UNIVERSITI PUTRA MALAYSIA

NEWCASTLE DISEASE IN QUAIL

MASJUKI B. HJ. MOHD. MUSURI

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BY

MASJUKI B. HJ. MOHD. MUSURI

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SUPERVISOR : DR. AINI IDERIS

ABSTRACT

This is a preliminary study on the ecology of Newcastle Disease virus (NDV) in quail, especially the carrier status and the susceptibility of the birds to NDV.

Cloacal swabs were collected from three quail farms and isolation of NDV was attempted. Very small number of isolates (4 %) were obtained, but it could still indicate that the virus is present in the farm, and quails could carry the virus. The serology results show that the vaccinated birds have a higher antibody titer as compared to nonvaccinated birds.

Two groups of nonvaccinated birds were challenged with 2 different doses of virulent NDV by three different route of challenge namely intramuscular, intranasal and incontact. The results revealed that quail is resistant to artificial infection with ND. The serological challenge response was higher in birds challenged with high dose of virus and those challenged by intranasal route. There is no significant difference in serological response of birds challenged by 3 different route with low dose of virus.

No virus was isolated from a small proportion of cloacal swabs collected post challenge.

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OLEH : MASJUKI HJ. MOHD. MUSURI

PENYELIA : DR. AINI IDERIS

ABSTRAK

Kajian permulaan ini telah dibuat untuk mengkaji ekologi virus penyakit sampar pada burung puyuh dengan tujuan untuk mengetahui samada burung puyuh boleh membawa kuman sampar dan samada puyuh boleh mendapat penyakit sampar seperti yang berlaku pada ayam.

Sampel-sampel sapuan dubur telah dipungut dan diuji untuk mengesan adanya virus. Jumlah yang dikesan adalah kecil (4 %) tapi ia membayangkan status pembawa kepada burung ini terhadap kuman sampar. Keputusan dari ujian serologi menunjukkan purata kandungan antibodi jelas tinggi pada burung-burung yang menerima vaksin.

Dua kumpulan burung yang tidak menerima vaksin telah didedahkan kepada virus sampar. Tiap kumpulan menerima jumlah kuman yang berlainan dan mereka dibahagikan lagi kepada tiga kumpulan mengikut 3 cara pemberian iaitu melalui suntikan otot, titisan hidung dan melalui kontak (contact). Keputusan ujian menunjukkan tidak terdapat tanda-tanda klinikal dan kematian dan ini menunjukkan puyuh mempunyai ketahanan terhadap penyakit ini. Ujian serologi menunjukkan kumpulan yang menerima jumlah kuman yang tinggi dan yang diberi melalui titisan hidung memberikan tindakbalas yang tinggi. Dari sejumlah contoh sapuan dubur yang diuji selepas pendedahan kuman, tiada kuman dapat dikesan.

1.0 INTRODUCTION

Newcastle Disease (ND) is an acute febrile infectious disease of fowls, somewhat resembling fowl plague but caused by a separate and immunologically distinct virus. The name, Newcastle was given because it was first recorded near Newcastle Town, England (Doyle, 1927). Other nomenclatures include pneumoencephalitis avium, pseudopestis avium, Ranikhet disease, fowl pest and 'Sampar ayam'.

This disease is caused by paramyxovirus, of the family Paramyxoviridae (Fenner, 1976). Waterson (1977) discovered that all strains of ND virus are similar in their gross morphology and serology. They have a wide spectrum of virulence in the natural host as well as cytopathogenicity in cell culture. Transmission of the virus can be via excretion from infected birds including aerosols coughed into the air, faecal contamination of feed, water and environment. A number of vaccines are available and used worldwide for control of this disease.

Newcastle disease has a widespread occurance in domestic and wild birds and it causes a serious and often continuous economic effect. Lancaster (1977), stated that in domestic poultry, the economic importance may be considered under four main headings ;

a) mortality and loss in egg and meat production,

b) cost to the poultry industry of a vaccination programme and other control measures designed to reduce the economic loss.

c) cost to governments adopting quarantine and other

measures to prevent the introduction of the virus or to control outbreaks within the country,

d) loss of export markets for poultry and poultry products.

In wild birds, the importance of ND is the ability of these birds to carry the virus and transmit the disease to chicken, turkey or other species of birds (Zarauelo et al.,1969). Studies on Newcastle disease in quails have been done by several workers such as Higgins (1968), Zarzuelo (1969), Corrado (1970), Aitken (1974) and Inooka (1982). However, so far the studies done on quails in Malaysia are mainly on the production aspect and there is no report of any disease work. Nevertheless, some farms practice vaccination whereas others do not. Since, the quail industry is developing progressively in Malaysia, and ND is endemic, it is therefore important to know the ecology of Newcastle disease in quails in Malaysia, and whether the birds can harbour the virus and transmit the disease to other birds as what is reported, or whether they are susceptible to the disease.

Thus, this study was carried out with the following objectives ;

1. to study the carrier status of quails to NDV,
2. to study the immune status of quails to ND and to correlate the antibody titer with vaccination,
3. to study the susceptibility and the serological response of quails to artificial infection with NDV.

2.0 LITERATURE REVIEW

Susceptibility to natural or artificial exposure to Newcastle disease virus (NDV) depends on many factors, including the species of the birds, whether domestic or wild (Palmer and Trainer, 1971), the dose of infecting virus (Karzon and Bang, 1951), the route of infection (Beard and Easterday, 1967), the age of the birds (Lancaster, 1966) and the intensive method of management (Garside, 1966). The pathogenicity is also variable depending on the species of the birds (Palmer et al., 1971). In wild or cage birds ND often is inapparent. Signs when apparent are variable and nonspecific.

Wild birds are known as important carriers and disseminators of NDV, they include sparrows (Pesser domesticus), quail (Cortunix cortunix, Cortunix japonica), and linnets (Carduelis, 1969). Natural outbreak of ND in turkey have been reported by some workers from many countries in the world (Box and Helliwell, 1970). Reports on Newcastle disease in other avian species include salmon-crested cockatoos (Cacatua moluccensis), exotic parrot (Eaves and Grimes, 1978), Canada geese (Rosenberger et al., 1978; Splatin et al., 1974), ducks (Higgins, 1971), pigeon (Biancifiori et al., 1983; Wilson 1986) and Japanese quails (Higgins et al., 1968; Zarzuelo et al., 1969; Corrado, 1970; Aitken et al., 1974; Inooka et al., 1982).

Higgins and Wong (1968) reported an ND outbreak in a commercial flock of 2,000 quails which was characterised by

anorexia, diarrhoea and nervous signs. A large proportion of the flock had symptoms and about 40 % died. Edgar et al (1964) reported that quail were susceptible to Newcastle disease, however, Corrado (1970) in his study of susceptibility of Japanese quail to fowl pathogens reported that there was no infection developed in 112 quails injected with virulent NDV.

Inooka et al (1982) in their study on breeding and genetics reported that selective breeding can be done for high and low antibody responses to inactivated NDV in Japanese quails. Comparable responses were noted in quails and chickens injected with NDV and avian leukosis virus (Farrow et al., 1975).

Higgins (1972) found that NDV could grow in six-day fertile quail eggs as compared to nine-day fertile hen eggs. The mean embryo infective dose was higher and the mean embryo death time (MDT) was shorter in quail eggs than in hen eggs. The HA and EID_{50} titers of allantoic fluids were lower from quails eggs than from hen eggs.

The HI test is a convenient, economical and is a reliable serological technique used to evaluate the immune status of flocks for ND (Ibrahim et al., 1980; Chulan and Ibrahim, 1982; Nurizan, 1983). In chicken, there is a direct correlation between presence of serum antibody to NDV and resistance to development of clinical disease following challenge with a virulent strain of NDV (Westbury, 1984). However, serology alone cannot be viewed as a true or absolutely reliable measures of disease resistance. The most reliable and accurate measure of

immunity is by challenge-exposure with virulent NDV (Mac Brugh, 1984).

3.0 MATERIALS AND METHODS

3.1 Farm Visits

Three quail farms in Seremban, Negeri Sembilan, farm A, B and C were chosen for this study. Visits to the farm were made several times for the following purposes :

i. To obtain the history of management and flock health programme of the farm.

ii. To do collection of samples and to purchase birds for the challenge experiment.

3.2 Study of Carrier Status

This study was done by collecting samples of cloacal swabs for virus isolation in order to survey the presence of the virus in the farms. About 100 samples were collected randomly from 3 different farms at which 60 samples were from farm A and 20 samples each from farm B and C. Each swab was kept cool in 2 ml transport media (Hank's Solution) before bringing back to the laboratory.

In the laboratory, each cloacal swab was shaken well in the Hank's media before filtering using 0.45 um pore size microfilter. About 0.2 ml filterate was inoculated into each of

9 to 11 day old embryonated hen egg via allantoic route. Two to three eggs were used for each sample depending on the availability of the eggs. These eggs were then incubated at 37°C and candling was done daily. Those eggs with dead embryo on the first day after incubation were discarded. Allantoic fluid from any dead embryonated eggs after 2 days of incubation was tested for haemagglutination (HA) using 1 % chicken red blood cells (RBC). On the fifth day after incubation, allantoic fluid from all the eggs were tested for HA. Positive samples were further tested for haemagglutination inhibition (HI) to confirm the virus. Second passage were done for all the negative samples and this passage was repeated up to 3 times before the samples were considered negative to both tests.

3.3 Study of Immune Status

For this purpose, 57 blood samples were collected from vaccinated birds and 56 samples from nonvaccinated birds. This was to survey the antibody titer in those groups of birds in the farms. In the laboratory, serum was collected from each of the blood sample and HI test was done to determine the antibody titer of Newcastle disease (Allan and Gough, 1974).

3.4 Challenge Experiment

The challenge experiment was carried out in an isolation unit. Two groups of nonvaccinated birds were brought in and challenged with velogenic viscerotropic Newcastle Disease virus

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(VVNDV) designated Ipoh AF 2240 which has already been proved as a virulent strain (VVNDV) by Lai Chooi May (1986).

Group 1 consisted of 29 birds, and was again divided into 3 subgroups according to 3 different routes of challenge, namely intramuscular (i/m), intranasal (i/n) and incontact (i/c). A high dose of virus that is 10^{8} EID_{50} per bird was given to this group which is about 1,000 times higher than the dose for group 11.

Group 11 consisted of 27 birds and the challenge was carried out as for group 1 except that they received a lower dose of virus that is $10^{5.3}$ EID_{50} per bird, which is a normal challenge dose for chicken (Ibrahim et al., 1983). Before challenge, the birds were wing-tagged for identification purposes and blood samples were collected from all the birds. Incontact group of birds act as control for the experiment.

All the birds from both groups were observed for clinical signs and mortality for a period of 2 weeks post challenge. Post-mortem examination was done on dead bird and samples of lung and trachea were collected for virus isolation. At the end of challenge period (on 15 th day), cloacal swabs and blood samples were collected from all birds that survived. HI test was done on all serum samples and the results were recorded individually. As for the cloacal swabs, 3 samples from each group were processed and innoculated into eggs as described earlier in 3.2.

4.0 RESULTS

4.1 Relevant History of the Flock

Farm A and B consist of 5,000 birds each and 20,000 birds for farm C. These are all commercial farms which produce eggs and meat. About 10 birds are kept in one small cage of about 0.5 m^2 wide and 10 cm high. Male and female birds are mixed only when they are used for breeding, otherwise they are kept separately. Female birds were vaccinated twice with Ranikhet F vaccine intranasally at 1 week old and Ranikhet S vaccine intramuscularly at 28 days of age. All male birds were not vaccinated.

4.2 Study of Carrier Status

For this study, the results ere as shown in table 1. Out of 100 samples of cloacal swabs tested, only 21 samples give positive result to rapid HA test. However when confirmatory test was done by rapid HI test, only 4 of them were confirmed positive to NDV. The number of isolates is not statistically significant when tested by T-test ($P < 0.05$).

4.3 Study of Immune Status

The summary of the results for this study is shown in table 2. The average HI titer is higher in vaccinated birds, that is $\log_2$ 2.37 $\pm$ 1.3 as compared to nonvaccinated birds which have only $\log_2$ 1 $\pm$ 1.2. The distribution of HI titers shows that 47 %

of vaccinated birds have high level of antibody (more or equal to $\log_2$ 3) as compared to nonvaccinated birds which is only 16 %.

4.4 Challenge Experiment

The results for challenge experiments are summarised in table 3. The average serological response is higher in the group of birds that received high dose of virus than the low dose group. The difference of serological response was also statistically significant between two dose groups, since the response of low dose group was only $\log_2$ 0.7 + 1.9 and the high dose group was $\log_2$ 2.0 + 2.1.

Among three different routes of challenge, intranasal seems to give higher response ($\log_2$ 2.4) as compared to the other routes. This figure was clearly observed in high dose group. In low dose group, the response from the whole group was not statistically significant.

For the clinical challenge response, only one bird died and it came from the low dose group. Upon post-mortem, the lesion observed was only congestion of the lung and when the samples of lung and trachea were tested, no virus was isolated. Out of 18 samples of cloacal swabs collected post challenge, there was also no isolate obtained.

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Table 1 :

The number of virus isolation from the samples of cloacal swabs taken from three quail farms.

FARMS	NO. SAMPLES	NO (+) HA	NO. (+) NDHI	% (+)NDHI
A	60	12	2	3.3 %
B	20	6	1	5.0 %
C	20	3	1	5.0 %
TOTAL	100	21	4	4.0 %

- NO (+) HA is the number of samples that give positive result to haemagglutination (HA) test.
- NO (+) NDHI is the number of samples which is positive to HA test as well as haemagglutination inhibition (HI) test.

Table 2 :

Distribution of NDHI titer in vaccinated and nonvaccinated birds.

VACCINATION STATUS	NO. SAMPLES	NDHI TITER (LOG_2) 0	1	2	3	4	5	6	%HI $\geq LOG_2$ 3	AVERAGE
VACCINATED	55	6	5	18	15	9	2	0	47	2.37 ± 1.3
NONVACCINATED	56	28	12	7	7	1	1	0	16	1.00 ± 1.2

Table 3

Clinical and serological response of quails challenged with two different dose of virulent Newcastle disease virus by three different routes of challenge.

DOSE	ROUTE	bTITRE*	aTITRE*	DIFFERENCE*	MORTALITY
HIGH $10^8 EID_{50}$	I/M(10)	0.6 ± 1.2	2.8 ± 1.3	2.0 ± 1.9	0
	I/N(10)	0.8 ± 1.2	3.3 ± 1.5	2.4 ± 2.4	0
	I/C (9)	0.7 ± 1.2	2.1 ± 1.6	1.4 ± 2.3	0
total/ave.	(29)	0.7 + 1.2	2.8 ± 1.6	2.0 ± 2.1	0
LOW $10^5 EID_{50}$	I/M(10)	1.4 ± 1.6	2.5 ± 1.1	1.1 ± 2.2	0
	I/N(10)	1.3 ± 1.1	1.7 ± 1.3	0.3 ± 2.0	1
	I/C (7)	1.3 ± 1.1	1.7 ± 2.2	0.4 ± 1.4	0
total/ave.	(27)	1.3 ± 1.3	2.0 ± 1.5	0.7 ± 1.9	1

* Values given as (mean ± SD) log_2.

bTITRE - titre before challenge.

aTITRE - titre after challenge.

DIFFERENCE - the serological response.

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5.0 DISCUSSION AND RECOMMENDATION

5.1 Flock Management

From the observation and personal communication, it was found that the farmers do not pay very much attention on the disease prevention measures and flock health programme (FHP). For ND vaccination itself, it seems that the farmer do not know anything about the vaccine, including the type of disease the vaccine is given for. In this study, the history of vaccination was obtained from the State Veterinary Department. There was also no record kept by the farmers (Baitun, 1987).

Therefore the farmers are recommended to improve their FHP such as vaccination procedure and disease prevention measures such as proper sanitation and ventilation of the house. They are also recommended to keep a good record and to do proper analysis on the record.

5.2 Study of Carrier Status

A small number of Newcastle disease virus (4 %) could be isolated from the cloacal swabs taken during the field survey but not from the challenged birds. However , in the farm, the samples were collected randomly, so the samples may come either from vaccinated or nonvaccinated birds. Since the type of virus was not determined, there is a posibility that these virus comes from the vaccine virus. Anyway, since the vaccinated birds at

the time of study are already old (11 months) and the last dose of vaccination was about 10 months back (at 28 days old), it indicates that the virus isolated comes from natural infection.

There is no virus isolated after challenge probably because the number of samples tested were too small due to time constraint. The result also could not determine the degree of carrier status of the birds. Nevertheless, we can still say that the birds do transmit the virus and this finding seems to be quite similar with that reported by many workers such as Zarzuelo (1969) who reported that quails is one of many other species of wild birds that can carry the Newcastle disease virus.

More detail work should be carried out to determine when and for how long the virus is shed after infection. This is because there is a possibility that the virus is shed for a certain period of time after exposure or they may be latent in normal condition and being excreted when the birds are stressed.

Out of 21 haemagglutinating agents isolated, only 4 were positive for NDV. This indicates that there may be some other diseases present in the farm, especially those disease agents that can also produce haemagglutination to chicken RBC, such as influenza, parainfluenza and other paramyxoviruses. Therefore, further study may be required to investigate the type of virus commonly infecting quails in Malaysia.

5.3 Study of Immune Status

The average antibody titer is higher in vaccinated group and many of them have a high level of antibody titer. It is therefore important to study and to evaluate the vaccination programme, including the type of vaccine, the correct time of vaccination, and to evaluate whether the vaccine is protective or whether the vaccination is neccessary at all.

5.40 Challenge Response

Corrado (1970) reported that out of 112 quails challenged with virulent NDV, no infection was developed. Similar finding was obtained from this trial, whereby, none of the birds showed sign of infection or mortality after challenged with virulent NDV. The death of one of the birds was not confirmed to be due to ND, since no ND virus was isolated, no typical symptom of NDV from autopsy and it was from the low dose group. However, the serological challenge response was observed from this trial.

Since the antibody titre to ND at time of challenge was relatively low ($\log_2$ 1.0 $\pm$ 1.2) and yet none of the birds came down with the disease, we could say that quail is resistant to artificial infection of ND. However, the resistance is still uncertain for natural infection, since a natural Newcastle disease outbreak has been reported by Higgins (1968) in Hong Kong and as claimed by many other workers that quail is susceptible to NDV.

5.41 Comparison between Dose

Karzon and Bang (1951) have reported that the susceptibility to natural and artificial exposure to NDV depends on the dose of virus. Quite similar finding was found in this trial, that is the higher the dose the higher the serological response (Table 3). However, the result of the mortality seems to be questionable since the one that died was from the low dose group, and no NDV isolate was obtained to confirm that it died of Newcastle Disease.

5.42 Comparison Between routes

Beard and Easterday (1967) observed that the susceptibility of the birds to NDV depends on the routes of infection. Although the mortality pattern was not clear in this trial, but the serological response seem to agree with the statement whereby the higher response was observed from intranasal route as compared to the other routes.

5.3 Recommendation

It is recommended that another challenge experiment is done on both vaccinated and non vaccinated birds by using different age and sex groups. This is because Lancaster (1966) reported that the age of the birds affects the susceptibility to NDV, whereas in this trial, only the young birds were available for nonvaccinated group, and it so happened that they were all male birds, although so far there is no report which says that sex can influence the susceptibility of the birds to NDV.

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6.0 CONCLUSIONS

The results obtained, shows that quails do carry NDV, although it may not be a very important source of carriers. From the serological survey, the antibody to ND could be detected even from nonvaccinated birds and the titer seems to be significantly higher in vaccinated birds as compared to nonvaccinated birds.

The challenge experiment reveals that there is poor serological response unless if the birds are given very high dose of virus. There is significant difference in the response between dose and route of challenge. The most important finding is that quail is quite resistant to artificial infection of Newcastle disease which is consistent to that observed by Corrado (1970) who found that no infection developed from quail challenged with virulent NDV.

The FHP of the farm need to be improved and further study is needed to investigate in detail the ecology of NDV as well as other diseases in quails.

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APPENDIX I: Distribution and average of HI titres in quails before and after challenged with 2 different doses of virulent NDV by three different routes of challenge.

	DOSE	ROUTE	NO. BIRDS	TITRE (LOG_2) 0	1	2	3	4	5	6	% HI*	AVERAGE
BEFORE	HIGH	I/M	10	6	0	2	2	0	0	0	20	0.6 ± 0.8
		I/N	10	7	1	1	0	0	1	0	10	0.8 + 1.6
		I/C	9	6	1	1	1	0	0	0	11	0.7 ± 1.1
	Total/ave		29	19	4	4	1	0	1	0	7	0.7 ± 1.2
	LOW	I/M	10	5	1	0	3	1	0	0	40	1.4 + 1.7
		I/N	10	2	5	1	2	0	0	0	20	1.3 + 1.1
		I/C	7	2	2	2	1	0	0	0	14	1.3 + 1.1
	Total/ave.		27	9	8	3	6	1	0	0	26	1.3 + 1.3
AFTER	HIGH	I/M	10	0	2	2	3	2	1	0	60	2.5 + 1.0
		I/N	9	0	1	1	4	1	1	1	78	1.7 + 1.3
		I/C	8	2	1	3	0	1	0	1	25	1.7 + 2.0
	Total/ave.		27	2	4	6	7	4	2	2	56	2.8 + 1.6
	LOW	I/M	10	0	1	5	3	0	1	0	40	2.5 + 1.1
		I/N	9	0	6	2	0	0	1	0	11	1.7 + 1.3
		I/C	7	3	1	1	1	0	0	1	29	1.7 + 2.2
	Total/ave.		26	3	8	8	4	0	2	1	27	2.0 + 1.5

I/M - intramuscular

I/N - intranasal

I/C - incontact.

% HI* - percentage of the birds having more or equal to log_2 3.

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APPENDIX II : Method For Washing of Blood for HA and HI

1. Blood was collected from the wing vein at a ratio of 1 ml ACD to 4 ml blood in a 10 ml syringe.
2. Blood was poured into the centrifuge tube and was made up to 10 ml using PBS.
3. It was centrifuged at 1,5000 r.p.m. for 10 minutes.
4. The supernatant was removed and the volume was made up to 10 ml using PBS and centrifuged again. This was repeated thrice.
5. For the last centrifugation, it was centrifuged at 2,000 r.p.m. for 10 minutes.
6. The supernatant was removed and the volume was made up to 10 ml using PBS. The PCV was determined and 10 % RBC suspension was prepared.

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APPENDIX III : Haemagglutination Inhibition (HI) Test

1. 0.025 ml PBS was placed in well no. 2 to 12.
2. 0.05 ml serum was placed in well no. 1.
3. Serial two fold dilution of serum was done using 0.025 ml micro diluter from well no. 1 to 11.
4. 0.025 ml of appropriate antigen containing 4 HA units was placed in wells no 2 to 11. (Well no 1 received no antigen and served as a control of nonspecific agglutination).
5. Then it was mixed well on the jogger and was left at room temperature for 15 minutes.
6. 0.05 ml of 0.5 % chicken RBC suspension was added to each well and was maintained at room temperature for about 30 minutes.
7. The titre was read about 30 minutes later. Positive HI was indicated by the 'tear drop' shape of the button.

NOTE :

Before carrying out HI, an antigen back titration (haemagglutination) was performed to make certain that it contained at least 4 HA units.

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APPENDIX IV: Collecting, Handling and Processing Specimen for Virological Study of NDV.

a) Cloacal Swabs

1. Sterile swabs which were first dampened (but not wetted) in a sterile diluent namely Hank's Balanced Salt Solution (BSS) were used. The swabs were inserted into the cloaca and rotated gently.
2. Then, the swabs were immediately deposited into a sterile bijou bottles containing about 2 ml Hank's Solution and the extra length of swab sticks were snapped off before replacing the cap.
3. The bottles with the swabs were kept cool in a flask containing ice.
4. At the laboratory, the bottles were shakened using an electric shaker. The solution were then filtered by using 0.45 um pore size microfilter.
5. Sterility check was made by streaking the filterate onto blood agar and incubated at 37°C for 24-36 hours and the solution ready for egg inoculation.

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b) Autopsy Materials (lung and Trachea)

1. Lung and trachea were collected from dead birds. Samples were taken immediately and aseptically.
2. Samples were labelled individually and were kept in refrigerator (at about 4°C).
3. At virology laboratory, the samples were ground in a mortar using sterile alundum.
4. About 7 ml PBS was added, mixed and poured into centrifuge tube. Then they were centrifuged at about 2,000 r.p.m. for about 10 minutes.
5. The supernatants were collected and filtered by using 0.45 um pore size microfilter. Sterility check was done and the fluid was ready for inoculation.

APPENDIX V: Cultivation of Virus in Embryonated eggs

a) Inoculation of Eggs by the Allantoic Route

Materials - 9 to 11 day old embryonated eggs.

- 1 ml tuberculin syringes.
- NDV or suspected materials.
- Paraffin (wax) and swabs.
- 70 % alcohol.

Procedure

1. Eggs were candled and the air sac was marked with a spot on a nonvascular part near embryo.
2. Then the eggs were labelled individually.
3. The 'air sac end' of the eggs and area of marked spot were sterilized by spraying 70 % alcohol.
4. A hole was made at the marked point by a modified pin.
5. A sterile syringe was then filled with sample materials using a sterile technique.
6. The syringes were held at a slight angle from horizontal and the needles were inserted into the hole over embryo to a depth of about 3 mm. About 0.2 ml of the sample materials were inoculated into each egg and 2 to 3 eggs were used for each sample depending on availability of the eggs.
7. After inoculation of eggs the syringes were discarded into a pan of water and the holes on the eggs were sealed with wax.
8. The eggs were then incubated at 37°C for about 2 to 5 days.
9. The eggs were candled daily and all eggs with dead embryo on first day after incubation were discarded.

b) Harvesting and Testing Allantoic Fluids

Materials
- pasteur pippetes
- rubber bulb
- forceps
- 1 % chicken RBC.
- 2 ml vials
- alcohol
- blood agar plate

Procedure

1. At the end of the incubation period, the embryo were chilled in cold room for at least 4 hour.
2. The eggs were then sprayed with alcohol and allowed to dry before cracking the air sac end with blunt forceps. The shell above the air sac was removed.
3. A sterile pasteur pippete equipped with a rubber bulb was used for each egg to withdraw the allantoic fluids.
4. The fluids were placed into 2 ml vials and labelled individually.
5. Sterility check were performed by streaking the fluids on blood agar and incubated at 37°C for 1 to 2 days. Meantime the fluids were tested by using rapid HA test. A drop of 1 % chicken RBC was placed on an enamel plate for every sample and same amount of the allantoic fluids were mixed. The plate were rotated gently and the presence of haemagglutinin after 1 minute indicates a positive HA test.
6. To identify the virus, rapid HI test was done on positive HA samples of fluid. A drop of known ND antisera (256 HI titer) was mixed with same amount of fluid on enamel plate and then 1 drop of 1 % RBC was added, mixed and rotated. The absence of haemagglutinin indicates the presence of NDV.