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Pathogenesis of infectious bronchitis virus with different routes of inoculation and the effect of *in vivo* serial passage in nephropathogenicity using cloacal infection

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Abstract

In this study, we wanted to determine if the respirotropic JMK strain of infectious bronchitis virus(IBV), which has a spike glycoprotein gene that is 99% similar to the nephropathogenic Gray strain of IBV, could adapt and cause lesions in the kidney following intracloacal passage in chickens. Two day old specific pathogen free(SPF) chickens were infected with Gray and JMK strains by the intraocular and cloacal route. Several tissue samples were collected at various times. Viruses were recovered from more tissues and earlier in the infection from chickens infected cloacally than chickens infected intraocularly. Virus was isolated from the kidney of chickens infected with Gray by the intraocular route and JMK by the intracloacal route, but not from chicken given JMK by the intraocular route. Histopathologically, interstitial nephritis was observed in Gray infected chickens. However, viral RNA or antigen were not detected in the kidney by in situ hybridization and immunohistochemistry. We further passaged the JMK strain ten times in two day old SPF chickens using cloacal inoculation. We examined the virus titer and histopathological change in the kidney at each passage level. The amount of virus recovered from the kidney was stable throughout this serial passage and the passaged virus did not caused renal damage. Further, virus could not be isolated from the kidney when chickens were infected with the passaged virus by the intraocular route. We conclude that the JMK strain has a strict upper respiratory tract tropism since cloacal passage did not produce nephrotropism or nephropathogenicity.

Key words : JMK, Infectious bronchitis virus, Intracloacal passage, Nephrotropism

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Introduction

Infectious bronchitis(IB) is a worldwide disease of poultry caused by infectious bronchitis virus(IBV). Strains of IBV have wide and variable tissue tropism, and the clinical expressions of IB are highly variable[1]. Nephritis in chickens infected with IBV was first reported in 1960s in the United States[2]. Since then, nephropathogenic IBV has been reported worldwide[3~7]. Usually, nephropathogenicity has been associated with certain strains of IBV. However, it was also suggested that nephropathogenic IBV arose from strains causing respiratory disease[8]. Uenaka et al[9] succeeded in inducing nephrotropism in a non-nephropathogenic strain of IBV by successive passage in kidneys using cloacal inoculation.

Virus replication in the kidneys has been detected by virus isolation, immunofluorescence assay, in situ hybridization(ISH), and immunohistochemistry(IHC)[10~12]. The infected kidneys appear swollen and pale. In urolithiasis, the ureters associated with atrophied kidneys are distended with urates and often contain large calculi composed of urates. Despite a lack of gross lesions microscopic changes of nephritis may still be present. It has been proposed that IBV-induced renal lesions can be considered to be a ductotubular interstitial nephritis[13].

IBV is the first coronavirus described and sequenced completely[14]. The genome of IBV is approximately 27 kb in length and encodes four structural proteins : spike(S), envelope (E), membrane(M), and nucleocapsid(N) protein in the 5′ to 3′ direction[15]. The precursor S protein is post-translationally cleaved into S1 and S2 subunits[16]. Neutralizing and serotype specific epitopes are associated with the S1 subunit[17,18]. The variations in tissue tropism among coronaviruses are in large part attributable to variations in the spike glycoprotein. Large insertions or deletions that occur near specific sites in the S1 subunit and S2 substitution mutation have been associated with different tissue tropism[19]. However, information concerning a molecular basis of IBV tissue tropism is limited. Gray and JMK strain of IBV are the same serotype and share more than 99% nucleotide sequence similarity in the S gene[20]. However, they differ in tissue tropism. The Gray is characterized as a moderate nephropathogenic strain and the JMK is a respirotropic strain[21].

In this study, we compared the pathogenesis of IBV strains JMK and Gray following different route of inoculation. We utilized ISH and IHC together with virus isolation and histopathologic data to examine the pathogenesis in the upper respiratory tract and the kidney. We further passaged the JMK strain in chickens by the intracloacal route of infection to determine if that strain could change tissue tropism.

Materials and Methods

Viruses, eggs and chickens

Gray and JMK strains were used in this study. These strains are maintained as allantoic fluid stocks at the University of Georgia. Titrations were done as previously described[22]. Specific pathogen free(SPF) embryonated chicken eggs were obtained from Merial Select Laboratories(Gainesville, GA).

Experiment 1. Intraocular and cloacal infection of chickens with Gray and JMK strains

Ninety six 2 day old SPF chickens were housed in positive-pressure isolators. Groups of 24 chickens were individually inoculated via eyedrop with 0.1 mℓ($10^{5.0}$$EID_{50}$) of inoculum of one of the Gray or JMK strains. One group of 24 birds were cloacally inoculated with 40 μℓ($10^{5.0}$$EID_{50}$) of JMK strain. Another group of 24 birds served as noninoculated controls. Animals were monitored daily by visual observation for clinical signs. Four birds in each group were euthanatized at 12 hours, 1, 3, 5, 7, and 10 days postinfection(dpi).

(1) Pathology

The following tissues were collected immediately post-mortem and fixed by immersion in 10% neutral buffered formalin for 24 hours : trachea, lung, cecal tonsil, spleen, kidney, intestine, bursa, and cecal tonsil. Tissues were routinely processed into paraffin, and 3 μm sections were cut for hematoxylin and eosin staining, ISH, and IHC.

(2) Virus isolation

Tissues were collected in a tube that contained 1 mℓ of PBS containing gentamicin sulfate(200 mg/mℓ). Tissues were minced and centrifuged at 1,500 ×g for 20 min, and 0.1 mℓ of undiluted supernatant was inoculated into each of three 9~11 day old SPF embryonated chicken eggs. After 2 days of incubation, allantoic fluid was harvested. Conformation of IBV was determined by reverse transcriptase-polymerase chain reaction(RT-PCR) and restriction fragment length polymorphism(RFLP) as described[23].

(3) ISH and IHC

A riboprobe for ISH was created from the 484 bp hypervariable region of the S1 gene from the JMK strain. The 484 bp gene was inserted into the pGEM-T vector(Promega, Madison, WI). Based on sequence information from GenBank, this region shares 98% sequence similarity with the Gray strain. *In vitro* transcription was performed using SP6-RNA polymerase (Roche, Indianapolis, IN) with DIG-labeled NTPs to make antisense RNA complementary to mRNA. The concentration of DIG-labeled riboprobes was determined by dot-blot comparison with a known standard DIG-labeled RNA. ISH was done as previously described[10].

The immunostaining was performed with DAKO EnVision™ System, Peroxidase (DAKO Corporation, Carpinteria, CA) with an automated immunostainer(LEICA ST5050, Leica instruments GmbH, Nussloch, Germany) as previously described[24]. Briefly, the immunostaining was conducted by 5 min of blocking with peroxidase blocking reagent, 10 min of treatment with hydrogen peroxide, 5 min of digestion with proteinase K, 30 min of incubation with diluted(1 : 100) Mab 9C1C6[26], 30 min of incubation with peroxidase labelled polymer, and 5 min of developing with substrate and diaminobenzidine tetrahydrochloride(DAB) chromogen.

Experiment 2. *In vivo* passage of the JMK strain by cloacal infection

Inocula were prepared from kidneys of chicks inoculated with JMK by the cloaca. The inoculum for the first passage was obtained from Experiment 1. The kidney suspension was inoculated into the cloaca of five 2 day old SPF chicks. The chicks were euthanatized at 5 dpi. Necropsies were performed immediately postmortem and kidneys were collected for virus isolation and histopathology. The virus was passaged a total of 10 times. The virus titer in each inoculum was determined as previously described[22].

We also inoculated 2 day old chicks by

intraocular route with the parent virus, passage 3, passage 5, and the 10th passage of the JMK virus. All chicks were euthanatized at 5 dpi. Trachea and kidneys were collected for virus recovery and histopathological comparison.

Results

Experiment 1. Intraocular and cloacal infection of chickens with Gray and JMK strains

(1) Pathology

Clinical signs were not obvious in chickens infected with Gray and JMK either by intraocular or intracloacal route. In birds infected with the Gray strain by intraocular route, kidneys were slightly swollen and urates were observed in the ureters at 5 and 7 dpi. No gross kidney lesions were observed in JMK infected chickens either by intraocular or cloacal routes. No clinical signs or gross pathology were observed in uninfected control chickens.

There were no IBV infection associated histological changes in intestine and bursa. Compared to controls, cecal tonsils of chickens infected with JMK by the cloacal route showed lymphoid development at 3 and 5 dpi. Changes in the trachea were severe in chickens infected with JMK by the intraocular route with loss of cilia and sloughed epithelial cells as early as 3 dpi. In chickens infected with Gray by the intraocular route and chickens infected with JMK by the cloacal route, similar but milder tracheal lesions were observed at 5 dpi. Multifocal lymphocyte infiltrate and diffuse hyperplasia were evident at 7 and 10 dpi in chickens infected with either Gray or JMK. In the lung, lymphocyte infiltrations in the parabronchus were observed at 3 and 5 dpi in chickens infected with JMK by the intraocular route. In the kidney, multifocal lymphocytic nodules in the interstitium were evident in Gray infected chickens at 5 and 7 dpi(Fig 1).

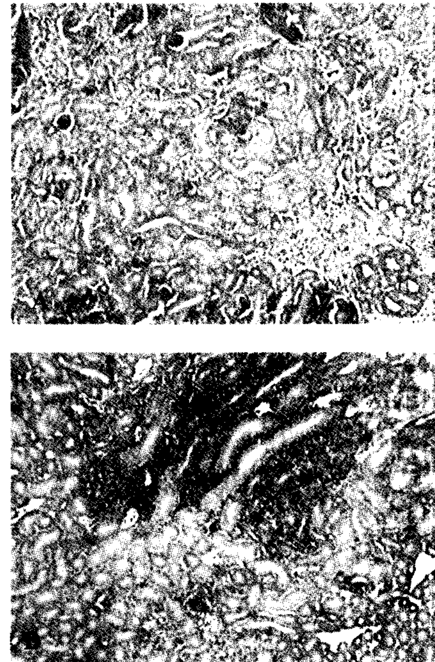

Fig 1. Kidneys (10×). A. Uninoculated control chicken killed at 7 days after infection, B. Chicken infected with Gray strain 7 days previously ; an extensive infiltrate of mononuclear cells in the interstitium is evident.

(2) Virus Isolation

In chickens infected with Gray by the intraocular route, virus was isolated in all tissues examined in this study. In trachea, virus was isolated as early as 12 hours PI and remained in that tissue throughout the experiment. In the lung, virus was isolated in the early phase of infection(1 and 3 dpi). In the late phase of infection(5, 7, and 10 dpi), virus was isolated in the kidney, intestine, cecal tonsil, and bursa.

In chickens infected with JMK by the intraocular route, virus was isolated from the trachea, lung, and cecal tonsil, which was similar to Gray infected chickens. However, virus was not isolated from the kidney, intestine and bursa at any time point.

In chickens infected with JMK by the cloacal route, virus was isolated in all tissues. Compared to Gray infection, virus was isolated from kidney, intestine, cecal tonsil and bursa in the early phase of infection. Results are summarized in Table 1.

(3) ISH and IHC

Viral RNA or antigen were detected in the trachea of different groups. In Gray infected chickens, positive staining for viral RNA and antigen was detected in bursa and cecal tonsil at 5 and 7 dpi(Fig 2). In chickens infected with JMK by intraocular route, only tracheal tissue showed positive staining for viral RNA and antigen(Fig 2). In chickens infected with JMK by the cloacal route, positive staining was observed in the bursa as early as 12 hours PI and remained throughout the experiment(Fig 2). No staining was observed in the kidney from chickens infected with Gray or JMK. The extent of viral replication is summarized in Table 2.

Experiment 2. In vivo passage of the JMK strain by cloacal infection

JMK strain was passaged ten times in chickens by cloacal infection of kidney tissue clinical or postmortem signs were observed.

Table 1. Comparison of virus isolation

Virus	Route	Tissue	Hours or days post infection					
			12 hr	1 day	3 day	5 day	7 day	10 day
Gray	Intranasal	Trachea	+	+	+	+	+	+
		Lung	−	+	+	−	−	−
		Kidney	−	−	−	+	+	+
		Intestine	−	−	−	−	+	−
		Cecal tonsil	−	−	−	+	+	+
		Bursa	−	−	−	+	+	+
JMK	Intranasal	Trachea	+	+	+	+	+	−
		Lung	+	+	+	+	−	−
		Kidney	−	−	−	−	−	−
		Intestine	−	−	−	−	−	−
		Cecal tonsil	−	−	−	−	+	+
		Bursa	−	−	−	−	−	−
	Intracloacal	Trachea	−	+	+	+	+	+
		Lung	−	+	+	+	−	−
		Kidney	+	+	+	+	+	+
		Intestine	−	+	+	−	+	+
		Cecal tonsil	−	+	+	+	+	+
		Bursa	+	+	+	+	+	+

Fig 2. ISH and IHC. Positive staining can be observed as a dark area in the cytoplasm of epithelial cells. A. Trachea(20×). 1 days postinfection with the JMK strain by the intraocular route. ISH, B. Bursa(10×). 5 days postinfection with the JMK strain by the cloacal route. IHC, C. Cecal tonsil (40×). 5 days postinfection with the Gray strain by the intraocular route. IHC, D. Bursa(20×). 7 days postinfection with the Gray strain by the intraocular route. ISH. All the sections were counterstained with Mayers hematoxylin.

Table 2. Summary of ISH and IHC results

Virus	Route	Tissue	Hours or days post infection					
			12 hr	1 day	3 day	5 day	7 day	10 day
Gray	Intranasal	Trachea	-	-	++	++	++	+
		Bursae	-	-	-	+	+	-
		Cecal tonsil	-	-	-	+++	+++	-
		Kidney	-	-	-	-	-	-
JMK	Intranasal	Trachea	-	+++	+++	++	+	-
		Bursae	-	-	-	-	-	-
		Cecal tonsil	-	-	-	-	-	-
		Kidney	-	-	-	-	-	-
	Intracloacal	Trachea	-	-	-	+	+	-
		Bursae	+++	+++	+++	+++	+++	++
		Cecal tonsil	-	-	-	-	-	-
		Kidney	-	-	-	-	-	-

+++ = >5 positive cells per high-power field (400×); ++ = >1 and <5 positive cells per high-power field; + = 1 positive cell per high power field; - = no positive cells.

Histopathologically, there was no change in kidney from chickens infected with virus of different passage levels compared to that of the parental virus. Virus was successfully recovered from kidney at each passage. Virus titer at each passage level is presented in Fig 3. The amount of virus recovered was stable throughout serial passage. The titer was between $10^{3.00}$ and $10^{4.00}$ $log_{10}EID_{50}/0.1g$. We further infected the chickens intraocularly with parental, passage 3, passage 5, and passage 10. For each passage, we isolated the virus from the trachea, but not from the kidney of infected chickens.

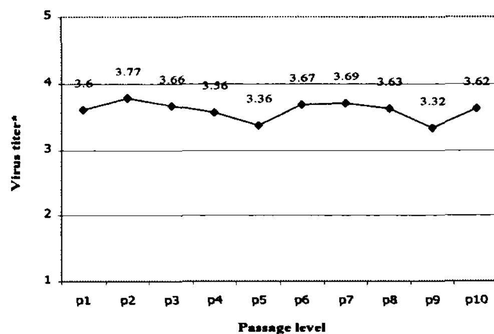

Fig 3. Profile of titers of virus recovered from kidneys in each serial passage by cloacal infection.(*Virus titer = $log_{10}EID_{50}/0.1g$)

Discussion

The variations in tissue tropism of coronaviruses are in large part due to differences in the spike glycoprotein. With a carefully constructed recombinant coronaviruses differing only in the spike gene, the importance of spike protein in determining the tissue tropism has been well established[19]. Nephropathogenic IBVs isolated worldwide have large difference in the S1 gene sequence with other non-nephropathogenic IBVs[5,26]. However, no relationship among nephropathogenic IBVs has been determined so far at a molecular level. Gray and JMK strains differ by only 10 amino acids in the S1 glycoprotein[20]. However, they differ in tissue tropism. Our results show that JMK replicates in the trachea and the replication of virus is strictly restricted to the upper-respiratory tract when chickens were infected by the intraocular route. On the contrary, Gray could be isolated in other tissues such as kidney and bursa as well as trachea following intraocular inoculation. The receptor on the cell surface for IBV is known to be a 2,3-linked N-acetylneuraminic acid[27]. However, binding of IBV to this molecule in itself is not likely the basis of viral cell tropism because sialic acid is a common cell surface carbohydrate residue. Thus, an additional molecule is probably required for Gray infection, but not JMK in the lower tissues. Based on the histopathology and ISH or IHC results, the JMK strain replicates more efficiently in the trachea· and produced more severe lesions than the Gray strain. Thus, it is also possible that the strong local immune function in the upper respiratory tract of JMK infected chickens may have prevented the spread of virus to the kidney and bursa.

There were some discrepancies between virus isolation and ISH or IHC. Virus was easily recovered from kidney of chickens infected with Gray and JMK by the cloacal route. However, no viral RNA or antigen were detected using ISH and IHC. Virus isolation indicates that viable virus was present in or on that tissue but does not imply that viral replication occurred within the cells of that organ. Thus, it is possible that the virus in the kidney is the result of viremia and the renal pathologic changes in Gray infected chickens are indirect. However, the lower sensitivity of ISH and IHC for

virus detection compared to virus isolation should not be overlooked.

The cloacal inoculation of the JMK strain resulted in virus isolation in the lower tissues including kidney. Previously, Uenaka et al[28] hypothesized that the cloaca is one of the most efficient natural inoculation routes to spread IBV to the kidney. They produced a nephropathogenic virus from a non-nephropathogenic strain(Connecticut A-5968) by *in vivo* serial passage using cloacal inoculation[9]. The passaged virus caused more severe lesions in the kidneys of infected chickens than parent virus. We applied same strategy in an attempt to change the tissue tropism of JMK. The amount of virus recovered in the kidney was stable up to the 10th serial passage Histopathologically, passaged virus did not caused renal damage. Thus, serial *in vivo* passage of the JMK virus did not result in nephrotropism or nephropathogenicity. The Connecticut strain used by Uenaka et al[9] is a non-nephrosis-causing virus. However, this strain was easily recovered in the kidney of chickens subjected to aerosol or intratracheal infection[9,29]. Thus, the increase in nephropathogenicity of the Connecticut strain seems to be a result of back passage in the chicken. Further studies using different strains of IBV should be pursued to clarify the effect of cloacal passage in tissue tropism.

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References

1. Cavanagh D, Naqi SA. 1997. *Infectious bronchitis.* In : Disease of poultry, 10th ed. Calnek BW, Barnes HJ, Beard CW, et al., Eds. Iowa State University Press, Ames, Iowa : 511~526.
2. Winterfield RW, Hitchner SB. 1962. Etiology of an infectious nephritis-nephrosis syndrome of chickens. *Am J Vet Res* 23 : 1273~1279.
3. Butcher GD, Winterfield RW, Shapiro DP. 1989. An outbreak of nephropathogenic H13 infectious bronchitis in commercial broilers. *Avian Dis* 33 : 823~826.
4. Cummings RC. 1962. The etiology of uraemia of chickens. *Aust Vet J* 38 : 554
5. Gelb J, Jr., Ladman BS, Nix WA, et al. 1999. Characteristics of nephropathogenic infectious bronchitis virus in Pennsylvania Poultry. *JAVMA* 215 : 1678.
6. Adzhar A, Gough RE, Haydon D, et al. 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian Pathol* 26 : 625~640
7. Song CS, Lee YJ, Lee CW, et al. 1998. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. *J Gen Virol* 79 : 719~723.
8. Otsuki K, Matsuo K, Maeda N, et al. 1990. *Selection of variants of avian infectious bronchitis virus showing tropism for different organs.* In : Coronaviruses and their diseases : Cavanagh D, Brown TDK, Eds. Plenum Press, New York : 379~384.
9. Uenaka T, Kishimoto I, Uemura T, et al. 1998. Cloacal inoculation with the Connecticut strain of avian infectious bronchitis virus : An attempt to produce nephropathogenic virus by *in vivo* pas-

sage using cloacal inoculation. *J Vet Med Sci* 60 : 495~502.
10. Lee CW. 2001. Study of genetic variation and tissue tropism of avian infectious bronchitis virus : *In situ* hybridization with antisense digoxigenin-labeled riboprobe for the detection of avian infectious bronchitis virus in formalin-fixed paraffin- embedded tissues. Dissertataion. University of Georgia : 164~180.
11. Jones RC. 1974. Nephrosis in laying chickens caused by Massachusetts-type infectious bronchitis virus. *Vet Rec* 95 : 319
12. Chen BY, Hosi S, Nunoya T, et al. 1996. Histopathology and immunohistochemistry of renal lesions due to infectious bronchitis virus in chicks. *Avian Pathol* 25 : 269~283.
13. Siller WG. 1981. Renal pathology of the fowl. *Avian Pathol* 10 : 187~262.
14. Boursnell ME, Brown TD, Foulds IJ, et al. 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *J Gen Virol* 68 : 57~77.
15. Lai MM, Cavanagh D. 1997. The molecular biology of coronaviruses. *Adv Virus Res* 148 : 1~100.
16. Cavanagh D, Davis PJ, Pappin DJC, et al. 1986. Coronavirus IBV : partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor polypeptide of IBV strains Beaudette and M41. *Virus Res* 4 : 133~143.
17. Cavanagh D, Davis PJ, Mockett A. 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV(Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. *Virus Res* 11 : 141~150.
18. Niesters H, Bleumink PN, Osterhaus A, et al. 1989. Epitopes on the peplomer protein of infectious bronchitis coronavirus strain M41 as defined by monoclonal antibodies. *Virology* 161 : 511~519.
19. Gallagher TM, Buchmeier MJ. 2001. Coronavirus spike proteins in viral entry and pathogenesis. *Virology* 279 : 371~374.
20. Kwon HM, Jackwood MW. 1995. Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. *Virus Genes* 9 : 219~229.
21. Albassam MA, Winterfield RW, Thacker HL. 1986. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. *Avian Dis* 30 : 468~476.
22. Villegas P. 1998. *Titration of biological suspensions*. In : A laboratory manual for the isolation and identification of avian pathogens. 4th ed., Swayne DE, Eds. American Association of Avian Pathologists. Kennett Square, PA : 248~254.
23. Kwon HM, Jackwood MW, Gelb J Jr. 1993. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction-fragment-length-polymorphism analysis. *Avian Dis* 37 : 194~202.
24. Lee CW. 2001. Study of genetic variation and tissue tropism of avian infectious bronchitis virus : Pathogenesis of infectious bronchitis virus in chickens experimentally infected with viruses of different tissue tropism. Dissertation. University of Georgia : 181~199.
25. Parr RL, Collisson EW. 1993. Epitopes on the spike protein of a nephropathogenic strain of infectious bronchitis virus. *Arch Virol* 133 : 369~383.

26. Shaw K, Britton P, Cavanagh D. 1996. Sequence of the spike protein of the Belgian B1648 isolate of nephropathogenic infectious bronchitis virus. *Avian Pathol* 25 : 607~611.
27. Holmes KV, Compton SR. 1995. *Coronavirus receptors.* In : The Coronaviridae. Siddell SG, Eds. Plenum Press, New York : 55~71.
28. Uenaka T, Kishimoto I, Sato S, et al. 1998. Intracloacal infection with avian infectious bronchitis virus. *Avian Pathol* 27 : 309~312.
29. Hofstad MS, Yoder HW. 1966. Avian infectious bronchitis. Virus distribution in tissues of chicks. *Avian Dis* 10 : 230~239.