

Dot-ELISA for Newcastle Disease, Infectious Bursal Disease and Mycoplasmosis

Jahan Alam,¹ Faiz Muhammad,¹ Muhammad Umar Siddiqui,¹ Shakeel A. Khan,¹ Shafqat Rehmani² and Aqeel Ahmad^{1*}

¹Department of Microbiology, University of Karachi, Karachi, Pakistan

²Sindh Poultry Vaccine Center, Poultry Research Institute, Korangi, Karachi, Pakistan

Abstract.- Newcastle disease (ND), Infectious bursal disease (IBD) and mycoplasmosis are common respiratory diseases of poultry affecting several billion rupees business in Pakistan. Prevalence of these diseases demands specific strategies to control their occurrence. Efforts were made to develop Dot-ELISA for detection of antibodies against NDV, IBD and *Mycoplasma gallisepticum* (MG). Dot-ELISA was performed on the sera samples collected from sick birds showing symptoms of ND, IBD and Mycoplasmosis. Antibodies were detected by dot-ELISA against NDV, IBDV and MG. Study showed that birds were highly exposed to MG in our region. Antibodies were detected against NDV, IBDV and MG in 37.2%, 32.1% and 54.7% infected birds, respectively. Present study also showed that the birds possessed antibodies against more than one pathogen tested: 5.1% of the infected birds possessed antibodies against NDV and IBDV; 9.5% against NDV and MG; and 11.7% against IBDV and MG. Data also showed that 2.9% of the infected birds were exposed to all the three pathogens that were tested. Antibodies were also detected in the sera obtained from healthy birds against all these three pathogens. Around 9.5%, 8.4% and 15.5% sera were found to contain antibodies against NDV, IBDV and MG, respectively. Present study also showed that the prevalence of co-infections are 1.9% (NDV and IBDV), 3% (NDV and MG) and 3.3% (IBDV and MG), respectively. Dot-ELISA using specific antigens from pathogens is very specific, highly sensitive, less time consuming with little false result. A diagnostic kit can be prepared for large number of pathogens; this combine approach of several antigens on a single strip can play an important role in the diagnosis of poultry disease at early stage, and help in controlling infectious diseases in poultry sector in Pakistan.

Key words: NDV, IBDV, *Mycoplasmosis gallisepticum*, Dot-ELISA.

INTRODUCTION

The poultry industry in Pakistan has recently gained importance due to the production of low-cost good quality animal protein with the creation of job opportunities to help poverty alleviation. Nevertheless, poultry farming is a high-risk business in Pakistan which is subjected to instability, uncertainty and insecurity due to a number of reasons like regular outbreaks of infectious diseases (Anjum, 1990; Mustafa and Ali, 2005; Khan *et al.*, 2009; Naeem *et al.* 2003; Bhatti, 1989; Kataria *et al.*, 2005). These outbreaks demand extensive field work especially in avian microbiology. Early diagnosis, effective treatment and prevention of diseases are of crucial importance to control outbreaks and are of immense economic values.

To control infectious diseases, there is a dire need for effective monitoring system to keep check on the microbial prevalence in the poultry-farms; which require effective strategies for their control. Serological studies could be helpful in monitoring the prevalence of pathogens. Since presence of antibodies, that are highly specific, indicate exposure to pathogens. So, detection of antibodies can help early diagnosis and guide to take prophylactic measures to control outbreaks.

A few common respiratory diseases of chicken in our regions are Newcastle disease (ND), infectious bursal disease (IBD), mycoplasmosis, infectious bronchitis (IB), and avian influenza (AI) (Tariq *et al.*, 1989; Naeem *et al.*, 2003; Mustafa and Ali, 2005). In spite of vaccination, ND and IBD are still the most common diseases in Pakistan (Numan *et al.*, 2005; Siddique *et al.*, 1986), causing great economic losses. Similarly mycoplasmosis caused by *Mycoplasma gallisepticum*, also affect poultry industry (Bradbury, 2001). High prevalence of these diseases demands development of cheap, reliable,

* Corresponding author: aahmad57@gmail.com
0030-9923/2012/0005-1301 \$ 8.00/0
Copyright 2012 Zoological Society of Pakistan.

and rapid serologic tests for monitoring infectious diseases in flocks and also for determining immune status of birds.

Serodiagnosis is considered to be effective and reliable methods for determining the exposure of birds to various infectious agents. There are many commercial kits available for diagnosis of poultry diseases, commonly based on ELISA. Dot-ELISA is an immunoblotting assay and is relatively cheaper, reproducible and cost effective than liquid ELISA. In the present study efforts were made to develop Dot-ELISA, for detection of antibodies against NDV, IBDV and MG in infected and healthy birds.

MATERIALS AND METHODS

Antigen preparation

Crude preparation of antigens was used to develop Dot-ELISA. Briefly, freshly grown culture of *Mycoplasma gallisepticum* (MG) was sonicated and protein content was determined and 10 μ l of 10 μ g/ml was used in the test. NDV and IBDV were grown in embryonated eggs.

DOT ELISA for IBDV, NDV and MG

In Dot-ELISA, the antigens were directly applied onto nitrocellulose membrane; the antigens become immobilized onto a solid support. Antigen reacts with the serum antibody which then reacts with the secondary enzyme-linked antibody. The amount of enzyme linked bound antibody is assayed by incubating the strip with an appropriate substrate, which is converted to a colored, insoluble product. The colored end product precipitates onto the strip in the area of enzyme activity, hence the name Dot-ELISA. The enzyme activity is indicated by intensity of colour, which is directly proportional to the antibody concentration.

Serum samples of infected and healthy birds were obtained from diagnostic labs (Microlab.), PRI (Poultry Research Institute, Karachi), slaughter shops (especially healthy birds' sample) and different farms located in Gaddap Town across the Northern bypass, and Hub.

Sera from infected and healthy birds were diluted (1:100 or 1:50 in diluting buffer, Phosphate buffer saline containing 0.05% Tween-20) and 1ml of each diluted sample was added in separate

troughs, and then antigens-dotted strips were dipped in the diluted serum and left for one hour at room temperature. After incubation, all strips were washed with washing buffer, saline containing 0.05% Tween-20 (3 times for 15 minutes each). Conjugate (Rabbit anti-chicken IgG.HRP 1:1000) were then applied onto the washed strips and again incubated at room temperature for another hour. Strips were thoroughly washed with washing buffer (3 times for 15 minutes each) and then the substrate, diaminobenzidine (Bio-Rad Cat. # 170-6535) (50mg DAB/100ml of 50mM Tris pH7.4+25 μ l of 30% H₂O₂) was added to develop colour dots.

RESULTS

In the present study, antibodies against MG, NDV, and IBDV were detected by Dot-ELISA to determine the extent of exposure to these agents in healthy and infected birds (Fig. 1). Positive sera, prepared in experimental birds and the sera obtained from PRI (Poultry Research Institute, Karachi) were used as control. Clinically sick birds, showing symptoms of ND, IBD and mycoplasmosis, were examined for the presence of respective antibodies.

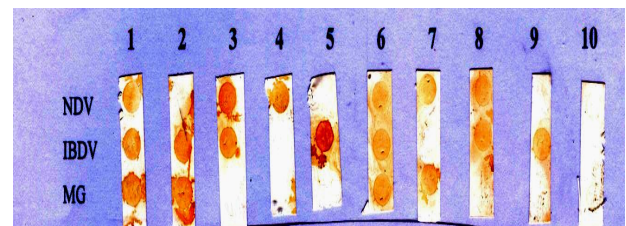


Fig. 1. Dot-Blot ELISA showing presence of antibodies against NDV, IBDV and MG. Antibodies were detected by blotting antigens of NDV, IBDV and MG onto nitrocellulose membrane. The blots were immersed into diluted serum. After proper washing, blots were then immersed in conjugate (rabbit anti-chicken Ig.HRP). Blots were washed and spots were developed by adding chromogenic substrate (diaminobenzidine). Dots show the presence of antibodies and intensity of colour is proportional to the amount of antibodies in the sera. Strip 1 and 10 are positive and negative control, respectively.

About 504 birds' sera samples (137 infected and 367 healthy sera) were tested for the presence of antibodies against *M. gallisepticum*, NDV and

IBDV by Dot-ELISA. The results in Tables I and II show that birds are highly exposed to MG in our region. Similar findings were also observed by ELISA in another study (Buim *et al.*, 2009). Around 54.7% infected sera were positive for antibodies against MG; while 37.2% and 32.1% sera were found to be positive for NDV and IBDV, respectively. Remaining birds may have been infected with other agents or antigenically different strains (Table I).

Table I.- Screening infected birds' sera by Dot-ELISA.

Serum samples (137)	NDV	IBDV	MG
NDV	27 (19.7%)	07 (5.1%)	13 (9.5%)
IBDV	07 (5.1%)	17 (12.4%)	16 (11.7%)
MG	13 (9.5%)	16 (11.7%)	42 (30.7%)
All 3 positive	04 (2.9%)	04 (2.9%)	04 (2.9%)
Total positive	51 (37.2%)	44 (32.1%)	75 (54.7%)
Total negative	86 (62.8%)	93 (67.9%)	62 (42.3%)
All negative		11 (8.0%)	

Antibodies were detected by Dot-ELISA in 137 serum samples obtained from infected birds showing symptoms of ND, IBD and Mycoplasmosis. Table shows that birds were infected with more than one pathogen (NDV, Newcastle disease virus; IBDV, Infectious bursal disease virus; MG, *Mycoplasma gallisepticum*).

Table II.- Screening healthy birds' sera by Dot-ELISA.

Serum samples (376)	NDV	IBDV	MG
NDV	14 (3.8%)	07 (1.9%)	11 (3.0%)
IBDV	07 (1.9%)	09 (2.5%)	12 (3.3%)
MG	11 (3.0%)	12 (3.3%)	31 (8.4%)
All 3 positive	03 (0.8%)	03 (0.8%)	03 (0.8%)
Total positive	35 (9.5%)	31 (8.4%)	57 (15.5%)
Total negative	332 (90.5%)	336 (91.6%)	310 (84.5%)
All negative		278 (75.7%)	

Antibodies were detected by Dot-ELISA in 367 serum samples obtained from healthy birds showing no symptoms of ND, IBD or Mycoplasmosis. Table shows that birds were exposed to more than one pathogen. However, a significant numbers do not contain detectable level of antibodies against NDV, IBDV or MG.

For abbreviations see Table I.

Present study also showed that birds are exposed to more than one pathogen: *e.g.*, 5.1% birds

were exposed to both ND and IBD; 9.5% to ND and MG and 11.7% to IBD and MG, respectively. Moreover, it was found that 2.9% were infected with all three pathogens that were tested.

Antibodies were also detected in the sera obtained from healthy birds against all the three pathogens (Table II). Around 9.5%, 8.4% and 15.5% sera were found to contain detectable levels of antibodies against NDV, IBDV and MG, respectively. These results indicate that birds are subclinically infected with these organisms. Present study also showed that the prevalence of co-infections in healthy birds is 1.9% for NDV and IBDV, 3% for NDV and MG and 3.3% IBDV and MG, respectively. Moreover, it was also found that 0.8% of healthy birds were infected with all the three pathogens tested. However, 75.7% of healthy birds showed no detectable antibodies against the test pathogens.

DISCUSSION

In Pakistan, the investment in poultry sector is more than eighty billion rupees. Infectious diseases are one of the main problems of poultry industry, causing high economic losses in term of high mortality and morbidity, stress, decreased egg-production and hatchability all over the world, including Pakistan. ND, IB, IBD, mycoplasmosis, avian influenza and few others are major respiratory infectious diseases of the poultry.

Acute respiratory-tract infections are of significant concern in the poultry industry. NDV, IBV and MG are the most important pathogens responsible for causing high mortality, and the higher percentages of cull-birds adversely affecting the economy. To minimize the economic damages to poultry farmers and consequently to the GDP, identification and differentiation between infected and healthy birds is necessary to prevent the spread of infections, and such practices could help to improve prophylactic measures and to control outbreaks.

One of the important ways to reduce the loss is to diagnose the diseases at an early stage. Secondly, constant surveillance is required to control infectious diseases in this region. Early diagnosis is a key for effective treatment and

prevention of diseases and to control outbreaks.

To address these objectives, there is a dire need for effective monitoring system to keep a vigil on microbial prevalence in the poultry-farms and to devise effective control strategies. Serological studies could be helpful in monitoring the pathogen prevalence. Since presence of antibodies, that are highly specific, indicate about an exposure to pathogens. So, detection of antibodies can be of great help in early diagnosis and to guide relevant authorities to take appropriate prophylactic measures.

In this study Dot-ELISA was developed and its performance was evaluated. Dot-ELISA is a solid-phase immunoassay that can be used to detect antibody-levels against more than one pathogen on a single strip at a time. Dot-ELISA was used to measure antibody status against three pathogens from a single test serum. Strips were produced containing 3 spots of cross-linked NDV, IBDV and MG antigens. The number of antigen spots or dots could be increased and applied onto a single strip.

ND causes very high losses in egg-type layers; its presence is reported in both the layers and broilers in all seasons (Anjum, 1990; Qureshi *et al.*, 1981; Numan *et al.*, 2005). A number of outbreaks have been recorded even in vaccinated chicken flocks (Siddique *et al.*, 1986).

In the present study, NDV antibodies were detected in 9.5% healthy birds. While, in the sera obtained from diagnostic labs., and sick birds at the farms, 37% showed antibodies against NDV. Among the NDV-infected birds, 13.7% also showed antibodies against IBDV and 25.5% against MG; while, 7.8% had antibodies against all the three pathogens tested.

Recently, Ahmed *et al.* (2009) have reported that NDV is endemic in Pakistan affecting up to 40% birds. Amongst the developing countries like Bangladesh, a little low incidence of NDV was reported (Mozaffor, 2010), while in Jordan, it was reportedly 13% (Roussan *et al.*, 2008) and the birds were also positive for MG (5.2%).

IBD, Gumboro or AIDS like disease, is responsible for higher mortality (10-100%) in chickens in all age-groups, however; losses were higher in early age (Sah *et al.*, 1995; Lukert and Saif, 1997). IBD causes 20% mortality per annum

by destroying immune-system despite vaccination (Numan *et al.*, 2005; Siddique *et al.*, 1986). In a previous study the prevalence of IBD in Peshawar was 7.75% (8.085%) (Khan *et al.*, 2009). In the present study, prevalence of IBD was 32% in infected birds and 8.4% in healthy birds. However, Hussain *et al.* (2003) reported a very high incidence of IBD using indirect Haemagglutination (65%) in commercial broilers in Faisalabad.

Mycoplasmosis causes severe economic loss in egg-type layers in terms of reduced egg production and higher mortality (Mohammad *et al.*, 1987; Yoder, 1991; Bradbury, 2001). The disease is mostly transmitted through eggs from the infected or carrier birds to the newborn chickens. Prevalence of mycoplasmosis in various poultry-farms is very high. 54.7% infected birds are found to possess antibodies against *M. gallisepticum* by Dot-ELISA. High prevalence of MG is also detected by ELISA in another study (Buim *et al.*, 2009). Hanif and Najeeb (2007) reported 26% incidence of MG in Lahore using PCR. However, antibodies were also detected in healthy birds (15.5%).

Dot-ELISA is slightly less sensitive to ELISA, because more antibodies are required to give visible reaction, but is very specific, highly sensitive, less time consuming with little false-positive results. Dot-ELISA detects antibodies earlier than HI (Folitse *et al.*, 1998), hence could be used directly at the farms. A diagnostic kit can be prepared and used for large number of pathogens; this approach of using several antigens on a single strip may play an important role in the diagnosis of poultry disease at an early stage and help in controlling infectious diseases in Pakistan.

In future, we plan to develop a kit for detection of antibodies against several poultry pathogens in a single step. This kit will help poultry farmers to detect exposure to commonly known pathogens in the birds.

ACKNOWLEDGEMENTS

This work was conducted by the support of HEC under national Research Program for Universities. We are extremely thankful to all those who helped in this project, especially Dr. Muhammad Akram, Consultant, Micro Laboratories,

Karachi, Dr. Aslam Jalali, Poultry Production & Research, Government of Sindh, Mr. Ibnul Hassan Hashmi, Manhattan Pharma, Karachi, and Dr. Shahnawaz Janjua, American Soyabean Association for arranging serum samples and clinical materials from infected birds. All of them also extended full support, expertise, and cooperation during the study.

Publication charges have been paid from Dean's (Faculty of Science) Grant, University of Karachi.

REFERENCES

- AHMED, A., KHAN, T.A., KANWAL, B., RAZA, Y., AKRAM, M., REHMANI, S.F., LONE, N. AND KAZMI, S., 2009. Molecular identification of agents causing respiratory infections in chickens from southern region of Pakistan from October 2007 to February 2008. *Int. J. Agric. Biol.*, **11**:325-328.
- ANJUM, A.D., 1990. Prevalence of the poultry disease in and around Faisalabad and their relation to weather. *Pak. Vet. J.*, **10**: 42-48.
- BHATTI, B.M., 1989. Incidence of poultry diseases and their importance in poultry production in Pakistan. *Pak. Vet. J.*, **9**:194-197.
- BRADBURY, J.M., 2001. Avian mycoplasmas. In: *Poultry diseases* (eds. F. Jordan, M. Pattison, D. Alexander and T. Faragher), W.B Saunders, London, UK, pp. 178-193.
- BUIM, M. R., METTIFOGO, E., TIMENETSKY, J., KLEVEN, S. AND FERREIRA, A.J.P., 2009. Epidemiological survey on *Mycoplasma gallisepticum* and *M. synoviae* by multiplex PCR in commercial poultry. *Pesq. Vet. Bras.*, **29**:552-556.
- FOLITSE, R., HALVORSON, D.A. AND SIVANANDAS, 1998. A dot immunoblotting assay (Dot-blot ELISA) from early detection of Newcastle disease antibodies in chicken. *Avian Dis.*, **42**:14-19.
- HANIF, A. AND NAJEEB, M.I., 2007. Comparison of conventional bacterial isolation, rapid slide agglutination and polymerase chain reaction for detection of *Mycoplasma gallisepticum* in breeder flocks. *Pak. J. Life Soc. Sci.*, **5**:1-5.
- HUSSAIN, I., ZAHOR, M.A., RASOOL, M.H., MAHMOOD, M.S., MANSOOR, M.K. AND RIAZ, M.N., 2003. Detection of serum antibody levels against infectious bursal disease (IBD) virus using Indirect Hemagglutination (IHA) Test in commercial broilers. *Intl. J. Poult. Sci.*, **2**: 442-445.
- KATARIA, J.M., DASH, B.B., DHAMA, K. AND KUMAR, N.S., 2005. Common diseases of indigenous poultry flocks and their control. *Pakistan Poult.*, **26**:9-13.
- KHAN, R.J., KHAN, F.A., KHAN, I. AND TARIQ, M., 2009. Prevalence of infectious bursal disease in broiler in District Peshawar. *ARPN J. Agric. Biol. Sci.*, **4**: 1-5.
- LUKERT, P.D. AND SAIF, Y.M., 1997. Infectious bursal disease. In: *Diseases of poultry* (ed. B.W. Calnek), Iowa State University Press, Ames, Iowa, USA, pp. 721-738.
- MOHAMMED, H.O., CARPENTER, T.E. AND YAMAMOTO, R., 1987. Economic impact of *Mycoplasma gallisepticum* and *M. synoviae* in commercial layer flocks. *Avian Dis.*, **31**: 477-482.
- MOZAFFOR, H.K.M., 2010. Antibody levels against newcastle disease virus in chickens in Rajshahi and surrounding districts of Bangladesh. *Int. J. Biol.*, **2**:102
- MUSTAFA, M.Y. AND ALI, S.S., 2005. Prevalence of infectious diseases in local and Fayomi breeds of rural poultry (*Gallus domesticus*). *Punjab Univ. J. Zool.*, **20**:177-180.
- NAEEM, K., NAURIN, M., RASHID, S. AND BANO, S., 2003. Seroprevalence of avian influenza virus and its relationship with increased mortality and decreased egg production. *Avian Pathol.*, **32**:285-289.
- NUMAN, M., ZAHOR, M.A., KHAN, H.A. AND SIDDIQUE, M., 2005. Serologic status of Newcastle disease in broilers and layers in Faisalabad and surrounding districts. *Pak. Vet. J.*, **25**: 55-58.
- QURESHI, M.S., RANA, M. A. AND QURESHI, A., 1981. Bacterial and viral etiology of mortality in layers. *Pak. Vet. J.*, **1**: 115-116.
- ROUSSAN, D.A., HADDAD, R. AND KHAWALDEH, G., 2008. Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. *Poult. Sci.*, **87**: 444-448
- SAH, R.L., KATARIA, J. M., ARYA, S. C. AND VERMA, K. C., 1995. Outbreaks of acute infectious bursal disease causing high mortality in chicken. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.*, **16**: 7-13.
- SIDDIQUE, M., SABRI, M.A. AND KHAN, M.Z., 1986. Outbreaks of ND in vaccinated chicken flocks in and around Faisalabad. *Pak. Vet. J.*, **6**:41-45.
- TARIQ, N., JAVED, T. AND SIDDIQUE, M., 1989. Prevalence of respiratory problems in chicken in Faisalabad and surrounding districts. *Pak. Vet. J.*, **9**:162-164.
- YODER, H.W. JR., 1991. *Mycoplasma gallisepticum* infection. In: *Diseases of poultry* (eds. M.S. Hofstad, H.J. Barnes, B.W. Calnek, W.M. Reid and H.W. Yoder Jr.), Iowa State University Press, Ames IA, pp. 198-212.

(Received 28 July 2010, revised 10 November 2010)