Title: Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies

Author: Youlin Shen Anchun Cheng Mingshu Wang Shun Chen Renyong Jia Dekang Zhu Mafeng Liu Kunfeng Sun Qiao Yang Xiaoyue Chen

PII: S0166-0934(15)00296-7
DOI: http://dx.doi.org/doi:10.1016/j.jviromet.2015.08.016
Reference: VIRMET 12870

To appear in: *Journal of Virological Methods*

Received date: 15-4-2015
Revised date: 19-8-2015
Accepted date: 25-8-2015

Please cite this article as: Shen, Y., Cheng, A., Wang, M., Chen, S., Jia, R., Zhu, D., Liu, M., Sun, K., Yang, Q., Chen, X.,Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies, *Journal of Virological Methods* (2015), http://dx.doi.org/10.1016/j.jviromet.2015.08.016

Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies

Youlin Shen [a,b,c], Anchun Cheng [a,b,c,*], Mingshu Wang [a,b,c,*], Shun Chen [a,b,c], Renyong Jia [a,b,c], Dekang Zhu [b,c], Mafeng Liu [a,b,c], Kunfeng Sun [a,b,c], Qiao Yang [a,b,c], Xiaoyue Chen [b,c]

[a] Avian Diseases Research Center, College of Veterinary Medicine, Sichuan Agricultural University, Wenjiang, Chengdu City, Sichuan, 611130, P.R. China

[b] Key Laboratory of Animal Diseases and Human Health of Sichuan Province, Wenjiang, Chengdu City, Sichuan, 611130, P.R. China

[c] Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Wenjiang, Chengdu City, Sichuan, 611130, P.R. China

*Corresponding author at: Key Laboratory of Animal Disease and Human Health of Sichuan Province & Avian Disease Research Center, College of Veterinary Medicine of Sichuan Agricultural University, Wenjiang, Chengdu City, Sichuan, 611130, P.R. China

Tel.: +86 835 2885774; fax: +86 835 2885774

E-mail address: chenganchun@vip.163.com (A. Cheng); mshwang@163.com (M. Wang).

Summary

An indirect enzyme-linked immunosorbent assay (I-ELISA) based on the recombinant VP3 protein of duck hepatitis A virus type 1 (DHAV-1) was developed and evaluated in this study. The optimal antigen, serum and enzyme-labeled antibody dilutions were 1:160 (0.94 μg), 1:160 and 1:2000, respectively. The optimal blocking buffer was 1% gelatin. The cutoff value was determined to be 0.332, and the analytical sensitivity was 1:1280 ($OD_{450-630}$ = 0.37). The results of the specificity evaluation showed that no cross-reactivity existed between DHAV-1 antiserum and other common duck-sensitive pathogens, except for duck hepatitis A virus type 3 (DHAV-3), suggesting that this could be a common approach for the simultaneous detection of DHAV-1 and DHAV-3 antibodies. The coefficients of variation (CVs) for all of the tested samples were lower than 10%. The concordance between the I-ELISA based on the VP3 subunit of DHAV-1 and that based on the whole DHAV-1 virus particle was 96%. These results indicate that the VP3-based I-ELISA method has high sensitivity, specificity, and repeatability and is as effective as the DHAV-1-based I-ELISA method for sero-surveillance. Thus, it may be a convenient and novel method for DHAV antibody detection and epidemiological surveillance of DHAV prevalence.

Keywords

Duck hepatitis A virus type 1 (DHAV-1); VP3 protein; Indirect ELISA (I-ELISA); Antibody detection.

Highlights:

1. This is the first report of development of a VP3 based I-ELISA for detecting antibodies against DHAV-1.

2. The I-ELISA method are capable of dectecting DHAV-1 and DHAV-3 antibodies simultaneously.

3. The I-ELISA method had a coincidence rate of 96% with the I-ELISA method based on DHAV-1 whole viruses.

1. Introduction

Duck viral hepatitis (DVH) is a highly fatal and rapidly spreading contagious disease of ducklings that is mainly caused by duck hepatitis A virus (DHAV). It belongs to the genus *Avihepatovirus* of the family *Picornaviridae* (Knowles et al., 2012). It is an OIE notifiable disease infecting almost all ducklings less than 4 weeks of age, with a mortality rate as high as 50%-95% (Woolcock and Tsai, 2013). Thus, the timely and accurate detection of infection is vital for disease control. The neutralization test is the most classical method among the currently approved methods (Hwang, 1969; Kim et al., 2006), but it is laborious and skill dependent. Other assays, such as immunofluorescence technology and the immune colloidal gold technique, have much higher sensitivity and specificity but are costly. With increasing knowledge of molecular biology and following publication of the whole DHAV genome (Tseng et al., 2007), techniques based on PCR, such as RT-PCR and real-time

TaqMan RT-PCR, have become available (Cheng et al., 2009; Yang et al., 2008). Although they are efficient, accurate and rapid, they rely on equipment that is not available in all settings, and they are far less convenient than detecting a virus-specific antibody in the serum (Liu et al., 2010). The indirect enzyme-linked immunosorbent assay (I-ELISA) is a good alternative to these techniques, and utilizing recombinant protein produced by molecular biological assays to establish an ELISA method is becoming a new trend for the efficient diagnosis and epidemiological surveillance of DHAV.

DHAV has three genotypes: DHAV-1, DHAV-2 and DHAV-3. Only DHAV-1 and DHAV-3 are prevalent in mainland China, and DHAV-1 is the most prevalent genotype worldwide (Soliman et al., 2015; Erfan et al., 2015). The DHAV genome consists of only one single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs), and it is translated into three mature structural proteins, VP0, VP3 and VP1 (Kim et al., 2007; Kim et al., 2006; Tseng et al., 2007). Among them, VP1 is the most external and important immunogenic protein and is thus the most widely studied. However, VP3 is also a main capsid protein with numerous antigenic sites that can potentially trigger host immune responses. Moreover, VP3 has a more conserved genome sequence than VP1 (Kim et al., 2006; Tseng et al., 2007; Tseng and Tsai, 2007), which may enable the development of novel diagnostic agents.

In this study, an I-ELISA method based on the recombinant VP3 protein of DHAV-1 (VP3-DHAV I-ELISA) was established. The parameters were optimized, and the sensitivity, specificity and repeatability were evaluated. Furthermore, I-ELISA

based on DHAV-1 whole virus particles (DHAV I-ELISA) was compared with the novel VP3-DHAV I-ELISA method. The newly established method may be a supplement to or even a substitution for the DHAV I-ELISA method for DHAV antibody detection and the monitoring of DHAV prevalence.

2. Materials and methods

2.1. Viral strain and serum samples

DHAV-1 strain H was kept in our lab, and its complete genome is available in GenBank (JQ301467.1). Positive serum samples were collected from ducks that were artificially infected with DHAV-1, and the samples were stored at -80℃ until use. Negative serum samples were collected from DHAV-free healthy ducks, and their negative statuses were confirmed by RT-PCR. Confirmed antisera against several common duck-sensitive antigens were stored in our lab. Serum samples were obtained from naïve, DHAV-1-vaccinated and DHAV-1-infected ducks from different farms in Sichuan province, China, and they were used to compare VP3-DHAV I-ELISA with DHAV I-ELISA.

2.2. Antigen preparation

Recombinant VP3 protein of DHAV-1 was expressed using a pGEX-4T-1 vector in a prokaryotic expression host, BL21 (DE3), and purified by SDS-PAGE gel extraction by the author (results to be published). DHAV-1 particles were propagated in 11-day-old duck embryos (Liu et al., 2010) and purified by approaches described in a previous paper published by our lab (Jiang et al., 1989). The concentrations of both antigens were analyzed with a spectrophotometer (SmartSpec^{TM} 3000, Bio-Rad).

2.3. Establishment and optimization of VP3-DHAV I-ELISA

High-titer serum samples from artificially infected ducks and confirmed serum samples from DHAV-free healthy ducks were used as positive and negative working standards, respectively. The optimum dilutions of coating antigen and serum were determined by checkerboard titration in a 96-well microplate, according to an essentially classical indirect ELISA protocol (Crowther, 2009). The purified VP3 protein was serially diluted by two-fold (from 1:20 to 1:2560). The positive and negative serum samples were diluted from 1:40 to 1:1280 in the same manner in a separate plate. An HRP-labeled rabbit anti-duck IgG conjugate (1:5000 to 1:10000 recommended, Beijing Kangbiquan BioScience) was optimized by preparing two-fold serial dilutions (from 1:500 to 1:4000). The optimal blocking buffer (dissolved in PBS containing 0.05% Tween-20) was chosen from 1% BSA, 5% BSA, 1% gelatin, 5% gelatin and 5% skim milk. All of the samples were tested in triplicate and measured with a microplate spectrophotometer (Model680, Bio-Rad) at two wavelengths (OD_{450} and OD_{630}). A reaction with a corresponding positive value (P) of approximately 1.0, a negative value (N) of below 0.4, and the highest P/N value that was no less than 2.1, was considered optimal.

2.4. Validation of the method parameters

The cutoff value for the VP3-DHAV I-ELISA was determined using a panel of 40 negative serum samples under optimal conditions. The result was described as the mean of the total negative serum OD values plus three standard deviations (SDs) (Jia et al., 2009; Upadhyay et al., 2009). An OD value of above the cutoff value was

138 considered positive for the serum sample.

139 A positive serum sample was used to evaluate the lower detection limit by
140 performing end-point titration from 1:80 to 1:10240 (Mahajan et al., 2015). Each
141 dilution was tested three times. Positive and negative serum samples for DHAV-1
142 served as controls. The maximum dilution with the mean OD value that was above the
143 cutoff value was considered to indicate the analytical sensitivity of the method. The
144 analytical sensitivity of DHAV I-ELISA (mentioned below in section 2.5) was
145 evaluated in the same manner.

146 The specificity of VP3-DHAV I-ELISA was evaluated by an antigenic
147 cross-reactivity test (Liu et al., 2014) and blocking test. Confirmed antisera to
148 DHAV-1, DHAV-3, duck plague virus (DPV), avian influenza virus (AIV), duck
149 swollen head septicemia virus (DSHSV), *Riemerella anatipestifer* (*R.A*), *Salmonella*
150 *enterica* (*S.E*) and *Escherichia coli* (*E. coli*) were used to evaluate antigenic
151 cross-reactivity. Each antiserum was tested three times, and DHAV-1-negative serum
152 served as a control. A blocking test was conducted to further evaluate the specificity.
153 Briefly, antiserum to DHAV-1 was mixed with DPV and DHAV-1 antigen separately
154 at 10:1 (V/V), neutralized at 37ºC for 1 hr, and then diluted to the optimal
155 concentration and incubated as a primary antibody. Each serum sample was tested
156 three times, and DHAV-1-positive serum that was not mixed with antigen served as a
157 control. DHAV-3 antiserum was mixed with DHAV-1 antigen and neutralized in the
158 same manner. The blocking rate was calculated as the evaluation index.

159 The repeatability evaluation was conducted as follows. Antigens from

the same and different batches were used to test the same panel of positive (n = 3) and negative (n = 3) serum samples for DHAV-1. The intra-assay and inter-assay coefficients of variation (CVs) were calculated. Each serum sample was tested four times.

2.5. Comparison of VP3-DHAV I-ELISA and DHAV I-ELISA for antibody detection of DHAV-1 in clinical specimens

DHAV I-ELISA was conducted as previously described (Zhao et al., 1991) with some modifications. First, we prepared antigens as described in section 2.2. Second, we blocked an ELISA plate with 5% skim milk (dissolved in PBS containing 0.05% Tween-20) for 1.5 hr at 37ºC before adding diluted serum. Next, we adjusted the incubation time of the serum with an HRP-labeled rabbit anti-duck IgG conjugate to 45 min at 37ºC. Finally, we used a commercial TMB substrate and performed measurements at two wavelengths (OD_{450} and OD_{630}).

Serum samples from different sources were tested by VP3-DHAV I-ELISA and DHAV I-ELISA separately to evaluate the similarities between the two assays to gain insights into the clinical applicability of the established VP3-DHAV I-ELISA. The sensitivity and specificity of the new method were determined. The similarity rate was calculated as follows: the sum of the true positive and true negative serum samples divided by the total number of samples.

3. Results

3.1. Standardization of the VP3-DHAV I-ELISA procedure

Purified recombinant VP3 protein was prepared and identified to satisfy the

experimental requirements (data not shown). The optimal concentrations of VP3 protein, serum and rabbit anti-duck IgG conjugate were 1:160 (0.94 μg), 1:160 (Table 1) and 1:2000 (Table 2) , respectively. The optimal blocking buffer was 1% gelatin (data not shown). Low background, as well as significant differences, were found to exist between the positive and negative serum samples.

3.2. Validation of the method parameters

A panel of DHAV-1 antibody-negative serum samples was tested under the determined optimal conditions. The OD values ranged from 0.035 to 0.267, with a mean OD value of 0.122 and an SD of 0.070. The cutoff value was calculated using the following formula: $0.122 + 3 \times 0.07 = 0.332$.

The lower detection limit, also called the analytical sensitivity of the method, was evaluated by end-point titration and was determined to be 1:1280 ($OD_{450\text{-}630}$ = 0.37) (Fig. 1). The analytical sensitivity was 1:2560 ($OD_{450\text{-}630}$ = 0.42) for the DHAV I-ELISA (data not shown).

The results for the specificity evaluation showed that all of the serum samples, except for the DHAV-1- and DHAV-3-positive samples, were below the cutoff value (Fig. 2). The OD value of the DHAV-1-negative samples was 0.258 ± 0.010. The DPV- positive serum samples had the highest OD value (0.246 ± 0.023) among the tested samples, except for the DHAV-1- and DHAV-3-positive serum samples. A significant difference in OD values was detected between the seronegative and seropositive samples. For the blocking tests (data not shown), the OD values of the sera mixed with DHAV-1 and DPV particles were 0.230 ± 0.003 and 1.210 ± 0.003,

204 respectively, while that of the control was 1.246 ± 0.038. The calculated blocking rate
205 for DHAV-1 antiserum was 82%, indicating that the DHAV-1-positive serum reacted
206 specifically with DHAV-1 rather than with DPV. The blocking rate for DHAV-3
207 antiserum was 56%, indicating that it cross-reacted with DHAV-1.

208 The repeatability of the assay was evaluated by determining the average
209 intra-assay and inter-assay CVs. Serum samples with different OD values were tested
210 (Fig. 3). The intra-assay CV ranged from 0.73% to 8.64%, with a mean of 5.36%,
211 while the inter-assay CV ranged from 3.35% to 7.68%, with a mean of 5.53%. None
212 of the tested serum samples had a CV of over 10%, indicating the stability and
213 repeatability of the method.

214 *3.3. Comparison of efficacies of VP3-DHAV I-ELISA and DHAV I-ELISA for detecting*
215 *DHAV-1 antibody*

216 Serum samples of different origins were tested simultaneously using the
217 VP3-DHAV and DHAV-based I-ELISA for comparison. The cutoff value of the
218 DHAV I-ELISA determined in this study was 0.313 (data not shown). The results
219 (data not shown) showed that the positive rates for the VP3-DHAV and DHAV-based
220 I-ELISAs were 50% (50/100) and 52% (52/100), respectively. However, one sample
221 that tested negative using DHAV I-ELISA was found to be positive by VP3-DHAV
222 I-ELISA. In contrast, 3 samples that tested negative by VP3-DHAV I-ELISA were
223 found to be positive by DHAV I-ELISA. The diagnostic sensitivity and specificity of
224 the new method were 94.2% (49/52) and 97.9% (47/48), respectively. The
225 concordance between the two methods was 96% [(49+47)/100], indicating that the

efficacy of VP3-DHAV I-ELISA for antibody detection was almost equal to that of DHAV I-ELISA.

4. Discussion

The pathogenic characteristics of DVH make it an intractable disease that must be identified and prevented during the early stages. I-ELISA can be performed to test sera from duck flocks with suspected DHAV infections to efficiently monitor the antibody levels in ducks, and it can provide a guide for preventive measures for timely control of the disease. ELISA is commonly used for the detection of numerous picornaviruses (Liu et al., 2010; Ma et al., 2008; Mahajan et al., 2013; Mahajan et al., 2015; Mohapatra et al., 2014; Yu et al., 2012) and not merely for identification of DHAV.

In theory, using a whole virus as a coating antigen in indirect ELISA is more advantageous than using its subunits because whole viruses have more comprehensive antigenicity. This advantage may account for the higher detection limit (1:2560) of DHAV I-ELISA than that of VP3-DHAV I-ELISA (1:1280). Nevertheless, purification of DHAV particles is costly and difficult (Liu et al., 2010); thus, it is urgent to identify a substitution for this antigen. Recombinant protein is a reasonable choice because it is readily available, easy to purify to any required concentration, and capable of immunoreactivity. Moreover, ELISAs based on recombinant proteins have been widely used (Ma et al., 2008; Zhang et al., 2015). To our knowledge, however, no VP3-DHAV I-ELISA method for DHAV detection has been reported.

In this study, an indirect ELISA method based on the recombinant VP3 protein

248 was developed. A panel of DHAV-negative serum samples collected from healthy
249 ducklings was confirmed by RT-PCR (Cheng et al., 2009). Although the samples had
250 been filtered, the OD values varied greatly among them, possibly due to hemolysis or
251 complex components present in the serum. The analytical sensitivity of the method
252 was 1:1280 ($OD_{450-630}$ = 0.37). The results of antigenic cross-reactivity testing
253 indicated that the VP3-DHAV I-ELISA method failed to detect antisera to other
254 duck-sensitive antigens but was capable of detecting antisera to both DHAV-1 and
255 DHAV-3. This finding may be used to aid in the monitoring of DHAV antibodies, but
256 it fails to differentiate whether DHAV-1 or DHAV-3 is the pathogen, and it also
257 indicates the existence of cross-reactivity between the two genotypes. VP3 is
258 relatively conserved, and sequence BLAST analysis showed that the VP3 proteins of
259 DHAV-1 and DHAV-3 share 79%-80% identity, which is higher than that of the VP1
260 proteins (70.51%-72.81%). However, a published VP1-based I-ELISA method has
261 been demonstrated to be able to detect antisera to DHAV-1 and DHAV-3
262 simultaneously (Yang et al., 2014). The repeatability of the method was proven to be
263 good because both the intra-assay and inter-assay CVs were lower than 10%. Taken
264 together, these results indicate that the VP3-DHAV I-ELISA method is sensitive,
265 specific and stable and that it can be used as a common approach to detect DHAV-1
266 and DHAV-3 antibodies.

267 To evaluate the efficacy of the novel I-ELISA method, it's critical and necessary
268 to compare it with the standard method (Liu et al., 2014; Yang et al., 2014; Zhang et
269 al., 2015). The neutralization test is not suitable for massive serological detection of

the virus strain used in this study, which has not adapted to cell culture to date; moreover, no approved commercial ELISA kits for DHAV are available for clinical use. Even so, DHAV whole particle-based I-ELISA has been demonstrated to have good consistency according to the neutralization test (Zhao et al., 1991; Sun et al., 1997); therefore, a published DHAV I-ELISA method was modified and improved for use as a standard to evaluate the newly established VP3-DHAV I-ELISA method. To imitate field conditions, serum samples were collected from ducks naturally infected with DHAV, and they were also obtained from vaccinated and naïve ducks were at the clinic. The results revealed strong similarities between the efficacies of the two assays (96%), suggesting that the immunoreactivity of the VP3 subunit is comparable to that of the whole DHAV-1 virus particle. It demonstrates that the VP3 subunit, in addition to VP1 (Oberste et al., 1999; Zhang et al., 2014), can also act as an indicator of DHAV-1 infection. Compared with the published VP1-based ELISA method developed by Liu et al. (2010), this new assay has lower sensitivity but higher specificity. This difference may be related to the increased sequence conservation of VP3 compared with VP1. It is difficult to accurately compare our results with those of Liu et al. because their diagnostic specificity and sensitivity were based on the neutralization test, while ours were based on the DHAV whole particle I-ELISA. Taken together, these results demonstrate that the VP3-DHAV I-ELISA is capable of serological detection.

This study is far from perfect, and we still need to evaluate a larger number of samples and to quantify antibodies. Even so, this method shows potential for the

serological detection of DHAV antibodies.

In conclusion, we have developed a novel indirect ELISA method for detecting DHAV-1 and DHAV-3 antibodies cheaply, rapidly and accurately. The VP3-DHAV I-ELISA method has been demonstrated to be highly sensitive, specific, and equally capable of sero-surveillance compared with the DHAV I-ELISA method. It is a useful supplement for the monitoring of both vaccinated and non-vaccinated infected flocks.

Acknowledgements

This research was supported by the China Agricultural Research System (CARS-43–8), the National Science and Technology Support Program for Agriculture (2015BAD12B05/2011BAD34B03) and the Innovative Research Team Program of the Education Department of Sichuan Province (12TD005/2013TD0015).

References

Cheng, A., Wang, M., Xin, H., Zhu, D., Li, X., Chen, H., Jia, R., Yang, M., 2009. Development and application of a reverse transcriptase polymerase chain reaction to detect Chinese isolates of duck hepatitis virus type 1. J. Microbiol. Methods 77, 332-336.

Crowther, J. R., 2009. The ELISA guidebook, 2nd ed. Humana Press, New York.

Erfan, A.M., Selim, A.A., Moursi, M.K., Nasef, S.A., Abdelwhab, E.M., 2015. Epidemiology and molecular characterisation of duck hepatitis A virus from different duck breeds in Egypt. Vet. Microbiol. 177, 347-352.

Hwang, J., 1969. Duck hepatitis virus-neutralization test in chicken embryos. Am. J. Vet. Res. 30, 861-864.

Jia, R., Cheng, A., Wang, M., Qi, X., Zhu, D., Ge, H., Luo, Q., Liu, F., Guo, Y., Chen, X., 2009. Development and evaluation of an antigen-capture ELISA for detection

of the UL24 antigen of the duck enteritis virus, based on a polyclonal antibody against the UL24 expression protein. J. Virol. Methods 161, 38-43.

Jiang, W., Liao, D., Xie, J., 1989. Purification and electron microscope observation of duck hepatitis virus. Animal Husbandry & Veterinary Medicine (In Chinese) 21, 45-50.

Kim, M.C., Kwon, Y.K., Joh, S.J., Kim, S.J., Tolf, C., Kim, J.H., Sung, H.W., Lindberg, A.M., Kwon, J.H., 2007. Recent Korean isolates of duck hepatitis virus reveal the presence of a new geno- and serotype when compared to duck hepatitis virus type 1 type strains. Arch. Virol. 152, 2059-2072.

Kim, M.C., Kwon, Y.K., Joh, S.J., Lindberg, A.M., Kwon, J.H., Kim, J.H., Kim, S.J., 2006. Molecular analysis of duck hepatitis virus type 1 reveals a novel lineage close to the genus Parechovirus in the family Picornaviridae. J. Gen. Virol. 87, 3307-3316.

Knowles, N., Hovi, T., Hyypiä, T., King, A., Lindberg, A., Pallansch, M., Palmenberg, A., Simmonds, P., Skern, T., Stanway, G., Yamashita, T., Zell, R., 2012. Virus taxonomy: classification and nomenclature of viruses. In: Picornaviridae, Ninth Report of the International Committee on T axonomy of Viruses. Elsevier, San Diego, pp. 855-880.

Liu, M., Zhang, T., Zhang, Y., Meng, F., Li, X., Hou, Z., Feng, X., Kong, X., 2010. Development and evaluation of a VP1-ELISA for detection of antibodies to duck hepatitis type 1 virus. J. Virol. Methods 169, 66-69.

Liu, Q., Jia, R., Wang, M., Huang, J., Zhu, D., Chen, S., Yin, Z., Wang, Y., Chen, X.,

Cheng, A., 2014. Cloning, expression and purification of duck hepatitis B virus (DHBV) core protein and its use in the development of an indirect ELISA for serologic detection of DHBV infection. Arch. Virol. 159, 897-904.

Ma, X., Song, M., Yu, K., Liao, M., Xin, C., 2008. [Expression of VP1 gene and ELISA detection of antibodies against duck hepatitis virus]. Wei sheng wu xue bao = Acta microbiologica Sinica 48, 1110-1114.

Mahajan, S., Mohapatra, J.K., Pandey, L.K., Sharma, G.K., Pattnaik, B., 2013. Truncated recombinant non-structural protein 2C-based indirect ELISA for FMD sero-surveillance. J. Virol. Methods 193, 405-414.

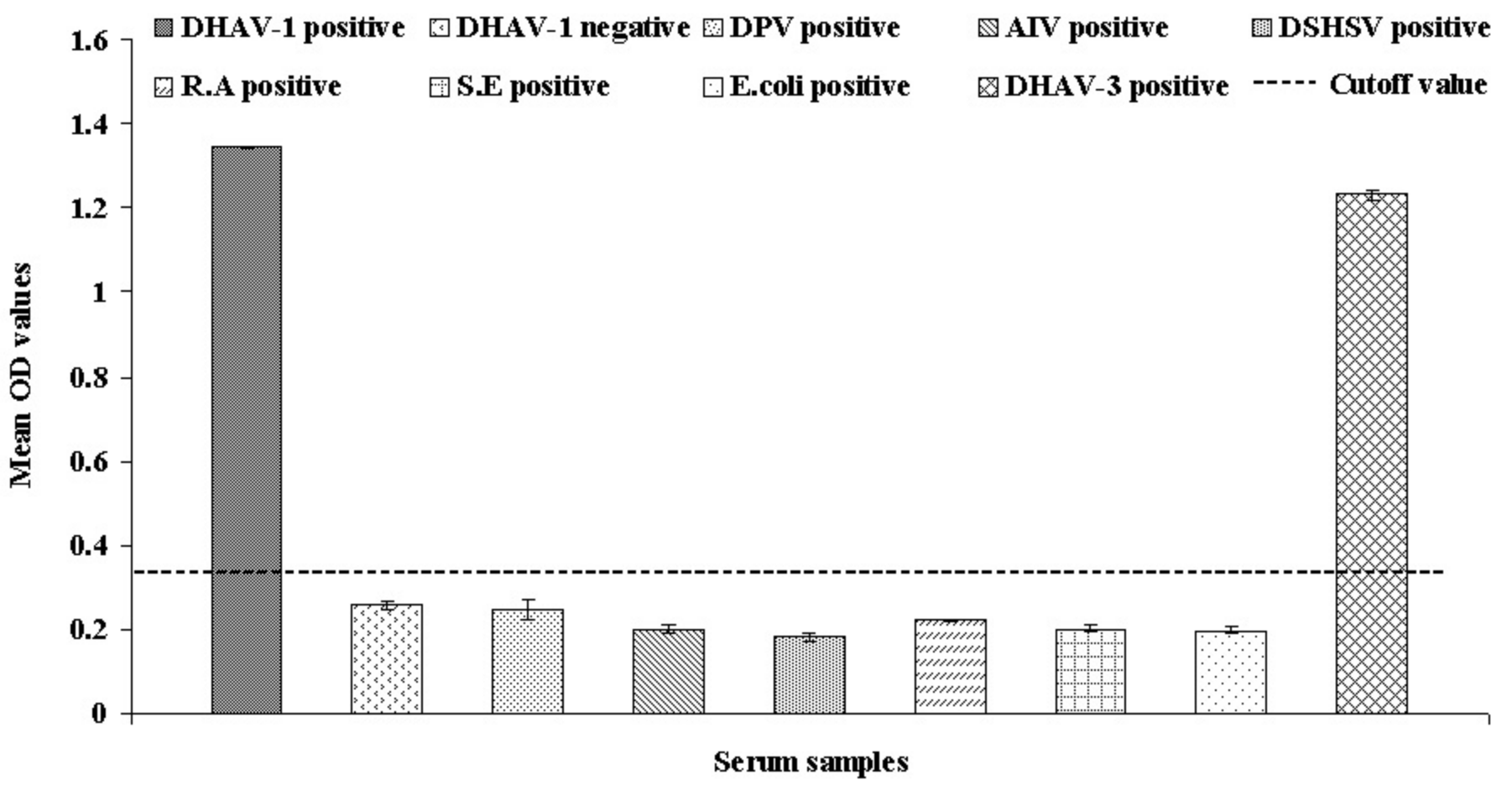
Mahajan, S., Mohapatra, J.K., Pandey, L.K., Sharma, G.K., Pattnaik, B., 2015. Indirect ELISA using recombinant nonstructural protein 3D to detect foot and mouth disease virus infection associated antibodies. Biologicals 43, 47-54.

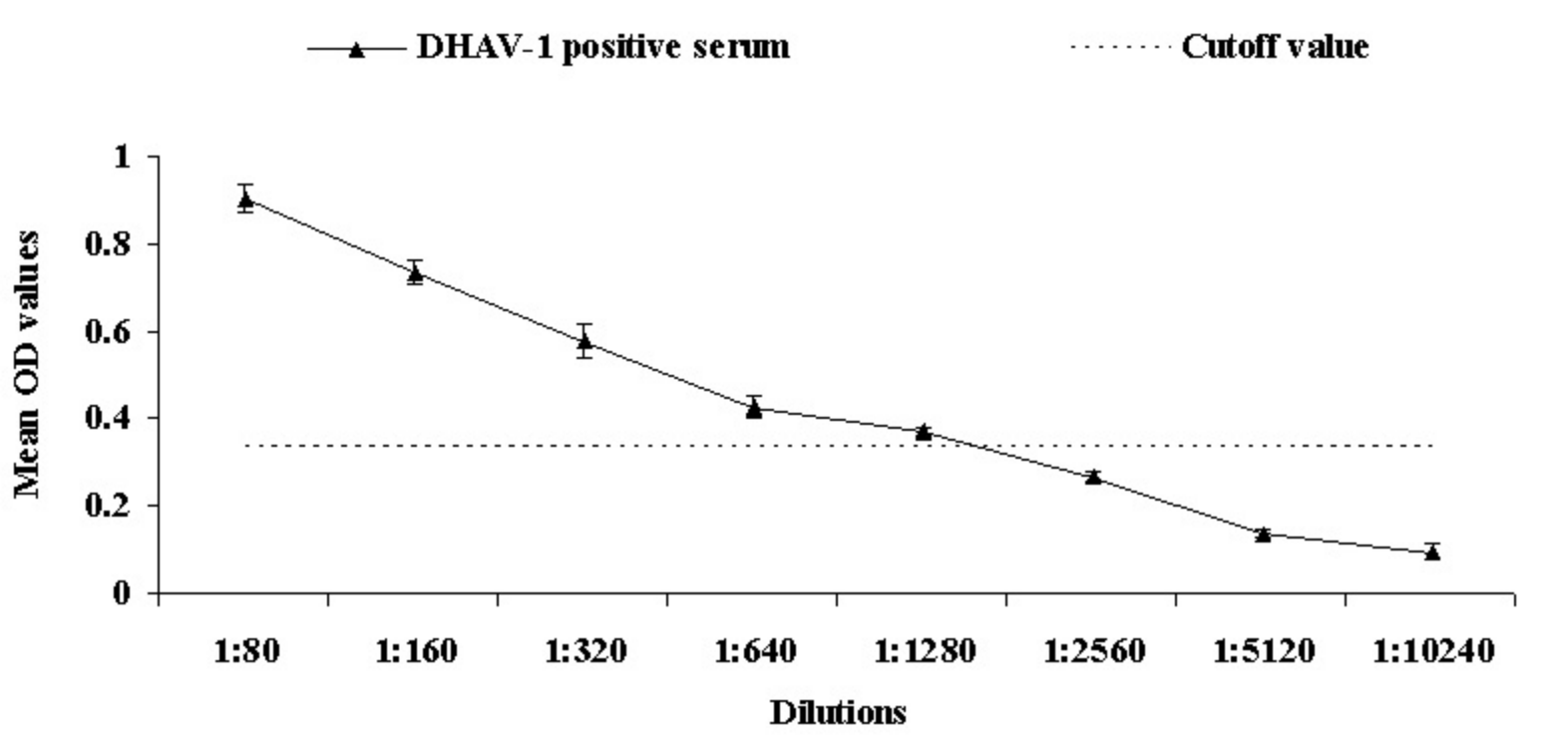
Mohapatra, A.K., Mohapatra, J.K., Pandey, L.K., Sanyal, A., Pattnaik, B., 2014. Diagnostic potential of recombinant nonstructural protein 3B to detect antibodies induced by foot-and-mouth disease virus infection in bovines. Arch. Virol. 159, 2359-2369.

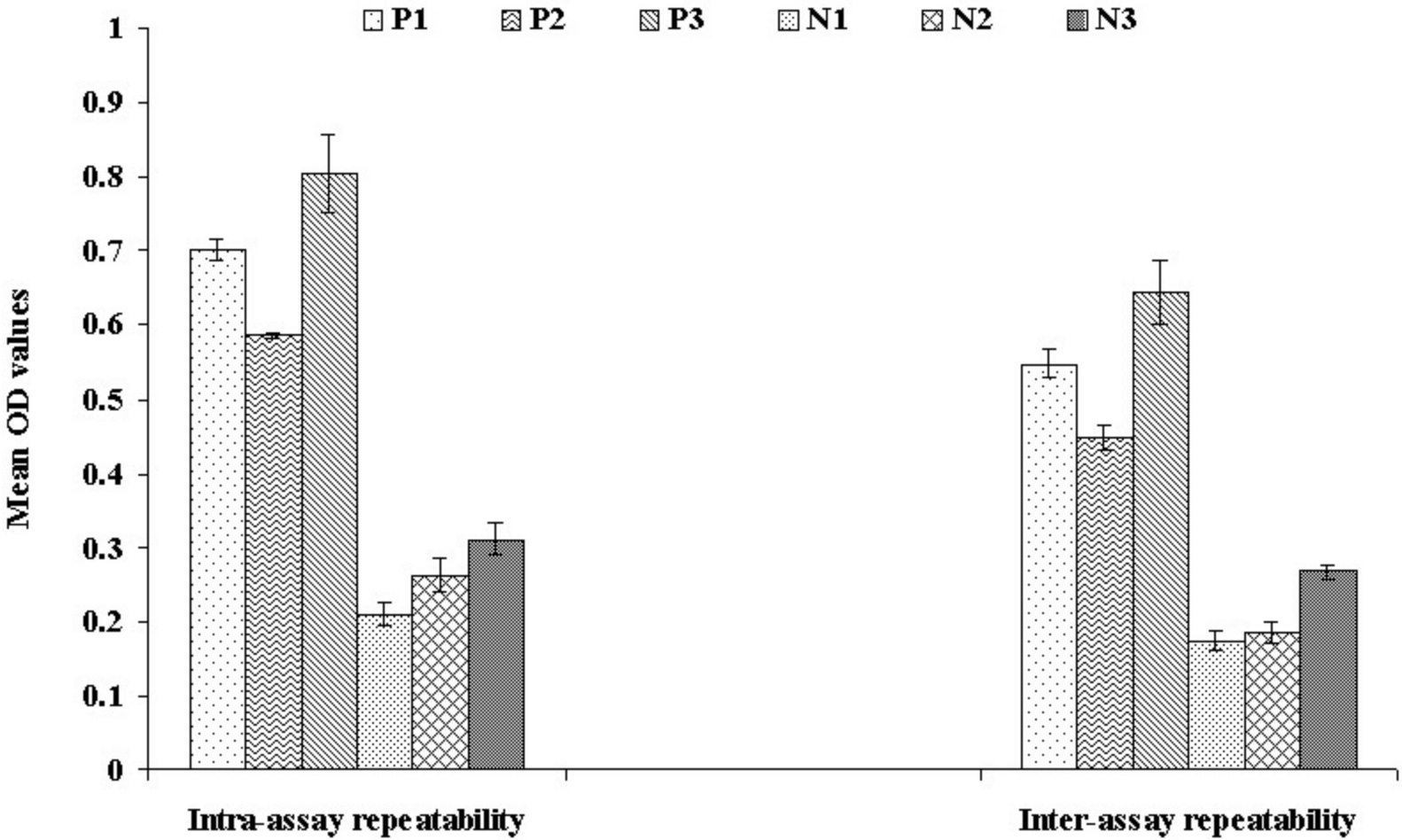
Oberste, M.S., Maher, K., Kilpatrick, D.R., Pallansch, M.A., 1999. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J. Virol. 73, 1941-1948.

Soliman, M., Alfajaro, M.M., Lee, M.H., Jeong, Y.J., Kim, D.S., Son, K.Y., Kwon, J., Choi, J.S., Lim, J.S., Choi, J.S., Lee, T.U., Cho, K.O., Kang, M.I., 2015. The

379 prevalence of duck hepatitis A virus types 1 and 3 on Korean duck farms. Arch.
380 Virol. 160, 493-498.
381 Sun, Q., Liu, H., Li, J., Cai, B., Tao, S., 1997. Study on the detection of antibodies
382 against duck hepatitis virus by an indirect enzyme-linked immunosorbent assay
383 (ELISA). Chinese Journal of Veterinary Scince (in Chinese) 17, 347-349.
384 Tseng, C.H., Knowles, N.J., Tsai, H.J., 2007. Molecular analysis of duck hepatitis
385 virus type 1 indicates that it should be assigned to a new genus. Virus Res. 123,
386 190-203.
387 Tseng, C.H., Tsai, H.J., 2007. Molecular characterization of a new serotype of duck
388 hepatitis virus. Virus Res. 126, 19-31.
389 Upadhyay, C., Ammayappan, A., Vakharia, V.N., 2009. Detection of NP, N3 and N7
390 antibodies to avian influenza virus by indirect ELISA using yeast-expressed
391 antigens. Virol J. 6, 158.
392 Woolcock, P.R., Tsai H.J., 2013. Duck hepatitis. In: Diseases of Poultry, 13th ed. John
393 Wiley & Sons, pp. 422-431.
394 Yang, F., Zhang, H., Cheng, F., Yue, H., Tang, C., 2014. Development of an indirect
395 ELISA for detecting antibodies against genotype A and C duck hepatitis A virus
396 based on recombinant VP1 protein. Acta Veterinaria et Zootechnica Sinica (In
397 Chinese) 45, 1148-1153.
398 Yang, M., Cheng, A., Wang, M., Xing, H., 2008. Development and application of a
399 one-step real-time Taqman RT-PCR assay for detection of Duck hepatitis virus
400 type1. J. Virol. Methods 153, 55-60.


DHAV-1 positive
DHAV-1 negative
DPV positive
AIV positive
DSHSV positive
R.A positive
S.E positive
E.coli positive
DHAV-3 positive
Cutoff value
Mean OD values
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Serum samples


DHAV-1 positive serum
Cutoff value
Mean OD values
1
0.8
0.6
0.4
0.2
0
1:80
1:160
1:320
1:640
1:1280
1:2560
1:5120
1:10240
Dilutions


P1
P2
P3
N1
N2
N3
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Mean OD values
Intra-assay repeatability
Inter-assay repeatability