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Virological assessment of hospitalized patients with COVID-2019

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Introduction

- Close genetic relatedness between severe acute respiratory syndrome coronavirus (**SARS-CoV**) and the causative agent of **COVID-19, SARS-CoV-2**
- Predominant ACE2 in the **lower respiratory tract** believed to determine natural history of SARS as a lower respiratory tract infection
- Whereas positive SARS-CoV-2 detection from the **upper respiratory tract** has been described

Introduction

- BUT not address principal differences between SARS and COVID-19 in terms of clinical pathology
- Patients enrolled because infections upon known close **contact to an index case**, thereby avoiding biases due to symptom-based case definitions
- Treated in a single hospital in Munich, Germany
- Virological testing by two closely-collaborating laboratories using the same standards for **RT-PCR** and virus isolation

Introduction

- Virological testing by two closely-collaborating laboratories using the same standards for RT-PCR and virus isolation
- Patients cluster occurred after January 23rd , 2020 in Munich, Germany, as discovered on January 27th
- Samples taken during the clinical course, as well as from initial diagnostic testing before admission

Methods

Clinical methods and viral load conversion

- Oro and nasopharyngeal throat swabs were preserved in 3ml of viral transport medium
- Viral loads in samples were projected to RNA copies
  - **Sputum: per 3ml**
  - **Stool: per gram**
  - **Throat swab: per 3ml**

Methods

RT-PCR for SARS-CoV-2 and other respiratory viruses

- RT-PCR used targets in the E- and RdRp genes
- Pre-formulated oligonucleotide mixture to make laboratory procedures more reproducible

Methods

All patients were also tested for other respiratory viruses

- Human coronaviruses (HCoV)

  -HKU1, -OC43, -NL63, -229E
- Influenza virus A and B
- Rhinovirus
- Enterovirus
- Respiratory syncytial virus
- Human Parainfluenza virus

  1-4
- Human metapneumovirus
- Adenovirus
- Human bocavirus

Methods

Virus isolation

- Isolation was done in two labs on Vero E6 cells
- Supernatant was harvested after **0,1,3 and 5 days** and used in RT-PCR analysis

Methods

Serology

- We performed recombinant immunofluorescence assays to determine the specific reactivity

Statistical analyses

- Statistical analyses were done using SPSS software or Grap-Pad Prism

Ethical approval statement

- All patients provided informed consent to the use of their data and clinical samples for the purposes of the present study

RT-PCR sensitivity, sites of replication, and correlates of infectivity based on aggregated data

All patients were also tested for other respiratory viruses

- Human coronaviruses (HCoV) - HKU1, -OC43, -NL63, -229E
- Influenza virus A and B
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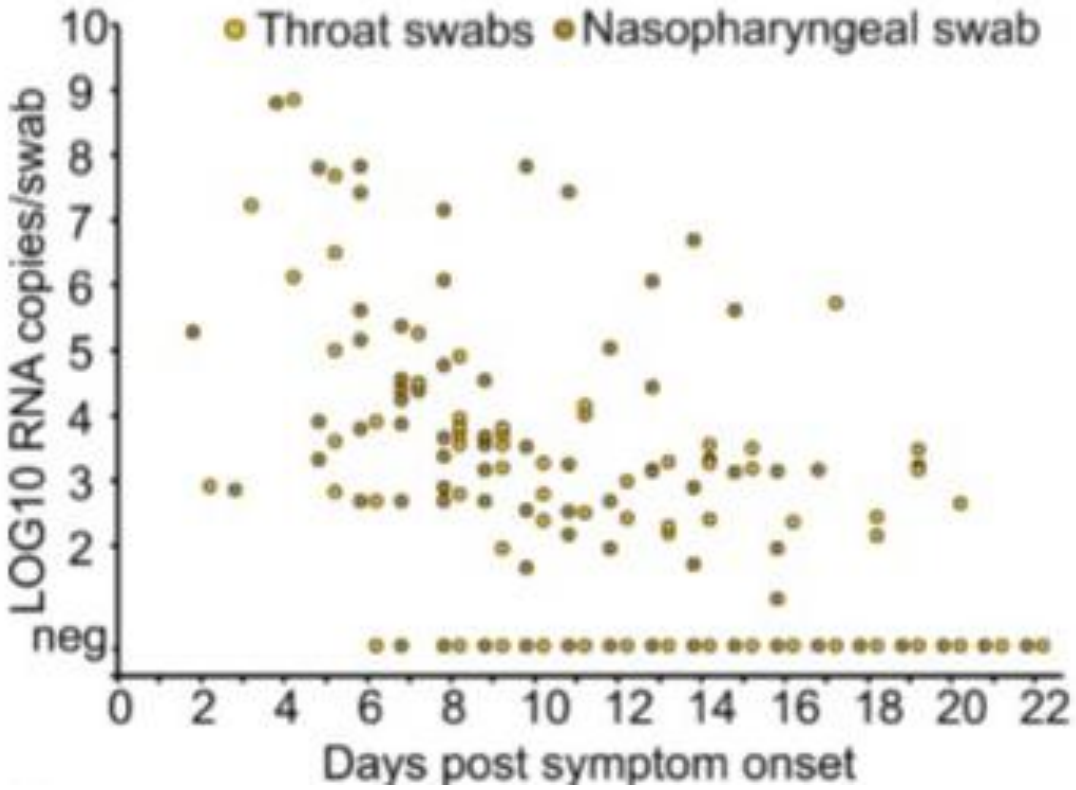


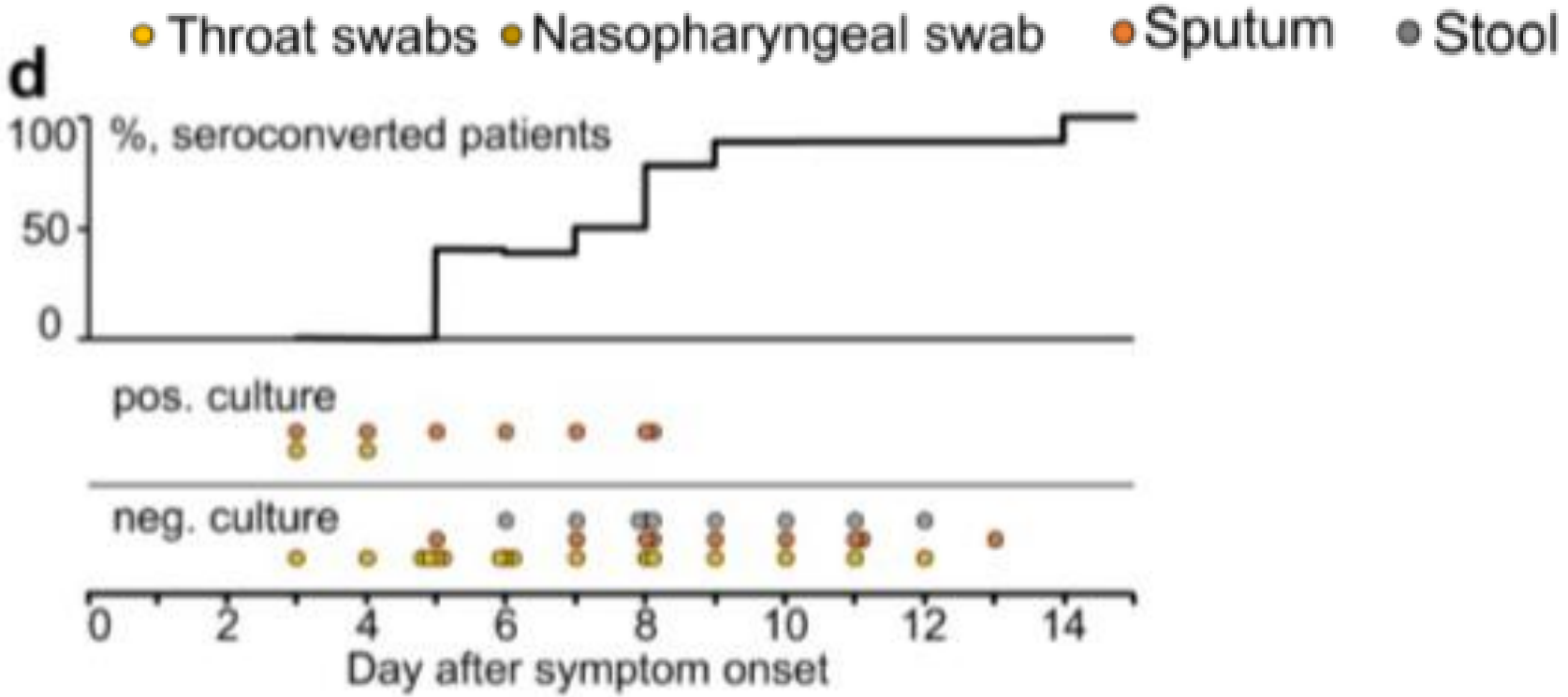
RT-PCR: Viral RNA concentrations in upper respiratory tract samples

- No discernible differences in viral loads or detection rates when comparing naso- vs. oropharyngeal swabs
- None of 27 urine samples and none of 31 serum samples were tested positive for SARS-CoV2 RNA

RT-PCR sensitivity, sites of replication, and correlates of infectivity based on aggregated data

Infectivity : Live viral isolation

- Stool samples was never successful, irrespective of viral RNA concentration
- Depended on viral load: samples containing <106 copies/mL (or copies per sample) never yielded an isolate



Infectivity : Live viral isolation

- Top panel shows fraction of seroconverted patients, bottom shows aggregated results of virus isolation trials

- No isolates were obtained from samples taken after day 8 in spite of ongoing high viral loads

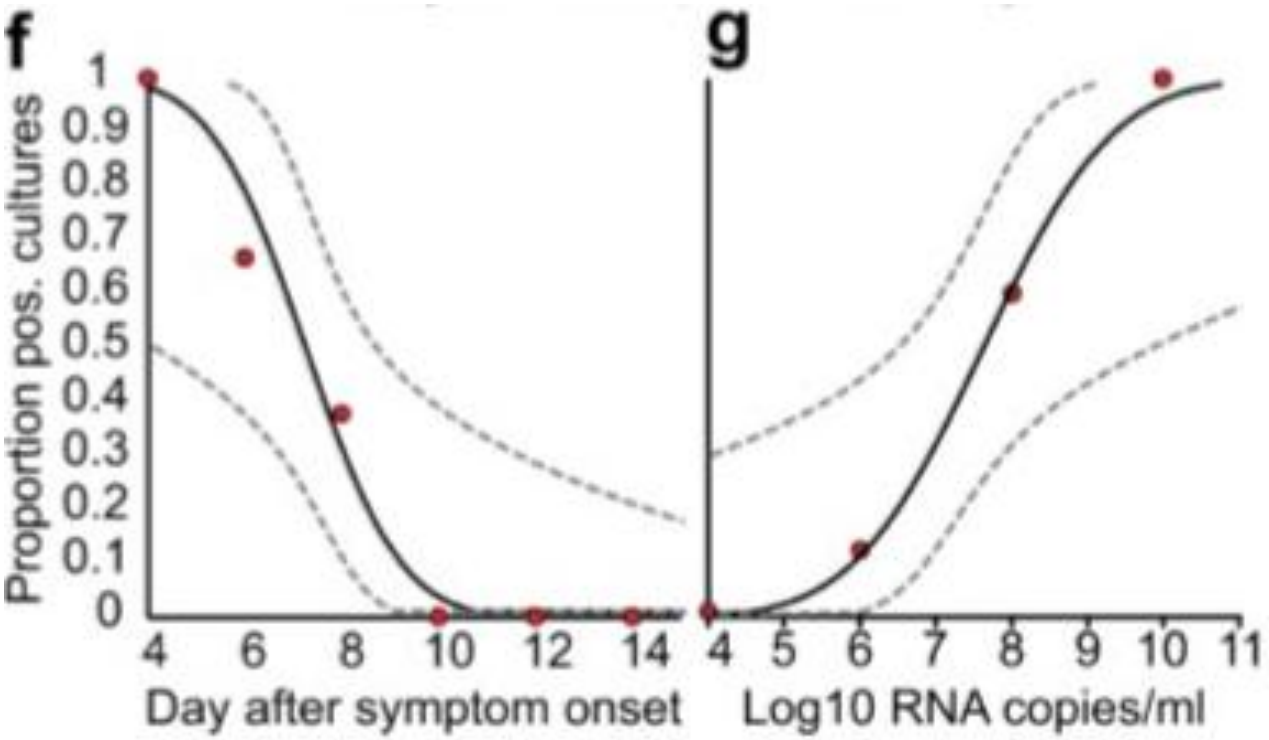
RT-PCR sensitivity, sites of replication, and correlates of infectivity based on aggregated data

Sites of replication

- High viral loads and successful isolation from early throat swabs **suggested potential virus replication in upper respiratory tract tissues**

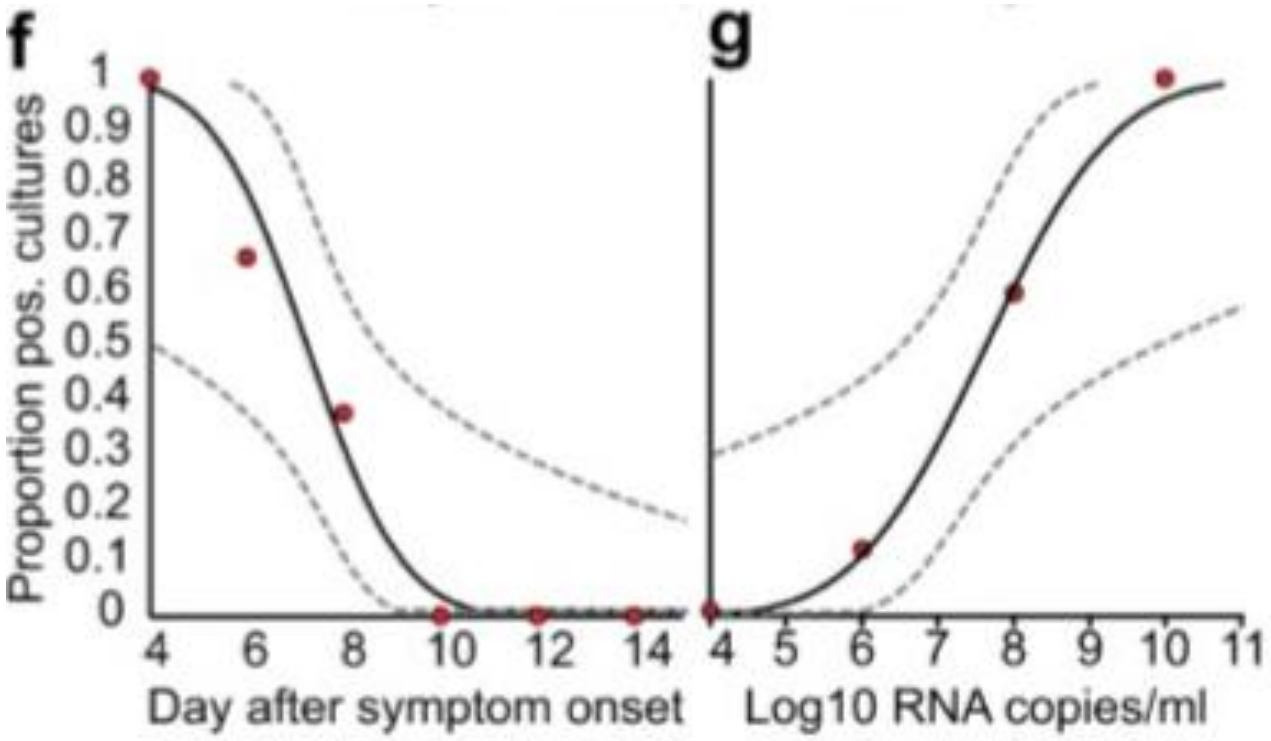
To obtain proof of active virus replication in absence of histopathology

- The arthors conducted RT-PCR tests :
  - Identify viral subgenomic messenger RNAs (sgRNA) directly in clinical samples
  - Viral sgRNA is only transcribed in infected cells and is not packaged into virions, therefore indicating the presence of actively-infected cells in samples.



Projected virus isolation success based on probit distributions

- The inner lines are probit curves (dose-response rule). The outer dotted lines are 95% CI.
- For less than 5% isolation success, the estimated day was **9.78** (95% CI: 8.45-21.78) **days post-onset** and the estimated RNA concentration for less than 5% isolation success was estimate to be **6.51 Log10 RNA/ml** (95% CI:-4,11-5.40
- Viral sgRNA was compared against viral genomic RNA in the same sample



Projected virus isolation success based on probit distributions

- In sputum samples taken on days 4/5, 6/7, and 8/9, a time in which active replication in sputum was obvious in all patients as per longitudinal viral load courses (see below), mean normalized sgRNA per genome ratios were ~0.4% A decline occurred over days 10/11.
- In throat swabs, samples taken up to day 5 were in the same range, while no sgRNA was detectable in swabs thereafter
- Together, these data **indicate active replication of SARS-CoV-2 in the throat during the first 5 days** after symptoms onset
- No, or only minimal, indication of replication in stool was obtained by the same method

Table 1 | Single nucleotide polymorphism at genome position 6446 in clinical samples from case #4

Day p.o.	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Swab	A		A				
Sputum		G	G	G	G>A		
Stool			G>A	A=G	A=G	G>A	A

- We sequenced full virus genomes from all patients
- The SNP was analyzed by RT-PCR and Sanger sequencing in all sequential samples available from that patient
- **A G6446A exchange** was first detected in one patient and later transmitted to other patients in the cluster
- The presence of separate genotypes in throat swabs and sputum **strongly supported our suspicion of independent virus replication in the throat**, rather than passive shedding there from the lung

Virus shedding, antibody response, and clinical correlation in individual course

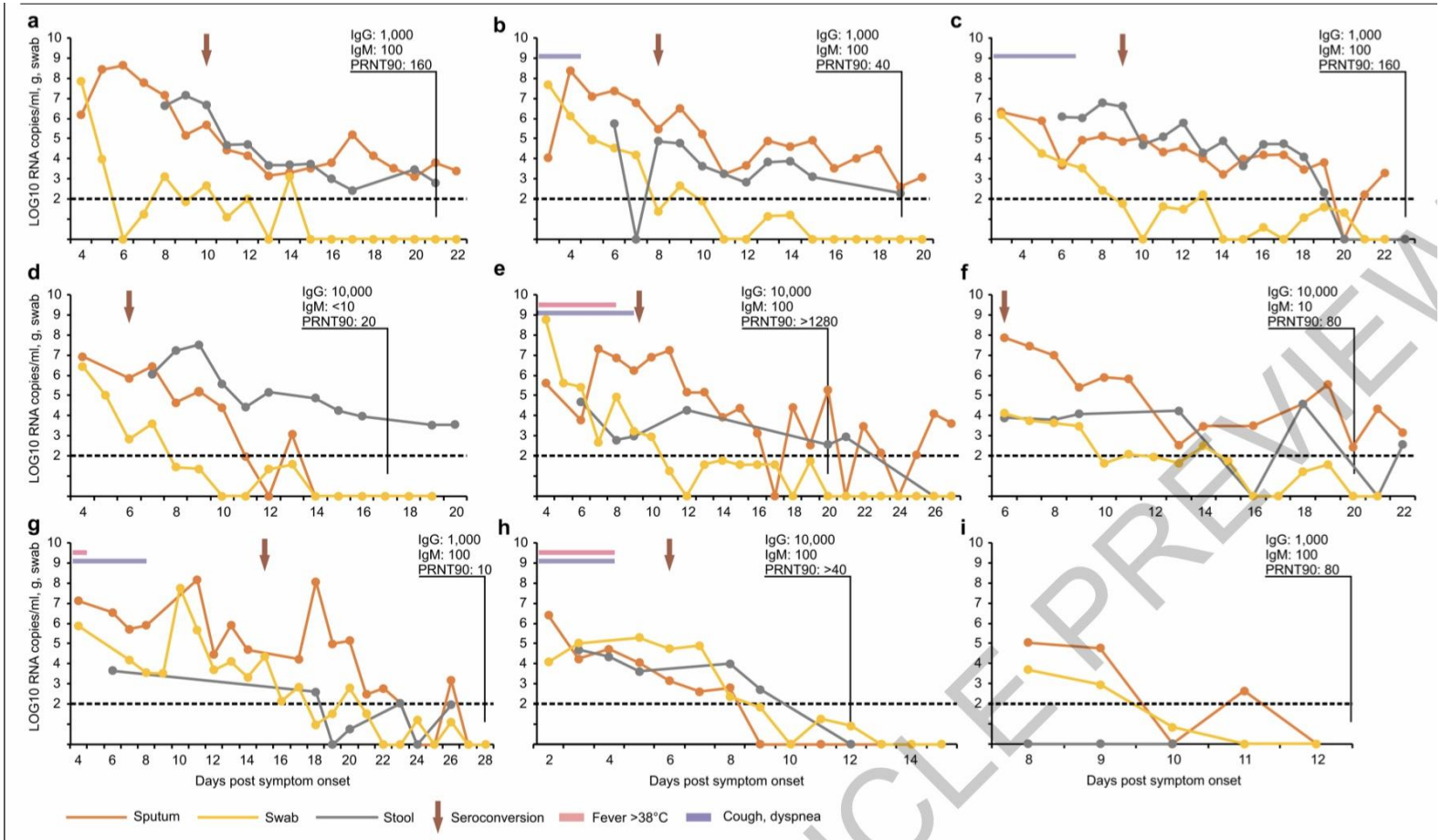
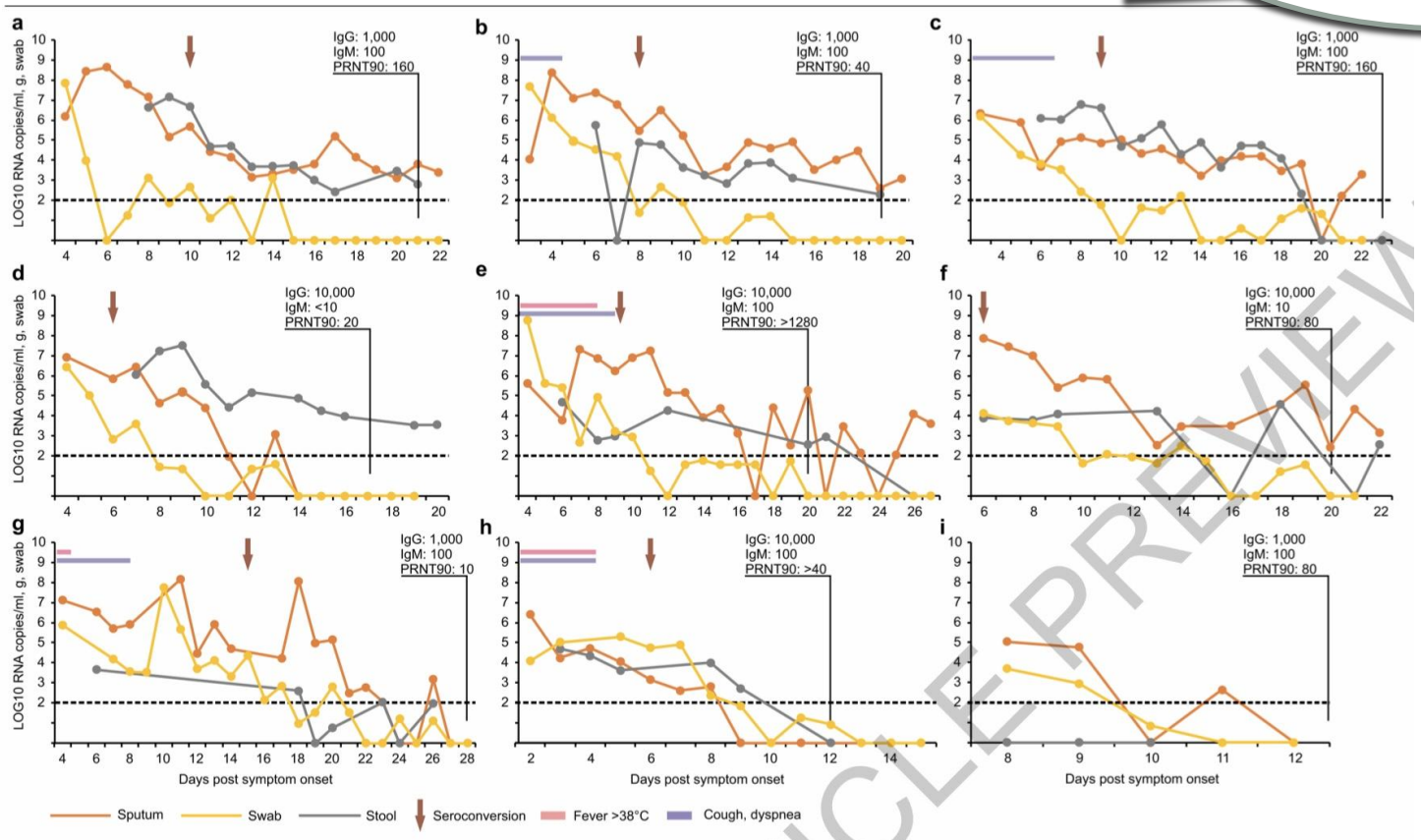


Fig. 2 | Viral load kinetics, seroconversion and clinical observations in individual cases. Panels A to I correspond to cases #1, #2, #3, #4, #7, #8, #10, #14, and #16 in Böhmer et al.[11] Dotted lines, limit of quantification. Experiments were performed in duplicate and the data presented are means of results obtained by two laboratories independently.

- Viral RNA concentrations very high in initial samples.
- In all patients except one, throat swab RNA concentrations already on decline at first presentation

- Sputum RNA concentrations declined more slowly, with a peak during the first week in 3/8 pts
- Stool RNA con. also high



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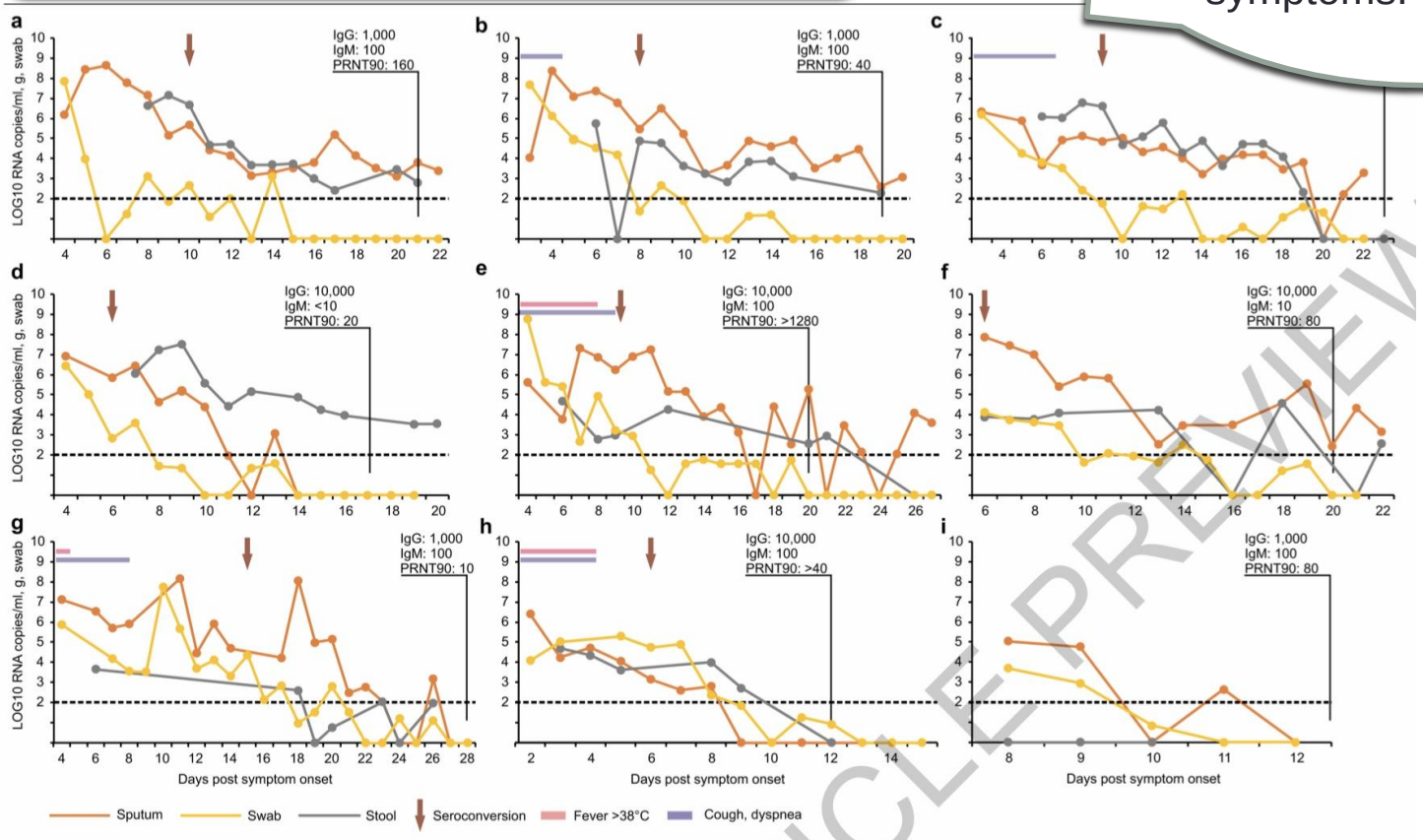
- Courses of viral RNA in stool reflect courses in sputum in many cases (e.g., Figure 2A, B, C)
- Only Figure 2D, independent replication in the intestinal tract -> stool RNA excretion

- Viral RNA detectable in throat swabs in 2nd wk.
- Stool and sputum remained RNA-positive over 3 wks in 6/9 pts, in spite of full resolution of symptoms.



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All cases had comparatively mild courses; symptoms mostly waned until the end of the first week

Table 2 | Clinical characteristics of all patients

PATIENT ID	COMORBIDITY	INITIAL SYMPTOMS	LATER SYMPTOMS	ANC/µl	ALC/µl	CRP (mg/l)	LDH (U/l)
#1	hypothyreoidism	cough, fever, diarrhea	diarrhoea	4870	1900	46	197
#2	none	sinusitis, cephalgia, cough,	hyposmia, ageusia	3040	1200	4.9	182
#3	COPD	arthralgia, sinusitis, cough,	dysosmia, dgeusia	5040	2600	1.3	191
#4	none	otitis, rhinitis,	hyposmia, hypogeusia	2420	2220	5.9	149
#7	hyper-cholesterinemia	rhinitis, cough,	fever, dyspnea, hyposmia, hypogeusia	4690	900	4.9	209
#8	none	sinusitis, cough		2500	1600	1.7	203
#10	none	sinusitis, cough,	fever, cough	2350	700	7.8	220
#14	none	fever, cough, diarrhea		5040	1500	9.8	220
#16	none	none		4620	900	0.5	201

Abbreviations: ANC = absolute neutrophil count, ALC = absolute lymphocyte count, CRP = C-reactive protein, LDH = lactate dehydrogenase, M = male, F= female

- Two pts (Figure 2F,G)who showed some signs of lung infection were the only cases sputum viral loads showed a late and high peak around day 10/11
- Whereas sputum viral loads on the decline by this time in all other patients 2F, G)

4/9 pts loss of taste and olfactory sensation, and described be stronger and more long-lasting than in common cold diseases.

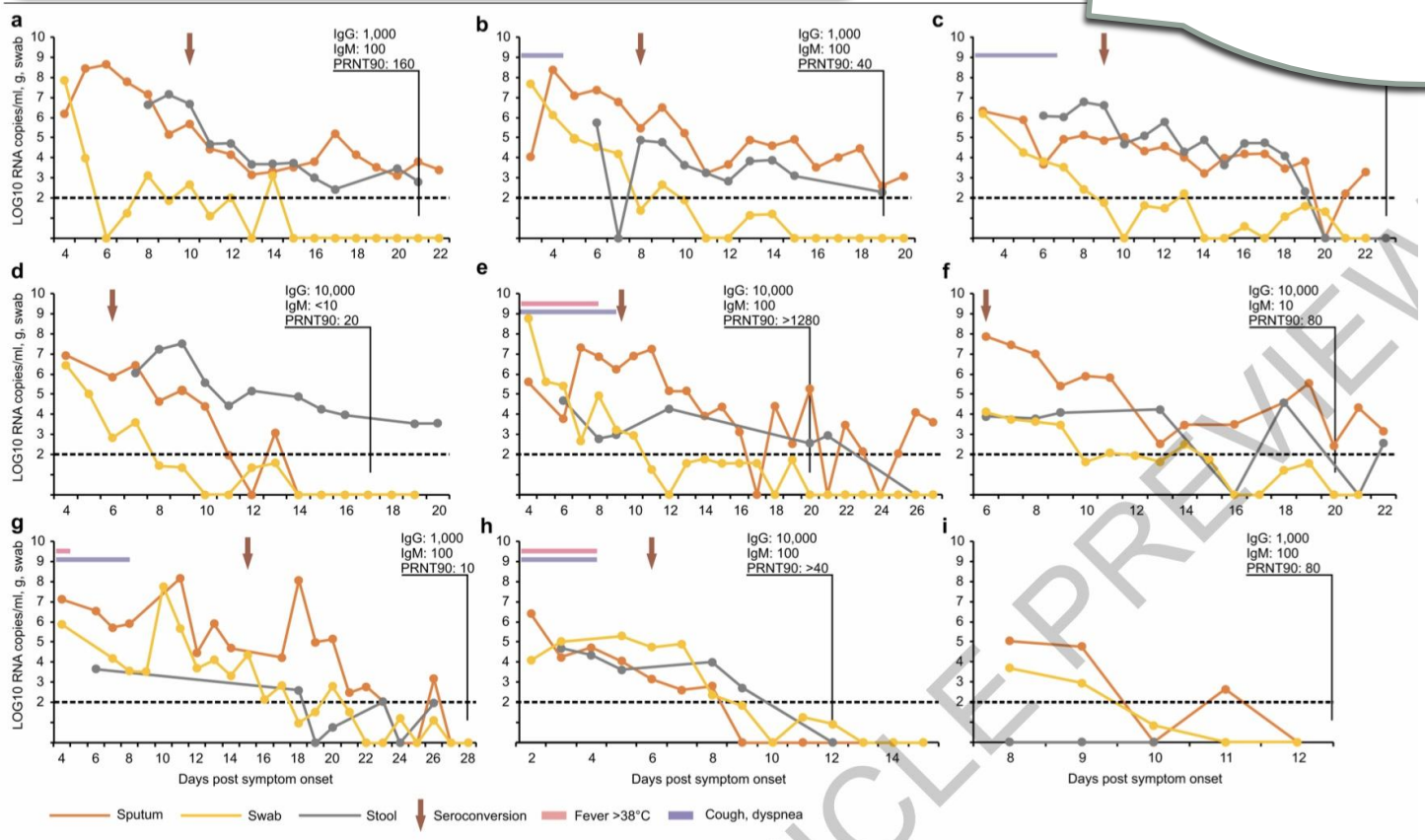

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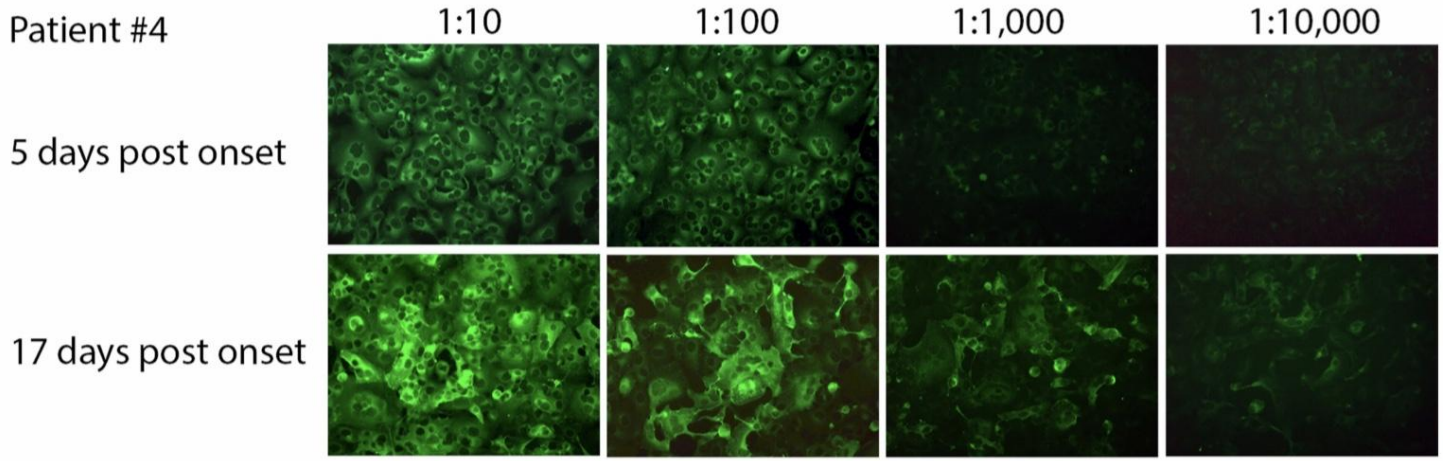
Table 3 | IgG and IgM immunofluorescence titers against SARS-CoV-2 of all patients

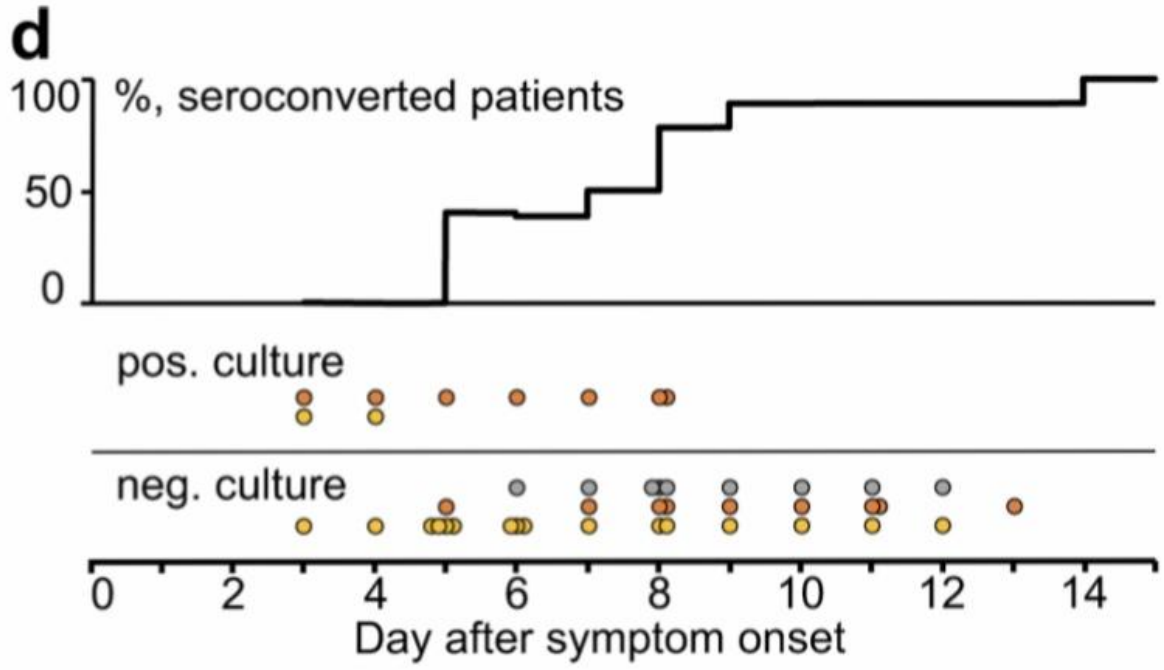
PATIENT ID	INITIAL SERUM		FINAL SERUM				
	Day p.o.	IgG	Day p.o.	IgG	IgM	$PRNT_{90}$	$PRNT_{50}$
#1	5	<10	21	1,000	100	160	>640
#2	4	<10	19	1,000	100	40	320
#3	3	<10	23	1,000	100	160	>640
#4	5	<10	17	10,000	<10	20	160
#7	6	<10	20	10,000	100	>1280	>1280
#8	6	10	20	10,000	10	80	>320
#10	6	<10	28	1,000	10	10	>40
#14	na	na	12	10,000	100	>40	>40
#16	na	na	13	1,000	100	80	>320

Seroconversion detected by IgG and IgM immunofluorescence using cells expressing the spike protein of SARS-CoV-2 **and** a virus neutralization assay using SARS-CoV-2

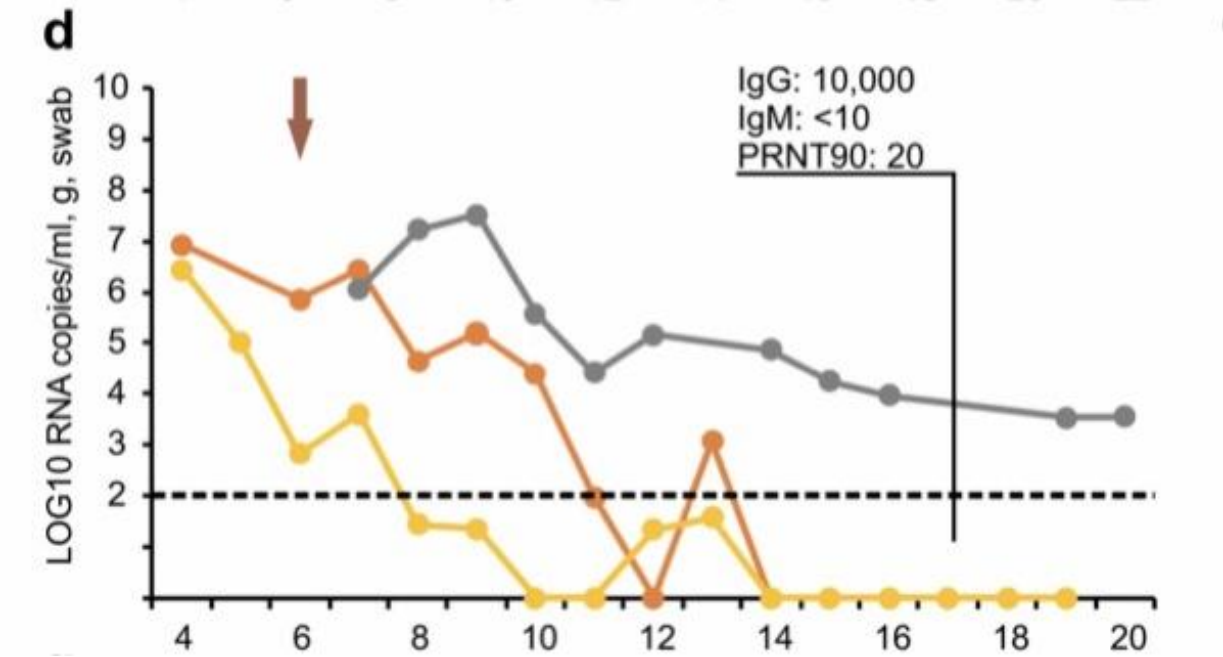


Extended Data Fig. 2 | Recombinant SARS-CoV-2 Spike-based immunofluorescence test shows seroconversion of patient #4. Representative outcome of a recombinant immunofluorescence test using serum dilutions 1:10, 1:100, 1:1000 and 1:10,000 of patient #4 at 5 and 17 days post onset of symptoms. Secondary detection was done by using a goat-anti human immunoglobulin labeled with Alexa488 (shown in green). Experiment was performed in duplicate.

- Seroconversion in 50% of patients occurred by day 7, all by day 14.
- No viruses were isolated after day 7
- All patients showed detectable neutralizing antibodies, the titers of which did not suggest close correlation with clinical courses



- Case #4, with the lowest virus neutralization titer at end of week 2, seemed to shed virus from stool over prolonged time



- Results on differential recombinant immunofluorescence assay indicated cross-reactivity or cross-stimulation against the four endemic human coronaviruses in several patients

Extended Data Table 1 | IgG immunofluorescence titers against endemic human CoVs

Patient ID	*Primary serum*					*Final serum*				
	Day p.o.	OC43	NL63	HKU1	229E	Day p.o.	OC43	NL63	HKU1	229E
#1	5	1,000	1,000	1,000	100	15	1,000	1,000	1,000	100
#2	4	1,000	1,000	100	100	13	**10,000**	100	**1,000**	10
#3	3	10,000	100	1,000	1000	16	10,000	**1,000**	**10,000**	1,000
#4	5	1,000	100	100	100	17	**10,000**	10	**1,000**	100
#7	6	1,000	100	1,000	1000	13	**10,000**	**1,000**	**10,000**	**10,000**
#8	6	1,000	100	1,000	1000	10	**10,000**	**1,000**	**10,000**	100
#10	6	1,000	100	100	1000	11	**10,000**	**1,000**	100	1,000
#14	na	na	na	na	na	5	100	100	100	100
#16	na	na	na	na	na	13	10,000	1,000	1,000	100

p.o. = post onset; na = not available; Increases of titer through the final serum are indicated by reciprocal titers in bold face

Conclusions

Study subjects

- Mild clinical courses
- Young- to middle-aged
- Without significant underlying disease
- Almost all were first tested when symptoms were still mild or in the prodromal stage

Conclusions

- Diagnostic testing: sensitivity of simple throat swabs is sufficient for diagnosing COVID-2019 infection, but not in SARS
- Peak viral load:
  - SARS-CoV: 7-10 days after onset
  - SARS-CoV-2 (present study): before day 5, and were more than 1000 times higher

Conclusions

Evidence for independent replication of SARS-CoV-2 in the upper respiratory tract

- Live virus isolation of SARS-CoV-2 from throat swabs is successful
- Presence of sgRNA transcribing cells, namely actively-infected cells, in throat swab samples
- Existence of a distinct virus in the throat from that in the lung in one of the patients

Conclusions

- Transmission of SARS-CoV-2 is more efficient than SARS-CoV through active pharyngeal viral shedding when symptoms are still mild
- SARS-CoV-2 also replicates in the lower respiratory tract later in the disease, as SARS-CoV does

Conclusions

- This study is limited in that no severe cases were observed
- Future studies including severe cases should look at the prognostic value of an increase of viral load beyond the end of week one

Conclusions

- One hypothesis to explain a potential extension of tropism to the throat is the presence of a polybasic furin-type cleavage site at the S1-S2 junction in the SARS-CoV-2 spike protein
- This leads to a gain of fusion activity that might result in increased viral entry in tissues with low density of ACE2 expression

Conclusions

Very high virus RNA concentrations

+

occasional detection of sgRNA-containing cells

higher detection rate as compared to MERS-coronavirus (14.6%)

⇒ **Active replication in the GI tract**

If…

- Passively present in stool → similar detection rates as for MERS-CoV
- Replication in the GI tract → SARS, CoV (regularly excreted in stool)

Failure to isolate live SARS-CoV-2 from stool may be due to…

the mild courses of cases, with only one case showing intermittent diarrhea

Conclusions

Initial results suggest …

measures to contain viral spread should aim at droplet-,

rather than fomite-based transmission

Conclusions

Prolonged viral shedding in sputum

1. Hospital infection control
2. Discharge management

- **Early discharge with home isolation** for

  > day 10 of symptoms + **< 100,000** **sputum viral RNA copies/ml**

- Both(based on cell culture) → little residual risk of infectivity

Conclusions

- **Seroconversion**

  similar to or slightly earlier than in SARS-CoV infection

  (during the **2nd week of symptoms**)

- As in SARS and MERS, IgM was not detected significantly earlier than IgG (technical reasons)

Conclusions

- **ELISA tests** as a screening test
- Neutralization testing is necessary

  for r/o cross-reactive antibodies directed against endemic

  coronaviruses

Conclusions

- **Viral load courses**

  no abrupt virus elimination at the time of seroconversion

  ⇒ slow but steady decline of sputum viral load

- In any case, vaccine → induction of antibody responses

  ⇒ induce particularly strong antibody responses in order to be effective