EVALUATION OF EFFICACY OF NIBRG-14 VACCINE AGAINST HIGHLY PATHOGENIC H5N1 VIRUSES ISOLATED DURING 2011 INFLUENZA OUTBREAKS IN VIETNAM

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ABSTRACT

Highly pathogenic avian influenza (HPAI) H5N1 viruses continue to be endemic in many Asian countries causing lethal infections in human. The vaccine virus (NIBRG-14) developed from a H5N1 virus strain (A/Vietnam/1194/2004) has been approved by WHO for use in human as well as poultry vaccine. It is well-known that the A/H5N1 viruses have diversified both genetically and antigenically allowing them to escape from the host immune surveillance system. Therefore, evaluation of the vaccine immunogenicity and its relationship to newly emerging viruses is crucially important. NIBRG-14 virus particles propagated in embryonated chicken eggs were inactivated with formalin and adjuvanted with mineral oil to form a water-in-oil emulsion. The resulting vaccine was injected subcutaneously into chickens and ducks. The vaccinated birds were challenged with the HPAI virus strains circulating in Vietnam including clade 1, clade 2.3.2.1a and 2.3.2.1b at day 21 post-vaccination (p. v.). We observed that vaccinated birds were protected from manifestation of disease signs upon challenge with HPAI clade 1 and clade 2.3.2.1a viruses; however, it did not confer protection against clade 2.3.2.1b challenge andstressing the need for development of new effective vaccines against the newly emerging viruses.

Keywords. Vaccine, Avian Influenza

1. INTRODUCTION

Since the first human case of H5N1 was reported in Hongkong - Special Administrative Region (SAR), H5N1 has spread among birds out of East Asia and in far west of England and West Africa, tend to spread into American and Australian continents. There are total of 565

human cases have been confirmed by WHO by August 2011, with a very high case fatality rate of 60 % [1, 2] and millions of poultry were killed and culled of influenza. The outbreakof avian influenza A **(**H5N1**)** in Vietnam over years has caused major morbidity and mortality, and severe economic consequences [3]. Therefore, prepandemic H5N1 vaccines have been thus developed and tested. Shortage of vaccine is of particular concern in developing countries. It is estimated that global production capacity of seasonal influenza vaccines is of approximately 350 million doses and mostly in production plants located in industrialized countries [4]. As part of the preparedness plan for an influenza pandemic in Vietnam, H5N1 vaccine was produced in our laboratory using e';/mbryonated chicken egg-based technology [5]. This vaccine strain (NIBRG-14) was produced from a human isolate (A/Vietnam/1194/2004 [H5N1]) of a virulent clade 1 influenza A (H5N1) virus by reverse genetic technology and now is being used in many countries including Vietnam to prevent H5N1 infections.

A recent review of H5N1 influenza A virus sublineages in Vietnam has shown the presence of emerging H5N1 virus clades. In the northern of Vietnam, two clades of 2.3.2.1a and 2.3.4 are known to be the most common ones. Meanwhile clade 1 still circulates and causes outbreaks in southern provinces of Vietnam [3, 6]. NCVD's systematic surveillance recently update the presenting of clade 7 [7], and clade 2.3.2.1b. According to CDC and WHO surveillance, H5N1 virus clade 2.3.2.1b is not new. It was first detected in poultry during 2009 in Vietnam and evolved from viruses that had previously been circulating in Vietnam since 2005. Till now, the clade 2.3.2.1b viruses are very highly pathogen with high resistance to almost vaccine strains and are spreading more widely in poultry and wild birds.

The FAO report indicated the fact that the H5N1 poultry vaccines currently being used in Vietnam do not protect poultry against the new H5N1 clades[8]. Here we aimed to assess whether the vaccine strain NIBRG-14 can induce protective immunity and protect poultry from clade 1, 2.3.2.1a and 2.3.2.1b strain infections.

2. MATERIALS AND METHODS

2.1. Virus andvaccine

A/H5N1 viruses (clade 1, clade 2.3.2.1a and 2.3.2.1b) were isolated from dead chickens during the outbreak of HPAI in Vietnam and provided by National Center for Veterinary Diagnosis. The vaccine used in this study was produced from the reverse genetics H5N1 master seed (called NIBRG-14) generated with the modified genes H5 and N1 from the strain A/Vietnam/1194/2004(H5N1) and the rest of the backbone segments (PB2, PB1, PA, NP, M, NS) from the A/Puerto Rico/8/34(H1N1) strain by the National Institute for Biological Standards and Control (NIBSC), UK [9]. Vaccine virus was propagated in the allantoic cavity of 10-day-old embryonic hen's egg. Allantoic fluid was harvested 72 hours post infection and was clarified at high speed (8000 rpm, 10 min, 4ºC, in a Sorval rotor) [10]. The virus was harvested and inactivated with formalin and adjuvanted with mineral oil to form a water-in-oil emulsion vaccine.

2.2. Chicken, Duck, and vaccine administration

Two week old specific antibody negative (SAN) chickens ISA Brown hybrid(body weight 100 ± 0.8 g) and ducks Super M (body weight 250.0 ± 1 g) were purchased from the Company of Poultry (Hanoi, Vietnam). The birds were divided into 3 groups of 10 birds each (10

experimental, 3 as control) for each of the chicken and duck groups, respectively (table 1). The experimental groups were vaccinated as follows: 1st dose (160 HAU in 0.5 ml/dose via the subcutaneous route) was given at 14 days old and the 2nd dose (booster dose) was given again in exactly the same way 21 days after the first injection. Blood was collected from the single vaccinated, booster vaccinated, and control groups at days 21 (before challenge). Evaluation was based on antibody titer by HI. A HI titer > 3.0 $\log_2$ is considered protective [11 - 14]

2.3. Challenge and clinical monitoring

The vaccinated birds were challenged with highly pathogenic A/H5N1 viruses (clade1, clade 2.3.2.1a and clade 2.3.2.1b) according to the World Health Organization manual [2]. Briefly, each of birds was inoculated with 0.1 ml of the infectious virus by the intranasal route at day 21 after vaccination; upon the single dose and booster dose, respectively. Clinical signs, mortality, and weight were monitored after the challenge. Serum samples were collected from all birds before challenge to test for the presence of antibodies against H5N1 clade 1, clade 2.3.2 and clade 2.3.2.1 by HI test.

2.4. HemagglutinationInhibition Test

Antibodies in the serum samples were determined by HI test according to WHO manual[15]. Briefly, a duplicate serial 2-fold dilution of each test serum (pretreated at 56^{o}C for 30 min to inactivate nonspecific inhibitors) was made. Sera in wells were then incubated with 4 HA units of the H5 antigen purchased from the National Institute of Veterinary Research, Hanoi, Vietnam (clade 1.0 of the A/H5N1 subtype; a dominant clade in Vietnam and the Southeast Asian countries), for 15 min at room temperature. Control wells were filled with phosphate-buffered saline (1× PBS). Then 0.5 % (v/v) suspension of bird red blood cells (RBC) was added to each well. The HI antibody titer was determined as described by [16]. Antibody titer corresponding to the reciprocal of the highest serum dilution that still inhibited hemagglutination was recorded as the realistic HI titer expressed as a log2 value. The geometric mean titer (GMT) of HI antibodies of each group was determined and compared, and titer equal to or greater than 3 $\log_2$ were considered positive [11, 17, 18].

2.5. Real-time RT-PCR assay

All vaccinated chickens and ducks were throat swabs collected at day 3 and after virus challenge. Total cellular RNA was isolated from the birds using TRIzol reagent (Invitrogen)according to the manufacturer's instructions with some modification [19]. Reverse transcription was carried out with the Superscript TM III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was then used as a template for PCR using H5 specific primers.

2.6. Statistical Analysis

The 10 birds are considered technical replicates. For the studies on the immunogenicity of vaccines, the titer in each chick was considered an independent experimental unit for analysis. Data are presented as Mean ± S. D. Comparisons between experimental groups were analyzed by Student's t test. A value of 0.05 was used to determine statistical significance in all analyses. P values < 0.05 were considered to be statistically significant.

3. RESULTS

3.1. Immune response of vaccinated birds to NIBRG-14

Two groups of SAN animals including 60 chickens and 60 ducks were used for the animal experiments of antibody response in animals. The vaccine (NIBRG-14) was injected into 2-week-old chickens and ducks as described above. We observed that 3-5 percent of vacinated birds exhibited signs of weakness in the first 3 days after vaccination but grown normally after the next 3 days. There was no inflammatory syndrome at injecting side; no fever or death was observed for the vaccinated birds.

Serum samples of the vaccinated birds collected every 3 weeks were examined for antibodies to the vaccine strain, NIBRG-14, by HI test using H5N1 antigen (A/chiken/Scotland/59).

Table 1. Immune responses of chickens and ducks against vaccine strain

Vaccination	Challenge virus	No. of chickens(Positive/ Total)	HAI Unit – GMTlog2		No. of ducks(Positive/ Total)	HAI unit – GMTlog2	
			Examined chicken	Control chicken		Examined duck	Control duck
1st dose	1	7/10	3.7	0	6/10	3.2	0
	2.3.2.1a	8/10	4.4	0	4/10	3.1	0
	2.3.2.1b	8/10	3.9	0	5/10	3.4	0
Total (%)		**23/30 (76.6 %)**	**4.0**	**0**	**15/30 (50 %)**	**3.2**	**0**
2nd dose	1	10/10	6.7	0	9/10	5.0	0
	2.3.2.1a	10/10	6.2	0	10/10	4.8	0
	2.3.2.1b	10/10	6.5	0	8/10	4.4	0
Total (%)		**30/30 (100 %)**	**6.46**	**0**	**27/30 (90 %)**	**4.7**	**0**

The result showed that two weeks after the 1st dose, 76 % of vaccinated chickens induced immune response to hemagglutinin antigen; average unit (GMT) was at 4 log2. However, 3 weeks after the 2nd boosting dose 100 % of examined chickens are positive to HA antibody; GMT was at 6.46 log2. Whilst they are 50 %, 3.2 log2 and 90 % and 4.7 log2 in vaccinated ducks after they received the one dose and two doses, respectively (table 1). Whereas, in the control birds who received PBS instead of vaccine, the immune response was not observed (table 1). This data indicated that the vaccine used in the study was protective. We also observed the body weight of immunized animals compared to the control group. No differences were recorded between the vaccinated animals and control subjects (data not shown). By HI, the vaccine induced seroconversion at rates exceeding the required criteria [11-14, 20] against the NIBRG-14 strain. Thus, vaccine quality could be considered good to use.

3.2. Challenge with H5N1 virus clade 1, clade 2.3.2.1a and clade2.3.2.1b

To evaluate the efficacy of NIBRG-14 vaccine against the most common H5N1 clades circulating in Vietnam, we performed two experiments (tables 2). The first determined the

efficacy of a single dose of vaccine followed by a challenge dose of the clade 1, clade 2.3.2.1a and clade 2.3.2.1b. In the second experiment, the birds received two doses of vaccine and challenged with the above HPAI viruses (table 2).

Two-week-old SPF chickens and ducks were vaccinated with a clade 1 whole-virus vaccine (NIBRG-14) and then challenged intranasally with the clade 1, clade 2.3.2.1a and clade 2.3.2.1b with $10^6 TCID_{50}$/unit. Ten chickens or ducks were used for challenging with each virus clades. Disease signs were observed post challenging (p.c). The survival rates of the chickens challenged with each of the three HPAI viruses were shown in table 2. In the chicken group which received only single dose of vaccine, 20 % of vaccinated chickens died within 5 days after challenge with clade 1 (H5N1); and they were 20 % and 100 % for those injected with clade 2.3.2.1a and clade 2.3.2.1b, respectively. In contrast, all of the control chickens died within 5 days p.c. Similarly, in the chicken group which received two doses of vaccine, we observed that the survival rates were 80 %, 100 % and 10 % as they were vaccinated with clade 1, clade 2.3.2.1a and clade 2.3.2.1b, respectively. The bird that died had the lowest HI titer to the challenge virus (data not shown).

Likewise, the ducks which received one dose of vaccine had survival rate of 90 %, 100 % and 10 % when challenged with clade 1, clade 2.3.2.1a and clade 2.3.2.1b, respectively (Table 2). All ducks who received 2 doses of vaccine induced immune response to HPAI viruses but with varied survival rates as 100 %, 100 % and 30 % to clade 1, clade 2.3.2.1a and clade 2.3.2.1b, respectively. Meanwhile in the control group these were 20 %, 60 % and 0 % when ducks were infected with clade 1, clade 2.3.2.1a and clade 2.3.2.1b, respectively. The results indicated that ducks were somehow more relatively resistant to HPAI viruses as compared to chickens. In other words, chickens were more susceptible to the H5N1 HPAI virus.

Table 2. Imunized chickens and ducks challenge with H5N1 virus clade 1, clade 2.3.2.1a and clade 2.3.2.1b

Vaccination	Challenge virus	Vaccinated group		Control group	
		No. of birds	Alive/total	Total	Dead/total
Chicken received single dose	1	10	8/10 (80 %)	5	5/5 (100 %)
	2.3.2.1a	10	8/10 (80 %)	5	5/5 (100 %)
	2.3.2.1b	10	0/10 (0 %)	5	5/5 (100 %)
Chicken received boosted	1	10	8/10 (80 %)	5	5/5 (100 %)
	2.3.2.1a	10	10/10 (100 %)	5	5/5 (100 %)
	2.3.2.1b	10	1/10 (10 %)	5	5/5 (100 %)
Duck received single dose	1	10	9/10 (90 %)	5	4/5 (80 %)
	2.3.2.1a	10	10/10 (100 %)	5	2/5 (40 %)
	2.3.2.1b	10	1/10 (10 %)	5	5/5 (100 %)
Duck received boosted	1	10	10/10 (100 %)	5	4/5 (80 %)
	2.3.2.1a	10	10/10 (100 %)	5	2/5 (40 %)
	2.3.2.1b	10	3/10 (30 %)	5	5/5 (100 %)

3.3. Detection of virus sheding by Real-time RT-PCR

It is known that once vaccinated, animals will be able to inhibit viral multiplication and then neutralize them. The sensitivity of chickens and ducks to H5N1 viruses are known to be different. A number of studies have revealed that ducks are more resistant to the viruses which are highly pathogenic for chickens[21-23].To assess the viral replication in the immunized animals and in the control group we used Real-time RT-PCR assay. The results are presented in Table 3. We observed that almost vaccinated animals were capable of inhibiting viral replication. The virus multification were significantly decreased (2-3 fold reductions) in all vaccinated birds compared to that of the control subjects. These results are well consistent with that of HI assay (table 1) which also showed the low virus titer in vaccinated birds and with the survival rates determined by challenging (table 2). The virus replicated very strongly in control subjects, especially in unvaccinated chickens (table 2).

Table 3. Virus multiplication in chickens and ducks 3 days post challenging determined by real-time PCR assay

Vaccination	Challenge virus	Average value of virus quantity			
		Examined chicken	Control chicken	Examined duck	Control duck
Bird received single dose of vaccine	1	1.3	3.4	1.1	1.8
	2.3.2.1a	2.0	3.6	1.4	2.8
	2.3.2.1b	2.7	3.0	3.2	3.4
Bird received boosted doses of vaccine	1	1.8	3.2	1.0	1.8
	2.3.2.1a	1.5	3.0	0.5	3.0
	2.3.2.1b	2.0	3.0	2.4	3.0

Value range from 0 to 5; 4 ultra high (Ct < 20); 3 is high (Ct = 20-25); 2 is normnal (Ct = 25-30); 1 is low (Ct = 30-35); and 0 is none (Ct >35).

4. DISCUSSION

Avian Influenza type A is highly pathogenic and is able to infect a wide host range, potentially resulting in epidemics and pandemics. The divergence of influenza H5N1 viruses into several clades challenges the efforts of finding antigenically appropriate vaccines to control. In reassortment, the unique, segmented nature of influenza viruses allows for the mixing and matching of genes that may lead to the creation of a novel subtype. Additionally, the emergence of a new clade with different antigenic properties and antiviral susceptibilities than previous outbreak strains in Vietnam, re-enforce the need for timely surveillance and development of new vaccine strains. It has been reported that prior to 2007, HPAI H5N1 viruses isolated from poultry and humans in Vietnam were mainly clade 1 viruses [24]. However in recent lines of evidences indicated that emerging clades (clade 2.3.2.1a, clade 2.3.2.1b) are frequently circulating in Vietnam.

To prevent H5N1 spreading and/or infections, effective vaccines are required. The NIBRG-14 vaccine strain belongs to clade 1. It is clear that the efficacy of the vaccine is contingent upon the degree of match that exists between the vaccine and challenge strains. In this study, we conducted the test to assess whether the vaccine strain (NIBRG-14), a reverse-genetics-derived

2:6 reassortant between A/Vietnam1994/2004 (H5N1) and A/Puerto Rico (PR)/8/34, provided by WHO can induce cross-reactive immunity to the most common HPAI viruses in Vietnam.

Our data suggested that NIBRG-14 vaccine provides sufficient immunogenicity and induces comparable amounts of anti-HA antibodies in vaccinated animals. The vaccine induced protective immunity and protected 80 % to 100 % chickens and ducks from clade 1 and clade 2.3.2.1a viruses challenge. In fact, we found that this vaccine is less or even no effective to clade 2.3.2.1b. At present we cannot speculate the exact mechanism of how viruses clade 2.3.2.1b escaped from its host immune surveillance system induced by NIBRG-14 vaccine. In other words, further studies need to be carried out to characterize H5N1 HPAIV clade 2.3.2.1b in order to understand why and how this strain is resistant to the NIBRG-14 vaccine strain.

Taken together, this study provides new insights into the protective immunity elicited by currently stockpiled H5N1 (NIBRG-14) vaccine and suggests that new clade 2.3.2.1b should be selected as a based source for vaccine seed viruses in Vietnam.

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TÓM TẮT

ĐÁNH GIÁ HIỆU LỰC VACCINE CÚM A H5N1 CHỦNG NIBRG-14 DỐI VỚI MỘT SỐ CLADE VIRUS CÚM A H5N1 PHÂN LẬP Ở VIỆT NAM NĂM 2011

Virus độc lực cao cúm A H5N1 liên tục gây ra các vụ đại dịch tạirất nhiều quốc gia châu Á trên gia cầm và lây lan sang người. Vaccine cúm A chủng NIBRG-14 phát triển dựa trên chủng virus độc lực cao A/Vietnam/1194/2004 được WHO khuyến nghị sử dụng trên gia cầm và người. Như chúng ta đã biết virus cúm A H5N1 có khả năng biến đổi đặc tính di truyền và kháng nguyên lien tục và đa dạng để thoát khỏi hệ thống miễn dịch của vật chủ. Chính vì vậy, việc đánh giá hiệu lực vaccine và khả năng đáp ứng với các chủng virus mới là thật sự cần thiết. Virus chủng NIBRG-14 được nhân lên trong phôi gà, bất hoạt bằng formalin và nhũ dầu để tạo vaccine, sau đó gây miễn dịch cho gà và vịt thí nghiệm qua đường tiêm dưới da cổ. Các động vật thí nghiệm được thử thách công cường độc bằng các virus độc lực cao thuộc clade 1, clade 2.3.2.1a và clade 2.3.2.1b vào ngày thứ 21 sau khi gây miễn dịch. Chúng tôi nhận thấy các động vật được tiêm chủng đều tạo đáp ứng miễn dịch tốt và có khả năng bảo hộ đối với các chủng virus thuộc clade 1 và 2.3.2.1a. Tuy nhiên vaccine này lại không hoặc bảo hộ kém đối với clade 2.3.2.1b. Kết quả của nghiên cứu này gợi ý rằng cần phải phát triển vaccine mới cho chủng virus cúm đang lưu hành tại Việt Nam

Từ khóa. Vắc xin, cúm gia cầm.