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Original Article

Clone Purification, Characterization and Standardization of LaSota Strain for Developing a Live Vaccine against Newcastle Disease Virus

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Abstract

Background and Aims: Newcastle disease (ND) is one of the most serious illnesses of chickens. Live vaccines are widely used to prevent chicken from the disease all over the world.

Materials and Methods: To access the effective and potentiate ND vaccine, a homogenous subpopulation from LaSota strain was selected following cultivation of the virus on primary chicken embryofibroblast (CEF) cells. Pathogenicity indices and molecular characterization of a several plaques at 3rd passage were analyzed and then the selected clone was candidate for vaccine development. ND vaccine was prepared according to the standard protocols.

Results: The immunogenicity of the live vaccine was examined in specific pathogen free (SPF) and commercial chickens. The geometric mean hemagglutination-inhibition (HI) titers induced in chickens vaccinated with the cloned vaccine were not significantly differ than those induced in chickens vaccinated with the similar ND clone vaccine.

Conclusion: Efficacy of the ND vaccine was estimated against the virus challenge. The results indicate that the cloned vaccine could confer a complete protection against NDV.

Keywords: Newcastle disease virus, LaSota, clone purification, live vaccine

Introduction

Newcastle disease (ND) is a highly contagious and widespread disease. The first outbreaks of NDV were reported during the mid 1920s in Java, Indonesia and Newcastle-upon-Tyne, England, and within a few years were occurring throughout the world (1). ND is caused by one single serotype of the avian Paramyxovirus serotype 1 (APMV-1). ND virus (NDV) strains can be grouped, in order of increasing pathogenicity, into five pathotypes as highly virulent (viscerotropic velogenic and neurotropic velogenic), intermediate (mesogenic), low virulent (lentogenic), and asymptomatic (2, 3). On the basis of molecular characterization, virulent virus can be confirmed by the presence of multiple basic amino acids at the C-terminus of the F2 subunit of the fusion protein (F) and phenylanine at residue 117, the N-terminus of the F1 subunit. In ND endemic areas, vaccination against ND is an effective method to keep the disease under control. Immunity is derived from neutralizing antibodies formed against the viral hemagglutinin and F glycoproteins, which are responsible for attachment and spread of the virus (4-6). The

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initial immune response to infection with NDV is cell mediated and may be detectable as early as 2-3 days after infection with live vaccine strains (7). The current vaccination programs include the use of lentogenic live vaccine and or inactivated vaccine to induce protective immunity while producing minimal adverse effects in chickens (8).

Several different types of live vaccines are commercially available. Lentogenic strains such as the Hitchner B1 and LaSota are widely used as live vaccines against ND (9). The vaccines are mixtures of sub-populations which differ significantly from each other in immunogenicity and pathogenicity (10, 11). These sub-populations may differ in plaque morphology which correlates with virus virulence. The ND strains show heterogenicity in size of plaques which may lead to the level of post-vaccination reactions usually associated with the use of live ND vaccines. Cloned vaccines which contain only one set of genetic information have the advantage of being more stable than mixed virus strain vaccines (11-13).

This study attempts to select a homogenous population from LaSota strain of NDV and to introduce it as a candidate for developing a live vaccine against ND. The immune responses elicited in both specific pathogen free (SPF) chickens and commercial chickens immunized with the cloned lentogenic live vaccine were evaluated by pathogenicity, safety, efficacy and other immunogenicity aspects.

Methods

Eggs. The fertile SPF white leghorn flock (Venkey,s Co., India) was the source of embryonated eggs for embryonic inoculation and chicken embryo fibroblast cell cultures.

Virus. The LaSota strain of NDV prepared in high titer at Razi Institute was used.

Plaque Purification

Cell culture. Primary chicken embryofibroblast (CEF) cell cultures were prepared from 10-day-old decapitated, eviscerated, crushed, and trypsinized SPF embryos. The cell suspension was washed 3 times with PBS 3-4 times then the tissue fragments were digested in 0.25% trypsin in dissociation medium at 37°C for 1 h. The trypan blue dye exclusion technique was used for determining the viable cell count and the cell concentration was adjusted to 2 × 106 cells/ml with Dulbecco's modified Eagle's medium (DMEM). Cells were plated into 6/12-well culture plates and maintained in DMEM with 10% fetal bovine serum (FBS) and antibiotic solution include 100U/ml penicillin, 100µg/ml streptomycin, and 250 µg /ml fungizone .

Virus dilution and infection. The CEF cells in six-well tissue culture plates were inoculated with 0.2 ml of a serially diluted viral suspension from 10-1 to 10-6. After adsorption for 1 h at 37°C, the monolayer was washed and covered with the overlay medium (DMED containing antibiotics, 0.3 µg/ml trypsin and 0.3% agarose) and incubated for 3 days in a 37°C with 5% CO2 incubator. The cytopathic effect (CPE) which appeared as plaques were observed daily. The virus was plaque purified by three rounds of plaque purification on CEF cells and named Clone12IR.

Cloned virus pathogenicity indices. The pathogenicity indices of the ND cloned strain were detected (14, 15) as followed :

Intra cerebral pathogenicity index (ICPI). The Clone12IR strain of NDV was injected into five 9- to 11-day-old embryonated specific-pathogen-free (SPF) eggs. The infected allantoic fluids were harvested and pooled (HA titre >16) and diluted 1/10 in pure sterile isotonic saline without any additives. 0.05 ml of the diluted virus was injected intracerebrally into each of ten 1-day-old chicks hatched from SPF eggs. The birds were examined every 24 hours for 8 days. At each observation, the birds were scored: 0 if normal, 1 if sick, and 2 if dead. The ICPI was calculated with the mean score per bird per observation over the 8-day period.

Intra venous pathogenicity index (IVPI). The Clone12IR strain of NDV was injected into five 9- to 11-day-old embryonated SPF eggs. The infected allantoic fluids were harvested and pooled (HA titre >16) then diluted 1/10 in pure sterile isotonic saline without any additives. 0.1 ml of the diluted

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virus was injected intramusculary into each of ten 6 weeks old chicks hatched from SPF eggs. The birds were examined every 24 hours for 10 days. At each observation, the birds were scored: 0 if normal, 1 if sick, 2 if paralysed or showing other nervous signs, and 3 if dead. The IVPI was calculated with the mean score per bird per observation over the 8-day period.

Mean death time (MDT). The Clone12IR strain of NDV was injected into five 9- to 11-day-old embryonated SPF eggs. The infected allantoic fluids were harvested and pooled then diluted in sterile saline to give a ten-fold dilution series between 10-6 and 10-9. For each dilution, 0.1 ml was inoculated into the allantoic cavity of each of five 10-day-old embryonated SPF eggs and incubated at 37°C. The remaining virus dilutions are retained at 4°C and another five eggs were inoculated with 0.1 ml of each dilution 8 hours later and incubated at 37°C. Each egg was examined twice daily for 7 days and the times of any embryo deaths were recorded (The highest virus dilution that causes all the embryos inoculated with that dilution to die, was the minimum lethal dose). The MDT was the mean time in hours for the minimum lethal dose to kill all the inoculated embryos.

Molecular characterization. The forward and reverse primers were designed for amplification of F gene in length of 1662 bp: FF: 5'-ATGGGCTCCAAACCTTCTAC-3' and FR: 5'-TCAGCTCTTGTAGTGGCTCTCATC-3‘ (16). RNA was extracted using High Pure RNA Isolation (Roche, Germany). The F gene of NDV was amplified by one-step Premix RT-PCR kit (iNtRON, South Korea). The RT-PCR reaction consisted of 1 cycle of 42°C for 60 min and 95°C for 5 min and 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min, followed by a final elongation step of 72°C for 10 min. The amplified fragment was cloned in TA cloning vector (pTZ57R/T, Thermo Fisher Scientific) and the extracted plasmid sequenced in both directions. The Clone12IR F gene sequence was deposited in GenBank under accession number KJ174522. Neighbor-Joining phylogenic tree was constructed with 1000 bootstrapping in MEGA4. The amino acids sequence of the cloned virus was determined on the cleavage site of fusion protein.

Vaccine seed preparation and standardization. According to serological, molecular, and pathogenicity analysis, one NDV clone was candidate for vaccine seed preparation. At first step, the master seed virus was prepared by inoculating of the selected isolate in 9-11-day-old embryonated SPF eggs. The amniallantoic fluids (AAF) were harvested and centrifuged for 30 min at 3000 rpm at 4°C. Then the sterility, safety, efficacy and purity, assays were managed on the AAFs according to the standard protocol (14).

Vaccine development and validation

Vaccine production. ND clone vaccine was developed according to the Office International des Epizooties (OIE) (3) and FAO (14) protocols.

Dose finding. Vaccine dose was determined by inoculation of 5, 5.5, 6, 6.5, and 7 EID50 log10 of the seed virus into five groups of 7-day-old SPF chickens (n=10) via eye-drop method. Serum samples were taken prior and three weeks post immunization (pi) and IBDV antibody titers evaluated using haemagglutination inhibition (HI) test.

Experimental procedure in SPF chickens. Eighty SPF chickens at one week of age were divided into two treatment groups compromising 30 birds. Chickens in group A were received Clone12IR vaccine and group B the same imported vaccine by eye-drop. The remaining 20 birds were kept as non-vaccinated control. At 1 to 8 weeks post-vaccination sera were collected and assayed for antibody levels against NDV by HI. Twenty chickens per groups were challenged by intramuscular with >105 EID50 of a vNDV Herts-33 strain at 3 weeks pi. For safety test, ten field doses of vaccine were administered by eye-drop to each of 15 SPF chickens of 1-wk-old age. The chickens were observed up to 21 days for any clinical symptom or mortality.

Experimental procedure in commercial chickens. A total number of 200 Ross-308 broiler chicks were divided into two treatment groups of 100 each and vaccinated with the same regimen. Twenty chickens were considered as non-vaccinated control group.

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Challenge and serological assessment were performed in the same manner as detailed above.

Statistical analysis. Serological data were analyzed by using t test. The data for challenge assays were compared using Pearson Chi-square test. $P<0.05$ was considered as meaningful differences.

Results

Virulence determination. The NDV strain was cloned after three rounds of plaque purification on CEF cells. The ICPI, IVPI and MDT were determined to analyze the ND-cloned virus pathogenicity. The Clone12IR virus exhibited ICPI, IVPI and MDT values of 0.32, 0, and 104 characterizing as lentogenic NDV .

Nucleotide and predicted amino acid sequence analysis. Nucleotide sequence of the Clone12IR F gene and deduced amino acid were determined. Phylogenic data (Figure 1) was revealed that Clone12IR clustered with LaSota and other lentogen ND viruses with >98% identity. The proteolytic cleavage site of the virus is characterized by five basic amino acids at the carboxy terminus of F2 and a phenylalanine at the amino terminus of F1 (112G-R-Q-G-R*L117) associated with the lentogenic NDV.

Immunogenicity and protective efficacy evaluation. Vaccine dose was determined by HI assay. The best virus titer Clone12IR vaccine was estimated >106 EID50/bird .

To determine if Clone12IR vaccine provided immunity against ND, SPF chickens were received the vaccine by eye-drop method. The NDV antibody titers in the sera of vaccinated and unvaccinated chicks were assayed before and after vaccination. The geometric mean HI antibody titers against NDV were raised in the treated groups compared with the control chicks. The mean of titers three weeks post vaccination were 5.26 for group A, 5.05 for group B, and 0.3 (negative) for control. The results were summarized in table 1.

In efficacy test, the signs observed were recorded and protection rates were calculated

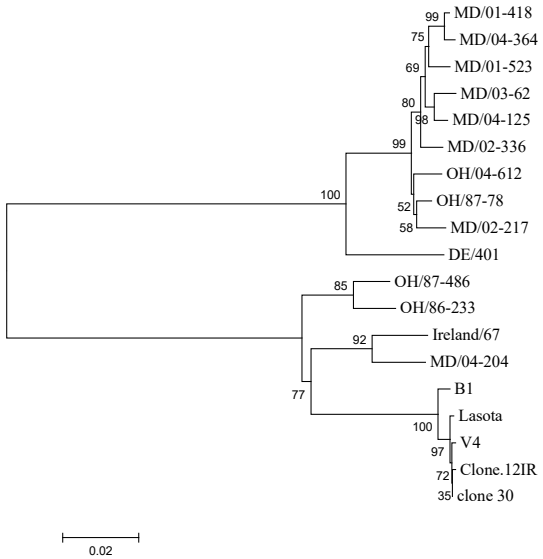

Fig. 1. The evolutionary history of Newcastle disease virus fusion protein (F) sequences was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.39127728 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1662 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

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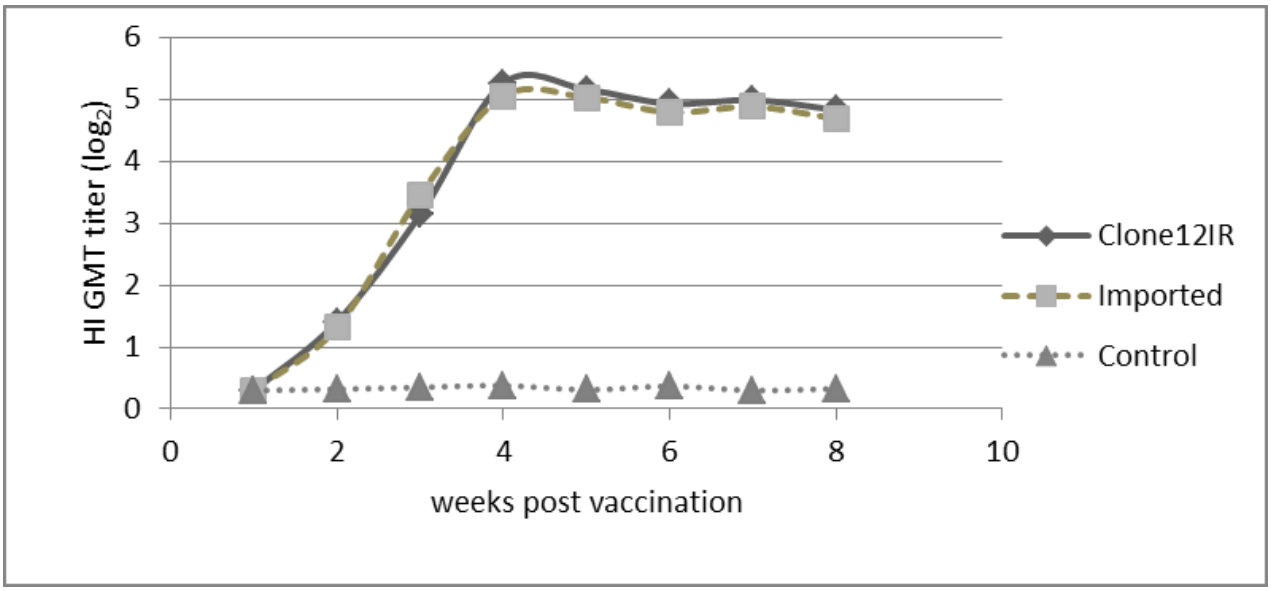

Fig. 2. Protective efficacy of Clone12IR ND vaccine in SPF chickens.

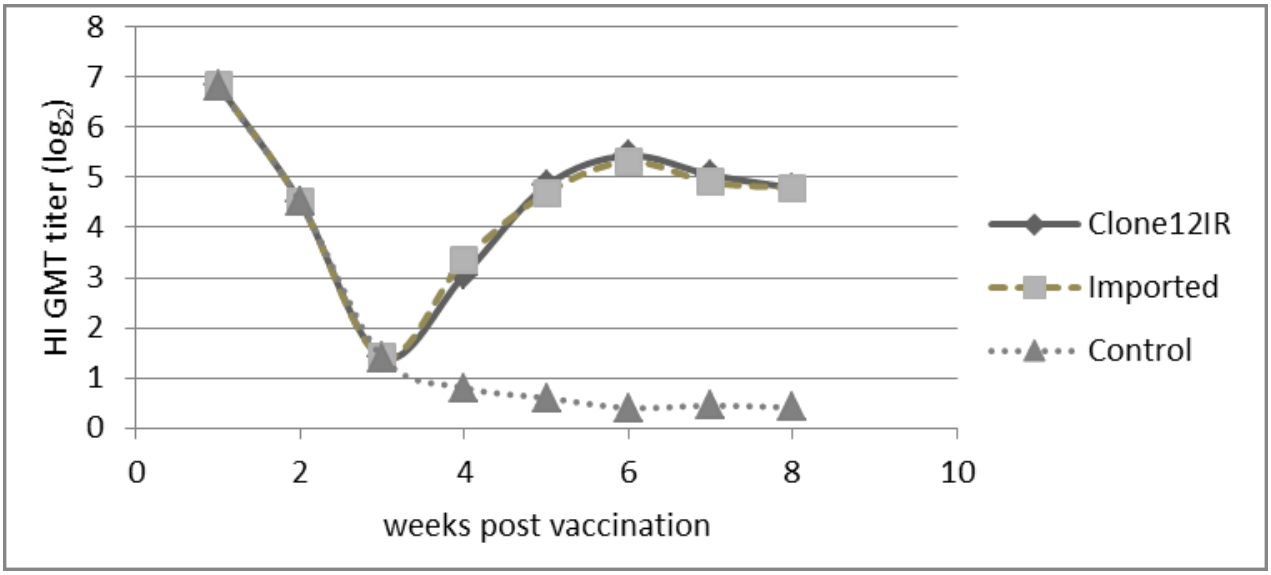

Fig. 3. Protective efficacy of Clone12IR ND vaccine in Ross-308 chickens.

(Figure 2). No sign of ND was seen in chickens of group A until the end of experiment. All chicks vaccinated with Clone12IR and 90% of the chickens vaccinated with the imported ND vaccine were protected from challenge. Seventeen chickens (85%) of control groups died and the remained were shown the marked signs of ND.

In commercial chicken trial, the HI antibody titers were raised in both vaccinated groups compared with the control chicks (Table 2). The mean of titers three weeks post vaccination were 5.43 for group A, 5.31 for group B, and 0.4 (negative) for control. In challenge trial chickens vaccinated with Clone12IR revealed 95% protection. Group B showed 90% protection and two birds showed mild clinical disease, while 55% chickens in control group dead and the remaining (n=9) showed the signs of ND disease (Figure 3).

Discussion

Without a doubt, ND disease is one of the most economically damaging diseases for the poultry sector and included in the OIE List A (3). From the first outbreak of ND to now, the

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Table 1: Vaccination of SPF chickens with Clone12IR ND vaccine. Serum HI titers against NDV are recorded.

Bleeding time (week)	Clone.12IR (group A)		Clone imported (group B)	
	HI	GMT	HI	GMT
1	1,1,1,0,0,0,0,0,0,0	0.30	1,1,1,0,0,0,0,0,0,0	0.30
2	2,2,2,2,2,1,1,1,1,0	1.41	2,2,2,2,1,1,1,1,1,0	1.32
3	4,4,4,3,3,3,3,3,3,2	3.14	5,4,4,4,4,4,3,3,3,3	3.64
4	7,6,5,5,5,5,5,5,5,5	5.26	6,6,6,5,5,5,5,5,4,4	5.05
5	6,6,6,5,5,5,5,5,5,4	5.16	6,6,6,6,5,5,5,4,4,4	5.05
6	6,6,6,5,5,5,5,4,4,4	4.93	6,6,6,5,5,5,5,4,4,3	4.79
7	7,6,6,6,5,5,4,4,4,4	4.99	7,6,6,6,5,5,4,4,4,3	4.85
8	6,6,6,5,5,5,4,4,4,4	4.83	6,6,6,5,5,5,4,4,4,3	4.69

Table 2: Vaccination of Ross chickens with Clone12IR ND vaccine. Serum HI titers against NDV are recorded.

Bleeding time (week)	Clone.12IR		Clone.imported	
	HI	GMT	HI	GMT
0	8,8,8,7,7,7,7,6,6,5	6.83	8,8,8,7,7,7,7,6,6,5	6.83
1	6,6,5,5,5,4,4,4,4,3	4.50	6,6,5,5,5,4,4,4,4,3	4.50
2	2,2,2,2,2,1,1,1,1,0	1.41	2,2,2,2,2,1,1,1,1,0	1.41
3	4,4,4,4,4,3,3,2,2,2	3.06	5,4,4,4,4,4,3,3,2,2	3.36
4	7,7,6,5,5,5,4,4,4,3	4.84	6,6,5,6,5,5,4,4,4,3	4.69
5	7,6,6,6,6,5,5,5,5,4	5.43	7,6,6,6,6,5,5,5,4,4	5.31
6	6,6,6,5,5,5,5,5,4,4	5.05	6,6,6,5,5,5,5,5,4,3	4.90
7	6,6,6,5,5,5,5,4,4,3	4.79	6,6,6,5,5,5,5,4,4,3	4.79
8	6,6,5,5,5,5,5,4,4,3	4.71	6,6,5,5,5,5,4,4,4,3	4.60

disease represents one of the most devastating poultry diseases. Despite having as etiological agent one single serotype, ND has continuously challenged poultry industries worldwide. High mortality rate, rapid spread to other flocks, great variation in type and severity of the disease have caused some problems for producers (1, 5). Types of commercial efficacious live ND vaccines are applied to prevent the serious economic losses. Some post-vaccination reactions on birds' performance are usually associated with the use of live ND vaccines (17-19). Avoid the negative impact and decease the level of vaccine reaction became a very important issue for poultry vaccine production companies.

Here, we focused to generate vaccine strain which would induces less post-vaccination reactions through the clone selection procedure and then produce an efficacious live ND vaccine from it. Some markers include plaque morphology, elution time of the virus from hemagglutinates, heat stability of the hemagglutinin, and the ability of NDV strains to hemagglutinate mammalian erythrocyte have been introduced to differentiate NDV strains (20-22). Among them plaque morphology represents virus virulence feature . The LaSota strain of NDV was varied in producing plaques when cultured in primary CEF cells. Based on the morphological properties of the plaques, subpopulations from the ND strain were defined. The heterogenicity of the plaques and the presence of large plaques have been associated with pathogenic indices (10, 22). Each selected plaque derived from one single virus particle was verified for pathogenicity. A clone which has the same molecular and pathogenicity characteristics as the original LaSota strain was candidate as vaccine strain. Immunogenicity and efficacy of the prepared homogenous cloned ND vaccine were then assayed in both SPF and commercial chickens. The results indicate that the cloned vaccine consists of a homogenous subpopulation can induce high and reproducible immunity with less vaccine reactions. Protection from disease correlated with the presence of antibody titers determined by HI. The geometric mean HI titers induced in

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chickens vaccinated with Clone12IR were not significantly differ than those induced in chickens vaccinated with the similar imported clone vaccine .

The development of a successful ND control program requires close collaboration between those involved in the production and testing of the ND vaccine. As the level of humoral antibodies increase in vaccinated birds, the number of infected birds and the amount of vNDV shed decreased. The effective levels of humoral antibodies could be raised by increasing the homogenicity of the virus vaccine strain. To re-evaluate the control and vaccination for protecting the commercial poultry from ND in endemic countries, cloned ND vaccines have been introduced in market in the 80's. The vaccines had a very good features include lower reactivity than LaSota strain, excellent immunogenicity, and does not revert to virulence. Therefore the strategies were applied in this project. Our studies indicate that Clone12IR vaccine has potential to provide superior protection in primary and re-vaccination against NDV in the control campaign.

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