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BURSAL DISEASE OUTBREAK IN VACCINATED YOUNG POULTRY FLOCKS IN SOUTHERN DISTRICTS OF BHUTAN

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ABSTRACT: Infectious Bursal Disease (IBD), also known as Gumboro disease caused by avibirnavirus belonging to the family Birnaviridae, is an economically significant poultry disease. Vaccination is considered as an important control measure depending on the strains of virus used in the vaccine. In Bhutan, with the intensification of poultry rearing activities in the country, IBD is considered economically important disease to prevent loss to poultry rearing farmers. In fiscal year 2014-15, southern districts of Bhutan bordering with India experienced series of IBD outbreaks. In an outbreak that lasted for about four months, a total of 13032 birds died in 47 farms in five districts with an average district mortality of 28.4±6.97 (2.5–44.0). An investigation on the outbreak was undertaken with the objectives to ascertain the possible factors attributing to the outbreak and provide technical recommendations to prevent outbreaks in future. The investigation found that the vaccine used before the outbreak failed to provide immune protection and confirmed the involvement of circulating virulent strain of IBD virus in the affected farms. Subsequently, the disease was controlled after vaccine was replaced.

Keywords: Infectious Bursal Disease; outbreak; investigation; vaccine; control.

1. INTRODUCTION

Infectious Bursal Disease (IBD) is a highly contagious acute viral disease that affects young chickens. The disease was first described by Cosgrove (1962) in Gumboro county, Delaware USA. Thus, this disease is also known as Gumboro disease. IBD is a serious concern in the poultry industry and is considered economically significant poultry disease (Shane et al. 1994). It is caused by a small, hardy avibirnavirus belonging to the family Birnaviridae. The virus is resistant to a great range of temperatures, disinfectants and pH (Van den Berg and Meulemans 1991; Winterfield et al. 1972). It is therefore very stable to environmental exposure and can survive up to four months. Disinfectant preparations belonging to formaldehyde, glutaraldehyde, chlorine and iodophore groups can effectively destroy the virus. The predilection site of IBD virus is the Bursa of Fabricius (BF) where the immature B lymphocytes are the target cells for virus replication. Immature B lymphocytes in BF differentiate into active immune cells responsible for immune protection. BF is the

immune defense power house responsible for protection of young birds where matured B lymphocytes are programmed to produce specific antibodies in response to infection or immunization. IBD usually affects young chickens when BF is in developmental stages with sufficiently large number of immature B lymphocytes. When BF is damaged, immature B lymphocytes are also destroyed, leading to severe histopathological changes in BF and other associated organs. Affected birds become inactive, ruffled feathered, droopy and excrete whitish diarrhoea. BF undergoes different stages of pathological changes depending upon the stages of infection. The lesions are predominantly haemorrhages and edema of BF and accumulation of transudate. Urate diathesis is also a commonly associated renal pathological change in IBD affected birds. A sudden mortality is observed after brief illness in a morbid flock, which may be as high as 20-30% (Ley et al. 1983). However, mortality in flocks challenged with virulent variant IBD virus (vvIBDV) is reported as high as 80% (Segal 2004). Vaccination is considered as an important control measure depending on the strains of virus used in vaccine (Van den Berg and Meulemans 1991). Maternal derived antibody (MDA) in young chicks plays important role of passive immunity and protection from infection. High maternal antibody titre in young chicks interferes with vaccine, leading to immunosuppression and predisposition to infection (Sharma et al. 1989; Naqi et al. 1983). In spite of practising different vaccination schedules, there are reports of frequent outbreaks of IBD (Moraes et al. 2005).

The level of MDA in young chicken plays an important role in determining the use of vaccine strain and vaccination time. Therefore, vaccination is usually not done until the MDA titre drops to breakthrough level depending on the strain of vaccine used. For IBD vaccination, breakthrough MDA titre levels of ~500 and ~200 are reported as vaccine breakthrough levels for intermediate plus and intermediate strains of commercial vaccine, respectively.

In Bhutan, during mid-1990s, there were reports of several IBD outbreaks, particularly in government poultry farms. Regular vaccination was done as a measure to control the outbreak. In the recent years, with the intensification of poultry rearing activities in the country, IBD control program was prioritised to prevent loss from disease outbreak. In 2014, a series of IBD outbreaks occurred in southern districts of Bhutan bordering India. Loss from such outbreak entailed serious economic impact to poultry industry. Therefore, the aim of this study was to undertake epidemiological investigation on the outbreak of IBD reported in the country, including ascertaining the possible risk factors attributing to the outbreak in order to provide technical recommendations and prevent outbreaks in the future.

2. MATERIAL AND METHODS

The investigation process involved visiting affected farms, interviewing farm owners, examination of affected flock for clinical manifestation, autopsy of dead and sacrificed sick birds, sample collection, performing serological tests and real time polymerase chain reaction tests. Samples were tested for differential diagnosis.

2.1 Interview, post-mortem, histopathology and sample collection

During the visit, farmers and government farm officials were asked about the clinical manifestation, onset of outbreak and mortality, breed and age affected birds, feeding and treatment regime, vaccination record and farm bio-security measures. Dead and sacrificed sick birds were examined for gross pathology. Lesions in affected organs were recorded. Tissue samples from affected organs were examined for histopathological changes. Swab samples from cloaca and trachea were collected while performing autopsy as well as from sick birds for molecular, immunological and virological analysis. Bursa of Fabricius was also collected for histopathological examination. Serum samples were collected from infected, uninfected and parent flocks for serological investigation.

2.2 Field rapid and laboratory tests

Antigen detection lateral flow immunoassay using commercial kit (Quicking Biotech, China) following manufacturer's instruction was performed to determine the presence of viruses that cause avian influenza type, Newcastle disease and IBD.

Sera samples were tested to detect level of antibody in vaccinated flocks (IBD affected and unaffected flocks) using enzyme-linked immunosorbent assay (ELISA) (IDEXX, Montpelier, France) following manufacturer's instruction. According to the test protocol, samples that revealed sample-to-positive (SP) ratio > 0.20 were considered positive due to the exposure to field strain IBD virus or immunisation through vaccination.

RT-PCR test was performed using bursal swab samples to detect the involvement of IBD virus. RT-PCR test for IBD was performed at National Institute for Animal Health, Bangkok, Thailand and National Institute of National Institute for High Security Animal Disease, Bhopal, India. Primers from the regions of gene segments A and B were

used for detection of IBD virus. RT-PCR tests for Flu A, subtype H5 and N1 and ND were performed at the National Centre for Animal Health, Serbithang, Bhutan, for differential diagnosis. Primers and probes for AI and ND were supplied by Australian Animal Health Laboratory (AAHL), Geelong, Australia.

IBD vaccine that was used in the infected flock (IBD intermediate strain, live, NLT 10^3 EID_{50}) and subsequently procured IBD vaccine containing same strain, manufactured by different company (IBD Intermediate strain, live, NLT $\geq 10^2$ EID_{50}) were compared for the presence of immunoreactive viral protein (VP2) using rapid test kit (Quicking Biotech, China). Briefly, for each vaccine, twenty vaccine vials were randomly picked from storage facility to be used for *in vitro* immunoreactivity test. Samples were prepared by reconstituting lyophilised vaccine in manufacturer recommended and supplied diluent. Initially, manufacturer recommended reconstitution using supplied diluent was prepared. Subsequently, series of dilutions at 1:10, 1:100, 1:200 and 1:400 were prepared. Three drops of each reconstituted or diluted vaccines were loaded onto the sample well of the rapid test card. The samples were allowed to react for 10 minutes and the result was read. Development of colour band at the designated test position "T" on test card was considered as positive reaction. On the other hand, absence of development of colour band at position "T" was considered as negative reaction. The test was considered valid if the colour band was developed at designated control positon "C" on test card.

2.3 Comparative immune response between two different vaccines

IBD vaccine, that was used prior to outbreak was immediately withdrawn after confirmation of involvement of IBD virus in the outbreak. Subsequently, same strain of IBD vaccine from different manufacturer was procured and put into use in all poultry establishment in the affected areas. After the introduction of new vaccine, two groups of serum samples were collected at same interval, 57 days post vaccination: a) group that received previous vaccine and b) group that received new vaccine. Two groups of serum samples were for immune protection using ELISA (IDEXX, Montpelier, France).

3. RESULTS

3.1 Clinical signs and post-mortem

The sick birds appeared exhausted, prostrated, ruffled feathered, huddled in large number, suffered with watery white diarrhoea and soiled vent region.

A total of 34 dead and sacrificed sick birds from different affected farms were examined for postmortem lesions (Figure 1). Bursa of Fabricius was swollen, oedematous and haemorrhagic with gelatinous yellowish transudate, covering the serosal surface. The bursa was filled with transudate and in one case the transudate was solidified taking the shape of bursa. Affected BF showed necrotic foci and petecheal haemorrhages on the mucosal surface. Haemorrhages were seen in thigh and pectoral muscles. The kidneys appeared swollen and undergone urate diathesis. Subcutaneous petecheal haemorrhages were also observed especially on the skin of back and lumbar region. Few cases of haemorrhages were found on the mucosal wall at the junction of proventriculus and gizzard.

3.2 Histopathology, rapid test and RT-PCR

The haematoxylin and eosin (H and E) stained tissue section from the affected bursa revealed histopathological changes of edema, haemorrhages, loss of follicles and lymphoid cell depletion (Figure 2).

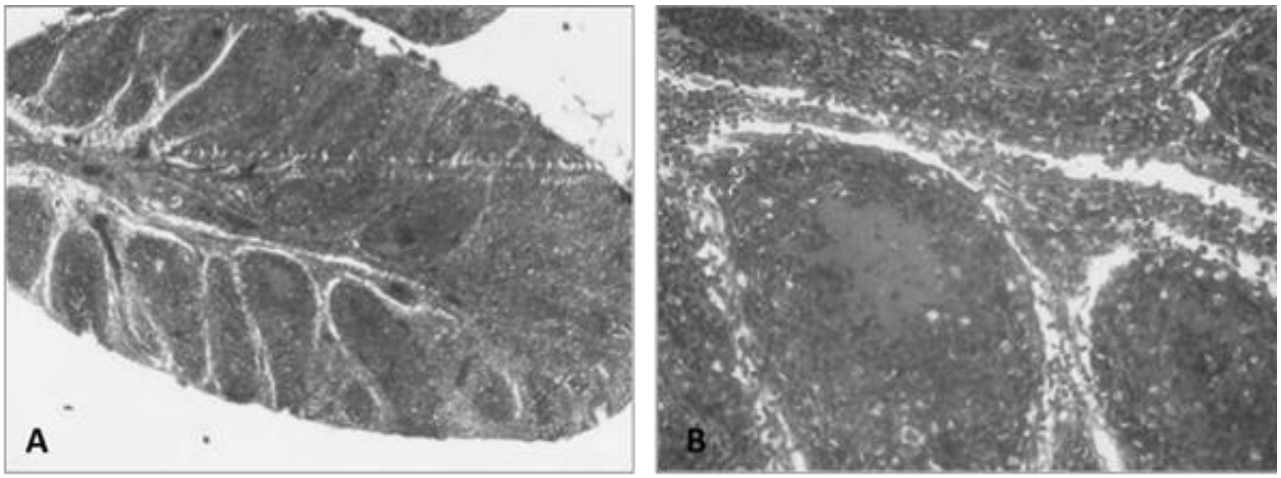


Figure 1: Gross pathological lesions in affected organs. Haemorrhagic lesions in pectoral (A) and thigh (B) muscles; oedematous BF (C); haemorrhagic BF (D) and (E); urate diathesis of kidney (F).

Bursal and cloacal swab samples revealed positive reaction in IBD rapid test, indicating the involvement of IBD virus (Table 1). The test reaction from bursal swab was stronger when compared with that of cloacal swab. Rapid field test and RT-PCR test did not detect the involvement of avian influenza and ND virus in swab samples.

3.3 Spatial distribution of IBD and epidemiological features

The first case of IBD was reported in the young layer stock of National Poultry Development Centre (NPDC) in Sarpang district, southern Bhutan. Three private farms located at close proximity to NPDC were also found to be affected by outbreak with huge mortality. In the next four months, the disease spread to another four districts north of Sarpang in the sequence of Tsirang, Wangdue, Punakha and Thimphu (Figure 3). The disease affected poultry farms in five districts with varying degrees of economic losses.

A total of 13032 birds, originating from 47 farms in the five districts, died of the disease causing an overall mortality percentage of 28.4±6.97 (2.5–44) within four months. The district level mortality rates ranged from as low as 2.5% in Thimphu district to as high as 44% in Tsirang district (Table 2).

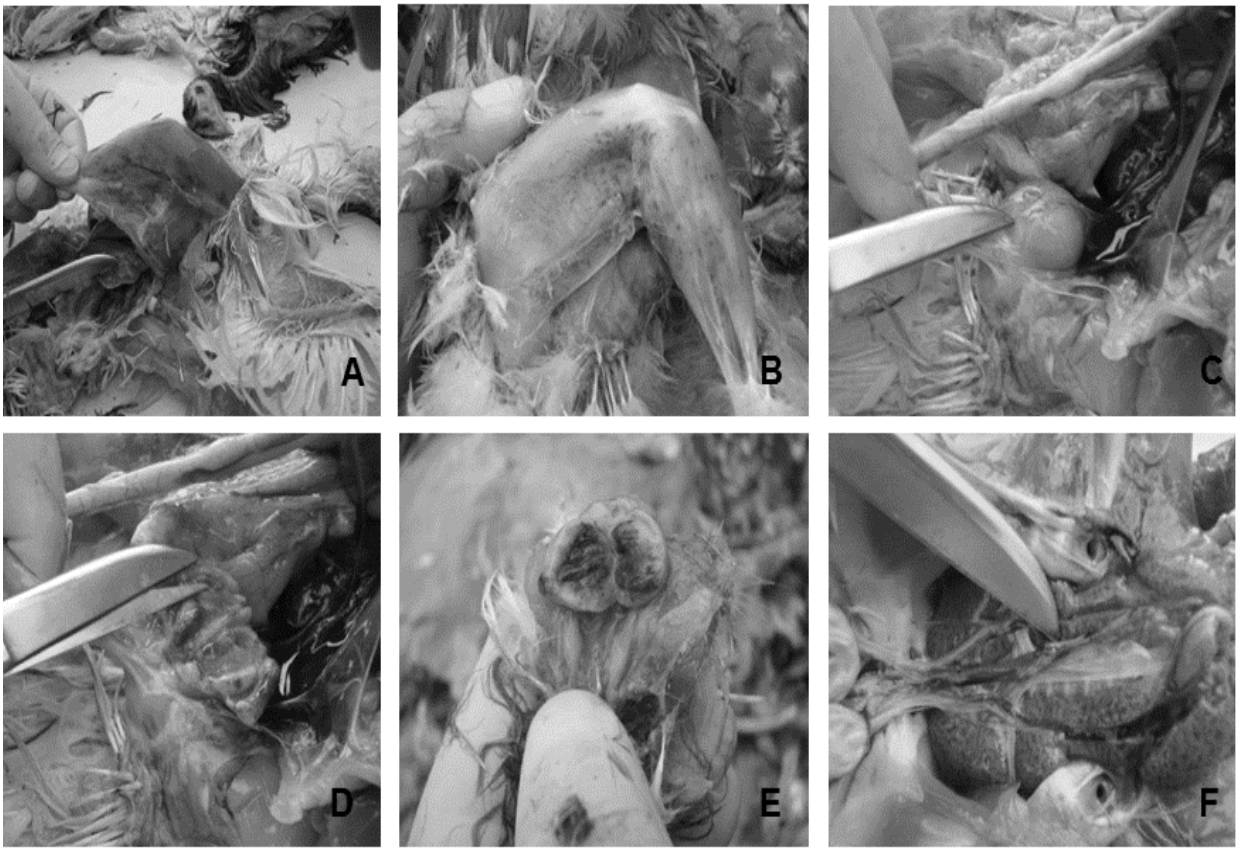
3.4 Disease evolution and mortality pattern in beginning of outbreak

In all affected farms, no mortality was reported after the 7th day of the onset of initial mortality. In general, mortality pattern followed a typical bell-shaped curve, in that the mortality peaked at half way through the curve (3rd to 4th day) with maximum mortality and rapidly dropping to zero after a week (Figure 4).

The mortality appeared to persist longer in bigger flock, compared with the smaller flock. All the affected birds were approximately six weeks old. The affected birds were Bovans Brown layer and Ross 344 broiler.

3.5 Diagnosis

Findings from the clinical signs and age of affected birds, disease evolution and mortality pattern, post-mortem lesions and test results from laboratory analysis of samples and differential diagnosis confirmed the involvement of wild strain of virulent IBD virus in young flock mortality at NPDC, Sarpang and private farms in Bhutan.



FIC: Lateral Flow Immunochromatography; RT-PCR: Real time PCR

Figure 2: H and E stained tissue sections of affected bursa. A: 100X resolution tissue section of bursa showing the distended interfollicular spaces and mild degree of lymphocytic depletion; B: 400X resolution tissue section with edema and congestion.

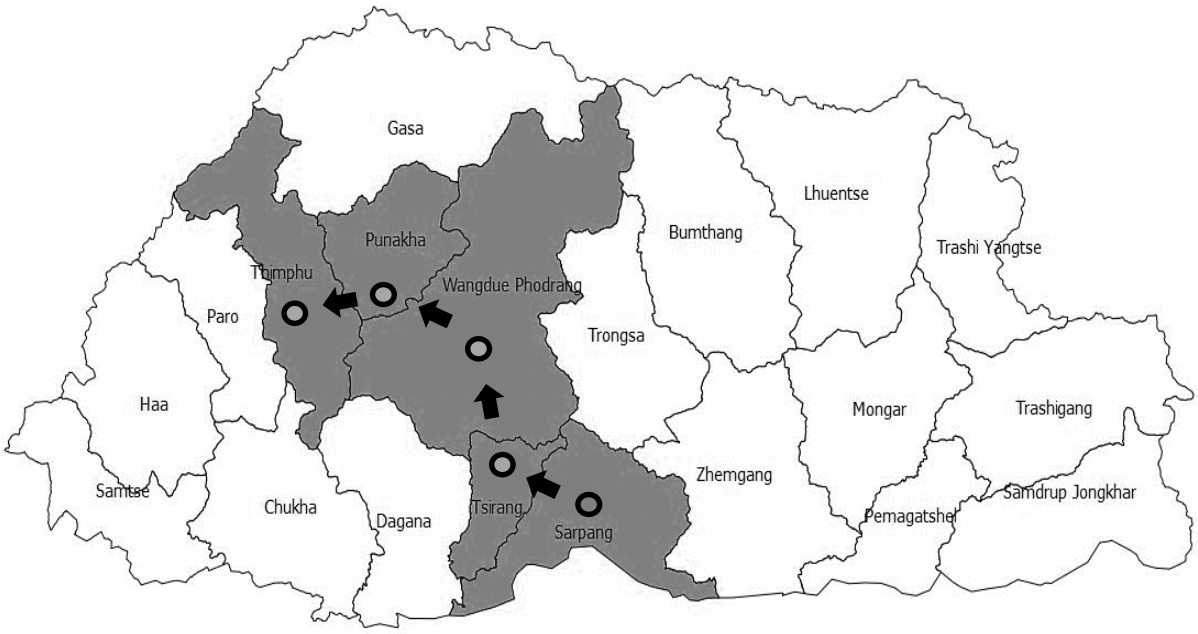


Figure 3: Map of Bhutan depicting the districts affected with IBD (the shaded districts are the IBD-affected districts; the circles depict the location of IBD outbreaks; and the direction of arrows indicates the likely direction of spread of disease).

Table 1: Laboratory test results.

Sample type	n	Agent detection	LFIC	RT-PCR
Tracheal swab	6	AI type A virus	Negative	Negative
Tracheal swab	6	AI subtype H5 virus	Negative	Negative
Tracheal swab	6	AI subtype N1 virus	Negative	Negative
Tracheal swab	6	ND virus	Negative	Negative
Cloacal swab	6	IBD virus	Positive	Positive
Bursal swab	6	IBD virus	Positive	Positive

3.6 Vaccine, vaccination and immune profile of vaccinated flock

The Day-Old Chicks (DOC) were supplied from Satara hatchery, a NPDC contracted private hatchery in Sarpang district. DOCs were vaccinated against Marek's disease on 0 day. After DOCs were lifted from hatchery, vaccination against ND, IBD and fowl pox were administered at respective farms. It was found that primary dose of IBD was administered when the chicks were 3 days old, followed by boosters on 14^{th} and 28^{th} days of age. Vaccine was administered via ocular route using dropper. The vaccine strain used for IBD vaccination was intermediate strain of IBD live virus. Parent flocks, which was the source of hatching eggs, were also found to be vaccinated against IBD. Based on the ELISA cut point SP ratio of 0.20, proportions of antibody positive birds in vaccinated-affected and vaccinated-unaffected flocks were 85% and 84%, respectively. At 6 weeks of age, the mean antibody $\log_{10}$ titre of vaccinated-affected flock (3.1±0.166, n=20) was not significantly different from that of vaccinated-unaffected flock (3.2±0.107, n=25). Previously published studies reported that the minimum antibody $\log_{10}$ titre required for protection from challenge by virulent wild strain IBD virus is 3.4 (Moraes et al. 2005).

With reference to this titre value, 45% of vaccinated-affected and 35% of vaccinated-unaffected birds were found to be unprotected. Corresponding antibody titres in affected and unaffected flock were 2665±451 (12–6911; 95% CI) and 2544±348 (104–5650; 95% CI), respectively. Coefficient of variations of antibody titre in affected and unaffected flock was 76% and 68%, respectively, indicating the heterogeneous nature of immune profile.

3.7 *In vitro* immunoreactivity of vaccine sample and response to new vaccine

At manufacturer recommended reconstitution, the newly procured IBD vaccine was able to produce strong colour band at position "T", indicating the presence of adequate amount of IBD virus immunoreactive protein.

Weak positive reactions were also noticed in further dilution series (1:10 and 1:100). In contrast, none of the dilutions or reconstitution of IBD vaccine used prior to outbreak was able to produce colour band at position "T" on test card, indicating lack of immunoreactive IBD virus proteins.

The mortality rates during the outbreak were 42.7% and 12.9% in Sarpang and Tsirang districts, respectively. With the use of new IBD vaccine, the mortality rate was dramatically reduced and no further IBD associated mortality was reported (Figure 5). Additionally, a comparative immune profile analysis indicated that the protective antibody titre of birds that received new vaccine was significantly higher ($p \leq 0.05$) than those received vaccine previously.

4. DISCUSSION

Findings from the investigation confirmed the unequivocal involvement of virulent strain of IBD virus in the outbreak that caused mortality of large number of young poultry birds in government and private farms in Bhutan. Differential diagnosis excluded involvement of AI and ND virus that cause similar pathological conditions and mortality in birds. Interestingly, all affected birds were vaccinated against IBD using intermediate strain of vaccine. Vaccination schedule followed by farm management was expected to protect these birds from challenge by IBD virus, which in this case had failed and resulted in IBD outbreak. It appeared the possibility of vaccine failure punctuated by bio-security breach that may have allowed the entry of virulent strain IBD virus into susceptible population. Live vaccines are available at different degree of attenuation and cause bursal atrophy and immunosuppression. Such intrinsic characteristics of IBD live vaccine and quite often failing to induce protection against very virulent strain of IBDV is not uncommon (Muller et al. 2012). With the recent development in IBD vaccine, research viral vector vaccine that uses VP2 protein could be a better option since such vaccines are known to have minimal effect on immunosuppression and interference with maternally derived antibody (Perozo et al. 2009; Bublot et al. 2007; Le Gros et al. 2009). Inadequate level of protective antibody

Table 2: Mortality data in the affected districts.

Affected district	Number of affected farm	Flock size	Number died	Mortality %: Mean ± SE
Sarpang	25	28835	8768	30.9 ± 4.3 (4.4 - 93.3)
Tsirang	18	7347	3114	44 ± 4.7 (5.3 - 92)
Punakha	2	1050	380	29.1 ± 16.4 (12.6 - 45.6)
Wangdue Phodrang	1	2066	734	35.5
Thimphu	1	1400	36	2.5

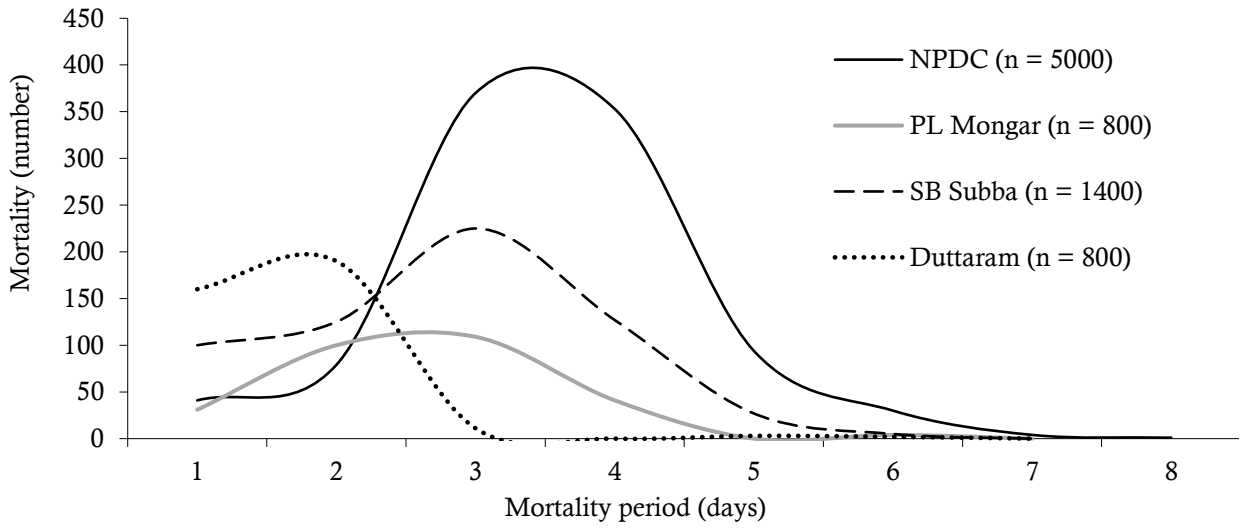


Figure 4: Disease evolution and mortality pattern. Numbers in parenthesis are the total stock before the outbreak**.**

titre in vaccinated birds and undetectable level of immunogenic viral protein in vaccine used before the outbreak clearly suggested that the vaccine did not serve the purpose.

Immunoassay detected high proportion of antibody positive (SP ratio>0.20) birds in vaccinated flocks. However, it is important to note that not all birds with an SP ratio>0.20 are protected from wild strain virus exposure. SP ratio is more of diagnostic significance than an indicator of protection from infection. Only the birds with an antibody $\log_{10}$ titre value >3.4 are considered protected.

An effective immunization programme changes the bird from being passively immune by MDA into an actively protected bird through the development of its own antibodies in response to vaccination. The correct timing of vaccination plays critical role in this operation. If the vaccine is applied too early, while the MDA titre is too high, the vaccine is neutralised and no protection is conferred (Naqi et al. 1983; Van den Berg et al. 1991). When the primary vaccine fails, following booster has to act as primary vaccine to elicit protective immune response within the expected time period (Goddard et al. 1994; Islam et al. 2005; Islam et al. 2008). On the other hand, if the vaccine is applied too late, then an opportunity is created for field virus to infect susceptible population and an outbreak is anticipated. Published study reported that the decay rate of MDA half-life is about 3-4 days (Segal 2004). Other studies have reported that MDA persists for as long as 15-30 days (Hitchner 1971; Wyeth and Cullen 1979; Iordanides et al. 1991; Yehuda et al. 2000; Paul et al. 2005). Therefore, an accurate identification of the window of opportunity is extremely important. In this investigation, it did not appear the vaccination time played a role in allowing the outbreak to occur.

At six weeks of age, affected and unaffected flocks that received three vaccinations were not able to achieve protective antibody $\log_{10}$ titre value of 3.4. It is critical to ensure adequate protection of young birds from IBD virus infection at that age, owing to the development of functional BF. However, it is to be noted that in the absence of circulating wild strain of IBD virus, even the susceptible flock would remain unaffected. For an outbreak to occur, IBD virus need to have access to susceptible population. This was the most likely case in this outbreak.

In vitro immunoreactivity test of vaccine confirmed that the vaccine used prior to outbreak lacked immunogenic protein. To investigate the quality of such vaccine in detail was beyond the scope of this study. Additionally, immune profile of birds vaccinated using such vaccine did not develop adequate level of protection and that may have been the reason for spread of disease in five districts despite continuous efforts in vaccination. With the introduction of new vaccine from another manufacturer and its ability to protect birds from infection clearly indicated that previous vaccine failed to protect birds from IBD. The use of new vaccine prevented further loss to the owners of poultry establishments.

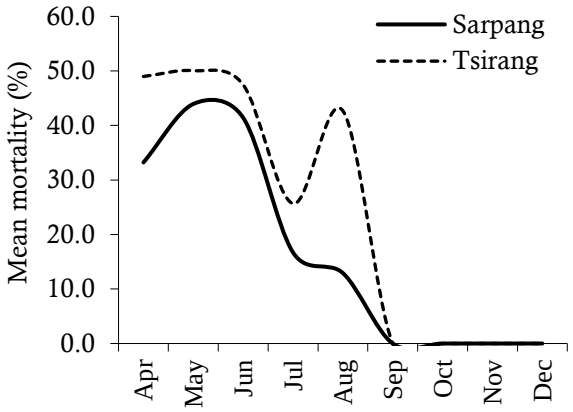


Figure 5: Response to vaccine replacement in flocks from mainly two affected districts. The bold bar in April represents the first case of mortality due to IBD; and the broken bar in August represents the time of vaccine replacement.

5. CONCLUSION

Based on the facts and findings from this study, there are some key areas needed to be considered in preventing future outbreak of IBD. All poultry establishments must adhere to strict bio-security practices while transporting farm supplies, equipment and consumables; control on vehicle movement and visitor; proper use of personal protective equipment and appropriate disinfectant. Concerned authority must put a system in place to ensure all poultry property owners have access to quality vaccine. Compromise in vaccine quality may lead to serious economic loss, not only from IBD but also from many other poultry diseases.

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Conflict of interest

The authors declare that there is no conflict of interest with regard to the findings of this study.

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