



## Research Article

# Velogenic Viscerotropic Newcastle Disease Virus Produces Variable Pathogenicity in Two Chicken Breeds

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### ABSTRACT

Newcastle disease has a high prevalence in Pakistan and outbreaks are reported even in vaccinated chickens. Disease resistance of various breeds to Newcastle disease needs to be studied for better husbandry and breeding decisions. The aim of the present study was to compare resistance of two chicken breeds to Velogenic Viscerotropic Newcastle Disease Virus (VVNDV) infection. It was carried by using different pathogenicity tests like mean death time (MDT) of chicken embryos, intracerebral pathogenicity index (ICPI) in day old chicks and intravenous pathogenicity (IVPI) in 6-week old chickens. MDT in VVNDV inoculated embryonated eggs of Fayoumi and White Leghorn breeds was 41.5±16 and 42.1±10.3 hours, respectively but difference was not statistically significant. In embryonated eggs of Fayoumi 0.6 log<sub>10</sub> higher EID<sub>50</sub> and significantly higher (0.8 log<sub>2</sub>) hemagglutination titers of VVND virus than White Leghorn breed was observed. The ICPI in Fayoumi and White Leghorn, was found to be 0.93 and 0.71 while IVPI in Fayoumi and White Leghorn was found to be 2.51 and 2.65, respectively. Average lesion scores for intravenously infected chickens were 4.8 and 4.9 while in contact exposed chickens scores were 6.6 and 8.4 for Fayoumi and White Leghorn breeds, respectively. Embryos and day-old chicks of Fayoumi were found more susceptible to VVNDV than White Leghorn as they showed lower MDT along with higher HA / EID<sub>50</sub> tiers and ICPI, scores than White Leghorn. Both intravenously inoculated as well as contact-exposed chickens of Fayoumi breed were found more resistant as they had lower IVPI and postmortem lesion scores.

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### INTRODUCTION

Newcastle disease (ND) is regarded as one of the most important diseases in the poultry industry of the world. Among the viral diseases affecting poultry, Newcastle disease (ND) has evolved into a greater challenge due to emergence of novel strains and ultimately vaccine failures. Since 2010, various outbreaks of Velogenic Viscerotropic Newcastle Disease (VVND) have been reported throughout Pakistan. Moreover, a failure of previously effective live vaccines in protecting the birds from current field isolates has also been reported. The velogenic form of ND continues to appear in both vaccinated flocks as well as unvaccinated commercial, wild captive and backyard poultry birds (Shabbir et al., 2013, Munir, et al., 2012a).

Genetic basis of disease resistance have been studied in chicken and genes responsible for genetic resistance to scrapie, Infectious Bursal Disease, Avian lymphoid leukosis, and Marek's disease have been discovered (Cheng, 2010). Moreover, some chickens can survive even challenged with NDV deserves further attention. Various breeds of poultry may vary in terms of resistance to infectious diseases like VVND. However effective quantifiable tests measures are needed.

In present study, pathogenicity of VVNDV has been evaluated in Fayoumi and White Leghorn (WLH) breeds through embryonic mean death time (MDT), intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI), VVNDV production and contact-exposure to the virus.

### MATERIALS AND METHODS

#### *Virus Isolation and Culture*

The VVNDV isolate, APMV-1/chicken/Multan /-19-06/2012, was propagated in the allantoic cavity of nine-day old embryonated chicken eggs. The amnioallantoic fluid (AAF) were obtained after the death of embryos (72 hours post infection). The AAF harvested from embryonated eggs was pooled, aliquoted and frozen at -80°C. This virus stock solution was used for all experimental trials.

#### *Embryo Lethal Dose<sub>50</sub> (ELD<sub>50</sub>), Embryo Infectious Dose<sub>50</sub> (EID<sub>50</sub>) and Mean Death Time (MDT) Determination*

EID<sub>50</sub>/ml and MDT of the VVNDV were determined in 09 days old embryonated eggs of two chicken breeds by the method as described by Alexander and Senne, 2008. Briefly, fresh AAF having VVNDV was serially (10 fold) diluted in sterile normal saline to give dilutions ranging from 10<sup>-1</sup> to

$10^{-25}$ . A volume of 0.1 ml of each dilution ( $10^{-5}$  to  $10^{-25}$ ) was inoculated through chorio-allantoic sac (CAS) route in embryonated chicken eggs (5 eggs were used for each dilution). The eggs were incubated at 37°C, were candled every eight hours daily for next 7 days and the time of embryo mortality was noted. The embryonic mean death time was determined as described by Alexander and Senne, 2008. All eggs showing embryo mortality were chilled for three hours, AAFs were harvested and tested by both the micro and rapid HA assay. Embryos not showing mortality up to 7 days post-inoculation were also opened and tested for VVNDV by HA test. Calculation  $EID_{50}$  was performed by using Reed and Muench method (Reed and Muench, 1938).

#### Rapid and Micro Hemagglutination (HA) Assay:

The micro HA titration was performed in 96-well micro titer plates while the rapid HA was performed on a marble stone as is already described (Alexander and Senne 2008).

#### Intracerebral Pathogenicity Index (ICPI)

ICPI was determined in day-old chicks by intracerebral injection of VVNDV as per the standard procedure described in OIE manual (OIE 2012). Briefly, freshly obtained AAF having VVNDV with an HA titer  $>1/16$  was tenfold diluted in normal saline solution. A volume of 50  $\mu$ l was intra-cerebrally inoculated into 10 day-old chicks of each breed. The sterile normal saline solution was also intra-cerebrally inoculated into ten chicks (of both breeds), which served as negative controls. The inoculated chicks were then monitored daily and scored as normal (0), sick or paralyzed (1), and dead (2), to compile an index for the 8 days observation period.

#### Intravenous Pathogenicity Index (IVPI)

Chickens of both breeds were raised at SPVC Karachi for up to six weeks of age. The standard vaccination schedule was followed except that these were not vaccinated against ND. Briefly, ten 6-week old chickens of each breed were inoculated with 0.1 ml of 10-fold diluted freshly obtained AAF containing VVNDV, in normal saline solution. Moreover, control groups comprising eight birds of each breed were also injected with sterile normal saline solution. The birds were examined daily for 10 days and observation for each bird was recorded and scored accordingly (0 if normal 1 if sick, 2 if paralyzed, and 3 if dead). The IVPI is the

mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period (Alexander and Senne 2008).

#### Study of VVNDV Induced Pathology in Two Breeds of Chickens

Ten 6-week old birds of both breeds were intravenously infected with VVNDV for determination of IVPI. While another group of ten birds of each breed with contact-exposed by intermixing with already intravenously infected chickens. The birds were observed daily for morbidity and mortality. The postmortem examination was performed of dead birds to study the gross lesions produced during the course of disease. On necropsy, any lesions on trachea, brain, bursa of Fabricius, eyelids, proventriculus, small intestine and caecal tonsils were examined and lesions were scored as none (0), mild (1) or severe (2). Scores per bird (0–14) were calculated by adding scores of all seven organs. Scores of all birds in each group were combined and means were determined.

#### Statistical Analysis

Analysis of variance (One way ANOVA) was performed to find out the significant differences among the data obtained for MDT and HA / HI titration, using the computer package Student Edition of Statistics (SXW), version. 8.1 (copy right 2005, Analytical Software, USA). The least significant differences of mean (LSD = 0.05) test was used to compare the significant differences between the groups

## RESULTS

#### VVNDV Inoculation of Embryonated Eggs of Two Breeds Produces Variable $EID_{50}$ and HA Titers

$EID_{50}$  / ml of virus stock solution in in Fayoumi embryonated eggs was  $10^{24.2}$  where as in WLH embryonated eggs it was  $10^{23.6}$  (Figure 1). The embryos of Fayoumi breed produced 06 times more VVND virus particles than WLH breed. The mean HA titers of VVNDV produced in embryonated eggs of Fayoumi and WLH were  $7.9 \pm 1.5$  and  $7.1 \pm 1.6$ , respectively (Figure 2). The differences of HA titers of two breeds were analyzed and were found statistically significant.

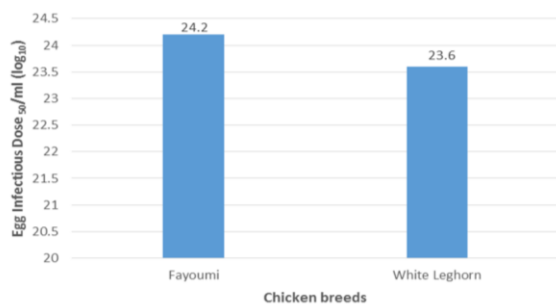


Figure 1:

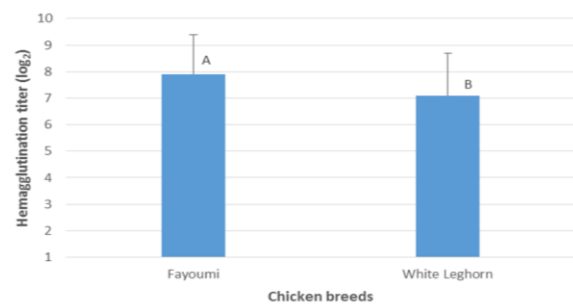


Figure 2:

Figure 1: Embryo infectious dose<sub>50</sub> ( $EID_{50}$ ); Figure 2: HA titers of VVNDV produced in embryonated eggs of two breeds of chicken; Means having superscript with similar letters on columns are not significantly different from one another; LSD (0.05) = 1.981

**Mean Death Time (MDT)**

The MDT of embryos of VVNDV infected Fayoumi and WLH was determined in 9-days old embryonated eggs. MDT in VVNDV inoculated embryonated eggs of Fayoumi and WLH breeds was 41.5±16 and 42.1 ±10.3 hours, respectively (Figure 3). MDT was higher in WLH while it was lower in Fayoumi but the difference between breeds was not statistically significant.

**Intracerebral Pathogenicity Index (ICPI)**

Intracerebral pathogenicity index (ICPI) in day-old chicks of Fayoumi and WLH breeds was found to be 0.93, and 0.71, respectively (Table 1). No morbidity or mortality was found in the control groups of two breeds when sterile PBS was injected intracerebrally. On day 8 post inoculation, all

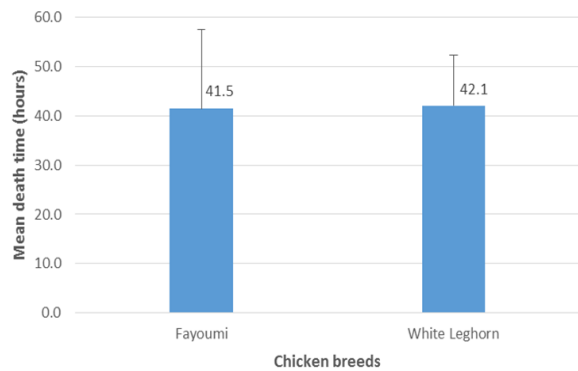


Figure 3:

surviving chicks were euthanized and blood samples were taken for determination of HI titers to check whether surviving birds are seroconverted or not. HI titers of the surviving birds were 8.25 and 9.2, in Fayoumi and WLH breeds, respectively (Figure 4). The difference in HI titers was not statistically significant.

**Intravenous Pathogenicity Index (IVPI)**

Intravenous pathogenicity index in Fayoumi and WLH was determined in 6 week-old chickens intravenously inoculated with VVNDV. The IVPI in Fayoumi and WLH was found to be 2.51 and 2.65 respectively (Table 2). No morbidity or mortality was observed in the control groups of 4-week old chickens of two breeds intravenously injected with sterile PBS.

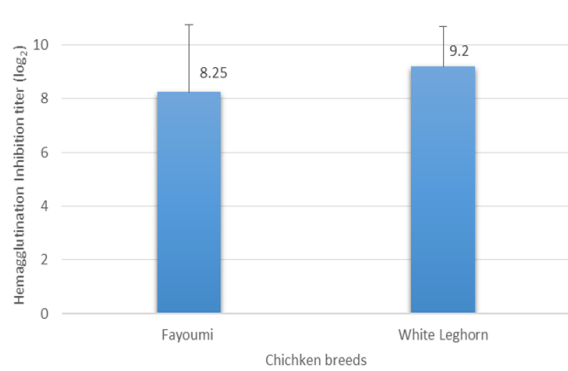


Figure 4:

Figure 3: Mean time of death (hours); Figure 4: HI titers in day old chicks of two breeds intra-cerebrally infected with VVNDV; Means having superscript with similar letters on columns are not significantly different from one another; LSD (0.05) = 2.365

Table 1: Intracerebral pathogenicity index for VVND virus infected day-old chicks

Groups	Clinical signs	Days								Total	Weight	Score
		1	2	3	4	5	6	7	8			
Fayoumi	Normal	10	7	4	4	4	4	4	4	41	0	0
	Sick	0	1	3	0	0	0	0	0	4	1	4
	Dead	0	2	3	6	6	6	6	6	35	2	70
										80		74/80= 0.93
White Leghorn	Normal	10	10	5	5	5	5	5	5	50	0	0
	Sick	0	0	3	0	0	0	0	0	3	1	3
	Dead	0	0	2	5	5	5	5	5	27	2	54
										80		57/80= 0.71

**Morbidity and Mortality Rates Post-Intravenous Inoculation and Contact-Exposure to VVNDV**

In intravenously inoculated chickens of both breeds, first clinical signs appeared day-2 post-intravenous inoculation of VVNDV while in sentinel chickens of both breeds the signs appeared on day-3 post-exposure. Mortality in both breeds started on day-2 post-intravenous infection and was 20% and 60% in Fayoumi and WLH. By day-3 post-intravenous inoculation, mortality rate reached 100% in both breeds. Mortality in sentinel birds of both breeds started on day-4 post exposure VVND and by day-6 mortality rate reached 100% (Figure 5). Mortality rates in sentinel birds were 40, 30, and 30 % in Fayoumi and 50, 30 and 20 % in WLH on day 4, 5 and 6, respectively.

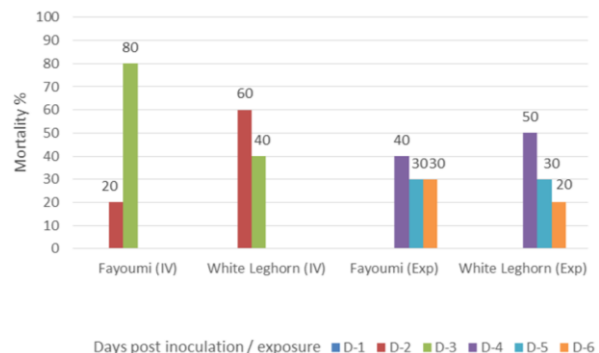


Figure 5: Mortality rates in 6-week old chickens of two breeds intravenously infected and contact exposed to VVNDV

### Necropsy Findings

On necropsy, the lesions included inflammation and petechial hemorrhages on trachea, brain, bursa of Fabricius and eyelids. Proventriculus, small intestine and caecal tonsils had multifocal, necrotic and hemorrhagic areas. The lesions in major organs including brain, bursa of Fabricius, eye lids, trachea, proventriculus and small intestine were scored as none (0), mild (1), moderate (2) and severe (3). Lesion scores per bird (0–18) were calculated by adding scores of all six organs. Scores of all birds in each group were combined and means were determined. Average lesion scores for intravenously infected chickens were recorded as 4.8, and 4.9 for Fayoumi and WLH breeds, respectively. Whereas, mean lesion scores for sentinel chickens were 6.6 and 8.4 Fayoumi and WLH, respectively (Figure 6).

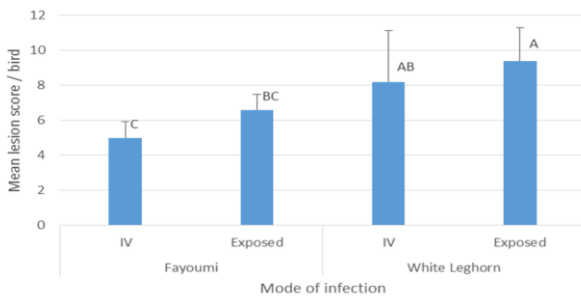


Figure 6: Mean lesion scores in 6-week old chickens of two breeds intravenously infected and contact exposed to VVNDV; Means having superscript with similar letters on columns are not significantly different from one another; LSD (0.05) = 2.060

### DISCUSSION

The study showed that in case of transmissibility of VVNDV Fayoumi is more resistant than White Leghorn (WLH). However in case of different pathogenicity indices it varies between Fayoumi and WLH  $EID_{50}$  was found  $10^{24.2}$  in Fayoumi, and  $10^{23.6}$  for WLH. The embryos of Fayoumi breed produced 06 times more VVNDV virus particles and were 0.6  $Log_{10}$  less susceptible to VVNDV than WLH breeds (Figure 1). Similarly, HA titers also significantly varied between two breeds. However, egg and embryo sizes of two breeds also vary which may have a role in variation.

The MDT was found higher in WLH (42.1 ±10.3) than Fayoumi breed (41.5±16) though the difference was not statistically significant. NDV strains having MDT lesser than 60 hours are considered velogenic (Parimal et al., 1997; Namita et al., 1995). The ND virus used in present study therefore, belongs to velogenic ND viruses. Khan et al., 2010 also determined MDT of eight isolates of velogenic NDV isolates from Karachi and found MDT ranging from 48 to 64 hours. Munir et al., 2012a determined MDT of a Pakistani isolate from apparently healthy backyard chicken which was 49.6. Munir et al., 2012b, determined the MDT of eight isolates from various outbreaks in the year 2010 from Punjab, Pakistan. The isolates belonged to a velogenic category and their MDT varied from 44.6 to 53.6.

Intracerebral pathogenicity index (ICPI) is considered by OIE as a gold standard for measuring viral virulence for ND strains. ICPI in current study was measured using day-old chicks of two breeds. This study shows a variation in intracerebral pathogenicity of VVNDV virus in two breeds which is evident from variation in ICPI scores which were 0.93 and 0.71, respectively observed in Fayoumi, and WLH breeds. Higher ICPI score in Fayoumi breed is probably due to a higher susceptibility of day old chicks to VVNDV infection than the chicks of WLH breed. ICPI of 0.7 or greater in day-old chicken or presence of three basic amino acids (R or K) at the F protein cleavage sites between residues 113 and 116 indicate the virulent (velogenic) form of NDV (Brown, 1999). According to OIE standards, the ICPI of the strain used in present study belongs to virulent or velogenic category. Munir 2012a et al., determined ICPI of an isolate from apparently healthy backyard chicken which was found to be 1.5. Munir et al., 2012b, in another study, determined the ICPI of eight isolates from various outbreaks in the year 2010 from Punjab, Pakistan which varied from 1.5 to 1.75. Reason for lower ICPI in present study could be due difference of strain of VVNDV under study, while technique, site and depth of intra-cerebral injection may also cause some variation in index. In intra-cerebrally infected day old chicks, morbidity and mortality started earlier in Fayoumi breed than WLH breed (Table 1). Moreover, in the day-old chicks surviving intra-cerebral inoculation of VVNDV, Fayoumi breed had on average lower antibody titers than WLH which may be responsible for higher susceptibility and more severe clinical signs (Table 1 and Figure 4).

Groups	Clinical signs	Days										Total	Weight	Score
		1	2	3	4	5	6	7	8	9	10			
Fayoumi	Normal	10	5	0	0	0	0	0	0	0	0	15	0	0
	Sick	0	1	0	0	0	0	0	0	0	0	1	1	1
	Paralyzed	0	2	0	0	0	0	0	0	0	0	2	2	4
	Dead	0	2	10	10	10	10	10	10	10	10	82	3	246
		100												251/100= 2.51
White Leghorn	Normal	10	0	0	0	0	0	0	0	0	0	10	0	0
	Sick	0	1	0	0	0	0	0	0	0	0	1	1	1
	Paralyzed	0	3	0	0	0	0	0	0	0	0	3	2	6
	Dead	0	6	10	10	10	10	10	10	10	10	86	3	258
		100												265/100= 2.65

Table 2: Intravenous pathogenicity index for VVNDV virus infected chickens

Intravenous pathogenicity index (IVPI) of VVNDV was measured in six weeks old chicken two breeds. Our study shows a variation in intravenous pathogenicity of VVNDV virus in two breeds which is evident from variation in IVPI

which were 2.51 and 2.65, for Fayoumi, and WLH breeds, respectively (Table 2). Velogenic strains possess IVPI ranging from 1.5 to 2.0 (Parimal et al., 1997, Namita et al., 1995). According to this criterion, the VVNDV virus used in



the present study belongs to virulent or velogenic category. First clinical signs appeared in both on day-2 post-intravenous inoculation of VVNDV whereas, mortality in both breeds started on day-2 and by day-3 post-intravenous inoculation of VVNDV and mortality rate reached 100% by day-3 (Figure 5). Interestingly, mortality rate was lower in Fayoumi on day-2 than the WLH breed. It was found 20% and 60% in Fayoumi and WLH, respectively. While on day-3 all birds of both breeds died due to VVNDV infection. Whereas, in contact exposed chickens, first clinical signs appeared in both breeds on day-3 post-exposure with VVNDV while mortality in both breeds started on day-4 and by day-6 post-exposure with VVNDV mortality rate reached 100%. The incubation period in this experiment was two days in intravenously inoculated group while it was three days in contact exposed group with total mortality being 100% for both groups. Hamid et al., (1990) studied an Indonesian strain of VVNDV and reported incubation periods of 2 to 16 days and 3 to 5 days in 7- and 20-week-old non-immune chickens, respectively while mortality rates were 85.3 and 100%. Age and breed of chickens and difference of virus strain may be responsible for difference in incubation period and mortality rates as compared to present study. All dying chickens were necropsied and lesions in major organs were scored. The average lesion scores for intravenously infected chickens infected chickens were similar for Fayoumi (4.8), and WLH (4.9) breeds (Figure 6). Whereas, average lesion scores for contact exposed chickens varied in Fayoumi (6.6), and WLH (8.4). Significantly higher lesion scores and IVPI scores in WLH as compared to Fayoumi breed point towards greater susceptibility of WLH to VVNDV infection at the age of 6 weeks.

Route of inoculation has impact not only upon appearance speed but also on intensity of lesions. Following inoculation through IV route, viruses directly enter into the circulation and subsequently distribute to different organs at faster rate (Flint et al., 2004). The hemorrhages in the proventriculus, intestines and cecal tonsils indicate that the isolate used in present study is velogenic viscerotropic strain of NDV (VVNDV). The nervous signs and lesions were also observed, but were found in fewer birds than the gastro-enteric lesions which predominated. The nervous involvement could be due to the systemic route used in the inoculation of the virus. Beard and Hanson (1981) reported that the intra-muscular, intravenous and intracerebral routes appeared to enhance the neurological signs as compared to the natural routes (oral, intraocular and nasal) of infection. Two chickens were also inoculated orally of with VVNDV which resulted in lesions primarily located in gastro-intestinal tract, upon necropsy (data not shown).

## CONCLUSION

Present study concludes that conventional virus pathogenicity tests like MDT, ICPI and IVPI may give variable results due to breed variation and therefore may be used evaluate breed resistance to ND. Embryos and day-old chicks of Fayoumi are more susceptible to VVNDV than White Leghorn as they show lower MDT along with higher HA / EID<sub>50</sub> tiers and ICPI, scores than White Leghorn. Both intravenously inoculated as well as contact-exposed 6-week old chickens of Fayoumi breed are more resistant as they have lower IVPI and postmortem lesion scores.

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