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A novel recombinant Meq protein based dot-ELISA for rapid and confirmatory diagnosis of Marek's disease induced lymphoma in poultry

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A B S T R A C T

Marek's disease (MD), is an economically important virus disease of poultry throughout the world. In this study, we for the first time reports development of a novel dot enzyme-linked immunosorbent assay (dot-ELISA) for the confirmatory diagnosis of lymphoma caused by Marek's Disease Virus (MDV). Suspected lymphoma tissue extracts from the diseased birds were used for the Meq oncoprotein antigen detection, which is expressed specifically in MDV lymphomas. Recombinant Meq oncoprotein was expressed using Expresso™ Rhamnose Sumo Cloning and Expression system and the hyperimmune serum was raised against it, which was used later while developing dot-ELISA. The dot-ELISA exhibited higher specificity (92%) in diagnosing MD lymphomas as compared to conventional PCR (40%), where later assay is unable to differentiate disease development (lymphoma) and/or infection. The developed dot-ELISA proved to be a specific, rapid and inexpensive technique detecting MDV lymphomas in poultry. Of the note, this new assay could be opted as a valuable diagnostic tool in the resource poor countries andcould further be used to differentiate from other tumor causing viruses in poultry.

1. Introduction

Marek's disease virus (MDV) belongs to the genus *Alphaherpesvirus* and causes Marek's disease (MD), a lymphoproliferative disease in chickens. It is an economically important virus disease of poultry (Couteaudier and Denesvre, 2014). MDV is amongst the leading causes of lymphoma in chickens, resulting in severe mononuclear cell infiltration in one or more of the tissues viz., peripheral nerves, gonads, spleen, iris, muscle, skin and other visceral organs. Marek's disease, once effectively controlled by vaccination, has been re-appearing throughout the world including India, even in vaccinated poultry flocks owing to evolution and increase in virulence of circulating MDVs (Nair, 2005; Kamaldeep et al., 2007; Arulmozhi et al., 2011; Muniyellappa et al., 2013; Mcpherson and Delany, 2016). The diagnosis of MD lymphomas is often difficult and has remained controversial, as quantification of the MDV genome cannot be established using standard PCR techniques (Davidson et al., 1995; Witter and Schat, 2003). Nonetheless, PCR could not differentiate MDV infection and the resulting overt disease. Further, it fails to discriminate transformation and latent infections. Thus, there appeared a need of a better test which could differentiate disease from infection. Marek's EcoRI Q-encoded protein or Meq is one of the most extensively studied MDV proteins. Meq is essential for oncogenicity as it is consistently expressed in MDV-induced lymphomas, and MDV induced transformed cell lines (Lupiani et al., 2004; Brown et al., 2006; Suchodolski et al., 2010). Deletion of the *meq* gene has resulted in loss of oncogenic ability and reduced virus replication (Kumar et al., 2012). Research on

Abbreviations: MEQ, MDV EcoRI-Q; HIS, Hyper Immune Serum; LB, Luria Broth; ELISA, Enzyme-Linked Immunosorbent Assay; PBS(T), phosphate buffered saline (tween 20); RIPA, buffer-radioimmunoprecipitation assay buffer; BSA, Bovine serum albumen; RT, room temperature; NCM, Nitrocellulose membrane; IBH, Inclusion Body hepatitis; PO, peroxidase; ALKP, alkaline phosphatase; LL, lymphoid leucosis; RE, reticuloendotheliosis; IBH, Inclusion Body hepatitis.

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the development of Meq oncogene or its protein based diagnostic for screening of commercial poultry farms for MD lymphomas has not been exercised so far. Meq protein is a 339 amino acid protein encoded in the MDV EcoRI-Q fragment of the serotype 1 MDV strain (Jones et al., 1992). It is a potent candidate which could delineate differential diagnosis of MDV induced lymphomas from other lymphoid tumor causing agents and also between various serotypes of MDV. At present agar gel precipitation test, ELISA and PCR test are available to detect either the presence of viral antigen or genome. It is the right time now to have efficient, specific and rapid diagnostic aids/kits for detection of MD lymphomas at the field level. Therefore, we intended herein to develop a Meq oncoprotein specific dot-ELISA for the quick and definitive diagnosis of oncogenic MDV induced lymphomas.

2. Materials and methods

2.1. Virus

The serotype 1 MDV was obtained from Avian Diseases Section, Division of Pathology, IVRI, Izatnagar (U.P.).

2.2. Cloning and transformation of Meq oncogene of MDV

Viral DNA from the serotype 1 MDV was extracted using the commercial DNeasy Blood & Tissue Kit (Qiagen, Germany) and used as a template for the amplification of partial length *meq* gene using the Cloning Primers that were custom designed with adapter sequence. The primers sequences used were pRham F-5′- CGCGAACAGATTGGAGGTTCTCAGGAGCCAGAGCCGGG-3′ and pRham-R-5′GTGGCGGCCGCTCTATTATTGGGAACCGGAGCAATGTG-3′, which amplified a partial length *meq* gene of length 507 bp. The amplification reaction was performed in 50 μl volume containing 10 μl of 5× Phusion HF Buffer, 1 μl of dNTP (10 mM), 10 pM each of forward and reverse primer and 1.0 U of Pfu polymerase and 5.0 μl of template DNA (5 ng). The PCR reaction was performed in a thermal cycler (QB 96 thermal cycler, Quanta biotech) following standard cycling procedure with an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 56 °C for 45, extension at 72 °C for 45 s and a final extension at 72 °C for 10 min. The amplified PCR products were purified using GeneJET PCR Purification Kit (Thermo Scientific, U.S.A.). Cloning and transformation was done as per Expresso™ Rhamnose Sumo Cloning and Expression System (Enzyme-free Cloning with the pRham™ N-His SUMO Vector and *E. cloni* 10G cells) (Lucigen, U.S.A.) following manufacturer's protocol. The transformed culture was plated on Luria Broth (LB) agar plates with kanamycin (30 μg/ml) and incubated at 37 °C over night. Screening for recombinants was done using Colony PCR with a mixture of 12.5 μl Master Mix (Thermo Scientific, U.S.A.), 0.5 μl SUMO forward primer (50 μM), 0.5 μl pETite reverse primer (50 μM) (primers included in the kit) and 11.5 μl nuclease free water following cycling procedures of the Expresso™ Rhamnose Sumo Cloning and Expression System (Lucigen, U.S.A).

2.3. Expression kinetics, confirmation, purification and quantification of recombinant Meq protein

One of the transformed clone screened by colony PCR was grown overnight in LB medium containing kanamycin (30 μg/ml) at 37 °C. The expression kinetics of the recombinant Meq protein was studied at different time intervals (8 h, 16 h, 24 h) atdifferent concentrations (0.05%, 0.1%, 0.15%, 0.2%) of rhamnose (inducer). The bacterial cells were harvested and subjected to lysis followed by protein separation and characterization by SDS-PAGE analysis. The purification of Meq protein was performed using Ni-NTA Fast Start Kit (Qiagen, Germany) following manufacturer's protocol. SDS-PAGE analysis of the purified His-tagged recombinant Meq protein was done to assure that the recombinant protein is separated alone and eluted without any contaminant protein. Following purification, the concentration of the recombinant protein was assessed by Lowry's protein estimation method following the manufacturer's protocol (GeNei, India). Western blotting of the purified Meq protein was performed according to the standard protocol's using anti-Meq monoclonal antibodies raised in mouse (Kindly gifted by Dr. Venugopal Nair, Head of the Viral Oncogenesis, Pirbright Institute, UK).

2.4. Raising of Hyper Immune Serum (HIS) against recombinant (r) Meq oncoprotein

Hyper immune serum (HIS) against the recombinant (r) Meq oncoprotein was raised in rabbits following standard methods (Leenaars and Hendriksen, 2005). Animal experimentation was performed as per the approved guidelines and due permission of the Institute Animal Ethics Committee (IAEC), ICAR-IVRI, Izatnagar (India). The rMeq protein preparations (@500 μg/animal) were mixed equally with Freund's incomplete adjuvant and injected subcutaneously at multiple sites in rabbits (n = 2). Rabbits were injected with two booster doses of 500 μg antigen at 2 weeks intervals. Blood (5 ml/rabbit) was harvested from the rabbits by cardiac puncture on day 0, 14, 21, 28, 35, 42, 49 and 56 post immunization and the serum was separated and stored in cryovials at −20 °C until further use. The immunoreactivity of the harvested HIS was ascertained by blotting Nitrocellulose membrane (NCM) strips of 0.45 μ pore size (Advanced Microdevices Pvt. Ltd.) with recombinant Meq antigen (500 ng) and performing dot-ELISA with the raised HIS as a source of primary antibodies and anti-Rabbit IgG–alkaline phosphatase antibody produced in goat as secondary antibodies (Sigma, U.S.A).

2.5. Sample preparation

The tissue samples were prepared for testing in the dot-ELISA by extracting nuclear protein (∼100 mg), as Meq protein is localized in the nucleus and nucleolus. Three methods were followed to facilitate the nuclear protein extraction namely, (i) simple tissue trituration (physical shearing using pestle and mortar), (iii) cellular protein extraction using radio-immuno precipitation assay (RIPA) buffer (Sigma-Aldrich, U.S.A) and (iii) cytoplasmic and nuclear fraction extraction using NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Scientific, U.S.A) following manufacturer's protocol. Before proceeding with the test procedures the endogenous enzymes were quenched. Cellular protein of spleen and liver samples from known positive and negative MD cases were extracted using the RIPA buffer method. Endogenous peroxidase (PO) and alkaline phosphatase (ALKP) were quenched in these tissue samples using 1 mM levamisole and 3% H_2O_2 as quenchers, respectively, to avoid false positive reaction. Briefly, 2–3 μl of the cellular protein extracts were dotted on to different NCM strips and allowed to dry for 15 min at room temeprature. The endogenous enzymes were quenched at different time intervals using quenchers, followed by addition of suitable chromogens/substrates (BCIP/NBT and DAB) to determine the optimal quenching time.

2.6. Standardization and development of dot-ELISA for detection of meq protein

2.6.1. Checker board titration

For developing dot-ELISA, the optimum concentrations of antigen, serum and conjugated secondary antibodies was determined by checker board titration method using their serial dilutions.

The expressed rMeq protein was used as antigen source and the pooled HIS was used as primary antibody. Anti-rabbit IgG (whole molecule)–peroxidase antibody (Sigma, U.S.A) and anti-rabbit IgG (whole molecule)–alkaline phosphatase antibody produced in goat (Sigma, U.S.A) were used as secondary antibodies. Four different concentrations of rMeq antigen diluted to 500 ng/μl, 250 ng/μl, 125 ng/μl and 62.5 ng/μl in phosphate buffered saline (PBS, pH 7.4) and eight different dilutions of HIS as 1 in 10, 1 in 20, 1 in 40, 1 in 80, 1 in 160, 1 in 320, 1 in 640 and 1 in 1280 were used in checker board standardization of dot-ELISA. The secondary antibodies (both peroxidase and alkaline phosphatase tagged) were used in dilutions of 1 in 1000, 1 in 2000, 1 in 4000, 1 in 8000 and 1 in 16,000, separately. The chromogen/substrate used for alkaline phosphatase was BCIP/NBT and for peroxidase was DAB (Sigma, USA). The different concentrations of rMeq antigen were dotted on to the NCM strips and incubated with various dilutions of HIS, followed by conjugated secondary antibodies, and substrate following standard protocol and positive reaction noted as blue and brown dots (Abdelmagid et al., 1998).

2.6.2. Dot-ELISA test procedure

The protocol for screening of field samples by dot-ELISA has been elaborately presented in results section. Briefly, it included the following steps, dispensing of tissue extracts on to the NCM strips, blocking with 5% Bovine serum albumen (BSA) solution, quenching of the endogenous enzymes with suitable quenchers, incubation with HIS and secondary antibodies, followed by washing of NCM strips with PBS-T three times after each step as above. Finally, a suitable chromogen/substrate solution according to the secondary antibody (BCIP/NBT) for IgG – alkaline phosphatase antibody and DAB for IgG – peroxidase antibody was added on to the NCM strips and incubated until the color development. The expected reaction of blue dot in case of BCIP/NBT and brown in DAB system was recorded. Suitable controls comprised of extract from known MD lymphoma tissue as positive control, neat RIPA buffer as vehicle control and extract from liver/spleen (MD negative) as quencher control.

2.7. Analytical specificity

To assess the analytical specificity of the in-house developed dot-ELISA, the raised HIS was tested for the presence of any cross-reactivity with the extracts of lymphoid leucosis (LL) and reticuloendotheliosis (RE) virus induced lymphoma tissues and Inclusion Body hepatitis (IBH) affected birds.

2.8. Screening of the lymphoma samples by dot-ELISA

Poultry carcasses presented to Post-mortem Facility, ICAR-Indian Veterinary Research Institute, Izatnagar (India) were necropsied and 150 cases presented with lymphoma were selected. The affected tissues (liver, spleen, feather follicles) from each case without any autolytic changes were collected and subjected for screening with developed dot-ELISA for Meq antigen detection.

2.9. Immunocytology for MDV Meq detection in lymphoma samples

Immunocytology was used to analyze the sensitivity and specificity of the developed dot-ELISA for detection of Meq antigen in lymphoma affected tissues of 150 necropsied cases of birds. Immunocytology was carried out on the liver and spleen touch impression smears made over the clean grease free glass slides, wet-fixed in chilled acetone for 5 min and washed with PBS (pH 7.4) for 5 min. Each step was preceded by washing thrice in PBS (5 min each). Endogenous peroxidase was quenched with 3% H_2O_2 for 15 min in dark at room temperature (RT). Non-specific antigen blocking was performed using 200 μl of 5% normal rabbit serum in PBS and the slides were incubated for 30 min at room temperature in a humidified chamber. The mouse anti-Meq mAb (1: 200 in 1% BSA) was applied over the slides and incubated for 1 h at room temperature. The imprints were reacted with 100–150 μl of anti-mouse IgG-HRP secondary antibody raised in rabbit (Sigma, USA) at the dilution of 1:100 in 1% PBS, followed by incubation in a humidified chamber at room temperature for 1 h. Finally, treated smears were reacted with DAB chromogen/substrate and immunodetection of Meq antigen was observed as brownish-red color intranuclear positive signals under the light microscope. In negative control, 1% BSA was added instead of primary antibody.

2.10. PCR detection of MDV *meq* gene in lymphoma samples

PCR detection of the MDV *meq* gene was carried out with the pooled liver and spleen samples collected from 150 necropsied cases of birds. The full *meq* gene with amplicon size of 1062 bp was amplified using PCR reaction mix including 12.5-μL of 2× DreamTaq PCR Master Mix, 0.4-μM each forward (M-S:5′TGTCTCAGGAGCCAGAGCCGGCGCT 3′) and reverse primer (M-AS:5′GGGGCATAGACGATGTGCTGCTGA 3′) (Lee et al., 2000), template DNA and nuclease free water to 25-μL. Cycling procedure followed was an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 10 min.

2.11. Comparison of sensitivity and specificity of dot-ELISA with immunocytology for detection of Meq antigen

The screening results of lymphoma samples using the dot-ELISA and immunocytology were compared for the detection of Meq antigen. Further, the sensitivity and specificity of dot-ELISA for definitive diagnosis of MDV induced lymphoma were calculated as per the method described by Parikh et al. (2008).

2.12. Calculation of sensitivity and specificity of conventional PCR with immunocytology for MD diagnosis and comparison with spleen based dot-ELISA

The screening results of lymphoma samples by PCR testing and immunocytology were compared and the sensitivity and specificity of PCR were calculated as per the method described by Parikh et al. (2008). The results obtained were compared with the sensitivity and specificity of dot-ELISA (spleen) obtained under Section 2.11.

3. Results

3.1. Cloning and transformation of Meq oncogene of MDV

The DNA extracted from serotype 1 MDV in PCR yielded a partial length *meq* gene with a single and specific amplicon of 540 bp (507 bp of *meq* gene + 33 bp of adapter sequence). Cloning and transformation of the amplified and purified partial MDV *meq* gene was achieved successfully using Expresso™ Rhamnose Sumo Cloning and Expression System. The transformed colonies on LB agar plates supplemented with kanamycin showed white colonies post incubation at 37 °C overnight. Colony PCR for screening the recombinants revealed an amplicon of 690 bp in 1.2% agarose gel run (Fig. 1).

3.2. Expression kinetics, confirmation, purification and quantification of recombinant Meq protein

The expression kinetics of the Meq protein was studied by SDS-PAGE analysis which revealed that after 16 h, rhamnose at a concentration of 0.05% and 0.1% shows good induction of the Meq protein (Fig. 2), while keeping these two concentrations of

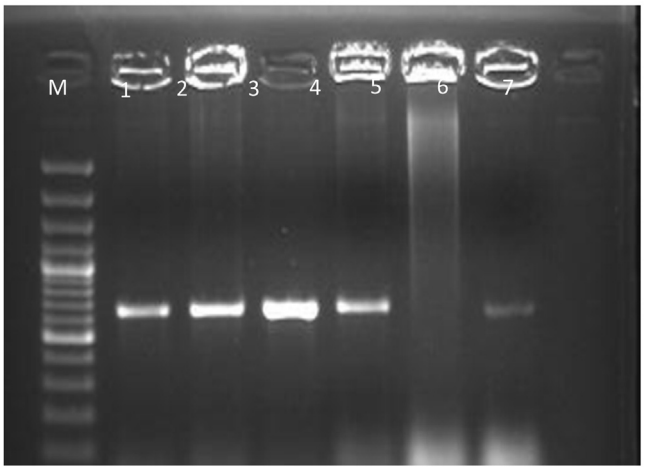

Fig. 1. Agarose gel electrophoresis, Colony PCR-Screening for Recombinants. Lane M–100 bp+ DNA ladder, Lane 1–4 and 6–recombinants with insert, Lane 5 and 7–recombinants without insert.

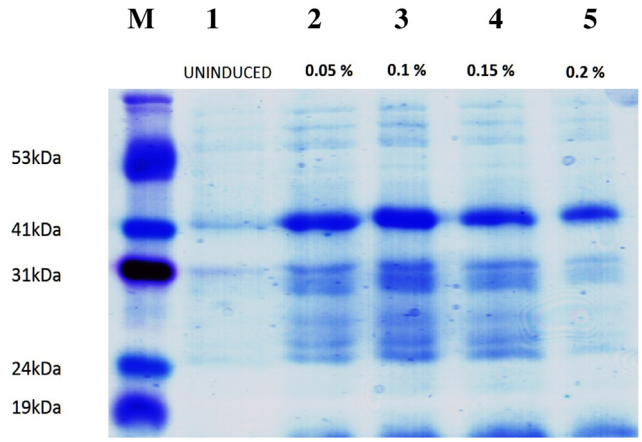

Fig. 2. SDS-PAGE analysis of recombinant Meq protein expression in *E. coli* with different concentrations of Rhamnose at fixed 16 h post induction. Lane M–Protein ladder, Lane 1–uninduced control, Lane 2–5–induced with different concentrations of Rhamnose showing protein expression at ~39 kDa in all concentrations.

rhamnose constant, induction of the Meq protein was best seen at 24 h (Fig. 3). The molecular size of the expressed Meq protein was approximately 39 kDa. Recombinant Meq protein was purified with Ni-NTA agarose column which revealed a single band of 39 KDa in SDS-PAGE analysis. Western blot analysis of the induced culture revealed a band of 39 kDa (Fig. 4). The concentration of the expressed protein was found to be 0.7 mg/ml.

3.3. Raising of Hyper Immune Serum (HIS) against rMeq oncoprotein

The dot-ELISA testing of the harvested HIS on days 0, 14, 21, 28, 35, 42, 49, 56 showed specific positive reaction of brown color dots on NCM except with 0th day HIS indicating that the rabbits immunized produced antibodies against the administered rMeq protein.

3.4. Sample preparation

Cellular proteins were extracted from known MDV induced lymphoma tissues and negative tissues by three methods as described in Section 2.5. Optimal quenching time was found to be 40 min and 30 min post incubation for endogenous alkaline phosphatase

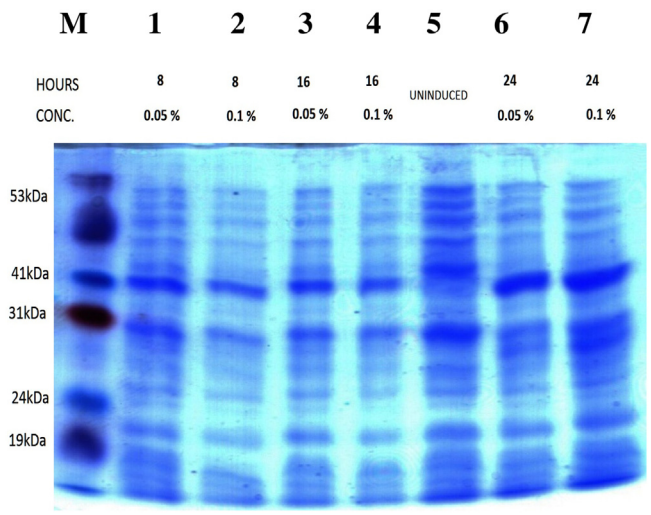

Fig. 3. SDS-PAGE analysis of Meq protein expressed in *E. coli* at different time intervals with two different concentration of Rhamnose, Lane M–protein ladder, Lane 5–uninduced control, Lane 1–7–induced with different concentrations of Rhamnose and time interval.

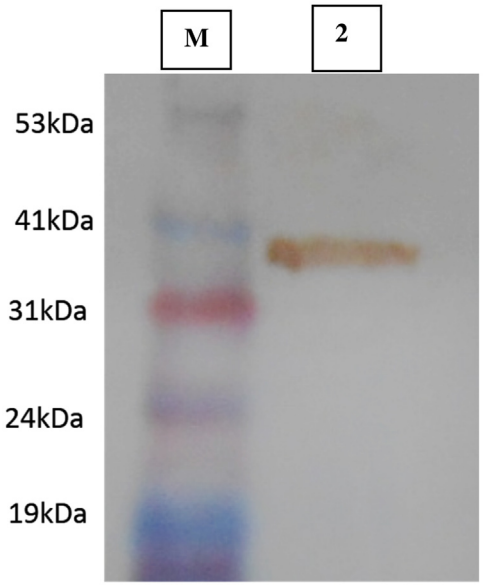

Fig. 4. Western Blot analysis of the recombinant Meq protein, Lane M–pre-stained protein ladder, Lane 2–expressed protein of size ~39 kDa.

and peroxidase, respectively (Fig. 5a and b). The efficacy of various methods in extracting cellular protein is shown in Fig. 6. Cellular protein extracted using the simple tissue trituration method yielded inconsistent results, out of the three NCM strips that were dotted with the same sample, only one yielded mild positive reaction while the rest two remained negative. The RIPA buffer based extraction method yielded consistent results characterized by definite blue dot indicating positive reaction. In NE-PER Nuclear and Cytoplasmic Extraction Reagent method, the cytoplasmic fraction was not reactive but the nuclear fraction showed positive reaction Thus RIPA buffer and NE-PER Nuclear and Cytoplasmic Extraction Reagent can be used efficiently for cellular protein extraction as both gave consistent results. RIPA buffer based extraction was preferred as it could be performed in 15 min in contrast to NE-PER Nuclear and Cytoplasmic Extraction Reagent based extraction which took around 2 h.

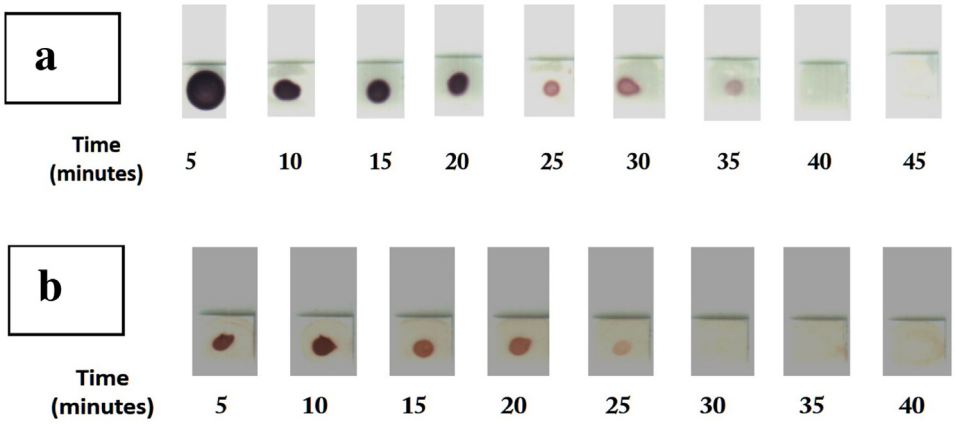

Fig. 5. (a) Oaptimization of quenching time for endogenous ALKP with 1 mM levamisole at different time intervals. Quenching was optimum at 40 min post incubation. (b) Obptimization of quenching time for endogenous PO with 3% H_2O_2 at different time intervals. Quenching was optimum at 30 min post incubation.

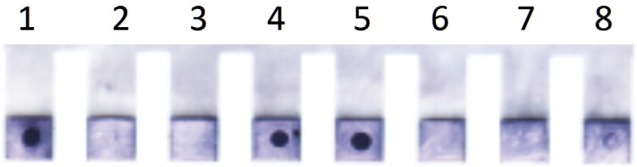

Fig. 6. NCM strip, Cellular Protein Extraction – Strip 1–Known positive. Strip 2–negative control (PBS), Strip 3–Cytoplasmic extract (negative), Strip 4–Nuclear extract (positive), Strip 5–RIPA buffer extracted (positive), Strip 6–8–Tissue triturate (6-positive; 7 and 8-negative).

3.5. Standardization and development of dot-ELISA for detection of Meq protein

3.5.1. Checker board titration

The checker board titration method for standardization of dot-ELISA revealed optimum positive results with expressed rMeq protein antigen at a concentration of 250 pg/μl, HIS at a dilution of 1: 320 and the secondary antibody (for both anti-rabbit IgG – peroxidase antibody and anti-rabbit IgG – alkaline phosphatase antibody) at a dilution of 1:16,000 (Fig. 7). The positive reaction was observed as blue dot in case of BCIP/NBT used as substrate/chromogen and brown dot in case of DAB substrate in dot-ELISA.

3.5.2. Dot-ELISA test procedure

The protocol standardized and optimized for the dot-ELISA included following steps: 3 μl (~250 pg) of extracted tissue antigen (cellular protein) was dispensed in the center of each square of the NCM strip kept in a petridish and allowed to dry at 37 °C for 15 min. About 10 ml of 5% BSA solution was poured over the NCM strip to block the unbound sites and the strip incubated for 1 h at 37 °C. The NCM strip was then washed 3 times with PBS-Tween 20 (PBS-T). Quenching of the endogenous enzymes was done with 1 mM levamisole for 35 min and/or 3% H_2O_2 for 30 min. After quenching, the NCM strip was washed 3 times with PBS-T. Then the strip was incubated with HIS (4 ml) at a dilution of 1:320 for 1 h at 37 °C. After incubation, the NCM strip was washed 3 times with PBS-T. Strip was then incubated with secondary antibody (4 ml) at a dilution of 1:16,000 for 37 °C for 45 min. The NCM was again washed 3 times with PBS-T. Finally, suitable chromogen/substrate solution (4 ml) according to the secondary antibody (BCIP/NBT for IgG – alkaline phosphatase antibody and DAB for IgG – peroxidase antibody) was poured onto the NCM strip and kept for 30 s for the color development, blue dot in case of BCIP/NBT and brown in DAB system (Fig. 8a and b). After the development of color, the reaction was stopped with distilled water.

Table 1
Screening of the lymphoma samples by dot-ELISA in tissue extracts of liver, spleen and feather follicles of field cases. (n = 150).

Organ	Positive	Negative
Liver	122	28
Spleen	120	30
Feather follicle	74	76

3.6. Analytical specificity

The HIS raised had reacted only with the MDV induced lymphoma cases characterized by formation of brown color dot while rest of the tissue samples from other disease conditions remained unreactive (Fig. 9). Thus, the HIS raised was found specific to detect only the Meq protein of MD and does not react with proteins expressed in other disease conditions (LL, RE, IBH).

3.7. Screening of the lymphoma samples by dot-ELISA

A total of 150 lymphoid tumor cases were tested using the in-house developed dot-ELISA. The results of the tests are presented in Table 1. By the in-house developed dot-ELISA, Meq antigen could be detected in 122 liver samples, 120 spleen and 74 feather follicle samples.

3.8. Immunocytology for MDV Meq detection in lymphoma samples

Moderate to strong, homogeneous nuclear immunostaining was observed in tumor cells. The cells were pleomorphic. About 60–90% of the tumor cells showed immunoreactivity to Meq protein which was localized in the nucleus (Fig. 10).

3.9. PCR detection of MDV meq gene in lymphoma samples

The MDV genome (*meq* gene) was detected in 140 lymphoid tumor samples (pooled liver and spleen) out of 150 cases employing PCR assay.

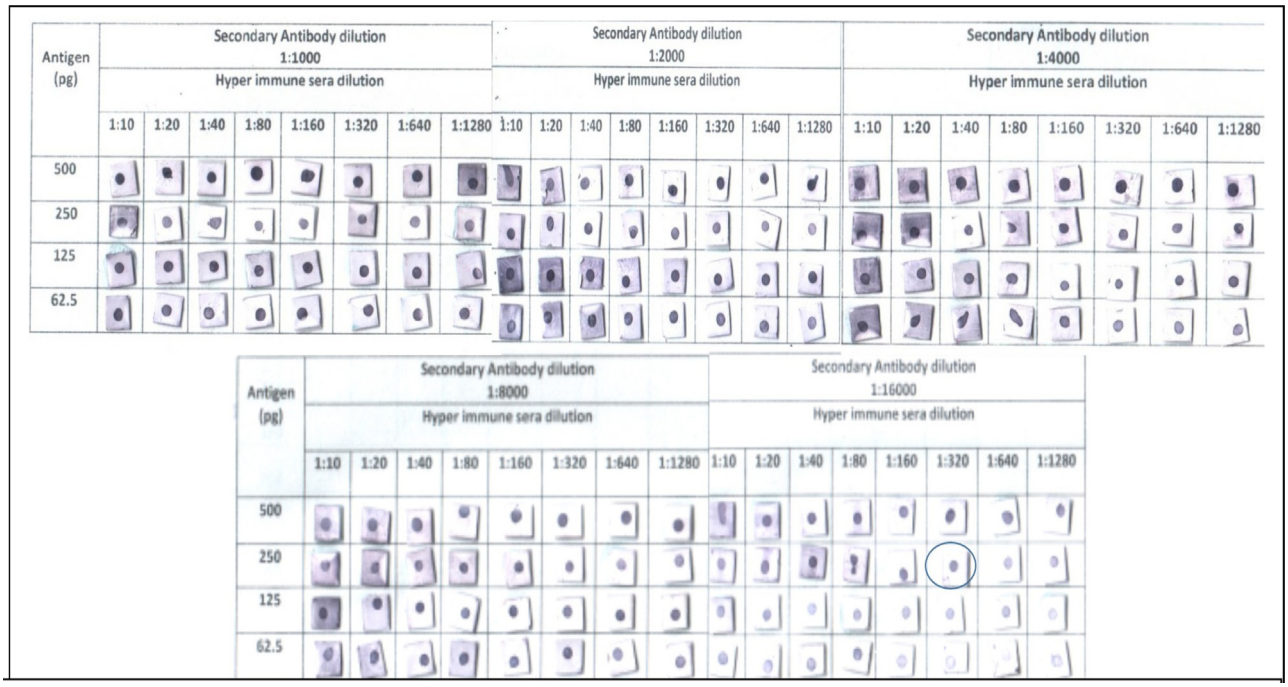

Fig. 7. Checker Board Titration – Optimal antigen concentration was 250 pg, HIS was 1:320 and secondary antibody at 1:16,000 (anti-rabbit IgG – alkaline phosphatase antibody).

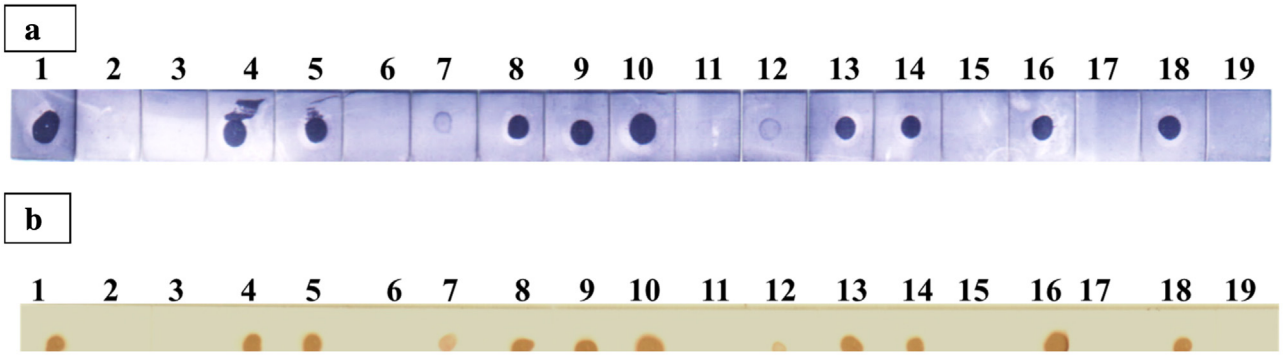

Fig. 8. (a) Daot-ELISA of field samples, 2^0 antibody tagged with ALKP, chromogen/substrate: BCIP/NBT, Screening of field samples. Strip 1–Known positive (MD lymphoma), Strip 2–vehicle control (RIPA buffer), Strip 3–quencher control (MD negative liver/spleen), Strips 4, 5, 7, 8, 9, 10, 12, 13, 14, 16, 18–positive for Meq antigen, Strips 6, 11, 15, 17, 19–Negative for Meq antigen. (b) Dbot-ELISA of field samples, 2^0 antibody tagged with HRPO, chromogen/substrate: DAB/PO. Screening of field samples. Strip 1–Known positive (MD lymphoma), Strip 2–vehicle control (RIPA buffer), Strip 3–quencher control (MD negative liver/spleen), Strips 4, 5, 7, 8, 9, 10, 12, 13, 14, 16, 18–positive for Meq antigen, Strips 6, 11, 15, 17, 19–Negative for Meq antigen.

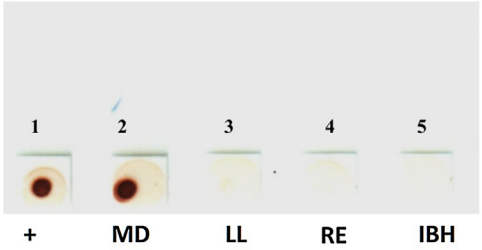

Fig. 9. NCM strip, Analytical specificity of the dot-ELISA. Strip 1–Known positive (recombinant Meq protein), Strip 2–MD lymphoma, Strip 3–LL lymphoma, Strip 4–RE lymphoma, Strip 5–IBH.

3.10. Comparison of sensitivity and specificity of dot-ELISA with immunocytology for detection of Meq antigen

By immunocytology, 125 out of 150 cases of lymphoid tumors were positive for Meq antigen and rest of the 25 were negative.

Table 2
Sensitivity and specificity of dot-ELISA with immunocytology for detection of Meq antigen.

Organ	Sensitivity (%)	Specificity (%)
Liver	95.20	88.00
Spleen	94.40	92.00
Feather follicle	56.80	88.00

The sensitivity and specificity of dot-ELISA on comparison with immunocytology are summarized in Table 2. Liver and spleen based dot-ELISA had a sensitivity of 95.2% and 94.4% respectively. Feather follicle based dot-ELISA had poor sensitivity (56.8%). Among all organs, spleen based dot-ELISA had highest specificity of 92%.

3.10. Calculation of sensitivity and specificity of conventional PCR with immunocytology for MD diagnosis and comparison with spleen based dot-ELISA

The sensitivity and specificity of the PCR in comparison with immunocytology was found to be 100% and 40%, respectively. The

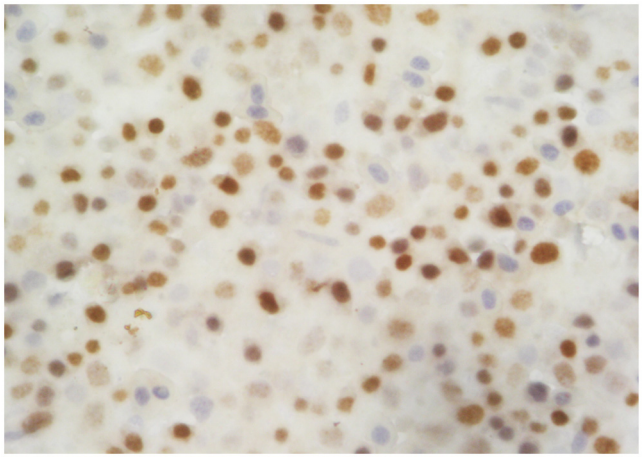

Fig. 10. Tumour imprint from liver: pleomorphic neoplastic cells with prominent nuclear positive immunostaining with Anti-Meq mAb. IP-DAB-MH × 1000.

Table 3
Comparison of Sensitivity and Specificity of conventional PCR and Spleen cellular protein based dot-ELISA.

Test	Sensitivity	Specificity
PCR	100%	40.00%
Spleen cellular protein based dot-ELISA	94.40%	92.00%

sensitivity and specificity of both PCR and spleen cellular protein based dot-ELISA are given in Table 3. PCR was more sensitive (100%) than spleen based dot-ELISA (94.4%) in diagnosing MDV induced lymphoma, while on the contrary spleen based dot-ELISA was more specific (92%) in diagnosing MDV induced lymphoma than PCR (40%).

4. Discussion

Diagnosis and differential diagnosis of lymphomas in poultry is a multistep process (Gimeno et al., 2005). Numerous criteria (age, organs affected, lymphoid tropism) and diagnostic techniques have been established previously by many workers, which would aid in the diagnosis of lymphomas (Singh et al., 2012; Woźniakowski and Samorek-Salamonowicz, 2014). Many of these criteria/techniques have their own limitations and even are not confirmatory always. In the cases suspected for MDV induced tumors, characterization of the cell type (B cells or T cells), quantification of MDV DNA (Islam et al., 2006 and Gimeno et al., 2008) or expression of Meq protein (Gimeno et al., 2005) are considered confirmatory for the MD diagnosis. There are few MDV unique genes such as Meq oncoprotein, MDV phosphoprotein 38 (pp38), Viral IL-8 (vIL-8), Viral-encoded telomerase RNA (vTR), Viral lipase (vLIP) (Cui et al., 1990; Jones et al., 1992; Parcells et al., 2001; Zhao et al., 2011), which get expressed at particular stage of the disease in birds. Among all these genes, the *meq* gene expression has been found to be abundant in MDV transformed T- cell lines and in tumors (Jones et al., 1992; Zhao et al., 2011). Further, among the genes unique to MDV, only Meq-null recombinants alone shows loss of oncogenic properties (Xie et al., 1996; Liu and Kung, 2000), while the knockout mutants of other viral genes viz., pp38 and vIL-8 results in attenuated virulence without any change in oncogenicity (Parcells et al., 2001; Reddy et al., 2002). In addition, analysis of MDV induced tumors and transformed cells revealed consistent expression of Meq as an oncogenic component of MDV (Kung et al., 2001). Meq protein interacts with itself forming homodimers and also forms heterodimers with other cellular proteins like c-Fos, c-Jun, JunB, SNF, ATF, CREB and C/EBP inturn transactivating its respective target genes. In addition, Meq interacts with cell cycle regulator proteins like p53, cyclin-dependent kinase 2, C-terminal binding protein-1 and retinoblastoma protein. Thus, Meq oncoprotein interaction with these cycle regulatory and transcriptional regulatory proteins in a complex way leads to lymphomagenesis (T-cell lymphomas) (Deng et al., 2010). In view of these findings, it is presumed that out of all the MDV proteins, only the Meq protein is more specific in causing tumorogenesis and is also consistently expressed in almost all the tumor cells in MD (Anobile et al., 2006), and thus its selection as a target protein could pave way forward in developing a valuable diagnostic test for quick, accurate detection and differentiation of MDV induced lymphomas from other lymphoma causing viruses (LL, RE, IBH).

The Meq protein has two major domains, the N-terminal bZIP domain and the C-terminal proline-rich domain (Kung et al., 2001), herein, the N-terminal portion of MEQ (aa 1–169) was selected as the target portion to be cloned and expressed (Qian et al., 1995; Liu et al., 1997). The optimum expression was obtained in Expresso™ Rhamnose Sumo Cloning and Expression System. Recombinant Meq proteins have also been produced using a Baculovirus expression system (Suchodolski et al., 2008). The dot-ELISA test has been adopted as a simple and appropriate method for the detection of either antigens or antibodies in numerous infectious diseases of humans and animals (Madhusudana et al., 2004; Manoharan et al., 2004; Bojanich et al., 2012). The procedure of dot-ELISA has a number of advantages over the standard ELISA including the capability of the NCM in binding more antigens than the microtiter plates and additionally does not require spectrophotometric readings (Burt et al., 1979; Prabha and Kocher, 2012). Most immunologic assays are influenced by antibody concentration and affinity but dot-ELISA is least affinity-dependent and a more sensitive method (Belo et al., 2010). Dot-ELISA has been employed in diagnosing various important poultry diseases (Charles et al., 1996; Manoharan et al., 2004; He et al., 2010; Alam et al., 2012). A mere detection of antigen or antibody in poultry against MDV could not give specific clues about the stage of infection and tumor development. Hence, the use of conventional ELISA in diagnosis of MDV induced lymphomas has limited practical applications of identifying the diseased birds specifically with tumor development as it detects only either MDV antigens or antibodies (Cheng et al., 1984; Davidson et al., 1986; Scholten et al., 1990; Zelnik, 2004). Thus, dot-ELISA method of antigen detection targeting oncogenic Meq protein was tried in the present study.

The present study aimed at detecting the Meq protein of MDV in morbid tissues, which is localized in the nucleolus and nucleoplasm of the nucleus in the transformed cells (Anobile et al., 2006), and thus it must be extracted from tissues, which is the important key step for using it as a diagnostic antigen. Out of the three extraction methods tried, RIPA buffer based protein extraction was found to be more suitable, enabling protein extraction from both the cytoplasm and nucleus, and the protein obtained was compatible for employing in several applications like protein assays, immunoassays and reporter assays (Shi et al., 2008). RIPA buffer contains SDS, which disrupts the protein–protein interaction (Su et al., 2010; Janes, 2015) and thus makes available the protein of interest (Meq protein) for detection purposes, even if it is bound to host proteins. Moreover, due to the presence of harsher detergent, RIPA buffer has potential to extract five times more total protein than the non-detergent buffer with greater protein solubilization (Cappione et al., 2012). The tissue extracts in RIPA based method contains both the host endogenous enzymes and other viral proteins, however the host endogenous enzymes needs to be quenched with suitable quencher to avoid false positive reaction (Buchwalow and Böcker, 2010; Suvarna et al., 2013). The raised HIS in this study

was found to be highly specific as it remained unreactive to the host proteins and proteins of other lymphoma causing viruses.

Cytology and special staining's have been used regularly for detection and differentiation of avian lymphoid tumors (Siccardi and Burmester, 1970; Schat and Nair, 2008). Imprint cytology is a well-recognized simple technique for preparing a surgical specimen for pathological assessment in humans (Bell et al., 2010) and has comparable accuracy to frozen sectioning (Scucchi et al., 1997; Azarpira et al., 2012). Herein, touch imprints from the suspected lymphoma cases were immune-stained to detect Meq protein. By immunocytology, 125 out of 150 lymphoid tumors showed reactivity to Meq protein and the rest 25 remained unreactive. The immunoreactivity was seen in the nucleus of MDV transformed lymphoid cells as described earlier (Anobile et al., 2006). The immunocytology was used as the standard test to compare the sensitivity and specificity of the developed dot-ELISA as it specifically detects the localized Meq protein within the cells.

Representative samples from liver, spleen and feather follicles of 150 spontaneous lymphoid tumor cases were tested for Meq protein detection using the developed dot-ELISA. Liver and spleen were selected because these organs develop lymphoma invariably in MDV affected birds. Feather follicles were selected with an idea that the developed test can be used for screening lymphoma in live birds as has been frequently reported by various workers (Denesvre, 2013; Couteaudier and Denesvre, 2014). As reported in literature, the tissues of liver, spleen and feather follicle reacted with HIS against Meq protein and rendered positive reaction in the developed dot-ELISA. Amongst the three organs tested, liver extract based dot-ELISA detected more lymphoma positive cases (122/150) followed by spleen and feather follicles, thus the sensitivity was better when liver extract was used as the test material. However, the specificity of spleen extract based dot-ELISA was more than liver and feather follicles. Feather follicle extract based dot-ELISA showed very poor sensitivity which limits its use in diagnosis of MDV induced lymphomas. The reason behind such lesser sensitivity is attributed to the low/no expression of Meq protein in the feather follicles despite a high viral load, and moreover, the Meq protein is not consistently expressed in feather follicles except in cutaneous form of MD (Couteaudier and Denesvre, 2014). From the above findings it is clear that spleen extract based dot-ELISA and liver extract based dot-ELISA can be used for rapid detection of MD induced lymphomas in birds. In general, the reduction in specificity in the developed dot-ELISA may be attributed to the bacterial protein contamination with the expressed Meq protein. It has been reported that some of the bacterial protein may weakly bind with the affinity column and get eluted along with the protein of interest (Structural Genomics Consortium et al., 2008), moreover it has been found that recombinant proteins expressed in *E. coli* also contain background proteins which can alter protein–protein interaction studies (Howell et al., 2006). By taking into consideration the above findings, when HIS was raised in rabbits there might be antibody raised against these non-specific/background proteins. Amongst numerous post-mortem invaders, *E. coli* is capable of invading tissues after death (Morris et al., 2006). The samples used in the present study were mostly morbid tissues and therefore, there could be every chance that these samples might have possessed invaders including *E. coli*. The HIS raised may react with these bacterial proteins extracted during the cellular protein extraction protocol and thus there is reduction in specificity. The specificity can be increased by purifying the recombinant protein without bacterial proteins and also if fresh tissues are used for detection.

In the present study, all the test samples (n = 150) were screened for the presence of MDV genome by using conventional PCR. The PCR testing results showed high sensitivity (100%) but a lower specificity (40%) in diagnosing MDV induced lymphoma. The specificity of PCR was lesser than the developed dot-ELISA which may be attributed to the fact that the PCR detects MDV genome irrespective of infection, or disease (lymphomas), but cannot confirm whether the lymphoma has been caused by MDV or other viruses in chickens. Owing to its ubiquitous nature, MDV genome may be present in infected birds without active form of the disease. Therefore, the diagnostic value of PCR in confirming MD lymphoma is not definite as reported earlier (Davidson et al., 1995; Silva and Witter, 1996; Witter and Schat, 2003). Since the tissue extract based dot-ELISA revealed better sensitivity and specificity than conventional PCR, the developed dot-ELISA can effectively be used for rapid diagnosis of MD lymphoma in diseased birds as well as has potential to be used at field level owing to its easiness, cost-effective applicability and doesn't require any sophisticated instruments. This test can also be used to rule out presence of MDV induced lymphoma in cases of other types of lymphomas induced by other viruses thus aiding in differential diagnosis of lymphoid tumors in poultry.

5. Conclusion

The diagnosis of MDV induced lymphomas based on the genomic studies is useful to detect MDV infection but its specificity in confirming the disease in chickens is very low. The present study for the first time reports the development of a highly sensitive, specific and novel dot ELISA targeting a specific oncoprotein (Meq) for the confirmatory detection of MDV lymphomas in tissues/organs of affected birds. This assay could also differentiate MDV infection and the disease as well as lymphomas of other viral etiologies. This simple, easy, economic, efficient and useful detection method of dot-ELISA could have wider field applicability for diagnosing and identifying diseased birds showing lymphomas of MD and hence its use in the MDV surveillance programs is suggested.

Conflicts of interest

We declare that none of the authors has a financial or personal relationship that could inappropriately influence or bias the manuscript's contents.

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