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Immunohistochemistry on Cryostat Tissue Sections Using Synthetic Peptide Produced Monospeciffic Anti-Sheep Pox Virus Antibody

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Abstract

An immunohistochemistry method on frozen tissue cryostat sections based on new polyclonal monospecific affinity-purified rabbit antibodies (PMAP) against 19-residue synthetic peptides (154-171), originated from the group-specific peptide P-32 for sheep poxvirus (SPV) was developed. PMAP were used as primary antibodies in an immunohistochemistry test on frozen ovine tissue for rapid laboratory diagnosis of SPV infection. This method is highly specific, reproducible, simple and inexpensive. The total time of the procedure was within 2 hours of receiving the samples in the laboratory.
Key words: cryosections, immunohistochemistry, polyclonal monospecific affinity purified antibodies, sheep poxvirus.

Резюме

Разработен е имунохистохимичен метод върху замразени криостатни срези на базата на нови поликлонални моно-специфични афинно пречистени заешки антитела (PMAP) срещу 19 синтетични пептиди (154-171), произхождащи от групата на специфичния пептид P-32 на вируса на шарката по овцете (SPV). PMAP се използват като първични антитела в имунохистохимичния тест на замразени тъкани от овце за бърза лабораторна диагностика на SPV инфекция. Този метод е много специфичен, възпроизводим, прост и не-скъп. Общото време на процедурата е до 2 часа от получаването на пробите в лабораторията.

Introduction

Sheep pox (SP) is an endemic infection in Africa, the Middle East, and Central Asia, as well as in Russia, China, Israel (Yeruham *et al.*, 2006) and India (OIE, 2000). The disease is highly contagious and causes mortality in young animals leading to significant economic losses (Kitching and Hammoud, 1991; OIE, 2000).The sheep poxvirus (SPV) belongs to the family *Poxviridae*, genus *Capripoxviruses* (OIE, 2000).

The diagnosis of SPV is still a problematic issue. The currently used diagnostic methods are with low sensitivity (Agar Gel Precipitation Test) and the differentiation between the poxviruses is difficult (Rao and Negi, 1997; Mangana-Vougiouka *et al.*, 1999; Mangana-Vougiouka *et al.*, 2000). The basic diagnostic methods are histopathology and virus isolation on cell culture. For confirmation of the infection, the virus neutralization test or immuno-fluorescence by using hyper immune anti-capripox serum are used (Rao and Bandyopadhyay, 2000). Due to the slow growth of SPV in cell culture (it takes more than two passages of visible cytopathic effect), the isolation and identification of the virus is a long procedure (Carn, 1995; Mangana-Vougiouka *et al.*, 1999; Gulbahar *et al.*, 2000). Transmission electron microscopy for identification of typical SPV particles is used as a rapid diagnostic method, but it is not applicable for the ordinarily laboratory practice (Gulbahar *et al.*, 2000; Rao and Bandyopadhyay, 2000). Recently, different techniques of PCR and ELISA have been developed in many laboratories (Carn, 1995; Tiwari *et al.*, 1996;

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Mighell *et al.*, 1998; Mangana-Vougiouka *et al.*, 1999; Mangana-Vougiouka *et al.*, 2000; Markoulatos *et al.*, 2000; OIE, 2000).

The aim of the study is to develop a highly specific, reproducible, inexpensive and rapid method for SPV diagnosis.

Materials and methods

Indirect immunohistochemistry methods (IHC) for detection of SP viral antigen in Vero cells or frozen tissue cryostat sections by using the Avidin-Biotin (AB) technique (Gulbahar *et al.*, 2000; Eligilashvili *et al.*, 2002) and polyclonal monospecific affinity-purified (PMAP) rabbit antibodies (Abs) against 19-residue synthetic peptides (154-171) (Vyberg and Nielsen, 1998; Eligilashvili *et al.*, 2002; Petrosyan *et al.*, 2002), originated from the group-specific peptide P-32 for sheep poxvirus (SPV) were developed.

Preparation of cell culture and tissue samples

Vero cells (ATCC CCL-81) were infected with the RM 65 vaccine strain of SPV. After observation of visible cytopathic effect (CPE) the cells were fixed and tested by IHC reaction at 24, 48, 72 h and after 5 days.

Small particles (8x5 mm) of skin originating from animals with clinical and pathological changes of SPV infection were frozen by using 2-Methylbutane in liquid nitrogen. The tissue samples were sectioned by Leica CM 1800 cryostat microtome (6 µm thick section at T -25° C) and were mounted on poly-L-lysine-coated glass microscope slides. After air-drying at room temperature (RT) for 20 min, the sections were fixed in cold acetone for 10 min. The endogenous peroxidase activity on frozen tissue section was blocked with peroxo-block (Zymed, USA) for 45 s at RT following the manufacture's instructions. After that the slices were washed three times x 2 min with phosphate buffered saline (PBS). Nonspecific background of Vero cells was blocked with 10% Normal Goat Serum for 10 min at RT without washing.

Preparation of polyclonal monospecific affinity-purified rabbit antibodies (PMAP)

The specificity of the used 19-residue synthetic peptides (154-171 ETFHNSNSRILFNQENNN) from the group-specific for SPV peptide P-32 (Vyberg and Nielsen, 1998; Eligilashvili *et al.*, 2002; Petrosyan *et al.*, 2002; Tulman *et al.*, 2002) was verified by Gene Runner, ClasteW multiply alignment and Blast software. Rabbits were immunized twice with a 22-day interval with the specific synthetic peptide attached to a carrier peptide (Zymed, USA). Forty days after the last inoculation the rabbits were bled. Specific antibodies for synthesized peptide were separated by affinity chromatography. Specificity and determination of optimal working conditions of purified Abs were determined by titration in ELISA (dilution from 1:10 to 1:1000) and IHC reaction (dilution from 1:50 to 1:500). Primary PMAP anti-SPV rabbit Abs, diluted 1:100 in PBS were added (50 µL) to the skin sections or tissue culture slides and were incubated for 30 min in a humid chamber at RT. After rinsing in PBS (three times x 2 min), they were treated for 10 min with secondary Goat-Antirabbit Biotinylated Abs, at RT according to the manufacture's protocol (Zymed, USA). After incubation with secondary antibodies the slides were washed with PBS and treated for 10 min at RT with enzyme conjugate (Streptavidin – Horse Radish Peroxidase). After that the substrate 3-amino 9-aethyl carbasol (AEC) was applied for 8-10 min at RT. The specimens were counterstained with Mayer's Hematoxylin 0.1% solution for 3 min and rinsed in tap water for 10 min. The slides were mounted with GVA-Mount with cover glass and were monitored under a light microscope Olympus CK-40 with a magnification of 100x or 200x (Eligilashvili *et al.*, 2002).

Controls

Uninfected Vero cells, skin from healthy sheep or sheep with other diseases peste de petit ruminants (PPR), border disease virus (BDV), enterotoxaemia, rabbit PMAP anti - Rift Valey Fever (RVF), west Nile fever (WNF) Abs and rabbit isotype control were used as negative controls.

Results

After investigation to determine the optimal working dilution and conditions of incubation, rich and dense coloring of the cytoplasm of the inoculated cell cultures at Abs dilution 1:50 was observed. At 1:200 dilution of PMAP Abs the coloring was with less density in comparison with the 1:100 dilution. The number of colored virus conglomerates was smaller than that at the 1:100 dilution. No difference in the number of colored virus conglomerates at dilutions 1:50 and 1:100 was found. The optimal dilution of PMAP was determined to be 1:100.

In IHC reaction on Vero cells infected with the SPV strain RM-65, coloring of the cells cytoplasm without any background was observed. IHC reaction was performed on infected cells at 24, 48, 72 and 120 h after infection. The number of colored cells containing SPV antigen was growing propor-

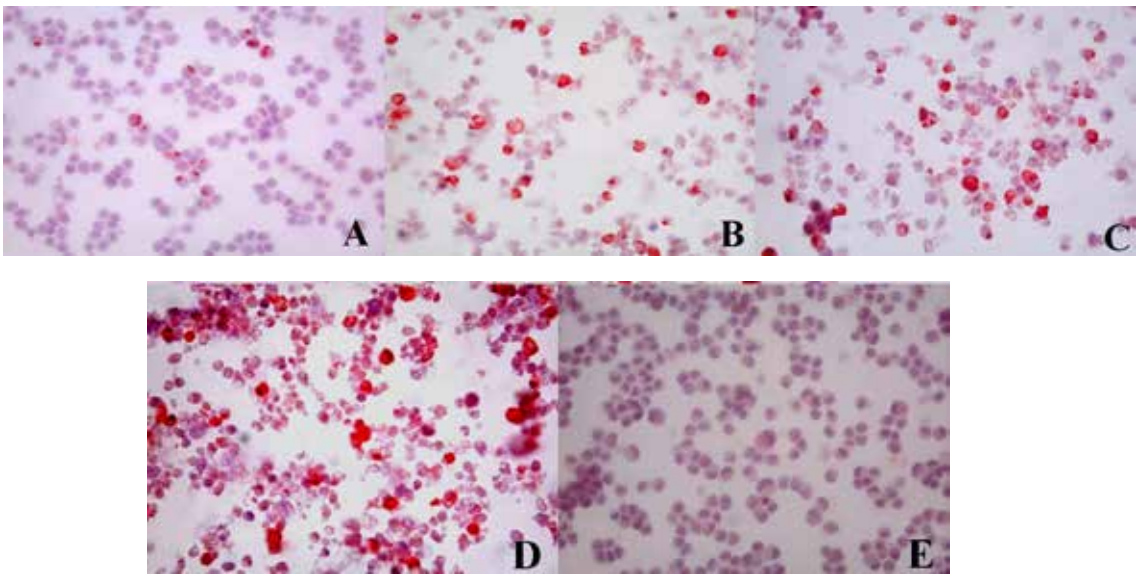

Fig. 1. Positive immunostaining in Vero cells inoculated with vaccine SPV RM-65 after infection at 24 (A), 48 (B), 72 (C) and 120 h (D). Uninfected cell culture (E). Counterstaining with Mayer's haematoxylin. Magnification x100.

tionally to the time after infection (Fig. 1 A, B, C and D). Background or cells with coloring of the cytoplasm were not observed in uninfected cell cultures or in cell cultures infected with viruses different than SPV (PPR, BDV, RVF, WNF and isotype specific rabbit antibodies) (Fig. 1 E).

The primary PMAP Abs used in IHC reaction for cell cultures were applied in the same concentration and by the same scheme onto organs or tissue cryosections from diseased animals or animals which died of SPV.

All frozen skin sections from SPV-infected sheep were positive in the cryo-IHC test using rabbit anti-SP PMAP Abs. In the cytoplasm of epidermal and dermal cells specific coloring without any background staining was observed. The staining of the cell cytoplasm was clear in both parallel and vertical sections of the skin surface (Fig.2 A and B). Background or staining in the cytoplasm were not found in the organs or tissue section from alive or infected with different than SPV viruses (PPR, BDV, RVF and WNF) animals (Fig. 2 C).

Discussion

The origin and procedures for obtaining immunogen are very important for the preparation of high quality reagents for the IHC reaction. An advantage of synthetic peptides is the knowledge of amino acid sequences. That information can be useful for the interpretation of the IHC reaction results according to the isoforms of the desired peptides and cross reactivity with similar peptide sequences in other proteins contained in the investigated tissues. Synthetic peptides also have potential disadvantages. The created synthetic peptides can have an altered three-dimensional structure of the native protein. Proteins similar to the desired peptides can exist in the investigated tissue. Both of these factors can lead to false negative results and unsuccessful detection of antigen (Mandel *et al.*, 1992; 1994).

For SPV detection we developed and used a peptide-based IHC method. The specificity of the IHC reaction depends on the specificity of the used components. The obtaining of new classes of polyclonal Abs based on studying the peptide structure of viral strains allows the creation of highly specific Abs (polyclonal monospecific) which can be concentrated and purified by affinity chromatography with the column containing gel with included synthetic peptide (Vyberg and Nielsen, 1998; Eligilashvili *et al.*, 2002; Petrosyan *et al.*, 2002).

Polyclonal Abs are produced by immunization of experimental animals with purified antigens. The immunized animals respond by a rise of Abs which specifically recognized and reacted with the antigens used for inoculation. Polyclonal Abs have high affinity and broad reactivity but lower specificity compared to monoclonal Abs (Hayat, 2002). Polyclonal Abs contain different Abs to the target antigen and nonspecific Abs with high concentration (up to 10 mg/mL) if they are not affinity purified (Mighell *et al.*, 1998; Elias, 2003).

The used nucleotide sequences of SPV p32 peptide (ETFHNSNSRILFNQENNN) were specific for the *Capripoxvirus* group. A confirmation of this fact is the absence of background in the IHC re-

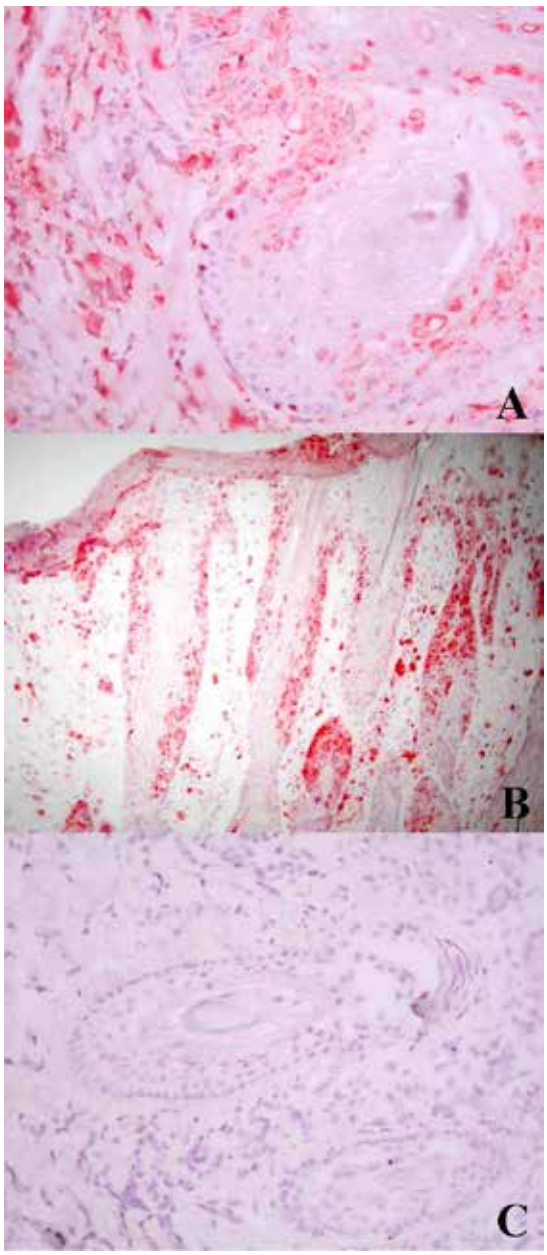

Fig. 2. Positive immunostaining in parallel (A) and vertical (B) cryo section of sheep skin epidermis with and without SPV infection (C). Counterstaining with Mayer's haematoxylin. Magnification x100.

action. In the other used methods background was observed due to endogen biotin or avidin (Vyberg and Nielsen, 1998; Sabattini *et al.*, 1998; Shi *et al.*, 1999; Petrosyan *et al.*, 2002; Taylor *et al.*, 2002). The evidence of IHC specificity and sensitivity is the observed virus antigen in infected Vero cells within the first 24 h after their infection.

It can be concluded that the developed cryo-IHC reaction is a suitable method for rapid recognition of SPV antigen in skin biopsies taken from live animals. This method can be used for rapid laboratory diagnosis of SPV in the early stages of the disease and could be a useful tool for studying the pathogenesis of SP infections (Gulbahar *et al.*, 2000; Kitching and Hammoud, 1991).

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