Turkey Rhinotracheitis Virus Isolated from Broiler Chicken with Swollen Head Syndrome in Japan

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ABSTRACT. Turkey rhinotracheitis (TRT) virus was first isolated from a commercial broiler chicken with swollen head syndrome (SHS) in Japan. At the same time, Newcastle disease virus (NDV), infectious bronchitis virus (IBV), avian reovirus (ARV), *Escherichia coli* (*E. coli*), *Morganella morganii*, and *Proteus mirabilis* were also isolated from the same broiler chicken. The presence of antibodies to TRT virus was confirmed in the sera of 34-day-old chickens of the flock with SHS, however the antibodies to TRT virus were undetectable in the sera of 17-day-old chickens. In this investigation, we confirmed avian pneumovirus infection in chickens in Japan, and the virus and other agents may be considered as a cause of SHS.—**KEY WORDS:** avian pneumovirus, SHS, TRT virus.

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The Pneumovirus, turkey rhinotracheitis (TRT) virus is considered to a factor in swollen head syndrome (SHS) in chickens [12, 15]. This virus has been isolated from chickens with SHS in France [12], South Africa [1], England [6], and Taiwan [8].

In Japan, SHS was first observed in Hyogo prefecture in 1989 [13], but its causative agents have not been identified in the chickens with SHS.

This paper describes first isolation and properties of the TRT and other viruses, as well as bacteria from a commercial broiler chicken with SHS in Japan.

An outbreak of SHS occurred in a commercial broiler farm in Miyazaki prefecture during the spring of 1994. Four to six-weeks-old broilers were affected with the syndrome, which initially began as snicking, conjunctivitis, swelling of the lachrymal gland, swelling around the eyes, over the head into the submandibular region, and subcutaneous oedema of the head followed. Chickens showing respiratory signs and stunting were also found in this flock.

To isolate viruses, 20% suspensions of pooled and homogenized tissues or organs were prepared from nasal secretions/exudate and eyelids (N & E), as well as the trachea and lung (T & L) of 17 and 34-day old live chickens, respectively. Antibiotics were added to the samples, which were filtered through a 450 nm membrane (Millipore, U.S.A.). Virus infection was examined in the filtrate by inoculation into the chorioallantoic cavity (CAC) and membrane (CAM) of 9-day-old specific pathogen free (SPF) chicken embryonated eggs obtained from a White Leghorn flock (Kyoto Biken, Japan) maintained in our laboratory [16]. Cell cultures of chick embryo fibroblast (CEF) and Vero cells were also inoculated. All samples were passaged twice. These and samples of other organs, heart, liver, spleen, and kidney, were tested for bacteriological infection.

Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) were isolated from chicken Nos. 1, 2 and 10 samples of N & E, and T & L in chicken eggs and CEF cell cultures, and only NDV was isolated from chicken No. 8 samples of N & E in CEF cell cultures. NDV was identified by the neutralization test using a hyperimmune antiserum to

the NDV-B1 strain. NDV is a lentogenic strain with a 120 hr survival in the chick embryo after chorioallantoic inoculation. IBV was identified by agar gel precipitation using a hyperimmune antiserum to the IBV-M41 strain and by the reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers [7] for amplifying the IBV-S1 gene. Avian reovirus (ARV) was isolated from chicken No.10 samples of T & L in chicken eggs and CEF cell cultures. ARV was identified by agar gel precipitation using a hyperimmune antiserum to the ARV-Uchida strain and by electron microscopy. The positive samples for virus isolation in embryonated eggs and CEF cell cultures, the samples were inoculated into Vero cells after neutralization with hyperimmune antisera against NDV, IBV and ARV. A non-neutralized agent was isolated from chicken No. 10 samples of T & L in Vero cell cultures (Table 1).

Escherichia coli (*E. coli*), *Morganella morganii*, and *Proteus mirabilis* were isolated from the same samples and other organs of affected chickens. Enterobacteria were identified using Bio test No.1 (Eiken, Japan).

The isolate designated strain 8597/CV94 produced characteristic cytopathic effects (CPE) upon syncytia after the second passage, six days after inoculation. The strain cloned 3 times by limiting dilution. Culture fluids lacked haemagglutination (HA) activities against chicken and goose erythrocytes. Viruses were characterized according to the following criteria: contains RNA, sensitive to lipid solvents, stable at pH 3.0 to 9.0, inactivates at 56°C after 30 min.

For electron microscopy, the culture fluid was separated by centrifugation at 5,000 × g for 20 min, and the supernatant was centrifuged at 100,000 × g for 90 min (Beckman XL-80, U.S.A.). The resulting pellet was resuspended in 0.01 M phosphate buffered saline and examined by negative stain electron microscopy (JEOL JEM100S, Japan) using 4% uranyl acetate. Electron micrographs of the virus revealed pleomorphic particles that were mostly spherical, 130–200 nm in diameter with spaced surface projections of about 13 nm (Fig. 1).

Spherical and filamentous shaped virions were similar in size and morphology to virus associated with TRT [2, 3, 12,

Table 1. Isolation of virus from broiler chickens with SHS

Chicken No.	Age in days	Inoculum	Route of inoculation to embryonated eggs										Cell culture			
			CAC				CAM						CEF		Vero	
			1 ^{a)}		2		1		2				1	2	1	2
			HA	Embryo	HA	Embryo	HA	Embryo	CAM	HA	Embryo	CAM	CPE		CPE	
1 ^{b)}	17	N&E	—	—	+	+	—	—	—	+	+	+	—	+	—	—
		T&L	—	+	+	+	—	—	—	+	+	+	—	+	—	—
2 ^{b)}	17	N&E	—	+	+	+	—	—	—	+	+	+	+	+	—	—
		T&L	—	—	—	—	—	—	—	+	+	+	+	+	—	—
3	17	N&E	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	17	N&E	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	34	N&E	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	34	N&E	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	34	N&E	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8 ^{b)}	34	N&E	—	—	—	—	—	—	—	—	—	—	—	+	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	34	N&E	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10 ^{b)}	34	N&E	+	+	+	+	+	+	+	+	+	+	+	+	—	—
		T&L	—	+	—	+	—	+	+	—	+	+	+	+	—	+
11	34	N&E	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		T&L	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Abbreviation: N&E; nasal secretions & eyelids T&L; trachea & lung CAC; chorioallantoic cavity CAM; chorioallantoic membrane CEF; chick embryo fibroblast HA; haemagglutination CPE; cytopathic effect

The symbols, + and —, represent positive or negative for HA, death or stunting of the embryo, CAM lesion and production of CPE.

a) Passage number.

b) NDV and IBV were isolated from specimens from chickens 1 and 2, and only NDV was isolated from chicken 8. In addition to these viruses, ARV and TRT virus were isolated from chicken 10. The TRT virus was isolated from specimens inoculated into Vero cells. The antibody to TRT virus was undetectable in the sera of chicken 10.

14]. These biological, physico-chemical and morphological features suggested that this virus belongs to the Pneumovirus genus of the family Paramyxoviridae.

The identity of the agent of TRT virus was confirmed by the neutralization test using a hyperimmune turkey antiserum, by indirect immunofluorescence staining of Vero cells four days after inoculation using anti-turkey IgG FITC-labelled antibody (Nordic Immunological Laboratories, Netherlands), and by the RT-PCR using specific primers (oligo PCRF, 1st and oligo F.GRH) [5] for amplifying the TRT-F gene.

Four 30-day-old SPF chickens were inoculated intraocularly with 0.1 ml of culture fluid containing about $10^{5.0}$ TCID₅₀ of the virus. No clinical signs of SHS were evident in these chickens for 2 weeks thereafter. However antibodies to TRT virus in all chickens appeared 4 weeks after inoculation.

Although the presence of antibodies to TRT virus was confirmed by the neutralization test in the sera of 34-day-old chickens of the flock with SHS, antibodies to TRT virus were undetectable in the sera of 17-day-old chickens, is suggesting that the virus spread through the flock at this time.

In other flocks, the seronegative 14-day-old broilers were affected with SHS at 3 to 4 weeks of age. They were converted positive for antibody to TRT at 29 to 34 days of age.

Due to the fastidious nature of TRT virus, the virus was isolated from tracheal organ cultures of the chicken or turkey embryo [3, 6, 8, 12]. The virus adapted to organ cultures replicated in Vero cells with characteristic syncytium formation [3, 8]. However, we directly isolated the virus with Vero cells. The reason for this success may be use of cloned Vero cells which are highly sensitive to bovine respiratory syncytial virus of the same genus as the family Paramyxoviridae.

SHS virus is associated with NDV, IBV [9], laryngotracheitis virus [6], adenovirus [9], reovirus [4] and infectious bursal disease virus [8, 9]. *E. coli* [4, 6, 8–11] is frequently isolated from SHS-affected chickens. We isolated other pathogens similar to the reported agents. TRT virus has been thought to be primary agents, but secondary agents such as *E. coli* and other viruses have been considered as relative factors in the development of the syndrome.

In this investigation, we confirmed avian pneumovirus

Fig. 1. Electron micrograph of isolate 8597/CV94 derived from infected Vero cell culture. The ultracentrifuged pellet was negatively stained with 4% uranyl acetate. The spherical virions ranged from 130 to 200 nm in diameter, and were covered in the surface projections about 13 nm long.

infection in Japan. This virus may play a significant role in the production of SHS, although the relationship remains unclear between the virus and other pathogens in production of the disease. Further studies on the pathogenicity of this virus and other pathogens in broiler and SPF chickens are needed.

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