大韓獸醫學會誌 (1990) 第30卷 第4號
Korean J Vet Sci (1990) 30(4):435~440

Avidin-biotin complex for immunohistochemical diagnosis of Aujeszky's disease and hog cholera

Soon-bok Kim, Jung-hyang Sur
College of Veterinary Medicine, Gyeongsang National University
Un-gyeong Moon
Gyeongnam Provincial Institute of Animal Health
(Receiued Aug. 14, 1990)

Avidin—biotin 복합체를 이용한 오제스키병과 돼지콜레라의 면역조직화학적 감별진단

김 순 복·서 정 향
경상대학교 수의과대학
문 운 경
경상남도 가축위생시험소
(1990. 8. 14 접수)

초록: 오제스키병바이러스 또는 돼지콜레라바이러스에 인공 또는 자연감염된 돼지 10두를 실험동물로 공시하였으며, 감염돈의 편도선, 비장, 대뇌 및 연층(buffy coat)의 냉동 및 파라핀절편에서, avidin-biotin-peroxidase complex(ABC)를 이용하여 이들 바이러스를 면역조직화학적으로 검출하였다.

오제스키병바이러스 항원은 임파구와 대식세포의 핵내 또는 세포질에서 검출되었으며, 돼지콜레라바이러스 항원은 이들 세포의 세포질에서 검출되었고, ABC법은 양성반응 부위에서 갈색의 색소침착을 일으켰다.

오제스키병바이러스 양성세포는 편도선과 대뇌에서 가장 빈번히 검출되었음에 반해 돼지콜레라바이러스는 비장에서 가장 빈번하였다. 그리고 연층에서도 이들 두가지 바이러스항원이 모두 검출되었다.

ABC법은 이들 바이러스의 면역조직화학적 검출에 있어 특이성이 높고 배경의 비특이염색성이 낮아, 바이러스 분리동정을 거치지않고 이들 질병을 확진할 수 있는 진단수단으로 활용될 수 있을 것으로 생각된다.

Key words: avidin-biotin, Aujeszky's disease, hog cholera, immunohistochemical diagnosis.

Introduction

Aujeszky's disease(AD) and hog cholera(HC) have long been recognized as severe, highly fatal infectious diseases of pigs, and in recent years a rising incidence of the diseases have been reported in this country.[1,2] Both causative viruses commonly affect the central nervous system producing nonsuppurative meningoencephalitis and show a similar clinico-pathological character in young pigs.[3-7] Therefore pat-

This paper was supported by NON DIRECTED RESEARCH FUND, Korea research foundation, 1989.

hologists have found some difficulty in making differential diagnosis from AD and HC.

Immunohistochemical method allows identification of cells and detection of viral antigen in tissue sections of infected organs, and can make confirmative diagnosis for viral diseases without boring virological laboratory works.[8,9]

Avidin-biotin-peroxidase complex(ABC), introduced by Hsu and Raine[10] in 1981, produces both excellent sensitivity and minimal background staining. The major advantage of ABC method over peroxidase-antiperoxidase(PAP) technique is its wide range of applicability. Any biotinylated substances can be detected by ABC. The method today has been used popularly for the simple and rapid diagnosis of various diseases.[11~15]

The purpose of this study was to identify cells and organs in which Aujeszky's disease virus(ADV) or hog cholera virus(HCV) was present by means of ABC method, and to establish a rapid immunohistochemical procedure for differential diagnosis of AD and HC.

Materials and Methods

Specimens: Five, 60-day-old, Landrace pigs were inoculated intranasally with 1ml of infected culture medium containing 10^7 $TCID_{50}$/0.1ml of the ADV; NYJ-1 strain (courtesy of Dr. Cho and Dr. Jun, Chungnam National Universiy), and these were anesthetized at the 7th day after virus inoculation. Another 5 Landrace pigs, 60 to 80 days old, naturally infected with HCV were also examined. Tonsils, spleens, cerebra and buffy coats were fixed in 10% neutral formalin for paraffin section or freezed in liquid nitrogen for frozen section. Whole blood were suspened in Alsever's solution and centrifuged to get buffy coat, and the pellets were embedded in 3% Bactoagar at 42°C for sectioning.

First antibodies: Polyvalent hyperimmune serum (HIS) to ADV or HCV was prepared in rabbits, and another HIS to ADV was obtained by courtesy of Dr. Ducatelle, University of Ghent, Belgium. HISs were used at a dilution of 1 : 100~1,000 in tris buffered saline(TBS) on tissue sections. Monoclonal antibodies(MCA) specific to ADV or HCV were obtained by courtesy of Dr. An, Institute of Veterinary Research, Rural Development Administration.

ABC immunostaining: All procedures were performed at room temperature and immunostanining was done as follows.

—sections were washed in TBS, 5 minutes
—pretreated with 0.6% hydrogen peroxide in methanol for cryosections, 3% hydrogen peroxide in distilled water for paraffin sections, 5 minutes
—preincubated with 5% normal goat serum, 20 minutes
—covered with first antibodies respectively; rabbit/ADV or HCV/HIS, 60 minutes; mouse/ADV or HCV/MCA, 30 minutes, in moisture chamber
—washed 2×3 minutes in TBS on slide shaker
—covered with second antibodies at a dilution of 30~600 in TBS, 20~30 minutes; biotinylated goat/rabbit IgG(Vector Lab) or rabbit/mouse IgG (Dakopatts)
—covered with avidin-biotin-peroxidase conjugate (Vector Lab) at a dilution of 1 : 200 or 1 : 300 in TBS, 30 minutes
—wash in TBS on slide shaker, 5 minutes
—substrate; 0.001% 3,3′-diaminobenzidine tetrahydrochloride(DAB, Sigma) in TBS, in dark chamber, 10~20 minutes
—washed quickly in TBS
—processed sequentially in graded alcohols and xylene
—mounted with balsam

When appropriate, two adjacent sections were cut and one was immunostained either with PAP,[16] immunoalkaline phosphatase(IAP)[17] or immunogold-silver(IGS) method,[18] and another stained with hematoxylin and eosin.

Results

The ABC-positive reactions were detected in sections from paraffin-embedded or frozen tissues, and specific staining and low background colouring were exhibited in both sections of this experiment. Cells containing viral antigens were mainly recognized in lymphocytes and macrophages of infected organs. Positive regions of the cells labelled with ABC ap-

peared clearly a areas of fine granular brown reaction product. Such brownish granules were not seen in negative controls. Most intense staining was observed with positive serum diluted at 1 : 200 and 1 : 300, and least intense at 1 : 1,000 for 1 antiserum to ADV. There were no positive reactions noted in tissues treated with TBS or negative sera, or when healthy tissue was used. A significant difference in reaction specificity between ABC and PAP methods was not noticed, however, staining intensity of ABC was better than PAP methods. Furthermore ABC method required less incubation time for first antibodies and DAB substrate than other methods tested.

ADV-positive reaction was found in the nucleus and throughout the cytoplasm of the infected cells. Positive cells were sometimes severely vacuolated, and as a result, the brown pigmentation was mainly lined around the cell membrane. Other times, the cytoplasm was stained homogenous brown. Positive cells were detected in all cases of 5 tonsils(Fig 1) and 5 cerebra(Fig 2) tested, and in each 3 of 5 spleens and 5 buffy coats.

HCV-positive reaction was primarily recognized in the cytoplasm of lymphocytes and macrophages. HCV-positive cells were most frequently detected in the spleen(Fig 3, 4). Of 5 cases tested, 5 spleens, 3 tonsils, 2 buffy coats(Fig 5), and 1 cerebrum(Fig 6) showed positive reaction.

By treatment with normal goat serum nonspecific bindings were remarkably reduced. However, some nonspecific staining of connective tissue persisted, and this staining could be differentiate from specific staining and did not confuse result interpretation. The hydrogen peroxide-methanol treatment for cryosections and —distilled water treatment for paraffin sections were proved to be effective in inhibiting endogenous peroxidase activity. Control sections were negative, with the exception of blood cells which showed an intense brown reaction product due to their endogenous peroxidase activity.

Discussion

AD in young pigs is likely to be confused with HC because of thier close resemblance in clinico-pathlogical character.[3~7] Confirmation of the diagnosis of viral diseases requires cultural identification of causative agents, however, positive identification of the agents is time-consuming and usually only done by individuals who work regularly with the viruses. The use of immunohistochemical methods in suspected cases should provide a diagnostic tool when culturing is not feasible, or as a subsidiary test even when culturing is possible.[8,9] Establishment of more rapid and accurate diagnosis of HC and AD should lead to earlier and more effective control measures which will, in turn, prevent a dissemination of the infections, and alleviate economical losses.

The purpose of this study was to establish an ABC procedures that would facilitate detection of ADV and HCV antigens in paraffin sections of the infected organs, and to select each optimal organs for differential diagnosis of AD and HC. Most reliable organs for ADV-antigen detection were tonsil and cerebrum, whereas, for HCV-antigen detection spleen was best, which were same as our previous observations in PAP and IGS methods.[16~18] Both virus antigens were also detected in the buffy coats, which suggested some possibilities in making diagnosis from living animals.

The ABC technique has become increasingly popular in recent years. The test was proved to be a highly sensitive, and specific diagnostic test, and have a wide range of applications in surgical pathology.[10~15] In this experiment the ABC technique seemed a highly specific method to detecting ADV and HCV antigens in both tissue sections, and PAP, IGS and IAP were also good for this purpose.

Immunohistochemistry applicable to paraffin sections could make an important contribution to detection and study of AD and HC. By allowing clear visualization of the viral antigens in the lesions, the amount of information derived from routine histologic examination was increased and a valuable pathogenesis tool was provided. Recently, Allan et al[19] delineated the direct immunoperoxidase technique on impression smears of brain and pharynx. And also immunofluorescence staining of fresh frozen sections will facilitate a more rapid diagnosis of the disease. However, the superior morphologic features

of tissue in paraffin sections allowed more precise identification of the infected areas most suitable for immunocytochemical staining. This selection is of particular importance when lesions at different stages or of different types are present in the same organs. By examination of paraffin blocks stored from previous years, retrospective diagnoses are possible.[20] This method is sensitive, and therefore can be used on ultrathin electron microscopic sections.[21] Immunohistochemistry of paraffin sections also will be a convenient diagnostic method when fluorescence facilities or fresh frozen tissues are not available.

Summary

Ten pigs infected with Aujeszky's disease virus (ADV) or hog cholera virus(HCV) were tested for the detection of virus antigens in frozens or paraffin-embedded sections by avidin-biotin-peroxidase complex(ABC) method. Tonsils, spleens, cerebra and buffy coats were examined for the immunohistochemical test.

Where ADV antigen was detected by ABC, a dark brown deposit occurred in both the nucleus and the cytoplasm of lymphocytes and macrophages, however, HCV antigen was demonstrated in the cytoplasm of the infected cells.

ADV-positive cells were most frequently detected in tonsils and cerebra, whereas, HCV-positive cells were frequently observed in spleens. And buffy coat were also good for both virus detection.

The results suggested that ABC method is considered as an excellent and reliable tool for confirmative diagnosis of these viral diseases.

Legends for figures

Fig 1. ABC-stained paraffin section of the tonsil infected with Aujeszky's disease virus(ADV). Positive reaction shows an accumulation of fine brown granules in both the cytoplasm and the nucleus. Positive cells are often aggregated within the tonsilar crypt. ×125.

Fig 2. ABC-stained paraffin section of the cerebral cortex infected with ADV. The nucleus is often more intensely stained than the cytoplasm. Counterstained with hematoxylin. ×50.

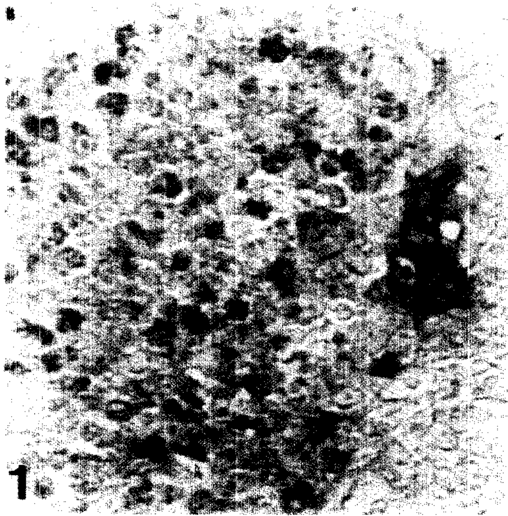
Fig 3. PAP-stained frozen section of the spleen infected with hog cholera virus(HCV). Brown-colored positive cells are seen. Counterstained with hematoxylin. ×125.

Fig 4. IAP-stained frozen section of the spleen infected with HCV. Red-colored positive cells are seen. ×125.

Fig 5. ABC-stained paraffin section of the buffy coat infected with HCV. Positive reaction is often found in lymphocytic cells. ×50.

Fig 6. IGS-stained frozen section of the cerebrum infected with HCV. A limited number of neurons are stained. Intense black deposits, indicating the presence of viral antigen, appears in the cytoplasm of a neuron. ×400.

Abbreviations of immunostaining methods; ABC: avidin-biotin-peroxidase complex, PAP: peroxidase-antiperoxidase, IAP: immunoalkaline phosphase, IGS: Immunogold-silver.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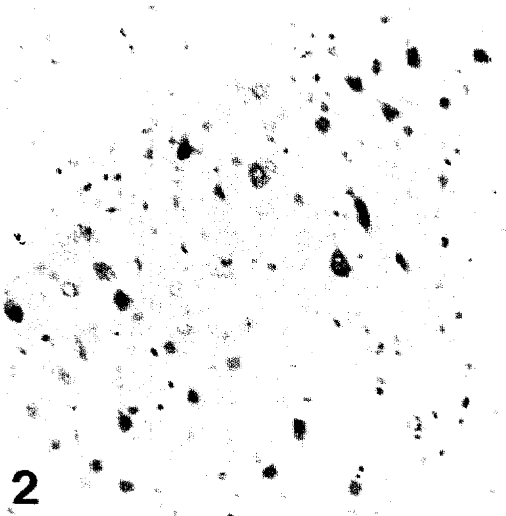
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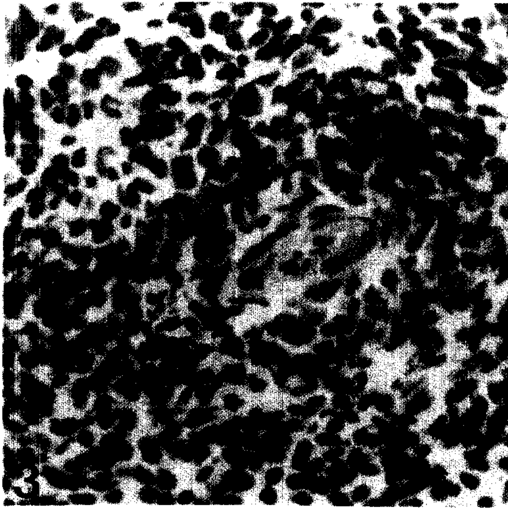
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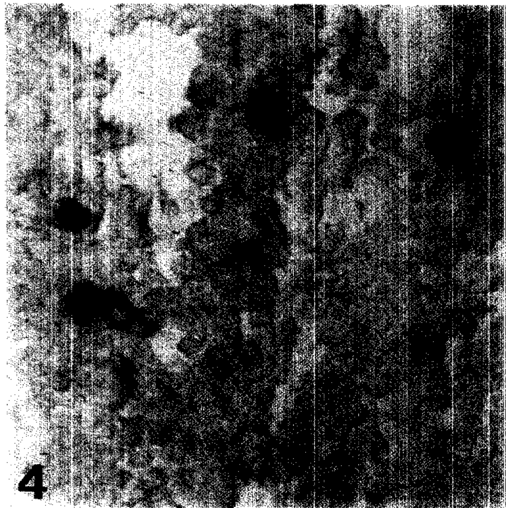
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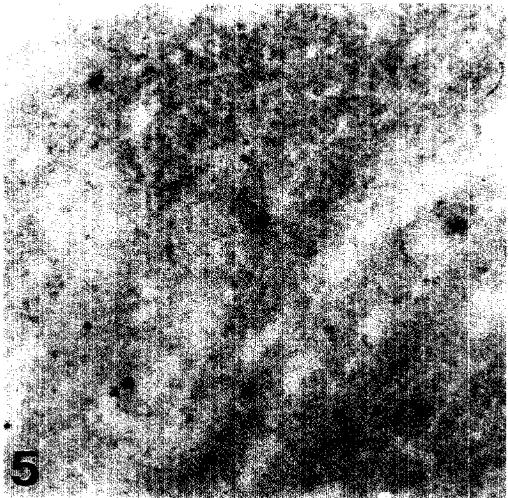
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