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Technical Report

Evaluation of a panel of antibodies for the immunohistochemical identification of immune cells in paraffin-embedded lymphoid tissues of new- and old-world camelids

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ABSTRACT

Different species of camelids play an important role in the epidemiology of various emerging infectious diseases such as Middle East respiratory syndrome. For precise investigations of the immunopathogenesis in these host species, appropriate immunohistochemical markers are highly needed in order to phenotype distinct immune cells populations in camelids. So far, specific immunohistochemical markers for camelid immune cells are rarely commercially available, and cross-reactivity studies are restricted to the use of frozen dromedary tissues. To bridge this gap, 14 commercially available primary antibodies were tested for their suitability to demonstrate immune cell populations on formalin fixed paraffin-embedded (FFPE) tissue sections of dromedaries, Bactrian camels, llamas, and alpacas in the present study. Out of these, 9 antibodies directed against CD3, CD20, CD79α, HLA-DR, Iba-1, myeloid/histiocyte antigen, CD204, CD208, and CD68 antigen exhibited distinct immunoreaction patterns to certain camelid immune cell subsets. The distribution of these antigens was comparatively evaluated in different anatomical compartments of thymus, spleen, mesenteric, and tracheobronchial lymph nodes. The presented results will provide a basis for further investigations in camelids, especially with respect to the role of the immune response in certain infectious diseases, which harbor a considerable risk to spill over to other species.

1. Introduction

The numbers of alpacas (*Vicugna pacos*) and llamas (*Lama glama*) housed as companion animals is substantially increasing (Riek et al., 2013). Similarly, dromedaries (*Camelus dromedarius*) represent an important livestock species in both Africa and Asia and especially on the Arabian Peninsula, where they serve as a major source of milk and meat (Burger, 2016).

In 2012 a single-stranded, positive-sensed beta-coronavirus was isolated in the Kingdom of Saudi Arabia (Chan et al., 2015; van Boheemen et al., 2012; Zaki et al., 2012). Until today *Middle East respiratory syndrome coronavirus (MERS-CoV)* spread to 27 countries and caused more than 1,800 laboratory-confirmed cases of pneumonia including 643 fatal progressions in man (World Health Organization (2016) *Middle East respiratory syndrome coronavirus. Available at* www.who.int/emergencies/mers-cov/en/. *Accessed October 06, 2016).* Recent studies indicate that dromedaries appear to play a pivotal epidemiological role in transmitting the virus to humans, and experimental studies with dromedaries illustrate shedding of *MERS-CoV* after intranasal inoculation (Adney et al., 2014; Haagmans et al., 2015; Reusken et al., 2013). However, there is a high demand for additional animal models, including the use of llamas and alpacas, which allow sophisticated pathological investigations in order to determine details of pathogenesis, cell tropism, and viral transmission of MERS-CoV. Besides MERS-CoV infections, dromedaries and Bactrian camels (*Camelus ferus*) also play a sig-

Abbreviations: CD, cluster of differentiation; DALT, dense anodular lymphoid tissue; DC(s), Dendritic cell(s); DLT, diffuse lymphoid tissue; FFPE, formalin-fixed paraffin-embedded; Foxp3, forkhead box P3; GAM, goat anti-mouse Immunoglobulin G; GAR, goat anti-rabbit Immunoglobulin G; H&E, hematoxylin and eosin; HLA, human leucocyte antigen; Iba-1, ionized calcium-binding adapter molecule 1; IgG, Immunoglobulin G; IHC, immunohistochemistry; LN, lymphoid nodules; mc, monoclonal; MERS-CoV, Middle East respiratory syndrome coronavirus; MUM1, multiple myeloma oncogene 1; PALS, periarteriolar lymphoid sheath; Pax-5, paired box protein 5; PBS, phosphate buffered saline; pc, polyclonal; RAR, rabbit anti-rat Immunoglobulin G; RT, room temperature.

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nificant epidemiological role in other emerging infectious diseases such as West Nile Virus encephalitis (Joseph et al., 2016), Hepatitis E Virus infections (Rasche et al., 2016), and rabies (Feng et al., 2014; Liu et al., 2016), demonstrating that naturally occurring and experimentally induced diseases in old and new world camelids might emerge as important animal models for studies upon the pathogenesis of such zoonotic infections.

The use of commercially available mono (mc)- and polyclonal (pc) antibodies in immunohistochemistry (IHC) has substantially improved our knowledge about the immunopathogenesis of pathological processes in a broad range of species and diseases (Duraiyan et al., 2012). Primary antibodies detecting specific human and murine antigens can often be used successfully for the detection of conserved epitopes on tissues of various animals, including both domestic and exotic wild life species (Faldyna et al., 2007; Heinrich et al., 2015; Salvadori et al., 2016; Seibel et al., 2010).

Earlier studies have tested a limited panel of antibodies, mainly for the detection of lymphocytes, and were carried out on frozen tissue in only one camelid species (dromedary; Zidan and Pabst, 2004, 2012; Zidan et al., 2000b). In another study, one B cell marker (Pax-5) and one T cell marker (CD3) were used on paraffin embedded gut associated lymphoid tissue (GALT) of alpacas, experimentally infected with bovine viral diarrhea virus (Steffen et al., 2014). However, the establishment of a broader panel of immunohistochemical markers on formalin fixed paraffin embedded (FFPE) tissue represents a prerequisite to elucidate the role of the immune response in diseases of camelids. Therefore, it was the aim of the present study to test the applicability of commercially available immunohistochemical markers for the detection of immune cells in new- and old-world camelids in FFPE tissue of different lymphoid organs to provide a solid fundament for further research on healthy and diseased camelids with special respect to prospective spill-over infections.

2. Materials and methods

2.1. Animals and tissue samples

Tissues used in the present study were taken from the archive of the Department of Pathology, University of Veterinary Medicine Hannover. A complete necropsy was performed and sampled organs were fixed in 10% non-neutral buffered formalin for 24 h and routinely embedded for histology. The archive was screened for cases of new- and old world camelids, in which lymphoid organs were available. Briefly, formalin fixed tissues were automatically dehydrated by an ascending alcohol series using the embedding machine Thermo Scientific™ Shandon™ Pathcentre™ (Thermo Fisher Scientific, Langenselbold, Germany) and subsequently embedded in paraffin. 4 μm thick paraffin sections of lymphoid organs were stained with H&E and examined by routine light microscopy. Lymphoid tissues affected by destruction of organotypic architecture and/or major lesions such as severe suppurative inflammation, abscess or granuloma formation, and neoplastic processes were excluded from the present study. Moreover, lymphoid organs with severe lymphoid depletion and marked auto- and heterolytic changes were omitted from further processing. Overall, the lowest number of lymphoid organs fulfilling the inclusion criteria was available from dromedaries. In particular, 5 spleens, 5 mesenteric lymph nodes, and 5 tracheobronchial lymph nodes of dromedary origin were obtainable. The number of lymphoid tissues from the remaining species (Bactrian camels, llamas, and alpacas) was consequently adjusted to n = 5 for each of the aforementioned organs. Accordingly, in total 20 spleens and 20 tracheobronchial and 20 mesenteric lymph nodes were investigated in the present study (5 per species for each organ). Additionally, thymus tissue was available from two young animals of each species (8 animals with a median age of 3 days).

2.2. Immunohistochemistry

For IHC a panel of different antibodies (n = 14) was applied on lymph nodes of each species to screen for their general suitability for camelid lymphoid tissue (Table 1). The tested antibodies have previously been reported to cross-react with canine and bovine immune cells, respectively (Ackermann et al., 1994; Alldinger et al., 1996; Fernandez et al., 2017; Kato et al., 2013; Pierezan et al., 2014; Ramos-Vara et al., 2007; Romero-Palomo et al., 2013; Spitzbarth et al., 2011; Wünschmann et al., 2000). Consequently, archived FFPE sections from mesenteric lymph nodes of a dog or an ox were used as positive controls. IHC was performed by use of the avidin-biotin-peroxidase complex method as described previously (Alldinger et al., 1996). Briefly, after dewaxing by incubating the sections for 5 min twice in Roticlear® (Roth C. GmbH & Co. KG, Karlsruhe, Germany) and rehydration in isopropanol and ethanol (96%; each for 5 min), endogenous peroxidase activity was blocked by incubation of sections in 85% ethanol with 0.5% H_2O_2 for 30 min at room temperature (RT). If necessary (Table 1), pretreatment was either done by incubating the sections in citrate buffer (2.1 g citric acid monohydrate in 1 l distilled water, adjusted with NaOH to pH = 6.0) for 20 min in a microwave (800 W) or with 20 min pronase treatment at 37 °C (pronase E, 50 mg in 100 ml phosphate buffered saline [PBS] and 0.1 g $CaCl_2 \times 2H_2O$ with pH adjusted to 7.2). Subsequently, sections were transferred to Shandon Coverplates™ (Thermo Electron GmbH, Dreieich, Germany). To block unspecific binding, inactivated normal rabbit serum (for CD208 and Foxp3) or normal goat serum (all other antibodies) was applied for 30 min in a dilution of 1:5 with PBS (pH 7.2). For appropriate negative controls, primary antibodies (Table 1) were replaced by ascites fluid from Balb/c mice (1:1000; CD79α, Pax-5, MUM1 protein, HLA-DR antigen, CD68, myeloid/histiocyte antigen, CD204, CD205), rabbit (1:3000; CD3, CD20, Iba-1, lysozyme), and rat serum (1:1000; Foxp3, CD208), respectively. After 90 min of incubation at RT and subsequent washing with PBS, the appropriate secondary biotinylated antibody was added in a dilution of 1:200 with PBS. Incubation for 60 min at RT was followed by treatment with the avidin-biotin-peroxidase complex (Vectastain ABC Kit Standard, Vector Laboratories, Burlingame, California, USA) according to the manufacturer's protocol. Visualization of the reaction was achieved by the chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB, 0.05%, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) with addition of 0.03% H_2O_2. Slides were finally slightly counterstained with Mayer's hematoxylin (Roth C. GmbH & Co KG, Karlsruhe, Germany).

2.3. Evaluation and quantification

Sections were viewed under an Olympus BX-51 digital camera microscope (Olympus Optical Co., Hamburg, Germany). Positive immunoreactivity was appreciated if cellular staining pattern and cellular morphology fitted with the expected distribution pattern and immunoreactivity appearance of the positive control. All antibodies, which produced positive results on camelid lymph nodes, were applied to serial sections of lymph node, spleen, and thymus of all examined camelid species and comparatively assessed according to a semiquantitative scoring system (Heinrich et al., 2015). For all camelid species, scoring was adapted to the anatomical compartments of dromedary lymph nodes, which are known to differ from other mammals in some aspects (Abdel-Magied et al., 2001; Zidan et al., 2000a; Zidan and Pabst, 2004; Zidan and Pabst, 2012). Within sections of spleens, positive immunoreactivity was differentially evaluated for the periarteriolar lymphoid sheaths (PALS), follicles,