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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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ABSTRACT

The highly pathogenic Middle East respiratory syndrome (MERS)-related coronavirus 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.

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1. Introduction

The Middle East respiratory syndrome-related coronavirus (MERS-CoV) causes the severe lung disease MERS (Zaki et al., 2012), which takes a fatal course in roughly ~35 % of infected patients (WHO, 2019). MERS-CoV is endemic in the Middle East, where the virus is transmitted from dromedary camels, the natural reservoir, to humans (Perera et al., 2013; Reusken et al., 2013). Human-to-human transmission is inefficient but resulted in several hospital outbreaks of MERS (Assiri et al., 2013; Harriman et al., 2013; Memish et al., 2013), and there is concern that the virus may adapt to humans and cause a pandemic.

Infection of dromedary camels with MERS-CoV is not limited to the Middle East. African camels are frequently infected with MERS-CoV (Ali et al., 2017a; Ali et al., 2017b; Chu et al., 2015; Chu et al., 2014; Chu et al., 2018; Corman et al., 2014; Deem et al., 2015; Kiambi et al., 2018; Miguel et al., 2017; Ommeh et al., 2018; Perera et al., 2013; Reusken et al., 2013; Reusken et al., 2014; van Doremalen et al., 2017) and the responsible viruses are genetically distinct from those circulating in the Middle East (Chu et al., 2018; Kiambi et al., 2018; Ommeh et al., 2018). Moreover, viruses isolated from animals in Morocco, Nigeria and Burkina Faso form a distinct phylogenetic subclade, C1, and exhibit reduced ability to replicate in human respiratory cells (Chu et al., 2018). In addition, MERS-CoV transmission from camels to humans has not been observed in North- and West-Africa (Munyua et al., 2017; So et al., 2018), although two livestock handlers in Kenya were shown to harbor antibodies against MERS-CoV (Liljander et al., 2016), Moreover, no MERS cases were documented in Africa. At present, the barrier(s) impeding efficient spread of African MERS-CoV in human cells and camel-human transmission of these viruses remain to be identified.

The MERS-CoV spike protein (S) is incorporated into the viral envelope and facilitates viral entry into target cells (Li, 2016). For this, the S protein binds to the cellular receptor dipeptidyl peptidase 4 (DPP4, CD26) (Raj et al., 2013) via its surface unit, S1, and

fuses the viral membrane with a target cell membrane via its transmembrane unit, S2 (Li, 2016). Binding of MERS-S to DPP4 is essential for MERS-CoV infection of cells and DPP4 expression and the S protein/DPP4 interface are major determinants of MERS-CoV cell and species tropism (Raj et al., 2013; van Doremalen et al., 2014). The S proteins of North- and West-African MERS-CoV of the C1 clade harbor 6-9 amino acid substitutions relative to MERS-CoV (Fig. 1A, Table 1) and these substitutions might reduce S protein-driven entry into target cells. However, this possibility has not been examined so far.

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2. Results

We employed a previously described vesicular stomatitis virus (VSV)-based pseudotyping system to study MERS-S-driven host cell entry (Kleine-Weber et al., 2018; Kleine-Weber et al., 2019). Pseudotyping systems are 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 Nigeria (camel/Nigeria/NV1657/2016, NI), Morocco (camel/Morocco/CIRAD-HKU213/2015, MO) 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; Kleine-Weber et al., 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 NI, MO and BF mediated entry into all cell lines with at least the same efficiency as MERS-S EMC (Fig. 3). Moreover, under conditions of low or medium DPP4 expression, entry mediated by MERS-S MO and BF was even more efficient than entry mediated by MERS-S EMC (Fig. 3), although these differences were not statistically significant.

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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 appreciably altered. Moreover, binding of North- and West-African S proteins to DPP4 was not diminished as compared to MERS-S EMC, despite the presence of at least one substitution in the receptor binding domain (RBD) in each S protein tested. This finding might not be unexpected since the substituted amino acid residues do not make direct contact with residues in DPP4 (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; Kleine-Weber et al., 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.

Confirmation of our findings with authentic viruses is pending and we cannot exclude that, for instance, the S protein modulates recognition of the virus by sensors of the interferon system, which cannot be measured with the assays available to us. Moreover, we note that a recent study examining two MERS-S sequences (C2 clade) from camels in Ethiopia demonstrated that these sequences, when inserted into MERS-CoV EMC, reduced viral entry

and replication and increased sensitivity to antibody-mediated neutralization (Shirato et al., 2019). The reduction in entry was observed for Vero and to a lesser degree for Vero-TMPRSS2 cells and was generally modest. Nevertheless, these results suggest that S proteins from viruses circulating in Ethiopia might harbor mutations that diminish entry into human cells and that are not present in the MERS-S proteins studied here. Amino acid residues I139, L515, E851 and S1302 in the spike protein are unique to Ethiopian MERS-CoV and warrant further analysis.

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.

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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; Kleine-Weber et al., 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), BF (Burkina Faso, camel/Burkina Faso/CIRAD-HKU785/2015, GenBank: MG923471.1) and NI (Nigeria, camel/Nigeria/NV1657/2016, GenBank: MG923475.1) (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% CO_2. 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-ß-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; Kleine-Weber et al., 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; Kleine-Weber et al., 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 an incubation period for 1 h at 4 °C, the cells were washed and incubated with AlexaFluor488-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 pseudoparticles (VSVpp) and transduction of 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; Kleine-Weber et al., 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; Kleine-Weber et al., 2019).

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446 **Figure legends**

447

448 **Fig. 1.** S proteins of North/West- and West-African MERS-CoV isolates from dromedary
449 camels are robustly expressed in human cells and efficiently incorporated into viral particles.
450 (A) Domain organization of the MERS-CoV S proteins studied here. Black arrowheads
451 indicate amino acid variations found in S proteins of African viruses as compared to the S
452 protein of the prototypic Arabian MERS-CoV strain, human betacoronavirus 2c EMC/2012
453 (GenBank: JX869059.2). Abbreviations: SP = signal peptide, RBD = receptor binding
454 domain, FP = fusion peptide, HR1/HR2 = heptad repeat 1/2, TD = transmembrane domain.
455 (B) The indicated S proteins were transiently expressed in 293T cells, whole cell lysates were
456 prepared at 48 h posttransfection and S protein expression was analyzed via Western blot,
457 using an antibody targeting the C-terminal V5-tag. Cells expressing no S protein at all were
458 used as negative control and detection of β-actin (ACTB) served as loading control. Similar
459 results were obtained in two separate experiments. (C) Rhabdoviral transduction vectors
460 (VSVpp) harboring the indicated S proteins were concentrated by centrifugation and,
461 following lysis, analyzed by Western blot for S protein incorporation, using an antibody
462 targeting the C-terminal V5-tag. Transduction vectors harboring no S protein were used as
463 negative controls and detection of vesicular stomatitis virus matrix protein (VSV-M) served
464 as loading control. Similar results were obtained in a separate experiment. Numbers on the
465 left side of each blot indicate the molecular weight in kilodalton (kDa). Further, bands
466 representing the precursor S protein (S0, black circle) and the S2 subunit of proteolytically
467 processed S protein (grey circle) are indicated.

468

469 **Fig. 2.** S proteins of North/West- and West-African MERS-CoV isolates from dromedary
470 camels efficiently bind to DPP4. 293T cells expressing the indicated S proteins or no S

471 protein at all (Control) were successively incubated with soluble DPP4 containing a C-
472 terminal Fc tag (sol-DPP4-Fc) and AlexaFluor488-conjugated anti-human antibody, before
473 DPP4 binding to the respective S protein was analyzed by flow cytometry. Presented are the
474 combined data of three independent experiments for which sol-DPP4-Fc binding to MERS-S
475 EMC was set as 100 %. Error bars indicate the standard error of the mean (SEM). Statistical
476 significance was tested by one-way analysis of variance with Sidak's posttest ($p > 0.05$, not
477 significant, ns; $p \leq 0.01$, **).
478
479 **Fig. 3.** Host cell entry driven by the S proteins of North/West- and West-African MERS-CoV
480 isolates from dromedary camels is robust. 293T, 293T transfected to express DPP4, Vero 76
481 and Caco-2 cells were inoculated with equal volumes of rhabdoviral transduction vectors
482 harboring the indicated S proteins or no S protein at all (Control). At 18 h posttransduction,
483 the activity of the virus-encoded luciferase, which served as an indicator for transduction
484 efficiency, was measured in cell lysates. Presented are the combined data of three independent
485 experiments for which transduction mediated by MERS-S EMC was set as 100 %. Error bars
486 indicate SEM. Statistical significance was tested by one-way analysis of variance (ANOVA)
487 with Sidak's posttest ($p > 0.05$, ns; $p \leq 0.01$, **; $p \leq 0.005$, ***).
488
489

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Table 1

Amino acid variations between MERS-S EMC and the S proteins of MERS-CoV of North/West- and West-African dromedary camels.

S protein	Variation[1]	Localization[2]
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

[1]Amino acid position (numbering according to MERS-S EMC), [2]Subunit / Functional domain (if applicable); Abbreviations: S1 = S1 subunit; S2 = S2 subunit; RBD = receptor binding domain, PS(S2') = priming site at the S2' position (884-RSAR-887), n/a = not applicable

Figure 1 Kleine-Weber *et al.*, 2019

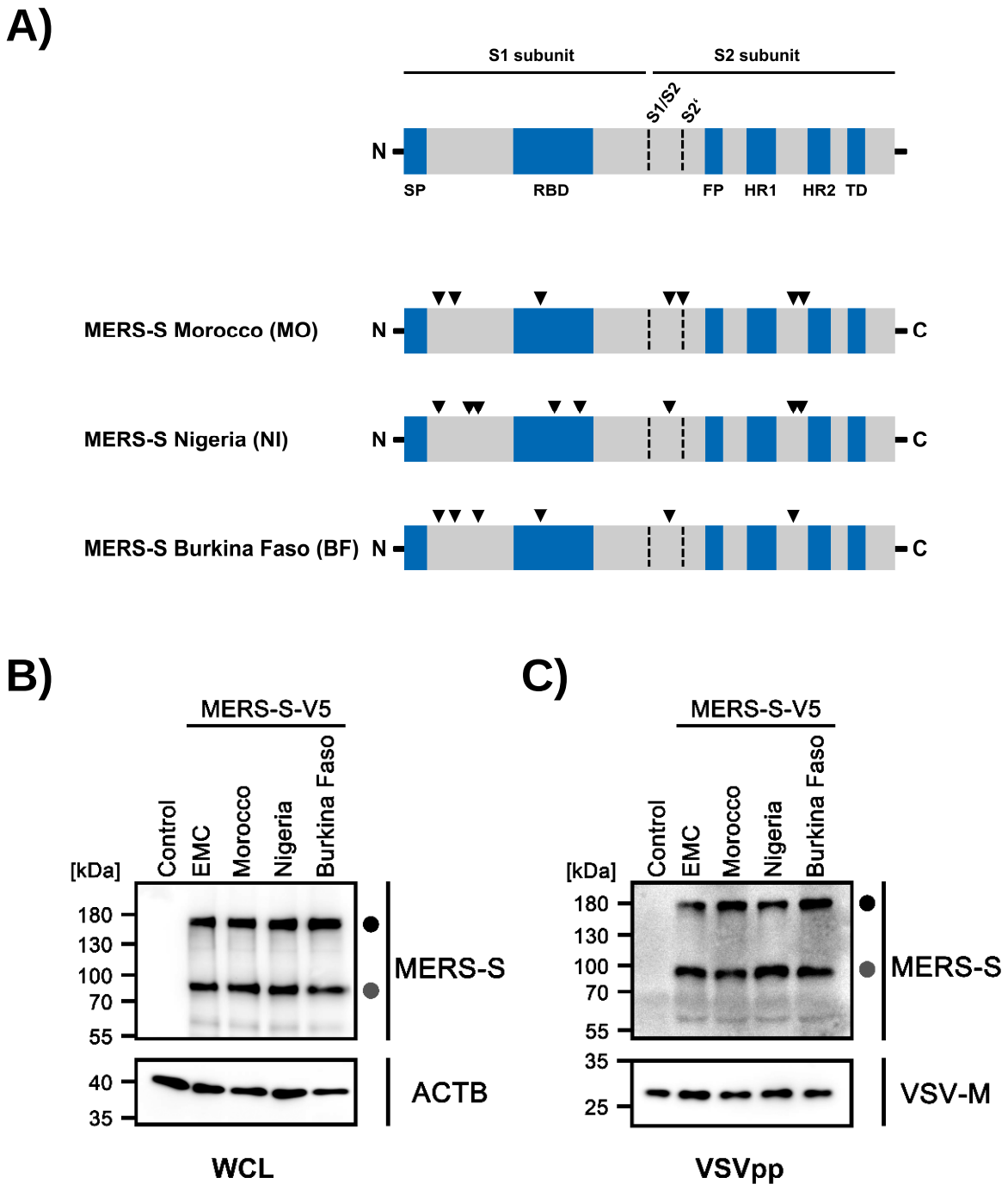


Figure 2

Kleine-Weber *et al.*, 2019

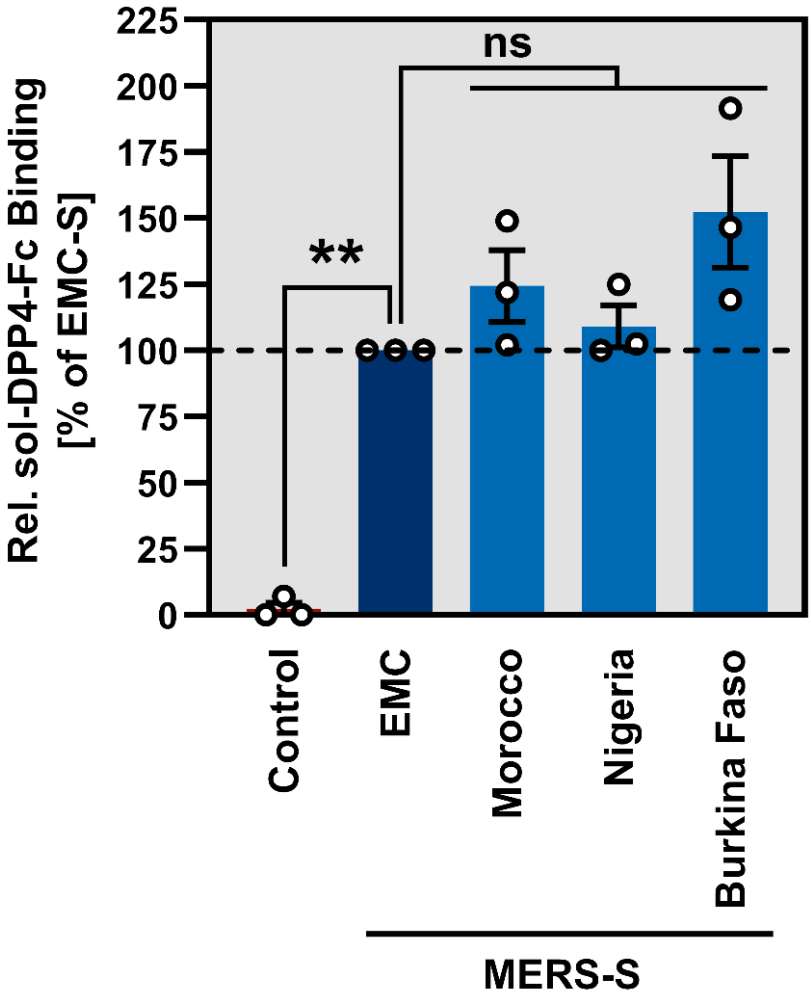
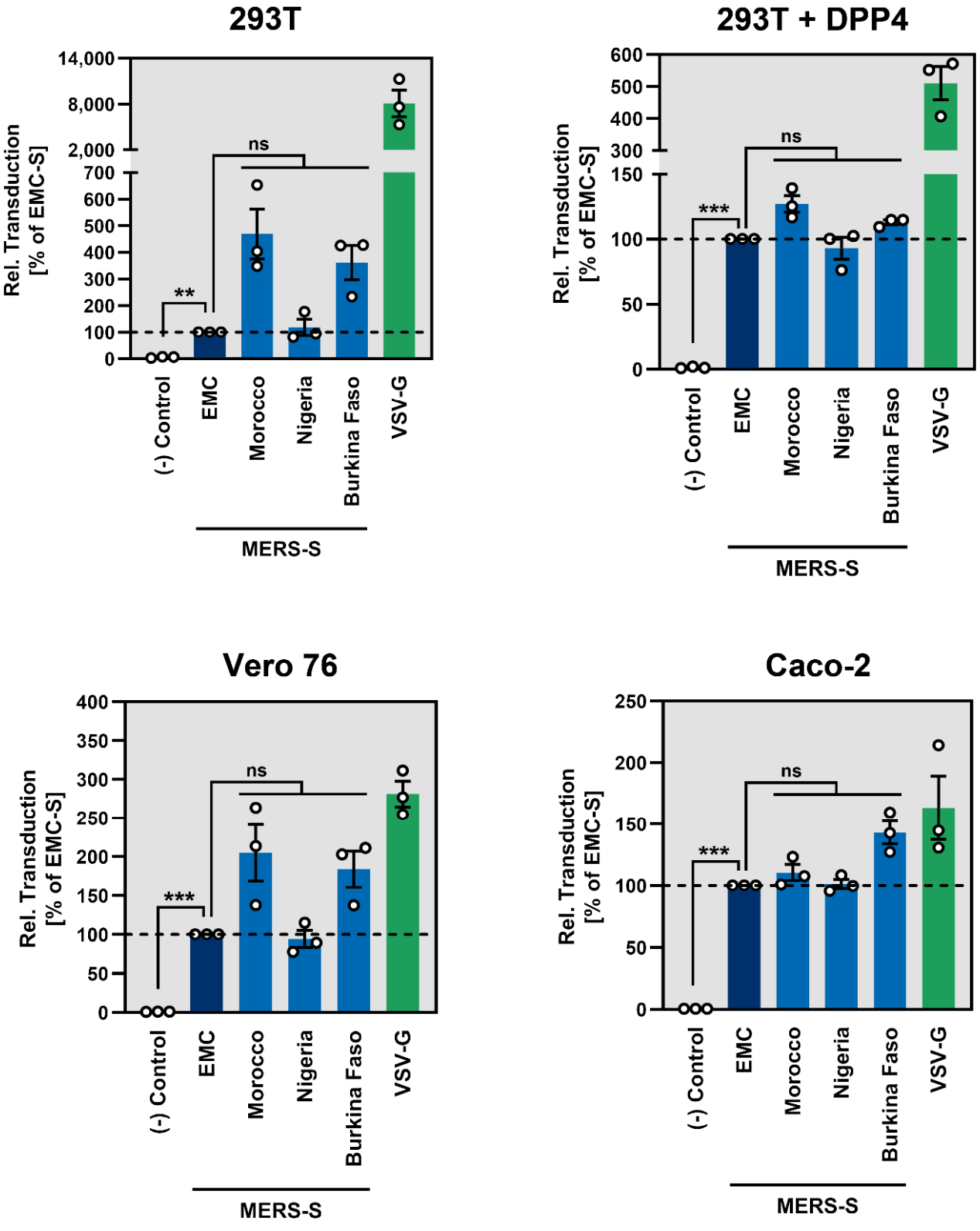


Figure 3 Kleine-Weber *et al.*, 2019



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Highlights:

- Spikes of MERS-CoV from African camels bind efficiently to human DPP4
- Spikes of MERS-CoV from African camels mediate efficient entry into human cells

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