American Journal of Immunology 3 (2): 31-34, 2007
ISSN 1553-619X

Immunohistochemistry Assay to Detect Turkey *Coronavirus* (TCoV) from Experimentally Infected Poults

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Abstract: The objective of this study was to develop a direct immunohistochemical assay to detect TCoV antigens in formalin-fixed paraffin-embedded sections prepared from experimentally infected poults. The sections of ileo, ileo-cecal junction and ceca regions from intestine were prepared and submitted to two different primary antibodies, first the non-biotin labeled polyclonal antibody for the indirect method, and second the biotin-labeled polyclonal antibody, both raised against IBV by immunized specific pathogen free chickens. All sections were submitted to immufluorescent assay (IFA), a conventional method, and the results compared. The direct immunohistochemical technique showed a higher frequency of antigen in tissues, especially from the ileo-cecal junction with no difference between results obtained by the conventional method. Finally, the immunofluorescence and all modalities of molecular approaches have been played an important role to the diagnosis and prevention of TCoV infections, although to be precise on infectious disease diagnosis, it is necessary complementary techniques. Here, was standardized the biotin labeled polyclonal antibody as reliable tool to be used as an alternative detection of Turkey *Coronavirus.*

Keywords: TCoV, PEMS, Immunohistochemistry, IFA.

INTRODUCTION

The Brazilian turkey industry ranks second producer in the world with 187 millions of carcases commercialised during 2006, corresponding to 29% of the international market. In spite of intensive production, turkey coronaviral enteritis, the most costly viral disease has been affected young poults in Brazil in early 2006 and, the respective outbreak has been classified as poult enteritis and mortality syndrome-PEMS, according to previous descriptions [1-4,9]. Elsewhere, the clinical signs are characterized as inappetence, wet droppings, ruffled feathers, decreased weight gain, growth depression, and uneven flock growth observed normally from affected breeders, symptoms also observed in Brazil, for the first time [5].

In fact, Coronaviruses are in the family *Coronaviridae*, which are enveloped, positive-stranded RNA virus that infects a wide range of mammalian and avian species [6]. So far, Turkey *Coronavirus* (TCoV) and infectious bronchitis virus (IBV) belong to antigenic group III and share antigenic similarity [7]. This biological characteristic allows using antigen, as well as, antibodies raises against IBV, to develop and apply immunological tools for the TCoV diagnosis [8-10].

The detection of coronavirus direct from intestinal contents has been usually done by direct electron microscopy (EM), indirect immunfluorescence antibody (IFA), ELISA approaches, and molecular techniques, as RT-PCR or multiplex RT-PCR. Actually, the IFA and the RT-PCRs are the most important control measures for the PEMS in the turkey industry described in USA and Great Britain [3,6] and more recently in Brazil [9].

Although, the IFA test is routinely applied on fresh or frozen tissues, it is labours and time consuming when a large number of specimens must be evaluated. In order to rapidly diagnose, as well as, effectively control turkey poult enteritis new techniques must be useful and specific for clinical samples, especially when the industry is experiencing an outbreak. In this report, we developed and applied a useful simple direct

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immunohistochemical technique to detect the TCoV from formalin-fixed tissues, comparing the results with those obtained by IFA.

MATERIAL AND METHODS

Experimental infection: Turkey embryos aged 23-25 days of incubation were obtained from commercial breeder and infected by Brazilian[9] isolate strain. After 72h post-infection the intestine was collected and divided into three portions: ileo, ileo-cecal junction and ceca.

Histopathology: Samples of ileo, ileo-cecal junction and ceca were collected and fixed in 10% neutral buffered formalin. Than, were embedded in paraffin blocks, sectioned at 2mm and stained with hematoxylin/eosin (HE) routine method, and finally examined using light microscope [9].

Primary antibody production: The IgG against IBV was produced by vaccination of 10 inbred C/O line white Legorns chickens at one day of age by intra-ocular route with purified M_{41} serotype as described before [11] with some modifications. After two weeks, the chickens received the second injection of commercial vaccine, 1ml per bird (H_{120}) by intra-muscular route, and 21 days after they were bled from the wing vein. The γ-globulin fraction was prepared by the salting-out procedure adding 35% (v/v) of ammonium sulfate (Sigma) and followed by IgY fraction purification using chromatography separation on Sephadex-G200 (Sigma).

Biotin labelled antibody production: The purified IgG was conjugated to biotin (Biotin disulfide N-hydroxysuccinide ester, Sigma) according to previous studies [19] with some modifications and used as primary antibody. The first step was to mix 1mg/ml of chicken IgG fraction with 250µg/ml of biotin dilute in sodium borate buffer (Ph 8,8) and left at 4°C during 4h. After the reaction, the mixture was dialyzed against phosphate saline buffer (PBS) Ph 7,2 48 h consecutively to eliminate the non-linked molecules and the antibody work dilution determined by direct ELISA.

Preparations of sections for staining: Unstained sections were used for the indirect and direct immunohistochemical examination just after submitted to deparafinisation, rehydratation and washes in buffered saline added by 0,1% Tween 80. First step, the heat-induced epitope retrieval with citrate buffer (Ph 6.1) for 15 min at 700W was the pre-treatment for viral antigen reactivation, normally damaged by formaldehyde fixation. Just before staining, slides were treated 3 times with hydrogen peroxide (Merck) 50% during 30 min, 2 times with mixture of hydrogen peroxide and methanol 30% during 30 min and once hydrogen peroxide 3% during 1 h to inactivated endogenous peroxidase, commonly found in inflammatory reactions. So, the slides were placed in saline buffered for 10 min 5 times, consecutively, to remove the residues between each step of the reaction. The unspecific bindings were blocked using a variety of blockers to decrease the background activity. First was tested dried 15% nonfat milk during 90 min, gelatin 5% during 60 min, serum albumin bovine (Sigma cat # A-9647) 5% during 120 min and normal rabbit serum 2% over night. After established the best condition of blocking the slides were submitted to procedure staining.

Indirect Immunohistochemistry: The primary antibody used in the indirect immunohistochemical analysis was the same described before, without biotin labeling. First, the antibody was applied to the slides at 1:100, 1:200 and 1:400 dilutions to determine the optimal work dilution, made in PBS plus 10% nonfat dried milk, and incubated over night at 4°C under dark. The next step was to performer 5 washes of 10 min each using PBS plus 0.5% Tween 80, and applied the specific anti-IgG conjugated to peroxidase (HRPO-Zymed goat anti-chicken linked to peroxidase) diluted 1:500 at PBS plus nonfat dried milk during 1h at 37°C. After incubation, the slides were washed and the substrate made fresh in the dark, by mixing equal volumes of 0.02% hydrogen peroxide and 0.6mg DAB (3,3´-diaminobenzidine tetrahydrochloride, Gibco BRL), was added to the slides for 30 min at room temperature. The reaction was stopped by washing with tap water and the specific brown color was revealed after counterstained with Meyer´s hematoxilin. An intensive dark red deposit was considered positive and the negative controls consisted of sections treated with buffered saline instead of primary antibody. The intensity of dark red deposit was arbitrarily rate on a scale of – (undetectable) to ++++ (the pattern present at its highest intensity). Omission of the primary antibody was used as a negative control for the reaction and the positive control, infected chicken kidney cells (CKC) infected by IBV were used.

Direct Immunohistochemistry: Viral antigen was demonstrated by avidin-biotin complex (ABC) direct immunoperoxidase method as described before [16] and with some modifications. The optimal primary biotin

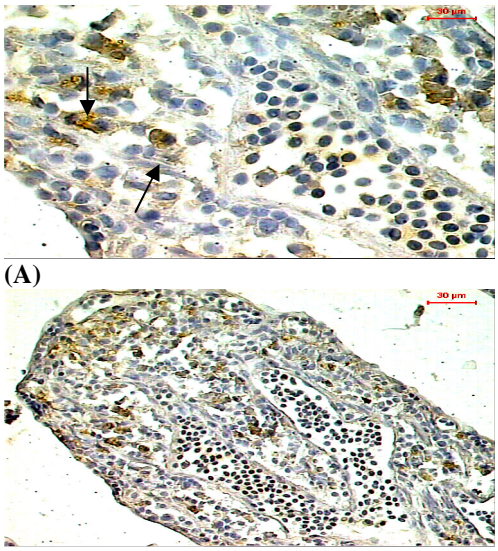
labeled antibody dilution was determined by titration (1:100, 1:200 and 1:400) made on PBS plus 10% of nonfat dried milk covered by 200µl of each dilution over-night at 4°C in a humidified chamber. After 5 washes, the 100µl/slide of streptavidin-peroxidase (Sigma cat # S-5512) complex was added and incubated 1h at 37°C. In addition, substrate made fresh in the dark, by mixing equal volumes of 0.02% hydrogen peroxide and 0.6mg DAB (3,3´- diaminobenzidine tetrahydrochloride, Gibco BRL), was added to the slides for 30 min at room temperature. The reaction was stopped by washing with tap water and the specific brown color was revealed after counterstained with Meyer´s hematoxilin. An intensive dark red deposit was considered positive and the negative controls consisted of sections treated with buffered saline instead of biotin labeled antibody and the results calculated as described before. For the parameters to be analyzed the same procedure described before was used. The IFA was performed according to previous reports [8].

Statistical Evaluation: Statistical comparisons of positive results among all the methods were performed with two-sample t-test and P value determined and origin 7.0 software were used for data analysis.

RESULTS AND DISCUSSION

Microscopical examination of the intestine (ileo, ileo-cecal junction and ceca) revealed marked degeneration, destruction of the villous epithelium, hyperactivation of the intestinal glands intestinal and lumen filled with desquamated epithelial cells as well as mucous exudates. In addition, basal lamina was infiltrated with mononuclear cells, presented submucosa oedema compatible with viral enteritis (results not shown). These findings have been described for PEMS, however they are common to other enteric pathogens [6-8]. Regarding to the intestines analyzed here, they were flaccid, thin-walled, and filled with loose contents, and the disease was acute, with symptoms appearing and lasting for about 3 weeks

In order to determine the efficiency of biotin-labeled antibody produced, the direct ELISA was performed and the IBV antigen reacted specific against the respective antibody, producing an optical density pf 1.0 (DO), whilst the non-labeled antibody produced any OD [9-12]. In this way, the immunohistochemical, indirect and direct methods, produced results that revealed a precise detection of TCoV antigens. The work dilution chosen was 1:200, diluted in PBS plus 10% nonfat dried milk for the both methods. In addition, the best blocking condition was the use of serum albumin bovine at 5%, which gave the best neutralization of non-specific reactions (Fig. 1A and B).



(A)

(B)

Figure 1: A) Ileo-cecal junction presented positive browm reaction, +++ cross, classified as intense (narrowhead); B) Ileo region showed positive brown reaction on the villus; bar 30µm.

The sections prepared from the ileo presented same positive results for the immunohistochemical analyze and IFA. Moreover, in previous reports, it has been described that ileo-cecal junction is the best region to detect TCoV from naturally and experimentally infected poults in agreement with those observed here [3, 5, 9-12].

In fact, the immunohistochemical test, using the peroxidase system offer advantages over the IFA method in that: 1) it does not require an ultraviolet microscope, and 2) the tissues were stained for immunohistochemical and then re-stained for microscope evaluation, allowing the observer to correlate the location, numbers, and intensity of stained cells with a normal microscopic for pathology examination.

Herein, the immunohistochemical methods using the polyclonal antibody demonstrated great advantage over IFA, where monoclonal antibodies have been routinely used for the same purpose. However, diagnosis of TCoV infections based on histopathology description is not reliable, because other infectious and non-infectious agents can cause similar symptoms and microscopic lesions [4].

CONCLUSION

Based on this fact, the RT-PCR and all modalities of molecular approaches have been played an important role to the diagnosis and prevention of TCoV infections. Thereafter, immunohistochemistry assay has demonstrated viral antigen and may be useful for differential diagnosis of enteric diseases as an alternative viable tool for TCoV diagnosis.

ACKNOWLEDGEMENTS

FAPESP - Fundação Amparao à Pesquisa do Estado de São Paulo (Grant number **05/51484-1** and **06/59769-8**). We are indebt to the technical assistance of Adriana Petrocelli whom supplied the samples.

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