AVIAN DISEASES vol. 25 no. 4

Isolation of Avian Reovirus as a Possible Etiologic Agent of Osteoporosis ("Brittle Bone Disease"; "Femoral Head Necrosis") in Broiler Chickens

L. van der Heide

Department of Pathobiology
University of Connecticut
Storrs, Connecticut 06268

D. Lütticken

Intervet International Laboratories
Boxmeer, The Netherlands

and

M. Horzinek

Department of Veterinary Virology
Faculty of Veterinary Medicine
University of Utrecht
Utrecht, The Netherlands

Received 27 March 1981

SUMMARY

Avian reovirus was isolated from intestines of 3-to-7-day-old broiler chickens with enteritis from broiler houses where osteoporosis was a problem. The virus was purified in a cesium chloride gradient (buoyant density 1.37 gm/ml) and identified as a reovirus by electron microscopy. Specific-pathogen-free (SPF) chickens and commercial broiler chickens with anti-reovirus maternal antibodies inoculated at 1 day of age with the reovirus isolate developed lesions of femoral head fractures and/or osteoporosis; reovirus could be reisolated from the bone marrow and intestinal tracts of experimentally infected SPF birds.

The reovirus isolate, although isolated from intestines, induced development of tenosynovitis lesions in SPF and commercial broiler chickens.

INTRODUCTION

Since 1977, the broiler industry of Western Europe has been observing a condition in broiler chickens in which fracturing of femoral heads (Fig. 1) and a brittle bone structure (osteoporosis) are the main clinical features (1,2,8,9).

The incidence of diarrhea in the first 2 weeks of life and the appearance of smaller-than-normal chicks with malpositioned feathers on the wings (so-called "helicopter" birds) appear to be a feature of this condition (Fig. 2). The disease was thought to be infectious based on experimental infection of chickens with intestinal contents of affected birds (1). It was hypothesized that early enteritis caused by an infectious agent results in malabsorption of such necessary nutrients as calcium, phosphorus, vitamin D_3, vitamin A, and vitamin E, which would lead to osteoporosis. Those clinical signs have been observed in other countries also, including the United States, South American countries and Australia (5,10).

To determine the etiology of "brittle bone disease," virus isolations were attempted in The Netherlands at the Virology Institute of the University of Utrecht, Faculty of Veterinary Medicine. This report contains a characterization of an isolate as avian reovirus by electron microscopy and cesium chloride density gradients and attempts to reproduce the condition in specific-pathogen-free (SPF) and commercial broiler chickens.

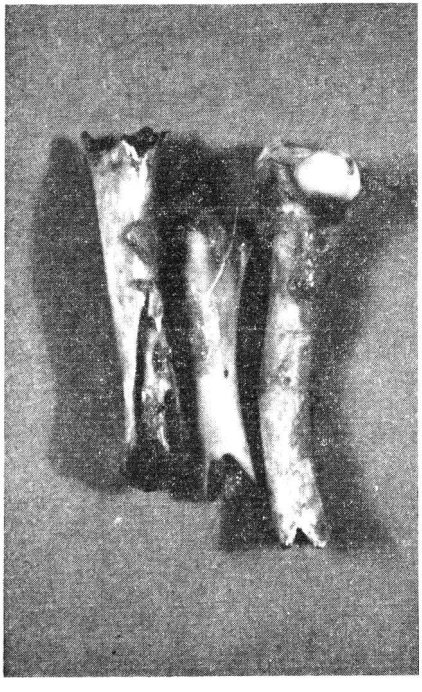

Fig. 1. Broken femur heads and brittle bone structure in chickens with "femoral head necrosis."

MATERIALS AND METHODS

Broiler chickens 3 and 7 days of age with clinical evidence of diarrhea were collected on a commercial broiler farm in the southern part of The Netherlands. Parts of the small intestinal tract of these chickens were pooled and ground in glass Ten Broeck grinders, and phosphate-buffered saline (PBS) was added to make a 10% suspension. After centrifugation of the suspension at 1,200 $\times$ *g* and 4,000 $\times$ *g* for 30 min each, the supernatant was mixed with penicillin (100 μg/ml) and streptomycin (50 mg/ml) and inoculated onto the chorioallantoic membranes (CAMs) of 15 ten-day embryonating SPF chicken eggs kindly supplied by Intervet International Laboratories, Boxmeer, The Netherlands. CAM tissues were harvested 3 to 4 days postinoculation, and 5 subsequent passages were made on the CAMs of 9-day embryonating SPF eggs.

Cesium chloride gradients. CAM material from the 5th chicken egg passage was ground in a glass Ten Broeck grinder, 3 to 4 ml PBS was added, and it was clarified by centrifugation at 1,900

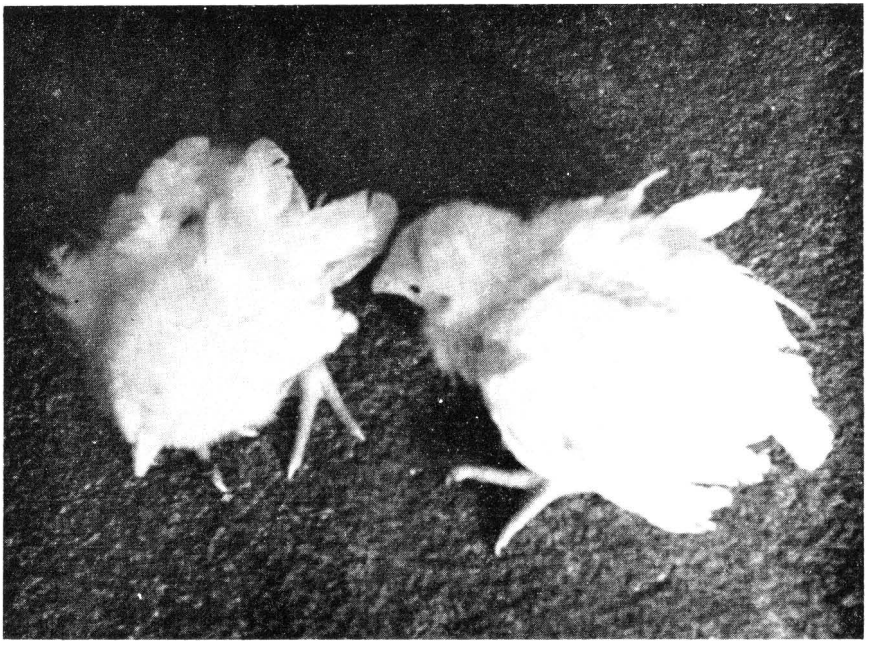

Fig. 2. Week-old broiler chickens with malpositioned feathers ("helicopter birds").

$\times$ g for 30 min. The supernatant was washed 3 times with ether. The aqueous phase was then centrifuged at 4,000 $\times$ g for 30 min. Cesium chloride gradients were prepared in plastic tubes by adding 2.5 ml saturated cesium chloride to 4.5 ml virus suspension, giving nD/25 = 1.3678 gm/ml. The cesium chloride gradient tubes were centrifuged at 41,000 rpm in a Beckman Ultracentrifuge, rotor 50.1, for 22 hr.

Cesum chloride gradient fractions, where bands appeared, were titrated on dropped CAMs of 9-day embryonating SPF chicken eggs, and titers were calculated as EID_{50} according to Reed and Muench (4).

Chickens and eggs. Day-old chicks in Expt. 1 were hatched from eggs of SPF chickens and lacked maternal antibodies to avian reovirus. Day-old chicks in Expts. 2 and 3 were broiler parent chicks from a commercial broiler breeder company and were demonstrated to have maternal antibodies to avian reovirus.

Eggs used for isolation of avian reovirus were from the SPF chicken flocks of Intervet International Laboratories, Boxmeer, The Netherlands.

Virus-neutralization tests. Five serum samples from day-old chicks of each experiment were pooled and examined for levels of anti-reovirus-neutralizing antibodies in a virus-neutralization (VN) test, using chicken embryo fibroblast (CEF) cultures in a microtiter system, as described previously (3).

Serial 10-fold dilutions of CEF-adapted S1133 strain reovirus (6) were mixed with equal amounts of serum, incubated 1 hr at 37 C on a shaker, and then inoculated in 0.2-ml amounts onto wells of CEF monolayers, 5 wells per virus dilution. Virus dilutions without serum added were treated in the same manner and inoculated in 0.1-ml amounts onto the CEF monolayers, 5 wells per dilution.

The cultures were assayed for cytopathic effect (CPE) 5 days postinfection. Fifty-percent endpoints were calculated according to the method of Reed and Muench (4), and the neutralization index (NI) was determined as the difference between the negative reciprocals of the 10 log titers of virus and virus-serum mixtures.

The virus isolate and its antiserum were tested in a VN test against standard S1133 strain antiserum and virus, respectively.

Electron microscopy. Fractions of banded material from cesium chloride gradients were mixed with a solution of 2% phosphotungstic acid (PTA) at pH 6.2 with KOH containing 0.01% bacitracin. They were observed with a JEOL 100C electron microscope

(Dr. D. Ellens, Central Veterinary Institute, Lelystad, The Netherlands).

Infection experiments. *Expt. 1.* Thirty SPF chicks were inoculated at 1 day of age orally and 30 were inoculated subcutaneously with 0.1 ml of a suspension of CAM material of the 15th chicken embryo passage of the viral isolate (approximately $10^{3.0}$ ELD_{50}/chicken). Twenty-one chicks were examined at 1 day of age for the presence of maternal anti-reovirus antibodies by agar-gel precipitation (AGP) and VN tests. Thirty-seven chicks were kept as uninoculated controls. Infected and control chickens were kept in separate brooder cages in an isolation room. At 38 days of age (37 days postinoculation) all chickens were bled and killed. Blood serum samples were individually examined by AGP tests for the presence of anti-reovirus antibodies.

The condition of the femur heads was examined by lateral deflection of both legs of each chicken. Bone marrow and intestinal tract tissue from several infected chickens with signs of femoral head fracture were ground in glass Ten Broeck grinders and inoculated onto CAMs of 9-day embryonating SPF chicken eggs.

Tarso-metatarsal tissues were fixed in 10% Formalin-saline and embedded in paraffin. Cross-sections 5 μm thick were cut from digital flexor tendons. Sections were stained with hematoxylin and eosin and examined by light microscopy for the presence of tenosynovitis lesions.

Expts. 2 and 3. In Expts. 2 and 3, 30 one-day-old commercial broiler chicks per experiment were inoculated subcutaneously with approximately $10^{5.7}$ and $10^{6.7}$ ELD_{50} per chicken, respectively, of CAM suspension of the 8th and 10th chicken embryo passage of the viral isolate. Blood serum samples from approximately 20 chicks per experiment were tested by both AGP and VN testing for the presence of maternal anti-reovirus antibodies. Thirty chickens per experiment were kept uninoculated as negative contro's. At 5 weeks of age, all chickens were bled and killed, and blood serum and legs were examined as described above. Cross-sections of the digital flexor tendons were treated as described in Expt. 1.

RESULTS

Three to 4 days after embryonating SPF eggs were inoculated with clarified intestinal suspensions, the CAMs had edema and gray thickening. In subsequent passages, the lesions became more pronounced, and embryo death at 3 days postinoculation was observed beginning with the first CAM passage.

Table 1. Development of AGP antibodies and gross and microscopic lesions in SPF and commercial broiler chickens after experimental infection at 1 day of age with an avian reovirus isolate.

Expt.	Chickens	Status	Route of inoculation	AGP test at 1 day	VN test 1 day	AGP test at 5 weeks	No. of chickens with femoral head lesions and/or osteoporosis	Microscopic lesions of tenosynovitis
1	SPF chickens	Inoculated	Subcutaneous			23/23	1/23	41/45 (91.0%)
			Oral			28/28	6/28	36/51 (70.5%)
		Control	Uninoculated	0/21	N1 = 1.0	0/34	0/34	0/66 (0.0%)
2	Commercial broilers	Inoculated	Subcutaneous			12/27	3/27	30/54 (55.5%)
		Control	Uninoculated	13/13	N1 = 3.1	4/25	0/27	4/49 (8.2%)
3	Commercial broilers	Inoculated	Subcutaneous			26/26	0/26	42/52 (80.7%)
		Control	Uninoculated	12/26	N1 = 2.3	7/20	0/20	0/40 (0.0%)

Cesium chloride density gradients. Four bands appeared in the cesium chloride gradients at densities of 1.361, 1.363, 1.365, and 1.37 gm/ml.

Electron microscopy. No virions were observed in the three fractions of the cesium chloride gradient containing material from the bands with densities of 1.361, 1.363, and 1.365 gm/ml. However, fractions 8 and 9 from the band with a density of 1.37 gm/ml contained a pure population of virus particles 70 nm in diameter with reovirus morphology (Fig. 3). Fractions 8 and 9 had a virus titer of $10^{6.5}$ EID_{50}/ml and $10^{5.4}$ EID_{50}/ml, respectively.

Virus-neutralization tests. Table 1 gives the results of VN tests of the day-old chick serum samples. SPF chickens had no maternal antibodies against reovirus at one day old (NI = 1.0), and maternally immune chickens had NI values of 3.1 and 2.3 in Expts. 2 and 3, respectively. The viral isolate was neutralized by anti-S1133 antiserum (NI = 3.6); standard S1133 strain reovirus was neutralized by antiserum against the virus isolate (NI = 3.7).

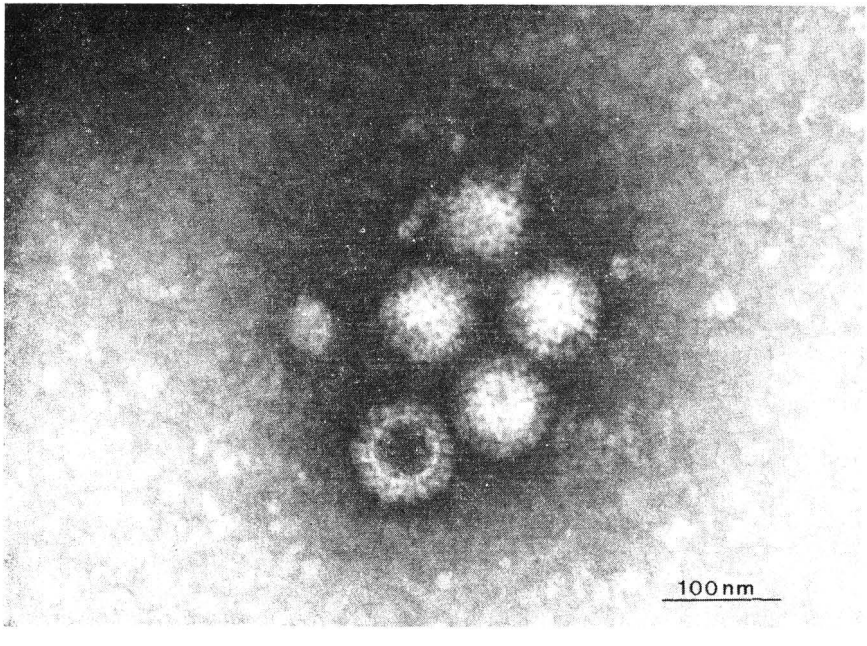

Fig. 3. Electron micrograph of reovirus particles in CsCl band with density of 1.37 gm/ml.

Infection experiments. *Expt. 1.* Six of 28 SPF chickens orally infected with the virus isolate at 1 day of age developed femoral head fractures and/or osteoporosis, whereas none of the uninoculated control chickens developed such lesions (Table 1). In the group of subcutaneously infected SPF chickens, only one chicken developed a femoral head fracture. Reovirus could be reisolated from bone marrow and intestinal tracts of chickens with lesions.

Feathering abnormalities were observed in two infected chickens.

Typical microscopic lesions of tenosynovitis as described (7) were found in 77 of 96 cross-sections of digital flexor tendons from orally and subcutaneously inoculated chickens, whereas none were found in the uninoculated control chickens (Table 1).

No clinical signs of diarrhea were observed.

Seven subcutaneously infected, 2 orally infected, and 3 uninfected control chickens died during the 4-week experimental period. However, no gross or microscopic lesions of reovirus infection were found in the livers or hearts, and deaths were considered to be from nonspecific causes.

Expt. 2. In broiler chickens with maternal antibodies against reovirus at 1 day of age, femur head fractures and/or osteoporosis developed in 3 of 7 infected chickens at 5 weeks of age but not in the uninfected control chickens.

Microscopic lesions of tenosynovitis were observed in 30 of 54 (55.5%) cross-sections from infected chickens and in 4 of 49 (8.2%) from uninfected control chickens.

Three infected and 3 control chickens died from nonspecific causes; microscopic examination of their heart and liver tissues revealed no lesions associated with reovirus infection. No clinical signs of diarrhea were observed.

Expt. 3. None of the infected or control chickens developed femur head lesions or osteoporosis. However, 42 of 52 metatarsal cross-sections (80.7%) from infected chickens had microscopic tenosynovitis lesions; none of the uninfected control chickens developed such lesions.

The average weight of 26 infected chickens was 297 grams, whereas the average of 20 control birds was 346 grams. Diarrhea was observed in several inoculated birds but not in the controls at 4 weeks of age. The presence of enteritis could not be confirmed microscopically.

DISCUSSION

The infectious nature of osteoporosis/brittle bone disease in broiler chickens in the field was corroborated in the laboratory by the isolation of an avian reovirus from the intestines of chickens with clinical diarrhea, a condition that usually preceded the osteoporotic condition. Furthermore, femoral head fractures and osteoporosis could be reproduced with the avian reovirus isolate, although the incidence was low.

The data suggest that malabsorption due to virus-induced enteritis may be a major cause of some observed cases of osteoporosis ("femoral head necrosis"; "brittle bone disease"). The incidence of vitamin E deficiency (pale bird syndrome), encephalomalacia, and other signs of vitamin deficiencies often observed in affected broiler chickens might also be due to the aforementioned malabsorption syndrome, as these clinical signs are often observed at the same time as "femoral head necrosis"/osteoporosis.

Some horizontal transmission of reovirus to uninoculated control birds did occur in Expts. 2 and 3, as indicated by development of AGP antibodies in a few birds in Expts. 2 and 3 and microscopic tenosynovitis lesions in a few birds in Expt. 2.

A reovirus isolate from the intestinal tract of young chickens with diarrhea was capable of inducing lesions of tenosynovitis and femoral head fractures and osteoporosis, thereby indicating that avian reovirus is a possible etiologic agent of "femoral head necrosis" and "brittle bone disease," although presumably indirectly by causing enteritis and malabsorption of essential nutrients as primary events.

The virus isolate appeared serologically related or even identical to standard Connecticut strain S1133 avian reovirus.

Although the reovirus isolate was isolated from intestinal contents of chickens with diarrhea that developed femoral head necrosis and/or osteoporosis, it was capable of inducing typical lesions of tenosynovitis. This observation is again an indication that avian reoviruses are not limited in their lesion development ("enteric," "respiratory," "tenosynovitis," etc.), but that a given reovirus isolate is capable of inducing several or all of the manifestations that have been described for avian reovirus infections.

REFERENCES

1. Kouwenhoven, B. Osteoporosis in broilers. Bull. Pluimvee Gezondheidszorg. November 1978.

2. Meens, M. H. C. C., and J. B. Litjens. Degeneration of the head of the femur in poultry. Tijdschr. Diergeneeskd. 103:907-909. 1978.

3. Rau, W. E., L. van der Heide, M. Kalbac, and T. Girshick. Onset of progeny immunity against viral arthritis/tenosynovitis after experimental vaccination of parent breeder chickens and cross-immunity against six reovirus isolate. Avian Dis. 24:648-657. 1980.

4. Reed, L. J., and H. Muench. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 28:493-497. 1938.

5. van der Heide, L. Controlling leg problems in the poultry meat industry. Proc. New Hampshire Poult. Health Conf. 1980.

6. van der Heide, L., and M. Kalbac. Infectious tenosynovitis (viral arthritis): characterization of a Connecticut viral isolant as a reovirus and evidence of viral egg transmission by reovirus-infected broiler breeders. Avian Dis. 19:683-688. 1975.

7. van der Heide, L., J. Geissler, and E. S. Bryant. Infectious tenosynovitis: serologic and histopathologic response after experimental infection with a Connecticut isolate. Avian Dis. 18:289-296. 1974.

8. Vertommen, M., J. H. H. van Eck, B. Kouwenhoven, and Nel van Kol. Infectious stunting and leg weakness in broilers. 1. Pathology and biochemical changes in blood plasma. Avian Pathol. 9:133-142. 1980.

9. Voeten, A. A., and A. A. van der Leest. Rickets-like changes in broiler chickens. Tijdschr. Diergeneeskd. 104:215-220. 1979.

10. Williams, W. P. Viral arthritis with a different twist. Poultry International, pp. 32-35. February 1979.