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Studies on the Cold Attenuation and Protective Effects of a Thermostable Newscastle Disease Virus Isolated from Korean Pheasants

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한국산 꿩으로부터 분리한 열안정성 뉴캣슬병 바이러스의 저온순화와 방어효과

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ABSTRACT : Newcastle disease virus, CBP-1 strain isolated from Korean pheasants was passaged for 173 times by 9-day-old specific pathogenic free(SPF) embryonated eggs at 37℃ (parent strain) and subsequently passaged for 15(cold attenuation (CA)-15) and 30(cold attenuation (CA)-30) times by 10-day-old of commercial broiler chicks embryonated eggs at 29℃, respectively. The physical and chemical properties(sensitivity to lipid solvents, low pH and thermostability), pathogenicity(mean death time, intracerebral pathogenic index and intravenous pathogenic index), safety, booster or protective effect and characterization of temperature sensitivity were measured in cold attenuated CA-15 or 30 strain and compared to those of parent CBP-1 strain. NDV, CBP-1 CA-30 strain acquired cold attenuation and decreased infectivity at 41℃ compared to those of parent strain grown at 37℃. It lost hemagglutination activity (HA) and cell infectivity at 56℃ for 30, 60, and 120 Min. CA-30 strain treated with ethyl ether also lost its HA and cell infectivity. Both CA-30 and parent strains exhibited a little resistant to HA at pH 3.0 glycine HCl buffer. Intracerebral pathogenic index(ICPI) and intravenous pathogenic index (IVPI) of parent strain were 1.12 and 1.45, but decreased to 0.75 and 0.00 in CA-30 strain, respectively. The safety was evaluated by mortality in chicks inoculated with $10^{4.0}EID_{50}$/0.1 ml. The mortalities of parent, CA-30 and commercial B1 strains were 17.5, 12.0 and 0.0%, respectively. The safety of CA-30 strain was higher than that of parent strain. The booster effects of CA-30 strain and parent strain performed in 4-week-old chicks after being vaccinated with primary commercial B1 strain. The mortality of CA-30 strain(10.0%) was higher than that of parent strain(7.5%). To investigate the protective effect, 4-week-old chicks vaccinated two times at 1-day-old and 2-week-old with CA-30 and parent strains, respectively, were challenged with velogenic NDV, Kyojeongwon strain. The mortality of CA-30 strain was 11.7% and parent strain was 20.0% at 6-week-old broiler chicks. After inoculating CA-15, CA-30 and commercial B1 strains at 1-day-old chicks, the antibody titers of these chicks exhibited $2^{5.00}$, $2^{4.95}$ and $2^{4.45}$ HI titer. Chicks inoculated with commercial B1 strain and reinoculated with CA-15 and CA-30 strain showed $2^{7.33}$ and $2^{6.90}$ HI titer. Chicks administrated with CA-15 and CA-30 strain twice at one day old and 2 weeks old, respectively, were $2^{7.95}$ and $2^{7.33}$ HI titer.

(Key words : cold attenuation, NDV, CBP-1, thermostability, Korean pheasants)

INTRODUCTION

Since Newcastle disease(ND)virus was reported by Kraneveld (1926), it has been the most important pathogen leading cause of severe viral disease in domestic poultry and other animals including human (Alexander, 1986). Outbreak of NDV was so severe that almost all of the affected flock died within 72 hrs without showing clinical signs. It belongs to a member of the genus *Rubelavirus* of the family *Paramyxoviru-dae* and possessed RNA of non-segmented, single-stranded genome of negative polarity (Lancaster, 1966; Park et al 1995).

ND has been controlled by oral, intranasal, intramuscular, intraocular and subcutaneous vaccination. These vaccine strains such as B1, La Sota and Ulsta strains have been generally used to control the ND. However, the biological properties of these vaccine strains would be destroyed by heat, moisture, acid, UV light when used by aerosol, water vaccine, and an erroneous preservation before administration (Alexander, 1997). Because these vaccines affected by those various factors were not enough to rise serum antibody level against ND, we attempted to develop NDV vaccine to keep the environmental resistance. Especially, an undeveloping country without cold chain system and delivery system will require to environmental resistant vaccine.

NDV, CBP-1 strain showed thermostability at 56℃ for 120 min was isolated by Park et al. (1995) from Korean pheasant and passaged. According to Han et al. (2000), CBP-1 strain passaged to 173rd generation kept mesogenic pathogenicity and thermostability at 56℃ for 120 min. We attempted to reduce its pathogenicity by cold attenuation. For those purposes, CBP-1 strain was selected and passaged at 29℃ for 30 times in commercial broiler chicks eggs.

According to Gelb et al. (1996; 1991), Govea et al. (1983) and Maassab (1967), the cold attenuation of virus has been proven to be less virulent than the parent strain, and Gelb et al. (1996; 1991) reported that cold adaptation of NDV had advantages to reduce its virulence without using the mutagen. Lentogenic commercial B1 vaccine strain and avian infectious bronchitis virus were practically passaged at low temperature by Gelb et al. (1996; 1991) and the virulence of vaccine strain was significantly reduced.

MATERIALS AND METHODS

1. Eggs and Chicks

Embryonated eggs of commercial broiler were purchased from Halim Co.(Iksan, Korea) and were incubated in egg incubator(Lyon, USA) at 37℃ for the first 10 days. They stayed at 29℃ for 24 hrs on 10th day (Gelb et al., 1996). They were also incubated at the same temperature for 72 hrs to produce NDV after inoculation at 11 days old. Commercial broiler chicks were hatched and housed in environmental controlled isolator(Myungjin Co, Korea) from 4 to 6 weeks of age (Han et al. 2000). All chicks were provided a commercial diet and drinking water, *ad libitum*.

2. Virus and Passage

NDV, CBP-1 parent stock strain which was subsequently passaged for 173 times in specific pathogenic free(SPF) chicken egg, obtained from Chungnam National University (Park et al., 1995). Parent strain virus diluted with 1/10 in Hank's balanced salt solution(Ca^{2+}, Mg^{2+} free HBSS, Gibco BRL, NY) was inoculated into chorioallantoic cavity(CAC) at 10-day-old embryonated eggs. They were incubated in 29℃ for 72 hrs. Chorioallantoic fluid was tested for hemagglutination activity(HA) to confirm the NDV CBP-1 strain. Virus was continuously passaged to 30 times from 173 to 202 passages. Commercial B1 strain was purchased from Daesung Microbiological Co. (Seoul, Korea). Kyojeongwon strain obtained from Veterinary Research and Quarantine Service(Anyang, Korea) and applied for challenge strain.

3. HA and HI test

The HA and HI were tested by conventional microtiter methods described as Alexander (1996).

4. Characterization of Temperature Sensitivity

Chorioallantoic fluids of parent, CA-15 and 30 strains were inoculated into primary chicken embryo fibroblast cells and then incubated at 29℃, 37℃ and 41℃ for 4 days, respectively. After incubation, cell infectivity of each chorioallantoic diluted fluid was observed and evaluated by $TCID_{50}$/0.1ml at respective temperature (Gelb et al., 1996; Maassab, 1967).

5. Sensitivity to Lipid Solvent and Low pH

The sensitivity of lipid solvent and resistance test to low pH of CBP-1 strains were performed by Burleson et al. (1992) method.

6. Pathogenicity

The MDT in eggs, ICPI in 1-day-old chicks and IVPI in 6-week-old chicks were completed as described Alexander (1997). MDT, ICPI and IVPI of eggs and chicks were observed for 7, 8 and 10 days, respectively. Sterile saline was used to control the negative .

7. Safety

21 days old broiler chicks were divided into three treatments. The first group was also inoculated with B1 strain at $10^{4.0}EID_{50}$/0.1 ml, The second group was intraorally inoculated with parent strain at $10^{6.8}$ EID_{50}/0.1 ml. and the third group was inoculated with CA-30 strain at $10^{4.0}EID_{50}$/0.1 ml. Each treatment was raised in environmental controlled system. Clinical signs and deaths of those birds were daily observed for 4 weeks and recorded.

8. Protective Effects

61 days old chicks were divided into two groups. The first group intraorally vaccinated with parent strain at $10^{6.8}EID_{50}$/0.1 ml per bird at 1 day and 2 weeks old. The second group intraorally vaccinated with CA-30 strain $10^{4.0}EID_{50}$/0.1 ml per bird at 1 day and 2 weeks old. The chickens of two group were intraorally challenged at 4 weeks old with velogenic Kyojeongwon strain at $10^{5.5}EID_{50}$/0.1 ml. The clinical signs and deaths of chicks were observed from 4 to 5 weeks and estimated the protective effects against velogenic strains.

RESULTS

1. Virus Passage

Heat stable Newcastle disease virus(NDV), CBP-1 parent(173rd) strain was subsequently passaged at 29 ℃ to 202nd generation in embryonated eggs of commercial broiler chicks.

2. Hemagglutination Titer of Parent, CA-15 and CA-30 Strain

HA titers of parent, CA-15 and CA-30 strain were 2^8, 2^6 and 2^6, respectively (Fig. 1). When parent strain was passaged in commercial chicken eggs at 29℃ for the first time, it decreased from 2^8 to 2^6 in CA-1 strain. These results may be due to rapid low temperature which inhibit the growth of parent strain. HA titer of CBP-1 showed consistency on the further passage and maintained at the level of 2^6 HA titer.

3. A Comparison of Temperature Sensitivity of Parent, CA-15 and CA-30 Strain

Table 1 showed that the cell infectivity of parent strain tended to be higher titer than that of other treatments at 29℃ ($10^{4.3}TCID_{50}$/0.1 ml), 37℃ ($10^{7.5}$

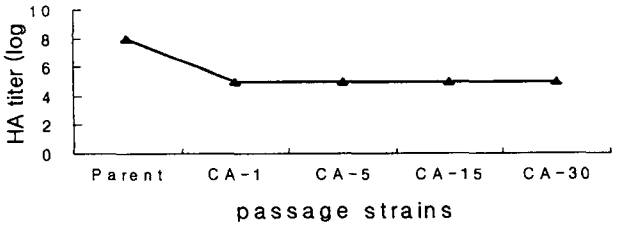

Fig. 1. HA titer change of NDV, CBP-1 strain.

Table 1. Comparison of $TCID_{50}$ of parent, CA-15 and CA-30 strains at various temperature

Treatments	29℃	37℃	41℃
Parent	4.3*	7.5	7.2
CA-15	4.2	4.5	4.0
CA-30	3.6	4.0	2.5

Table 2. Effects of various time treatments on thermostability of parent, CA-15 and 30 strains

NDV	0 min		30 min		60 min		120 min	
	HA	TCID	HA	TCID	HA	TCID	HA	TCID
Parent	512*	7.5**	512	0	512	0	256	0
CA-15	64	4.5	0	0	0	0	0	0
CA-30	64	4.0	0	0	0	0	0	0

* : Hemagglutination titer.

** : Tissue cultured infectious dose 50.

$TCID_{50}$/0.1ml) and at 41℃ ($10^{7.2}TCID_{50}$/0.1ml). The cell infectivity of CA-15 strain had similar tendency compared to those of CA-30 at 37℃, but reduced at 41℃ ($10^{4.2}$ $TCID_{50}$/0.1ml). However, CA-30 strain's cell infectivity and preferability seemed to decrease at 41℃ relative to those of parent strains.

4. Thermostability

Table 2 showed that parent strain maintained its HA activity at 56℃ for 30, 60 and 120 min, respectively. However, HA activity of CA-15 and 30 strains was not sustained at 56℃ for 30, 60 and 120 min. When parent, CA-15 and 30 were treated at several different time and temperature, all strains were not infective in CEF cell.

5. Sensitivity of Low pH and Lipid Solvent of CBP-1 Strain

Low pH response of parent, CA-15 and CA-30 strains were shown in Table 3. When the CBP-1 strains were treated with pH 3.0 Glycine HCl buffer, HA titers were decreased by low pH. The parent, CA-15 and 30 strains treated with ethyl ether also lost their hemagglutination(HA) activity and cell infectivity. As the results, CBP-1 strains confirmed to envelop virus in these experiments.

Table 3. Influence of low pH and lipid solvent on HA titer of parent, CA-15 and CA-30 strains

Glycine HCl	Parent	CA-15	CA-30
pH 3.0	2*	2	2
pH 7.4	8	4	4

* : Hemagglutination titer.

6. Pathogenicity

ICPI of parent, CA-15 and 30 strains was displayed to 1.12, 0.78 and 0.75, respectively(Table 4). It decreased in parent strain for cold passage compared to that of other strains.

The chicken embryo MDT of CA-30 strain was increased to 118 and showed higher than that of parent strain(112.4). IVPI of CA-30 strainwas remarkably lower than those of parent strain and CA-15 strain. According to Lancaster (1966), Hanson and Brandly (1955), the pathogenecity of CA-30 strain was not belong to previous pathogenic type. Thus, it would be classified as lentogenic strain of NDV.

7. Safety of Parent and CA-30 Strain

After vaccination with commercial B1 strain, parent and CA-30 strain at one day old chicks per os, all groups of them showed lameness, torticollis, respiratory sign and sudden death syndrome without clinical signs. However, clinical sign and mortality of CA-30 strain was safer than those of parent and similar to those of B1 strain.

Table 4. MDT, ICPI and IVPI of parent, CA-15 and CA-30 strains[1]

NDV	Pathogenic index of NDV		
	MDT	ICPI	IVPI
Parent	112.4	1.12	1.45
CA-15	62.0	0.78	0.33
CA-30	118.0	0.75	0.00

[1], MDT: mean death time to 9-day-old chickens embryo.
ICPI: intracerebral pathogenic index to 1-day-old chickens.
IVPI: intravenous pathogenic index to 6-week-old chickens.

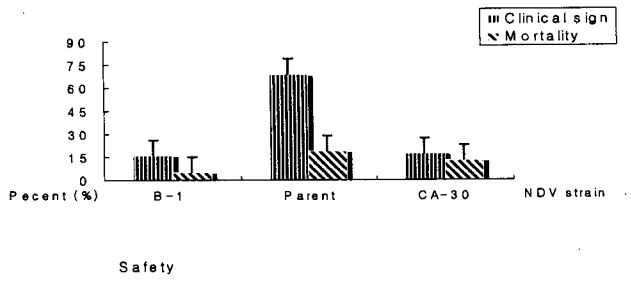

Fig. 2. Percentage of Clinical Sign and Mortality of CA-30 compared with Parent and B-1.

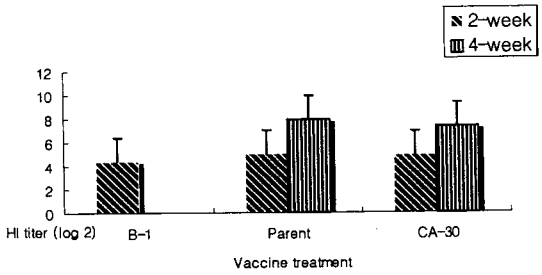

Fig. 4. HI titer of CA-30 compared with Parent and B-1.

8. Protective Effects

PPVaccination with Parent, CA-30 and B1 strain were performed at 1-day and 2-week old chicks. These chicks were orally challenged by NDV, Kyojeongwon strain at 4 weeks old and then observed for 2 weeks. Parent and CA-30 strain protected velogenic NDV, Kyojeongwon without clinical signs and death. When the chicks administered with B1 strain were orally challenged by Kyojeongwon strain at 4 weeks old, they appeared to weakness, leg paralysis, torticollis and prostration. Chicks vaccinated with CA-30 and parent strain were successfully protected from velogenic NDV, Kyojeongwon strain.

9. Immunogenicity of NDV Strain

HI titers of chicks exposed to commercial B1, parent and CA-30 strain at 1 day were $2^{4.40}$, $2^{5.00}$and $2^{4.95}$, respectively. Chicks injected with parent strain recorded higher HI titer than that of commercial B1 stain and CA-30 strains. HI titer of chicks inoculated with parent strain at 1-day and 2-week old was $2^{7.95}$ (mean titer) at 4 weeks and it was $2^{7.3}$ (mean titer) at 4 weeks old chicks administered with CA-30 strain at 1 day and 2 weeks of age. The results of these experiments confirmed that antibody responses of parent and CA-30 strain could be defended from velogenic NDV.

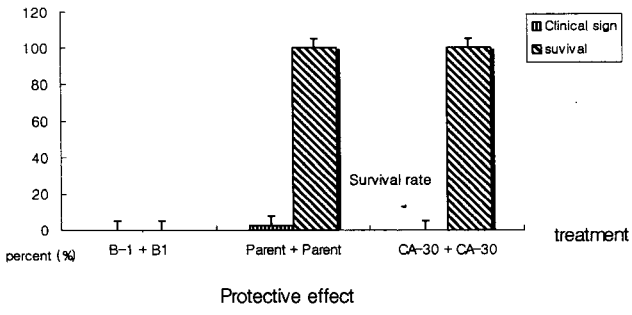

Fig. 3. Percentage of Clinical Sign and Survival Rate of CA-30 compared with Parent and B-1.

Discussion

NDV, CBP-1 parent strain was thermostable at 56℃ for 120 min and subsequently passaged in embryonated commercial broiler eggs for 30 times at 29℃.

Lancaster (1966) and Beard and Hanson (1984) reported that NDV was easily destroyed by physical and chemical treatments such as heat, UV-light, oxidation process, pH effects and various chemical compounds and showed resistance to those agents depended on the strain of virus, the length of the time of exposure, the quantity of virus and the nature of suspending medium. In those reports, any single treatment could not demonstrate the destruction of NDV.

In these experiments, the pathogenecities of CBP-1 strains were destroyed by heat and lipid solvent treatment except low pH treatment. Hanson et al. (1949) reported that hemagglutination (HA) activity and cell infectivity of chicken embryo were independently inactivated by those agents. The parent strain of this experiment was similar to Han et al. (2000). However, CA strain tended to lost their hemagglutination(HA) activity and cell infectivity at the same time when it was treated by 56℃. These results were dif-

ferent from previous report of Han et al. (2000).

To confirm the cold adaptation of CA strain, parent , CA-15 and 30 strain were inoculated into CEF-cell and incubated at 29℃, 37℃ and 41℃, respectively. CA strain, passaged for 30 times at 29℃, remarkably reduced cell infectivity at 41℃ compared to that of parent strain, grown at 37℃. The cell infectivity of parent strain grown at 37℃ was not remarkably decreased at 41℃. However, the growth of parent strain reduced to $10^{4.0}$ $TCID_{50}$/0.1 ml at 29℃. These results suggested that body temperature(41℃) of host(chicken) inhibited the growth of CA strain. Thus, it also seemed to be related with decreasing the pathogenecity.

Hanson and Brandly (1955) suggested that strains of NDV could be grouped as velogenic, mesogenic and lentogenic strain based on MDT in chicken eggs at less than 60 hrs, 60-90 hrs and more than 90 hrs, respectively.

The MDT of CA-30 was 118 hrs in this experiment and belong to be classified as a lentogenic strain. Alexander (1988; 1997), Alexander and Allan (1974) and Lancaster (1966) suggested that ICPI and IVPI could be classified as velogenic, mesogenic and lentogenic strain. So CA-30 strain would be classified into lentogenic strain by those reports.

MDT, ICPI and IVPI of CA-30 strain were reduced compared to those of parent strain, but remained more pathogenicity than that of other lentogenic strain such as Ulsta strain and B1 strain. Therefore, more passages will be needed to reduce the virulence in this experiment.

In protective effects and safety experiments, CA-strain showed less virulence and induced higher antibody titer against ND than that of parent strain. The results of these experiments indicated that chicks vaccinated with CA-30 strain could keep the constant antibody titer in serum.

Because CA-30 strain remained the resistance for low pH and practically induced to get high antibody titer and protective effects, it may be orally administered as a candidate vaccine against ND.

In conclusion, additional genetic and biological studies will be necessary to investigate the relationship between pathogenecity and cold attenuation of CBP-1 strain, and will estimate the differences compared to other commercial vaccine strains.

적 요

열 안정성을 가지고 있는 한국산 꿩에서 분리된 Newcastle disease virus CBP-1주는 9일령 SPF 계태아에 접종되어 37℃에서 배양하는 방법으로 173번(parent주) 누대 배양되었다. 37℃에서 173번 누대 배양된 NDV CBP-1 주를 10 일령 계태아에 접종한 후 저온에서 (29℃) 15번 (CA-15) 30번(CA-30) 누대 배양하였다. 저온순화주인 CA-15주와 CA-30주의 이화학적 성상검사 (열 안정성 실험, 지질 용매에 대한 감수성 실험, 산성 용매에 대한 감수성 실험)와 병원성 실험(MDT, ICPI, IVPI), 온도 감수성 실험, 안전성 실험, 부스터 효과 실험, 방어효과 등을 실험하였고, 37℃에서 173번 누대 배양된 parent주와 비교하였다. 29℃에 적응된 CA-30주는 37 ℃와 41℃에서 세포 감염력이 parent주와 비교할 때 감소하였다. CA-15주와 CA-30주를 56℃에서 30분, 60분, 120분 동안 처리하였을 때 이들 저온 순화주들은 혈구응집능과 세포 감염력을 상실하였다. parent주와 CA-15, CA-30주는 ethyl ether를 10분간 처리했을 때 혈구응집능과 세포 감염력을 모두 상실하였다. 그러나 parent주와 CA-15, CA-30주는 pH 3.0-glycine HCl 완충액에 60분간 처리하였을 때 혈구응집능을 가지고 있었다. Parent주의 대뇌 병원성 지수와 정맥내 병원성 지수는 각각 1.12, 1.45 이었다. 그러나 CA-30주의 대뇌 병원성 지수와 정맥내 병원성 지수는 각각 0.75, 0.00으로 감소하였다. CA-30주의 안전성은 1 일령 병아리에서 실시하였고 parent주와 B-1주와 비교였다. 이들의 안전성은 치사율을 가지고 평가하였다. parent주와 CA-30주와 B-1주의 치사율은 각각 17.5, 12.0. 0.0%이었다. CA-30주가 parent주에 비해 보다 높은 안전성을 보여 주었으나 B-1주에 비해서 아직 높은 치사율을 보여 주고 있었다. 보강 접종 효과 실험은 1 일령에 B-1주를 접종하고 2 주령에 CA-30주와 parent 주를 각각 접종한 후 4 주령에 강독주인 교정원주를 가지고 공격한 후 2 주간 치사율을 관찰하였다, CA-30주로 보강 접종한 군과 parent 주로 보강 접종한 군의 치사율은 10%와 7.5%를 각각 나타났다. CA-30주와 parent주의 방어 효과는 1 일령에 CA-30주와 parent 주를 각각 접종

하고, 2 주령에 다시 CA-30주와 parent주로 보강 접종된 4 주령 닭에 강독주인 교정원주를 접종하고 2 주간 치사율을 관찰하였다. CA-30주 처리군의 치사율은 11.7%이었고, parent주 처리군의 치사율은 20%를 기록하였다. 1 일령에 CA-15주와 CA-30주와 B-1주를 접종한 후, 2 주 후에 혈중항체가를 측정하였을 때 각각 $2^{5.00}$, $2^{9.50}$, $2^{4.45}$ HI 역가를 나타내었고, B-1주로 1 일령에 1차 면역된 병아리에 2 주에 다시 CA-15주와 CA-30주로 각각 면역시킨 닭에서의 혈중항체가는 $2^{7.33}$, $2^{6.90}$ HI 역가를 나타내었다. 1 일령에 CA-15주와 CA-30주로 기초 면역된 병아리에 2 주령에 다시 CA-15주와 CA-30주로 각각 2차 면역시킨 닭에서의 HI 혈중항체가는 각각 $2^{7.95}$, $2^{7.33}$로 나타났다.

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

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