Differential expression of the MERS-coronavirus receptor in the upper respiratory tract of humans and dromedary camels.

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Running title: DPP4 expression in camel and human respiratory tract.
Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) is not efficiently transmitted between humans, but it is highly prevalent in dromedary camels. Here we report that the MERS-CoV receptor - dipeptidyl peptidase 4 (DPP4) - is expressed in the upper respiratory tract epithelium of camels but not humans. Lack of DPP4 expression may be the primary cause of limited MERS-CoV replication in the human upper respiratory tract, hence restrict transmission.

Keywords: dipeptidyl peptidase-4 (DPP4), MERS-CoV, respiratory tract, humans, camels.
Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel coronavirus that causes pneumonia in humans, which may lead to acute respiratory distress syndrome (1). Currently more than 1500 confirmed cases have been reported with a relatively high case fatality rate. Although most MERS outbreaks have been reported in the Middle Eastern countries, travel-related cases may seed outbreaks in other regions, such as in South Korea (2). In principle, they can be controlled through implementation of early viral diagnostics, strict hygiene measures, and isolation of patients. However, there is still a lack of understanding how this virus is transmitted, both between humans and from camels to humans.

Dromedary camels are currently considered as the only zoonotic source of MERS-CoV. This is largely based on the fact that closely related viruses have only been isolated from this species thus far (3, 4). Although studies in the Middle East and several North Eastern African countries revealed a high percentage of serological positivity among dromedary camels (3, 5-8), there seems to be limited MERS-CoV transmission from camels-to-humans. Recent studies have shown that only 2-3% of persons in Saudi Arabia and Qatar that come into close contact with dromedary camels have neutralizing antibodies to MERS-CoV (5, 9). Additionally, most notified MERS patients to date did not report any contact with camels or other livestock animals, consistent with the fact that most outbreaks took place in hospitals (7, 8). On the other hand, studies in the hospital and household setting also reported a low percentage of confirmed MERS cases among patient contacts (10, 11). As a result, over a three year period the number of MERS cases is relatively low, providing evidence that MERS-CoV transmission to humans and between humans is relatively inefficient.
One factor considered to be critical for the transmission of MERS-CoV is the ability of the virus to replicate in the upper respiratory tract. Differences in viral shedding in dromedary camels and humans have been observed. Relatively high levels of infectious virus can be detected in nasal swabs of dromedaries infected with MERS-CoV, but not in MERS patients (12, 13). We hypothesized that a critical determinant of MERS-CoV replication in the respiratory tract of different hosts is the differential expression of the viral receptor. Dipeptidyl peptidase 4 (DPP4), a serine exopeptidase involved in various biological functions (14), has been shown to act as the functional MERS-CoV receptor (15). Although there is ample evidence that it is expressed in different tissues and cell types, including kidney, small intestines, and T lymphocytes (14, 16), its expression in the upper respiratory tract has not been investigated thus far. Here we addressed this gap of knowledge by analyzing the tissue localization of DPP4 along the human and dromedary camel respiratory tract.

We obtained 14 human respiratory tract and 3 human kidney formalin fixed paraffin embedded (FFPE) tissues from Erasmus MC Tissue Bank. These respiratory tract tissues were six nasal tissues (three superior and three inferior concha), two tracheas, three bronchi, and three lungs. These tissues were taken either from healthy donors or patients with non-malignant tumors. Kidney was used as positive control due to its abundant expression of DPP4 (14). These tissues were residual human biomaterials, which are collected, stored and issued by the Erasmus MC Tissue Bank under ISO 15189:2007 standard operating procedures. Use of these materials for research purposes is regulated according to the Human Tissue and Medical Research: code of conduct for responsible use (2011). Dromedary camel tissues were obtained from animals used in an experimental MERS-CoV infection (17). DPP4 immunohistochemistry staining was then performed on these FFPE tissue sections with 3 µm thickness. Antigen was retrieved by boiling
these sections in citric acid buffer 10.0 M pH 6 for 15 minutes using 600W microwave.

Endogenous peroxidase was blocked by incubating the slides with hydrogen peroxidase 3% for 10 minutes. DPP4 was detected using 5 µg/ml polyclonal goat IgG anti-human DPP4 antibody (R&D systems, Abingdon, UK), while negative controls were stained using normal goat serum (MP Biomedicals, Santa Ana, CA, USA) in equal concentration. This primary antibody staining was done overnight at 4°C. Secondary antibody staining was performed with peroxidase-labeled rabbit anti-goat IgG (DAKO, Glostrup, Denmark) in 1:200 dilution for 1 hour at room temperature. The sections were then treated with 3-amino-9-ethyl-carbazole (Sigma-Aldrich), counterstained with hematoxylin, and embedded in glycerol-gelatin (Merck, Darmstadt, Germany).

In the human respiratory tract tissues, DPP4 was detected in the lower part, i.e. alveolar epithelial cells and macrophages, but mostly type II alveolar epithelial cells (Fig. 1). In addition, DPP4 expression was also limitedly detected on the apical surface of the terminal bronchioles and bronchial epithelium of two lungs and one bronchus samples. In sharp contrast, DPP4 was not detected in the nasal respiratory and olfactory epithelium nor the trachea in any of our samples (Fig. 1). In the submucosal layer of these tissues, DPP4 was detected in the serous glandular epithelium, inflammatory cells, and vascular endothelium. In contrast to humans, DPP4 was detected in the ciliated epithelial cells of the upper respiratory tract epithelium of dromedary camels (Fig. 1). Additionally, it was also present in the ciliated epithelial cells of the tracheal and bronchial epithelium of these animals. However in the alveoli, it was mostly detected in the endothelial cells, and barely in the alveolar epithelial cells. Therefore, we conclude that there is a differential expression of DPP4 in the respiratory tract of humans and dromedary camels. The absence of DPP4 in the upper respiratory tract epithelium of humans
may limit MERS-CoV from replicating efficiently here. To confirm the localization of DPP4 expression, we performed in-situ hybridization to detect mRNA transcripts. Using the RNAscope® platform (18) with commercially available probes for DPP4, mRNA was detected in human submucosal glands, but not in the nasal epithelium of the nose (Fig. 2A and B). Probes for ubiquitin C and DapB (Advanced Cell Diagnostics, Hayward CA, USA) were used as positive and negative control, respectively. Ubiquitin C is a housekeeping gene abundantly present in human tissue, while Dap B is a bacterial gene that should not be present in healthy human tissue.

Alternatively, other yet unidentified MERS-CoV receptors may localize in the upper respiratory tract. To investigate the presence of such receptors, we performed immunohistochemistry staining using the spike S1 protein of MERS-CoV on frozen human tissue material. The spike protein is one of the structural proteins that forms the outer layer of the MERS-CoV particle and binds to DPP4 (15). By fusion of the MERS-CoV S1 protein to the mouse IgG2a Fc fragment (mFc-S1 MERS), binding of the S1 protein to cells or proteins in human tissue sections could be investigated. The S1 protein of coronavirus OC43 is used as a positive control, since this virus is commonly known to cause upper respiratory tract infection in humans (19). Meanwhile, as negative control we used S1 protein of porcine epidemic diarrhea virus (mFc-S1 PEDV) and mouse isotype antibodies (DAKO, Glostrup, Denmark). Additionally, immunohistochemistry with mouse monoclonal antibody against human DPP4 (MAb anti-DPP4) (Santa Cruz Biotechnology, Dallas, Texas, USA) was performed to further confirm the absence of the MERS-CoV receptor in the same nasal epithelium. Frozen human nose and kidney tissues for this experiment were also obtained from the Erasmus MC Tissue Bank and sections of 6 µm were cut. Kidney was again used as a DPP4 positive control. These sections were fixated in
acetone and incubated in room temperature for 1 hour with 1 µg/ml of either mFc-S1 MERS-CoV, mFc-S1 OC43, mFc-S1 PEDV, MAb anti-DPP4, or isotype mouse antibody. They were subsequently incubated with peroxidase-labeled goat anti-mouse IgG (DAKO, Glostrup, Denmark) in 1:100 dilution for 1 hour at room temperature and processed as described above. As shown, mFc-S1 OC43 bound to the surface of nasal epithelium, while mFc-S1 MERS and MAb anti-DPP4 did not. Similar to our results depicted in figure 1 and 2, mFc-S1 MERS-CoV and MAb anti-DPP4 bound to the nasal submucosal glands and kidney proximal tubuli. Meanwhile, our negative control, mFc-S1 PEDV and mouse isotype antibodies, did not show binding to either nasal or kidney tissues (Fig. 3). This result suggests that neither DPP4 nor any other alternative receptor is capable of binding spike protein of MERS-CoV in the upper respiratory tract epithelium of humans.

Here we report that the MERS-CoV receptor is expressed in the lower respiratory tract of humans but not in the upper respiratory tract epithelium. Similar results were recently reported by Meyerholz et al., using a different monoclonal antibody (20). Our results with respect to the localization of DPP4 in the human lower respiratory tract are consistent with earlier studies showing MERS-CoV tropism in the alveolar and bronchial epithelial cells of ex-vivo infected human lung tissues (21). The presence of the receptor at this location is also in line with clinical observations showing that MERS is considered in essence a lower respiratory tract infection and the fact that MERS-CoV RNA is detected at higher amounts in the tracheal aspirate and sputum samples of MERS patients than in the nasal or throat swabs (13, 22). The lack of DPP4 in the human upper respiratory tract epithelium may limit MERS-CoV infection and replication at this site, hence impede viral transmission. Expression of viral receptors in the upper respiratory tract epithelium has been shown to be critical in the transmission of viral infections, as exemplified by
respiratory infection caused by influenza viruses. Efficient airborne transmission of influenza
viruses between humans and ferrets requires binding to α2,6-sialic acid, which is highly
expressed in the upper respiratory tract. In contrast, influenza viruses which bind exclusively to
α2,3-sialic acid, that mostly expressed in the lower respiratory tract, are less likely to transmit
(23).

Although there is limited DPP4 expression in the human upper respiratory tract
epithelium we observed expression of the MERS-CoV receptor in glands located in the
submucosa of the upper respiratory tract. These glands have been shown to be targeted by other
coronaviruses, such as SARS-CoV and rat sialodacryoadenitis virus (24, 25). We therefore
cannot exclude that MERS-CoV may replicate in submucosal glands that are connected to the
respiratory epithelium by their secretory ducts. It remains to be investigated whether viral
replication in patients that have been shown to shed MERS-CoV for long period of time, could
be linked to the presence of virus at these locations. The susceptibility of these cells and their
capacity to support MERS-CoV replication needs to be investigated in future studies.

Although DPP4 is not expressed in the human upper respiratory tract epithelium in the
tissues analyzed in this study, it remains possible that the expression pattern could depend on
several factors. DPP4 expression in the lower respiratory tract seemed to vary between
individuals and as shown by previous studies in T lymphocytes, DPP4 is not stably expressed on
the cell surface but can be upregulated upon activation (16). Interestingly, one study
demonstrated that cultured primary human nasal epithelial cells expressed DPP4 (26), which
likely reflects upregulated expression as a result of cell division, as also observed in different cell
lines (27). Whether DPP4 expression in the respiratory tract tissues is regulated by certain host or
environmental factors remains to be studied. In general, our study highlights a critical difference
in the distribution of DPP4 expression between species, humans and camels. Future studies should investigate this DPP4 distribution in other species, which would be relevant to further understand the transmission of MERS-CoV.
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References


**Figure 1.** DPP4 expression in the upper respiratory tract of camels and humans. DPP4 immunohistochemistry staining was performed on human and dromedary camel respiratory tissues; kidney was used as the positive control. (Nose, trachea, bronchus, and kidney at a 200X magnification; Bronchiole, terminal bronchiole, and alveoli at a 400X magnification). Positive staining is revealed in red.

**Figure 2.** Presence of DPP4 mRNA and protein in the human nasal epithelium and the submucosal glandular epithelial cells. (A) DPP4 mRNA was detected in the kidney, but not in the nasal epithelium (200X magnification). (B) DPP4 mRNA (arrows) and protein were detected in the submucosal gland cells with in-situ hybridization (ISH) technique and immunohistochemistry (IHC), respectively (400x magnification). Positive signal in ISH was marked by red dots. Kidney was used as positive control both for ISH and IHC. For ISH, Ubiquitin C and DapB mRNA were used as positive and negative controls, respectively.

**Figure 3.** S1 protein of MERS-CoV (mFc-S1 MERS) binds to human kidney proximal tubuli and nasal submucosal glands but not nasal epithelium. Mouse monoclonal antibody against human DPP4 (MAb anti-DPP4) showed similar binding as mFc-S1 MERS-CoV. S1 protein of OC43 (mFc-S1 OC43) binds to the nasal epithelium (indicated by arrows) and was used as a positive control. As a negative control, S1 protein of porcine epidemic diarrhea virus (mFc-S1 PEDV) and mouse isotype antibodies (mouse IgG2a and IgG2b) were used. The figure for the
mouse isotype antibody is representation for the two isotype antibodies used in the experiment.

Positive staining is revealed in red. All figures were made at a 200X magnification.