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Original article

Insight into behavior of epithelial cells of the feline conjunctiva in chronic conjunctivitis as a possible limitation in detection of *Chlamydophila* spp.

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Abstract

The aims of this work was documentation of the reactivity of feline conjunctival epithelial cells in chronic conjunctivitis and the investigation of a possible correlation of histological findings in conjunctiva with a limitation in detection of the pathogen. In this observational study, conjunctival swab samples collected from six cats suffering from chronic conjunctivitis were monitored for *Chlamydophila spp.* infection for one month, every ten days. Chlamydophilosis was diagnosed by conventional PCR, and confirmed by sequencing analysis. A lack of coherence with results in subsequent studies using PCR did not allow an accurate diagnosis. Additional bioptat samples of conjunctiva were collected for diagnostic purposes and stained in haematoxylin and eosin following the Giemsa method for light microscopic analysis. Additionally the samples were incubated for 15 min with IMAGEN™ Chlamydia conjugate (IMAGEN™ Chlamydia reagent kit, Dako, UK), allowing immunofluorescence detection of *Chlamydophila* spp. Within the epithelium an increased number of goblet cells, as well as general enlargement of the epithelium and a reduced number of normal epithelial cells, was observed. Only in areas of low epithelium could structures similar to the elementary bodies of *Chlamydophila spp.* be distinguished. The presented data document a possible limitation in molecular evidence for chlamydophila infection in some naturally infected cats, taking into account histological conditions in conjunctiva at the same time.

Key words: conjunctivitis, cats, epithelial cells reactivity, *Chlamydophila* spp.

Introduction

Epithelial cells of the conjunctiva and/or respiratory tract are the target of *Chlamydophila* spp. replication in cats. Histological examination of scrapings obtained from normal conjunctiva shows that samples contain sheets of epithelial cells, small numbers of bacteria and occasional leukocytes. Goblet cells are not seen unless the sample was collected from the fornix. In the case of conjunctivitis an increased number of degenerating epithelial cells, fibrin, inflammatory cells, bacteria and inclusion bodies are visible

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in the sample (Lavach et al. 1977). Cytologic examination is one of the laboratory methods which allow estimation of infectious causes of conjunctivitis. In cytological examination of smears of conjunctival scrapings a large number of polymorphonuclear cells together with a few lymphocythes showing chlamydial inclusions were noted. These mostly large, dark-blue staining inclusions were seen in epithelial cells, appearing as intracytoplasmic, basophilic and discrete masses (Ohya et al. 2008). However, it should be noted that opinion on the utility of cytologic examination differs strongly. Some authors suggest a good correlation between cytologic examination and molecular detection of *Chlamydophila spp.* in cases of acute conjunctivitis, others describe a limited value of cytology in cats with conjunctivitis (Hillstrom et al. 2012). Currently, the most popular method of causal identification of chlamydial infection in cats is polymerase chain reaction (PCR, Rt-PCR). However, possibilities for the detection of *Chlamydophila* in the light of current histological conditions of conjunctiva structures should be taken into account. This study attempts to find and explain a possible correlation between the behavior of epithelial cells and histological findings of conjunctiva during chronic *Chlamydophila* spp. infection which may influence pathogen detection.

Materials and Methods

Six cats with chronic conjunctivitis were diagnosed with *Chlamydophila* spp. infection at the Department of Epizootiology with Clinic for Birds and Exotic Animals and the Department of Surgery, Faculty of Veterinary Medicine (Wrocław, Poland). An ophthalmic examination was performed on each cat; eyelash and cartilage abnormalities, and incorrect positioning of the eyelids were ruled out. Irregularities of the drainage system were eliminated with a 1% fluorescein test and by irrigation via a 26G catheter. Conjunctival swab samples were taken from the ventral conjunctival fornix, using sterile cotton-topped swabs. The ages of the cats ranged from 2 years to 12 years. Material was collected every 10 days for one month (2 conjunctival swabs from left and right eye/ sampling). In the history, recurrent conjunctivitis persisted longer than one year. Specimens were examined by polymerase chain reaction (PCR) based on the OMP 2 gene, characteristic for *Chlamydiaceae*, using the method described by Marsilio et al. (2004). The amplified product was purified using a DNA extraction and purification kit (Fermentas International Inc., Canada). The sequencing reaction of the PCR products was performed in Genomed Sp. z o.o. (Warsaw, Poland). None of the animals was considered to represent a constant positive result in all three, successively performed, PCR tests. A lack of coherent results in PCR within the study did not allow implementation of appropriate therapy. For diagnostic purposes, additional bioptat samples were collected on the next day after the last swabbing. Fragments of bulbar conjunctiva were taken aseptically under dissociative analgesia (ketamine hydrochloride 10 mg/kg, xylazine 1 mg/kg b.w.; i.m.) for histological examination. The conjunctiva was drawn up with tweezers, and a small fragment was dissected using microsurgery scissors. All manipulations were performed under an operative microscope (Operationsmikroskop Zeiss, Jena, Germany). Conjunctival fragments, after freezing, were cut in a cryostat perpendicular to their surface. Slides, containing 10 μm thick sections, were stained in haematoxylin and eosin following the Giemsa method for light microscopic analysis. Additionally they were incubated for 15 min with IMAGEN^{TM} Chlamydia conjugate (IMAGEN^{TM} Chlamydia reagent kit, Dako, UK), allowing immunofluorescence detection of *Chlamydophila* spp. in cats (Hartmann et al. 2010). All samples were analysed using a Nikon Eclipse 80i microscope equipped with Nomarski contrast and fluorescence.

Results

Conjunctival findings observed on histological examination of the biopsy specimens are shown in Figs. 1, 2 and 3. In the analysed material, the main changes were observed within the epithelium, where an increased number of goblet cells, as well as general enlargement of the epithelium, and a reduced number of normal epithelial cells, was observed. Goblet cells formed two to six rows and were filled with a mucous substance. In the fluorescence test, such regions of conjunctiva do not show the presence of elementary bodies, probably because of a lack of normal epithelial cells. Only in areas of low epithelium could structures similar to the elementary bodies (*Chlamydophila* spp.) be distinguished. These inclusions were found in biopsy specimens of cats positive for *Chlamydophila* spp. (PCR) as well as in cats with negative result in the PCR test. In the connective tissue associated with the conjunctiva enlarged capillary vessels were noted. No signs of proliferation or infiltration of immune cells were observed in the biopsy specimens.

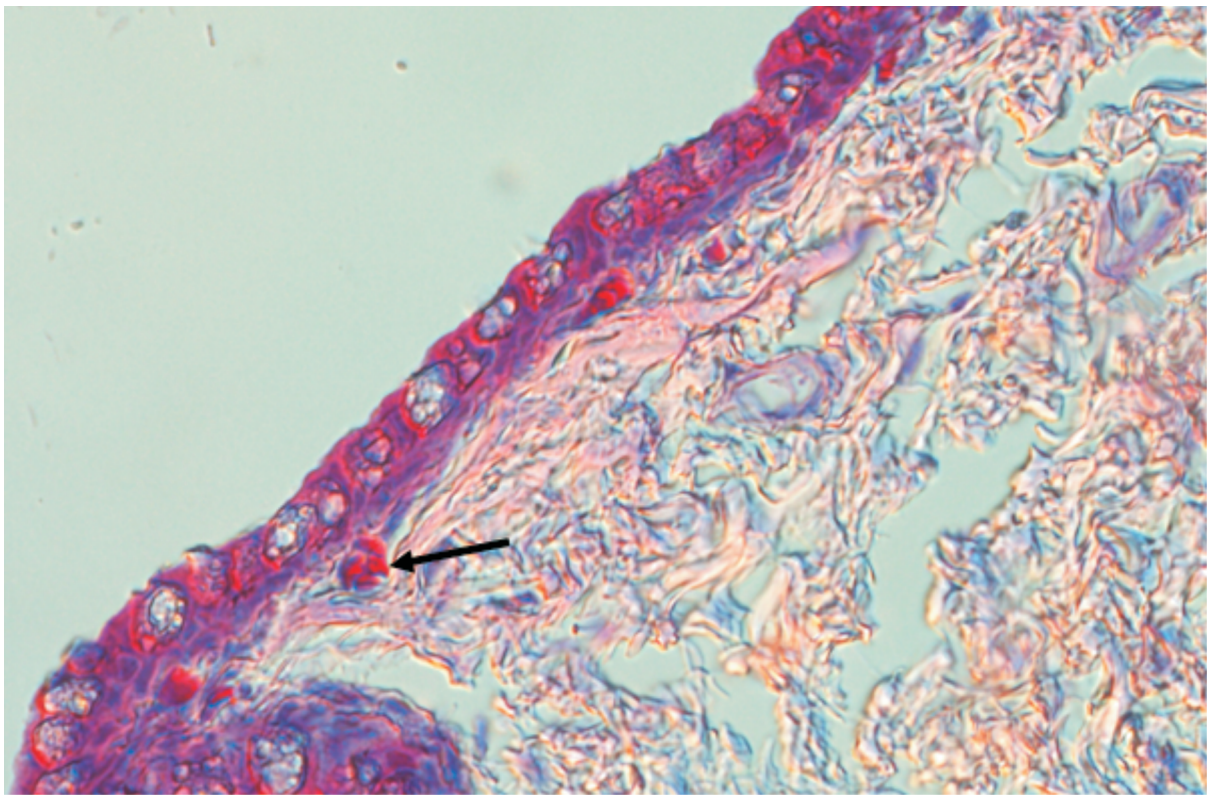

Fig. 1. Unchanged part of conjunctiva. Low epithelium containing sparse goblet cells. In connective tissue no signs of inflammation or increased proliferation are noted. Directly under the epithelium small capillaries are visible (arrow). Hematoxylin and eosin, Nomarski contrast (P. Kuropka). x600

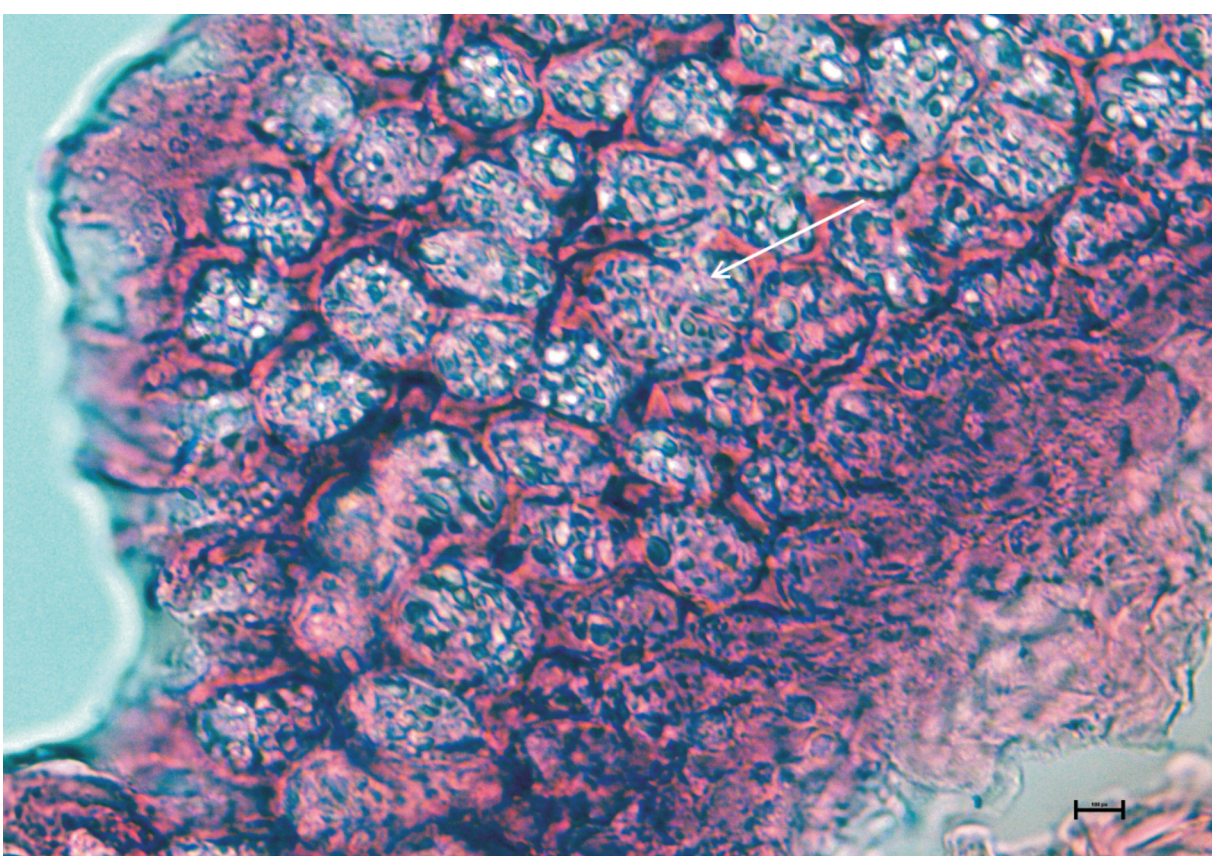

Fig. 2. Goblet cells. Epithelium and connective tissue of feline conjunctiva. High epithelium containing numerous goblet cells with mucous substance (arrow). Hematoxylin and eosin, Nomarski contrast (P. Kuropka). x600

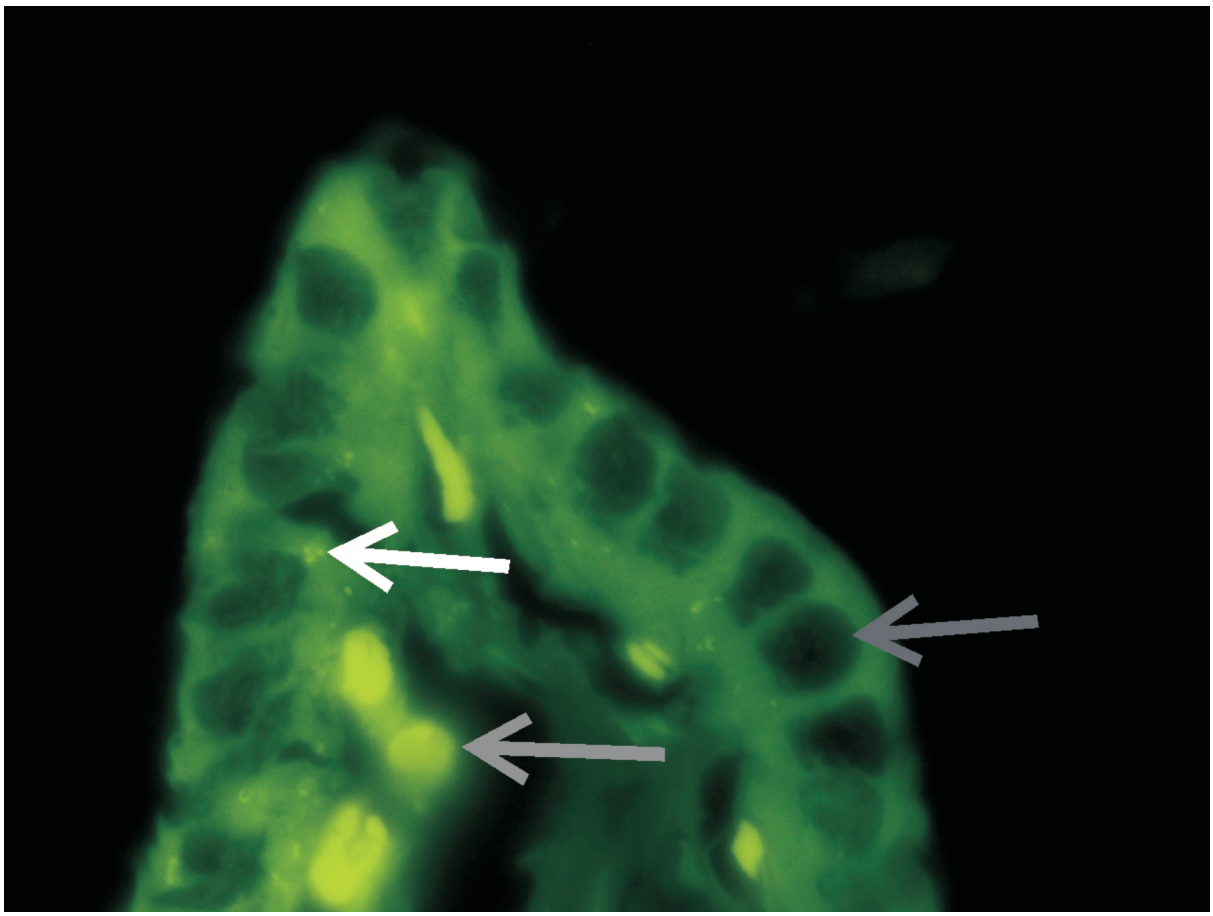

Fig. 3. Epithelium with structures which may suggest chlamydophilosis. Epithelium and connective tissue of feline conjunctiva. Low epithelium containing goblet cells (dark grey arrow). In the epithelial cells structures similar to the elementary bodies of *Chlamydophila* spp. are visible (white arrow). In the connective tissue below the epithelium erythrocytes in enlarged capillaries are present (light grey arrow). Immunofluorescence staining (P. Kuropka). x1000

Discussion

Chlamydophila spp., especially *Chlamydophila felis*, can occur in cats with clinical signs as well as in animals without any manifestation of disease; however some studies have noted that the presence of *Chlamydophila* was significantly associated with conjunctivitis ($p = 0.04$) (Rampazzo et al. 2003). A variety of tests and methods are available for the diagnosis of *Chlamydophila* infection. Isolation of the pathogen and specialized culture techniques was once the "gold standard" method. Today it is considered that maintenance of bacteria viability in the specimen is a limit to effectiveness (Sachse et al. 2009, Sykes 2005). Isolation is also time consuming, therefore routine diagnosis is dominated by methods which quickly provide a test result. Commercially available tests such as an immunoenzymatic assay (ELISA) or immunofluorescence assay (IFA) are based on family-specific lipopolisaccharide (LPS) antigens. The monoclonal antibodies used in the IFA are directed against a genus-specific epitope located on the chlamydial LPS (Ohya et al. 2008, Sachse et al. 2009).

In chronic conjunctivitis epithelium and goblet cells of conjunctiva may become hyperplastic and increase in number. Samples from changed conjunctiva show increased numbers of degenerating epithelial cells and fibrin (Lavach et al. 1977). Cytological staining and immunohistochemical analyses for chlamydial inclusions can be performed to demonstrate the presence of bacteria in tissue. Cytology can be useful in the identification of *Chlamydophila felis* by demonstrating intracytoplasmatic inclusion bodies in epithelial cells. However, studies on upper respiratory tract pathogens detection demonstrate that the choice of sample site could increase detection in low-shedding carriers (Veir et al. 2008).

Mild changes within the epithelium (metaplasia) and no changes in connective tissue may be the result of any chronic process. In such a situation the normal epithelial cells in which *Chlamydophila* replicates are in the minority and, in addition, they are covered by

an increased number of goblet cells which make *Chlamydophila* difficult for sampling in conjunctival swabs. Mucin, which is secreted by goblet cells, may bind chlamydial pathogens and facilitate their removal in the ocular discharge. On the other hand, an increased number of goblet cells makes the observation of elementary bodies in fragments of conjunctiva under fluorescent staining difficult. The observation of structures resembling elementary bodies was only possible in the basal layers of the epithelium. The enlarged epithelium seems to confirm the chronic changes which could be caused by the pathogen. Investigation of the utility of cytology and conjunctival smears for detection of infectious causes of feline conjunctivitis concluded that this method could be reliable for chlamydophilosis diagnosis when many typical inclusions are present (Hillström et al. 2012). In the light of this preliminary investigation, in some cats with chronic conjunctivitis the utility of cytology, specific staining (IFA) or even PCR for detection of chlamydial infection using conjunctival swabs could be unsatisfactory, taking into account histological conditions in the conjunctiva visible at the same time in biopsy specimens only. It is worth noting, that in chronic inflammation the presence of *Chlamydophila* spp. in the epithelial cells of conjunctiva can be detected in the areas of low epithelium which can be unreachable in the normal procedures of swabbing. All these factors may create false negative results in diagnostic methods. On the other hand, there are no logical reasons to collect samples from other places in the case of clinical conjunctivitis.

Acknowledgements

The study was performed in accordance with the ethical principles of the Ethics Committee of the Wrocław Univeristy of Environmental and Life Sciences.

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