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Attenuation and Protective Effects of a Thermostable Newcastle Disease Virus Isolated from Korean Pheasants

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한국산 꿩으로부터 분리한 열 안정성 뉴캣슬병 바이러스의 순화와 방어효과

한수철 · 곽길한 · 김태중 · 장경수[1] · 전무형[1] · 송희종

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ABSTRACT: The objective of these experiments was to develop an attenuated thermostable Newcastle disease virus (NDV), CBP-1 strain isolated from infected pheasants. Safety, pathogenicity and protective effects against velogenic NDV were also investigated to evaluate if the attenuated NDV, CBP-1 strain could be a candidate for a new NDV vaccine strain. CBP-1 strain was passaged up to the 173 times by nine days old embryonated eggs and chicken embryo fibroblast (CEF) cell cultures. Its sensitivity to lipid solvents and low pH, thermostability, mean death time (MDT), intracerebral pathogenicity index (ICPI) of one day old chicks and intravenous pathogenicity index (IVPI) of four weeks old chicks were examined. Safety, boosting and protective effects were tested by chicks mortality. CBP-1 NDV strain had significant thermostability at 56 ℃ for 30 minutes. by hemagglutinin activity and egg infectivity test, but was not resistant to lipid solvent. It showed possibility to use as a feed or water vaccine because of the resistance to low pH. MDT, ICPI and IVPI of CBP-1 were attenuated from 51.5, 1.96, 2.60 to 112.4, 1.12, 1.45. These results implied that the 173rd passages in embryonated egg and CEF cell cultures induced a substantial attenuation of the pathogenicity of the parent virus, changing the virulence from velogenic to intermediate between mesogenic and lentogenic. After vaccination with CBP-1 at one day old by drinking water mortality was 17.5 %. However, spray vaccination with B1 at one day old, CBP-1 at two weeks old and challenge with velogenic Kyojeongwon strain at four weeks old showed 93.5 % survival rate. Mortality of chicks, vaccination with 173rd passaged CBP-1 strain at one day old, two weeks old and challenge with Kyojeongwon strain at four weeks old, was 20.0 %. The results of these studies indicated that partial attenuated CBP-1 strain tended to be a low safety for ND of broiler chicks and would need to be more successive attenuation.

(Key words : Newcastle disease virus, attenuated CBP-1 strain, thermostability, safety, pathogenicity)

INTRODUCTION

Newcastle disease (ND) is caused by a group of closely related viruses which form the avian paramyxovirus type 1 (PMV-1) serotype and produced with varies from extremely pathogenic to inapparent in poultry, depending on strain and host infected. It was grouped into single-stranded, nonsegmented, negative-sense enveloped RNA virus (Melnik, 1982; Kolakofsky et al., 1974). Nine serogroups of avian paramyxoviruses (PMV) have been recognize as PMV-1 to 9. Especially, PMV-1 has been reported the most important pathogens to decrease the performance as one of poultry (Alexander, 1986). Since the first case of ND was reported in 1926 (Calnek et al., 1997), numerous types of ND has been reported in our country. Its clinical signs are not routine and predominant in recent poultry industry. They decreased egg production and developed abnormal respiratory or nervous signs in poultry.

A striking feature of NDV strains and isolates is their ability to cause quite distinct signs and severity of disease, even in the same host species. Based on the disease produced in chickens under laboratory conditions. NDVs have been

placed in five pathotypes: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic respiratory, and asymptomatic enteric (Jordan and Pattison, 1996).

As the result, to confirm the virulence of NDV mean death time (MDT), intracerebral pathogenicity index (ICPI) of one day old chicks' cerebrum, intravenous pathogenicity index (IVPI) of four weeks old chicks' jugular vein have been reported as an official method for standard diagnostic tests (Alexander and Allan, 1974; Kendal and Allan, 1970). Recently, Hitchner B-1 strain and LaSota strain of lentogenic pathotype have been used as a live vaccine against ND in Korea. However, those NDV showed high sensitivity to environmental temperature, humid, and depended on method of NDV vaccine preservation. It would also be destroyed or inactivated by spray, oral administration or food vaccine. As a result, those prescriptions would not be able to elevate the sufficient immune reaction.

Thus, these studies were conducted to investigate the physical, chemical characteristics and thermostability of heat-stable NDV, isolated from Korean pheasants and to measure the safety and pathogenicity of attenuated heat-stable NDV strain compared to those of other commercial vaccine strains against velogenic strains after oral administration to broiler chicks.

MATERIALS AND METHODS

1. Virus

The 153rd attenuated and heat stable CBP-1 strain (Microbiology Lab, Chungnam National University) was isolated from NDV of Korean pheasants (Park et al., 1997). Hitchner B-1, Ulster and Kyojeongwon strains were provided from National Veterinary Research and Quarantine Service (NVRQS, Anyang, Korea) (Park et al., 1995a, 1995b).

2. Chicken Embryo and Incubating Chicks

Nine to eleven days old of embryonated egg were purchased from Harim Co. (Iksan, Korea) and incubated at 37 ℃ in hatchery (LYON, USA). Experimental groups of chickens were raised under the environmental control system (Myungjin Co., Seoul, Korea).

3. Virus Passage

Virus was inoculated and passaged via embryonated egg and chicken embryo fibroblast (CEF) cell. Hemagglutination (HA) test was conducted to confirm the virus proliferation by using 1 % chicken red blood cell (cRBC) (Park et al, 1997).

4. Thermostability of Hemagglutinin

NDV, CBP-1 strain were heated at 56 ℃ for 0, 30, 60 and 120 minutes, respectively. HA test was conducted to certify the thermostability with chicken RBC (Burnet, 1942).

5. Sensitivity to Lipid Solvents and Low pH

The sensitivity of CBP-1 strain was tested with lipid solvents and low pH by the method of Burleson et al., (1992). Each NDV control were treated by 0.1 ml non-treated CBP-1 and 0.5 ml chloroform with 0.5 ml HBSS.

Low pH test of CBP-1 were performed to confirm the tolerance in acidic conditions of the gastrointestinal tract. Each 0.9 ml of pH 3.0, 7.4 of glycine-HCl buffer and HBSS, respectively. Stability of CBP-1 strain were also mixed for 60 minutes and then inoculated into monolayed CEF cell. Titer was measured by tissue culture infective dose ($TCID_{50}$).

6. Tissue Culture Infective Dose ($TCID_{50}$)

NDV diluted 10^{-3} to 10^{-9} were inoculated into monolayed CEF cell and observed CPE for 4 days at 37 ℃. Titer was measured by the method of Reed and Muench (Burleson et al., 1992).

7. Embryo Infective Dose (EID_{50})

CBP-1 diluted with 10 fold was inoculated into CAC of embryonated egg at 37 ℃ for 48 hours and chilled at 4 ℃ for 4 to 24 hours. Virus was harvested from CAF and practiced by HA test. Titer was calculated by the method of Reed and Muench calculation (Burleson et al, 1992).

8. Mean Death Time (MDT)

CBP-1 strain was diluted up to 10 fold dilution from 10^{-6} to 10^{-9} in HBSS. Each dilution was inoculated into the CAC of each of five 9 to 11 days old embryonated eggs and incubated at 37 ℃. CBP-1 strain was inoculated another five

eggs with 0.1 ml of each dilution 8 hours later and place at 37 ℃. Each egg was examined twice at 9 am and 6 pm daily for one week. The MDT of all embryos were recorded and used to classify the velogenic, mesogenic and lentogenic types of NDV depend on the basis of death rate of chicks (Hanson and Brandly, 1955; Jordan and Pattison, 1996).

9. Intracerebral Pathogeity Index (ICPI)

The 2^4 HA titer of CBP-1 strain was diluted one-tenth in sterile isotonic saline. 0.05 ml of CBP-1 strain was intracerebrally injected to one day old of ten chicks. They were examined at every 24 hours for 8 days under environmental control system. Chicks were scored : 0 (normal), 1 (sick), and 2 (dead). The ICPI was meaured by the previous report (Park et al., 1997).

10. Intravenous Pathogenicity Index (IVPI)

The 2^4 HA titer of CBP-1 strain was diluted one-tenth in sterile isotonic saline. The 0.1ml of diluted CBP-1 strain was injected into ten chicks intravenously at four weeks old. They were examined at every 24 hours for 10 days and scored to 0 (normal), 1 (sick), 2 (paralysed or showing other nervous signs), and 3 (dead) (Park et al., 1997; Jordan and Pattison, 1996).

11. Protective Effects

Three experiments were conducted to evaluate the protective effects of CBP-1 and commercial NDV on their safety and booster of broiler chicks. Forty chicks were placed into isolator in each experiment for six weeks.

12. Experiment 1

Safety test of 173rd passaged CBP-1 were orally administered with EID_{50} at one day old chicks and examined for four weeks. Clinical signs and death rate were recorded.

13. Experiment 2

One day old chicks were sprayed by B-1 strains. CBP-1 was orally administered with $10^{6.8}$ EID_{50}/0.1 ml at two weeks old of chicks. Booster vaccine effect of CBP-1 strain was evaluated at six weeks old. Velogenic, Kyojeongwon strain was challenged on those chicks at four weeks old and observed and record for the rest of two weeks.

14. Experiment 3

Protective effect of CBP-1 strain was investigated. 0.1 ml of the 173rd passaged CBP-1 strain were vaccinated with confirmed EID_{50} titer per os into one day old commercial broiler chicks. After the first two weeks following the primary vaccine, the 173rd passaged CBP-1 strain were revaccinated per os again. Challenge was performed with velogenic Kyojeongwon strain at four weeks and observed for the rest of two weeks.

RESULTS AND DISCUSSION

1. Thermostability of CBP-1 Strain

Many researchers have reported that NDV could be destroyed by heat, UV light, oxidizer and alkaline (Lancaster et al., 1966; Beard and Hanson, 1984). Decreased rate of viral infectivity is depended on virus strain, exposure and quantity of time. No single treatment could control the NDV completely and remained its low infectivity (Lancaster et al., 1966; Beard and Hanson, 1984). In this experiment, hemagglutination (HA) activity of the 173rd-passaged CBP-1 strain was 427 HA titer at 56 ℃ for 120 minutes (Table 1). Although, CBP-1 strain has been passaged until the 173rd, the thermostability remained similar to that of parent strain. Thus, CBP-1 strain of this experiment was thought to be developed as an another ND vaccine strain.

2. Physical and Chemical Characteristic of CBP-1 Strain

$TCID_{50}$ titer of sensitivity to lipid solvent was under detection level (10^0) when CBP-1 strain was exposed to chloroform for 10 minutes at room temperature. $TCID_{50}$ titer of CBP-1 strain with pH 3.0 glycine-HCl buffer for 60 minutes was $10^{5.0}$. It decreased in log 2 titer and showed less stable than that of pH 7.4 conditions which were $10^{7.0}TCID_{50}$

Table 1. Thermostability of CBP-1 strain

Passages	0 min	30 min	60 min	120 min
3rd	683[1]	512	512	427
173rd	512	512	512	427

[1] Hemagglutination titer.

Table 2. $TCID_{50}$ titer of 173rd passaged NDV, CBP-1 strain[1] at different treatments

Treatments	$TCID_{50}$ titer
Control	10
Chloroform	10
pH 3.0	10
pH 7.4	10

[1] Embryo infective dose (EID_{50}/0.1ml) of 173rd passaged NDV, CBP-1 strain was presented $10^{9.5}$.

(Table 2). The results of this experiment confirmed that enveloped virus would be destroyed by lipid solvent. In pH test, CBP-1 exhibited $10^{5.0}TCID_{50}$ and identified the resistance to low pH. As a result, oral or feeding administrate of CBP-1 strain seemed to keep the infectivity of intestinal lymphoid cell.

3. Pathogenecity of CBP-1 Strain

Pathogenicity of the 3rd passaged NDV was velogenic which was 51.5 hours MDT, but was 112.4 hours on the 173rd passaged CBP-1 strain. MDT increased on the 173rd passaged one compared to that of the 3rd passaged strain. This result confirmed that CBP-1 strain would be classified as a lentogenic pathotypes (Table 3).

ICPI of the 3rd passaged one was 1.96, whereas it was 1.12 on the 173rd passaged CBP-1 strain. These results indicated that the 173rd passaged CBP-1 strain decreased their pathogenicity by the successive passage (Table 3).

IVPI of the 3rd-passaged CBP-1 strain was 2.60 which was belong to high virulence, but decreased to 1.45 on the 173rd- passaged CBP-1 strain (Table 3).

Table 3. Pathogenicity of NDV, CBP-1 strain at different passage[1]

AV \ Passage	3rd	173rd
MDT	51.5	112.4
ICPI	1.96	1.12
IVPI	2.60	1.45

[1] Abbreviations are AV, assessment of virulence ; MDT, mean death time ; ICPI, intracerebral pathogenicity index and IVPI, intravenous pathogenicity index.

Hanson and Brandly (1955) suggested that NDV strains could be divided into velogenic, mesogenic and lentogenic ones based on MDT at <60, 60~90, and >90 hours, respectively. Thus, MDT of the 173rd passaged CBP-1 strain of these studies was 112.4 hours and confirmed to lentogenic pathogenicity. ICPI and IVPI of the 173rd passaged CBP-1 was 1.12 and 1.45, respectively. They were higher than those of Hitchner B-1 and Ulster strain (Calnek et al., 1997) that were 0.2, 0 and 0, 0, respectively. Lentogenic strain has been used as a primary vaccine, whereas mesogenic strain has been used as a booster vaccine (Calnek et al., 1997). The results of this experiment implied that if CBP-1 strain were changed from velogenic to mesogenic pathotype, it was possible to use for secondary or booster vaccine.

4. Protective Effects of CBP-1 Strain

When the 173rd-passaged CBP-1 strain was administered into one-day-old chicks per os in expt 1, clinical symptoms and mortalities were recorded to 17.5 % for four weeks (Table 4).

After Ulster strain was sprayed into one-day-old chicks, the 173rd-passaged CBP-1 strain was boostered at two weeks old and challenged by Kyojeongwon, velogenic pathotype at four weeks old in expt 2. The lentogenic commercial vaccine was first inoculated, and then attenuated CBP-1 strain was inoculated into the broiler chicks. Mortalities of the 173rd passaged CBP-1 strain were 7.5 %. Thus, CBP-1 strain would be used as the secondary and booster vaccine. The results of expt 1 and 2 suggested that CBP-1 strain should be more attenuated because commercial vaccine had a lower pathogenicity than attenuated CBP-1 strain.

In expt 3, the 173rd passaged CBP-1 strain were administered into one day and two weeks old chicks *per os*, respectively and then challenged with velogenic strain, Kyojeongwon at four weeks old. Mortality was increased upto 20.0 (Table 4) (Hanson et al., 1949). These results indicated that it was not possible to substitute CBP-1 strain for primary vaccine.

Thus, further studies need to conduct the comparative experiments among commercial strains, live NDV, inactivated NDV, live recombinant NDV and inactivated recombinant

Table 4. Safety, boosting and protective effects of 173rd NDV, CBP-1 strain[1]

Expt	1	2	3	4	5	6	Mortality(%)
	---------- Weeks ----------						
1	5/18 (35)	1/5 (34)	1/3 (33)	0/0 (33)	-	-	17.5
2	2/7 (38)	0/0 (38)[2]	0/0 (38)	0/0 (38)[3]	1/1 (37)	0/0 (37)	7.5
3	4/19 (36)	2/7 (34)[2]	2/3 (32)	0/0 (32)[3]	0/2 (32)	0/0 (32)	20.0

[1] No. of dead chicks/no. of chicks with clinical signs are presented in each experiments. The values in the ( ) are the number of survivors.

[2] Booster injection with NDV CBP-1 173rd : $10^{6.8}EID_{50}$/0.1 ml (1 dose).

[3] Challenged with Kyojeongwon strain : $10^{5.5}EID_{50}$/0.1ml (1 dose).

NDV to confirm the safety of CBP-1. CBP-1 strain also need to describe how it would be related with immunogenicity when it's combined with infectious bronchitis virus (IBV) vaccine or infectious bursal disease virus (IBDV) vaccine.

적 요

본 시험은 한국산 꿩에서 분리한 열안정성이 있는 뉴캣슬병 바이러스인 CBP-1을 약독순화시킨 후에 환경 저항성, 안정성, 병원성 및 보강접종 후 뉴캣슬병 바이러스의 강독주에 대한 방어효과를 측정하고 또한 새로운 백신주로 육계에 적용 가능성을 연구하고자 시행하였다. 뉴캣슬병 바이러스 CBP-1주는 9일령의 발육계란과 계태아섬유아세포를 이용하여 173대까지 계대 배양하였다. 조사항목은 실험주의 지질용매와 낮은 pH에 대한에 안전성, 열안정성, 평균 계태아 치사시간(MDT), 1일령 병아리의 뇌내 병원성 지수(ICPI) 및 4주령 정맥내 병원성 지수(IVPI)를 측정하였다. 이외에도 계태아 50 % 감염지수(EID_{50}), 배양세포 50 % 감염지수($TCID_{50}$), 안전성, 보강접종 및 방어효과를 측정하였다. 173대까지 약독순화된 CBP-1 뉴캣슬병 바이러스의 열안정성은 56 ℃에서 30분간 열처리한 후에도 혈구응집능과 계태아 감염성이 인정되었다. 본 실험에서 이용된 CBP-1은 지질용매에 대한 저항성은 없었으나 산성용매에 대하여 저항성을 나타냈으므로 음수나 사료첨가용 백신으로 적용 가능성을 보였다. CBP-1의 병원성 실험으로 MDT, ICPI, IVPI를 측정한 결과 강독주였던 최초 분리주는 51.5, 1.96, 2.60에서 112.4, 1.12, 1.45로 순화되었다. 본 실험에서 이용된 173대 CBP-1을 1일령 병아리에 음수접종하고 4주간 관찰한 결과 17.5 % 폐사율을 나타냈으며, 1일령 병아리에 약독주인 B1을 분무접종하고 2주령에 173대 CBP-1을 보강접종한 후 4주령에 강독주인 교정원주로 공격접종하였을 때 생존율이 93.5 %였다. 이외에도 1일령과 2주령에 173대 CBP-1을 접종한 후 4주령에 강독주인 교정원주로 공격접종하였을 때 생존율은 80.0 %을 나타냈다. 본 실험 결과 173대까지 계대 배양한 CBP-1은 백신주로서 안전성이 낮으므로 지속적인 계대순화가 필요할 것으로 사료된다.

(색인어 : 뉴캣슬병바이러스, CBP-1주, 병원성, 방어효과, 열안정성, 약독순화)

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