Comparative Analysis of Early Chicken Response Following Infection with Genotype VII and Lentogenic Strains of Newcastle Disease Virus

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ABSTRACT

The study of the host immune response of Newcastle disease virus (NDV) infected chickens and the relation between the innate immune response and the intensity of disease during infection in vivo can explain the pathogenesis of NDV. Sixty specific pathogen free (SPF) chickens were divided into four groups. (G1, G2 and G3) were inoculated intra nasally by three different strains of NDV; highly virulent velogenic viscerotropic (VVNDV) (NDV.VII.1.1/Egy-Matr/ELH.5/2018), velogenic neurotropic (VNNDV) (NDV.VII.1.1/Egy-Elbeh/ELH.1/2020) and lentogenic strain (LaSota). The fourth group was used as negative control group. The virus shedding, cytokine measurement, clinical signs, mortality rate, and pathological lesions were compared among these different groups. Our findings revealed that the amount of IFN-α and IFN-γ were increased at 48- and 72-hour post-infection by 2-3 folds in the VVNDV group more than VNNDV group. In addition, the VVNDV group showed higher level of IFN-α and IFN-γ more than LaSota group by 5-6 folds. The course of the disease was severe and rapid 2–4 days in the VVNDV group and much longer 3-6 days in the VNNDV group ending in 100% mortality in both groups but no signs or mortality in the LaSota group. VNNDV group displayed neurological lesions in brain including non-suppurative encephalitis, gliosis, and perivascular cuffing. The three-group infected with velogenic and lentogenic shed the virus till the 4th day post infection. In conclusion, we found comparative changes in the pathogenesis and cytokine expression in birds infected with velogenic viscerotropic, velogenic neurotropic, and lentogenic NDV strains.

Keywords: Cytokines, Lentogenic, NDV, Velogenic neurotropic and Velogenic viscerotropic.

INTRODUCTION

Newcastle disease (ND) is a highly contagious disease that affects poultry industry through worldwide distribution and excessive flock losses. NDV is also called avian Orthoavulavirus 1 (AOaV-1). It is a single-stranded, non-segmented RNA virus, its family is (Paramyxoviridae) and its genus is (Avulavirus) (Alexander and Senne, 2008). Nucleo-protein, phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large protein are the six structural proteins encoded by the RNA genome (Chambers et al., 1986; Alexander and Senne, 2008). V and W proteins are other
proteins that are produced by an RNA-editing event during the transcription of the P gene (Harrison et al., 2011). Five different pathotypes for NDV could be identified based on the pathogenicity; velogenic (high virulence) which could be further subdivided into velogenic viscerotropic and velogenic neurotropic, mesogenic (moderate virulence), lentogenic (low virulence), and finally symptomatic (OIE, 2021). In most endemic regions, immunization against NDV is routinely practiced by using available commercial live or inactivated vaccines as the major means of prevention against virulent ND strains (Absalon et al., 2019). There is plenty of knowledge on the role of adaptive immunity (particularly humoral and cell-mediated immunity) in NDV infection protection (Kapczynski et al., 2013). On the contrary, the possible role of host innate immune responses in ND pathogenesis remains to be fully explained. The most significant innate immune response and initial line of defense against viral infections is interferon (IFN) production. (Santhakumar et al., 2017). Innate immune systems are the first steps of antimicrobial invasion and restricting pathogen spread. When the innate immune system is unable to control infections, the host initiates a specialized immunological response (adaptive immunity). There are two antigens (F and HN surface glycoproteins) that appear to be required for a protective avian immune response to NDV (Seal et al., 2000). The IFNs (particularly IFNα and IFN-β) have antiviral properties that affect many viral infections in birds (Munir et al., 2005; Kapczynski et al., 2013; Qu, H. 2013., Santhakumar et al., 2017). In-vitro study carried by (Lomniczi et al., 1973), revealed that IFN induction is stronger for mesogenic and velogenic strains of NDV as compared to lentogenic viruses. In macrophages, NDV can generate IFN-α and IFN-β mRNA, as well as IFN-γ mRNA in peripheral blood mononuclear cells (Sick et al., 1998). In this study, we compared the early chicken response following infection with genotype VII and lentogenic strains of NDV.

MATERIALS AND METHODS

Virus strain:
Two strains of Newcastle disease virus were isolated and propagated in 10-day-old specific-pathogen-free (SPF) chicken eggs in the National Laboratory of veterinary quality control on poultry production (NLQP), Egypt. Their EID<sub>50</sub> was calculated as 10<sup>6</sup> /ml. The allantoic fluids were extracted from dead eggs and preserved at -70°C. LaSota (ND LaSota MLV, Co. IFT. Reg. No.547) was propagated using the same method.

Birds:
Sixty SPF chickens three weeks old were gotten from a farm of eggs devoid of pathogens in Kom Oshim, Fayoum Governorate, Egypt. Chickens were kept in biosafety level 3 (BSL3) isolators with ad libitum access to feed and water and were subjected to the same standard management condition, NLQP-animal health research institute (AHRI). Throughout the experiment, the experimental and control groups were housed in different rooms to avoid cross-contamination.

Experimental design:
SPF chickens were divided randomly into four groups at three weeks old (n = 15 per group), group 1 (G1-control group) were inoculated intranasally with 0.1 ml phosphate buffered saline (PBS), group 2 (G2-LaSota group) were inoculated intranasally with NDV lentogenic strain (LaSota) at a dose of 10<sup>6</sup> EID<sub>50</sub> in 0.1 ml, group 3 (G3-neutrotropic infected group were inoculated intranasally with NDV velogenic strain (NDV.VII.1.1/EgyElbeh/ELH.1/2020) at a dose of 10<sup>6</sup> EID<sub>50</sub> in 0.1 ml and group 4 (G4-viscerotropic infected group) were injected intra nasally with NDV velogenic strain (NDV.VII.1.1/Egy-Matr/ELH.5/2018) at a dose of 10<sup>6</sup> EID<sub>50</sub> in 0.1 ml. Daily clinical signs observation and mortality rate were recorded. Necropsy was carried out on freshly dead birds for PM examination. On the 2<sup>nd</sup> and 3<sup>rd</sup> day post-infection (dpi), heparinized peripheral blood was obtained from three birds in each group randomly and the samples were put into iced water immediately and...
kept at -20°C for PCR till the total RNA was isolated for IFNs (IFNα and IFN-γ). Cloacal swabs were collected at 4th (dpi) for titrating the virus shedding by qRT-PCR.

**Measurement of Cytokines by qRT-PCR:**
On days 2nd and 3rd dpi RNA levels of IFN-α and IFN-γ were determined by qRT-PCR in three birds from each group. In the first, the total RNA was extracted from theuffy coat according to RNeasy Mini Kit instructions. The thermal cycler conditions were performed by the following steps: one cycle for 30 min at 50 °C, one cycle for 10 min at 94 °C, 40 cycles for 15 seconds at 94 °C, and a final cycle of extension for 1min at 60 °C. Table (1) shows the primers for these cytokine genes. The Stratagene MX3005P software was used to analyze qRT-PCR results, CT values, and amplification curves. The CT of each sample was compared to that of the negative control group to evaluate the variation of gene expression on the RNA of the different samples.

**Detection of NDV shedding by qRT-PCR:**
On the 4th dpi cloacal swabs from 3 birds of each control, VNDV and LaSota infected group were collected in 2 mL of transport media with a final concentration of 100 units/mL of penicillin G 10 µg/mL of gentamicin, and 56 µg/mL of amphotericin B and stored at −70 °C until its process. Viral RNA Mini Kit (QIAGEN) and QIAamp were used to extract the RNA. Then the qRT-PCR targeting the M gene was performed (wise et al., 2004). A standard curve for virus quantification was established by titration of RNA extraction 10-fold serial dilutions in nuclease-free water.

**Histopathological examination:**
Tissues for histopathological examination were collected from the four groups on the 3rd dpi. Sections from the brain, lung, spleen, proventriculus, and cecal tonsil were fixed in 10% neutral buffered formalin, sectioned, stained with hematoxylin and eosin (H & E), and histologically examined (Bancroft and Gamble, 2008).

**RESULTS**

**Clinical signs and mortality rate:**

In G1 and G2 groups, there were no clinical signs or mortalities observed on chickens till the end of the experiment. All inoculated chickens in G4 showed early signs from the 2nd dpi such as depression, listlessness, greenish diarrhea, ruffled feathers, conjunctivitis, and reduced feed intake, the signs became more severe with 50% mortality at the 3rd dpi which reached 100% at 4th dpi. In G3 the signs started mildly at the 3rd dpi in the form of depression, mild respiratory signs, lateral recumbency, and unable to right themselves, the signs became more severe at the 4th dpi, and the mortality was 50% at the 5th dpi reached 100% at 6th dpi (Fig. 1).

**Expression of Cytokines (IFN-α gene & IFN-γ) in the Buffy coat:**
The results showed that the level of IFN-α has increased following infection with NDV isolates. At 48-hour post-infection (hpi) 2-fold in G4 more than G3, and 2-fold in G3 more than G2, but at 72 hpi, there was a 2-fold increase than 48 hpi in G4 and G3 with the lowest changes in G2 (Fig. 2 A).

IFN-γ RNA amounts were increased by the two NDV isolates more than IFN-α within the same group. Expression values of IFN-γ at 48 hpi increased by 3-fold than G3 and 5-fold than G2. The increased expression level of the IFN-γ gene was up to 10-fold at 72 hpi and a high value 3-fold increase in G4, 2-fold in G3, with the lowest change in IFN-γ expression was found in G2 (Fig. 2 B).

**Virus shedding:**
All the cloacal swabs collected from VNDV and LaSota infected groups were positive for NDV by qRT-PCR targeting the M gene during the entire period of the experiment. Regarding shedding of NDV from experimentally infected chicken (Fig. 3), it was found that 100% of collected samples from experimentally infected chicken (G3, G4, and LaSota G2) were found to be positive for NDV with CT (19, 23, and 27) respectively. On the other side, none of the samples collected from control (non-
infected) chicken showed positive results for the ND virus.

**Gross pathology:**
No gross lesions were recorded in G1 and G2. Severe lesions were observed in the inoculated birds of G4 as severe congestion and edema of the lungs, a hemorrhagic necrotizing area in the proventriculus, and cecal tonsils with a greenish film of bile exudates and plaques coating the intestinal mucosa around the cecal tonsils, the spleen was enlarged and mottled. While the inoculated birds in G3 showed mild lesions than G4 with mild petechial hemorrhages in the cerebrum and cerebellum.

**Histopathology:**
There were no lesions in groups G1 and G2. Lesions were more severe in G4 than G3 except for the lesion of the brain was more severe in G3 than G4 which showed moderate lesions. Brain suffered from non-suppurative encephalitis as multifocal satellitosis, gliosis, neuronal degeneration, and necrosis with vacuolation of the neuropil and multifocal perivascular cuffing and vacuolation (Fig.4 A-B). Cecal tonsil was affected in G3 by mild focal necrosis and inflammatory cells infiltration in the lamina propria (Fig.4 C), while in G4 affected by the destruction of intestinal lymphoid areas, hemorrhages, and inflammatory cell infiltration in the associated lymphatic nodules (Fig.4 D). Lung in G3 showed a mild focal area of congestion and hemorrhages (Fig. 4 E) while G4 showed severe congestion and hemorrhages with ruptured alveoli (hemorrhagic pneumonia) (Fig. 4 F). Also, there was tracheitis in both groups with severe desquamation and deciliation of the epithelial mucous membrane, edema, and infiltration of inflammatory cells. Proventriculus in G3 showed focal necrosis and lymphocytic infiltration of mucosal folds (Fig. 4 G) and in G4 severe necrosis and desquamation of glandular epithelial cells and mucosal folds with infiltration of inflammatory cells (Fig. 4 H). Spleen showed lymphoid depletion in both groups and apoptosis of lymphocytes (Fig. 4 K). See table 2.

**Table (1):** Primers used for cytokine expression:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN α Fw: GACAGCCAACGCCAAAGC</td>
<td>Eldaghayes et al., 2006</td>
<td></td>
</tr>
<tr>
<td>Rev: GTCGCTGCTGTCAAGCATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(FAM) CTCAACCGATCCACCGCTACACC (TAMRA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28SrRNA Fw. GGCGAAGCCAGAGGAACT</td>
<td>Kaiser, 2000</td>
<td></td>
</tr>
<tr>
<td>Rev: GACGACCGATTITGACGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(FAM) AGGACCGCTACGGACCTCCACCA (TAMRA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN γ Fw: AAACAAACCTTCCTGATGGCGT</td>
<td>Kaiser, 2000</td>
<td></td>
</tr>
<tr>
<td>Rev: CTGGATTCTCAAGTCGCATCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(FAM) TGAAAGATATCATGGACCTCCAGCTC (TAMRA)</td>
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</tbody>
</table>

**Table (2):** Pathological lesions of different organs in G3 and G4:

<table>
<thead>
<tr>
<th>Organs</th>
<th>Lesions</th>
<th>G3 (velogenic viscerotropic (VVNDV))</th>
<th>G4 (velogenic neurotropic (VNNDV))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Neuronal degeneration, Satellitosis and gliosis</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Organ</td>
<td>Description</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Necrosis with vacuolation of the neuropil</td>
<td></td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Multifocal perivascular cuffing and vacuolation</td>
<td></td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Cecal tonsils</td>
<td>Necrosis and inflammatory cells infiltration in the lamina propria</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Destruction of intestinal lymphoid areas</td>
<td>_</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Hemorrhages, and inflammatory cell infiltration in the associated lymphatic nodules</td>
<td>_</td>
<td>+++</td>
</tr>
<tr>
<td>Lung</td>
<td>Congestion and hemorrhages with ruptured alveoli</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Trachea</td>
<td>Desquamation and deciliation of the epithelial mucous membrane</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Edema, and infiltration of inflammatory cells</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Proventriculus</td>
<td>Necrosis and lymphocytic infiltration of mucosal folds</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Desquamation of glandular epithelial cells and mucosal folds</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Spleen</td>
<td>Lymphoid depletion and apoptosis of lymphocytes</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

+++ = >5 positive cells per high-power field (400x); ++ = 1 and <5 positive cells per high-power field; + = 1 positive cell per high-power field and _ = no positive cells

Figure (1): Mortality rate after inoculation of chickens with NDV in different groups of the experiment.
Figure (2): Changes of IFN-α (A) and IFN-γ (B) levels in the Buffy coat of the chickens in different experimental groups during the experiment.

Figure (3): Shedding of NDV from the chickens in different experimental groups during the experiment.
Figure (4): Histopathological lesions of groups 3 & 4. **Brain**, (A) G3 showed degenerated neurons, gliosis and satellitosis with vacuolation (arrow) perivascular edema (asterisk). (B) G4 showed moderate gliosis(arrow). **Cecal tonsils**, (C) G3 showed focal necrosis and inflammatory cell infiltration in the lamina propria (arrow). (D) G4 showed excessive hemorrhages and inflammatory cells infiltration in the lymphatic nodules (arrow). **Lung**, (E) G3 showed diffuse hemorrhages. (F) G4 showed severe congestion and hemorrhages with ruptured alveoli (hemorrhagic pneumonia). **Proventriculus**, (G) G3 showed focal necrosis and lymphocytic infiltration of mucosal folds (arrow). (H) G4 showed hemorrhages and edema in the inter follicular space (asterisk), necrosis and destruction of glandular epithelial cells and mucosal folds with inflammatory cells infiltration (arrow). (H&E X100 & 200). **Spleen** (K) lymphoid depletion in both groups (thick arrow) (H&E X100) apoptosis of lymphocytes (thin arrow) (H&E X400).

**DISCUSSION**

For understanding the early pathogenesis of different pathotypes of NDV by utilizing different virulence strains (velogenic and lentogenic). Two cytokines IFN-γ and IFN-α RNA expression and their relationship with the severity of NDV. The velogenic strain of NDV is classified as viscerotrophic and neurotropic depending upon their behavior after infection (Alexander and Senne, 2008). When an infection is still in its early stages, NDV replicates in macrophages and
lymphocytes of different lymphoid organs, particularly after infection with the velogenic strains (Brown et al., 1999; Kommers et al., 2002). Numerous cytokines are produced mostly by macrophages and lymphocytes (Lowenthal et al., 1995). These lymphoid organs get activated after infection, resulting in a significant increase in IFN- production, which enhances the destruction of cell-associated pathogens. It is also a major pro-inflammatory cytokine that increases nitric oxide production, which has a harmful effect on the host (Abbaset al., 2010) and decreases the replication of the virus (Djeraba et al., 2002; Parviziet al., 2009). When macrophages are excessively activated, they produce lysosomal enzymes, reactive oxygen species, and nitric oxide, which can harm normal host tissues. These antimicrobial components (reactive peroxy nitrite radicals) cannot differentiate between self and invading antigens. So, if these components are released into the extracellular environment, they might cause tissue damage. (Abbaset al., 2010). Also, necrotic cells leak their cytoplasmic contents into the extracellular fluid, producing an inflammatory response. (Lam et al., 1996). Because NDV is an RNA virus, type I interferons are expected to be highly expressed early in infection. Type I IFN (α and β subtypes) is produced mostly by macrophages, it is the host’s natural defensive system against viral infection (Samuel, 2001) and it contributes to the protection of surrounding cells from viral infection by causing these cells to produce antiviral products (interferon-stimulated genes-ISGs) that prevent viral replication (Krishnamurthy et al., 2006). High levels of cytokines, particularly IFN- γ, were associated with severe clinical signs and depression in birds infected with the velogenic viscerotrophic virus. (Samuel, 2001; Degenet al., 2005).

In this experiment, IFN- γ and IFN -α RNA levels were found to be higher in G4 chickens infected with (NDV.VII.1.1/Egy-Elbeh/ELH.1/2020) at 2nd and 3rd dpi by two and three folds more than birds of G3 infected with (NDV.VII.1.1/Egy-Elbeh/ELH.1/2020) and by four and five folds more than birds of LaSota G2. These differences were explained by (Abbas et al., 2010) Who found that macrophages infected with the typical VVND strains get activated, resulting in a significant increase in IFN- γ production as well as the formation of reactive intermediates that cause tissue damage. On the other hand, other strains (VNND and lentogenic) may not penetrate macrophages or lymphocytes to the same extent. Therefore, even while type I interferon is still inhibited, the animal can survive longer as the innate response is not very harmful, so the viruses can spread to other organs. This explains why the virus shedding of VNND was found at early CT than VVND.

NDV has been shown in some investigations to be able to stop type I IFN production. To prevent the host immunological reaction, NDV uses an accessory protein generated from the P protein called V protein. The V protein stimulates the disintegration of the signal transducers and activators of transcription (STAT-1) protein, resulting in ISG transcriptional inactivation and type I IFN inhibition. (Huang et al., 2003; Munir et al., 2005).

The results we found in the level of IFN- α and IFN- γ were parallel to clinical, gross, and microscopic findings, as there were not any lesions or mortalities in Lasota G2, this corresponds with Brown et al. (1999), who reported no overt disease in birds inoculated with lentogenic pathotypes. The course of the disease was rapid 2–4 days in G4 and much longer 3-6 days in G3 ending in 100% mortality in both groups. The clinical signs were more severe in G4 than G3 as depression, ruffled feathers, and mild respiratory signs with greenish diarrhea in G4 while G3 suffered from mild neurological signs restricted on lateral recumbency and unable to right themselves because most neurological symptoms appear between 5 and 10 dpi. (Brown et al. 1999). These results were like the findings of (Brown, et al. 1999; Kommers, et al.2003 and Susta, et al. 2010).
Genotype VII NDV strains are genotype-specific phenotype resulting in viral deregulation and proliferation in host cells affecting lesion severity (Zenglei et al. 2012). This explains the severity of visceral lesions in G4 and neurological lesions in G3. Respiratory impairment was observed early after intranasal inoculation. The virus uses the sialic acid receptor to bind to the respiratory epithelial cell (Wen et al. 2016) producing congestion and increased mucus secretion into the trachea, where the body will try to remove virus antigen from the respiratory system, protecting the epithelial surface against virus attachment and invasion (Kothlow et al. 2008) leading to tracheitis, desquamation of epithelial cells with lymphocytic infiltration which is similar to finding of (Alaa et al. 2020). Following primary viremia, NDV reaches different organs through blood circulation where it replicates causing vascular injury (Wen et al. 2016). So, there were congestion, hemorrhages, and edema in some internal organs early after infection followed by degeneration, necrosis, and infiltration of inflammatory cells. In the lung there was hemorrhagic pneumonia with ruptured alveoli in both velogenic groups. These results are in accordance with (Alaa et al. 2020) while (Kommers, et al.2003 and Susta, et al. 2010) reported no pulmonary effect caused by VVND but only by VNND.

Intestinal lesions were prominent in the liver, intestine, and proventriculus which it starts at the point where the proventriculus and the gizzard meet (the site of lymphoid aggregate) which appear as desquamation of glandular epithelial cells and fusion of mucosal folds. Similar lesions were reported by (Alaa, et al. 2020 and Congriev, et al. 2020). Lymphoid organs were affected especially cecal tonsils that were coated by a greenish film of bile exudates and plaques, spleen enlarged and mottled. Lymphoid follicles showed severe lymphoid depletion, necrosis, and apoptosis. These results agree with (Brown, et al. 1999, Susta, et al. 2010 and Congriev, et al. 2020). As NDV can replicate in macrophages, it can cause functional changes and subsequent viral spread (Brown et al., 1999) while apoptosis is stimulated by immune response rather than by direct viral damage (Moss et al., 2004 and Schobesberger et al., 2005). Apoptosis and necrosis may not be the only reasons for lymphoid depletion in immunological organs, and cell migration from these organs could also be a factor in the observed lymphoid depletion. (Anis.et al., 2013). The observed lesions in the neurological system were because of the presence of NDV in the brain, which causes vascular and neuron damage, resulting in an inflammatory reaction. The inflammatory response started with macrophages and then spread in perivascular cuffing from which it expanded to adjacent astrocytes and microglia (Zachary, et al., 2012).

CONCLUSION
In this investigation, we found changes in cytokine RNA expression in birds infected with velogenic viscerotropic, velogenic neurotropic, and lentogenic NDV strains. During the very early stages of infection, the expression levels of IFN-γ and IFN-α RNA are elevated at 48 and 72 hpi in velogenic viscerotropic viruses when compared to lentogenic and velogenic neurotropic viruses. These cytokines were found to be at higher levels when the birds were displaying severe clinical symptoms. Combining both arms of the adaptive immune response provides the best protection for birds while also lowering the danger of transmission to susceptible birds.

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