Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Therapeutic Efficacy of the Combined Extract of Herbal Medicine Against Infectious Bursal Disease in Chickens

Changbo Ou, Ningning Shi[1], Qing Pan, Deyu Tian, Wenshu Zeng and Cheng He*

College of Veterinary Medicine, China Agricultural University, Beijing 100193, China; [1] College of Life Sciences, Agricultural University of Hebei, Baoding 071000, China
*Corresponding author: hecheng@cau.edu.cn

ARTICLE HISTORY

Received: July 19, 2012
Revised: October 18, 2012
Accepted: January 21, 2013

Key words:
Astragalus polysaccharide
Crassirhizomatis
Fructus mume
Infectious bursal disease virus
Rhizoma Dryopteridis
Therapeutic efficacy

ABSTRACT

Currently, infectious bursal disease (IBD) is a highly contagious disease leading to huge economic losses in poultry industry. Our objective was to investigate potential therapeutic effects of the combined extracts of Rhizoma *Dryopteridis crassirhizomatis* and *Fructus mume* (RDCFM) against IBDV infection. Seventy-two 4-week-old SPF chickens were randomly divided into six groups and inoculated intranasally with 0.2 ml of $10^{2.5}$ EID_{50} of IBDV strain CJ801. Twenty-four hours post infection, the birds were orally administered with 400, 200 and 100 mg/kg BW of RDCFM, respectively, 125 mg/kg *Astragalus polysaccharide* (ASP) and saline water, respectively for 5 days and then monitored daily for 10 days. Finally, the remaining birds were euthanized to collect the sera for detecting antibodies and immune organs for determining the immune organs index as well as virus loads. The herbal extracts improved survival rate and relative body gain rate. Virus loads in bursa of Fabricius in herbal treated groups decreased significantly while higher antibody levels were detected in the three RDCFM groups as compared to those of ASP and infection group. These results implied that chickens administered with 100-200 mg/kg of RDCFM for 5 days could improve protection against IBDV infection and RDCFM may be a promising alternative to ASP and egg yolk antibody.

To Cite This Article: Ou C, N Shi, Q Pan, D Tian, W Zeng and C He, 2013. Therapeutic efficacy of the combined extract of herbal medicine against infectious bursal disease in chickens. Pak Vet J, 33(3): 304-308.

INTRODUCTION

Rhizoma Dryopteridis crassirhizomatis (RDC) and *Fructus mume* (FM) have been used in some herbal prescriptions since ancient times in China. In particular, RDC has been widely applied to formulate antiviral granules for severe patients during the severe acute respiratory syndrome (SARS) outbreak in 2003 (Zhao *et al.*, 2007). Furthermore, RDC has been shown to possess anti-cancer properties (Chang *et al.*, 2010) and anti-viral applications (Lee *et al.*, 2008; Min *et al.*, 2001). On the other hand, FM juice has also been shown to inhibit human influenza-A virus infection by inhibiting viral hemagglutinin attachment to host cell surfaces (Yingsakmongkon *et al.*, 2008; Zhao *et al.*, 2007). In recent reports, the combination of FM, *Schizandrae Fructus* or *Coptidis Rhizoma* has shown an effective antimicrobial activity against enterohemorrhagic *Escherichia coli* infection (Lee and Stein, 2011). The active substances (4-hydroxycinnamic acid and protocatechuic acid) isolated from FM were confirmed to induce a better protection and improve the immune response for chickens inoculated with infectious bursal disease virus (IBDV) (Ou *et al.*, 2011; Ou *et al.*, 2012; Okwor *et al.*, 2012). However, the combined extracts of RDC and FM haven't been formulated to apply in poultry industry and its potential therapeutic efficacy on SPF chickens infected with IBDV should be evaluated.

Infectious bursal disease is an acute, highly contagious disease for young chickens and is prevalent in chicken farms throughout the world (Eterradossi and Saif, 2008; Lone *et al*., 2012). Moreover, IBD leads to severe economic losses due to high morbidity and mortality in an acute form or severe immunosuppression or secondary infections (Muller *et al.*, 2003). However, immunity failure occurs at any time, thereby leading to tremendous difficulties in controlling IBD due to the presence of variant strains (Craft *et al.*, 1990). IBDV vaccine and drug development must be accelerated due to the increasing prevalence of IBDV. Therefore, the cheap RDC was used

for formulating a prescription with FM in this study and our objective was to evaluate whether RDCFM could provide a better protection against IBDV infection by increasing the immune response in chickens.

MATERIALS AND METHODS

Herbal medicines: Astragalus polysaccharide (ASP) (batch no. 20110309) was purchased from the Institute of Veterinary Drug and Control, China and the concentration of ASP was determined to be 55.0% using HPLC method (Chen *et al.*, 2003). RDC and FM were purchased from a pharmacy (Xiyuan Traditional Medicine Hospital, Beijing, China) and authenticated by Professor Jianyong Si, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College.

Preparation of herb extracts: A heat reflux method was employed for herb extraction. Briefly, RDC was ground to a powder using a mill and was then mixed with FM at a ratio of 6:4 (w/w). The mixture was soaked with an ethanol/water (75:25, v/v) solution (1:10, w/v) for 24 hours. Finally, the mixture was boiled for 2 hours and filtered to collect the liquid extracts. Subsequently, the solvent was removed under vacuum using a rotary evaporator. The yield of the dried syrup accounted for 23±1% of the dried herbs and the preparation was kept at 4^{o}C until use.

Quality control of herb extracts: Citric acid was used to control the FM quality according to the protocol of Chinese Pharmacopoeia while Dryocrassine ABBA was considered as the main active ingredients and quality control reference in RDC. Therefore, the quality control of RDCFM was evaluated by the quantitative determination of citric acid and Dryocrassine ABBA using HPLC and visible spectrophotometry (Gao and Su, 2009; Li *et al.*, 2010).

Virus: A classical IBDV strain CJ801 was donated by Prof. Liu Jue, Beijing Institute of Husbandry and Veterinary Research. The LD_{50} of IBDV CJ801 strain for SPF chickens was determined to be $10^{2.5}$ EID_{50} in 0.2 ml (Zhou *et al.*, 2010).

Chickens and experimental design: All the studies were performed on 4-week-old specific-pathogen-free (SPF) chickens (Vital Bridge Co. Ltd., Beijing, China). The experimental procedures and animal management were approved by the Institutional Animal Care and Use Committee at China Agricultural University. Seventy-two chickens were randomly divided into 6 groups and challenged intranasally with 0.2 ml IBDV CJ801 strain solution except the healthy control group. 24h post challenge, the birds were orally administered with 400 mg/kg (high dosage group), 200 mg/kg (medium dosage group), 100 mg/kg body weight RDCFM (low dosage group), 125 mg/kg body weight ASP (positive control group), the same volume of solvent (infectious control group) and the no-treatment group (healthy control group). Each group was maintained in individual negative pressure isolators. All chickens were provided feed and water *ad libitum*. The body gain and mortality among all groups were monitored for 10 days. On day 10, the birds were euthanized and sacrificed to collect the serum samples for the detection of antibody and immune organs for determining the virus loads as well as the immune organs index.

Quantification of IBDV in the bursa of Fabricius: The total RNA was isolated from the homogenated bursa of Fabricius using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA was then reverse transcribed into cDNA and amplified and quantified using the QuantiTect SYBR Green Kit (Qiagen, Germany). *β*-actin was used as a loading control. The primers were designed to amplify the VP2 gene and *β*-actin as previously described: IBDV-P1: 5'-AGA AGC ACA CTC TCA GGT CA-3', IBDV-P2: 5'-AGT GTG CTT GAC CTC ACT GT-3'; *β*-actin-P1: 5'-CAT CAC CAT TGG CAA TGA GAGG-3', *β*-actin-P2: 5'-GCA AGC AGG AGT ACG ATG AATC-3' (Tham *et al.*, 1995; Khatri and Sharma, 2006). Each reaction was performed in triplicate using the above primers. The real-time PCR procedure was briefly described as follows: an initial incubation for 5 min at 94°C, 40 cycles of 30s at 94°C, 30s at 68°C and 30s at 60°C. The quantification was based on the fluorescence detection by real-time PCR (Bio-Rad, USA). The viral amount in the samples was evaluated by relative quantification.

Antibody detection: The antibody levels against IBDV were measured using a commercial test kit (BioChek, Holland). All procedures were performed according to the manufacturer's instructions. Briefly, 100µl of diluted samples were added into the appropriate wells of IBDV-coated plates. The positive serums and negative samples were also added to the wells. After several washes, the conjugate reagent and substrate reagent were added to react with above samples. Finally, 100µl of stop solution was added to terminate the reactions and the OD values were recorded at 405 nm.

Statistical analysis: All data were expressed as the mean ± standard error. The two-tailed one-way ANOVA was employed for all statistical analyses using SPSS 13.0. The results were considered significantly different at the level of $P<0.05$ or $P<0.01$.

RESULTS

Determination of citric acid and ABBA in RDCFM extract: The chromatograms were shown in Fig. 1, including methanol control, positive citric acid control, RDCFM sample and standard curve**.** The retention time for citric acid was approximately 7.17 min while the calibration curve arranged from 0.2 to 2.4 mg/ml. The citric acid concentration was determined to be 120 mg per gram extract based on the regression equation Y=(1E-06) X+0.0072 (R^2=0.9997). As for the concentration of Dryocrassine ABBA, the regression equation was A=0.0703C+0.0436 and OD value was 0.568. Finally, the concentration of Dryocrassine ABBA was 7.459 mg per one gram of the RDCFM extract.

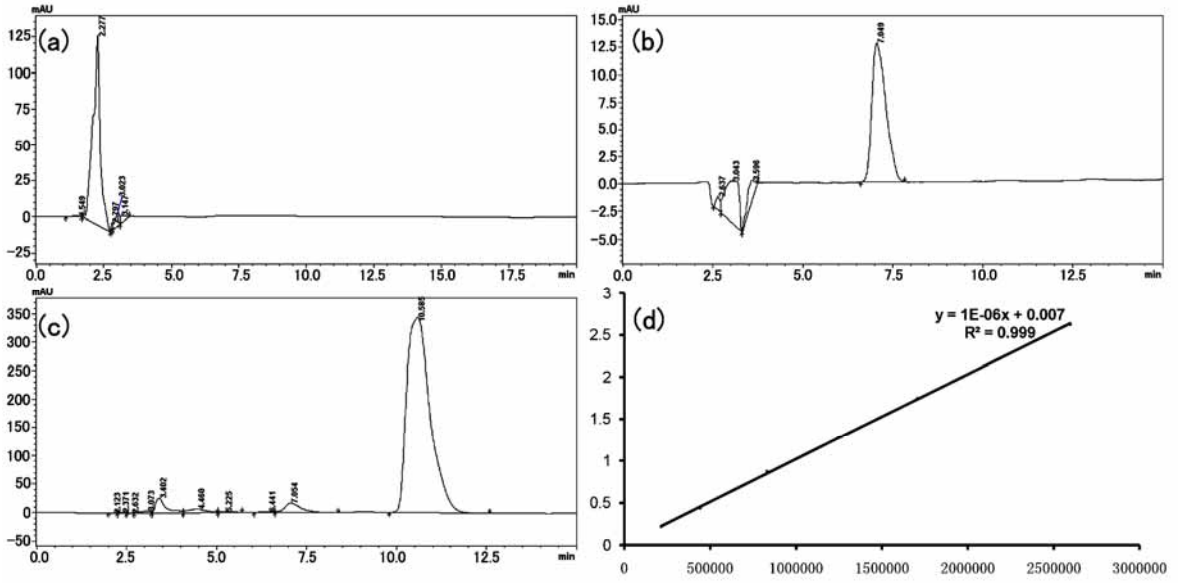

Fig. 1: The content of citric acid was used to evaluate the quality of RDCFM. HPLC method was established with a VP-ODS C18 column and the mobile phase consisted of 0.02M potassium dihydrogen phosphate-methanol (93:7) at a flow rate of 1.0 ml/min. (a) Methanol was used as solvent control at the above condition, (b) the retention time for citric acid was approximately 7.049 min in the same condition, (c) the citric acid peak for sample solution appeared at 7.054 min, (d) the calibration curve arranged from 0.2 to 2.4 mg/ml and the regression equation was Y= (1E-06) X+0.0072 (R2=0.9997).

Clinical performance and survival rate: After inoculated with IBDV, the chickens showed classical symptoms, such as decreased feed intake, fluffed feathers and diarrhea. Chickens died on day 3 post challenge and the mortality amounted to the peak on day 4. The remaining birds gradually recovered on day 7 and returned to normal activities on day 10.

The survival rate, relative body gain rate and immune organs index were listed in Table 1. The survival rates were 66.7, 75.0 and 75.0% in the three RDCFM-treated groups, which were higher than those in the ASP and infection groups (58.0 and 50.0%). The relative weight gain rates in drug treatment groups (67.06, 67.92, 62.2 and 64.96%) were significantly higher than that of the infection group (52.88%) ($P<0.05$).

The RDCFM improved the spleen index in the three groups (3.06, 3.02 and 2.99) while only the bursa index in the 200 mg/kg group (up to 1.41) was higher than those in ASP and infection groups.

Virus loads in bursa of Fabricius: The virus clearance was evaluated by the quantification of the IBDV genome in bursa organs. Based on a standard curve, the relative amount of viral RNA was calculated by $\log_{10}C_T$ in Fig. 2. The virus loads of the bursal organs in herbal groups were significantly lower than those of the ASP and infection groups ($P<0.05$). However, there were no significant differences among the three herbal groups.

Antibody detection: The antibody levels for RDCFM groups, ASP group and infection group were shown in Fig. 3. Anti-IBDV antibody levels of the medium- and low-dosage groups were significantly higher ($P<0.01$) than those of the ASP and infection groups. Additionally, antibody level for the high-dosage group was also significantly higher than that of the ASP group ($P<0.05$).

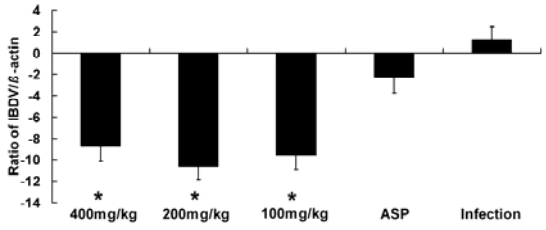

Fig. 2: Quantification of IBDV loads in the bursa of Fabricius in the experimental groups (400, 200 and 100 mg/kg BW, ASP and the infection group) using real-time RT-PCR. The data were expressed as the average ratio of IBDV/*β*-actin. * Indicates $P<0.05$ when the three herbal medicine-treated groups were compared to the ASP or infection group.

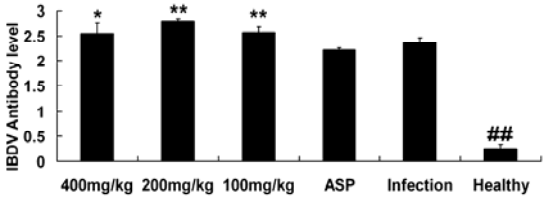

Fig. 3: Serum samples were collected from chickens in all groups (400, 200 and 100 mg/kg BW, ASP, infection and healthy group) at days10 post challenge and antibody levels against IBDV were measured by ELISA kit. ** Indicates $P<0.01$ when the medium- and low-dosage groups were compared to the ASP or infection group at days 10 post challenge. * Indicates $P<0.05$ when the high dosage group was compared to the ASP or infection group at days 10 post challenge. **##** Indicates $P<0.01$ when the healthy group was compared to the ASP or infection group at days 10 post challenge day.

DISCUSSION

In the present study, the survival rate amounted to 75% both in the medium and low concentration of RDCFM. Meanwhile, body weight gains, antibody level, virus clearances and immune organ index in the three

Table 1: The effects of herbal extracts on chickens' performance post challenge with IBDV

Groups	No.	Initial BW	Final BW	BWG	SN	SR (%)	BI	SI
400mg/kg	12	210.4±24.2	333.8±10.1*	123.3	8	66.7	1.20±0.301	3.06±0.624
200mg/kg	12	210.1±23.1	335.1±6.3*	125.0	9	75.0	1.41±0.478	3.02±0.824
100mg/kg	12	211.5±21.7	325.9±9.7*	124.4	9	75.0	1.19±0.422	2.99±0.841
ASP	12	210.7±25.0	330.1±10.1*	119.5	7	58.3	1.23±0.591	2.86±0.409
Infection control	12	211.1±23.3	309.0±8.7	98.9	6	50.0	1.24±0.490	2.53±0.481
Healthy control	12	210.3±23.3	394.2±31.6**	183.9	12	100	1.73±0.811	3.07±0.967

* P<0.05, **P<0.01; BW: body weight; BWG: body weight gain; SN: survival number; SR: survival rate; ASP: Astragalus polysaccharide BWG = Final BW-Initial BW; SR = SN/No ×100%; BI= weight of bursa (mg)/Final BW×1000; SI= weight of spleen (mg)/Final BW×1000

RDCFM -treated groups were found to be improved post challenge with IBDV as compared to those of the infectious and ASP groups. The quality control of RDCFM was also evaluated by determining the content of citric acid and dryocrassine ABBA. Thus, the RDCFM could be produced in large number under the strict quality control. Based on the above evidence, RDCFM might be a promising substance against IBDV in the poultry industry as an alternative medicate to ASP and egg yolk antibody.

The hemorrhagic lesions in immune organs have been recorded post infection with several IBDV isolates and mortality ranged from 30 to 100% in chickens (Aricibasi *et al.*, 2010). In this experiment, the protection rate was an objective index to evaluate the efficacy of the combined extracts. A significant survival rate was observed both in the medium group and in the lower herbal-treated group as compared to ASP group, which implied that RDCFM had the therapeutic efficacy against IBDV infection. In the previous reports, spleen and bursa are the target organs of IBDV (Zierenberg *et al.*, 2004). Therefore, the immune organs indexes are used for determining the lesions in spleen and bursa post IBDV infection. Obviously, 200 mg/kg herbal extract was found to have a significant increase of spleen index as well as a good protection of bursa in comparison with ASP and infection groups.

The high levels of antibody responses would induce the significant protection against the virulent IBDV infection (Hsieh *et al.*, 2010). In our experiment, higher antibody levels in the three herbal extract groups were corresponded with the higher body weight gain, the increasing survival rate, lower virus load and better immune organ indexes post IBDV infection. In this sense, the infected chickens could get protection due to improving birds' appetite and humoral immunity. In previous reports, chickens administered with ASP were able to get a good protection against IBDV infection due to the increasing erythrocyte-C3b receptor and the immune complex rosette rate (Li *et al.*, 2007). Hence, the combined herbal extracts might involve the protection of B cells in the bursa of Fabricius and induce higher antibody levels post IBDV infection.

Until recently, both the egg yolk antibody and ASP have been accredited for legal veterinary drugs against IBDV in China. Particularly, these substances are complementary approach for vaccine failure, early subclinical infections and the occurrence of the virulent IBDV strains in the field (Kibenge *et al.*, 1988). Nevertheless, the application of egg yolk antibody caused multiple adverse effects, such as the risk of secondary infection and immune depression (Hatta *et al.*, 2008). Furthermore, the egg yolk antibody is not suitable for clinical applications in large poultry flocks due to the intensive labor requirement. Currently, Chinese farmers turn to large amounts of ASP in routine treatments for antiviral activity. As a result, forthcoming viral resistance will be inevitable in the poultry industry. The potential outbreak of new very virulent strains will challenge the health of poultry. Therefore, RDCFM might be an alternative to ASP and egg yolk antibody as a new medicatement against IBDV infection.

Based on the evidence presented here, we conclude that RDCFM is a promising medication against IBDV infection by improving antibody level and decreasing virus load in the targeted organs. Further investigations will be performed to understand the mechanism of the herbal extract and expand the potential application of RDCFM against other avian viral diseases.

Acknowledgement: This work is partly supported by the National Hi-tech Research and Development Program (No. 2006AA10A208-3-2), and the Ministry of Education of Guangdong Province Combination Program (No. 2008B090500155).

REFERENCES

Aricibasi M, A Jung, ED Heller and S Rautenschlein, 2010. Differences in genetic background influence the induction of innate and acquired immune responses in chickens depending on the virulence of the infecting infectious bursal disease virus (IBDV) strain. Vet Immunol Immunopathol, 135: 79-92.

Chang SH, JH Bae, DP Hong, KD Choi, SC Kim, E Her, SH Kim and CD Kang, 2010. Dryopteris crassirhizoma has anti-cancer effects through both extrinsic and intrinsic apoptotic pathways and G0/G1 phase arrest in human prostate cancer cells. J Ethnopharmacol, 130: 248-254.

Chen HL, DF Li, BY Chang, LM Gong, JG Dai and GF Yi, 2003. Effects of Chinese herbal polysaccharides on the immunity and growth performance of young broilers. Poult Sci, 82: 364-370.

Craft DW, J Brown and PD Lukert, 1990. Effects of standard and variant strains of infectious bursal disease virus on infections of chickens. Am J Vet Res, 51: 1192-1197.

Eterradossi N and Y Saif, 2008. Infectious Bursal Disease. In: Diseases of Poultry, 12 ed (Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, DE S, eds). Ames, IA: Iowa State Press; 185-208.

Gao Z and Y Su, 2009. Quantitative determination of total phloroglucinol in commercial Rhizoma Dryopteris. J Beijing Univ Trad Chinese Med, 4: 259-262.

Hatta H, MP Kapoor and J Lr, 2008. Bioactive components in egg yolk. In: Egg Bioscience and Biotechnology (Mine Y, ed). New York: John Wiley Sons, pp: 185-237.

Hsieh MK, CC Wu and TL Lin, 2010. DNA-mediated vaccination conferring protection against infectious bursal disease in broiler chickens in the presence of maternal antibody. Vaccine, 28: 3936-3943.

Khatri M and JM Sharma, 2006. Infectious bursal disease virus infection induces macrophage activation via p38 MAPK and NF-kappaB pathways. Virus Res, 118: 70-77.

Kibenge FS, AS Dhillon and RG Russell, 1988. Biochemistry and immunology of infectious bursal disease virus. J Gen Virol, 69(8): 1757-1775.

Lee JH and BD Stein, 2011. Antimicrobial activity of a combination of Mume fructus, Schizandrae fructus and Coptidis rhizoma on

enterohemorrhagic Escherichia coli O26, O111, and O157 and its effect on Shiga toxin releases. Foodborne Pathog Dis, 8: 643-646.

Lee JS, H Miyashiro, N Nakamura and M Hattori, 2008. Two new triterpenes from the Rhizome of Dryopteris crassirhizoma and inhibitory activities of its constituents on human immunodeficiency virus-1 protease. Chem Pharm Bull (Tokyo), 56: 711-714.

Li H, R Lloyd and J Wang, 2007. Effect of Astragalus polysaccharides on erythrocyte immune adherence of chickens inoculated with infectious bursal disease virus. Agric Sci China, 6: 1402-1408.

Li S, Y Li, F Zhang, C Ou, H Chen and C He, 2010. Quality control of fructus mume & semen ziziphi spinosae oral liquid. Lishizhen Medicine and Materia Medica Research, 21: 3101-3104.

Lone NA, SF Rehmani, TA Khan and SU Kazmi, 2012. Efficacy of live attenuated and inactivated oil emulsion infectious bursal disease virus vaccines in broiler chicks. Pak Vet J, 32: 539-542.

Min BS, M Tomiyama, CM Ma, N Nakamura and M Hattori, 2001. Kaempferol acetylrhamnosides from the rhizome of Dryopteris crassirhizoma and their inhibitory effects on three different activities of human immunodeficiency virus-1 reverse transcriptase. Chem Pharm Bull (Tokyo), 49: 546-550.

Muller H, MR Islam and R Raue, 2003. Research on infectious bursal disease-the past, the present and the future. Vet Microbiol, 97: 153-165.

Okwor EC, DC Eze and K Okonkwo, 2012. Serum antibody levels against infectious bursal disease virus in Nigerian village chickens using indirect hemagglutination test. Pak Vet J, 32: 286-287.

Ou C, Q Pan, X Chen, N Hou and C He, 2012. Protocatechuic acid, a new active substance against the challenge of avian infectious bursal disease virus. Poult Sci, 91: 1604-1609.

Ou C, Z Pu, S Li, Q Pan, N Hou, F Zhang and C He, 2011. Effect of 4-hydroxycinnamic acid on chickens infected with infectious bursal disease virus. J Anim Vet Adv, 10: 2292-2296.

Tham KM, LW Young and CD Moon, 1995. Detection of infectious bursal disease virus by reverse transcription-polymerase chain reaction amplification of the virus segment A gene. J Virol Methods, 53: 201-212.

Yingsakmongkon S, D Miyamoto, N Sriwilaijaroen, K Fujita, K Matsumoto, W Jampangern, H Hiramatsu, CT Guo, T Sawada, T Takahashi, K Hidari, T Suzuki, M Ito, Y Ito and Y Suzuki, 2008. In vitro inhibition of human influenza A virus infection by fruit-juice concentrate of Japanese plum (Prunus mume SIEB. et ZUCC). Biol Pharm Bull, 31: 511-515.

Zhao ZL, CH Leng and ZT Wang, 2007. Identification of Dryopteris crassirhizoma and the adulterant species based on cpDNA rbcL and translated amino acid sequences. Planta Med, 73: 1230-1233.

Zhou X, D Wang, J Xiong, P Zhang, Y Li and R She, 2010. Protection of chickens, with or without maternal antibodies, against IBDV infection by a recombinant IBDV-VP2 protein. Vaccine, 28: 3990-3996.

Zierenberg K, R Raue, H Nieper, MR Islam, N Eterradossi, D Toquin and H Müller, 2004. Generation of serotype 1/serotype 2 reassortant viruses of the infectious bursal disease virus and their investigation in vitro and in vivo. Virus Res, 105: 23-34.