Development of Dot – Enzyme Linked Immunosorbent Assay for Detection of Antibodies to Infectious Bursal Disease, Hydropericardium Syndrome and Chicken Anaemia Viruses

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ABSTRACT
A rapid and sensitive dot-ELISA test was developed for the simultaneous detection of antibodies against infectious bursal disease (IBD), hydropericardium syndrome (HPS) and chicken anaemia (CAV) viruses in a single serum sample. The developed dot-ELISA was compared with the virus neutralization test (VNT). For IBD and HPS, viruses isolated and purified from field outbreak materials were used while VP1 ORF3 recombinant protein expressed in DH5α E.coli was used for CAV as coating antigens. Sera samples from 302 breeder chicken were screened for antibodies to the three viruses. A total of 195, 205 and 104 serum samples were found positive for IBDV, HPSV and CAV respectively. By comparing with VNT sensitivity, specificity and accuracy of the test were calculated. The dot-ELISA test was found to be highly sensitive, specific with a K value of 0.99 and hence could be very useful for serological diagnosis of IBDV, HPS and CAV in a single serum sample.

Keywords: Chicken anaemia virus - Diagnosis - Dot-Enzyme linked immune assay - Hydropericardium syndrome virus - Infectious bursal disease virus - Virus neutralization test

1. INTRODUCTION
The important immunosuppressive viral infections of poultry viz., infectious bursal disease virus (IBDV) and chicken anaemia virus (CAV) are paving path for so many other infections. Infection with IBDV causes impaired immune response to many viral infections like Newcastle disease virus [1-2], making the chickens highly susceptible to other viral infections like inclusion body hepatitis [3], Marek’s disease [4-5], infectious bronchitis [6], infectious laryngotracheitis [7], chicken anaemia virus [8] and reoviruses [9]. Along with viral infections, IBDV infection also increases susceptibility to secondary bacterial infections like S.typhimurium and E.coli [10], S.aureus [11], and coccidial infections [12-13]. Infectious bursal disease (IBD) causes high mortality in 3 to 6 week old chicken, where as less acute or subclinical disease is common in 0 to 3 week old chicken [14] and IBDV is immunosuppressive at an early age. CAV causes disease in 2 to 4 weeks old chicken and affected flocks show retarded growth and mortality is generally between 10-20% [15-18]. The appropriate breeder flock hyperimmunization will transfer adequate levels of maternal antibodies to hatchlings until chicks develop age resistance. The fine-tuning of breeder flock immunization can be achieved by screening with serological tests. Serological diagnosis based on ELISA has been a rapid and reliable method of screening large number of samples. However, these methods can only be performed under laboratory conditions causing delay in serological screening of large flocks. There is a need for a simple, rapid yet accurate method of diagnosis that can be performed at farm level. Hence, an immunocomb based dot –ELISA was developed with specially designed trough was developed to assess the antibody status for IBD, HPS and CAV in single test sera.

2. MATERIALS AND METHODS

VIRUSES AND ANTIGENS
Virus isolation and Purification:
The field isolate of IBDV from an outbreak in the Namakkal poultry belt of Tamil Nadu, India was used and the virus was isolated as per the method described by Thangavelu [19]. Briefly, the bursae collected from the disease outbreak were made into 50 per cent (w/v) suspension in phosphate buffered saline (PBS, pH 7.2) by triturating in sterile sand and the homogenate was centrifuged at low speed for 20 minutes. The virus was extracted from the supernatants with chloroform [20]. After treating the clear aqueous phase with antibiotics, the virus presence was confirmed by agar gel immunodiffusion (AGID) and counter immune electrophoresis (CIE) and stored in small
aliquots at -70°C until further use. The virus obtained from 50% bursal suspension was further propagated in 12 day old specific pathogen free (SPF) embryonated chicken eggs by chorioallantoic membrane route (CAM) and in chicken embryo fibroblast (CEF) cultures obtained from SPF eggs. The virus material has produced characteristic lesions in embryos by 3 to 5 days and the harvest was further adapted to CEF cultures which has produced characteristic cytopathic effects (CPE). The cell culture virus was further purified as per the method described by Dobos et al [21] with few modifications. The purified viral proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE) as described by Laemmli [22]. The 50% and 25% hydropericardium syndrome virus suspensions were extracted with chloroform from the livers of affected chicken in an outbreak near Coimbatore poultry belt of Tamil Nadu, India. The clear extract supernatants were used for further work after treating with penicillin (5000 LU/ml) and streptomycin (5000 µg/ml) and the virus was confirmed by both AGID and CIE as per method described by Roy et al [23]. Further, the HPSV was adapted to chicken embryo liver cell (CEL) culture from SPF eggs [24] and the CEL adapted HPSV was purified as described by Ahmad and Burgess [25] and purified viral proteins were analyzed by SDS – PAGE [22]. The CAV VP1 recombinant clones were obtained from department of animal biotechnology, Madras Veterinary College, TANUVAS, Chennai, India. PCR for CAV:
The VP1 ORF3 was constructed in pPROEXHTb plasmid and expressed in DH5α E.coli. The clones were further confirmed for the presence of CAV VP1 insert by extracting the plasmid DNA by alkali lysis method [26]. The plasmid DNA was amplified for the VP1 gene of CAV in a 50 µl reaction by polymerase chain reaction (PCR). The forward primer is 5'- TGG CAA GAC GAG CTC GCA GAC C-3’ and reverse primer is 5’- CCC AGT ACA TGG TGC TGT TCG-3’. The cycle conditions employed were 94°C for 2 minutes, 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes for 34 cycles and a final extension of 72°C for 10 minutes followed by holding at 4°C. The PCR product was electrophoresed on 1% agarose with DNA marker.
Restriction digestion of CAV:
The PCR product was subjected to restriction enzymes digestion using Sal I and Xho I enzymes after purification by EZ – 10 spin column PCR products purification kit (Bio Basic Inc., Canada). After confirmation of VP1 clones, the recombinant protein was induced in bulk with 1% isopropylthio-D-galactoside (IPTG) and the expressed recombinant protein was purified by using Ni CL agarose columns (Genei, Bangalore, India) and western blot was performed with CAV specific antiserum from CR Laboratories, USA.

ANTIBODIES AND ANTISERA
A group of 6 chickens each for IBDV and HPSV were immunized by mixing 500 mg/100 µl quantity of both viruses separately with 1 ml of Freund’s complete adjuvant (Sigma, USA) subcutaneously by keeping 4 chickens each for both viruses as control. After one week first booster was given and second booster was given 14 days after 1st booster dose with Freund’s incomplete adjuvant (Sigma, USA). The presence of antibodies was observed by test bleeding the birds after two weeks of last injection by AGID and CIE. Then, a large amount of blood was collected from chicken and the separated serum was stored in small aliquots at -20°C until further use. Antiserum against CAV VP1 was raised in rabbits, approximately 1 mg per ml of purified recombinant VP1 was mixed thoroughly with an equal volume of Freund’s complete adjuvant and the total quantity was given into two rabbits subcutaneously at different places. One week of first dose, a second dose was given with Freund’s incomplete adjuvant and rabbits were test bled one week after giving second booster and the serum was separated and stored until further use.

TEST SERUM SAMPLES
A total of 302 serum samples were collected from the broiler breeder flocks located around Coimbatore of Tamil Nadu state, India and these serum samples were inactivated at 56°C for 30 minutes and stored at -80°C until further screening by VNT and dot-ELISA.

DESIGN OF IMMUNOCOMB AND DOT-ELISA
The antibodies to IBDV, HPSV and CAV at a time in a single serum sample by dot - ELISA were detected as per the method described by Manoharan et al [27]. The purified antigens of IBDV, HPSV and CAV at 1 µl volume (150 ng / µl) were spotted onto a nitrocellulose membrane of immunocomb having 3 rows on 12 flanges. The purified antigens of three viruses were spotted on the first, second, and third rows respectively and the strips were incubated at 37°C for 1 hour in a humid chamber. One hour after incubation the combs were washed three times with wash buffer (PBS) and incubated for 60 minutes in blocking or serum dilution buffer. In the present study, a 7.5% of skim milk powder (Himedia, India) in PBST was used instead of routine 5%. The combs were again washed three
times with PBST and then incubated at 37°C for 45 minutes with serum samples from the field diluted 1:100 in serum dilution buffer. After incubating with serum samples washed three times in PBST and incubated with anti-chicken Ig Y HRP conjugate (Sigma, USA) diluted 1:1000 in conjugate diluent. Finally, the combs were exposed to the substrate (5 mg of diaminobenzidine (Sigma, USA) in 10 ml of distilled water and 10 µl of hydrogen peroxide) for 10 minutes. The enzymatic reaction was stopped by washing the combs with tap water. The positive reaction was indicated by the appearance of a brown dot within 10 min. A special trough was designed and fabricated with acrylic material that allowed the immersion of dot-ELISA comb completely.

VALIDATION OF THE TEST
Virus neutralization test:
The 302 serum samples collected from broiler breeders were screened for the presence of antibodies to IBDV and HPSV by VNT. The VNT for detection of IBDV antibodies was carried out as per the procedure described [14]. Briefly, 50 µl of virus containing 100 TCID₅₀ was placed in each well of a tissue-culture microtitre plate (Nunclon). The test serum samples were serially diluted by two fold with the virus placed in wells. After incubating for 30 minutes at 37°C, 0.2 ml of chicken embryo fibroblast cell suspension, with a cell density allowing confluent monolayer to be obtained after 24 hours of incubation, was dispensed into each well. Plates were sealed and incubated at 37°C for 4-5 days, after which the monolayers were observed microscopically for typical CPE. The end-point is expressed as the reciprocal of the highest serum dilution that did not show CPE.
The VNT for HPSV was carried out with chicken embryo liver cell culture [28]. Initially, doubling dilutions of serum were made in tissue culture medium and then 50 µl of each dilution was added to microtitre plate already containing 100 TCID₅₀ units of virus. Following reaction time of 1 hour at 37°C 100 µl of freshly prepared CEL culture was added to all wells. Plates were sealed and incubated at 37°C for 4-5 days, after which the monolayers were observed microscopically for typical CPE. The end-point was expressed as the reciprocal of the highest serum dilution that did not show CPE.

STATISTICAL ANALYSIS
The sensitivity, specificity and accuracy of dot ELISA, flow through were compared with the virus neutralization test as described by Venkatesh [29].

\[
\begin{align*}
\text{Sensitivity:} & \quad a / (a+c) \\
\text{Specificity:} & \quad d / (b+d) \\
\text{Accuracy:} & \quad a+d / (a+b+c+d)
\end{align*}
\]

The results obtained from the tests were analyzed for the percentage of agreement with virus neutralization test with the use of Kappa statistics. The kappa statistics is a decimal measure of agreement between two tests, especially in the absence of a standard and is defined as kapp or K. K = (a+d – P) / 1 - P, where P= (a+b) (a+c) + (c+d) (b+d) and P is the probability, a: is the number of samples positive by both i.e., test to be compared and gold standard test, b: is the number of samples negative by standard test whereas negative by test to be compared, c: is the number of samples negative by standard test and positive by test to be compared and d: is the number of samples negative by both.

Guide lines for interpretation of K values are as follows:

- k value < 0.1 indicates poor agreement
- k value between 0.1 and 0.2 indicates slight agreement
- k value between 0.21 and 0.4 indicates fair agreement
- k value between 0.41 and 0.6 indicates moderate agreement
- k value between 0.61 and 0.8 indicates substantial agreement
- k value > 0.81 indicates perfect agreement

3. RESULTS
The IBDV showed five structural polypeptides with molecular weights of 90,000 Da (VP1) a RNA dependent RNA polymerase of the virus playing key role in encapsidation of the viral particles, 40,000 Da (VP2) had been identified as host protective antigen and induced the neutralizing antibodies, 32,000 Da (VP3) a group specific antigen and recognised by non-neutralizing antibodies, 28,000 Da (VP4) a minor, non-structural polypeptide and involved in the auto-processing of the polyprotein as a virus-encoded protease producing VP2a, VP3 and VP4 itself and a precursor polypeptide of 49,000 Da (VPx) were detected in the SDS-PAGE [21 and 30]. The purified viral proteins of IBDV were shown in Fig.1.
In the present study, the polypeptides of purified virus HPSV were resolved by SDS-PAGE. In 12.5% acrylamide gel, the purified virus yielded 12 polypeptides. This finding was in agreement with some of the workers Rajesh Kumar and Rajesh Chandra [31]. On the contrary, some workers got 8 polypeptides on resolution in 10% gel [32]. This difference in number of polypeptides might be attributed to difference in concentration of resolving gel and various other physical conditions. The purified viral proteins of HPSV were shown in Fig.2.

The VP1 insert PCR amplified product of CAV was of approximately 1.3 kbp Fig.3. The restriction enzyme digestion of VP1 cloned plasmid of CAV yielded approximately 6 kbp and 1.3 kbp size fragments Fig.4. The peak expression of VP1 recombinant protein was noticed after 5 hours of expression Fig.5 and the purified recombinant protein was of approximately 51 kilodaltons (kDa) Fig.6. The Western blot analysis of CAV VP1 recombinant protein was performed using CAV specific antiserum. The recombinant protein blotted nitrocellulose membrane after reacting with CAV antiserum showed a single band with a size of 51kDa as shown in Fig.7.
Figure 3
Agarose gel electrophoresis of PCR amplified product of VP1 gene of CAV

Lane M: 1 kbp marker
Lane 1: Plasmid DNA

Figure 4
Agarose gel electrophoresis analysis of restriction enzyme digested products of cloned plasmid

Lane M: 1 kbp marker
Lane 1: Enzyme digested products

Figure 5
Cloning of coding sequence of VP1 gene of CAV
Analysis of time point samples for checking recombinant protein by SDS-PAGE

Lane M: Molecular weight marker
Lane 1: 0 hour before induction with IPTG
Lane 2 to 5: Gradual increase in expression
Lane 6: Final hour

Lane 1: 66 kDa
Lane 2: 51 kDa
Lane 3: 43 kDa
Lane 4: 29 kDa
Lane 5: 20.1 kDa
Lane 6: 14.3 kDa
A total of 302 serum samples were screened for the presence of IBDV and HPSV antibodies by VNT. The serum samples were diluted serially from 1:10, 20, 40, 80, 160, 320 and 640 (log$_2$ 3.3 to log$_2$ 9.3). The neutralization titres less than 4.9 on log$_2$ scale were considered as negative and above 5.0 as positive for IBDV [33]. The results are presented in Table 1. Out of 302 serum samples screened 182 serum samples were positive and 120 serum samples were negative. In case of HPSV, the neutralization titres less than 5.9 on log$_2$ scale were negative and above 6.0 were positive [34]. A total of 165 serum samples were positive for HPSV antibodies and 137 were found to be negative as presented in Table 2. As the MDCC MSB cell line was not available, VNT for CAV antibodies was not carried out and compared with that of dot-ELISA.

### Table 1: Screening of serum samples by VNT for IBDV antibodies

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Numerical dilution</th>
<th>Log$_2$ dilution</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/10</td>
<td>3.3</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>1/20</td>
<td>4.3</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>1/40</td>
<td>5.3</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>1/80</td>
<td>6.3</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>1/160</td>
<td>7.3</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td>1/320</td>
<td>8.3</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>1/640</td>
<td>9.3</td>
<td>6</td>
</tr>
</tbody>
</table>
TABLE 2: Screening of serum samples by VNT for HPSV antibodies

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Numerical dilution</th>
<th>Log(_2) dilution</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/10</td>
<td>3.3</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>1/20</td>
<td>4.3</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>1/40</td>
<td>5.3</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>1/80</td>
<td>6.3</td>
<td>84</td>
</tr>
<tr>
<td>5</td>
<td>1/160</td>
<td>7.3</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>1/320</td>
<td>8.3</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>1/640</td>
<td>9.3</td>
<td>5</td>
</tr>
</tbody>
</table>

The same 302 serum samples were screened for the presence of antibodies to three viral antigens viz., IBDV, HPSV and CAV by dot-ELISA and the results were presented in Table 3. A total of 195, 205 and 104 serum samples gave positive reaction in dot-ELISA for IBDV, HPSV and CAV antibodies respectively Fig.8. The comparison of dot – ELISA with VNT (for IBDV and HPSV) was provided in Tables 4 & 5, along with statistical analysis. The trough specially designed to carry out dot – ELISA comb was provided as Fig. 9.

Figure 8
Dot ELISA results for IBDV, HPSV and CAV antibodies

![Dot ELISA results for IBDV, HPSV and CAV antibodies](image)

PC - Positive Control  NC - Negative Control

Figure 9
Trough used for Dot ELISA combs

![Trough used for Dot ELISA combs](image)
TABLE 3: Screening of serum samples by Dot ELISA

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBDV</td>
<td>195</td>
<td>107</td>
</tr>
<tr>
<td>HPSV</td>
<td>205</td>
<td>97</td>
</tr>
<tr>
<td>CAV</td>
<td>104</td>
<td>198</td>
</tr>
</tbody>
</table>

TABLE 4: Comparison of VNT and Dot ELISA for IBDV antibodies

<table>
<thead>
<tr>
<th>Dot-ELISA</th>
<th>VNT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>164(^a)</td>
<td>31(^b)</td>
</tr>
<tr>
<td>Negative</td>
<td>18(^c)</td>
<td>89(^d)</td>
</tr>
<tr>
<td>Total</td>
<td>182</td>
<td>120</td>
</tr>
</tbody>
</table>

- Specificity : 90.1%
- Sensitivity  : 74.1%
- Accuracy     : 83.7%
- K value      : 0.99

TABLE 5: Comparison of VNT and Dot ELISA for HPSV antibodies

<table>
<thead>
<tr>
<th>Dot-ELISA</th>
<th>VNT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>160(^a)</td>
<td>45(^b)</td>
</tr>
<tr>
<td>Negative</td>
<td>5(^c)</td>
<td>92(^d)</td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
<td>137</td>
</tr>
</tbody>
</table>

- Specificity : 96.9%
- Sensitivity  : 67.1%
- Accuracy     : 83.4%
- K value      : 0.99

**DISCUSSION**

The objective of the present study was to develop immunodiagnostics for the detection of antibodies to IBDV, HPSV and CAV in poultry flocks. The early chick mortality is an important problem to be looked into and the breeder flock vaccination plays a crucial role in curtailing the early chick mortality. Age limiting diseases like IBD and CAV can be controlled by the transfer of enough antibodies to the chicks and this is achieved by hyper immunization of breeder flocks. The vaccination schedule of breeder flocks can be fine tuned based on the antibody status. The conventional serological tests are time consuming and cumbersome and a pen-side test is highly suitable for this purpose. Hence, the dot-ELISA was developed for screening antibodies to three viruses’ viz. IBDV, HPSV and CAV at a time. The combination of these three viruses is so important in that the IBDV and CAV cause immunosuppression and the adenoviruses, especially the HPSV, take upper hand during the phases of immunosuppression further worsening the situation. The monitoring of breeder flock antibody status reveals optimum protection to infections, to know the improvement of vaccination programme and to have an idea regarding the infections status of flock. For these reasons, seromonitoring has to be done on a permanent basis [35].

The virus neutralization test is the reliable serological test which besides differentiating IBDV isolates into antigenic serotypes and subtypes [36], the time of vaccination of breeder flocks can also be determined [37]. The IBDV neutralizing antibodies were detected as early as 3 or 4 days post infection [38] and even low levels of circulating antibodies (titres of 16 to 64) can be detected by VNT. In spite of the sensitivity of neutralization test, it is time consuming, demanding sophisticated facilities, requires expertise both for testing and interpretation. In the present case the dot-ELISA offers a rapid and economical procedure.
study, out of 302 serum samples screened for antibodies to IBDV by VNT, 182 serum samples were positive and 120 serum samples were negative (Table 1). With respect to the HPSV, in the present study, 302 serum samples were screened and 165 serum samples were found to be positive where as 137 serum samples were negative for HPSV by VNT (Table 2). The neutralization assay gives information on both serotype and neutralization titre and most of the neutralization assays with regard to adenoviruses were done for serotyping of isolates [39] and classification [40] but not for obtaining neutralization titres.

It could be surmised that the chicken with negative titre must have not transferred enough immunity to their hatchlings which will be under constant threat to IBDV and HPSV infection till they cross the susceptible age. The chicken with ‘just’ positive titres also must be considered as they were in border line. The breeder flock seromonitoring is the only solution in such situations and the tests that are equally sensitive to that of VNT were the need of the hour which saves time, reagent cost and easy reading of results.

The previous study for detection of antibodies to three viruses’ *viz.*, Newcastle disease (ND), IBD and infectious bronchitis (IB) by dot-ELISA, is a semi-quantitative method developed by Manoharan et al [27], but the results were not compared with any gold standard test. The positive samples could be classified as strong, moderate and weak positives by comparing the colour reaction given by the known strong and weak positive controls. In the present study, dot-ELISA test for detecting the antibodies to three viruses gave results that were in good agreement with VNT (for IBDV and HPSV only). Since the MSB1 cell line was not available, the dot-ELISA results of CAV were not compared and just detected CAV antibodies using recombinant antigen. The kappa (K) value for both IBDV and HPSV when compared with VNT was 0.99 (Tables 4 & 5), which indicated that they were in perfect agreement.

The purified antigens used in the present study should have reduced the non-specific reactions. In comparison to plate ELISA, the developed dot-ELISA can be performed without sophisticated equipment like ELISA reader and the dot-ELISA can be performed at farm level with minimum reagents without any specialized instruments. The farm personnel can perform the test as per the protocol and read the results without much effort. Moreover, antibodies to three viruses can be screened at a time and which reduces the cost of the dot-ELISA. The dot-ELISA is a semi-quantitative assay in which the antibodies level can be quantified up to some extent based on the colour intensity of the dot. Even a score card can be constructed with different intensities of dot, coding for the corresponding antibody level with VNT. By knowing the approximate antibody concentration, the breeder flock vaccination can be rescheduled so as to get uniform, high level of maternal antibodies in all hatchlings.

4. REFERENCES:


