

SHORT REPORT

Open Access



# Evaluation of alpaca tracheal explants as an ex vivo model for the study of Middle East respiratory syndrome coronavirus (MERS-CoV) infection

Nigeer Te<sup>1,2,4†</sup>, Jordi Rodon<sup>1,2†</sup>, Rhea Creve<sup>1,2</sup>, Mónica Pérez<sup>1,2</sup>, Joaquim Segalés<sup>1,3</sup>, Júlia Vergara-Alert<sup>1,2\*</sup>  and Albert Bensaid<sup>1,2</sup>

## Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) poses a serious threat to public health. Here, we established an ex vivo alpaca tracheal explant (ATE) model using an air-liquid interface culture system to gain insights into MERS-CoV infection in the camelid lower respiratory tract. ATE can be infected by MERS-CoV, being  $10^3$  TCID<sub>50</sub>/mL the minimum viral dosage required to establish a productive infection. IFNs and antiviral ISGs were not induced in ATE cultures in response to MERS-CoV infection, strongly suggesting that ISGs expression observed in vivo is rather a consequence of the IFN induction occurring in the nasal mucosa of camelids.

**Keywords:** Air-liquid interface, alpaca, camelid, ex vivo model, MERS-CoV, tracheal explants

## Introduction, methods, and results

Middle East respiratory syndrome coronavirus (MERS-CoV) is the etiological agent causing a respiratory disease that emerged in The Kingdom of Saudi Arabia in 2012 [1]. Early reports from the Arabian Peninsula indicated high case-fatality rates associated with the disease [2–4]. As of April 2022, the World Health Organization has reported 2585 infections and 890 fatalities (~34.4% case-fatality rate) globally in 27 countries across four continents [5]. The primary manifestation of MERS in humans ranges from asymptomatic or mild respiratory symptoms to pneumonia leading to acute respiratory distress syndrome [4]. Dromedary camels are reservoir/intermediate

hosts of MERS-CoV [6], since viral neutralizing antibodies have been reported in this species [7–13], and the virus does not require mutations to jump from dromedaries to humans [14]. Besides, all studied camelids are susceptible to MERS-CoV infection under both experimental and natural conditions [15–24].

Innate immunity, the first host defense system against viral infections, is thought to play a key role in MERS pathogenesis. Studies of MERS-CoV infections on numerous human cell types led to the conclusion that type I and III interferons (IFNs) are largely inhibited or delayed [25–27]. Furthermore, high and persistent secretions of inflammatory cytokines by lung macrophages and the inhibition of innate immune responses at the mucosal level are likely to contribute to a more severe infection in humans [28–31]. By contrast, camelids only show subclinical disease in response to MERS-CoV [15–24]. Such an outcome is apparently due to the action of type I and III IFNs generated by the infected nasal epithelia as suggested by recent work performed in alpacas

<sup>†</sup>Nigeer Te and Jordi Rodon contributed equally to this work

\*Correspondence: [julia.vergara@irta.cat](mailto:julia.vergara@irta.cat)

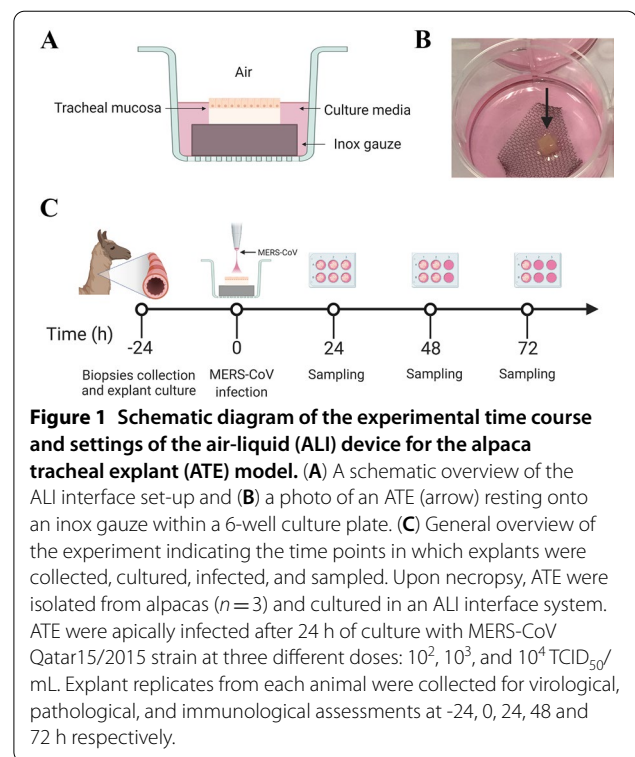
<sup>1</sup> Unitat Mixta d'investigació IRTA-UAB en Sanitat Animal, Centre de Recerca en Sanitat Animal (CRESA), Campus de la Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Catalonia, Spain  
Full list of author information is available at the end of the article



[24]. Infected epithelium produced IFNs, unlike distant uninfected cells, while the expression of interferon-stimulated genes (ISGs) was elevated in both infected and uninfected epithelial cells, suggesting that IFNs could act in a paracrine fashion to induce ISGs expression in uninfected cells [24]. Similarly, IFNs produced at the nasal epithelium might also be sensed via an endocrine pathway, as evidenced by the production of ISGs in the lower respiratory tract (LRT) in absence of detectable expression of IFNs in these tissues [22, 24].

Besides the effective innate immune response of camelid hosts, another factor that may contribute to the mild pathogenesis of MERS-CoV in these species is the ability of the virus to moderately replicate within cells from the LRT. Previous research showed that very few infected cells were found in trachea upon intranasal MERS-CoV inoculation [15, 19, 20, 22, 24], although dipeptidyl peptidase 4 (DPP4) expression levels are comparable to that of nasal mucosa in camelids [32, 33]. It is unclear how MERS-CoV replication towards the LRT is restrained. Thus, in the present study, we established an ex vivo tracheal organ culture using an ALI system to fulfill these knowledge gaps.

Three 10 to 12-month-old alpacas (*Vicugna pacos*) were purchased by private sale, housed at IRTA farm facilities at Alcarràs (Catalonia, Spain) during the acclimation period and transferred to the Autonomous University of Barcelona, in Barcelona (Spain), for necropsy procedures. Animals were euthanized with an overdose of pentobarbital injected into the jugular vein. The middle portion of tracheas ( $n=3$ ) was aseptically collected and transported to the laboratory in pre-heated transport medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Lonza) and Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100  $\mu$ g/mL), L-glutamine (10 mM); all of them from ThermoFisher Scientific (Life Technologies, Waltham, USA) and amphotericin (2  $\mu$ g/mL; Sigma-Aldrich). Obtained tracheal tissues were washed by immersion into fresh warm transport medium three times, followed by the isolation of ATE according to a previously published protocol [34] with some modifications. Briefly, the tracheal mucosa was stripped from the tracheal rings. The cartilage was carefully discarded, and each segment was then cut into squares of 25 mm<sup>2</sup>, prior to transfer to fine meshed inox steel gauzes placed in 6-well culture plates with the epithelium facing up. Each well contained 2 mL of culture media (CM) consisting of transport media without amphotericin. ATE were cultivated in ALI interface, so the epithelium was slightly immersed in fluid with cilia exposed to the air (Figures 1A, B). Explants were cultured in a humidified incubator for up to 4 days at 37 °C and



5% CO<sub>2</sub>. The ciliary beating was checked daily (each 24 h) under a light microscope.

A passage-3 of the clade B MERS-CoV Qatar15/2015 strain (GenBank Accession MK280984) stock was propagated in Vero E6 cells and titrated by calculating the dilution that caused cytopathic effect in 50% of the inoculated cell cultures (50% tissue culture infectious dose endpoint [TCID<sub>50</sub>]), as previously described [19]. The MERS-CoV Qatar 15/2015 strain was selected on the basis that only clade B strains are currently circulating in the Arabian Peninsula [35] and it has enhanced in vivo replicative capabilities in alpacas compared to the prototypical clade A MERS-CoV EMC/2012 strain [22]. ATE were inoculated with the MERS-CoV Qatar15/2015 strain at three different doses:  $10^2$ ,  $10^3$ , and  $10^4$  TCID<sub>50</sub>/mL in CM (Figure 1C). Briefly, tracheal explants were prepared in triplicates for each time point per animal ( $n=3$ ) onto sterile stainless-steel gauzes placed on 6-well culture plates with the epithelial surface upwards (Figures 1A, B). After 24 h of culture, explants were then transferred to a new 6-well culture plate prior to infection with each MERS-CoV dose apically. CM alone was used for mock-infected controls. After 1 h of viral exposure in a humidified incubator at 37 °C and 5% CO<sub>2</sub>, explants were washed three times with warm PBS and placed back to the gauze at the original 6-well plates containing CM. Explants were collected at 24, 48 and 72 h post-inoculation (hpi) and

introduced in tubes containing 1) 10% formalin for morphometric, histological and immunohistochemical analysis (two of the triplicates); or 2) TRI-Reagent (Zymo Research, California, USA) for RNA virus quantification as well as innate immune response analysis.

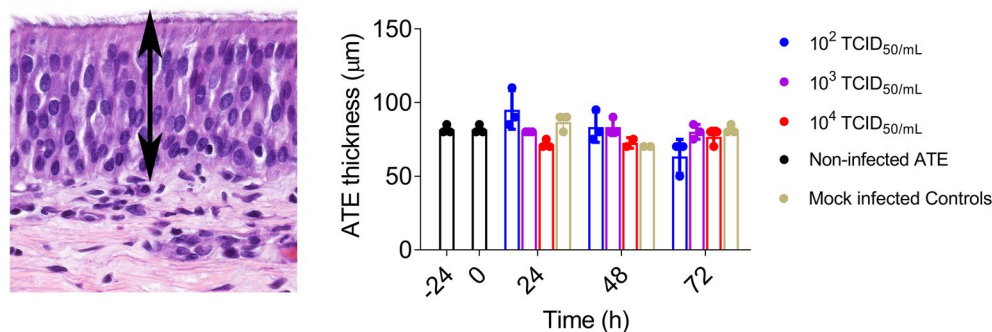
Explants were fixed by immersion in 10% neutral-buffered formalin for 5 days, embedded in paraffin blocks and processed for histological analysis (hematoxylin and eosin stain, H&E). Besides, the thickness of the tracheal epithelium was assessed by measuring five randomly selected fields across each trachea section. A monoclonal mouse anti-MERS-CoV N protein antibody (Sino Biological Inc., Beijing, China) was used to detect the presence of MERS-CoV antigen, following a previously established protocol [33]. A grading system for immunohistochemistry (IHC) was established by a board-certified veterinary pathologist (–, no positive cells detected; ±, less than 10 positive cells per tissue section; +, 10–50 positive cells per tissue section; ++, 50 to 150 positive cells per tissue section).

Total RNA from ATE samples collected at different time points was extracted and converted into cDNA following a previous standard protocol [24]. A microfluidic RT-qPCR assay was utilized to relatively quantify the expression of immune related genes. The selection of innate immune genes and specific pairs of primers to amplify their transcriptional products were described in previous works [22, 24]. In addition, specific primers for the detection of genomic (UpE) and subgenomic (M) viral RNA were added to the assay (Sheet A in Additional file 1) [24]. Data were analyzed with the Real-Time PCR Analysis 4.1.3 software (Fluidigm Corporation, South San Francisco, USA) and the DAG expression software 1.0.5. 6, as previously described [24]. *HPRT1*, *GAPDH* and *UBC* genes were used as normalizer genes, and

values obtained from infected explants collected at 24, 48 and 72 hpi were compared to those obtained from non-infected ATE at 0 hpi. The up- or down-regulated expression of each cytokine gene was expressed in fold-change values (Sheet B in Additional file 1).

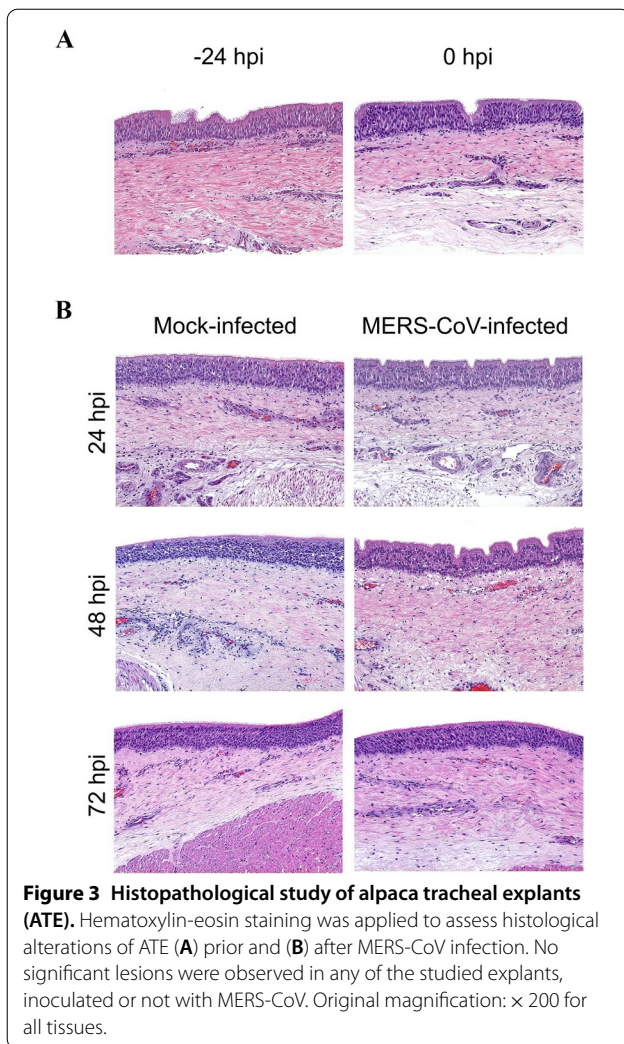
MERS-CoV infection did not significantly alter the average thickness of the epithelium layer (ranging between 63.3 to 86.7  $\mu\text{m}$ ) (Figure 2) nor caused obvious histopathological changes in ATE for the duration of the entire ex vivo experiment (Figures 3A, B). All cultured ATE maintained nearly intact cellular and tissular morphology despite vacuolation of very few cells within the epithelium and the lamina propria from 48 hpi onwards (Figure 3B). Figure 4A shows the kinetics of MERS-CoV yields after infection with three different viral doses ( $10^2$ ,  $10^3$  and  $10^4$  TCID<sub>50</sub>/mL, respectively). Viral genomic RNA loads in ATE were dose- and time-dependent (Figure 4A left). An infection dose of  $10^4$  TCID<sub>50</sub>/mL led to the highest viral RNA detection in ATE and a plateau was reached at 48 hpi onwards. To ascertain active viral gene transcription within ATE, the presence of MERS-CoV subgenomic RNA (M gene) was also assessed (Figure 4A right). M mRNA loads were only detected in ATE infected with  $10^3$  or  $10^4$  TCID<sub>50</sub>/mL MERS-CoV, while no M mRNA transcription occurred upon inoculation with  $10^2$  TCID<sub>50</sub>/mL doses. In agreement with the results obtained by viral RNA quantification, IHC positive labeling was detected in ATE infected with either  $10^3$  or  $10^4$  TCID<sub>50</sub>/mL sampled at 48 or 72 hpi (Figure 4A and Additional file 2). Specific staining was mostly located in the cytoplasm of tracheal epithelial cells (Figure 4B).

To explore antiviral and inflammatory pathways activated upon MERS-CoV infection in ATE, the relative mRNA expression levels of 39 innate immune response genes were monitored in mock-treated

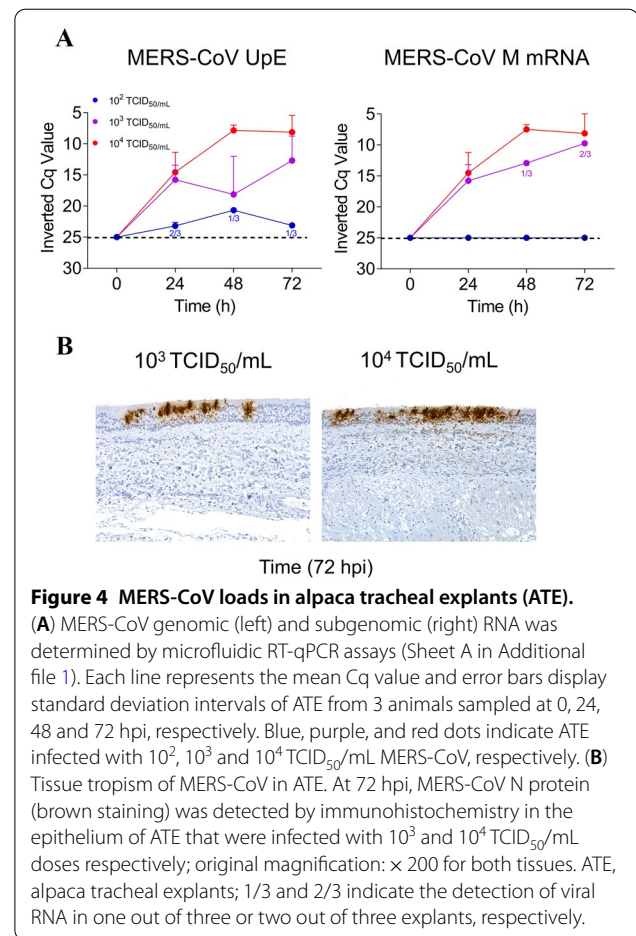


**Figure 2** Evaluation of alpaca tracheal explants (ATE) thickness after ex vivo culture by means of light photomicrographs. The trachea was harvested upon necropsy, followed by infection at different doses of MERS-CoV (right of the figure). ATE were collected at different time points and were fixed in 10% neutral-buffered formalin. Sections of each formalin-fixed paraffin embedded ATE were stained by hematoxylin-eosin to evaluate the thickness of the epithelium by measuring five randomly selected fields across each section (indicated with a black arrow at the left) at -24, 0, 24, 48 and 72 hpi, respectively.



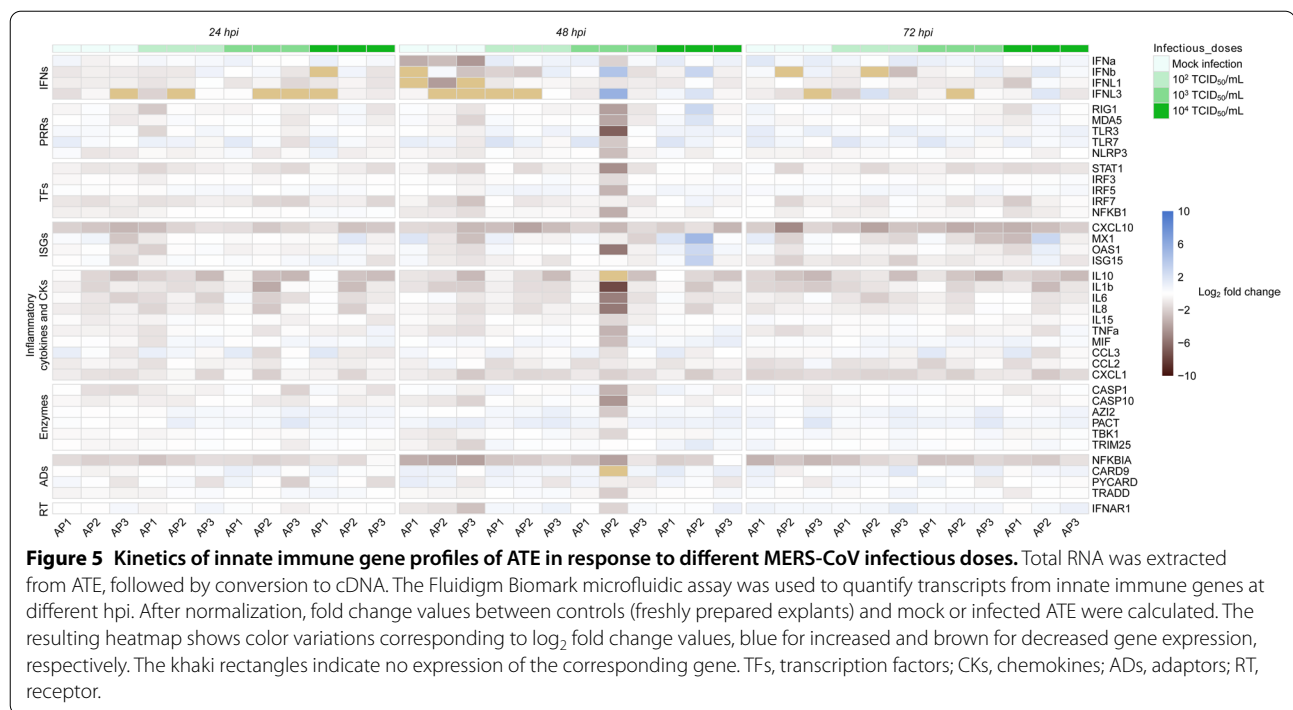


and viral-infected ATE. Three highly stable normalizer genes were included in the assay. All genes were detected at basal levels in non-infected ATE controls collected at 0 hpi, thus, allowing normalization of the whole data sets against this reference time point. Transcription of pro-inflammatory cytokines, chemokines, and transcription factors (except for IRF5 that remained unaltered) showed a slight down-regulation trend upon infection of ATE with any of the MERS-CoV dosages (Figure 5 and Sheet B in Additional file 1). The rest of the genes, including IFNs, ISGs, pattern recognition receptors, downstream signaling enzymes, adaptors, and receptors, fluctuated around basal levels (Figure 5 and Sheet B in Additional file 1), suggesting inhibition of antiviral responses in ATE upon MERS-CoV infection.



## Discussion

The pathogenesis of MERS-CoV in the intermediate host is yet to be fully understood. Although camelids can be experimentally infected, such an approach represents a significant financial cost, ethical concern, safety risk and requires special household facilities under a biosafety level-3 (BSL-3) environment. Thus, we developed an ex vivo ATE model to address these hurdles. ATE retained the ciliary integrity during the course of the experiment, indicating that the coordination and modulation of the ciliary beating of tracheal epithelium remained functional. Morphometric and histological assessments demonstrated that ATE remained integral with good cellular and tissular morphology for at least 72 hpi, showing great resemblance to the freshly collected trachea. Of note, a massive ciliary loss occurs in the nasal epithelia of dromedary camels experimentally infected with MERS-CoV [36] but not in llamas [33] or alpacas [24].



In the present study, ATE were unable to express IFNs in agreement with previous observations on the trachea of alpacas infected with MERS-CoV [22, 24]. In addition, ISGs were not induced in ATE upon MERS-CoV infection, supporting the fact that upregulation of ISGs in tracheal mucosa observed in vivo is due likely to the paracrine action of IFNs produced by the nasal epithelia [24]. Recently, pseudostratified airway epithelial cell (AEC) culture models for llamas and Bactrian camels have been developed. These AEC were generated from the tracheobronchial tract, supported the growth of several MERS-CoV lineages and were sensitive to the antiviral effect of IFNs [37, 38]. However, in these experiments, the production of IFNs and cytokines upon MERS-CoV infection were not checked. Nevertheless, when treated with IFNs, camelid tracheal cells can produce ISGs, denoting a functional innate immune system [38]. Both, ATE and AEC could be exploited to test agonists and antagonists of several innate immune pathways leading to MERS-CoV clearance.

The exclusive use of camelid tracheal cells to study host-MERS-CoV interactions might not be sufficient since, at least in vivo, infected nasal epithelial cells are able to notably up regulate the transcriptional expression of IFN genes [22, 24]. Therefore, the development of nasal explants and well differentiated nasal epithelial cell models will be necessary for future studies to fully appreciate the interplay between the epithelial barrier

and MERS-CoV infection in camelids. In that respect, explant models might better reflect an in vivo scenario since mucosa and submucosa are both co-cultured maintaining the tissue structure. Nonetheless, AEC and tracheal explant models overcome natural physiological drawbacks inherent to epithelial cell lines including differences in cell polarization, coordinated ciliary activity and apical contact with air [39]. Furthermore, tissue pieces obtained from a single animal can be used in numerous replicates, thereby reducing the experimental variability and the number of animals used, in line with the 3Rs principle [40].

In conclusion, a novel ex vivo culture model was developed using ATE, which is suitable to study MERS-CoV infection and replication as it occurs in natural reservoir hosts. We demonstrated as a proof of principle that ATE can be infected by MERS-CoV without triggering innate antiviral responses. Besides, ATE could also be suitable to study other respiratory pathogens of camelids by reducing the burden of animals used for experimentation.

#### Abbreviations

AEC: pseudostratified airway epithelial cell; ATE: alpaca tracheal explant; BSL-3: biosafety level-3; CM: culture media; DPP4: dipeptidyl peptidase 4; hpi: hours post-inoculation; IFN: interferon; IHC: immunohistochemistry; ISG: interferon-stimulated genes; LRT: lower respiratory tract; MERS-CoV: Middle East respiratory syndrome coronavirus; TCID<sub>50</sub>: 50% tissue culture infectious dose endpoint.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13567-022-01084-3>.

**Additional file 1: Viral loads (UpE and M mRNA) and Fc of innate immune response genes in ATE.**

**Additional file 2: MERS-CoV N protein detection by IHC in ATE.**

### Acknowledgements

We thank Dr Bart L. Haagmans from Erasmus Medical Center (EMC, Rotterdam) for providing the MERS-CoV Qatar-15/2015 strain. We also thank Dr Hans Nauwynck and staff from his lab (Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University) for providing advanced training on ex vivo cultures. We are particularly indebted to the staff of the BSL-3 biocontainment animal facility at IRTA-CReSA (Cerdanyola del Vallès, Barcelona).

### Authors' contributions

NT, JR, JS, JV-A, and AB designed research; NT, JR, MP, JS, and JV-A performed research; NT, JR, RC, MP, JS, JV-A, and AB analyzed data; and NT, and AB wrote the paper. All authors read and approved the final manuscript.

### Funding

This research was supported by European Commission: [Call: H2020-INFRAIA-2016–2017 grant N°: 731014, Innovative Medicines initiative (IMI) grant N°115760]. IRTA is supported by CERCA Programme / Generalitat de Catalunya. N.T. is a recipient of a China Scholarship Council grant (CSC NO. 201608150108). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Availability of data and materials

The data that support the findings of this study are available from the authors upon reasonable request.

### Declarations

#### Ethics approval and consent to participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All animal experiments were approved by the Ethical and Animal Welfare Committee of IRTA (CEEA-IRTA) and by the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia (file No. CEA-OH/10943/1). The work involving MERS-CoV was performed at the BSL-3 facilities of the Biocontainment Unit of IRTA-CReSA in Barcelona, Spain.

#### Competing interests

The authors declare that they have no competing interests.

#### Author details

<sup>1</sup>Unitat Mixta d'Investigació IRTA-UAB en Sanitat Animal, Centre de Recerca en Sanitat Animal (CReSA), Campus de la Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Catalonia, Spain. <sup>2</sup>IRTA, Programa de Sanitat Animal, Centre de Recerca en Sanitat Animal (CReSA), Campus de la Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Catalonia, Spain. <sup>3</sup>Departament de Sanitat i Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona (UAB), Campus de la UAB, 08193 Bellaterra, Catalonia, Spain. <sup>4</sup>School of Public Health, Li Ka Shing, Faculty of Medicine, The University of Hong Kong, Hong Kong, China.

Received: 22 April 2022 Accepted: 12 July 2022

Published online: 02 September 2022

### References

- Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM (2012) Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 367:1814–1820. <https://doi.org/10.1056/nejmoa1211721>
- Arabi YM, Arifi AA, Balkhy HH, Najm H, Aldawood AS, Ghabashi A, Hawa H, Allothman A, Khaldi A, Al Raiy B (2014) Clinical course and outcomes of critically ill patients with Middle East respiratory syndrome coronavirus infection. *Ann Intern Med* 160:389–397. <https://doi.org/10.7326/M13-2486>
- Assiri A, Al-Tawfiq JA, Al-Rabeeh AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, Flemban H, Al-Nassir WN, Balkhy HH, Al-Hakeem RF, Makhdoom HQ, Zumla AI, Memish ZA (2013) Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. *Lancet Infect Dis* 13:752–761. [https://doi.org/10.1016/S1473-3099\(13\)70204-4](https://doi.org/10.1016/S1473-3099(13)70204-4)
- Zumla A, Hui DS, Perlman S (2015) Middle East respiratory syndrome. *Lancet* 386:995–1007. [https://doi.org/10.1016/S0140-6736\(15\)60454-8](https://doi.org/10.1016/S0140-6736(15)60454-8)
- WHO | Middle East respiratory syndrome coronavirus (MERS-CoV). <https://www.who.int/emergencies/mers-cov/en/>. Accessed 18 May 2022
- Sabir JSM, Lam TT-Y, Ahmed MMM, Li L, Shen Y, Abo-Aba SEM, Qureshi MI, Abu-Zeid M, Zhang Y, Khiyami MA, Alharbi NS, Hajrah NH, Sabir MJ, Mutwakil MHZ, Kabli SA, Alsulaimany FAS, Obaid AY, Zhou B, Smith DK, Holmes EC, Zhu H, Guan Y (2016) Co-circulation of three camel coronavirus species and recombination of MERS-CoVs in Saudi Arabia. *Science* 351:81–84. <https://doi.org/10.1126/science.aac8608>
- Corman VM, Jores J, Meyer B, Younan M, Liljander A, Said MY, Gluecks I, Lattwein E, Bosch BJ, Drexler JF, Bornstein S, Drosten C, Müller MA (2014) Antibodies against MERS coronavirus in dromedary camels, Kenya, 1992–2013. *Emerg Infect Dis* 20:1319–1322. <https://doi.org/10.3201/eid2008.140596>
- Drosten C, Meyer B, Müller MA, Corman VM, Al-Masri M, Hossain R, Madani H, Sieberg A, Bosch BJ, Lattwein E, Alhakeem RF, Assiri AM, Hajomar W, Albarrak AM, Al-Tawfiq JA, Zumla AI, Memish ZA (2014) Transmission of MERS-coronavirus in household contacts. *N Engl J Med* 371:828–835. <https://doi.org/10.1056/NEJMoa1405858>
- Farag EABA, Reusken CBEM, Haagmans BL, Mohran KA, Stalin Raj V, Pas SD, Voermans J, Smits SL, Godeke G-J, Al-Hajri MM, Alhajri FH, Al-Romaihi HE, Ghobashy H, El-Maghraby MM, El-Sayed AM, Al-Thani MHJ, Al-Marri S, Koopmans MPG (2015) High proportion of MERS-CoV shedding dromedaries at slaughterhouse with a potential epidemiological link to human cases, Qatar 2014. *Infect Ecol Epidemiol* 5:28305. <https://doi.org/10.3402/iee.v5.28305>
- Reusken CB, Ababneh M, Raj VS, Meyer B, Eljarah A, Abutarbush S, Godeke GJ, Bestebroer TM, Zutt I, Muller MA, Bosch BJ, Rottier PJ, Osterhaus AD, Drosten C, Haagmans BL, Koopmans MP (2013) Middle East Respiratory Syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013. *Euro Surveill* 18:20662. <https://doi.org/10.2807/1560-7917.es2013.18.50.20662>
- Reusken CB, Farag EA, Jonges M, Godeke GJ, El-Sayed AM, Pas SD, Raj VS, Mohran KA, Moussa HA, Ghobashy H, Alhajri F, Ibrahim AK, Bosch BJ, Pasha SK, Al-Romaihi HE, Al-Thani M, Al-Marri SA, Alhajri MM, Haagmans BL, Koopmans MP (2014) Middle East respiratory syndrome coronavirus (MERS-CoV) RNA and neutralising antibodies in milk collected according to local customs from dromedary camels, Qatar, April 2014. *Euro Surveill* 19:20829. <https://doi.org/10.2807/1560-7917.es2014.19.23.20829>
- Raj VS, Osterhaus ADME, Fouchier RAM, Haagmans BL (2014) MERS: emergence of a novel human coronavirus. *Curr Opin Virol* 5:58–62. <https://doi.org/10.1016/j.coviro.2014.01.010>
- Reusken CBEM, Haagmans BL, Müller MA, Gutierrez C, Godeke G-J, Meyer B, Muth D, Raj VS, Smits-De Vries L, Corman VM, Drexler J-F, Smits SL, El Tahir YE, De Sousa R, van Beek J, Nowotny N, van Maanen K, Hidalgo-Hermoso E, Bosch B-J, Rottier P, Osterhaus A, Gortázar-Schmidt C, Drosten C, Koopmans MP (2013) Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. *Lancet Infect Dis* 13:859–866. [https://doi.org/10.1016/S1473-3099\(13\)70164-6](https://doi.org/10.1016/S1473-3099(13)70164-6)
- Hemida MG, Chu DKW, Poon LLM, Perera RAPM, Alhammadi MA, Ng H, Siu LY, Guan Y, Alnaeem A, Peiris M (2014) MERS coronavirus in dromedary camel herd. *Saudi Arabia Emerg Infect Dis*. <https://doi.org/10.3201/eid2007.140571>
- Adney DR, Bielefeldt-Ohmann H, Hartwig AE, Bowen RA (2016) Infection, replication, and transmission of Middle East respiratory syndrome coronavirus in alpacas. *Emerg Infect Dis* 22:1031–1037. <https://doi.org/10.3201/2206.160192>

16. Cramer G, Durr PA, Klein R, Foord A, Yu M, Riddell S, Haining J, Johnson D, Hemida MG, Barr J, Peiris M, Middleton D, Wang L-F (2016) Experimental infection and response to rechallenge of alpacas with Middle East respiratory syndrome coronavirus. *Emerg Infect Dis* 22:1071–1074. <https://doi.org/10.3201/eid2206.160007>
17. Reusken CBEM, Schilp C, Raj VS, De Bruin E, Kohl RHG, Farag EABA, Haagmans BL, Al-Romaihi H, Le Grange F, Bosch B-J, Koopmans MPG (2016) MERS-CoV infection of alpaca in a region where MERS-CoV is endemic. *Emerg Infect Dis* 22:1129–1131. <https://doi.org/10.3201/eid2206.152113>
18. David D, Rotenberg D, Khinich E, Erster O, Bardenstein S, van Straten M, Okba NMA, Raj SV, Haagmans BL, Miculitzki M, Davidson I (2018) Middle East respiratory syndrome coronavirus specific antibodies in naturally exposed Israeli llamas, alpacas and camels. *One Health* 5:65–68. <https://doi.org/10.1016/j.onehlt.2018.05.002>
19. Vergara-Alert J, van den Brand JMA, Widagdo W, Muñoz M, Raj S, Schipper D, Solanes D, Cordon I, Bensaid A, Haagmans BL, Segalés J (2017) Live-stock susceptibility to infection with Middle East respiratory syndrome coronavirus. *Emerg Infect Dis* 23:232–240. <https://doi.org/10.3201/eid2302.161239>
20. Adney DR, Letko M, Ragan IK, Scott D, van Doremalen N, Bowen RA, Munster VJ (2019) Bactrian camels shed large quantities of Middle East respiratory syndrome coronavirus (MERS-CoV) after experimental infection. *Emerg Microbes Infect* 8:717–723. <https://doi.org/10.1080/22221751.2019.1618687>
21. Rodon J, Okba NMA, Te N, van Dieren B, Bosch BJ, Bensaid A, Segalés J, Haagmans BL, Vergara-Alert J (2019) Blocking transmission of Middle East respiratory syndrome coronavirus (MERS-CoV) in llamas by vaccination with a recombinant spike protein. *Emerg Microbes Infect* 8:1593–1603. <https://doi.org/10.1080/22221751.2019.1685912>
22. Te N, Rodon J, Pérez M, Segalés J, Vergara-Alert J, Bensaid A (2022) Enhanced replication fitness of MERS-CoV clade B over clade A strains in camelids explains the dominance of clade B strains in the Arabian Peninsula. *Emerg Microbes Infect* 11:260–274. <https://doi.org/10.1080/22221751.2021.2019559>
23. Te N, Ciurkiewicz M, van den Brand JMA, Rodon J, Haverkamp A-K, Vergara-Alert J, Bensaid A, Haagmans BL, Baumgartner W, Segalés J (2022) Middle East respiratory syndrome coronavirus infection in camelids. *Vet Pathol*. <https://doi.org/10.1177/03009858211069120>
24. Te N, Rodon J, Ballester M, Pérez M, Pailler-García L, Segalés J, Vergara-Alert J, Bensaid A (2021) Type I and III IFNs produced by the nasal epithelia and dimmed inflammation are features of alpacas resolving MERS-CoV infection. *PLoS Pathog* 17:e1009229. <https://doi.org/10.1371/journal.ppat.1009229>
25. Comar CE, Goldstein SA, Li Y, Yount B, Baric RS, Weiss SR (2019) Antagonism of dsRNA-induced innate immune pathways by NS4a and NS4b accessory proteins during MERS coronavirus infection. *MBio* 10:e00319–e419. <https://doi.org/10.1128/mBio.00319-19>
26. Gutierrez-FJ, Perlman S, Sanchez-aparicio MT, Garci A, Enjuanes L, Sola I (2018) MERS-CoV 4b protein interferes with the NF- $\kappa$ B-dependent innate immune response during infection. *PLoS Pathog* 4:e1006838. <https://doi.org/10.1371/journal.ppat.1006838>
27. Rabouw HH, Langereis MA, Knaap RCM, Dalebout TJ, Canton J, Sola I, Enjuanes L, Bredenoord PJ, Kikkert M, de Groot RJ, van Kuppeveld FJM (2016) Middle East respiratory coronavirus accessory protein 4a inhibits PKR-mediated antiviral stress responses. *PLoS Pathog* 12:e1005982. <https://doi.org/10.1371/journal.ppat.1005982>
28. Alosaimi B, Hamed ME, Naeem A, Alsharif AA, AlQahtani SY, AlDosari KM, Alamri AA, Al-Eisa K, Khojah T, Assiri AM, Enani MA (2020) MERS-CoV infection is associated with downregulation of genes encoding Th1 and Th2 cytokines/chemokines and elevated inflammatory innate immune response in the lower respiratory tract. *Cytokine* 126:154895. <https://doi.org/10.1016/j.cyto.2019.154895>
29. Zhou J, Chu H, Li C, Wong BHY, Cheng ZS, Poon VKM, Sun T, Lau CCY, Wong KKY, Chan JYW, Chan JFW, To KKW, Chan KH, Zheng BJ, Yuen KY (2014) Active replication of middle east respiratory syndrome coronavirus and aberrant induction of inflammatory cytokines and chemokines in human macrophages: implications for pathogenesis. *J Infect Dis* 209:1331–1342. <https://doi.org/10.1093/infdis/jit504>
30. Zhao X, Chu H, Wong BHY, Chiu MC, Wang D, Li C, Liu X, Yang D, Poon VKM, Cai J, Chan JFW, To KKW, Zhou J, Yuen KY (2020) Activation of C-type lectin receptor and (RIG)-I-like receptors contributes to proinflammatory response in Middle East respiratory syndrome coronavirus-infected macrophages. *J Infect Dis* 221:647–659. <https://doi.org/10.1093/infdis/jiz483>
31. Kindler E, Jónsdóttir HR, Muth D, Hamming OJ, Hartmann R, Rodriguez R, Geffers R, Fouchier RAM, Drosten C, Müller MA, Dijkman R, Thiel V (2013) Efficient replication of the novel human betacoronavirus EMC on primary human epithelium highlights its zoonotic potential. *MBio* 4:00611–00612. <https://doi.org/10.1128/mBio.00611-12>
32. Widagdo W, Raj VS, Schipper D, Kolijn K, van Leenders GJLH, Bosch BJ, Bensaid A, Segalés J, Baumgärtner W, Osterhaus ADME, Koopmans MP, van den Brand JMA, Haagmans BL (2016) Differential expression of the Middle East respiratory syndrome coronavirus receptor in the upper respiratory tracts of humans and dromedary camels. *J Virol* 90:4838–4842. <https://doi.org/10.1128/JVI.02994-15>
33. Te N, Vergara-Alert J, Lehmbecker A, Pérez M, Haagmans BL, Baumgärtner W, Bensaid A, Segalés J (2019) Co-localization of Middle East respiratory syndrome coronavirus (MERS-CoV) and dipeptidyl peptidase-4 in the respiratory tract and lymphoid tissues of pigs and llamas. *Transbound Emerg Dis* 66:831–841. <https://doi.org/10.1111/tbed.13092>
34. Glorieux S, Van den Broeck W, van der Meulen KM, Van Reeth K, Favoreel HW, Nauwynck HJ (2007) In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract. *J Virol Methods* 142:105–112. <https://doi.org/10.1016/j.jvromet.2007.01.018>
35. El-Kafrawy SA, Corman VM, Tolah AM, Al Masaudi SB, Hassan AM, Müller MA, Bleicker T, Harakeh SM, Alzahrani AA, Alsaaidi GA, Alagili AN, Hashem AM, Zumla A, Drosten C, Azhar El (2019) Zoonotic patterns of Middle East respiratory syndrome coronavirus in imported African and local Arabian dromedary camels: a prospective genomic study. *Lancet Planet Heal* 3:e521–e528. [https://doi.org/10.1016/S2542-5196\(19\)30243-8](https://doi.org/10.1016/S2542-5196(19)30243-8)
36. Haverkamp A-K, Lehmbecker A, Spitzbarth I, Widagdo W, Haagmans BL, Segalés J, Vergara-Alert J, Bensaid A, van den Brand JMA, Osterhaus ADME, Baumgärtner W (2018) Experimental infection of dromedaries with Middle East respiratory syndrome-Coronavirus is accompanied by massive ciliary loss and depletion of the cell surface receptor dipeptidyl peptidase 4. *Sci Rep* 8:9778. <https://doi.org/10.1038/s41598-018-28109-2>
37. Schroeder S, Mache C, Kleine-Weber H, Corman VM, Muth D, Richter A, Fatykhova D, Memish ZA, Stanifer ML, Boulant S, Gultom M, Dijkman R, Eggeling S, Hocke A, Hippenstiel S, Thiel V, Pöhlmann S, Wolff T, Müller MA, Drosten C (2021) Functional comparison of MERS-coronavirus lineages reveals increased replicative fitness of the recombinant lineage 5. *Nat Commun* 12:5324. <https://doi.org/10.1038/s41467-021-25519-1>
38. Gultom M, Kratzel A, Portmann J, Stalder H, Chanfon Bätzner A, Gantenbein H, Gurtner C, Ebert N, Gad HH, Hartmann R, Posthaus H, Zanolari P, Pfaender S, Thiel V, Dijkman R (2022) Establishment of well-differentiated camelid airway cultures to study Middle East respiratory syndrome coronavirus. *Sci Rep* 12:10340. <https://doi.org/10.1038/s41598-022-13777-y>
39. Nunes SF, Murcia PR, Tiley LS, Brown IH, Tucker AW, Maskell DJ, Wood JLN (2010) An ex vivo swine tracheal organ culture for the study of influenza infection. *Influenza Other Respi Viruses* 4:7–15. <https://doi.org/10.1111/j.1750-2659.2009.00119.x>
40. Russell WMS (1995) The development of the three Rs Concept. *Altern to Lab Anim* 23:298–304. <https://doi.org/10.1177/026119299502300306>

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.