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Short communication

Detection of MERS-CoV antigen on formalin-fixed paraffin-embedded nasal tissue of alpacas by immunohistochemistry using human monoclonal antibodies directed against different epitopes of the spike protein

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ABSTRACT

Middle East respiratory syndrome (MERS) represents an important respiratory disease accompanied by lethal outcome in one third of human patients. In recent years, several investigators developed protective antibodies which could be used as prophylaxis in prospective human epidemics. In the current study, eight human monoclonal antibodies (mAbs) with neutralizing and non-neutralizing capabilities, directed against different epitopes of the MERS-coronavirus (MERS-CoV) spike (MERS-S) protein, were investigated with regard to their ability to immunohistochemically detect respective epitopes on formalin-fixed paraffin-embedded (FFPE) nasal tissue sections of MERS-CoV experimentally infected alpacas. The most intense immunoreaction was detected using a neutralizing antibody directed against the receptor binding domain S1B of the MERS-S protein, which produced an immunosignal in the cytoplasm of ciliated respiratory epithelium and along the apical membranous region. A similar staining was obtained by two other mAbs which recognize the sialic acid-binding domain and the ectodomain of the membrane fusion subunit S2, respectively. Five mAbs lacked immunoreactivity for MERS-CoV antigen on FFPE tissue, even though they belong, at least in part, to the same epitope group. In summary, three tested human mAbs demonstrated capacity for detection of MERS-CoV antigen on FFPE samples and may be implemented in double or triple immunohistochemical methods.

1. Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel lineage C betacoronavirus, which was first identified in a patient from the Kingdom of Saudi Arabia in June 2012 (Zaki et al., 2012). As of May 2019, MERS-CoV has been detected in 27 countries and infected more than 2300 patients (World Health Organization (2019) Middle East respiratory syndrome coronavirus, available at <https://www.who.int/csr/don/24-April-2019-mers-saudi-arabia/en/>). Although the disease vanished almost completely from headlines and newspapers, it is still a potential threat to human health since approximately one third of all affected patients succumb to acute respiratory distress syndrome and renal dysfunction (Zumla et al., 2015).

Even though MERS-CoV is most likely derived from an ancestral reservoir in bats (Corman et al., 2014; Cui et al., 2013; de Wit and Munster, 2013; Memish et al., 2013; Munster et al., 2016; van Boheemen et al., 2012), transmission to humans occurs mainly via dromedaries, which represent a major reservoir for the virus in large parts of the Middle East, the Canary Islands, Africa, and Southern Asia (Falzarano et al., 2017; Haagmans et al., 2014; Hemida et al., 2017; Saqib et al., 2017). Infection has also been demonstrated in farmed alpacas from a region of Qatar where MERS-CoV is endemic (Reusken et al., 2016), but not in any other livestock species (Reusken et al., 2013). Recently, experimental inoculation of pigs, alpacas, and llamas with MERS-CoV resulted in low levels of viral replication in pigs and moderate to high levels of viral replication and shedding in the latter

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Table 1
Antibodies, clonality, species, antigen retrieval, dilution, secondary antibodies and results.

Antibody	Clonality, host species (lot number)	Protein content [$\mu\text{g/ml}$]	Antigen retrieval	Dilution	Secondary antibody	Result
1.2g5	mc, human	0.23	#	1:5, 1:10, 1:20	GaH-b	+
1.10f3		0.27	#	1:5, 1:10, 1:20		(+)
1.6c7		0.27	citrate	1:5		(+)
1.6f9		0.23		1:5		-
4.6e10		0.43		1:5		-
7.7g6		0.77		1:5		-
1.8e5		0.25		1:5		-
3.5g6		0.91		1:5		-
MERS-CoV nucleocapsid (Sino Biological)	mc, mouse (HB10AP1804-B)	1.0		1:70	GaM-b	+++
MERS-CoV nucleocapsid (Sino Biological)	pc, rabbit (HB07AP1208)	1.0		1:2000	GaR-b	+++

GaH-b, goat anti-human IgG biotinylated; GaM-b, goat anti-mouse IgG biotinylated; GaR-b, goat anti-rabbit IgG biotinylated; mc, monoclonal; MERS-CoV, Middle East respiratory syndrome coronavirus; pc, polyclonal; #, antigen retrieval was performed with boiling citrate buffer, proteinase K and omission of pretreatment; +++ , intense antigen specific signal in several cells, minimal background staining (Fig. 1C, 1D); + , moderately intense antigen specific signal in fewer cells, minimal background staining (Fig. 2A); (+) , moderately intense antigen specific signal in fewer cells, strong background staining (Fig. 2C, D); -, no antigen specific signal, varying degrees of background staining.

species, respectively (Adney et al., 2016a; de Wit et al., 2017; Vergara-Alert et al., 2017). Similarly, minimal virus shedding was detected in goats, sheep, and horses after experimental infection, but only goats developed neutralizing antibodies (Adney et al., 2016b). Due to the close interactions of humans with potentially susceptible and contagious livestock species in certain parts of the world, the development of an effective vaccination strategy against MERS-CoV was proposed early after its initial discovery and was recently followed up by the development of protective antibodies by different protocols (Agrawal et al., 2016; Corti et al., 2015; Houser et al., 2016; Jiang et al., 2014; Johnson et al., 2016; Li et al., 2015; Pascal et al., 2015; Qiu et al., 2016; Tang et al., 2014; Widjaja et al., 2019). Widjaja and colleagues (2019) developed a set of protective neutralizing and non-neutralizing human monoclonal antibodies (mAbs) which target different epitopes of the MERS-CoV spike (MERS-S) protein. The MERS-S protein is known to represent a key target for the development of new therapeutics and consists of a receptor-binding subunit S1 and a membrane-fusion subunit S2 (Du et al., 2017). The subunit S1 is, in turn, composed of four different core domains. The domain S1B binds to the host-cell receptor dipeptidylpeptidase 4 (DPP4; Lu et al., 2013; Raj et al., 2013; Wang et al., 2013), while the domain S1A binds to sialoglycans which enhances infection of human lung cells by MERS-CoV (Li et al., 2017). The subunit S2 comprises the fusion peptide, two heptad repeats and a transmembrane domain, which mediate fusion of the virus with the cell membrane (Lu et al., 2013; Pallesen et al., 2017; Raj et al., 2013; Wang et al., 2013).

In the present study, these mAbs were tested for immunohistochemistry to detect respective epitopes on formalin-fixed paraffin-embedded (FFPE) nasal tissue sections of MERS-CoV infected alpacas. Immunohistochemistry is a well-established and powerful method which is frequently used for diagnostic purposes and evaluation of animal experiments. Simple protocols allow precise correlation between the presence and severity of lesions and the amount of viral antigen in the respective localization (Duraiyan et al., 2012). Even though there are several mono- and polyclonal antibodies available for the detection of the MERS-CoV nucleocapsid and spike protein on FFPE tissue (Haverkamp et al., 2018), these antibodies are all derived from rabbits and mice, respectively (<https://www.biocompare.com>, accessed March 11, 2019). However, for double or triple immunohistochemistry and -fluorescence labeling, it can be useful to have access to epitope-specific antibodies from different species, which can be used to visualize viral antigen in tissue sections. It was therefore the aim of the present study to evaluate the suitability of different human anti-MERS-S mAbs for detection of the respective antigen on FFPE material.

2. Materials and methods

2.1. Human monoclonal antibodies

The development of the eight diverse human mAbs directed against different epitopes of MERS-S protein has been published previously (Widjaja et al., 2019). Shortly, generation of anti-MERS-S H2L2 mAbs was achieved by recurrent immunization of transgenic H2L2 mice, which encode the human immunoglobulin variable regions, with the respective antigens and adjuvants (for preparation of antigens see original publications (Li et al., 2017; Mou et al., 2013; Walls et al., 2016; Widjaja et al., 2019)). At day 74, four days after the last immunization, spleen and lymph nodes were harvested and hybridoma cell lines were generated using the SP2/0 myeloma cell line. For production of recombinant mAbs, cDNA's encoding the variable heavy and light chain regions of anti-MERS-S H2L2 mAbs were cloned into expression plasmids containing the human IgG1 heavy chain and Ig kappa light chain constant regions, respectively. Recombinant human anti-MERS-S mAbs were produced in HEK-293 T cells following co-transfection of the IgG1 heavy and light chain expression plasmids (Invivogen) according to manufacturers' protocols (Widjaja et al., 2019). Antibodies were purified from tissue culture supernatants using Protein-A affinity chromatography. Purified antibodies were quantified using UV absorbance (280 nm) and Coomassie stained SDS-PAGE analysis was applied in addition. Antibodies were stored at 4 °C until further use. After screening for MERS-S reactivity and neutralizing capacity eight different mAbs (Table 1) were selected based on epitope distribution and neutralization.

2.2. Tissue

Formalin-fixed tissue of nasal turbinates from two experimentally infected alpacas was obtained from an animal trial recently implemented at the Research Center for Animal Health (*Centre de Recerca en Sanitat Animal*, CReSA, Barcelona, Spain). The mucosa was carefully removed from underlying bone and embedded in paraffin according to a routinely used protocol. Serial sections of 4 μm thickness were mounted on coated glass slides (Superfrost Plus[®], Menzel Co.) and the first section was stained with HE for evaluation by light microscopy and subsequent sections were processed for immunohistochemistry. For negative control, nasal turbinate of a non-infected alpaca (collected during routine necropsy at the Department of Pathology, University of Veterinary Medicine Hannover, Germany) was used. The animal died due to cachexia related to non-respiratory disease.

2.3. Immunohistochemistry

Sections were dewaxed in Rotoclear® (Roth C. GmbH & Co. KG) and subsequently rehydrated in isopropanol and 96% ethanol (Roth C. GmbH & Co. KG). Endogenous peroxidase activity was blocked by incubation of sections in 85% ethanol with 0.5% H₂O₂ (VWR™ International GmbH) for 30 min at room temperature and antigen retrieval was performed by incubation 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). In a preliminary experiment two of the eight antibodies (1.10f3 and 1.2g5) were additionally tested without pretreatment and with enzymatic digestion by Proteinase K antigen retrieval solution (Merck KGaA) according to the manufacturer's protocol. All sections were transferred to Shandon Coverplates™ (Thermo Electron GmbH) and nonspecific binding was blocked by inactivated 20% goat serum diluted in phosphate buffered saline (PBS) including 1% bovine serum albumin (BSA; PBS/BSA) for 30 min. Afterwards sections were incubated with the primary antibody (1.2g5, 1.6c7, 1.10f3, 1.6f9, 4.6e10, 7.7g6, 3.5g6, and 1.8e5) in their respective dilution (Table 1) for 90 min at room temperature and the secondary biotinylated antibody diluted in PBS (1:200) was added. Incubation for 60 min at room temperature was followed by treatment with the avidin-biotin-peroxidase complex (Vectastain ABC Kit Standard, Vector Laboratories) according to the manufacturer's protocol. Visualization of the reaction was achieved by addition of chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.05%, Sigma Aldrich Chemie GmbH) and 0.03% H₂O₂. Sections were finally slightly counterstained with Mayer's hematoxylin (Roth C. GmbH & Co KG). For positive control and elucidation of virus distribution, additional sections were stained with two different commercially available, previously published monoclonal mouse and polyclonal rabbit anti-MERS-CoV nucleocapsid antibodies (Sino Biological Inc.; Haagmans et al., 2016; Haverkamp et al., 2018), respectively. For negative controls, the primary antibody was replaced by ascites fluid from Balb/c mice (1:1,000) and normal rabbit serum (1:3,000), respectively. Moreover, all antibodies were applied to tissue of a non-infected alpaca (section 2.2).

3. Results and discussion

Evaluation of HE stained slides by light microscopy revealed a mild to moderate, multifocal, lymphoplasmahistiocytic and suppurative rhinitis characterized by low numbers of intraepithelial neutrophils and lymphocytes and infiltration of the lamina propria and submucosa by low to moderate numbers of lymphocytes, single plasma cells, and macrophages (Fig. 1A, B). These findings are reminiscent of previous observations in other large animal species though lesions in alpacas are relatively mild and lack the necrotizing component that has been described for dromedaries, llamas, and pigs (Adney et al., 2014; Haagmans et al., 2016; Vergara-Alert et al., 2017). The commercially available monoclonal and polyclonal anti-MERS-CoV nucleocapsid antibodies, which were used as positive controls, revealed a strong intracytoplasmic and cilia-associated staining pattern in numerous cells (Fig. 1C, D) according to formerly published results (Haagmans et al., 2016; Haverkamp et al., 2018). A specific staining was absent in all negative controls as expected.

For evaluation of human mAbs by immunohistochemistry, two of the tested antibodies (1.2g5 and 1.10f3) were applied in three different dilutions and three different pretreatments in a preliminary experiment to identify the best method for the use of human mAbs on FFPE tissue (Table 1). As a result, heat-induced antigen retrieval and application of the antibody in the lowest dilution revealed the best staining effects. According to these results, all other antibodies (1.6c7, 1.6f9, 4.6e10, 7.7g6, 3.5g6, 1.8e5) were tested with heat-induced antigen retrieval and in the lowest dilution.

The use of the 1.2g5 antibody in a 1:5 dilution with heat-induced

antigen retrieval showed the strongest signal of all tested non-commercially available antibodies. Detected antigen was present multifocally in the cytoplasm of ciliated respiratory epithelium and segmentally along the apical membranous region of few infected cells (Fig. 2A). The observed staining pattern was comparable to descriptions by others investigating the distribution of MERS-CoV in tissue sections of different infected animal species (Adney et al., 2014; Haagmans et al., 2016; Haverkamp et al., 2018; Vergara-Alert et al., 2017) but the number of labeled cells was considerably lower. Staining with the same antibody without pretreatment revealed a similar distribution of viral antigen in a comparable number of cells but a less prominent expression pattern (Fig. 2B), which is most likely linked to the lack of antigen unmasking which takes place during antigen retrieval (Shi et al., 2011). Detection of MERS-CoV antigen by the 1.2g5 antibody was almost completely absent with pretreatment by enzymatic digestion in all three dilutions, resulting only in a pale brownish signal interpreted as non-specific background staining (data not shown). The antibody 1.10f3 showed a strong non-specific background staining in all dilutions and pretreatments. A moderately intense antigen-specific signal was detected multifocally in cilia of few cells using the lowest dilution and citrate pretreatment (Fig. 2C).

Of the six remaining antibodies (1.6c7, 1.6f9, 4.6e10, 7.7g6, 3.5g6, 1.8e5), only antibody 1.6c7 showed a mild multifocal staining of cilia accompanied by a strong background reactivity (Fig. 2D). The other five antibodies (1.6f9, 4.6e10, 7.7g6, 1.8e5, 3.5g6) did not reveal any recognizable specific staining of MERS-CoV antigen on FFPE tissue along with no to very mild (1.6f9, 4.6e10, Fig. 3A, B), mild (7.7g6, 1.8e5, Fig. 3C, D) and strong (3.5g6, Fig. 3E) non-specific background staining. By contrast, all antibodies displayed a distinct signal by immunofluorescence staining of MERS-CoV-infected Huh-7 cells in culture (Widjaja et al., 2019). Possible explanations for the lack of immunoreactivity on FFPE tissue include effacement of conformational epitopes by formalin fixation and/or antigen retrieval protocols (Smedley et al., 2007).

The tested antibodies in the present study were derived from transgenic mice expressing chimeric antibodies containing human variable domains and constant antibody domains of rat origin. For generation of fully human antibodies the human variable heavy and light chain encoding gene segments of the antibodies isolated from MERS-S immunized mice transgenic for human V genes were cloned into human IgG1 heavy and light chain expression vectors, respectively (Widjaja et al., 2019). All these antibodies are directed against MERS-S and belong to six distinct epitope groups that interfere with three critical entry functions *in vivo*. In particular, 1.2g5, 7.7g6, 1.6f9, 1.8e5, and 4.6e10 are neutralizing antibodies directed against the receptor binding domain (RBD) S1B of the MERS-S protein (Widjaja et al., 2019), which facilitates virus binding to the DPP4 receptor on the host cell surface (Mou et al., 2013; Raj et al., 2013). The neutralizing antibodies 1.6c7 and 3.5g6 bind to the ectodomain of the membrane fusion subunit S2 which mediates fusion of the viral and cellular membranes (Li et al., 2017; Widjaja et al., 2019). By contrast, antibody 1.10f3 is a non-neutralizing antibody that recognizes the sialic acid binding domain (Widjaja et al., 2019). Since the positive staining antibodies 1.2g5, 1.10f3 and 1.6c7 recognize epitopes that belong to different functional groups (DPP4 binding, sialic acid binding and membrane fusion, respectively), the suitability of mAbs for use in immunohistochemistry cannot be predicted from the function of their target epitopes *in vivo*.

4. Conclusion

The present study shows that three tested human mAbs, designated 1.2g5, 1.10f3, and 1.6c7, are suitable to detect MERS-CoV antigen on FFPE tissue sections of experimentally infected alpacas, though to a limited extent. The best immunolabelling results for all three antibodies were achieved at the lowest dilution (1:5) with heat-induced antigen retrieval. However, results demonstrate that the suitability of

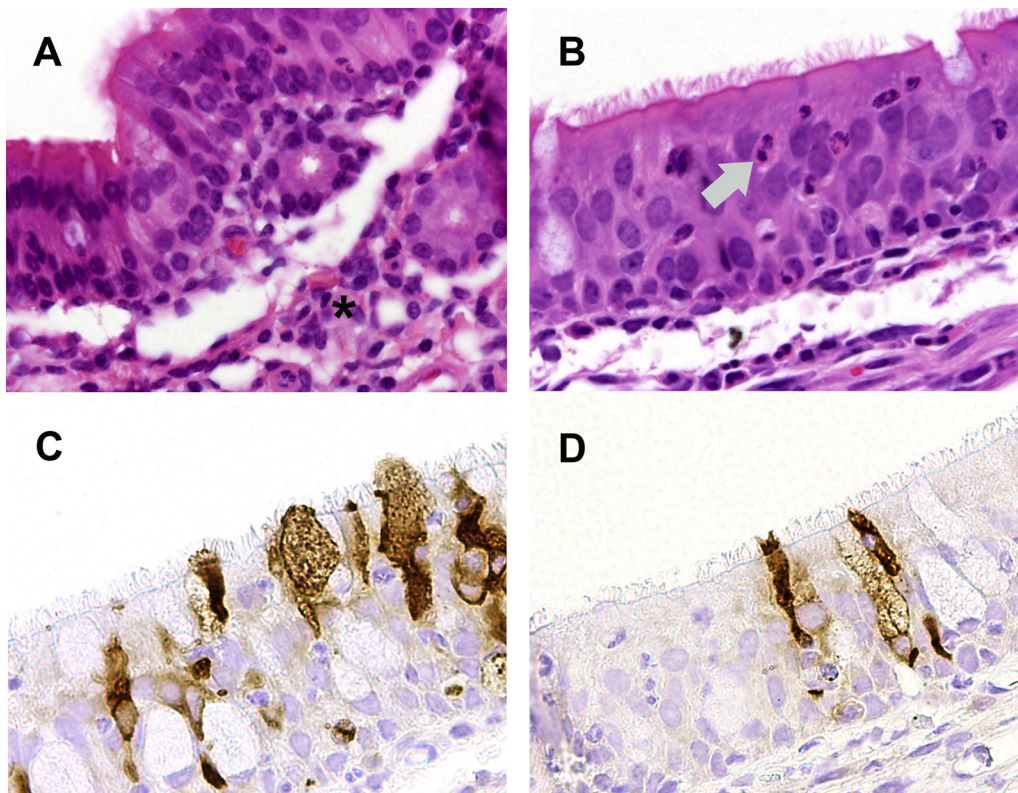


Fig. 1. Representative HE findings in respiratory epithelium of the nasal turbinates of MERS-CoV-infected alpaca and immunohistochemical reactions of the commercially available positive controls for comparison. (A) Multifocal infiltration of lamina propria and submucosa by moderate numbers of lymphocytes, macrophages, and single neutrophilic granulocytes (asterisk). (B) Exocytosis of single neutrophilic granulocytes (grey arrow). (C, D) Abundant viral antigen was detected multifocally in the cytoplasm and along the apical membranous region of epithelial cells using a monoclonal mouse (C, Sino Biological Inc.) and polyclonal rabbit anti-MERS-CoV nucleocapsid antibody (D, Sino Biological Inc.) with citrate pretreatment, in a dilution of 1:70 and 1:2,000, respectively. Both antibodies exhibited a similar staining intensity. (A, B) HE staining; 400x, (C, D) Avidin-biotin-peroxidase complex method with 3,3'-diaminobenzidine as chromogen; 400x.

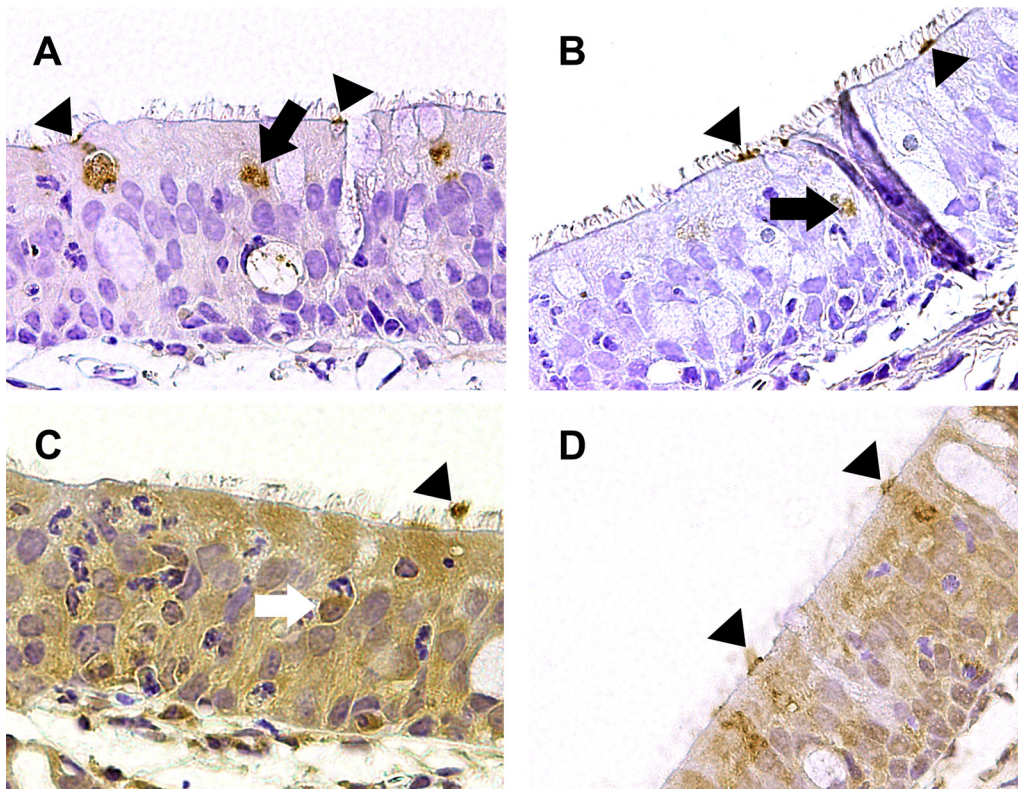


Fig. 2. Representative positive immunohistochemical reactions for human monoclonal antibodies. (A) Antibody 1.2g5 with citrate pretreatment exhibited a strong multifocal MERS-CoV antigen specific signal in the cytoplasm of ciliated respiratory epithelial cells (arrow) and segmentally along the apical membranous region (arrow heads). (B) The same protocol without pretreatment revealed a much fainter but similarly distributed staining of cytoplasm (arrow) and ciliary base (arrow heads). (C) Antibody 1.10f3 with citrate pretreatment displayed a strong diffuse background staining and only few positively stained cilia (arrowhead). Single intraepithelial plasma cells displayed a strong false positive intracytoplasmic staining (white arrow). (D) Antibody 1.6c7 with citrate pretreatment exhibited also a strong diffuse background staining and only few positive staining cilia (arrow heads). (A–D) Avidin-biotin-peroxidase complex method with 3,3'-diaminobenzidine as chromogen; 400x.

the antibodies was unrelated to the function of their target epitopes *in vivo* and that commercially available monoclonal and polyclonal anti-nucleocapsid antibodies generally appear more suitable to detect MERS-CoV antigen on FFPE sections in routine diagnostics. However, for certain purposes such as double or triple immunohistochemistry,

human mAbs might represent a promising alternative.

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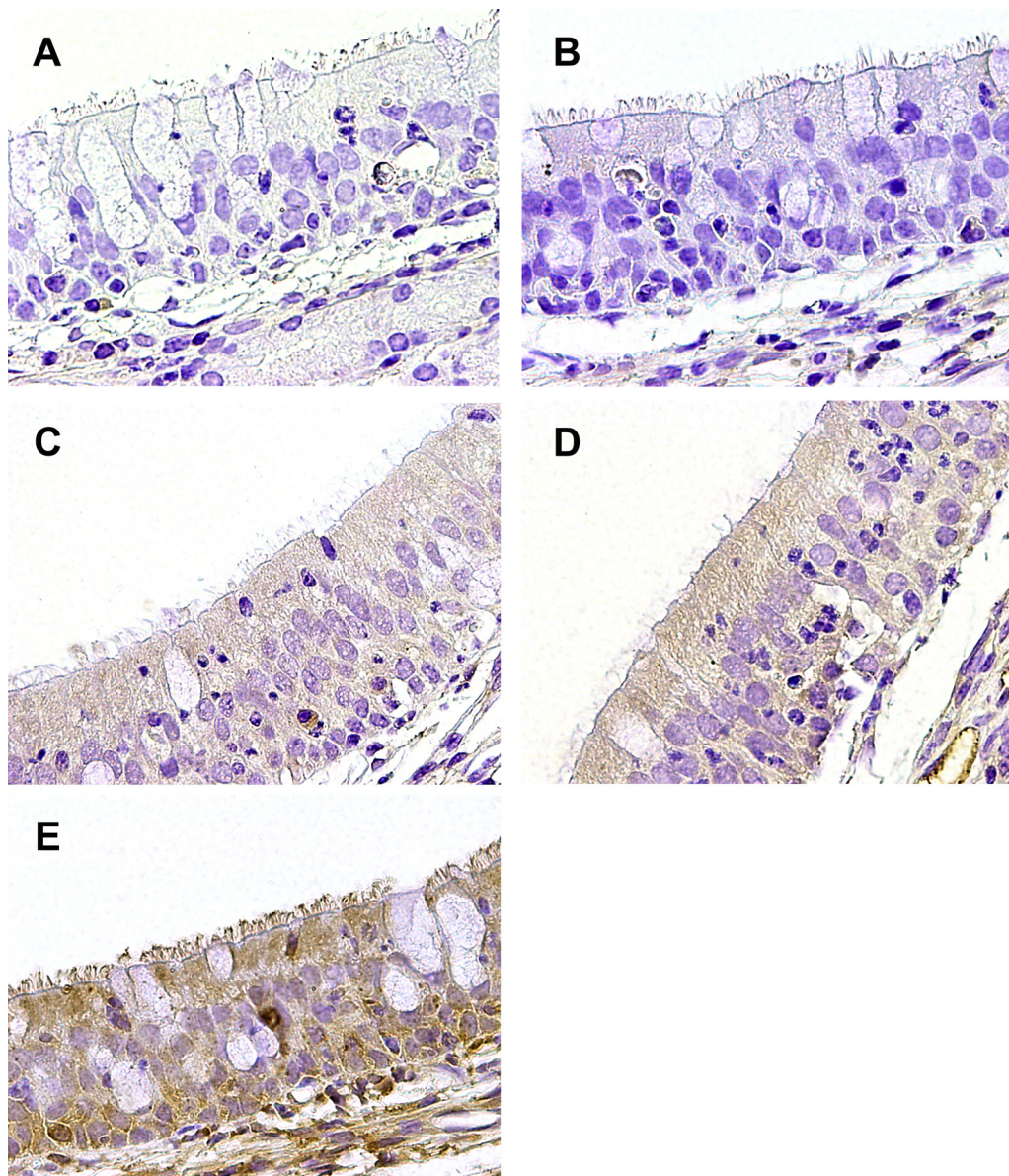


Fig. 3. Representative negative immunohistochemical reactions for human monoclonal antibodies. (A–E) Specific staining for MERS-CoV antigen was absent for the antibodies 1.6f9 (A), 4.6e10 (B), 7.7g6 (C), 1.8e5 (D), and 3.5g6 (E), respectively. Reactions were accompanied by mild to severe non-specific, intracytoplasmic background staining. (A–E) Avidin-biotin-peroxidase complex method with 3,3'-diaminobenzidine as chromogen; 400x.

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