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Author(s): Rüdiger Hauck , Rodrigo A. Gallardo , Peter R. Woolcock , H. L. Shivaprasad
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Case Report—

A Coronavirus Associated with Runting Stunting Syndrome in Broiler Chickens

Rüdiger Hauck,[A] Rodrigo A. Gallardo,[AD] Peter R. Woolcock,[B] and H. L. Shivaprasad[C]

[A]Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA
[B]University of California, California Animal Health and Food Safety Laboratory System, 620 West Health Science Dr., Davis, CA 95616
[C]University of California, California Animal Health and Food Safety Laboratory System, 18830 Rd. 112, Tulare, CA 93274

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SUMMARY. Runting stunting syndrome (RSS) is a disease condition that affects broilers and causes impaired growth and poor feed conversion because of enteritis characterized by pale and distended small intestines with watery contents. The etiology of the disease is multifactorial, and a large variety of viral agents have been implicated. Here we describe the detection and isolation of an infectious bronchitis virus (IBV) -like coronavirus from the intestines of a flock of 60,000 14-day-old brown/red broiler chicks. The birds showed typical clinical signs of RSS including stunting and uneven growth. At necropsy, the small intestines were pale and distended with watery contents. Histopathology of the intestines revealed increased cellularity of the lamina propria, blunting of villi, and cystic changes in the crypts. Negative stain electron microscopy of the intestinal contents revealed coronavirus particles. Transmission electron microscopy of the intestine confirmed coronavirus in the cytoplasm of enterocytes. Using immunohistochemistry (IHC), IBV antigen was detected in the intestinal epithelial cells as well as in the proventriculus and pancreas. There were no lesions in the respiratory system, and no IBV antigen was detected in trachea, lung, air sac, conjunctiva, and cecal tonsils. A coronavirus was isolated from the intestine of chicken embryos but not from the allantoic sac inoculated with the intestinal contents of the broiler chicks. Sequencing of the S1 gene showed nucleic acid sequence identities of 93.8% to the corresponding region of IBV California 99 and of 85.7% to IBV Arkansas. Nucleic acid sequence identities to other IBV genotypes were lower. The histopathologic lesions in the intestines were reproduced after experimental infection of specific-pathogen-free chickens inoculated in the conjunctiva and nares. Five days after infection, six of nine investigated birds showed enteritis associated with IBV antigen as detected by IHC. In contrast to the field infection, birds in the experimental group showed clear respiratory signs and lesions in the upper respiratory tract. The results suggest a broader tissue tropism of this isolate, which might be related to the mutations in the S1 gene.

RESUMEN. *Reporte de caso-* Coronavirus asociado con un síndrome de retraso del crecimiento y enanismo en pollos de engorde.
El síndrome de retraso en el crecimiento y enanismo (con las siglas en inglés RSS) es una enfermedad que afecta a los pollos de engorde que causa alteraciones en el crecimiento y mala conversión alimenticia debido a la enteritis caracterizada por la presencia intestinos delgados, pálidos con paredes distendidas y con contenido acuoso. La etiología de la enfermedad es multifactorial y una gran variedad de agentes virales han sido implicados. Aquí se describe la detección y el aislamiento de un virus parecido al coronavirus de la bronquitis infecciosa (con las siglas en inglés IBV) a partir de una parvada 60,000 pollos de engorda de 14 días de edad, de color marrón. Las aves mostraron signos clínicos típicos del síndrome de enanismo y retraso en el crecimiento, incluyendo el retraso del crecimiento o crecimiento desigual. A la necropsia, los intestinos delgados estaban pálidos y distendidos con contenido acuoso. La histopatología de los intestinos reveló un aumento de la celularidad en la lámina propria, acortamiento de las vellosidades y cambios quísticos en las criptas. La microscopía electrónica con tinción negativa de los contenidos intestinales reveló partículas de coronavirus. La microscopía electrónica de transmisión del intestino confirmó coronavirus en el citoplasma de los enterocitos. Mediante el uso de inmunohistoquímica (IHC), se detectó antígeno del virus de la bronquitis infecciosa en las células epiteliales intestinales, así como en el proventrículo y el páncreas. No hubo lesiones en el sistema respiratorio y no se detectó el antígeno del virus de la bronquitis infecciosa en la tráquea, el pulmón, los sacos aéreos, las conjuntivas o de las tonsilas cecales. Se aisló un coronavirus del intestino de los embriones de pollo, pero no del saco alantoideo inoculado con el contenido intestinal de los pollos de engorde. La secuenciación del gene S1 mostró identidades en las secuencias de nucleótidos de 93.8% con la región correspondiente al serotipo California 99 y de 85.7% con el virus Arkansas. Las identidades en las secuencias de nucleótidos con otros genotipos del virus de la bronquitis infecciosa fueron menores. Las lesiones histopatológicas en los intestinos fueron reproducidas después de la infección experimental de los pollos libres de patógenos específicos inoculados en la conjuntiva y en las fosas nasales. Cinco días después de la infección, seis de las nueve aves inoculadas mostraron enteritis asociada con antígeno de bronquitis infecciosa como se detecta por inmunohistoquímica. En contraste con la infección de campo, las aves en el grupo experimental mostraron signos respiratorios claros y lesiones en el tracto respiratorio superior. Los resultados sugieren un tropismo tisular más amplio de este aislamiento, lo cual podría estar relacionado con las mutaciones en el gene S1.

Key words: malabsorption syndrome, enteritis, pathogenesis, genotype

Abbreviations: bp = base pair; CAM = chorioallantoic membrane; dpi = days postinoculation; EM = electron microscopy; FA = fluorescent antibody test; H&E = hematoxylin and eosin; IBD = infectious bursal disease; IBV = infectious bronchitis virus; IHC = immunohistochemistry; NDV = Newcastle disease virus; PRCoV = porcine respiratory coronavirus; RSS = runting stunting syndrome; S = spike; SPF = specific-pathogen-free; TGEV = transmissible gastroenteritis virus; ToCV = turkey coronavirus

[D]Corresponding author. E-mail: ragallardo@ucdavis.edu

Broiler chickens worldwide, during their first 3 wk of life, often suffer from a disease characterized by diarrhea associated with impaired growth and poor feed conversion, but with only low mortality. The most prominent gross lesions at postmortem are pale and distended small intestines with watery or mucoid contents (42). Histopathologic lesions in the affected intestines comprise cyst formation in the crypts of Lieberkühn, caused by degeneration and detachment of the cells lining the lumen, and atrophy of the intestinal villi (26,46,50). Depending on other observed symptoms and the preferences of various authors, the condition has been given several different names, including runting stunting syndrome (RSS), malabsorption syndrome, infectious stunting syndrome, pale bird syndrome, or helicopter syndrome (15).

The etiology of the disease is multifactorial, and a large variety of viral agents like parvoviruses (28,51), calicivirus (48), rotavirus (40), astrovirus (5,45), reovirus (12,29), and birnavirus (34,39) have been implicated.

Infectious bronchitis virus (IBV) is known to replicate and persist in the cecal tonsils without causing lesions and thus often is isolated from cloacal swabs (1,43). Isolates from birds with respiratory signs, including reference strain M41, were also occasionally isolated from duodenum and jejunum after experimental infection without causing grossly visible intestinal lesions (33). Montgomery *et al.* (36) isolated an IBV from the intestines of commercial broilers with RSS, together with reovirus and bacterial pathogens. While none of these pathogens alone or combined reproduced the characteristic lesions of the disease, chickens infected with IBV alone or combined with other pathogens showed weight depression.

Another IBV isolate derived from broilers with respiratory signs was isolated from duodenum, jejunum, and ileum after experimental infection (16). This isolate also caused mild lesions like desquamation of epithelial cells from the tips of villi and congestion of the submucosal areas especially of the rectum but not in the small intestines (2,3). This virus did grow in embryonated chicken eggs, but not in organ cultures of duodenum, jejunum, and ileum (6). More recently, two IBV isolates from broiler flocks with enteric and respiratory problems, respectively, were compared. Both isolates were detected in the intestines but did not cause lesions in the intestines (11).

Here we describe the detection and isolation of an IBV-like coronavirus from the intestines obtained from broiler chickens showing RSS symptoms. Subsequently the obtained isolate caused RSS-like lesions after experimental infections of specific-pathogen-free (SPF) chickens.

MATERIALS AND METHODS

Case history. In a flock of 60,000 14-day-old brown/red broiler chicks, clinical symptoms suggestive of RSS-like anorexia and uneven growth were observed. The birds had been vaccinated against Marek's disease and infectious bursal disease (IBD) *in ovo* and against coccidia and Newcastle disease and IBV with Mass 41 and Conn at 1 day of age. At 42 days of age, the mortality including culls had reached 8% in the flock. Since 2012, several flocks on this ranch had shown similar signs.

Pathology and immunohistochemistry (IHC). Clinical examination of eight live birds submitted was conducted. The birds were weighed, and postmortem examination of the birds was performed according to usual procedures. Samples of various tissues, including conjunctiva, proventriculus, intestines including cecal tonsils, pancreas, bursa of Fabricius, thymus, liver, spleen, kidneys, crop, gizzard, brain, larynx, trachea, and lungs, were collected from a varying number of birds. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, mounted on glass slides, and stained with hematoxylin and eosin (H&E).

IBV antigen detection was attempted by IHC using routine methods as described previously (17). Briefly, a combination of two IBV monoclonal antibodies 9.19 and 9.04 directed against the matrix and spike (S) protein, respectively, were used for the antigen detection (27,38).

Electron microscopy. For direct electron (negative-stain) microscopy (EM), intestinal samples and its contents were examined by using the methodology described previously (49). For transmission electron microscopy, formalin fixed small intestinal specimens were postfixed in 2.5% glutaraldehyde followed by osmium tetroxide before embedding and sectioning according to the procedure described previously (8).

Virology. Virus isolation was performed as described by França *et al.* (17). Briefly, the intestines were homogenized, clarified by centrifugation, and filtered. SPF eggs of 10 days of embryonation were inoculated with this suspension via the allantoic cavity (SPAFAS, Storrs, CT). Chorioallantoic membranes (CAMs) were screened for IBV antigens by a fluorescent antibody test (FA). Furthermore, allantoic fluid and embryonary intestines were tested for the presence of IBV using EM and RT-PCR.

Bacteriology. Liver samples were streaked on 3% sheep blood and MacConkey agar (Remel, Lenexa, KS) and incubated at 37 C in 7.5% CO_2 for 24 to 48 hr. For the detection of salmonella, swabs from the ceca of several birds were selectively enriched in selenite enrichment broth (Remel) at 35 C for 18 to 24 hr. Following enrichment, selenite broth was plated on brilliant green with novobiocin and XLT4 agars (Remel) and incubated at 37 C for an additional 24 hr. Isolates were identified following standard methods.

Serology. Sera from all birds were tested for IBV, Newcastle disease virus (NDV), and IBDV by ELISA (IDEXX Laboratories Inc., Westbrook, ME). S/P ratios greater than 0.2 were considered positive.

Experimental infection. Forty chickens were hatched from SPF eggs (Sunrise Farms, Catskill, NY). At 14 days of age, 30 chickens were inoculated with 200 μl homogenate of embryonic intestines containing the viral isolate. Fifty microliters were given into each eye and nostril. The additional 10 chicks served as uninfected controls.

At 2, 4, and 5 days postinoculation (dpi), 10 experimental and three control chickens were evaluated individually by a blinded investigator for respiratory signs as described previously (47). Severity scores were recorded as no signs (score 0), mild signs, characterized by nasal rales or upper respiratory tract sounds (score 1), moderate signs, characterized by tracheal rales (score 2), or severe respiratory sounds audible from 20 cm distance (score 3). A respiratory signs index was calculated following the formula: $\{\Sigma (\text{Pos} \times \text{Severity}) / (\text{Hypothetical pos max} \times \text{Severity})\}$ in which "Pos" are the chickens showing clinical signs on each of the severity levels and "Hypothetical pos max" is the maximum possible score obtained when all the chickens are positive for the maximum severity. The same birds were euthanatized, and at postmortem samples of conjunctiva, sinus/turbinates, trachea, duodenum, jejunum, ileum, cecal tonsils, colon, and Harderian glands as well as tears were collected. Organs were investigated by histopathology and IHC. Tears, trachea, duodenum, and cecal tonsils were investigated by RT-PCR for the presence of IBV-specific RNA.

Molecular biology. RNA was extracted from samples using the QIAamp RNA Mini kit (Qiagen, Valencia, CA) as described by the manufacturer. Samples were investigated for IBV-specific RNA using primers S17F and S18R to detect an 818 base pair (bp) fragment of the S gene (20). Negative samples were double checked by RT-PCR using primers IBVN(+) and IBVN(–) to detect a 453 bp fragment of the nucleocapsid gene (22).

The inoculum for the experimental infection was checked for parvovirus of chickens using primers PVF1 and PVR1 (52), for avian

Table 1. Primers used for detection of IBV, chicken parvovirus, avian reovirus, avian rotavirus, and chicken astrovirus as well as for sequencing of the Spike 1 gene of IBV

Name	Sequence	Target virus	Target gene	Reference
S17F	TGA AAA CTG AAC AAA AGA CCG ACT TAG	IBV	Spike 1	20
S18R	GGA TAG AAG CCA TCT GAA AAA TTG C	IBV	Spike 1	20
(S18F)	(GCA ATT TTT CAG ATG GCT TCT ATC C)			
S1OLIG03	CAT AAC TAA CAT AAG GGC AA	IBV	Spike 1	30
S17F	TGA AAA CTG AAC AAA AGA CCG ACT TAG	IBV	Spike 1	20
S20R	TGC AGT CAT GGC TAT TGA GG	IBV	Spike 1	-[A]
S21R	CAC GAG GAC CGT TGT ATG AA	IBV	Spike 1	-
S22F	ATA CAA CGG TCC TCG TGC TT	IBV	Spike 1	-
IBVN(+)	GAA GAA AAC CAG TCC CAG ATG CTT GG	IBV	Nucleocapsid	22
IBVN(−)	GTT GGA ATA GTG CGC TTG CAA TAC CG	IBV	Nucleocapsid	22
PVF1	TTC TAA TAA CGA TAT CAC TCA AGT TTC	Chicken parvovirus	Nonstructural	52
PVR1	TTT GCG CTT GCG GTG AAG TCT GGC TCG	Chicken parvovirus	Nonstructural	52
ARV S4 P4	GTG CGT GTT GGA GTT TC	Avian reovirus	S 4	9
ARV S4 P5	ACA AAG CCA GCC AT R AT	Avian reovirus	S 4	9
NSP4 F30	GCC CGT GCG GAA AGA TGG AGA AC	Avian rotavirus	NSP 4	14
NSP4 R660	TCG GGT TGG GGT ACC AGG GAT TAA	Avian rotavirus	NSP 4	14
CAS pol 1F	GAY CAR CGA ATG CGR AGR TTG	Chicken astrovirus	Polymerase	14
CAS pol 1R	TCA GTG GAA GTG GGK ART CTA C	Chicken astrovirus	Polymerase	14

[A]Designed for this study.

reovirus using primers ARV S4 P4 and ARV S4 P5 (9), for avian rotavirus using primers NSP4 F30 and NSP4 R660 (14), and for chicken astrovirus using primers CAS pol 1F and CAS pol 1R (14). Sequences of all primers are listed in Table 1. RT-PCRs were done using the Qiagen One step RT-PCR kit (Qiagen); PCR for detection of parvoviruses was done using the Taq PCR Core Kit (Qiagen). Positive controls were kindly provided by Dr. M. Day, Southeast Poultry Research Laboratory, Athens, GA.

Sequencing of the S1 gene. The S1 gene was amplified by RT-PCR and primers S1OLIG03 (30) and S17F (20). The 1720 bp amplicon was purified using the QIAquick PCR purification kit (Qiagen) and sequenced directly using primers S1OLIG03, S17F, S18R (20), S18F, S20R, S21R, and S22F by the UCDNA Sequencing Facility (Davis, CA, USA). The sequence was assembled using cap3 (23). Sequence identities to other IBV types and a neighbor-joining tree without distance corrections were calculated using Clustal Omega (31).

RESULTS

Natural infection. Eight 14-day-old birds were submitted for necropsy and further investigation. The birds were lethargic and had ruffled feathers. Their body weights ranged between 42 and 138 g, and the average weight was 74 g. The normal expected body weight for this strain of chickens at 14 days of age was 175 g. At necropsy, the serosa of the small intestines of seven birds was pale, and the lumens were distended (Fig. 1). The contents were watery. The ceca of five birds were distended and their contents frothy. Furthermore, proventriculi of four birds were enlarged and had a pale or mottled pale serosa. Kidneys of five birds were pale. A few birds had retained yolk sacs. There were no gross lesions in other organs, including the respiratory system.

Histopathologic investigation of the intestines showed increased cellularity of the lamina propria, mild blunting, and fusion of villi and cystic dilation of the crypts lined by flattened epithelial cells (Fig. 2). In sections of the proventriculus, there was infiltration of a large numbers of lymphocytes in the glandular part. A few sections showed acute necrosis of the epithelium of the glands, and one section had mild to severe dilation of the deeper glands. The mucosa was mildly to moderately inflamed with lymphocytes. Kidneys in a few birds had multifocal dilatation and mild mineralization of the tubules. There was mild diffuse atrophy of acinar cells in the pancreas. In the other organs investigated, no lesions were observed.

IHC of various organs revealed IBV antigen in the cytoplasm of duodenum, jejunum, rectum/cloaca, proventriculus, and pancreas (Table 2). No IBV antigen was detected in three sections each of trachea, lung, air sac, conjunctiva, and cecal tonsils, as well as in the liver, spleen, kidney, bursa, esophagus/crop, heart, brain, gizzard, and gonads. In the small intestines, IBV antigen was located in the cytoplasm of enterocytes mostly at the tips of the villi throughout the intestine (Fig. 3a,b).

Direct electron microscopy of the intestine and its contents revealed the presence of rotavirus and coronavirus. Coronavirus measured about 90 nm in diameter and had typical club-shaped surface projections (Fig. 4a). Transmission electron microscopy of the intestine revealed typical coronavirus particles about 85 nm in diameter with club-shaped surface projections in the cytoplasm of enterocytes (Fig. 4b).

When virus isolation from the intestinal samples was attempted in chicken embryos, no IBV antigen was detected in the CAMs and allantoic fluid using FA, EM, and/or RT-PCR. However, the embryo's intestines were positive for IBV by EM and RT-PCR. Embryo intestines were used for one subsequent passage in eggs, collected, and stored as viral stock.

Coagulase-negative *Staphylococcus aureus* was isolated from the livers of three birds and group C *Salmonella* from the ceca.

In the eight investigated sera from submitted birds, no antibodies against IBV, NDV, and IBDV were detected.

Sequencing of the S1 gene. Of the 1720 bp amplicon (accession number KU311691), 1631 bases for which corresponding sequences of the S1 gene of all selected IBV variants were available were analyzed. The most closely related IBV types were IBV California 99 and IBV Arkansas DPI. Nucleic acid sequence identities were 93.8% to the corresponding region of IBV California 99 and 85.7% to IBV Arkansas. Other IBV genotypes were clearly set apart in the phylogenetic tree (Fig. 5).

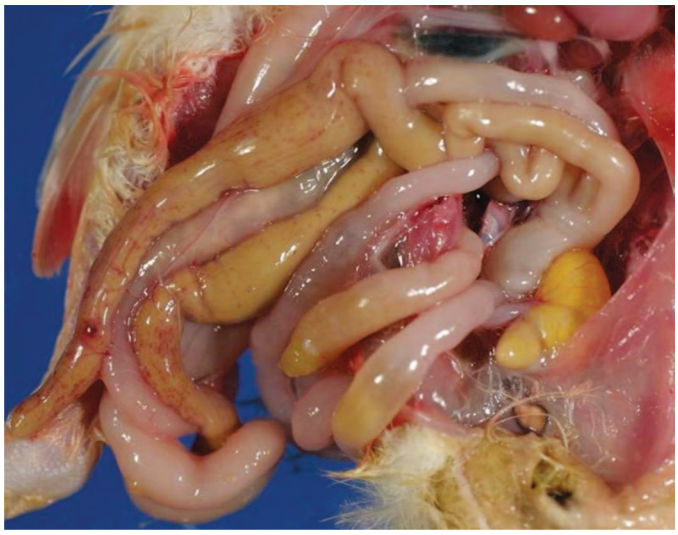

Fig. 1. Intestines of a 14-day-old bird with RSS; note pale serosa and distended lumens; small intestine contents were watery and cecal contents frothy.

Experimental infection. PCR or RT-PCR, respectively, did not detect parvovirus of chickens, avian reovirus, avian rotavirus, and chicken astrovirus in the inoculum.

SPF birds were inoculated using the oculonasal route with the obtained IBV isolate at 2 wk of age. At 2 dpi, a few birds had mild respiratory signs with a calculated respiratory signs index of 20%. At 4 dpi the index increased up to 50%. Finally, at 5 dpi the severity of respiratory signs decreased, and the calculated index was 33%.

Two, 4, and 5 dpi, mild inflammation was observed in almost all investigated sections of conjunctiva, sinus, and trachea (Table 3). In conjunctiva and sinus, IBV antigen was detected by IHC on all occasions, in the trachea only at 4 and 5 dpi. Similarly as in the intestines of the naturally infected birds, increased cellularity of the lamina propria, mild blunting, and fusion of villi and cystic dilation of the crypts lined by flattened epithelial cells was observed 4 dpi and more clearly 5 dpi.

On all occasions, IBV RNA was detected in the tears of all birds. Two dpi in all investigated trachea IBV was found, and 4 dpi five out of six tracheae and 5 dpi only four out of six tracheae were positive. The number of positive duodenum decreased likewise from two at 2 dpi to none at 5 dpi, while IBV RNA was detected in four out of six cecal tonsils 2 and 4 dpi and in three out of six cecal tonsils 5 dpi (Table 4).

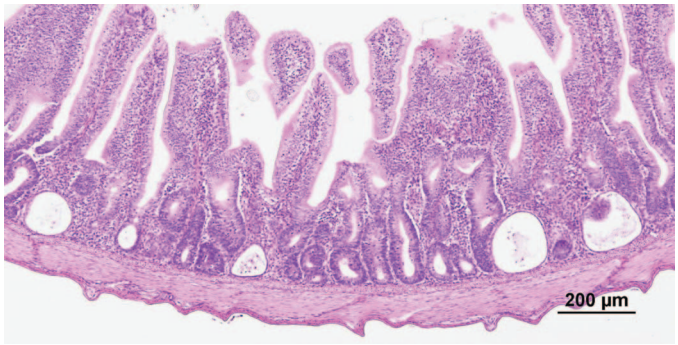

Fig. 2. H.E. stained intestine showing increased cellularity of the lamina propria, mild blunting and fusion of villi and cystic enteritis in the jejunum of a 14-day-old brown/red broiler chick naturally infected with IBV.

Table 2. Detection of IBV antigen by IHC in organs of 14-day-old broilers with RSS.

Organ	Samples investigated	Samples positive
Duodenum	8	6
Jejunum	6	3
Rectum/cloaca	5	2
Proventriculus	5	2
Pancreas	8	2

DISCUSSION

RSS is a common disease in broilers, which can be caused by several different viruses. In this report, we describe the detection and isolation of a coronavirus from broiler chickens with RSS. Signs and gross lesions observed in the infected flock included lethargy, delayed and uneven growth, and pale and distended small intestines with watery contents, as well as cystic enteritis. These lesions are consistent with those described for RSS (26,42,46,50).

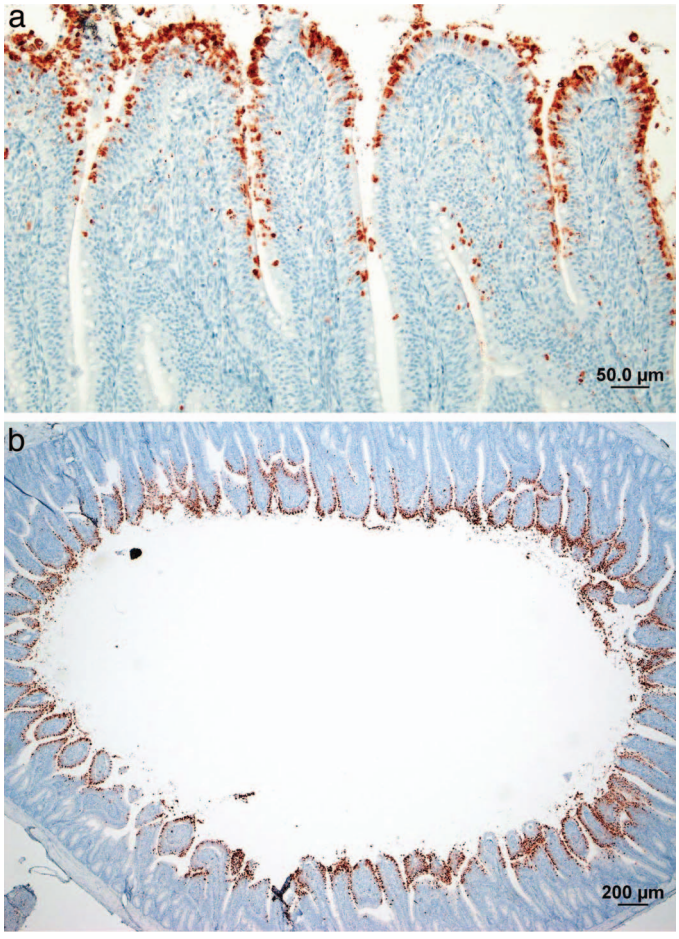

Fig. 3. Detection of IBV antigen by immunohistochemistry in the enterocytes at the tips of the villi of the jejunum of a 14-day-old brown/red broiler chick naturally infected with IBV. a. Shows higher magnification of the tips of the villi, b. shows distribution of IBV in the tips of the villi across the intestinal lumen.

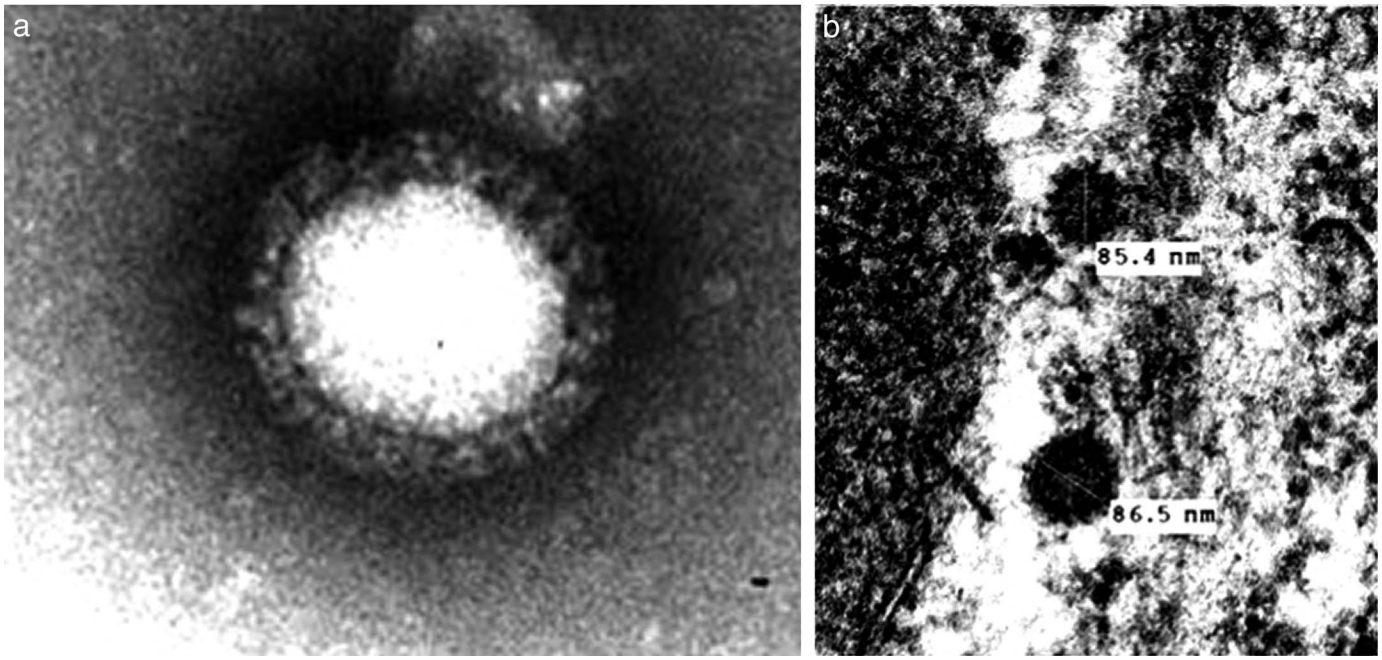

Fig. 4. (a) negative stain and (b) transmission electron micrograph of coronavirus found in the intestines of chickens with RSS; the virus measures 85–90 nm in diameter and has typical club-shaped surface projections; edited for contrast and brightness

Direct electron microscopy of the intestines showed rotaviruses and coronaviruses; however, only a coronavirus was isolated in embryonated chicken eggs. The standard method for isolation of avian rotaviruses is using cell cultures, and little is known about the replication of avian rotaviruses in embryonated chicken eggs (13), which explains why the rotavirus was not isolated.

IBV mainly infects the respiratory tract and the urogenital tract, but it has long been known that IBV can be isolated from the intestines or cloacal swabs and persists in the cecal tonsils. However, only very rarely has IBV been associated with lesions in the intestines (2,3,36). In contrast, turkey coronavirus (ToCV), which causes intestinal disease in turkeys, does not cause lesions in chickens after infection (21,24).

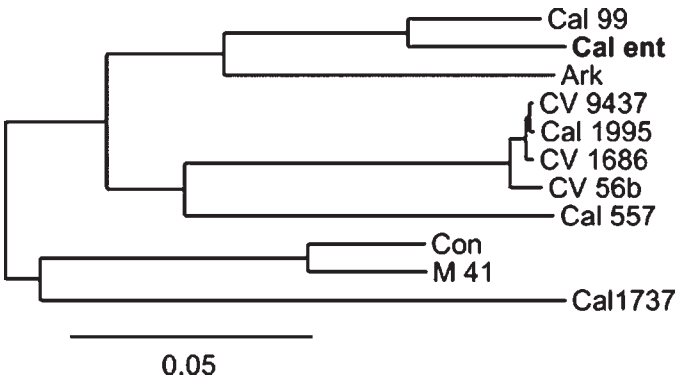

Fig. 5. Neighbor-joining tree without distance corrections of 1631 bases of the S1 gene of enteric IBV from California (Cal ent, accession no. KU311691) and IBV variants California 99 (Cal 99, accession no. AY514485.1), Arkansas (Ark, acc. no. GQ504720.1), CV 9437 (acc. no. AF027510.1), Cal 1995 (acc. no. FJ904714.1), CV 1686 (acc. no. AF027511.1), CV 56b (acc. no. AF027509.1), Cal 557 (acc. no. FJ904715.1), Connecticut (Con, acc. no. KF696629.1), Massachusetts (M 41, acc. no. GQ219712.1), and Cal 1737 (acc. no. EU925393.1).

Infection with our isolate reproduced lesions in the small intestines of the infected birds, suggesting a role of this coronavirus in the enteric pathology. Other authors report infection with other IBV-like isolates causing lesions only in the rectum (2,3). Interestingly, detection of IBV in the intestines by IHC was more sensitive than detection by RT-PCR, perhaps due to the presence of PCR inhibitors in the ingesta.

Additionally, lesions in the proventriculi similar to those caused by the transmittable viral proventriculitis virus were observed and IBV antigen was detected, while IBV has been isolated from proventriculi of experimentally infected chickens (33). On the other hand, it is reasonable that lesions in the proventriculus, regardless of the causative agent, contribute to RSS (39).

The experimental infection also showed that the isolate replicated in the upper respiratory tract causing lesions. This was in contrast to the observations in the field case, where no respiratory signs were present and no IBV antigen was detected in sections of trachea and conjunctiva. There are at least three possible reasons for this discrepancy: First, the inoculum was given into eyes and nostrils, and it is possible that an oral infection bypassing the upper respiratory tract might not have caused respiratory disease. Second, it

Table 3. Presence of lesions detected in tissue sections stained with H&E and IBV antigen detection by IHC at 2, 4, and 5 days after infection of SPF chickens with IBV isolated from the intestines of broilers with RSS.

DPI	Staining	Conjunctiva	Sinus/turbinate	Trachea	Intestines
2	H&E	5/5[A]	5/5	3/5	0/5
	IHC	1/2	2/2	0/2	0/5
4	H&E	4/4	4/5	5/5	3/5
	IHC	1/2	2/2	2/2	1/2
5	H&E	4/5	4/5	6/7	6/9
	IHC	1/2	2/2	2/2	6/9

[A]Positive samples/investigated samples.

Table 4. Detection of IBV RNA in tears, tracheas, duodena and cecal tonsils at 2, 4, and 5 dpi of SPF chickens with IBV isolated from the intestines of broilers with RSS.

dpi	Tears	Trachea	Duodenum	Cecal tonsils
2	6/6[A]	6/6	2/6	4/6
4	6/6	5/6[B]	1/6	4/6
5	6/6	4/6	0/6	3/6

[A]Positive samples/investigated samples.
[B]One sample yielded an unclear result.

is also possible that different genetics of the chicks in the field case and of the chicks used in the experimental infection are responsible for the differences in tropism of the virus. Genetics of chickens play an important role in the differences in the susceptibility and tropism of IBV (4). And third, in the field case IBV in the upper respiratory tract might have been missed, because no higher sensitivity test such as RT-PCR was used.

However, the detection of the isolate in the upper respiratory tract after experimental infection showed that the isolate did not lose its tissue tropism to respiratory organs. The additional tropism was also demonstrated by the isolation of the virus from the intestines of the embryo but not from the allantoic fluid or the CAM.

Coronaviruses such as ToCV and transmissible gastroenteritis virus (TGEV) of swine infect enterocytes. The S genes of ToCV and IBV have a sequence homology of less than 40%, while the rest of the genome has a homology between 85% and 90% (10,25,32). In swine, comparison of the genome of TGEV and porcine respiratory coronavirus (PRCoV) showed a homology of 96% with three deletions in the PRCoV genome, one of which is located in the N-terminus of the S protein (44). These comparisons corroborate that the S protein has an important role in determining tissue tropism (25,44). Sequencing of the S1 gene of the isolate in the present case did not show deletions or insertions in comparison to closely related IBV types, but it showed a distance to the rest of the genotypes that might explain the tissue tropism change.

The lesions of crypt dilation shown in the intestine in the present case resemble lesions caused by bovine coronavirus (18). It is interesting that bovine coronaviruses are also found in the respiratory tract and intestine and cause respiratory and enteric diseases in cattle (7). In contrast to bovine coronavirus, the crypts in the present case did not stain for IBV as demonstrated by IHC. The reason for this is not known. But the superficial villi of the small intestine in the present case were positive for IBV by IHC as expected since coronavirus tends to multiply in the mature enterocytes. Another interesting observation is that the lesions in the crypts of the chicks in the present study resemble lesions caused by parvoviruses of dogs and cats (41). Parvoviruses have a predilection for rapidly dividing immature cells like in the crypts. But the chicks in the present case were negative for parvovirus by EM as well as the inoculum used in the experimental trial by RT-PCR. Further the relationship between enteritis with lesions in the crypts in chickens and parvovirus has not been established. In general the pathogenesis of enteric lesions associated various viruses and RSS and in chickens is not known.

The isolate in the present case was clearly set apart from other IBV types that are used as vaccines in California like Massachusetts, Arkansas DPI and Connecticut, but also from California variants of IBV (35,37). The closest relative genotype was IBV California 99. This genetic and geographic proximity indicates that the isolate might have evolved from California 99, which is known to mutate more easily than IBV California 1737, the second most prevalent variant in California (19).

In conclusion, based on the information collected from the field case and during an experimental infection and characterization, the IBV strain isolated differs from other known IBV types genetically, based on the S1 gene sequence, and by its extended tissue tropism. Further studies such as full genome sequencing modification of the experimental protocol such as oral infection in broiler chicks are necessary to clarify the role of this virus in the development of intestinal lesions and the genetic bases of its differences in tissue tropism.

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