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Spike proteins of novel MERS-coronavirus isolates from North- and West-African dromedary camels mediate robust viral entry into human target cells

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1. Introduction

The highly pathogenic Middle East respiratory syndrome (MERS)-related coronavirus (CoV) is transmitted from dromedary camels, the natural reservoir, to humans. For at present unclear reasons, MERS cases have so far only been observed in the Arabian Peninsula, although MERS-CoV also circulates in African dromedary camels. A recent study showed that MERS-CoV found in North/West-Morocco and West-African (Burkina Faso and Nigeria) dromedary camels are genetically distinct from Arabian viruses and have reduced replicative capacity in human cells, potentially due to amino acid changes in one or more viral proteins. Here, we show that the spike (S) proteins of the prototypic Arabian MERS-CoV strain, human betacoronavirus 2c EMC/2012, and the above stated African MERS-CoV variants do not appreciably differ in expression, DPP4 binding and ability to drive entry into target cells. Thus, virus-host-interactions at the entry stage may not limit spread of North- and West-African MERS-CoV in human cells.
known to adequately model key aspects of the coronavirus entry process. In order to study host cell entry driven by S proteins from the C1 subclade, we employed PCR-based mutagenesis to generate expression constructs for the S proteins of MERS-CoV from Morocco (camel/Morocco/CIRAD-HKU213/2015, MO), Nigeria (camel/Nigeria/NV1657/2016, NI) and Burkina Faso (camel/Burkina Faso/CIRAD-HKU785/2015, BF), using a published expression construct for MERS-CoV EMC S protein as template (Kleine-Weber et al., 2018, 2019). Moreover, expression constructs for all S proteins were generated that encoded a C-terminal V5 antigenic tag. Western blot analysis of cells transfected to express the S proteins under study revealed that MERS-S EMC, MO, NI and BF were expressed and proteolytically processed to comparable levels (Fig. 1B). Moreover, these S proteins were incorporated into VSV particles with similar efficiency (Fig. 1C). These results suggest that mutations present in North- and West-African MERS-S of the C1 subclade do not reduce S protein expression and proteolytic processing in human cells.

We next asked whether DPP4 binding of North- and West-African MERS-S was altered. For this, 293T cells transfected to express the S proteins under study were incubated with soluble DPP4 fused to the Fc portion of human immunoglobulin and binding was quantified by flow cytometry, as described previously (Kleine-Weber et al., 2019). The results showed that MERS-S EMC, MO, NI, and BF bound to DPP4 robustly and with comparable efficiency while DPP4 binding to cells expressing no S protein was within the background range (Fig. 2). Finally, we tested whether the robust binding to DPP4 translated into efficient S protein-driven entry. For this, cell lines were selected that were shown to express low levels (293T), intermediate levels (Vero 76) or high levels of DPP4 (Caco-2, 293T + DPP4) (Kleine-Weber et al., 2019). MERS-S MO, NI and BF mediated entry into all cell lines with at least the same efficiency as MERS-S EMC (Fig. 3), although these differences were not statistically significant.

3. Discussion

Our results show that amino acid substitutions present in North- and West-African MERS-S proteins relative to MERS-S EMC do not compromise S protein expression in human cells, at least when transfected cells are examined. Similarly, proteolytic processing of the S proteins in the constitutive secretory pathway, which is known to be carried out by furin (Gierer et al., 2015; Millet and Whittaker, 2014), was not
protein was analyzed by conjugated anti-human antibody, before DPP4 binding to the respective S soluble DPP4 containing a C-terminal Fc tag (sol-DPP4-Fc) and AlexaFluor488-S proteins or no S protein at all (Control) were successively incubated with the S2 unit; S2 = S2 subunit; RBD = receptor binding domain, PS(S2)′ = priming site at the S2′ position (884-RSAR-887), n/a = not applicable.

### Table 1

| S protein | Variation | Localization |
|-----------|-----------|--------------|
| MERS-S MO camel/Morocco/CIRAD-HKU213/2015 GenBank: MG923469.1 | V26A | S1 / n/a |
| A89S | S1 / n/a |
| T424I | S1 / RBD |
| S856Y | S2 / n/a |
| R884L | S2 / PS(S2)′ |
| A1158S | S2 / n/a |
| V1209L | S2 / n/a |
| MERS-S NI camel/Nigeria/NV1657/2016 GenBank: MG923475.1 | V26A | S1 / n/a |
| H167Y | S1 / n/a |
| H194Y | S1 / n/a |
| L495F | S1 / RBD |
| L588F | S1 / RBD |
| S856Y | S2 / n/a |
| A1158L | S2 / n/a |
| L1200F | S2 / n/a |
| MERS-S BF camel/Burkina Faso/CIRAD-HKU785/2015 GenBank: MG923471.1 | V26A | S1 / n/a |
| A89S | S1 / n/a |
| H194Y | S1 / n/a |
| T424I | S1 / RBD |
| S856Y | S2 / n/a |
| A1158S | S2 / n/a |

3. Results

3.1. Binding of North- and West-African MERS-CoV S proteins to DPP4

MERS-CoV and its S protein have been shown to mediate human infection and cell entry (Lu et al., 2013). In keeping with these observations, all African S proteins mediated robust viral entry into non-human primate (Vero 76) and human cell lines (293T, Caco-2) expressing different levels of DPP4 (Kleine-Weber et al., 2019). In fact, MERS-S MO- and BF-driven entry into cell lines expressing low or intermediate levels of DPP4 was augmented as compared to MERS-S EMC, in keeping with these S proteins showing slightly enhanced DPP4 binding as compared to MERS-S EMC. Finally, it is noteworthy that MERS-S activation in Caco-2 cells mainly depends on the cellular serine protease TMPRSS2 while activation in 293T and Vero 76 cells is mediated by the cellular cysteine protease cathepsin L (Kleine-Weber et al., 2018, 2019). Thus, North- and West-African MERS-S proteins seem to be able to use both pathways available for S protein activation in human cells.

Fig. 2. S proteins of North/West- and West-African dromedary camels isofluently bind to DPP4. 293T cells expressing the indicated S proteins or no S protein at all (Control) were successively incubated with soluble DPP4 containing a C-terminal Fc tag (sol-DPP4-Fc) and AlexaFluor488-conjugated anti-human antibody, before DPP4 binding to the respective S protein was analyzed by flow cytometry. Presented are the combined data of three independent experiments for which sol-DPP4-Fc binding to MERS-S EMC was set as 100%. Error bars indicate the standard error of the mean (SEM). Statistical significance was tested by one-way analysis of variance with Sidak’s posttest (p > 0.05, not significant, ns; p ≤ 0.01, **).

Collectively, our results suggest that amino acid substitutions present in the S proteins of North- and West-African MERS-CoV do not compromise the ability of these viruses to enter human cells. Thus, future efforts to understand why North- and West-African MERS-CoV isolates show reduced replicative potential in human cells should be focused on other aspects of the MERS-CoV lifecycle than S protein-mediated host cell entry.

4. Materials and methods

4.1. Plasmids

Expression plasmids, based on the vector pCAGGS, for VSV-G and MERS-S EMC were previously described (Kleine-Weber et al., 2018, 2019). The MERS-S EMC plasmid was used as template for PCR-based mutagenesis to introduce the mutations found in MERS-S MO (Morocco, camel/Morocco/CIRAD-HKU213/2015, GenBank: MG923469.1), NI (Nigeria, camel/Nigeria/NV1657/2016, GenBank: MG923475.1) and BF (Burkina Faso, camel/Burkina Faso/CIRAD-HKU785/2015, GenBank: MG923471.1) (Table 1). In addition, PCR-based mutagenesis was used to equip the constructs with a C-terminal V5 antigenic tag. The integrity of all sequences was verified using automated sequence analysis.

4.2. Cell culture

293T (human embryonal kidney) and Vero 76 (African green monkey kidney) cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; PAN Biotech). The human colorectal adenocarcinoma cell line Caco-2 was grown in Minimum Essential Media (MEM, Life Technologies). All media were supplemented with 10% fetal bovine serum (FBS, PAN Biotech) and 1x penicillin and streptomycin from a 100x stock solution (PAN Biotech). The cells were incubated under humid conditions at 37°C and 5% CO₂. For transfection of 293T cells the calcium-phosphate precipitation method was used.
4.3. Antibodies and DPP4-Fc fusion protein

For Western blot analysis, anti-V5 (mouse, 1:2,500; ThermoFisher Scientific), anti-β-actin (mouse, 1:2,500; Sigma-Aldrich), anti-VSV-M (mouse, 1:2,500; Kerafast) were used as primary antibodies and anti-mouse HRP (horse radish peroxidase) conjugated antibody (goat, 1:2,500; Dianova) was used as secondary antibody. Antibodies were diluted in phosphate buffered saline [PBS] containing 0.5% Tween 20 [PBS-T] supplemented with 5% skim milk powder. For flow cytometry, a recombinant fusion protein of the ectodomain of DPP4 fused to the Fc fragment of human immunoglobulin (sol-DPP4-Fc, 1:200, ACROBiosystems) and an AlexaFlour488-conjugated anti-human antibody (goat, 1:500; ThermoFisher Scientific) were used (ligand and antibody were diluted in PBS containing 1% bovine serum albumin).

4.4. Immunoblot analysis of MERS-S expression and particle incorporation

For analysis of S protein expression, 293T cells were transfected with expression plasmid for MERS-S proteins harboring a C-terminal V5 tag, as described (Kleine-Weber et al., 2018, 2019). To investigate MERS-S incorporation into VSVpp, equal volumes of supernatants containing VSVpp bearing S proteins with V5 tag were centrifuged through a 20% sucrose cushion at 25,000 g for 120 min. Subsequently, cells and VSVpp pellets were lysed and analyzed by immunoblot, following an established protocol (Kleine-Weber et al., 2018, 2019).

4.5. Analysis of DPP4 binding efficiency

DPP4 binding was analyzed as described (Kleine-Weber et al., 2019). In brief, 293T cells were transfected with expression plasmids for MERS-S proteins and empty plasmid as negative control. At 48 h posttransfection, the cells were washed with PBS, pelleted and resuspended in PBS containing 1% BSA and soluble human DPP4-Fc fusion protein at a final dilution of 1:200. After incubation for 1 h at 4°C, the cells were washed and incubated with AlexaFlour488-conjugated anti-mouse antibody at a dilution of 1:500. Finally, the cells were fixed with 4% paraformaldehyde and analyzed by flow cytometry using an LSR II flow cytometer and the FACS Diva software (both BD Biosciences).
4.6. Production of VSV pseudovirions (VSVpp) and target cells

Transduction vectors based on a replication-deficient VSV (Berger Rentsch and Zimmer, 2011) and pseudotyped with the indicated viral glycoproteins (VSVpp) were generated according to a published protocol (Kleine-Weber et al., 2018, 2019). Target cells were transduced with equal volumes of supernatants containing VSVpp and transduction efficiency was quantified at 16 h posttransduction by measuring the activity of virus-encoded firefly luciferase in cell lysates as previously described (Kleine-Weber et al., 2018, 2019).

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References

Ali, M., El-Shesheny, R., Kandeil, A., Shehata, M., Elsokary, B., Gomaa, M., Hassan, N., El, 2011. A vesicular stomatitis virus replicon-based cell line. N. Engl. J. Med. 367, 1814–1823.

Kleine-Weber, H., Elzayat, M.T., Wang, L., Graham, B.S., Muth, D., Muth, A., 2014. Antibodies against MERS coronavirus in young dromedary camels in Jordan. Vector Borne Zoonotic Dis. 17, 1049–1053.

Drukker, I., Fassi-Fihri, O., Faye, B., Fekadu, G., Grosbois, V., Ng, B.C., Peiris, R.A., So, Y.T., Tawee, A., Roger, F., Peiris, M., 2017. Risk factors for MERS coronavirus infection in dromedary camels in Burkina Faso, Ethiopia, and Morocco. Euro Surveill. 22.

Millet, J.K., Whittaker, G.R., 2014. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. Proc. Natl. Acad. Sci. U. S. A. 111, 15214–15219.

Murray, P., Corman, V.M., Bieb, A., Osero, M., Be, B., Muth, D., Muth, A., Lattwein, E., Bosch, B.J., Huang, D., Raj, V.S., Mou, H., Smits-De Vries, L., Corman, V.M., Drexler, J.F., Smits, S.L., El Tahir, Y.E., De Souza, R., van Beek, J., Nowoyn, N., van Maanen, K., Hidalgo-Hermoso, E., Perera, R.A., Rottier, P., Osterhaus, A., Gortazar-Schmidt, C., Drosten, C., Koopmans, M.P., 2013. Middle East respiratory syndrome coronavirus neutralising serum antibodies reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro Surveill. 18 pii=20574.

Raj, V.S., Mos, H., Smits, S.L., Dekkers, D.H., Muth, M., Dijkman, R., Pöhlmann, S., 2018. Lack of serological evidence of Middle East respiratory syndrome coronavirus (MERS-CoV) and widespread seroprevalence of potential cleavage sites in the MERS-coronavirus spike protein. Sci. Rep. 8, 16597.

Kleine-Weber, H., Elzayat, M.T., Graham, B.S., Muth, D., Muth, A., 2014. Middle East respiratory syndrome coronavirus infections. N. Engl. J. Med. 369, 1761–1762.

Millet, J.K., Whittaker, G.R., 2014. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. Proc. Natl. Acad. Sci. U. S. A. 111, 15214–15219.

Murray, P., Corman, V.M., Bieb, A., Osero, M., Be, B., Muth, D., Muth, A., Lattwein, E., Bosch, B.J., Huang, D., Raj, V.S., Mou, H., Smits-De Vries, L., Corman, V.M., Drexler, J.F., Smits, S.L., El Tahir, Y.E., De Souza, R., van Beek, J., Nowoyn, N., van Maanen, K., Hidalgo-Hermoso, E., Perera, R.A., Rottier, P., Osterhaus, A., Gortazar-Schmidt, C., Drosten, C., Koopmans, M.P., 2013. Middle East respiratory syndrome coronavirus neutralising serum antibodies reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro Surveill. 18 pii=20574.

Raj, V.S., Mos, H., Smits, S.L., Dekkers, D.H., Muth, M., Dijkman, R., Pöhlmann, S., 2018. Lack of serological evidence of Middle East respiratory syndrome coronavirus (MERS-CoV) and widespread seroprevalence of potential cleavage sites in the MERS-coronavirus spike protein. Sci. Rep. 8, 16597.