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Establishment of Persistent Avian Infectious Bronchitis Virus Infection in Antibody-Free and Antibody-Positive Chickens

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SUMMARY. Avian infectious bronchitis virus (IBV) causes a highly contagious and economically significant disease in chickens. Establishment of a carrier state in IBV infection and the potential for the persistent virus to undergo mutations and recombination in chicken tissues have important consequences for disease management. Nevertheless, whether chickens can maintain persistent IBV infection in the absence of reinfection from exogenous sources or the presence of antibody in the host can modulate virus persistence remains unclear. Indeed, whether or not IBV genome can undergo genetic changes during *in vivo* infection has not been demonstrated experimentally.

In the present study, IBV shedding and tissue persistence were monitored in individual chickens maintained under strict isolation that precluded reinfection from exogenous sources. In the first of two experiments, intranasal exposure of 6-wk-old antibody-free chickens to IBV vaccine virus resulted in intermittent shedding of the virus from both trachea and cloaca of individual birds for up to 63 days. Also, the virus was recovered from the internal organs (spleen, gonad, kidney, lung, cecal tonsil, and cloacal bursa) of six of eight birds killed at various intervals between 27 and 163 days postinoculation (DPI). In the second experiment, IBV exposure of 1-day-old maternal antibody–positive chicks led to periodic virus shedding from the trachea and cloaca in all chickens until 77 days; however, internal organs (lungs and kidneys) of only one of seven birds (killed at 175 DPI) were virus positive, suggesting that presence of antibody at the time of infection protects internal organs from IBV infection. When the lung and kidney isolates of IBV from the latter experiment were compared with the parent-vaccine virus, no changes in their antigenicity, tissue tropism, or the nucleotide sequence of the S1 glycoprotein gene were observed. These findings indicate that, unlike the mammalian coronaviruses, propensity for frequent genetic change may not be inherent in the IBV genome.

RESUMEN. Establecimiento de una infección persistente por el virus de bronquitis infecciosa aviar en pollos con y sin anticuerpos.

El virus de bronquitis infecciosa aviar ocasiona una enfermedad altamente contagiosa de importancia económica significativa en pollos. El establecimiento de un estado portador en la infección por el virus de bronquitis infecciosa y el potencial por parte del virus persistente de experimentar mutaciones y recombinaciones en tejidos de pollos tiene consecuencias importantes para el manejo de la enfermedad. Sin embargo, no es claro si los pollos pueden mantener una infección persistente ocasionada por el virus de bronquitis infecciosa en ausencia de reinfección proveniente de fuentes externas o si la presencia de anticuerpos en el huésped puede modular la persistencia del virus. No se ha demostrado experimentalmente si el genoma del virus de bronquitis puede o no experimentar cambios genéticos durante una infección *in vivo.* Se evaluó la excreción y la persistencia del virus de bronquitis infecciosa en tejidos de pollos individuales mantenidos bajo estricto aislamiento, eliminando la posibilidad de reinfección por fuentes externas. En el primero de dos experimentos, la exposición al virus vacunal de bronquitis infecciosa por vía intranasal en pollos de 6 semanas sin anticuerpos resultó en una excreción intermitente del virus en la tráquea y cloaca de aves individuales hasta los 63 días. Se recuperó el virus a partir de órganos internos (bazo, gónadas, riñones, pulmón, tonsilas cecales y bolsa de Fabricio) en seis de ocho aves sacrificadas a varios intervalos entre los 27 y 163 días posteriores a la

inoculación. En el segundo experimento, la exposición al virus de bronquitis infecciosa en aves de un día con anticuerpos maternales condujo a una excreción periódica del virus en la tráquea y cloaca en la totalidad de las aves hasta los 77 días. Sin embargo, solo una de siete aves (sacrificadas a los 175 días posteriores a la infección) fue positiva al virus en sus órganos internos (pulmones y riñones), sugiriendo que la presencia de anticuerpos al momento de la infección protege los órganos internos de la infección por el virus de bronquitis infecciosa. No se observaron cambios en la antigenicidad, tropismo o secuencia de nucleótidos del gen de la glicoproteína S1 en los aislamientos del virus de bronquitis infecciosa obtenidos a partir de pulmón y riñón en el último experimento al ser comparados con el virus vacunal original. Estos hallazgos indican que, a diferencia de los coronavirus de los mamíferos, la propensión a cambios genéticos frecuentes puede no ser inherente al genoma del virus de bronquitis infecciosa.

Key words: antibody-free chickens, antibody-positive chickens, coronavirus, infectious bronchitis virus, serotype, spike protein gene, sequence, virus persistence

Abbreviations: DPI = days postinoculation; EID_{50} = mean embryo infective dose; ELISA = enzyme-linked immunosorbent assay; IBV = infectious bronchitis virus; N = nucleocapsid; PCR = polymerase chain reaction; RT = reverse transcription; S = spike; SPF = specific-pathogen free

Infectious bronchitis is a highly contagious disease of chickens that inflicts major economic losses. The etiologic agent, infectious bronchitis virus (IBV), is a coronavirus, which is worldwide in distribution. IBV has a single-stranded, positive-sense RNA genome that is 27.6 kb long and encodes three major structural proteins. These proteins include the spike (S) glycoprotein (S protein), which consists of an outer N-terminal-half S1 and a membrane-anchoring C-terminal S2 portion, membrane glycoprotein, and the nucleocapsid (N) protein (6,26). In addition, a small membrane protein is also associated with the virion envelope (27). Of these proteins, the S1 is biologically the most important because it mediates virus infectivity and membrane fusion (3) and carries serotype-specific antigenic determinants that induce virus-neutralizing antibodies (5).

The two properties of the mammalian coronaviruses that have been the focus of great interest and investigation are 1) the ability to establish persistent infection of host tissues and 2) the propensity to undergo genetic change during *in vivo* and *in vitro* replication (1,9,24). However, in the case of avian coronaviruses, neither of these properties has been demonstrated compellingly. For example, IBV outbreaks in vaccinated chickens often have been linked to antigenic and pathogenic variants of the virus, yet, whether IBV vaccine viruses establish persistent infection or contribute to variant virus populations in the field has not been established convincingly. IBV-infected birds have been shown to intermittently excrete the virus (15). Early studies in experimentally infected chickens led to the conclusion that IBV persistence in chickens is through continual reinfection from exogenous sources and not through establishment of a carrier state in individual birds (7). A number of subsequent studies, all conducted in group-housed and not individually isolated chickens, failed to clarify the question of whether persistence or reinfection leads to sustained IBV excretion (2,8,11,16). However, a study in which chickens inoculated with IBV at 1 day of age stopped shedding virus after about 7 wk postinoculation and resumed virus excretion at sexual maturity strongly suggested existence of persistent or chronic IBV infection (15). Although it is evident that IBV persistence and excretion have enormous implications for the control of this infection, the understanding of these phenomena is incomplete at best.

The present study, the first to be conducted in individually isolated birds, was aimed at addressing three fundamental questions: 1) can IBV vaccine viruses establish persistent infection, b) does the immune status of the host at the time of infection have a modulating effect on virus persistence, and 3) can the postulated genetic alteration of IBV during persistent infection be experimentally demonstrated.

MATERIALS AND METHODS

Chickens and housing. White leghorn-type chickens were used in both experiments (Expts. 1 and 2). The 6-wk-old antibody-negative chickens used in Expt. 1 were derived from our departmental breeder flocks, whereas the 1-day-old maternal antibody–positive chickens were obtained from a commercial hatchery. Chickens were individually housed in P3-level isolators during the entire study. The inside of each isolator was cleaned periodically (without breaking the

integrity of the unit) with dilute Clorox solution to inactivate any IBV that might be excreted by the bird and could cause reinfection.

Virus. A Massachusetts-serotype vaccine virus was used in both experiments. The virus was titrated in chicken embryos (23), and one drop of virus inoculum containing approximately 10^5 mean embryo infective dose (EID_{50}) was administered to individual birds through the ocular route.

Expt. 1. Nine 6-wk-old chickens were individually housed in separate isolator units. Eight were inoculated with IBV as described above, and one bird was maintained as an uninoculated control. An additional bird was placed in a cage within the room in which the isolators were located in order to monitor the environment for the presence of IBV. Cloacal and tracheal swabs were collected for virus isolation from each inoculated bird every 3–4 days for the first 27 days and subsequently at weekly intervals until 163 days postinoculation (DPI). On days 27, 48, 70, 91, 112, 126, 154, and 163, a bird was removed from the isolator, bled, and euthanatized for collection of tissues including brain, spleen, gonad, kidney, trachea, lung, cecal tonsil, and cloacal bursa. Tissues from the two uninoculated control chickens, one housed in the isolator and the other in the cage, were collected at 91 days and 134 days, respectively, from the start of the experiment. All collected tissues were used for virus isolation.

Expt. 2. Fifty 1-day-old chicks were wing-banded for identification, and 200–400 μl of blood was obtained from the jugular vein of each. An enzyme-linked immunosorbent assay (ELISA) was performed on individual serum samples to assess levels of maternal antibody to IBV (17). Eight birds with matching antibody levels were individually inoculated with IBV and placed in individual isolators as described for Expt. 1. One bird that died of nonspecific causes 48 hr after the start of the experiment was not replaced. Tracheal and cloacal swabs were collected from the remaining seven birds for virus isolation at weekly intervals starting at 3 wk until 25 weeks postinoculation. At 98, 140, 147, 154, 161, 168, and 175 DPI, birds were removed from the isolator, bled, and killed for tissue collection and virus isolation, as described for Expt. 1.

Virus isolation. Standard procedures were used for sample preparation and IBV isolation in embryonating chicken eggs (10). At least two 9-to-11-day-old chicken embryos were inoculated per sample via the allantoic sac route. After 40 hr of incubation, embryos were chilled overnight at 4 C, and allantoic fluid was harvested for further embryo passage. Allantoic fluids from the second and third embryo passages were centrifuged ($1000 \times g$ for 15 min), and the cell pellets were resuspended in phosphate-buffered saline, pH 7.4. Presence of IBV in the allantoic cells and allantoic fluids was investigated by immunohistochemistry with IBV-specific monoclonal antibody (21) and antigen-capture ELISA (22), respectively.

Determination of S1 sequence. S1 gene sequence was determined by a procedure described previously (18,19). Briefly, IBV genomic RNA was extracted from 250 μl of infected allantoic fluid with TRIzol LS reagent (Gibco BRL, Grand Island, NY) and resuspended in 3 μl of RNAse-free water. Amplification of the S1 gene was performed by reverse transcription (RT)–polymerase chain reaction (PCR) with the primers NewS1oligo5′ (5′TGAAACTGAACAAAAG-AC3′) and Degenerate3′ (5′CCATAAGTAACATAA-GGRCRA3′) (12,19). The RT-PCR was performed with the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). cDNA was synthesized from 3 μl of RNA with random hexamer primer at 42 C for 30 min, and the mixture was then heated for 5 min at 94 C to stop the reaction. Both the RT reaction and PCR were conducted in an MJ Research thermal cycler (PTC-100; MJ Research, Inc., Watertown, MA). For the PCR reaction, 2 μl (15 μM) of each primer, NewS1oligo5′ and Degenerate3′, was added in a 100-μl reaction volume. The first cycle of PCR amplification was carried out for 90 sec at 94 C, 30 sec at 50 C, and 2 min at 72 C. The remaining 34 cycles were carried out for 30 sec at 94 C, 30 sec at 50 C, and 2 min at 72 C with a final elongation step of 15 min at 72 C. PCR products were visualized by electrophoresis in 1% agarose gel, followed by staining with ethidium bromide (0.5 μg/ml).

We performed direct sequencing of the PCR products (18). We used a combination of flanking and internal primers to sequence both strands of cDNA in their entirety. Assembly of sequencing contigs, translation into amino acid sequence, and initial multiple-sequence alignment were performed with the BioEdit software version 5.0 (North Carolina State University, Raleigh, NC).

Challenge of immunity and tissue tropism studies. We examined two persistent IBV isolates recovered at 175 DPI from lung and kidney tissues, respectively, to determine whether their antigenicity and tissue tropism had changed compared with their parent-vaccine virus. Forty-seven specific-pathogen-free (SPF) chickens were vaccinated twice, first at 1 day of age and then at 14 days with the same vaccine virus used in the study. A group of 25 SPF chickens of the same age was maintained as unvaccinated controls in a separate isolation room. At 21 days of age (i.e., 7 days post second vaccination), vaccinated and unvaccinated chickens were separated into three groups each and housed in separate rooms. The three vaccinated groups with 19, 19, and 9 chickens, respectively, were intranasally inoculated with the persistent lung isolate, the persistent kidney isolate, and homologous vaccine virus, respectively; each bird received 1000 EID_{50} of the respective virus. The three groups of unvaccinated chickens, consisting of 10, 10, and 5 birds, were similarly inoculated with the three viruses as described for the vaccinated groups. Five days after the virus inoculation, all birds were euthanatized and tracheas

Table 1. IBV isolation from the tracheal/cloacal swabs of chickens collected between 3 and 154 DPI (Expt. 1).

	DPI													
Bird no.	3	6	10	13	17	20	24	27	34	41	48	57	63	70–163[A]
1	+/+	+/+	+/−	−/−	−/−	−/−	−/−	−/−						
2	+/+	+/+	+/+	−/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−			
3	+/−	+/+	−/+	−/−	−/−	−/−	−/−	−/−	+/+	+/−	+/−	−/+	+/+	−/−
4	+/+	+/+	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/+	−/−	−/−	−/−	−/−
5	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
6	+/−	+/+	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
7	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/+	+/−	−/−	−/−
8	−/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−

[A]Tracheal and cloacal swabs obtained between 70 and 154 DPI were IBV negative.

were collected from each for virus isolation by chicken embryo inoculation as described above.

To study tissue tropism, we inoculated the lung and the kidney isolates and the vaccine virus into separate groups of 27, 26, and 24 1-day-old chickens, respectively. During the subsequent 21 days, chickens were observed for signs of clinical disease including anorexia, respiratory signs (open-mouth breathing and sneezing), and mortality. At 21 DPI, all birds were euthanatized, and lung and kidney tissues from individuals were collected for virus isolation in chicken embryos.

RESULTS

The virus excretion pattern observed in Expt. 1 is presented in Table 1. All inoculated chickens excreted IBV from the trachea and/or cloaca during the first 6–10 DPI. Subsequently, birds 1, 5, 6, and 8 did not excrete IBV for the rest of the experiment, whereas birds 2, 3, 4, and 7 resumed virus shedding after 17, 34, 41, and 48 days, respectively. In the latter group, whereas chickens 2, 4, and 7 excreted IBV once or twice, bird 3 excreted the virus over a 29-day period.

When we examined the internal organs of the chickens in Expt. 1, six of the eight chickens killed between 27 and 163 DPI were IBV positive (Table 2). Among the organs from which IBV was isolated at various intervals were cloacal bursa, cecal tonsil, spleen, gonad, kidney, and lung.

The tracheal and cloacal swabs, as well as the internal organs of the two uninoculated control chickens (maintained to monitor possible escape of the virus into the environment), were free of IBV (data not shown).

In Expt. 2, IBV shedding was monitored from 21 DPI until 175 DPI (Table 3). Between 21 and 35 DPI, most chickens excreted the virus from either trachea or cloaca, although tracheal excretion was more common. Intermittent virus shedding was observed from birds 2, 4, 6, and 7, with bird 4 shedding IBV until 70 DPI and bird 7 until 77 DPI. Tracheal and cloacal swabs collected between 84 and 175 DPI were negative for IBV. Individual birds in Expt. 2 were killed for virus isolation between 98 and 175 DPI. Of the seven birds examined in this period, only one (bird 7), killed at 175 DPI, carried IBV in two internal organs, lung and kidney (Table 4).

A comparison of the S1 gene sequences revealed a 100% match between the two persistent lung and kidney isolates and their parent-vaccine virus (data not shown).

Table 2. IBV isolation from chickens inoculated at 6 wk of age and tissues harvested at various intervals between 27 and 163 DPI (Expt. 1).

Bird no.	DPI	Bursa of Fabricius	Cecal tonsil	Spleen	Gonad	Kidney	Lung	Trachea
1	27	+	−	−	−	−	−	−
2	48	−	−	−	+	+	−	−
3	70	−	−	−	−	−	−	−
4	91	−	−	−	−	+	−	−
5	112	+	−	+	−	−	−	−
6	126	−	−	−	−	−	−	−
7	154	−	−	−	−	−	+	−
8	163	+	+	−	−	−	+	−

Table 3. IBV isolation from tracheal/cloacal swabs of maternal antibody–positive chickens inoculated at 1 day of age (Expt. 2).

	DPI									
Bird no.	21	28	35	42	49	56	63	70	77	84–175[A]
1	+/−	+/−	+/+	+/−	−/−	−/−	−/−	−/−	−/−	−/−
2	+/−	−/−	+/−	−/−	+/−	−/−	−/−	−/−	−/−	−/−
3	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
4	+/−	+/−	+/−	−/−	−/−	−/−	−/−	+/−	−/−	−/−
5	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
6	+/+	+/−	−/−	±/−	−/−	−/−	+/+	−/−	−/−	−/−
7	+/−	−/+	+/−	−/−	−/−	−/+	+/+	−/−	−/+	−/−

[A]Tracheal and cloacal swabs obtained between 84 and 175 DPI were IBV negative.

In the antigenic comparison studies, none of the vaccinated chickens intranasally challenged with the lung isolate and only one of the 19 birds challenged with the kidney isolate yielded IBV from their tracheas 5 days after challenge, showing close antigenic homology between vaccine virus and the two persistent viruses (Table 5). On the other hand, the two groups of 10 nonvaccinated chickens that were respectively inoculated with the two persistent IBV isolates were all positive for IBV.

Table 6 summarizes the results of pathogenicity and tissue tropism studies. The chickens in the three groups, inoculated with the lung isolate, kidney isolate, and vaccine virus, respectively, suffered no mortality and no apparent respiratory disease or other signs of illness such as dullness, huddling near the heat source, and reduced feed intake. When virus isolation was attempted from the lung and kidney tissues at 21 DPI, 10 of 27 chickens inoculated with the lung isolate and 11 of 26 inoculated with the kidney isolate yielded IBV from the lung tissues. None of the kidney samples from those chickens was virus positive. On the other hand, of the 24 chickens that received the vaccine virus, 12 yielded the virus from lungs and three from the kidneys.

DISCUSSION

In this study, we examined IBV shedding from the trachea and cloaca and IBV persistence in internal organs of chickens that were antibody free or carried maternally derived antibody at the time of virus inoculation. To our knowledge, it is the first study in which IBV persistence has been monitored in individual birds kept under strict isolation that eliminated reinfection from exogenous sources. Also, it is the first report that addresses the possible modulating role of circulating antibody on virus persistence. In this study, although we focused primarily on virus isolation in embryonating chicken eggs as the method for detecting IBV persistence in tissues, we screened limited numbers of virus-negative tissues for the presence of viral RNA by RT-PCR with universal S1 and N gene primers (12,19,25) (data not shown). Those tissues that were IBV negative by the embryo inoculation method were also negative for viral RNA by RT-PCR.

We studied the persistence of a vaccine virus in this study because of two considerations: first, because the vaccine virus is egg-adapted, we believed it would be readily detected by embryo inoculation

Table 4. IBV isolation from tissues of maternal antibody–positive chickens inoculated at 1 day of age and killed between 98 and 175 DPI (Expt. 2).

Bird no.	DPI	Bursa of Fabricius	Cecal tonsil	Spleen	Gonad	Kidney	Lung	Trachea
1	98	−	−	−	−	−	−	−
2	140	−	−	−	−	−	−	−
3	147	−	−	−	−	−	−	−
4	154	−	−	−	−	−	−	−
5	161	−	−	−	−	−	−	−
6	168	−	−	−	−	−	−	−
7	175	−	−	−	−	+	+	−

Table 5. Recovery of IBV from the tracheas of vaccinated and nonvaccinated chickens 5 days after intranasal challenge with the lung and kidney isolates from persistently infected chickens and the homologous vaccine virus.

Challenge virus	Virus recovery from trachea 5 days postchallenge	
	Vaccinated[A]	Nonvaccinated
Lung isolate	0/19[B]	10/10
Kidney isolate	1/19	10/10
Vaccine virus	0/9	5/5

[A]Vaccine was administered intranasally at 1 day and 14 days of age.
[B]Number of birds positive for IBV/total number of birds challenged.

and, second, because of the vaccine virus's postulated role in establishing persistent infection and giving rise to variants in the field, we believed that the data would have applied significance.

The virus excretion data in Tables 1 and 3 show that IBV antibody-free and antibody-positive chickens shed the virus for up to 63 and 77 days, respectively, after the initial exposure. It is interesting to note that virus excretion was not continuous, and some of the birds re-excreted virus after a pause in shedding of up to 42 days. Although an earlier report indicated that persistently infected chickens might resume IBV shedding on reaching sexual maturity (15), we did not observe this in the present study.

When antibody-free chickens were exposed to IBV, the virus was isolated from the internal organs of six of eight chickens killed between 27 and 154 days after the initial exposure. Also, the longest period between virus shedding and virus isolation from the internal organs was 157 days. Interestingly, the virus was widely distributed in the internal organs of these birds, including cloacal bursa, cecal tonsil, spleen, gonad, kidney, and lung (Table 2). In antibody-positive birds, although the virus shedding was similar to that observed in antibody-free chickens (Table 3), the virus was found in the internal organs of only one of seven birds (Table 4). These observations suggest that presence of circulating antibody in the latter group at the time of virus inoculation might have blocked the virus from reaching the internal organs. On the other hand, because antibody in circulation is unlikely to influence virus infection in the respiratory and digestive tracts, we assume that the infection there remained unaffected by the systemic antibody.

Characterization of the two persistent tissue isolates of IBV from Expt. 2 revealed no change in their S1 gene sequence, antigenicity, pathogenicity, or tissue tropism when compared with the parent-vaccine virus (Tables 5, 6). This finding is quite in contrast to the mammalian coronaviruses that have been shown to undergo frequent genetic change during *in vivo* persistence (1). Indeed, a number of variants of murine hepatitis virus with deletions in the S glycoprotein gene have been isolated from persistently infected mice (24). In one study, 11 of 20 persistently infected mice harbored spike-deletion variants, indicating that deletions are common during persistent infection (24). Interestingly, in the latter study, mice with the most severe and persistent neurologic disease harbored the most prevalent and diverse quasispecies of spike-deletion mutants.

In the present study, we did examine tissues by RT-PCR for the presence of S1 and N gene-associated RNA in those cases where no IBV could be isolated from tissues by the embryo inoculation method. Those attempts failed to demonstrate presence of IBV or the respective RNA species (data not shown). Although the present study is limited by the fact that only two persistent viruses were examined in detail, it provides evidence that persistence in the case of IBV is not necessarily associated with a change in virus genetic and antigenic properties. It is also interesting to note that in a recent study in our laboratory, Mass isolates from the 1940s differed only marginally (2% in the S1 gene sequence) from Mass serotype strain M41, a laboratory strain of IBV that has undergone

Table 6. IBV recovery from the lung and kidney tissues of chickens at 21 days of age after intranasal inoculation at 1 day of age.

Virus	No. birds	No. birds positive for IBV in		Mortality
		Lung	Kidney	
Lung isolate	27	10	0	0
Kidney isolate	26	11	0	0
Vaccine	24	12	3	0

countless passages in chickens and chicken embryos (14). These and similar observations reported by others (4) indicate that genetic change that is a hallmark of mammalian coronaviruses may not be a constant feature of all IBV strains. Nevertheless, it can be argued that, despite the possible low rate of mutation in the IBV RNA, viral persistence may still be a significant factor in the emergence of variant viruses because a percentage of the billions of chickens that are vaccinated each year with IBV is likely to harbor the virus for long periods, which is certain to increase the probability of development of variant viruses through both mutation and recombination (13,20,28).

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