a clear dose dependency observed for LL-37 with significant reductions in IBV-Beaudette infection starting at 1.25 μM while CATH-2 and

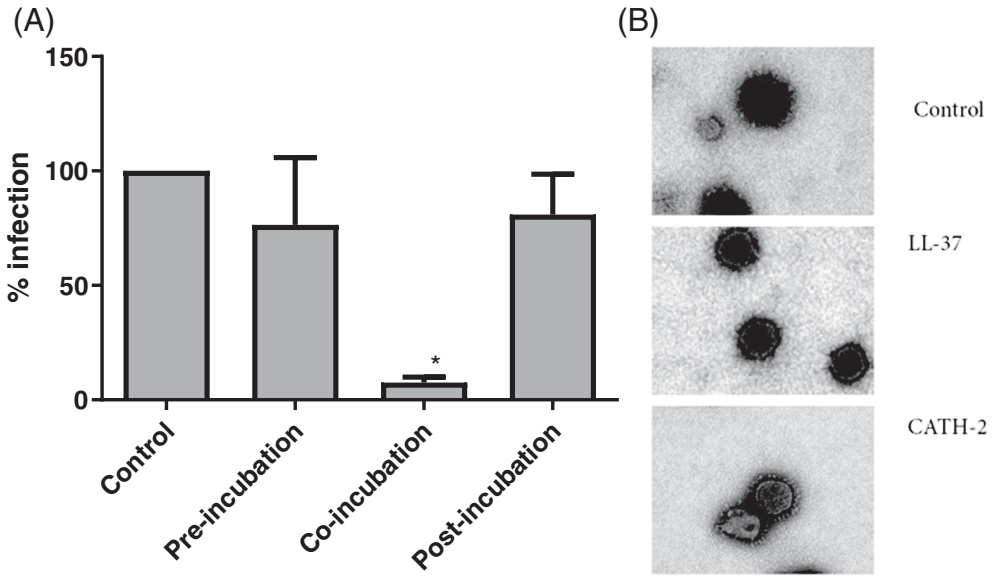

FIGURE 2 LL-37 antiviral activity is dependent on interaction with the IBV virion. A, BHK-21 cells were infected with IBV-Beaudette. LL-37 was added either 2 hours before infection (pre), together with the virus (co) or 2 hours after (post) addition of virus. Viral replication was measured after 8 hours using qPCR. B, Transmission electron microscopy of viral particles incubated with 40 μM CATH-2 or LL-37. Data (2A) are shown as mean ± SEM of at least three independent experiments for each group (in triplicate). * indicates significant difference ($P \leq .05$) compared to the no peptide control

CRAMP had no effect on the amount of viral RNA present in the cells at any concentration (Figure 1B).

To rule out that the inhibitory effect of LL-37 was due to toxicity to the cells thereby negatively affecting the replication of IBV, cell viability was measured after incubation with different concentrations of HDPs. These studies showed that none of the previously used concentrations of LL-37, but also not higher concentrations (10 μM), affected cell viability (Figure 1C). Only CATH-2 had a slight tendency toward cytotoxicity at the highest concentration tested.

In conclusion, IBV-Beaudette infectivity of BHK-21 cells can be inhibited by human cathelicidin LL-37 in a concentration-dependent manner.

3.2 | LL-37 has a direct antiviral effect but does not affect IBV's structure

To reveal whether LL-37 has a direct antiviral action or exerts its mode of action via previously described immunomodulatory effects on cells, LL-37 was added to the cells at different times during the infection. BHK-21 cells were either preincubated for 2 hours with LL-37 ("pre"), virus and LL-37 were mixed and applied to the cells together ("co"), or LL-37 was applied to cells 2 hours after incubation with IBV Beaudette ("post"). At 8 hpi, cells were lysed and viral RNA was determined by RT-qPCR (Figure 2A). The results clearly showed that LL-37 had the ability to reduce viral RNA content only upon co-infection, suggesting that the peptide had a direct effect on the virus, rather than on cellular processes that are critical for virus replication.

To understand the mode of action of LL-37 against IBV-Beaudette, we first elucidated whether its antiviral activity was due to disruption of the viral envelope since HDPs are known to exert their antibacterial activity through penetration of membranes. To investigate whether this is also the basis of its antiviral activity, negative staining electron microscopy was performed on IBV-Beaudette incubated with 40 μM of LL-37, and CATH-2 was taken along as a control. Figure 2B shows that the structure of the IBV virion is unaffected by the presence of either HDP, indicating that the direct action of LL-37 on viral activity is not likely through disruption of the viral membrane.

In addition, we performed isothermal titration calorimetry (ITC) to determine potential binding between IBV Beaudette viral particles

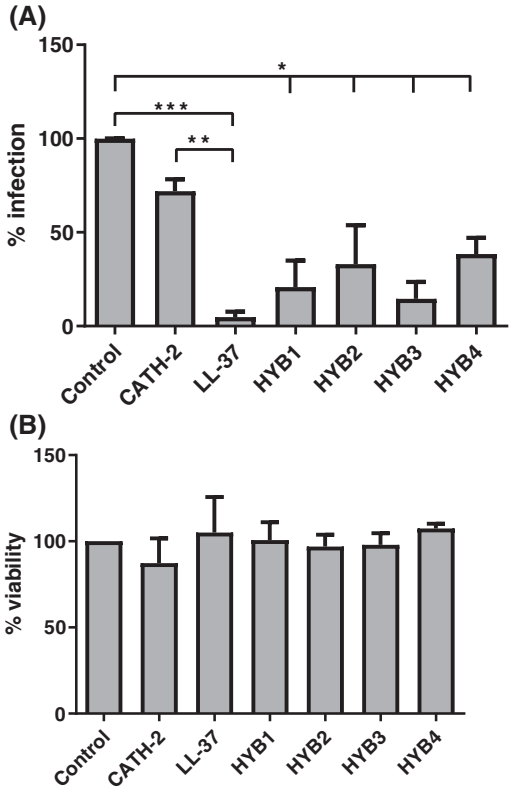

FIGURE 3 Introduction of CATH-2-specific structural elements lowers the antiviral activity of LL-37. A, BHK-21 cells were infected with IBV-Beaudette in the presence of chimeric LL-37/CATH-2 peptides. After 8 hours viral replication was determined using qPCR. B, Effect of chimeric LL-37/CATH-2 peptides on metabolic activity of BHK-21 cells measured by WST-1 reduction assay. Data are shown as mean ± SEM of at least three independent experiments for each group (in triplicate). * indicates significant difference ($P \leq .05$) between connected groups

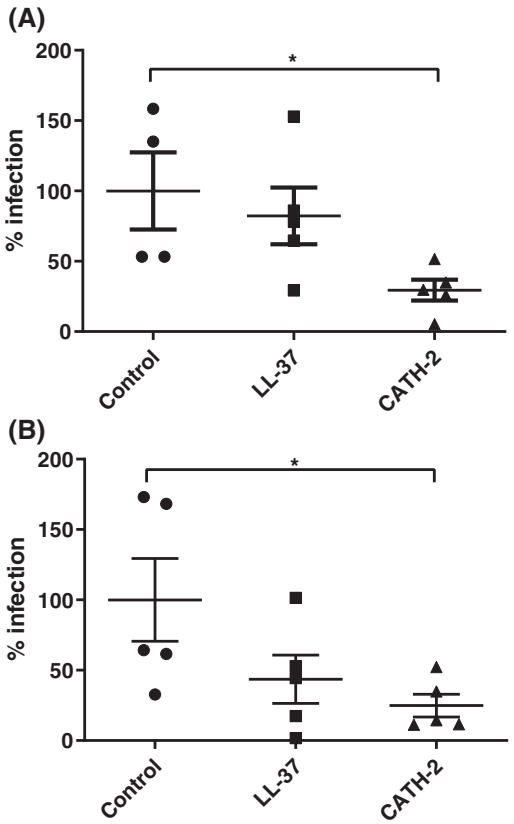

FIGURE 4 CATH-2 is a stronger inhibitor than LL-37 of IBV infection *in ovo*. Ten-day-old embryonated chicken eggs were inoculated into the allantoic cavity with 100 EID_{50} IBV-Beaudette or IBV-M41 premixed with peptides at 5 μM. At 12 hours pi viral load in the allantoic fluid (AF) was determined using RT-qPCR. A, IBV-Beaudette, B, IBV-M41. * indicates significant difference ($P \leq .05$) between connected groups

and HDPs. However, no binding could be detected using this methodology (data not shown).

3.3 | Antiviral activity of Chimeric LL-37/CATH-2 is lower than LL-37

To further understand why human LL-37, but not avian CATH-2, has antiviral activity against IBV-Beaudette, chimera-peptides containing structural elements of both LL-37 and CATH-2 were tested for their ability to reduce IBV infection of BHK-21 cells. All chimeras with an N-terminal segment of CATH-2 (HYB1, HYB2, HYB3; Table 1) were seemingly less potent than LL-37 in inhibiting the infection (Figure 3A), but also HYB4, which is basically the LL-37 sequence, with a proline-induced (CATH-2 like) kink in the helix showed reduced activity. Overall, these observations show that elements of CATH-2 within the peptide sequence of LL-37 negatively affected antiviral activity. None of the chimeric HDPs affected the cell viability up to 10 μM (Figure 3B).

3.4 | CATH-2, but not LL-37 inhibits IBV infection *in ovo*

IBV-Beaudette is a cell culture adapted IBV strain, known to have lost its ability to cause disease in chickens.[25] To further expand our knowledge on the antiviral capacity of HDPs against IBV, an infection model was used in which infection and replication of both IBV-Beaudette and IBV-M41, a virulent prototype Massachusetts IBV field strain, are supported. To this end, embryonated chicken eggs (ECE) were inoculated via the allantoic route with either IBV-Beaudette or IBV-M41 in the presence or absence of 5 μM CATH-2 and LL-37 in the inoculum (Figure 4). At 12 hpi, the allantoic fluid of the ECEs was collected and analyzed for the presence of viral RNA. Surprisingly, while LL-37 only had a minor effect on IBV-Beaudette infection, CATH-2 inhibited viral infection of ECEs by 70% (Figure 4A). For IBV-M41 comparable results were obtained, with CATH-2 significantly reducing infection by 75%, while LL-37 at most showed a (nonsignificant) tendency to lower infection by IBV-M41 (Figure 4B).

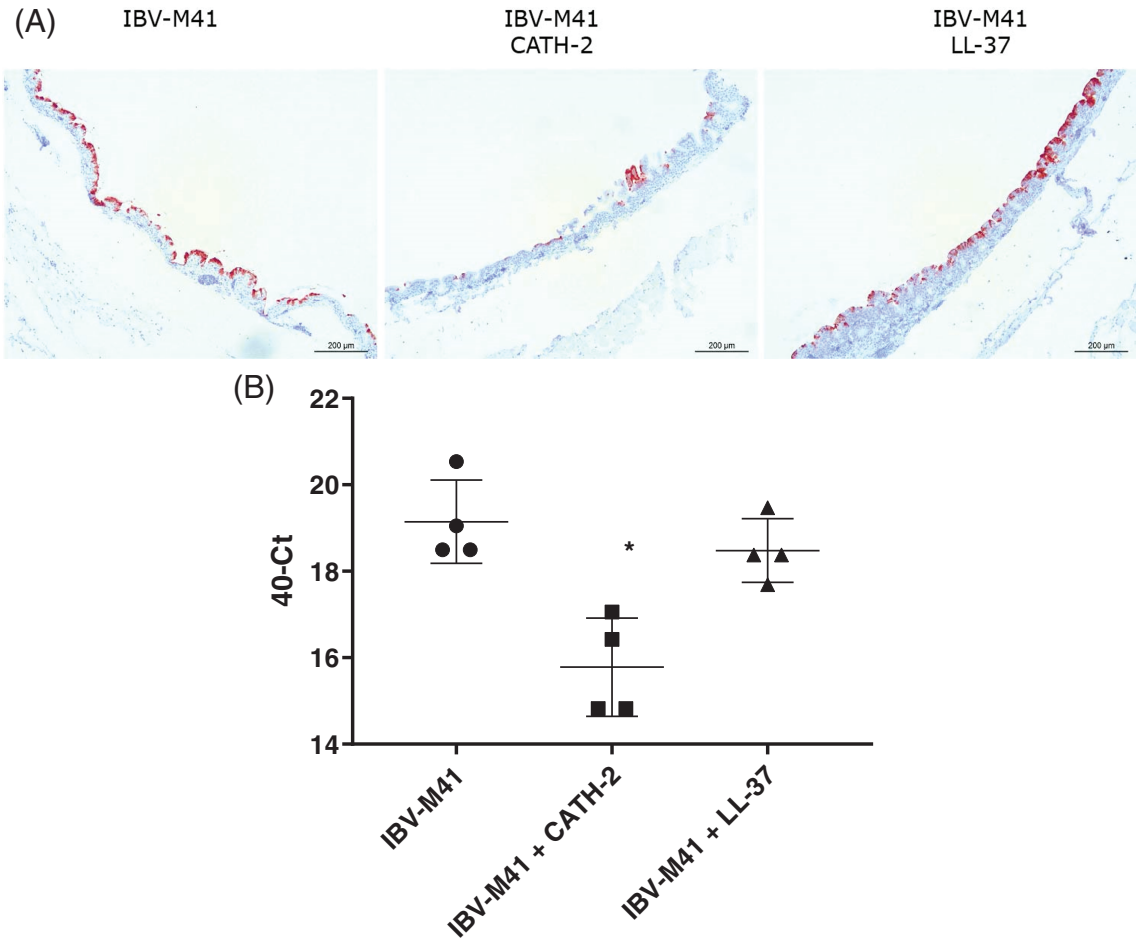

FIGURE 5 CATH-2, but not LL-37, inhibits IBV-M41 infection in TOCs. TOCs were inoculated with IBV-M41 (10^4 EID_{50}) in the absence or presence of CATH-2 or LL-37 at a concentration of 5 μM. A, Immunohistochemistry of TOCs fixed at 16 hours pi and stained using anti-S2 IBV antibody. B, qPCR on culture supernatant to measure relative viral replication at 16 hours pi. Data are shown as mean ± SEM of four independent experiments for each group (in triplicate). * indicates significant difference ($P \leq .05$) compared to the virus only control

3.5 | CATH-2, but not LL-37 inhibits IBV infection in tracheal organ cultures

Adult tracheal organ cultures (TOCs) morphology resembles that of the chicken trachea upon culture for 5 days (Weerts *et al*., unpublished data), in contrast to the often used embryonic TOCs and therefore allowed us to study the effect of the HDPs in a relevant ex vivo model. Unfortunately, IBV-Beaudette does not infect these TOCs, as repeatedly no expression of viral proteins could be observed after inoculation unlike infection of embryonic TOCs (control experiment, data not shown). Inoculation of TOCs with 10^4 EID_{50} IBV-M41 showed 80-90% IBV-antigen positive epithelial cells at 16 hours pi (Figure 5A). Inoculation of TOCs in the presence of 5 μM CATH-2, but not with LL-37, resulted in a clear reduction in number of infected cells, as demonstrated by immunohistochemistry (Figure 5A). RT-qPCR on viral RNA isolated from the culture supernatant of infected TOCs confirmed that there was a 10-fold decrease ($P < .005$) in viral RNA in the presence of CATH-2 but not with LL-37 (Figure 5B). Comparable results were obtained when TOCs were inoculated with 10^3 EID50, resulting in about 10% IBV-positive cells without treatment and again significant inhibition of viral RNA replication of CATH-2, but not LL-37 (data not shown). Taken together, in multicellular organ systems, the chicken CATH-2 has strong antiviral activity against virulent IBV.

4 | DISCUSSION

We here described that both human LL-37 and chicken CATH-2 demonstrate antiviral activity against infectious bronchitis virus. The antiviral activity of cathelicidins against IBV is strain and model dependent. In particular, while the infection of IBV Beaudette in cell culture cells could be inhibited only by LL-37, in tracheal organ cultures, infection by virulent IBV-M41 was inhibited by CATH-2 only. Subsequently in ECE, CATH-2 also showed a stronger, although not significant, antiviral activity than LL-37.

Antiviral activity of HDPs against viruses has been acknowledged for some time. For LL-37, several studies have shown that it can inhibit HIV,[29] RSV and vaccinia virus,[12,30,31] HSV-1,[32,33] PRRSV,[15] and Influenza virus[13,34] *in vitro*. To the best of our knowledge, however, this is the first study reporting antiviral activity of a chicken cathelicidin against avian viruses and the first report of antiviral effect of cathelicidins against a coronavirus.

The antiviral activity of CATH-2 and LL-37 seems to be dependent on the IBV strain and, consequently, the model studied. Whereas it is clear for IBV Beaudette that LL-37 inhibits entry or very early steps in infection in cell culture cells, deciphering this in complex models needed for virulent IBV strains is much more difficult. Direct binding of the LL-37 peptide to the viral particle has been shown for other viruses, including RSV,[30] influenza A virus[13] and vaccinia virus,[31] resulting in disruption of the viral membrane and clear morphological changes of the viral particle. In our studies, we also observed a requirement for co-incubation of LL-37 and IBV-Beaudette, and thus a likely requirement for direct interaction between the peptide and the virus for inhibiting activity. However, for IBV-Beaudette no disruption of the virus membrane or aggregation of virus particles was observed upon incubation with LL-37 (or CATH-2). ITC also failed to detect direct interaction between HDPs and IBV Beaudette. However, ITC requires relatively high concentrations of binding components and since it is unclear what component of IBV is (potentially) bound by HDPs, this technique might lack sensitivity to detect binding.

As we observed differences in antiviral action across models, it might well be that, in addition to direct binding of HDPs to viral particles, they modulate the antiviral response of host cells differently across cell types. In that respect it is interesting to note that the chicken HDP was more active in the *ex vivo* model using chicken TOCs. Cathelicidins have many immunomodulatory functions that could aid in the antiviral response. For example, these peptides have been shown to induce cytokine production in immune cells but can also act indirectly *in vivo* through chemokine production leading to increased recruitment of immune cells to the site of infection.[35,36] Such cellular effects might affect the virus infectious cycle at different stages. Here, we focused on analyzing antiviral activity at the earliest time point feasible for detection of viral RNA, that is after one single viral replication cycle. While our data demonstrate that inhibition of IBV-Beaudette in BHK21 cells only occurs upon co-incubation, suggesting that at least in this model direct immunoregulatory roles likely do not contribute to antiviral activity, the role of host cell responses in TOCs and ECEs cannot be excluded. Indeed, differences in responses to LL-37 between cell systems have been observed for other viruses, including RSV.[37] However, it is clear that the small tool set that is available for IBV research at the moment limits the possibilities to unravel the exact modes of anti-IBV activities of cathelicidins.

LL-37 and CATH-2 both belong to the cathelicidin family of host defense peptides, but this classification is somewhat misleading since the mature (active) cathelicidin peptides are often structurally very different. Both LL-37 and CATH-2 are cationic and mainly helical but there is no sequence homology.[38,39] In addition, CATH-2 contains a proline residue in its sequence that results in a break of the helix that is the dominating structural element of the tested HDPs. In order to obtain an initial indication for the structural requirements of activity we performed some antiviral activity assays with IBV-Beaudette with LL-37 / CATH-2 hybrid peptides. The hybrid peptides either contained the N-terminal part of CATH-2 (HYB1-3), which is the active domain for antibacterial activity, while in HYB 4, the characteristic proline residue was introduced into the LL-37 sequence. All of these peptides were less efficient in inhibition of infectivity, showing that both primary and secondary structural elements are a main determinant for activity. Obviously for a full structure—(antiviral) activity relationship study, testing of many more peptides would be required. However, this is of limited value using the cell culture adapted IBV-Beaudette strain given the observed strain dependency for antiviral activity, while the more complex models used for testing virulent IBV strains are very low throughput systems. Overall though, it would be interesting to test a much larger array of cathelicidins or derived peptides in order to be able to appoint peptide determinants for antiviral activity. Such structure activity studies are very common for HDP's antibacterial activity but almost completely lacking for antiviral studies.

5 | CONCLUSIONS

The initial lack of antiviral activity for chicken CATH-2 using IBV-Beaudette did not correlate with its activity against the virulent IBV strain M41. CATH-2 was actually more potent than LL-37 in experimental models (*in ovo*, and in TOCs) where IBV M41 can be used for infection. These results indicate a potential antiviral role for CATH-2 upon IBV infection *in vivo*.

ACKNOWLEDGMENTS

The authors would like Hanne Tjeerdsma and Kim Bouwman for their help in several parts of the experiments. This work was financially supported by the Immuno Valley ALTANT ASIA2 program of the Dutch Ministry of Economic Affairs.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

M. Hélène Verheije wrote the manuscript and provided supervision to most of the co-authors. Maarten Coorens designed the study, analyzed data and performed *in vitro* infection studies. Erik A. W. S. Weerts performed the TOC experiments. Steven J. van Beurden performed the *in ovo* experiments. Soledad R. Ordonez performed the EM studies, Alinda J. Berends, Marloes Angel, Jannetje Kooij, Albert van Dijk, and Roel M. van Harten performed all other experiments. Henk P. Haagsman and Edwin J. A. Veldhuizen supervised the project, interpreted the data and wrote and critically analyzed the manuscript.

DATA AVAILABILITY STATEMENT

All data are shown in the manuscript and also available upon request.

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How to cite this article: M. H. Verheije, M. Coorens, E. A. W. S. Weerts, A. J. Berends, R. M. van Harten, M. Angel, J. Kooij, S. R. Ordonez, S. J. van Beurden, A. van Dijk, H. P. Haagsman, E. J. A. Veldhuizen, *Peptide Science* **2022**, *114*(1), e24234. https://doi.org/10.1002/pep2.24234