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Full Length Research Paper

Avian influenza virus H_5N_1 subtype A diagnosed in sick and dead wild and domestic birds in Pavlodar oblast, Republic of Kazakhstan

Mamadaliyev S. M. [1], Koshemetov Z. K.[1], Matveyeva V.M.[1], Kydyrbayev Z. K.[2]

[1]Research Institute for Biological Safety Problems, Gvardeiskiy, Republic of Kazakhstan.
[2]Institute of Microbiology and Virology ME&S RK, Almaty, Republic of Kazakhstan.

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This work describes laboratory tests conducted on biological material taken from sick and dead birds from unknown infection in the Pavlodar oblast, Republic of Kazakhstan. In the course of pathological material laboratory tests electron microscopy revealed virion of type A avian influenza virus. Type A AI virus common protein is recognized with the help of "Directigen Flu A" test system. The results of HAIT showed hemagglutinin agent H5, and NAIT results showed neuraminidase agent N1. On the basis of laboratory tests results it was determined that the cause of sickness and death of birds in the territory of Pavlodar oblast Irtysh region farm "NAN" was H5N1 type A AI virus.

Key words: HAT, HAIT, NAIT, H5N1 type A avian influenza.

INTRODUCTION

Epizootic situation of the last decade on the territory of CIS was marked by outbreaks of such especially dangerous virus infections as rinderpest, PPR, sheep-pox, Aujeszky's disease and avian influenza. The reasons were uncontrolled movement of agricultural animals and their products through customs as well as migration of wild fauna (Ayanot, 1996; Mamadaliyev et al., 2000; Saryglar et al., 2000; Assanov and Mussina, 2002). The above mentioned circumstances stipulate the necessity of fur - ther improvement on laboratory diagnostic methods, and demand for conducting purposeful prophylactic and monitoring studies.

Avian influenza has significant place among all dangerous viral infections. Avian influenza is an exceptionally dangerous infection. At the present time epizooties of this disease cover many countries of Asian, American, European and African continents. Avian influenza was found in Great Britain and Italy. There is information that during outbreak of this disease in Mexico, 17 millions of poultry the cost of which was 64.0 mln USD was dead or killed.

First human case of avian influenza was reported from Hong Kong in 1997 when strain H5N1 caused infection in 18 persons, 6 of which died. 1.5 mln of chickens were killed to annihilate the source of virus. In 1999 in Hong Kong two children were infected by avian influenza virus type A strain H9N2. Epidemic caused by H5N1 strain started in 2003 and covered nine Asian countries: Vietnam, Cambodia, China, Indonesia, Lao, Pakistan, Republic of Korea, Thailand, Taiwan and Japan. According to WHO data since 2003 almost 100 persons became victims of avian influenza virus (more than 50% died).

*Corresponding author. E-mail: mkopochenya@srai.kz

Abbreviations: HAT: Hemagglutination Test; HAIT: Hemagglutination Inhibition Test, NAIT: Neuraminidase Inhibition Test, PPR; Peste des Petits Ruminants; WHO: World Health Organization; RIBSP: Research Institute for Biological Safety Problems; ME&S: Ministry of Education and Science; RK: The Republic of Kazakhstan; CIS: Commonwealth of Independent States; ECE: Embryonating Chicken Egg.

In 2003 there were two cases of AI virus strain H5N1 infection in a family which came to Hong Kong from China. In 2003 in Netherlands during AI outbreak there were two cases of AI virus strain H7N7 infection among poultry farm staff and their families. Virus was detected in 86 persons taking care about infected poultry and in 3 persons among the members of their families. In the same year in Hong Kong, virus strain H9N2 was detected in a child. In 2004–2005, 37 persons from Vietnam, China and Thailand were infected by AI virus strain H5N1.

At 1st of August 2005, 67 cases of AI infection have been registered all over the world (60 cases in Vietnam, 4 in Cambodia, 3 in Indonesia). Fatal outcomes averaged 25 cases (37.7%) (Litvinova et al., 1997; Gendon, 1998; Slepushkin et al., 1998; Mamadaliyev et al., 2000).

AI epizooty did not pass by our country. During the period from 22, August till 17, September 2005 7 pestholes of AI among birds were registered in Pavlodar, Akmolinsk, Karaganda and North-Kazakhstan oblasts. During 25 days the disease spread all over northern and central parts of North-Kazakhstan. Human AI cases were not registered in Kazakhstan. At the instance of Veterinary Department of the Ministry of Agriculture of the Republic of Kazakhstan RIBSP staff was sent to Pavlodar oblast aiming at samples collection from sick and dead domestic and wild birds and further laboratory study of the samples.

In this paper we report results of a study aimed at elucidating cause of mortality and morbidity of wild and domestic birds in farms of Pavlodar oblast, the Republic of Kazakhstan.

MATERIALS AND METHODS

Studied samples

List of pathology material taken from poultry and wild birds in the farm "NAN" in Irtysh region in Pavlodar oblast:

1. No.1 domestic goose samples – brain, lungs, trachea, liver.
2. No.2 wild duck samples – brain, lungs, trachea, liver, intestine, proventriculus.
3. No.3 domestic goose samples – brain, liver, heart, lungs, trachea.
4. No.4 domestic goose samples – lungs, trachea, and liver.
5. Sera of sick and relatively healthy birds – 38 samples.

Samples No.1 and No.2 were delivered to RIBSP in liquid nitrogen at the temperature – 196 ^{0} C. Samples No.3 and No.4 were delivered to RIBSP 50% glycerin solution. Sera from sick birds were delivered in thermal box with refrigerating medium.

Materials used

- Haemagglutinating specific sera used for AI virus type A subtypes $_{1-15}$ diagnostic in HAIT;
- Type A "N_1-N_9" strain neuraminidase specific sera to AI virus for Neuraminidase Activity Inhibition Test (NAIT);
- Test - system «Directigen Flu A» for AI virus type detection. The set is produced in USA;
- 10–11-days-old embryonating eggs.

Works were conducted following the rules of veterinary and sanitary regime developed in RIBSP. Samples preparations for viral research and test – setting were conducted in category III class biosafety cabinet.

Pathology material preparation for virus research

Samples (brain, trachea, lungs, intestine, spleen, liver, and kidney) were grinded with concentrated antibiotics in sterile physiological solution in mortar and then 20% suspension was prepared. Obtained suspensions were centrifuged at 3000 – 4000 revolutions per minute for 30 minutes. Supernatant liquid was used for cell culture and embryonating eggs infection, bioassay conduction, electron-microscopic study and performance of serological reaction.

Test-systems for detection of virus, virus antigens and antibodies to virus

1. To detect haemagglutinating agents in homogenates of dead poultry organs we conducted haemagglutination reaction with 0.75% hen erythrocytes.
2. We used « $_5N_3$», « $_7N_1$», «BOR-73» and VGNKI Newcastle disease strains for conducting haemagglutination inhibition test (HAIT). Here we aimed at the detection of antibodies to AI viruses in sera of sick poultry. Before conducting haemagglutination inhibition reaction poultry sera delivered from Pavlodar oblast were treated with receptor destroying enzyme in ratio 1:4 for 18 h at 37°C in thermostat. Then samples were inactivated at 56°C for 30 min. After inactivation sera were diluted with salt solution in proportion 1:10.
3. Test – system "Directigen Flu A+B" was used to detect general AI virus type A and B antigen protein from 20% suspension prepared from pathologic material.
4. Extraction of haemagglutinating agent from embryonating chicken eggs. Three 10-days-old embryonating chicken eggs were infected with 0.2 sm^2 20% organ suspensions in allantoic cavity.
5. Electron microscopy. Pathologic material samples taken from sick poultry from Pavlodar oblast were clarified by low-speed centrifuging and concentrated by ultracentrifugation. Obtained samples were contrasted by phosphotungstic acid, applied to a grid and examined in electronic microscope for presence of virus particles.
6. Neuraminidase agent detection in studied samples was conducted in Neuraminidase Activity Inhibition Test (NAIT) according commonly accepted methodic with neuraminidase sera N_1-N_9.

Laboratory results

20% suspensions were prepared from all organ- tissue material delivered from the farm "NAN" in Irtysh region in Pavlodar oblast. We used also birds' sera taken from the whole flock which was the nidus of epizooty of unknown infection with common characteristics. Organ- tissue materials were used for haemagglutinating agent detection with the help of haemagglutination reaction. The results are presented in Table 1.

Table 1 show that 20% suspension from intestine sample taken from wild duck has haemagglutinating agent with activity in HAT in whole. The rest samples had negative results. Furthermore this organ was tested on AI virus antigen presence. For this test we used test-system "Directigen Flu A" which detects general AI type A protein. As the result of "Directigen Flu A" application we got positive result on AI virus type A.

Aiming at increase of virus activity we used samples taken from poultry as antigen for 10-days-old embryonating chicken eggs' infection. Table 2 presents the list of tested materials applied for infection.

Table 1. Hemagglutination test results on the detection of virus of unknown etiology in pathologic material taken from dead poultry.

No.	Tested samples	HAT Results
1	Brain	-
2	Lungs + trachea	-
3	Liver	-
4	Brain	-
5	Lungs + trachea	-
6	Liver	-
7	Intestine	In the whole
8	Proventriculus	-
9	Brain	-
10	Liver	-
11	Heart	-
12	Lungs + trachea	-
13	Lungs + trachea	-
14	Liver	-

Note: "-"– negative result

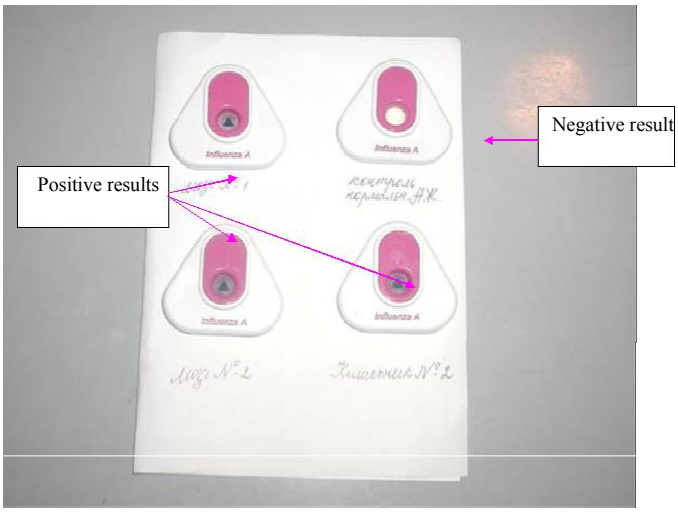

Figure 1. "Directigen Flu A" conduction results.

After 10-days -old embryonating chicken eggs were infected by 20% pathologic material suspension allantoic fluid samples were gathered beginning with 12 hours after embryos death. Obtained materials of allantoic fluid were tested on haemagglutinating agent presence in HAT. The results are shown in Table 3.

Table 3 shows that after we infected allantoic fluid by pathologic material taken from dead poultry it showed the presence of virus - haemagglutinating agent with activity in HAT in whole and till 1:64. The most active samples in HAT are brain No. 1, brain No. 2, and intestine No. 2. These samples were assayed in test-system "Directigen Flu A" to identify the agent. Study results are presented on the Figure 1.

Conduction of test- system "Directigen Flu A" revealed AI virus type A protein antigen in virus containing allantoic fluid infected with 20% suspension of domestic goose brain No.1 and wild duck Brain No.2 and intestine No.2.

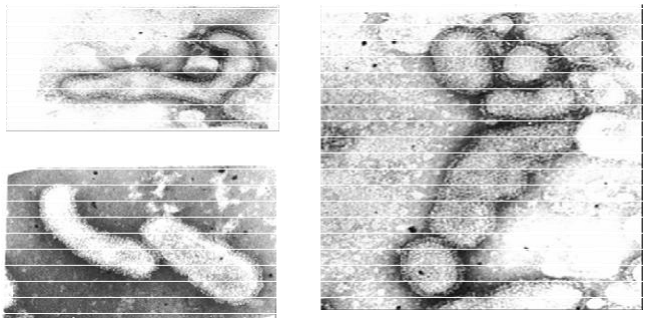

Figure 2. Electron-microscopy of pathology material samples (magnification 200000, RIBSP, RK ME&S)

Results of Haemagglutination inhibition test (HAIT)

Different specific sera of AI virus type A subtypes were used (Table 4). For performing the study we took samples of embryonating chicken eggs' allantoic fluid infected with 20% suspension of domestic goose brain No.1 and wild duck brain No.2. HAIT results on AI virus type A subtypes detection are presented in Table 4.

According to results in Table 4, isolate extracted with the help of haemagglutination agent belongs to subtype H_5 because tested samples produced positive results with such specific sera as $_5N_1$, $_5N_1$, $_5N_2$, $_5N_2$, $_5N_3$, $_5N_9$ with activity 1:20 – 1:320. Samples tested with other AI virus subtypes such as $_{1-4}$, $_{6-16}$ showed negative results.

We performed NAIT aiming at the detection of AI virus type A subtype H_5 neuraminidase agent type. To perform NAIT we took samples of embryonating chicken eggs allantoic fluid infected with 20% suspension of domestic goose brain No.1 and wild duck brain No.2. Test results are presented in Table 5.

The results presented in Table 5 show that tested samples contain neuraminidase N_1 with activity in NAIT 1:100 in two cases. The other samples produced negative results.

On the basis of laboratory tests data we may conclude that the cause of infection and death of wild and domestic birds in the farm "NAN" in Irtysh region of Pavlodar oblast is AI virus type A subtype H_5N_1.

Electron microscopy results

Pathologic material samples taken from sick poultry from Pavlodar oblast were clarified by low-speed centrifuging and concentrated by ultracentrifugation. Obtained samples were contrasted by phosphotungstic acid, applied to a grid and examined in electronic microscope for presence of virus particles. Results of electron microscopy reveled that samples of pathology material contain AI virus type A 5N1 (Figure 2) . Antibody detection against AI and Newcastle disease virusesWe used sera taken from sick and relatively healthy poultry from "NAN" in Irtysh region of Pavlodar oblast in HAIT to detect presence of antibodies to AI and Newcastle disease viruses. Results are presented in Table 6.

Table 6 shows that AI virus strain $_5N_3$ antibodies are detected in 7 of 38 tested samples th activity in HAIT 1:20 – 1:320. Newcastle disease antibodies are detected in 4 samples ctivi HAIT 1:20. Antibodies specific for AI virus strain 7N1 we t de ted in sera of sick poultry.

DISCUSSION

The scientists of RIBSP together with the Microbiology and Virology Institute staff took samples from birds died

Table 2. Materials used for embryos infection

No.	Samples taken from sick poultry	Materials used for infection of embryos allantoic fluid	Material for laboratory test-systems
1	Domestic goose	Lungs+trachea No.1	Embryos allantoic fluid
2		Liver No.1	
3		Brain No.1	
4	Wild duck	Brain No.2	
5		Lungs+trachea No.2	
6		Intestine No.2	
7	Domestic goose	Lungs+trachea No.3	
8	Domestic goose	Lungs+trachea No.4	

Table 3. HAT results on the detection of haemagglutinating agent of unknown etiology in allantoic fluid.

No.	Tested samples	HAT Results
1	Lungs + trachea No.1	1:4
2	Liver No.1	In whole
3	Brain No.1	1:8
4	Brain No.2	1:64
5	Lungs + trachea No.2	-
6	Intestine No.2	1:16
7	Lungs + trachea No.3	-
8	Lungs + trachea No.4	-

Note: "-"– negative result.

Table 4. HAIT results on AI virus type A haemagglutination agent detection

No.	Immune sera to reference strains	Tested samples	
		No.1 brain sample test result in HAIT	No.2 brain sample test result in HAIT
1	/Puerto-Rico/8/34 ($_1N_1$)	0	0
2	/Fort Mount/1/47 ($_1N_1$)	0	0
3	/pig/Shoup/56 ($_1N_1$)	0	0
4	/duck/Albert/35/76 ($_1N_1$)	0	0
5	/DK/Hon Kong/196/77 ($_1N_2$) Jan 85	0	0
6	/Singapore/1/57 ($_2N_2$)	0	0
7	/ Chalmers port/1/73 ($_3N_2$)	0	0
8	/horse/Miami/1/63 ($_3N_8$)	0	0
9	/duck/Ukraine/63 ($_3N_8$)	0	0
10	PD 620/89 ($_4N_3$) June 89	0	0
11	/duck/Czechoslovakia/1/56 ($_1N_1$)	0	0
12	/tern/S.Africa/1/72($_5N_3$)	1:20	1:80
13	/duck/Hong Kong/205/77 ($_5N_2$)	0	1:20
14	/Ostrich/Denmark-Q/72420/96 ($_5N_2$) Antiserum April 98	1:160	1:320
15	A/Turk/ENG/50/92 ($_5N_1$) Oct 94	1:40	1:80
16	A/Turkey/Ontario/7732/66 ($_5N_9$) Prep 06/91	1:40	1:80
17	/Vietnam/2004 ($_5N_1$)	1:20	1:40

Table 4. Contd.

18	/turkey/Massachusetts/1/65 ($_6N_5$)	0	0
19	/petrel/Australia/1/72 ($_6N_5$)	0	0
20	/horse/Prague/1/54 ($_7N_7$)	0	0
21	/seal/ Massachusetts/1/80 ($_7N_7$)	0	0
22	/turkey/Ontario/6118/68 ($_8N_4$)	0	0
23	/turkey/Wisconsin/1/66 ($_9N_2$)	0	0
24	/Chicken/Germany/N/49 ($_{10}N_7$)	0	0
25	///56 ($_{11}N_6$)	0	0
26	/duck/Memphis/546/74 ($_{11}N_9$)	0	0
27	/duck/Albert/60/76 ($_{12}N_5$)	0	0
28	/black-headed gull/Sweden/1/99 ($_{13}N_6$)	0	0
29	/gull/Maryland/704/77 ($_{13}N_6$)	0	0
30	/gull/Guriev/263/83 ($_{14}N_4$)	0	0
31	/duck/Australia/341/83 ($_{15}N_8$)	0	0
32	/petrel/Western Australia/2576/79 ($_{15}N_9$)	0	0
33	/black-headed gull/Sweeden/2/99 ($_{16}N_3$)	0	0

Note: “0” - negative result

Table 5. NAIT results on the detection of AI virus type A neuraminidase agent type in the tested samples

Antigens	**Specific neuraminidase AI sera**								
	N_1	**N_2**	**N_3**	**N_4**	**N_5**	**N_6**	**N_7**	**N_8**	**N_9**
AF No.1 (domestic goose brain)	1:100	0	0	0	0	0	0	0	0
AF No.2 (wild duck brain)	1:100	0	0	0	0	0	0	0	0

Notes: “AF” – Allantoic fluid; “0” – negative result

of unknown infectious disease in Pavlodar oblast and conducted laboratory test Laboratory expertise disclosed the following:

- Type A AI virus antigen was detected with the help of “Directigen Flu A” test-system
- Type A AI virus hemagglutinin agent H5 was revealed with the help of HAIT.
- Neuraminidase inhibition test confirmed neuraminidase agent N1 of type A AI virus
- Electron – microscopic study of the pathological material from Pavlodar oblast detected type A H5N1 AI virus.

38 samples of blood serum taken from sick and conditionally healthy birds in Pavlodar oblast were studied for the presence of antibodies to H5N3, H7N1 and to Newcastle disease. Antibodies to H5N3 with activity 1:20 – 1:320 were revealed in 7 samples. Antibodies to Newcastle disease with the activity 1:20 were detected in 4 samples. Specific antibodies to H7N1 were not revealed in blood serum samples taken from sick birds.

The expertise conducted in RIBSP resulted in the conclusion that the cause of illness and death of domestic and wild birds in Pavlodar oblast was AI virus of type A.

Conclusion

The present study revealed that Avian Influenza virus H5N1 subtype A has been responsible for the death of poultry in the farm “NAN” in Irtysh region of Pavlodar oblast, Republic of Kazakhstan. AI virus strain $_5N_3$ antibodies have been detected in 7 of the 38 tested samples with activity in HAIT 1:20 – 1:320. Newcastle disease antibodies are detected in 4 samples with activity in HAIT 1:20. Antibodies specific for AI virus strain 7N1 were not detected in sera of sick poultry. On the basis of results of

Table 6. HAIT results for antibodies presence in sera taken from sick and relatively healthy poultry.

	HAIT results			Serum numbers	HAIT results		
	AI virus strains		Newcastle disease strains		AI virus strains		Newcastle disease strains
	$_5N_3$	$_7N_1$	Bor-73, VGNKI		$_5N_3$	$_7N_1$	Bor-73, VGNKI
1	-	-	-	20	-	-	-
2	-	-	-	21	-	-	-
3	-	-	-	22	-	-	-
4	1:40	-	-	23	1:160	-	-
5	-	-	1:40	24	-	-	-
6	-	-	-	25	-	-	-
7	-	-	-	26	-	-	1:40
8	-	-	-	27	-	-	-
9	-	-	-	28	1:40	-	-
10	-	-	-	29	1:160	-	-
11	-	-	-	30	1:40	-	-
12	-	-	-	31	-	-	-
13	-	-	1:20	32	-	-	-
14	-	-	-	33	-	-	-
15	-	-	-	34	-	-	-
16	-	-	-	35	-	-	-
17	1:40	-	1:20	36	1:40	-	-
18	-	-	-	37	-	-	-
19	-	-	-	38	-	-	-

Note: "-"- negative result

laboratory tests we may conclude that the cause of infection and death of wild ducks and poultry on the territory of Pavlodar oblast is avian influenza virus.

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