

Effects of the Soil-Derived Microorganism BX-1 on Chicken Newcastle Disease

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Abstract

In recent years, effective microorganisms (EMs) have been administered to humans and domestic animals, and their usefulness has been recognized for promoting health and enhancing immunity. For example, the preventative effects against flu are enhanced by ingestion of *Lactobacillus* by humans, and symptom relief of atopic dermatitis has been reported, with EMs actually used in commercial products. In addition, EM preparations are being used in livestock to prevent infections (e.g. *Salmonella* and *Escherichia coli* infection). In poultry, avian influenza and Newcastle disease are terrible and fatal infectious diseases that cause significant economic damage. Furthermore, countries designated as contaminated with these pathogens can experience major trade problems. Given the above, how to protect livestock from infections safely and at low cost without using disinfectants, antibiotics and vaccines is a major issue. In the present study, we examined whether or not Newcastle disease could be suppressed by feeding chickens BX-1 as an EM feed. A field strain of Newcastle virus was cloned from cloaca swabs of large numbers of dying chickens in a poultry farm in Indonesia by polymerase chain reaction (PCR) and hemagglutination assays. Chicken kidney cells and embryonated eggs were highly sensitive to this virus, and high titers of virus were able to be collected. The experimental viral inoculated to chickens showed a high mortality rate, with high pathogenicity in birds. Conventional chickens were also raised on a diet supplemented with BX-1 and directly infected with the Newcastle virus. The mortality was decreased in these infected birds. Even the low dose of BX-1 had an inhibitory effect on the lethality of the infection. These results suggest that BX-1 intake through an EM diet is effective in controlling Newcastle disease.

*Equally contributed authors.

Keywords

Newcastle Disease, Effective Microorganisms, BX-1, Chicken

1. Introduction

Newcastle disease (ND) is a viral infection to which various species of bird, including poultry, are susceptible [1]. ND outbreaks occur in many countries, including Japan, and the disease is highly contagious exerting a significant economic impact. In Japan, ND is designated an infectious diseases under the livestock infectious disease prevention law for chickens, ducks, quails and turkeys [2]. Other infectious diseases mentioned in this law for poultry include highly pathogenic avian influenza, poultry cholera and salmonella infections.

ND was first discovered in 1926 in Southeast Asia but only named when it was rediscovered in 1927 in Newcastle upon Tyne, UK. The pathogen ND virus (NDV) is a single-stranded RNA virus classified into the *Paramyxoviridae* family along with measles virus, mumps virus, distemper virus, Sendai virus and others. NDV infection occurs through feed, water, equipment and clothing contaminated with feces of affected birds [3]. Symptoms vary greatly depending on the NDV strain, host species, health status and age, among other factors, but common symptoms include those of the respiratory system (dyspnea, cough) and nervous system (depression, loss of appetite, wing weakness, paralysis), swelling of the eyes and neck, diarrhea, abnormal egg shells and decreased egg production [4].

NDV strains are divided into highly toxic, additive and attenuated strains. The highly toxic type is further divided into an Asian type and an American toxic type [1]. Severely virulent strains cause serious respiratory and neurological symptoms, are highly infectious and have a mortality rate of over 80%. Addictive strains cause coughing and egg-laying abnormalities, resulting in a mortality rate of about 10%. The mortality of attenuated strains is negligible. Since the countries that use ND vaccines are designated as contaminated countries, their export of poultry products, such as chicken and eggs, to clean countries, such as the USA and Canada, is not permitted. If a novel ND prevention method could be developed instead of a vaccine, the livestock industry would benefit from a trade perspective.

We established a virus infection prevention method using ostrich antibodies instead of vaccines and antibiotics [5] [6]. In the present study, we attempted to control the viral infection in chickens using effective microorganisms (EMs), a feed additive. In humans, yogurt and beverages containing *Lactobacillus* stabilize the intestinal microflora and suppress the colonization of pathogens, relieve symptoms of atopic dermatitis and block pathogen infection by enhancing immunity [7] [8] [9] [10]. In livestock, various EM agents are used to promote growth and prevent infectious diseases. In chickens, microbial competitive ex-

clusion has been set for preventing food poisoning caused by *Salmonella* and *E. coli* [11] [12] [13].

As a probiotic, the mixed microorganism agent BX-1, which is mainly composed of *Lactobacillus* and yeasts removed from soil, has been commercialized (Kawashima Co., Ltd.) [14]. Since BX-1 is a mixture of soil microorganisms, it does not seem to exert the same effect as the natural intestinal microflora; nevertheless, it can still be expected to be useful as a member of the bacterial microflora with many physiological activities that can benefit the living body. BX-1 is a group of aerobic bacteria, and since it is in a dry powder form, it is easy to handle and inexpensive. BX-1 has been suggested not only to suppress the colonization of intestinal pathogens, such as *Salmonella* and *Escherichia coli*, but also to enhance the vitality of the living body, particularly the immunity.

We herein report the results of our infection experiment using a highly pathogenic field strain of NDV obtained from a poultry farm in Indonesia.

2. Materials and Methods

Newcastle disease virus (ND)

Swab samples from the cloaca of dying chickens on an Indonesia poultry farm were analyzed. RNA was extracted from the chorioallantoic fluid (CAF) of embryonated chicken eggs and culture media of chicken kidney cells inoculated with the swab samples and ND vaccine strain using a viral RNA extraction kit. Mixtures of 2 μ L of RNA, 1 μ L of 5-pmol oligo (dT), 4 μ L of 5 \times RTbuffer, 2 μ L of 10 mM dNTPs, 1 μ L of Rever-Tra Ace and 10 μ L of DEPC water were reverse-transcribed at 42°C; 60 minutes, 99°C; 5 minutes and further, 15°C. Polymerase chain reaction (PCR) was carried out using cDNA, 2 μ L of the cDNA, 12.5 μ L of 2 \times pre-mix Taq, 9.5 μ L of sterilized distilled water and 1 μ L of NDV F1/M1 primer (F1: 5' CAT-CTT-CCC-AAC-TGC-CAC-TG 3', M1: 5' TTC-TCT-AGC-AGT-GGG-ACA-GC 3') [2] under the following conditions: 94°C for 7 minutes and 34 amplification rounds at 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute, annealing at 72°C for 7 minutes and then held at 4°C. The PCR product was used for electrophoresis on an agarose gel with a 100-bp ladder.

Hemaggregation test

The blood samples from chickens were mixed with an equal volume of Alseba solution and centrifuged at 2000 rpm for 7 minutes. The supernatant was removed, and Alseba solution was added again; the mixture was then centrifuged at 2000 rpm for 7 min. The washed blood cells were added to phosphate-buffered saline (PBS) to prepare an erythrocyte solution. The serial dilution of virus solution of the NDV vaccine and farm strain was added to a round-bottom 96-well microplate, and then prepared erythrocytes were placed in each well and incubated at room temperature with pre-immune antibody or anti-NDV antibody for 45 minutes.

Infection experiments in chicken kidney cells

The viral solution of NDV farm strain in Dulbecco's modified medium was added to chicken kidney cells (CKs), and the cytopathic effect (CPE) in the cultures was examined at 48 h post-viral inoculation.

Infection experiment in embryonated eggs

A 100- μ L aliquot of the virus solution of NDV farm strain was injected into the 10-day-old embryonated eggs, and viability was judged 72 h later. The CAF was sampled and used for the *in vivo* infection examination in chickens.

NDV infection experiment using chickens

Ten white Leghorn male chicks at 10 days old were inoculated intranasally with 8 HA of NDV farm strain. The birds were raised under free water and feeding conditions in a BSL-2 facility, and the mortality rate was confirmed by the 10th day. For histopathology, the organs were removed from dying chickens, fixed in formalin, embedded in paraffin, sectioned at 3 μ m and stained with Hematoxylin-Eosin (HE) according to the conventional method. Thereafter, the sections were examined under a microscope.

Effects of BX-1 intake in chickens infected with NDV farm strain

BX-1 powder mixed at 0.02%, 0.2% and 2% (w/w) in commercial chicken formula feed was fed to 10-day-old white leghorn chickens. Over 10 individuals were used for each BX-1 formulation dose. As control animals, other chickens were fed the same commercial diet without BX-1 powder (0%). At one week of BX-1 feeding, 8 HA of the NDV farm strain was been inoculated intranasally into each chicken. These animals were raised under free water and feeding conditions in a BSL-2 facility, and the mortality rate was confirmed by the 10th day.

3. Results

Organisms in BX-1

The species of organisms in BX-1 were confirmed by a third-party laboratory in Japan and listed in **Table 1**. Various microorganisms were found in BX-1, with the *Staphylococcus* group dominant.

Newcastle virus

Embryo deaths were observed in all of the eggs inoculated with cloaca swabs of dying chickens from an Indonesian poultry farm. In addition, when inoculated into CK, the typical CPE appeared in culture cells. PCR using primers specific to NDV showed that the pathogen obtained from the chicken farm in Indonesia was Newcastle virus: An assumed 957-bp single band was detected in both the farm samples and vaccine strain used as a positive control (**Figure 1**). In addition, the solution from farm could aggregate the chicken erythrocytes which were inhibited by the addition of anti-NDV antibody (**Figure 2**). Over 80% of chickens died following intra-nasal inoculation of the CAF. Histopathologically, ND-specific findings, such as severe acute pneumonia and tracheitis, myocarditis and acute enteritis, were observed (**Figure 3**). Accordingly, the pathogens obtained from the Indonesian poultry farm were confirmed to be a highly pathogenic strain of NDV.

Table 1. Micro-organisms in BX-1.

Taxon name	colony Count/dish	Proportion (%)
<i>Staphylococcus saprophyticus</i> group	12,400	59.5524
<i>Staphylococcus kloosii</i>	6787	32.5953
<i>Weissella paramesenteroides</i> group	432	2.0747
<i>Staphylococcus aureus</i> group	272	1.3063
<i>Kocuria koreensis</i>	246	1.1814
<i>Staphylococcus pettenkoferi</i> group	148	0.7108
<i>Bacillus cereus</i> group	110	0.5283
<i>Leuconostoc pseudomesenteroides</i> group	67	0.3218
<i>Pediococcus acidilactici</i> group	24	0.1153
<i>Kocuria kristinae</i>	19	0.0912
<i>Staphylococcus sciuri</i> group	15	0.0720
<i>Corynebacterium variabile</i> group	14	0.0672
<i>Enterococcus italicus</i> group	14	0.0672
<i>Brachybacterium faecium</i> group	11	0.0528
<i>Brevibacterium</i> DL489154_s	10	0.0480
<i>Leuconostoc mesenteroides</i> group	8	0.0384
<i>Corynebacterium nuruki</i> group	7	0.0336
<i>Citricoccus muralis</i> group	5	0.0240
<i>Corynebacterium flavescens</i>	4	0.0192
<i>Corynebacterium minutissimum</i> group	4	0.0192
<i>Enterococcus faecium</i> group	4	0.0192
<i>Bacillus megaterium</i> group	3	0.0144
<i>Lactobacillus acidipiscis</i>	3	0.0144
<i>Lactococcus lactis</i> group	3	0.0144
<i>Actinomyces slackii</i>	2	0.0096
<i>Arthrobacter agilis</i> group	2	0.0096
<i>Arthrobacter echini</i>	2	0.0096
<i>Blautia wexlerae</i>	2	0.0096
<i>Clavibacter michiganensis</i> group	2	0.0096
<i>Kocuria rhizophila</i> group	2	0.0096
<i>Neomicrococcus lactis</i>	2	0.0096
<i>Ruminococcus faecis</i>	2	0.0096
<i>Actinomyces provencensis</i> group	1	0.0048
<i>Anaerostipes hadrus</i> group	1	0.0048
<i>Bacillus carboniphilus</i> group	1	0.0048
<i>Bacillus smithii</i>	1	0.0048
<i>Bifidobacterium adolescentis</i> group	1	0.0048
<i>Blautia faecis</i>	1	0.0048
<i>Brevibacterium iodinum</i> group	1	0.0048

Continued

<i>Caenibacillus caldisaponilyticus</i>	1	0.0048
<i>Caldibacillus debilis</i>	1	0.0048
<i>Corynebacterium tuberculostearicum</i>	1	0.0048
<i>Corynebacterium xerosis</i> group	1	0.0048
<i>Dorea formicigenerans</i>	1	0.0048
<i>Enterococcus saccharolyticus</i> group	1	0.0048
<i>Fusicatenibacter saccharivorans</i>	1	0.0048
<i>Geobacillus stearothermophilus</i> group	1	0.0048
<i>Geobacillus thermoleovorans</i> group	1	0.0048
<i>Geobacillus toebii</i> group	1	0.0048
<i>Kurthia zopfii</i> group	1	0.0048
<i>Lactobacillus dextrinicus</i>	1	0.0048
<i>Lactobacillus sakei</i> group	1	0.0048
<i>Lactococcus taiwanensis</i>	1	0.0048
<i>Listeria grayi</i>	1	0.0048
Dorea PAC000479_s	1	0.0048
Sporobacter PAC001162_s	1	0.0048
Sporobacter PAC001306_s	1	0.0048
PAC001201_g PAC002029_s	1	0.0048
<i>Romboutsia timonensis</i>	1	0.0048
<i>Rummeliibacillus pycnus</i>	1	0.0048
<i>Staphylococcus succinus</i> group	1	0.0048
<i>Weissella ghanensis</i> group	1	0.0048

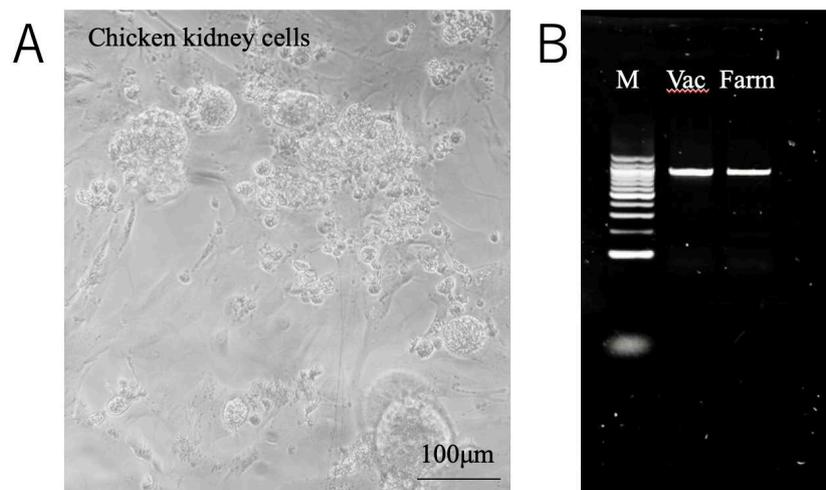


Figure 1. Chicken kidney cells inoculated with a cloaca swab from dead chickens from an Indonesian poultry farm. Cytopathic effects were observed at two days after the inoculation (A). PCR using NDV-specific primers showed a single clear band around 900 bp in the products from both the vaccine strain of NDV and the chorioallantoic fluid from a cloaca swab; the farm chicken sample was found to be NDV. M, marker of a 100-bp ladder, Vac, vaccine strain of NDV; Farm, chicken cloaca swab sample from poultry farm.

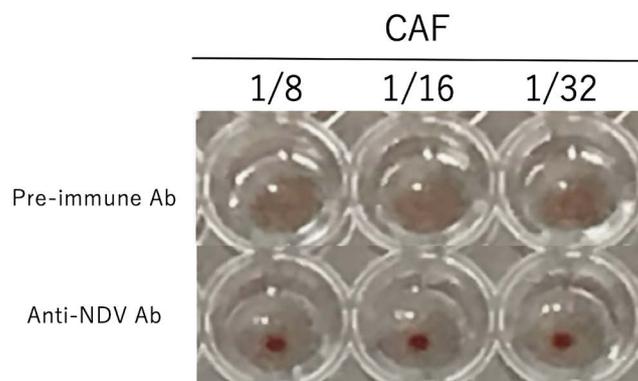


Figure 2. HA test using chicken erythrocytes. The chorioallantoic fluid (CAF) (diluted 8- to 32-fold) after injection with a cloacal swab was mixed with erythrocytes in the wells of a microplate. Hemagglutination was observed with the pre-immune antibody (Ad) as a negative control antibody (A). However, the addition of a specific antibody against NDV (anti-NDV Ab) blocked hemagglutination (B). Accordingly, CAF was found to contain amplified NDV.

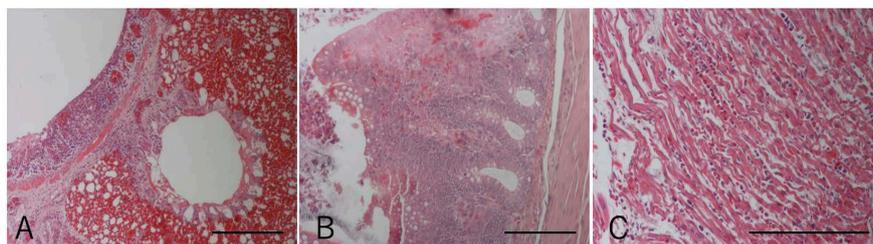


Figure 3. Histopathological findings of chicken lung, intestine and myocardium inoculated intranasally with ND field strain. In the lung, necrosis of the tracheal mucosal epithelium and inflammatory cell infiltration (mainly heterophils), inflammation accompanied by high hyperemia and congestion of the lung, and cell debris and mucus are observed in the lumen of the para-bronchi (A). In the heart, marked inflammation is observed with mucosal epithelial necrosis and heterophil infiltration (B). In the heart, extensive myocardial necrosis and interstitial inflammatory cell infiltration (such as heterophils) are observed (C). Bar, 100 μ m.

Effects of BX-1 intake on ND in chickens

Viral solution (8 HA) of NDV farm strain was inoculated into the nasal cavity of 10-day-old chickens, and the number of deaths was counted for the next 10 days, with or without BX-1. As a result, the mortality rate of ND-infected chickens drastically changed in the flock fed the BX-1 diet for one week before virus inoculation (**Figure 4**). Even the lowest dose of BX-1 at 0.02% exerted an inhibitory effect on mortality. Furthermore, higher doses of BX-1 tended to slow the deaths of birds. Given the lack of any marked differences in the mortality among BX-1 dosages at day 10, implementation of BX-1 for practical use at a poultry farm was considered feasible even at a dose of 0.02%, with consequent cost reduction expected. Since 0.2% - 0.5% BX-1 is used as the commercial products to prevent *Salmonella* colonization in chicken intestinal tracts, this commercial product is effective on the prevention for chickens by both bacterial and viral infections [14].

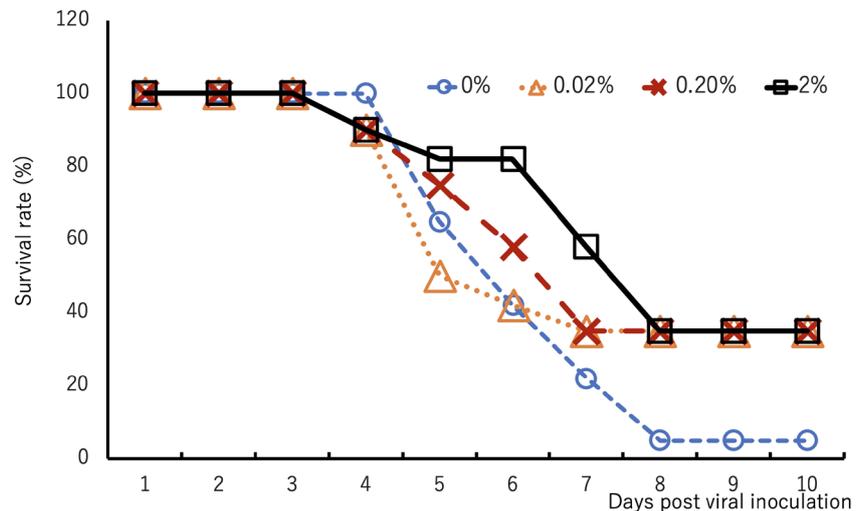


Figure 4. Effect of diet mixed with BX-1 on the survival of NDV field-infected chickens. BX-1 formulations (0%, 0.02%, 0.2%, 2%) were fed freely to chickens, and 1 week later, NDV field strains were inoculated intranasally into the chickens. The survival rates up to 10 days after virus inoculation are indicated in the graph.

4. Discussion

One particularly interesting and potentially important finding from this study was the observation that BX-1, a soil microbial EM agent, was able to control not only bacterial infections, but also viral infections: the intake of soil bacterial preparation BX-1 reduced the mortality rate of chickens infected with highly pathogenic NDV. Soil bacterial EM preparations can be mass-produced, are excellent for storage and transportation and can be supplied at low cost [13]. Therefore, EM is considered useful as a feed additive for livestock.

Since ND is a viral disease and thus operates differently from bacterial diseases, the soil bacterial preparations were unlikely to have contributed directly to the suppression of pathogen colonization in the gastrointestinal tract. Soil bacteria might therefore improve the biological function of chickens and suppress viral infection or reduce the disease severity as found in human atrophic dermatitis [9] [10] [14]. Indeed, there have been reports that the intake of lactic acid bacteria suppresses the flu virus, suggesting a relationship between the microflora in the digestive tract and immunity: the ingestion of bacteria is thought to activate natural killer (NK) cells, resulting in the suppression of virus infection and relief of symptoms [7] [8]. More detailed cellular and molecular analyses will be required in order to verify the mechanism underlying the ND-suppressive effects of the soil bacterial group BX-1.

In nature, birds commonly ingest soil and thereby consume various microorganisms from the environment. In poultry, however, breeding conditions lacking any windows or having only half-windows are becoming increasingly common, and therefore chickens often no longer consume soil. The construction of a ground-based poultry farming system might be useful as a countermeasure against infectious diseases.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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