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Full Length Research Paper

Isolation and characterization of Newcastle disease virus from ostriches in Iran

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Newcastle disease (ND) is a highly contagious infection of poultry that causes nervous signs and mortality in young ostriches. This study has been done during two years from 2008 to 2010 in Iran, in order to explore ND in ostriches died following nervous signs, and carcasses of these ostriches examined by different methods including histopathology, bacteriology and virology. No histopathological sign was found in the samples. In bacteriological study no significant bacterial agents were isolated. In virology tests, Newcastle disease virus (NDV) was isolated from 4 brain samples. Intracerebral pathogenicity index (ICPI) and mean death time of chicken embryo (MDT) values of these isolates were between 1.7-1.9 and 38-42 h, respectively, that indicated virulence of these viruses. The reverse transcription-polymerase chain reaction (RT-PCR) test confirmed NDV isolation from samples and its virulence. This study is the first report of NDV isolation from the Iranian commercial ostrich farms. With the respect to high virulence of isolated viruses and endemic pattern of virus in Iran, control program should be organized.

Key words: Newcastle disease virus, ostrich, Iran, MDT, ICPI, RT-PCR.

INTRODUCTION

Commercial rearing of the ostriches (*Struthio camelus*) was introduced to Iran 10 years ago and at present, ostriches are kept in many farms all over country. There is little information about the Infectious diseases that occur in commercial ostriches in Iran. One of the most serious infectious diseases in ostriches is Newcastle disease. Newcastle disease is caused by Avian paramyxovirus type-1(APMV-1) which is classified with the other avian paramyxoviruses in the genus Avulavirus, subfamily Paramyxovirinae, family Paramyxoviridae, order Monanegavirals. NDV is an enveloped virus, has a negative sense single strand RNA genome (Lamb et al., 2005). The genome contains six genes: $3'$ NP-P-M-F-, HN-L-$5'$ that encode six major proteins (Chamber et al., 1986; Alexander, 2008). NDV is categorized into lentogenic, mesogenic, velogenic and asymptomathic pathotype on the basis of virulence for chickens (Alexander, 2008). In the laboratory, the pathogenicity of NDV strains are estimated by different tests. The most widely used tests are the ICPI in day- old chicks and MDT in 9-day- old embryonated specific pathogen free (SPF) eggs. ICPI values may vary between 0 (Lentogenic) and 2 (Velognic) and MDT values may vary between ≤60 (velogenic) to 90≥ in lentognic strains (Alexander, 1997).

Recently, the molecular methods such as RT-PCR and amino acid sequencing are used for diagnosis of ND and amino acid sequence of the cleavage site of F protein of NDV strains which is considered as a virulence criterion (Panda et al., 2004; Alexander, 2008). NDV is able to infect over 241 species of birds of all age groups, but considering the variation in pathogenicity of different strains of viruses, diseases severity is highly dependent

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Abbreviations: ND, Newcastle disease; **NDV,** newcastle disease virus; **ICPI,** intracerebral pathogenicity index; **MDT,** mean death time of chicken embryo; **RT-PCR,** reverse transcription-polymerase chain reaction; **HA,** heamagglutination **HI,** heamagglutination inhibition; **EM,** electronmicrocopy; SPF, specific pathogen free.

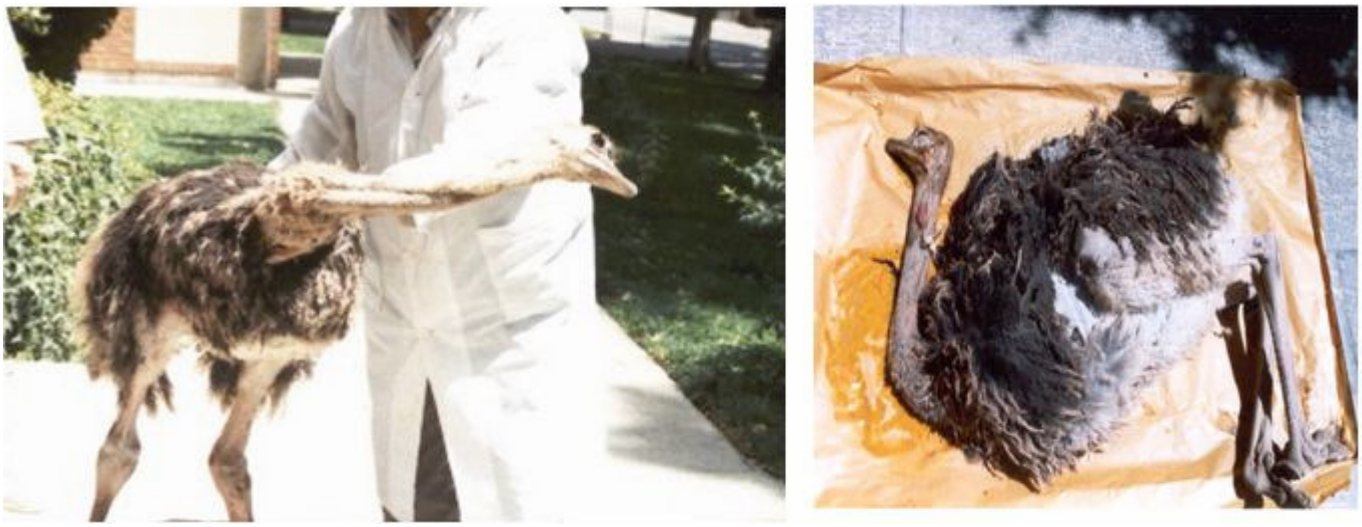

Figure 1. Typical signs of ND in ostriches referred to Razi institute.

on the host species (Kaleta and Baldauf, 1988; Alexander, 2008). NDV can cause nervous disease and high mortality in non-vaccinated ostriches. The clinical signs and mortality seen varied considerably with age, and disease affects young ostriches up to one year of age more often, although, virulent viruses may also affect adult birds (Alexander, 2000). The most important signs of ND in ostriches are depression and nervous signs.

Newcastle disease in ostriches was first reported in zoo birds in 1950s from Africa (Alexander, 2000). The outbreak of ND in commercial ostriches was first reported in 1989 by Samberg in Israel (Samberg et al., 1989). Huchzermeyer isolated NDV from three outbreaks of ND in ostriches with low mortality (Huchzermeyer et al., 1995). Jorgensen isolated virulent strain of NDV from a flock of unaffected ostriches and emus in Denmark (Jorgensen et al., 1998). Characterization of ND viruses isolated during outbreaks in ostriches has shown them to be indistinguishable from viruses infecting chickens in the locality (Alexander, 2000). Although, ND is endemic in Iran there is not any report of disease in ostriches and this study is the first report of NDV isolation from the Iranian commercial ostrich farms.

MATERIALS AND METHODS

Within two years of this study (2008 to 2010), 15 ostrich's carcasses in all ages died following nervous symptoms such as depression, muscular tremors, limp neck, torticollosis, paralysis of leg and wing, inability to stand up, submitted to the laboratory of poultry diseases department of Razi vaccine and serum research institute, Karaj-Iran, (Figure 1) and post-mortem examinations were performed on samples obtained from bone marrow, liver, brain, and intestine. Samples were taken for histopathology, bacteriology and virology examination. Histopathological slides were prepared from tissues.

For bacteriological examination, samples were streaked on specific media to study on presence of pathogenic bacterial agents causing nervous signs. Virological examination was carried out according to guidelines of the OIE (2008). Briefly, each specimen was suspended to a concentration of 20% PBS containing antibiotic and incubated for 2 h at room temperature then the suspension was centrifuged at 1000 g for 10 min and clarified supernatant was harvested and 0.2 ml of that was inoculated into the allantoic cavity of four, nine-day-old SPF chick embryos. The eggs incubated at 37°C for 5 days and were candled daily to monitor for embryo death.

At the end of the incubation period the allantoic fluids harvested from eggs with dead embryos and examined for heamagglutination (HA) activity. Heamagglutination Inhibition (HI) test was performed on HA positive allantoic fluids. HA and HI tests were performed by using 1% chicken red blood cell and reference chicken antisera according to OIE procedures (OIE, 2008). Harvested infectious allantoic fluid was examined by electronmicrocopy (EM) for detection of presence of virus's particles. APMV-1 isolates were tested for pathogenicity by means of ICPI in one-day old SPF chicks and MDT in embryonated SPF eggs according to guidelines of the manual for the isolation of avian pathogen (Alexander, 1997).

Molecular characterization

To confirm biological results RT-PCR reaction was performed using three pair's oligonucleotide primers designed by (Kant et al., 1997) (Table 1). The RT-PCR using primer pair A+B results in a 362 bp fragment with all NDV strain, primer pair A+C results in a fragment of 254 bp only with RNA from virulent NDV, and using primer pair A+D ends in a 254 bp fragment with non-virulent NDV strains. Viral RNA was extracted from 200 µl Volumes of clarified infected allantoic fluid using, Roch: High Pure Viral nucleic acid kit. The two step RT-PCR reaction was carried out using Revert Aid (M-Mulv). For cDNA synthesis 5 µl of extracted RNA was mixed with 2 µl (200 p mol) of specific primer A and 2 µl of dNTPs mix, then it was carried out in a 20 µl reaction volume containing: 4 µl RT 5x Buffer, 0.25 µl RNase inhibitor, 1 µl (200 unit) M-Mulv Enzyme and 5.75 µl DNase, RNase free distilled water. After this, reaction mixture was incubated at 42°C for 40 min.

For PCR 7 µl cDNA was amplified using the PCR mastermix for a reaction volume of 25 µl containing: 0.15 µl of each primer (15 p mol), 2.5 µl 10x PCR Buffer (with 1.5 mM $Mgcl_2$), 0.5 µl (10 Mm) dNTPs mix, 1 U Taq polymerase and 13.7 µl DNase, RNase

Table 1. Sense, sequence and location of used primers (Kant et al., 1997).

Code	Sense	Sequence	Location
A	+	5'-TTGATGGCAGGCCTCFFGC-3'	141-159
B	-	5'-GGAGGATGTTGGCAGCATT-3'	503-485
C	-	5'-AGCGT(C/T)TCTGTCTCCT3'	395-380
D	-	5'-G(A/G)CG(A/G)CCCTGT(C/T)TCCC-3'	395-380

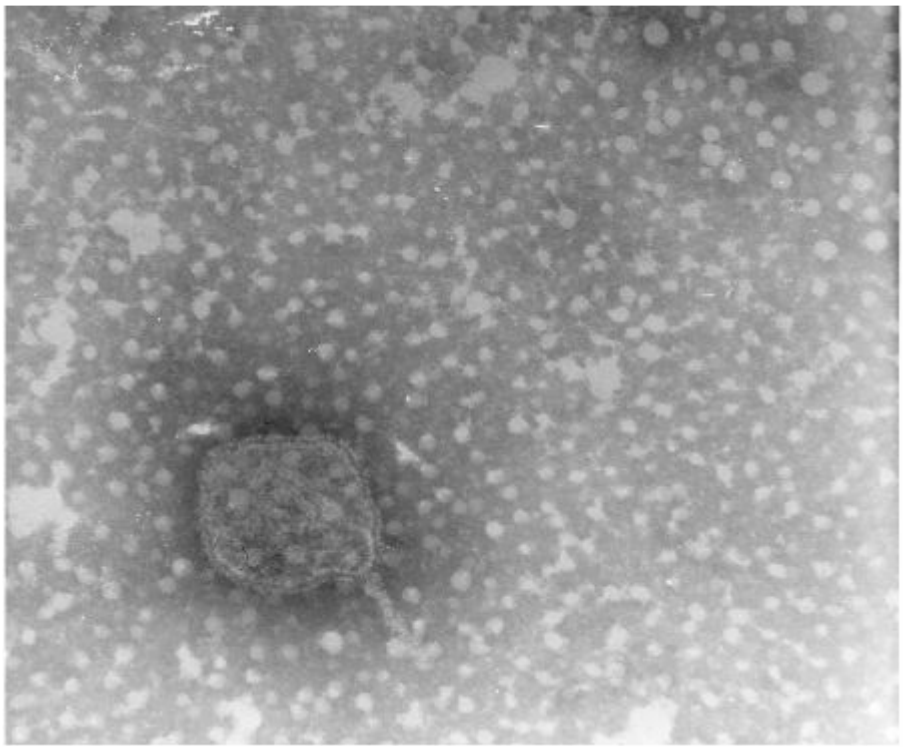

Figure 2. Electron micrograph of negatively stained PMV particles following inoculation of brain samples into chorioallantoic sacs of SPF eggs.

distilled water. Amplification was carried out in a thermal cycler with an initial denaturation at 94°C for 5 min followed by a sequence of 35 cycle (denaturation 94°C for 1 min, annealing 58°C for 1 min (53°C for primer pair A+C), extension 72°C for 1min) and final extension hold at 72°C for 10 min. The PCR products were detected and analyzed by electrophoresis on 1% agarose gel containing ethidium bromaide.

RESULTS

From all 15 transmitted ostrich's carcasses to laboratory, in necropsy no detectible signs of Newcastle disease or other infectious disease were seen. In histopathological observation, we did not find any specific changes for ND. In bacteriology no significant bacterial agents were isolated either. According to virological study NDV isolated from four brain samples, while isolation of virus from other tissues was unsuccessful. All NDV isolated ostriches were in range 3 - 5 monthes of age. While in adult died ones no virus was found. None of these birds were previously vaccinated against Newcastle disease. Virus particles that are characteristics of PMVs were observed by EM examination of the allantoic fluid (Figure 2). ICPI values for these isolates were in range 1.8 - 1.9 and MDT values were in range 37 to 42 h. RT-PCR with primer pair A+B confirmed isolation of NDV from brain samples (Figure 3). Reaction with primer pair A+C showed these isolates were virulent for chickens (Figure 4 and the test with primer pair A+D confirmed these isolates as non vaccinal strains (Figure 5)

DISCUSSION

Many reports have been published on ND in ostriches and other ratites. First report of ND in ostriches was kept

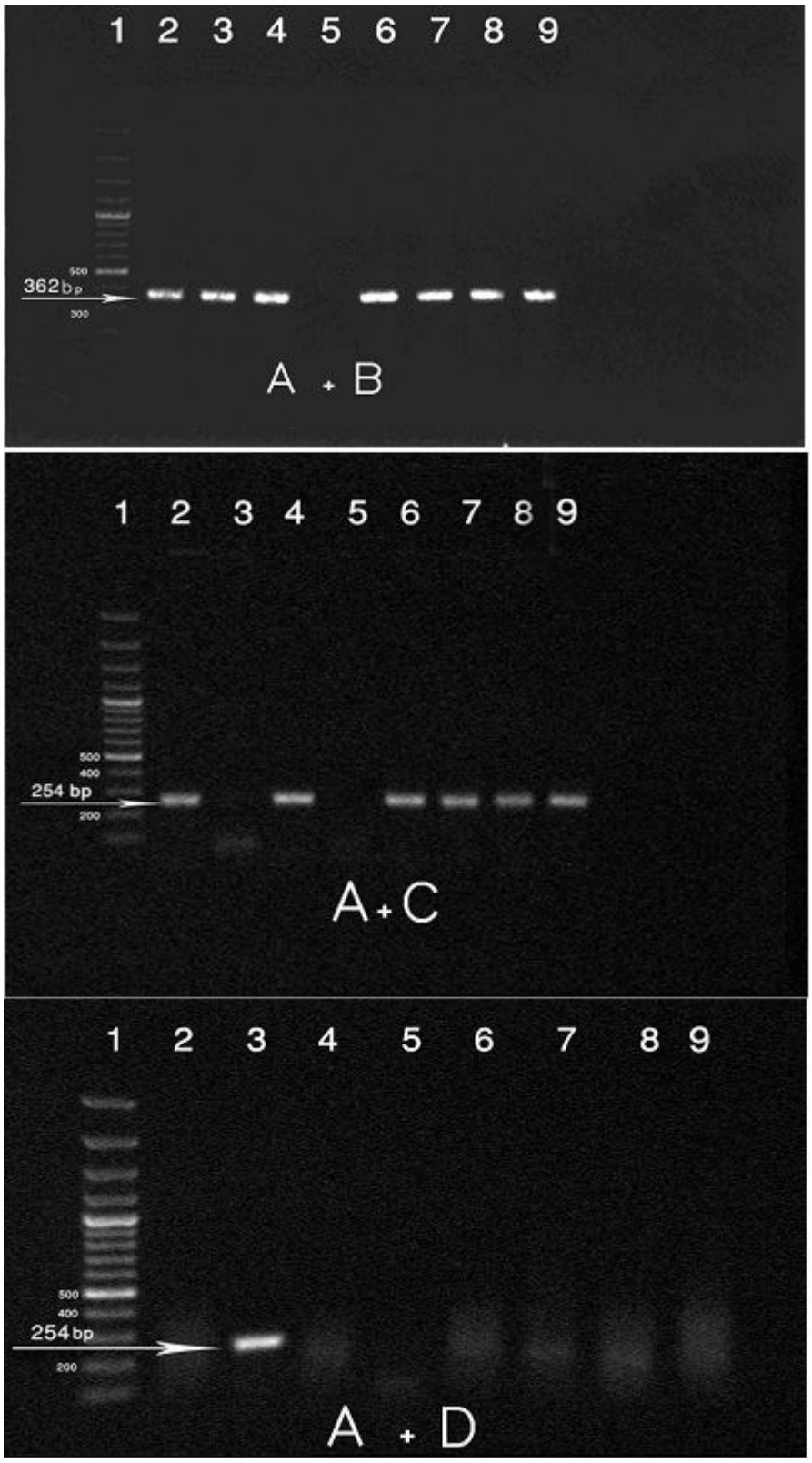

Figure 3. A+B: result of RT-PCR using primer pair A+B specific for all NDV,A+C: using primer pair A+C specific for virulent NDV and A+D: using primer pair A+D specific for non-virulent NDV. (lane1) ladder 100 Fermentase (lane2) Herts33 virulent strain, (line3) B_1, (line4) NR43: a virulent NDV strain isolated from fowl in Iran, (Lane5) negative control, (lane6-9) isolated viruses from ostriches in this study.

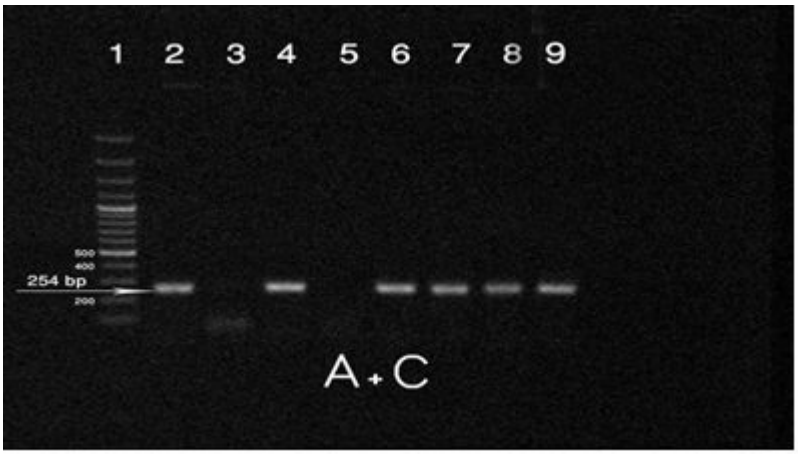

Figure 4. Using primer pair A+C specific for virulent NDV strains; (lane1) ladder 100 Fermentase (lane2) Herts33 virulent strains, (lane3) B_1, (lane4) NR43: a virulent NDV strain isolated from fowl in Iran, (Lane5) negative control, (lane6-9) isolated viruses from ostriches in this study.

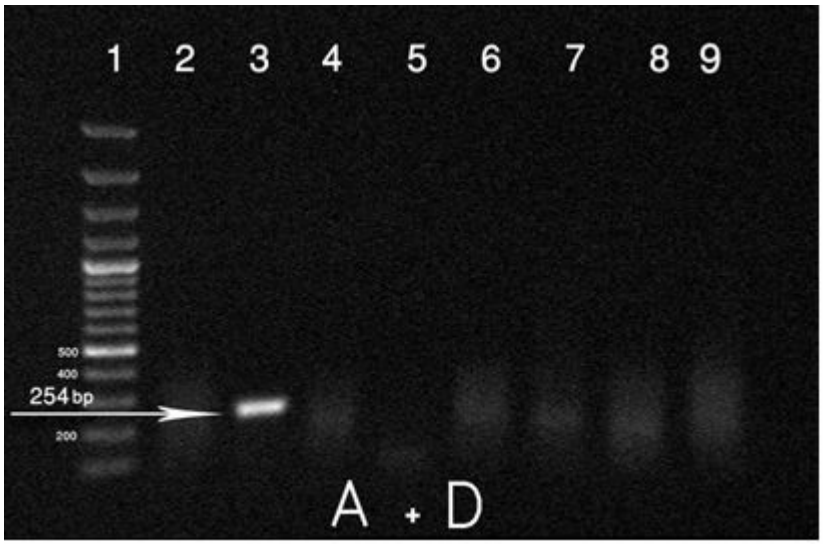

Figure 5. Using primer pair A+D specific for non -virulent NDV strains; (lane1) ladder 100 Fermentase (lane2) Herts33 virulent strains, (lane3) B_1, (lane4) NR43: a virulent NDV strain isolated from fowl in Iran, (Lane5) negative control, (lane6-9) isolated viruses from ostriches in this study.

in zoos in 1950s (Alexander, 2000). In commercial flock, Samberg reported 28% mortality in 5 to 9 months old ostriches following nervous signs in Israel for the first time (Samberg et al., 1989). Allwright recorded a series of outbreaks in southern African countries in farmed ostriches during a widespread ND epizootic in fowl between 1993 and 1995 (Allwright, 1996). Huchzermeyer reported nervous signs in infected ostrich chicks (Huchzermeyer, 1993). In these studies a marked difference in clinical signs and mortality were seen between young ostriches and adults, and they could isolate the virus from brain sample in natural infection. The absence of the clinical disease in the older ostriches may be explained by age resistance to ND virus. In present study, the clinical signs and virus isolation from brain in young ostriches corresponds with previous reports (Samberg et al., 1998; Huchzermeyer, 1993; Allwright, 1996). In contrast, Jorgensen reported isolation of NDV from intestinal tissue of died ostriches while held in quarantine in Denmark (Jorgensen et al., 1998) while we could not isolated virus from other tissues samples. ICPI values of isolates in our study were in range 1.8 to 1.9 and MDT values were in range 37 to 42 and these values predicated the high pathogenicity of these viruses

in fowl. In prior studies these indexes did not measured.

RT-PCR test with Kant's designed primers confirmed virus isolation from brain samples and its virulence, these findings confirmed prior experiments. Molecular detection using these primers was done for the first time, about ostrich isolates which showed the potential of this test for fast detection of virus in ostrich flock. The probable source of the infection in the under study ostriches is unknown and might be due to commercial poultry farms established near ostrich farms. This is in agreement with Samberg study. Since ostriches are kept in free-range, feral birds are considered as a common source of infection. However, transmission of NDV between ostrich and other birds and recognition of the source of the infection needs more studies. Because the virus could not be isolated from the other carcasses in this study, it is essential to look for the other viruses which cause neural symptoms like Borna and encephalitis. Because ND outbreaks have been occurred in Iran, further surveillance of NDV in ostriches is recommended. According to experimental challenge, successful protection against ND reported with using vaccine regimens in ostriches (Vrewoerd, 1997; Alexander, 2000). Therefore, in order to control the disease of legal provision such as; isolation of the farms, certification of ostrich movement, and performing routine vaccine programs are recommended. In conclusion, despite the isolation of NDV in this study, further molecular and nucleotide sequencing studies for identifying separated viruses are inevitable.

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