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Exploring the Extended Pathogenesis of Infectious Bronchitis Virus Strain M41 Insights from Expected and Novel Tissue Targets in Chicks

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Abstract: Infectious Bronchitis Virus (IBV) strain M41 exhibits a broad tissue tropism, but its potential to persist in non-traditional sites remains poorly understood. This study investigated the pathogenesis of IBV M41 in specific pathogen-free (SPF) chicks, focusing on both expected (trachea, lung, kidney, bursa of Fabricius, rectum, and caecal tonsil) and unexpected (brain, thymus, bone marrow, and testes) tissues. One-day-old chicks were inoculated intranasally and intraocularly with IBV M41 (4.9 log10 EID50/mL) and monitored for clinical signs, viral distribution, and immune responses over 14 days. Clinical signs, including respiratory distress and depression, peaked by day 10, with recovery by day 14. Post-mortem lesions revealed tracheal congestion, renal swelling, and lung congestion. Virus isolation and RT-PCR detected IBV in all expected tissues up to day 10, while the caecal tonsil and testes remained positive until day 14. Immunofluorescence confirmed active viral replication in tracheal, renal, and bursal epithelia, but bone marrow was consistently negative. Serological analysis demonstrated specific IgM and IgG responses by day 14. These findings highlight the caecal tonsil and testes as potential sites for prolonged IBV presence, warranting further investigation into their roles in viral persistence. The study underscores the utility of combining virological, molecular, and serological methods to elucidate IBV pathogenesis and informs future research on tissue-specific viral dynamics.
Infectious Bronchitis Virus (IBV)

Keywords: Tissue tropism, Pathogenesis, Caecal tonsil, Viral persistence, Immune responses.

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1. INTRODUCTION

In the previous experiment we compared two strains of IBV, namely Massachusetts M41 and Moroccan G and the result obtained suggested that strain M41 should be used for further studies on virus persistence rather than strain G. Consequently, we decided to choose strain M41 for this experiment [1]. The present study describes a slightly extended approach to the pathogencity of strain M41 of IBV in SPF chicks following infection at one day old. In this chapter, we re-examined these tissues where we expected to find virus (trachea, lung, kidney, rectum, bursa of Fabricius and caecal tonsil) - 'expected' tissues, together with 'unexpected' tissues that have not been fully investigated as possibly playing a part in IBV pathogenesis, such as brain, thymus, bone marrow and testes [2]. If any of these appear likely as sites for viral persistence, they could be incorporated in the persistence study including immunosuppression described in the next chapter [3].

2. MATERIALS AND METHODS

2.1. Viruses

Strain M41 of IBV-virus was used in this experiment as The Moroccan G and Massachusetts M41 strains used in this study were maintained at Leahurst, University of Liverpool. Each underwent three passages in specific pathogen-free (SPF) eggs before being titrated and adjusted to 2.5 EID_{50}/mL [4]. Cross-neutralization assays were conducted using 100 $TCID_{50}$ of each virus and serial twofold dilutions of antisera [5]. The titre was 4.9 log10 EID50/ml.

2.2. Chickens

Thirty-one-day-old SPF chicks obtained from a commercial source were hatched in our laboratory and the chicks maintained in strict isolation [6].

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2.3. Experimental design

Twenty chicks were each inoculated intranasally and intraocularly with a total 1.0 ml of strain M41 virus suspension. The infected group was maintained in one pen and 10 uninfected controls in another. Birds were examined daily for clinical signs.

At intervals post infection (days 3, 7, 10, and 14) five birds were euthanased by injection of pentobarbital sodium (Euthatal) via the wing vein. At autopsy, pieces of the following 'expected' tissue were taken aseptically: trachea, lung, kidney, cecal tonsil, rectum, duodenum, and bursa fabricius. Tissues normally not normally recognised as playing a role in IBV pathogenesis, here referred to as 'unexpected' tissues, were similarly taken thus: thymus, upper spinal cord, brain, bone marrow and testes. In addition, dry swabs were taken from the same tissues for detection of virus by RT-PCR.Adjacent samples from each tissue were processed for virus isolation and/or virus titration, and for immunofluorescence staining. Three controls were taken and sampled similarly each time.

2.4. Virus detection by TOC, RT-PCR and immunofluorescence

These were all done as described in RNA was reverse-transcribed using Superscript enzyme (Invitrogen, USA) with outer IBV oligonucleotide primers (SX2-). The first PCR used SX1+ (equimolar mixture of 5'cacctagaggtttgttagcatg3'and 5'cacctagaggtttgcttgcatg3') and SX2- (equimolar mixture of 5'tccacctctataaacacccttac 5'tccacctctataaacacctttac3'and 3) primers with Taq polymerase (Promega, USA), followed by a second amplification using universal IBV primers for detecting any IBV strain. except for upper spinal cord, which was not examined by IF.

2.5. Blood samples

At 14 p.i. blood samples were collected from the brachial veins of 5 chickens and used for detection of specific serum antibodies.

2.6. Enzyme linked immunosorbent assay (ELISA)

This was described in an indirect ELISA was employed to quantify IBV specific isotypic antibody responses to IgM and IgG in serum, following the protocols described by Mockett *et al.,* [7]. Additionally, an indirect ELISA was conducted to assess anti-IBV antibody responses in serum and local secretions, as per the method outlined by Elhafi *et al.,* [8].

5.3. RESULTS

5.3.1. Clinical signs

As early as 2 days post infection, the birds appeared depressed with ruffled feathers, with sneezing, coughing and tracheal răles characterized respiratory distress [9]. The respiratory distress persisted until day 10 post infection [10]. By day 14-post infection, at the end of the experiment, all infected birds had recovered clinically from the disease [11].The control birds remained normal throughout the experimental period

5.3.2. Post mortem lesions

Tracheas were congested and had excess mucus in the lumen on day 3,7 and 10 post infection. Lesions of euthanased birds revealed small white deposits along the urethers on day 7 and 10. By the day 7-post infection, the kidneys were slightly enlarged and pale. Some lung congested was seen on all occasions except day 14 [12.]

5.3.3. Virus isolation and PCR

Table 5.1 shows the virus isolation results for these tissues. In all cases except bone marrow, virus was isolated from all the tissues for the three occasions up to 10 days, although bone marrow, testes, rectum and caecal tonsil were not examined on day 7 [13]. On day 14 pi, testes and caecal tonsil were positive also.In almost all cases, at least two passages in TOC were needed for isolation.

Table 5.2 shows the RT-PCR results for the same tissues / organs. Detection of virus by this method was identical to virus isolation results except for virus detection in bone marrow on day 3[14]. All except bone marrow were positive up to 10 days but testes and caecal tonsils were also positive on day 14 pi [15].

Table-1: Virus isolation from pooled 'expected' and 'unexpected tissues or organs from IBV-infected birds

Tissue/organ	Days pi			
	3	7	10	14
Trachea	3a	3	3	0
Lung	3	3	2	0
Kidney	3	3	2	0
Rectum	1	n	2	0
Bursa of Fabricius	2	3	3	0
Thymus	2	3	2	0
Brain	3	3	2	0
Bone marrow	2	n	0	0
Testes	3	n	n	2
Caecal tonsil	N	n	2	2

a: number of passages in TOC needed to isolate virus from the tissue pool from 5 birds each time; 0: no virus isolated; n: not examined

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Table-2: Virus detection by RT-PCR from 'expected' and 'unexpected' tissues or organs of IBV-infected birds

Tissue/organ	Days pi			
	3	7	10	14
Trachea	+	+	+	–
Lung	+	+	+	–
Kidney	+	+	+	–
Rectum	+	n	+	–
Bursa of Fabricius	+	+	+	–
Thymus	+	+	+	–
Brain	+	+	+	–
Bone marrow	–	n	–	–
Testes	+	n	n	+
Caecal tonsil	N	n	+	+

+: virus detected by RT-PCR in tissue pool; -: no virus detected;
n: not examined

3.4. Immunofluorescence

At various times after infection, specific viral antigen was detected in the trachea (Figure.1), lung (Figur.2), kidney (Figure.3), bursa (Figure.4), caecal tonsil, brain and thymus but surprisingly, no virus was detected in rectal sections [17]. Virus was present in the epithelium of the trachea on day 3, 7 and 10 pi, and in the kidneys, viral antigen was found lining the damaged collecting tubules on days 7, 10 and 14 [18]. In other tissues, the fluorescence was amorphous, and it was not possible to define the cellular architecture of infected cells. The fluorescence was seen in the bursa on the epithelium on all occasions.

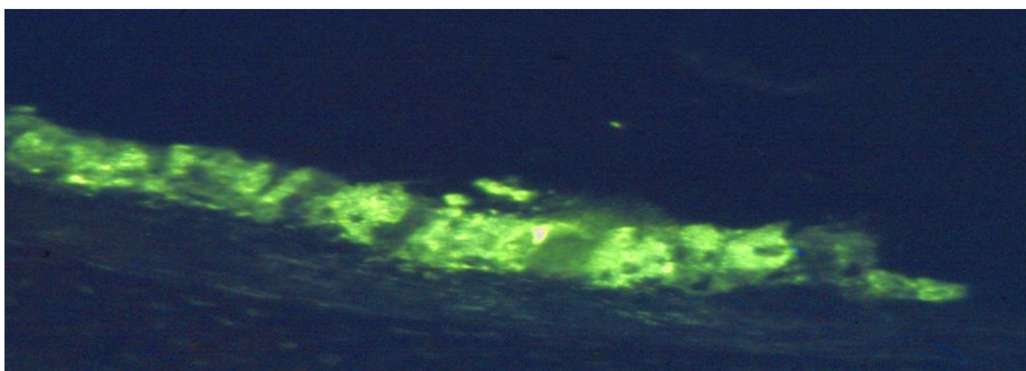

Figure-1: Immunofluorescence staining of IBV in trachea, day 3 p.i. Strong cytoplasmic staining in most epithelial cells. 400x

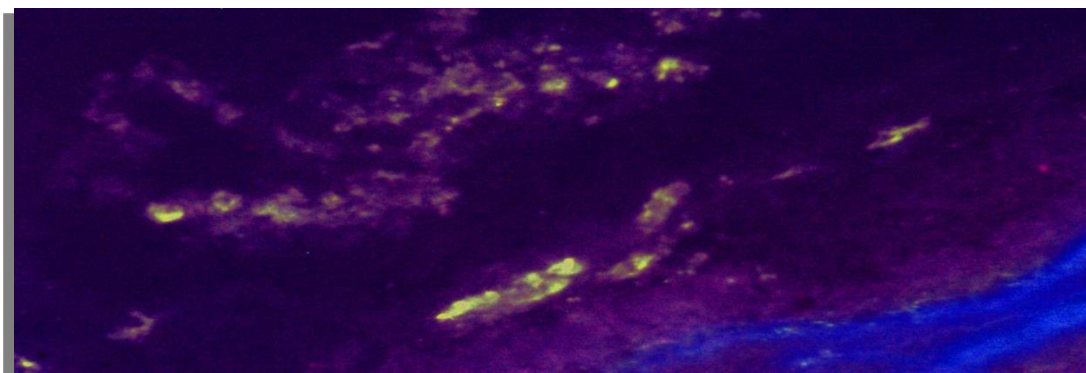

Figure-2: Immunofluorescence staining of IBV in lung, day 3 p.i. Virus in epithelial cells either side of a bronchus. 400x.

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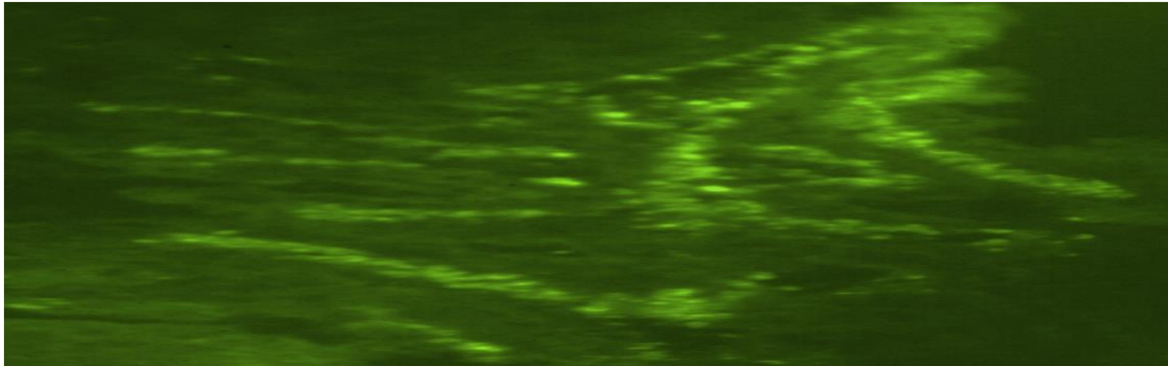

Figure-3: Immunofluorescence staining of kidney, day 7 Virus present in collecting tubules. 250x.

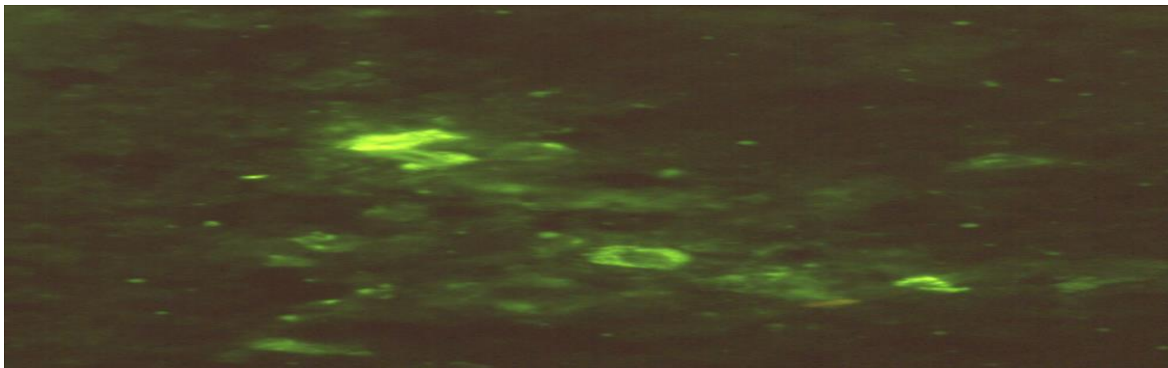

Figure-4: Isolated infected cells in the bursa of Fabricius showing cytoplasmic fluorescence. 400x.

3.5. ELISA: virus-specific IgM and IgG

Both were detected at 14 days pi in a pool of serum taken from the IBV-infected birds (Figure.5,.6). Control sera remained negative [16].

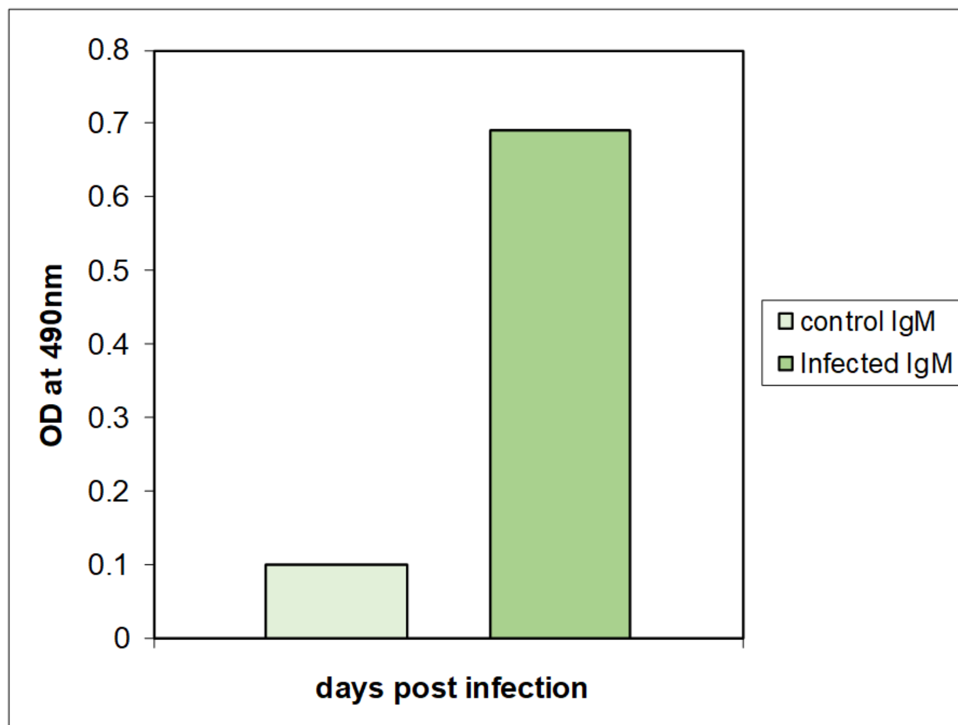

Figure-5: IBV-specific IgM in serum of chickens at 14 days pi.

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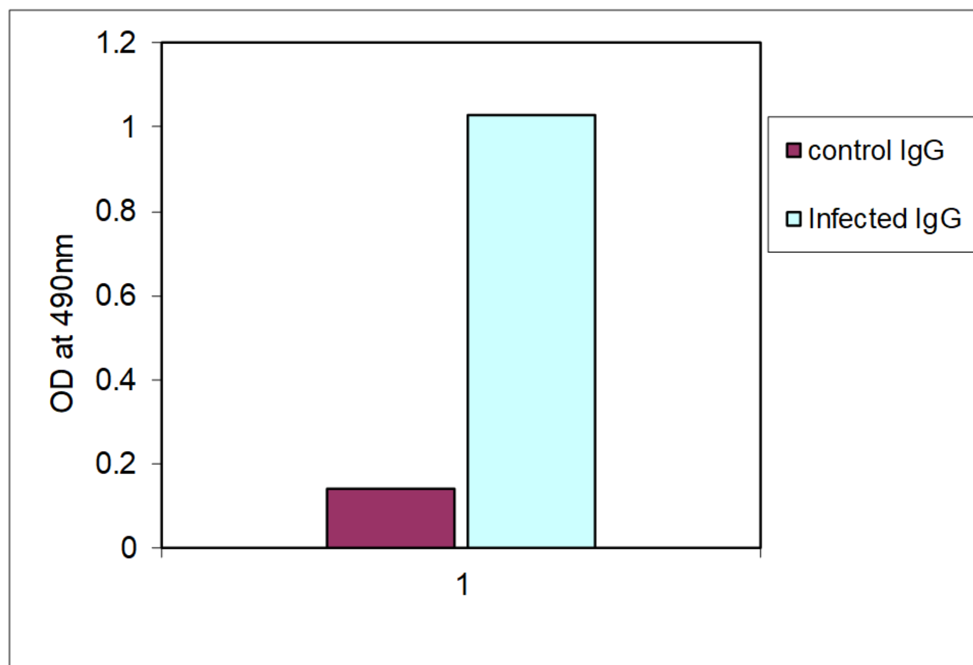

Figure-6: IBV-specific IgG in serum of chickens at 14 days pi.

4. DISCUSSION

The work described in this chapter was done to examine some tissues/organs, which are not usually implicated in the pathogenesis of IB. The 'expected' tissues, namely trachea, lung, kidney and rectum were examined together with bursa of Fabricius, thymus, brain, testes and caecal tonsil, the latter sometimes being considered as a site of long-term persistence [19]. Should any of them give an indication of potential viral persistence, they could be included in longer studies. However, because of the time-consuming and complicated nature of isolation of IBV, whether in eggs or TOC, it is preferable to examine as few tissues or an organ as is necessary in such a study [20]. Several reports have indicated that IB viruses have the potential to infect a large number of tissues and organs in the chicken [21,22], but relatively few play a part in the natural infection. The clinical disease, and more importantly, pattern of virus in the 'expected' tissues was different from that in the previous chapter, almost certainly because the dose of virus was greater this time - 4.9 log10 EID50 as compared with 2.5 log10 EID50 per bird before. Thus, virus persisted for at least 10 days in this the acute phase of infection [23].

Overall, virus persistence in the other tissues was similar, with the exception of testes and caecal tonsil, where virus was detected until day 14 pi.It is worth considering the other 'unexpected' tissues in turn. The bursa and thymus are the two major lymphoid tissues of the immune system and it might have been expected that virus would be present at some stage in the infection. El Houadfi *et al.* [5] and Cook *et al.,* [24] found virus in the bursa of Fabricius and suggested that other lymphoid organs should be examined. While all were positive by isolation and PCR, IF showed viral antigen clearly in the cytoplasm of scattered cells in the bursa [25]. This might have been IBV in lymphocytes ready from processing. While the thymus was also positive for virus for the same period, the IF staining was far less distinct. The bone marrow was also examined as a possible site for virus as it is established that some viruses are able to persist in blood cells but this was found to be negative by VI and RT-PCR, so it seems likely that this tissue plays little or no part in IB pathogenesis [26]. Virus detection in the caecal tonsil suggests support for the theory that this is an (or even the) site for persistence, as has been suggested by some workers. Although our previous work [27] did no confirm this, it would still be worthwhile including it in longer-term investigations. It is worthwhile mentioning the different virus detection methods used here. Virus isolation will detect infectious virus in tissues, but will not indicate if the virus detected is causing damage [28]. Virus detected in tissues this way could merely be viraemic virus in non-infected tissues. RT-PCR in turn detects viral nucleic acid, but again does not indicate if virus is damaging tissues. Immunofluorescence, on the other hand, is able to demonstrate active replication in cells, which is likely to result in tissue damage, although not always. For example, although Ambali & Jones [29] reported abundant replication of IBV in the intestinal villi of infected chickens, there was no apparent effect on gut function and histology revealed little damage. Testes were included in this study, because there happened to be a preponderance of males among the birds sampled. The finding of virus in this organ is interesting and potentially important. There is of course, abundant information on the effects of IBV on the functioning of the reproductive tract of the mature female chicken, causing reduced egg production and quality in [30-32]. In addition, it is known that in

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antibody-free female chicks, early infection with IBV can cause abnormalities of the oviduct, which result in 'silent layers', i.e. non-layers, when they reach maturity [33]. However, there have been no similar studies in males, probably because unlike in females, any likely consequences are not obvious. Should IBV have the capacity to seriously damage the testes, then this might be a cause of infertility in cockerels. This is an area of study that merits further investigation. However, that will need to be done another time and, of course with a flock of males only. Interestingly, in the present study, virus isolation and RT-PCR appeared to have similar sensitivities, although most isolation was made after three passages of material in TOC. Immunofluorescence was somewhat similar, although in some tissues, it was not easy to determine which cells were infected. This experiment was relatively short and it did not really give any indication of potential long-term persistence of IBV in these other tissues [34]. It was concluded that future longer-term studies on persistence probably need not include the 'unexpected' tissues examined here and attention should be paid mainly to the traditional or 'expected' ones.

5. CONCLUSION

The present study provides a comprehensive examination of the pathogenesis of Infectious Bronchitis Virus (IBV) strain M41 in SPF chicks, focusing on both expected and unexpected tissue targets. Our findings confirm the tropism of IBV M41 for traditional sites such as the trachea, lung, kidney, and bursa of Fabricius, where viral replication was consistently detected up to 10 days post-infection [8]. Notably, the caecal tonsil and testes emerged as potential sites for prolonged viral presence, with virus detectable until day 14, suggesting their possible role in viral persistence.

The use of multiple detection methods—virus isolation, RT-PCR, and immunofluorescence—highlighted the strengths and limitations of each technique. While virus isolation and RT-PCR demonstrated similar sensitivities, immunofluorescence provided critical insights into active viral replication within specific cell types, even in tissues like the bursa and thymus. However, the absence of virus in bone marrow and the inconsistent detection in other unexpected tissues suggest these may not play a significant role in IBV pathogenesis [32]. The clinical and pathological observations align with previous reports, emphasizing respiratory and renal involvement, while the detection of virus in the testes opens new avenues for research into potential reproductive impacts in male chickens. Although this short-term study did not definitively identify long-term persistence sites, it underscores the importance of including the caecal tonsil in future investigations.

In conclusion, while the unexpected tissues examined here did not strongly indicate a role in persistence, the findings reinforce the need for further studies, particularly on the reproductive implications of IBV in males and the caecal tonsil's role in viral dynamics. Future research should prioritize these areas to deepen our understanding of IBV pathogenesis and persistence.

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