Evaluation of the suitability of the RT-qPCR technique for the detection of possible infection and infectivity of individuals with respect to SARS-CoV-2.

Expert opinion

By:

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Statement
I assure that I have prepared the expert opinion impartially and to the best of my knowledge and belief and based on valuable and official scientific and public sources.

Würzburg, 23.02.2023 Ulrike Kämmerer

Prefix

The polymerase chain reaction (PCR) is a fantastic molecular method for detecting minute traces of a sought-after nucleic acid (DNA or RNA, depending on the PCR variant) in the laboratory. This makes the technique a valuable aid in the analysis of gene patterns in minute samples in research but also in routine diagnostics such as forensic trace evidence, contamination monitoring of large batches of food and beverages, trace detection of unauthorized animal species e.g. in meat/sausage products (keyword horse meat in minced meat) or monitoring of blood products for viral genomes of HIV and hepatitis viruses.

However, this extreme sensitivity also makes the technique very susceptible to contamination (see "Phantom of Heilbronn" under point 3.5) or over interpretation of results if PCR is used as the sole criterion without any further context. Thus, a gene signature of a wanted person found at a crime scene can only be regarded as an indication and thus can neither prove with certainty that the person was personally present there, nor whether the gene trace found originates from a living or a dead person. For comparison: the gene detection by means of PCR is as sensitive as if an alcohol measuring device could still detect 0.0000000008- per mille of alcohol in the blood and pass it off as "alcoholized" and thus put a motorist in distress during a control, although he does not consume a drop of alcohol and also has no signs of alcohol consumption.

However, the technique of PCR (also RT-PCR or RT-qPCR), regardless of the extreme sensitivity, can only ever amplify and detect the gene fragment being sought, but whether this originates from a viable or replicable organism cannot be clarified with the technique.

The **r**everse **t**ranscriptase-**q**uantitative **p**olymerase **c**hain **r**eaction (**RT-qPCR**) used in the SARS-CoV-2 case search is a variant of PCR in which the starting material, RNA, is first transcribed into DNA and then amplified in PCR in such a way that light signals in each round of copying provide an indication of the amount of genome duplicated.

As the sole instrument for the diagnosis of an active infection or even infectivity with SARS-CoV-2, this molecular technique is already unsuitable for mass testing for numerous reasons. However, the PCR technique can be used to support a differential diagnosis in the presence of symptoms by detecting the gene signature of a possible pathogen, which can then be correlated by the clinician with the patient's symptoms. However, PCR must not and can never be the sole diagnostic tool for a possible disease.

In the discussion about the suitability of RT-qPCR for the identification of Covid-19 patients, a positive PCR test in an asymptomatic healthy person is often used to refer to this person as an "asymptomatic patient". However, according to generally accepted definitions of the term, a person without clinical symptoms ("asymptomatic") is neither infected nor a patient, see **ANNEX 1.** (definitions of terms) and points 1.5 and 1.6.

Outline:

1. Definition and description of important terms

1.1. Polymerase chain reaction (PCR)

In a **polymerase chain reaction** (**PCR),** a defined short piece of deoxyribonucleic acid (DNA; usually 100-1000 bases) is amplified using the enzyme polymerase. The piece of DNA to be amplified is narrowed down with the help of two very short single-stranded DNA segments, the "primers".

These **primers** usually consist of a defined sequence of 18-25 nucleic acid bases (the primer sequence) that specifically match the regions on the DNA that flank the section to be amplified. To ensure PCR specificity, these primers must explicitly match only this flanking region and no other region of a DNA.
With the aid of large gene databases and corresponding software programs (e.g. Primer Blast, https://www.ncbi.nlm.nih.gov/tools/primer-blast/), these primers can be selected highly specifically in the PCR design. Specialized companies then synthesize the molecular chains from the submitted primer sequences and deliver them to the PCR laboratory or the manufacturer of PCR kits. Here, these primers must then be tested with valid positive and negative controls under a wide variety of experimental conditions and optimized in use. This ensures that only the DNA to be searched for is detected and amplified with the primer pair used, and that no other similar DNA segments are detected.

Once the primers have been found and are proven to be specific, the DNA to be amplified can be mixed with the primer pair, various auxiliary chemicals and the polymerase enzyme in a reaction batch and the chain reaction started.

PCR procedure: This runs in cyclic repetitions of the following individual steps:

1. The mixture is boiled at over 90°C (denatured). This separates the DNA strands, which are usually present as a double strand, into single strands to enable the subsequent attachment of the primers.

2. During subsequent cooling to the so-called "**annealing temperature**", the primers can attach to their matching regions on the separated DNA strands. The binding of the primers, the annealing, only occurs in a narrowly limited temperature range, the so-called melting temperature. This depends mainly on the base composition of the primers and therefore their sequence will ideally always be chosen so that both primers have the same melting temperature of about 60°C. The annealed primers form the DNA strands. The attached primers form the starting point for the polymerase.

3. Starting from the primers, this polymerase completes the single-stranded DNA present due to heating back to a matching double strand (**elongation**) usually at approx. 72°C.

Due to the position of the two primers on the flanking sides of the sought DNA segment, the elongation reactions on the single strands are in opposite directions, since the polymerase always works in one direction only. At the end of this step, two identical double-stranded DNA molecules have now been created from an original double-stranded DNA, which are separated again by boiling, then amplified into four identical DNA molecules with the aid of the primer addition and the polymerase, and so on.

Each PCR cycle consisting of boiling-annealing-elongation causes a doubling of the DNA segment being searched for, so that the amplification takes place in the logarithm of 2 and thus an extremely high number of copies of the original starting material is available very quickly.

Thus, after 10 PCR cycles, one DNA strand already becomes 2^{10} = 1,024 DNA copies, after 20 cycles already more than 1 million (1,048,576) and after 30 cycles more than 1 billion (1,073,741,824) copies.

1.2. Quantitative PCR (qPCR)

In the **quantitative PCR** (**qPCR**) technique, as currently used worldwide mainly for the detection of genomic RNA from SARS-CoV-2, a third short piece of DNA, similar to the two primers, is used, which can bind appropriately in the middle of the sought DNA section, the "**probe".** Unlike the two primers, this probe is connected to two molecules, a fluorescent dye at one end and another molecule (quencher), which can prevent the emission of fluorescence as long as both are simultaneously (i.e. in close proximity to each other) on the sample. During the elongation step, the polymerase now degrades this probe. This separates the quencher and the fluorescence molecule can now emit its color signal. This color signal is detected and measured in the device performing the PCR (thermocycler). Thus, with each PCR cycle, more and more fluorescence signals are released according to the increasing number of copies, the probe "glows" more and more. And the curve of color signal intensity increases with each cycle. At a certain value, the curve then exceeds the background noise (threshold) and is considered positive. The number of cycles at which this threshold is exceeded is referred to as the **CT value** (CT stands for "cycle threshold").

The faster the fluorescence rises above the set threshold (low CT), the more starting copies of the sought DNA were present in the PCR approach. Since neither the primers nor the enzyme polymerase always work 100% specifically, in each PCR- approach, a fraction of non-specific DNA is also copied. Thus, the more cycles the PCR runs through, the greater is the risk that even these few non-specific reactions will then exceed the threshold value. Therefore, from a CT value of 40, a false positive signal due to non-specific starting materials must be assumed with the greatest probability. A reliable PCR should therefore require no more than 30-35 cycles to generate a clear "positive" signal; in the case of active infections with sought-after viruses, a sufficient number of cycles of 25-30 can be assumed (see also point 3.2.).

1.3. Reverse transcriptase (RT) reaction

The **reverse transcriptase reaction** (**RT**) is required if the starting nucleic acid to be amplified is not present as DNA but as ribonucleic acid (RNA), as is the case with SARS-CoV-2 as an RNA virus. Since only DNA can be amplified by PCR, RNA must first be converted into DNA.
This is done with the help of the enzyme "reverse transcriptase", which creates a complementary copy strand of DNA from RNA, which then serves as the starting material for PCR.

In order to evaluate the reliability of a result obtained by RT-qPCR or even PCR, the sensitivity and specificity of the test system used are assessed using defined samples of diluted correct target genes (e.g. RNA of the virus sought) and very similar but not sought target genes (e.g. closely related viruses).

1.4. Sensitivity and specificity

In the case of PCR (in all variations), sensitivity indicates how sensitively the test can detect even the smallest amounts of the target gene sought; **specificity** describes how reliably the test excludes the possibility of other, closely related genes also leading to a positive result (**false positive**). The higher the specificity, the more certain it is that false positive results will not be obtained by the PCR system itself.

However, this does not exclude **false positive events,** which can be caused by **laboratory contamination** with target genes, contamination **of test chemicals** and **contamination directly during sample collection.** These contamination-related false positive results can be excluded or at least greatly minimized by rigorous quality assurance and standard operating procedures (SOPs), the use of specially trained personnel and permanent external control in the form of interlaboratory comparisons (Round robin tests).

1.5. Infection

An infection is defined as a situation in which at least the following three aspects occur together:

- Penetration of microorganisms (germs) such as bacteria or viruses into the body
- These invaded microorganisms multiply in the body
- And the body reacts to them (symptoms)

According to the CDC, symptoms of infection with the SARS-CoV-2 virus include. (https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/symptoms.html)

- Fever or chills
- Cough
- Shortness of breath or difficulty breathing
- Fatigue
- Muscle or body pain
- Headache
- New loss of taste or smell
- Sore throat
- Stuffy or runny nose
- Nausea and/or vomiting
- Diarrhea

For additional source descriptions of the generally applicable definition of infection, see **ANNEX 1.**

1.6. Patient

A patient, by definition, is a person under the care of professional health care providers who exhibits symptoms of illness or injury or demonstrates other limitations of complete health.

Thus, by definition, a healthy "asymptomatic" person without medical problems cannot be called a "patient." For further source descriptions of this generally applicable definition of the term "patient", see **ANNEX 1.**

2. Basic information on the diagnostic significance with regard to the question "infectivity

The inventor of the PCR test, Nobel Prize winner Kary Mullis, who died in August 2019, repeatedly pointed out that his test is solely suitable for making a molecule (deoxyribonucleic acid, DNA) or fragment of DNA, which is otherwise invisible to the human eye, visible by amplification. But not to allow a statement on whether what has been made visible is dangerous or causes illness.

In particular, a PCR test - even if performed correctly - cannot provide any information on whether a person is infected with an active pathogen or not. This is because the test cannot distinguish between "dead" matter*, such as a completely harmless genome fragment as a remnant of the body's own immune system's fight against a cold or flu (such genome fragments can still be found many months after the immune system has "dealt with" the problem), and "living" matter, i.e. a "fresh", reproducible virus.

* For example, PCR is also used in forensics to amplify residual DNA from hair residues or other trace materials by means of PCR in such a way that the genetic origin of the perpetrator(s) can be identified ("genetic fingerprint").

2.1. Official statements of important official institutions / experts on the uselessness of PCR as the sole diagnostic test for the detection of infectiousness or risk of infection.

A) Explicitly, the information sheet of the Swiss Federal Office for Civil Protection FOCP Spiez Laboratory lists the disadvantage of PCR as follows: "*Only pathogens whose gene sequence is known can be detected.* ***Whether a pathogen is infectious (virulent, "alive") or not remains unknown.***
The Original page is in the meantime not more findable, but in the Web archive (https://web.archive.Org/web/20210706132230/https://www.labor-spiez.ch//pdf/en/dok/pos/88 021 Plakate PCRe.pdf) still available.
The document is attached as **ANNEX 2.**

B) A key statement about the suitability of the PCR test as a parameter for infectious disease transmission risk was made by Antony Fauci, leading "disease expert and government advisor" in the U.S., in a 12/30/2021 MSNBC broadcast (of The Rachel Maddow Show) (Washington DC 9:04 PM; https://www.youtube.com/watch?v=bAICMQ1D5F8; From minute 6:35)

Reporter's Question:
"...is a PCR test not a good parameter either for transmissibility and isolation? How can people actually tell if they are contagious in the cycle of having covid? How do you measure that if not with either a PCR test or an antigen test? "

Dr. Fauci
"Yes, that is a very good question ***because PCR doesn't measure replication competent virus*** *it measures viral particles, nucleic acid. So in other words I could be infected, have cleared the replication competent virus from me but* ***I can continue to be positive with the PCR for several days after recovering and not being transmissible at all****. So a PCR is good to tell you if you are - if I am infected yes I am infected but the very fact that it is positive - the CDC director said for several days and even weeks later* ***it doesn't give you any indication of whether or not you are transmissible.***
And I think that's the understandable confusion that people have about testing. Testing say whether you are infected or not versus are you infected plus transmissible.
The only way you can tell if it is transmissible is if you can show that there really is life replication virus in you and the test don't measure that *(7:38) They measure the presence or absence of the virus and the virus can be dead inactive virus that doesn't transmit*".

C) Also Marion Koopmanns, director of the Department of Viral Sciences at Erasmus University and expert advisor to WHO, thus one of the central virologists of the corona question and at the same time co-author on the RT-qPCR publication of Corman/Drosten in Eurosurveillance confirms in an interview with NPO Radio 1 (26.11.2020 in the context of her podcast "Virusfeiten" https://www.nporadio1.nl/podcasts/virusfeiten/46542/4-blijvend- moe-na-corona-misschien-helpt-een-aspirientje) **that PCR is not suitable about the stratus infectivity todecide.** (minute 0:09 in https://www.youtube.com/watch?v=flsF7trvq2c)

Explicitly the crucial passage of the interview with Marion Koopmanns (MK):

"*MK: ...there are some stories circulating that say, well, the PCR test is not good.*
Interviewer: At least it doesn't necessarily show that you're contagious.
MK: Yes, exactly. And that's also true. Because the PCR shows that you have the viral RNA with you. That's literally what the PCR does. ***And whether that RNA is in a virus particle that is still intact and also infectious. Or whether it's just residual RNA that can be detected long after infection. There is no way to distinguish between the two.*** *You can get a feel for it by looking up "How much is there?". But you can't tell that difference very well. That is: this test is great for saying "you've had it,"* ***but this test is less good for saying "at this point, you're still infectious."***
Interviewer: You are just talking about the PCR test, aren't you?
MK: Yes.

In the original:
"MK: ...er ciruleren wat verhalen waarin gezegd wordt, nou ja, de PCR test ist niet goed.
Interviewer: Althans die toont niet persee aan dat je besmettelijk bent.
MK: Yes precies. En dat klopt ook. Want de PCR toont aan dat jij het virus RNA bij je hebt. Dat is letterlijk wat de PCR doet. En of dat RNA in een virus deeltje zit dat nog intact is en ook besmettelijk is. Of dat het gewoon restjes RNA zijn, die je nog een tijd lang nadat iemand

geinfecteerd is geweest, kunt aantonen, dat onderscheid zie je niet. Je kunt een beetje een gevoel krijgen door te kijken "hoeveel is het?". Maar dat verschil is niet goed te maken. Dat betekent, die test is prima om te zeggen "je hebt het gehad", maar die test is minder geschikt om te zeggen "op dit moment ben je nog besmettelijk".
Interviewer: Over de PCR test heb je het nu, huh?
MK: yes"

D) The Swedish Ministry of Health states on its official website (https://www.folkhalsomyndigheten.se/publicerat-material/publikationsarkiv/v/vagledning-om-kriterier-for-bedomning-av-smittfrihet-vid-covid-19/): "*The PCR technology used in tests used to detect viruses cannot distinguish between viruses that are capable of infecting cells and viruses that have been rendered harmless by the immune system,* ***and therefore these tests cannot be used to determine whether or not someone is infectious.*** *RNA from viruses can often be detected weeks (sometimes months) after infection, but does not mean a person is still infectious."*

In the original
"PCR-tekniken som anvands i test fdr att pavisa virus kan inte skilja pa virus med fdrmaga att infektera celler och virus som oskadliggjorts av immunfdrsvaret och darfdr kan man inte anvanda dessa test fdr att avgdra om nagon ar smittsam eller inte. RNA fran virus kan ofta pavisas i veckor (ibland manader) efter insjuknandet men innebar inte att man fortfarande ar smittsam." This assessment was confirmed on 04/19/2021.

E) Also back in May 2020, a position paper was published by the National Centre for Infectious Disease in Singapore, pointing out in item 5 that it is important to note that detection of viral RNA by PCR is not associated with infectivity or viable virus equate (https://www.ncid.sg/Documents/Period%20of%20Infectivity%20Position%20Statement v2.p df).
From the original document:

"... ***it is important to note that viral RNA detection by PCR does not equate to infectiousness or viable virus***".

F) In a Nature publication describing the analysis of the first Covid-19 cases in Germany (https://doi.org/10.1038/s41586-020-2196-x), the authors (including R. Wölfel, C. Drosten, and V. Corman) identified the SARS-CoV-2-positive cases by TIB- Molbiol/Roche PCR for the detection of the E and RdRp genes and compared the PCR results with the gold standard: virus isolation in cell culture."

With regard to PCR, the authors establish their own detection system based on RT-PCR in order *"To obtain proof of active virus replication in the absence of histopathology, we conducted RT-PCR tests to identify viral subgenomic RNAs directly in clinical samples. (....) Viral subgenomic mRNA is transcribed only in infected cells and is not packaged into virions, and*

therefore indicates the presence of actively infected cells in samples".

This means that it was known and published very early on, that **the usual RT-qPCRs** detecting genomic RNA of SARS-CoV-2 do **not allow a decision on whether an actively replicating virus may be present in the sample**.

Thus, by this time at the latest, all WHO PCR recommendations should have been changed to detection of subgenomic RNA - which would be significantly better than genomic detection, but still only indicates the likelihood of virus replication at the RNA level and is not definitive evidence of an infectious virus.

G) In a review paper published on December 2, 2022 in Nature Reviews Microbiology (https://doi.org/10.1038/s41579-022-00822-w), it is explicitly pointed out in several places that RT-PCR is neither suitable for detecting infectious viruses nor for reliably diagnosing infectious (contagious) people. It is noteworthy that the senior author of this publication, Isabella Eckerle, is a close colleague of Christian Drosten.

Quotes in detail:

Introduction, first paragraph:
"*Although detection of viral RNA in respiratory specimens by RT-PCR is highly sensitive and specific,* ***it does not distinguish between replication-competent virus and residual RNA."*** *....*
"This is ***because viral RNA (which would be picked up by RT-PCR) remains detectable in the absence of infectious virus,*** *whereas positivity of Ag-RDTs better correlates with the presence of infectious virus.*"

Under the heading:" Detection of RNA viral load".
*"****Although RT-PCR cannot directly determine infectiousness owing to its inability to differentiate between replication-competent (infectious) virus and residual (non-infectious) viral RNA****, a correlation between RNA viral load and the presence of infectious virus has been sought."*

Under the heading, "SARS-CoV-2 diagnostic in public health."
"*Unfortunately,* ***no point-of-care diagnostic test currently exists to determine infectious SARS- CoV-2 in a patient sample****, and virus culture as described above is not suited for diagnostic purposes. Thus, a range of approaches have been suggested to find a proxy for infectiousness to guide isolation periods."*

Note: it should be noted, this statement is from December 2022, almost 3 years after the introduction of RT-qPCR for direct testing (point of care) of presumptively infectious individuals in testing centers and as the basis of incidences, R-values and resulting measures!!!! This statement is even explicitly reinforced again in the conclusion of this review:

In the Conclusions section:
"*Although much progress has been made during the pandemic in the field of diagnostics,* ***to date, no diagnostic tests exist that reliably determine the presence of infectious virus.***"

2.2. Sample preparation precludes detection of replication-capable viruses

Another important aspect in assessing whether an RT-qPCR test relates to a statement about the infectivity of a person who tests positive, i.e., the extent to which the positive RT-qPCR result indicates the presence of replicable viruses, is the preparation of the sample for RT-qPCR.

The RNA of the gene sought (here: SARS-CoV-2 virus genome) must be isolated from the smear material in order to be usable for gene detection in RT-qPCR. A crucial step in this process is the complete denaturation of all biological material and separation of the main components protein, lipids and nucleic acids in order to finally have the RNA available as a starting base for RT-qPCR. The original protocol by Chomszynski and Sacci from 1987 (https://pubmed.ncbi.nlm.nih.gov/2440339/; https://pubmed.ncbi.nlm.nih.gov/17406285/) is still a component of almost all protocols for the purification of biological material for RNA isolation, whether produced in the laboratory or in purchased "extraction kits". Components of the original extraction solution are phenol/chloroform and isoamyl alcohol, and in various modified commercial solutions similar acting but less toxic substances. All have in common that they completely destroy any living or reproducible biological structure.

This means: **in the laboratory process of preparing a smear sample, which is mandatory preceeding the RT-qPCR, any biological material, be it a vital cell, a replicable virus or even just cell debris and gene residues, is denatured in such a way, that it is no longer possible to say whether the material originates from an intact or even replication competent organism or from samples that have already been damaged or destroyed.** Due to this extraction and preparation process, a positive RT-qPCR that detects genome fragments cannot be used to infer the presence of replication-capable viruses in the smear sample; only the isolated RNA can be detected, regardless of the source.

2.3. Interim Conclusion:

Thus, even if the PCR, including all preparatory steps (PCR design and establishment, sample collection, preparation and PCR performance), is carried out "correctly", and the test is

positive, i.e.: detects a genome sequence which may also exist in one or even the specific "Corona" virus (SARS-CoV-2), **this technique can under no circumstances prove that the person who tested positive could be infected with a replicating SARS-CoV-2 and consequently infectious = dangerous for other persons.**

A better plausibility of a positive PCR result with regard to a virus infection and especially a virus replicating in the tissue would have been the detection of the subgenomic RNA, already described in the publication mentioned under point 2.1.F. The detection of the subgenomic RNA would have been a better proof of a virus infection. **Since this sgRNA only appears during the formation of new viruses in an infected cell, it can at least be used as an indication (indicator) if not as proof of an active virus infection.** For this purpose, the publication listed under point 2.1.G very well describes under the point "SARS-CoV-2 diagnostics in public health":

"One example is the detection of sgRNA transcripts, which are generated during virus replication, and specifically the synthesis of negative-strand RNA. Although sgRNAs are transcribed in infected cells, they are not packaged in the virions and can therefore ***serve as an indicator of active replication and thus of infectious virus.*** *Specific RT-PCR assays were developed to detect sgRNAs in addition to the diagnostic detection of genomic SARS-CoV-2 RNA,* ***but such assays have not made their way into routine diagnostic use owing to their lower sensitivity than conventional RT-PCR assays****.*
[...]
Thus, although the absence of sgRNA would indicate absence of viral replication, ***the presence of sgRNA does not necessarily indicate infectiousnes"***

In general, for the detection of an active infection and the assessment of whether a person "produces" and releases infectious viruses, further, concrete diagnostic methods such as the isolation of interrogable viruses must be used in addition to the clinical symptom assessment (gold standard). The following correct statement can be found under the heading "Detection of infectious virus" in the publication listed in section 2.1.G:

"***The gold standard for determining the presence of infectious (that is, replication competent) virus in respiratory specimens is the recovery of virus in cell culture, a procedure that is commonly termed virus isolation****."*

2.4. Figure

For a better overview, Figure 1 is taken from this work (https://doi.org/10.1038/s41579-022-00822-w), in which the various detection methods are shown visually. The detection of viral nucleic acid (RNA) by means of RT-qPCR (referred to here as qRT-PCR), the detection of viral protein components by means of the antigen rapid test (lateral-flow assay) and the detection infectious viruses by means of different isolation methods in cell culture.

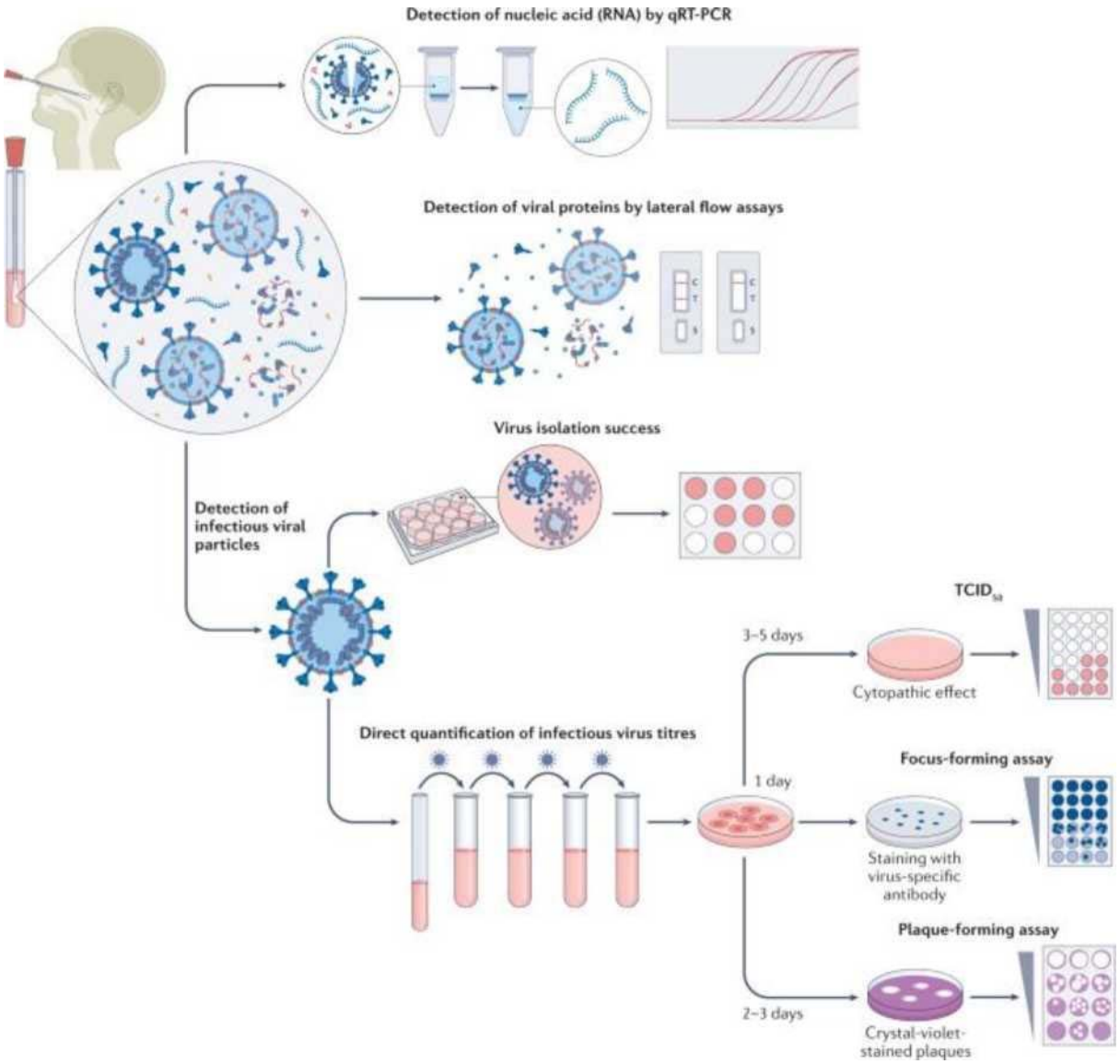


Fig. 1 | Methods to measure infectious virus and RNA viral load. Swab specimens from the nasopharynx or oropharynx are used for detection of SARS-CoV-2 viral loads. Detection of viral nucleic acids (RNA) is performed by quantitative real-time PCR (qRT-PCR). Viral RNA is extracted from lysed virus, reverse transcribed and amplified by qPCR using primers specific for one or more target regions in the viral genome. The amplification cycle at which samples cross the threshold (cycle threshold) defines the amount of viral RNA. RNA viral load can be expressed as the number of viral RNA copies per millilitre, or y the arbitrary test-specific cycle threshold value. Lateral flow assays detect the presence of specific viral proteins in the lysed viral particles. SARS-CoV-2 nucleocapsid is used in most antigen-detecting (rapid) diagnostic tests. The presence of infectious (replication-competent) virus in respiratory specimens can only be determined by the recovery of virus in cell culture by isolation or by quantification of infectious virus titres using 50% tissue culture infectious dose (TCID50), focus-forming assays or plaque-forming assays. Virus isolation is performed by applying infectious medium on the monolayer of cells; isolation success is determined by the presence of a cytopathic effect approximately 3–5 days post-infection. White colour indicates the presence of a cytopathic effect in cells. For quantification of infectious virus titres, serial dilutions of respiratory samples are performed and used for inoculation on the monolayer of cells. In TCID50, 3–5 days post-infection, viral-induced cytopathic effect is classically defined using microscopy. In focus-forming assays, cells are fixed 1 day post-infection and immunostaining with virus-specific antibodies is performed to detect groups of infected cells (foci). The foci, indicating the presence of infectious virus, are displayed in blue. In plaque-forming assays, plates are fixed 2–3 days post-infection and stained with crystal violet; wells with individual plaques are used to determine viral titres. The plaques, indicating the presence of infectious virus, are displayed in white.

3. Factors influencing the reliability of the PCR test

In fact, however, the results of a PCR test depend on a number of parameters which, on the one hand, cause considerable uncertainties and, on the other hand, can be influenced in such a way that many or few (apparently) positive results are obtained.

3.1. PCR design and specificity

A PCR can be designed very comfortable with the help of databases (e.g. Genbank, GISAID) and established software (so-called primer search programs). Here, the objective (what is to be detected and how precisely) determines the requirements for the target region used and the accuracy of fit of the primers.
If a rough search for representatives of a large group of similar viruses (e.g. all coronaviruses) is to be aimed for, the primers are placed in so-called conserved group-specific regions. This is similar to telling software to reliably detect all red cars in a parking lot (but not trucks, motorcycles, or any other colors). This can be used to search a sample to see if there are any gene traces at all from a representative of the virus group being searched for (e.g. coronaviruses).
If one wants to find only one subgroup (e.g. sarbecoviruses within the coronaviruses), corresponding to a car brand within the group of all red cars (in the example), then the primers can be designed subgroup-specific. This was used in the case of the WHO recommended PCR from Charite for the so-called "E gene" but also for the RdRp-from Corman/Drosten to the group of Sarbeco viruses, to which SARS-CcoV-2 belongs. However, it is also possible (not always, but often) to design a highly specific PCR that detects and amplifies ONLY the specifically sought gene (in this case, viral gene of SARS-CoV-2).
Analogous to this would be the detection of a specific model within the group of red cars of a brand in the example. To do this, the design must include that the region being used has no homologies to closely related viruses (e.g., SARS1 or bat viruses to SARS-CoV-2) or even any other already known gene signature (human genome, other organisms).
The search programs can do this very reliably. In qPCR, a third piece of the gene is added, which is specifically allowed to react only with the gene region being searched for, this is the "sample".

In this respect: a good PCR design includes for normal PCR two and for qPCR three highly specific primers/probes recognizing only the desired target sequence and can then indeed detect highly specific only the desired target gene.

Such a specific PCR design would also have been possible for SARS-CoV-2, but was omitted from the original and authoritative WHO protocols.

After design**, it is then mandatory to verify the primer pair and probe on real samples in the**

laboratory. For this purpose, safe samples with the genetic material to be identified (here: Samples of SARS-CoV-2 infected persons or even a completely characterized virus isolate) are required, as well as a broad spectrum of viruses and other pathogens, which in principle can cause similar symptoms in patients and which must not give a signal with the PCR used.

If all these conditions are met: The PCR exactly finds the target gene sought by the designer - either as intended of a group or just highly specific of a single pathogen - and this is experimentally proven beyond doubt with comprehensive positive and negative controls in the laboratory, then this design can also be reliably used as specific as a protocol to support a clinical diagnosis.

3.2. Number of independent target genes ("targets")

The protocol "***Diagnostic detection of Wuhan coronavirus 2019 by real-time PCR***" (https://www.who.int/docs/default-source/coronaviruse/wuhan-virus-assay-v1991527e5122341d99287a1b17c111902.pdf), originally published by WHO on Jan. 13, 2020, describes the sequence of PCR detections of three independent subgenes of the virus later renamed SARS-CoV-2. The sequence referred to the E gene, the RdRp gene, and then the N gene.

Already on 17.01.2020 a change followed by the WHO with the protocol. "***Diagnostic detectionof 2019-nCoVby real time PCR***" (https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c2) in which the N gene was removed as a detection and thus only two gene segments were recommended instead of the original three target regions.

On March 02, 2020, an again updated test protocol of the WHO "***Laboratory testing for coronavirus disease 2019 (COVID-19) in suspected human cases***" (https://apps.who.int/iris/bitstream/handle/10665/331329/WHO-COVID-19-laboratory-2020.4-eng.pdf?sequence=1&isAllowed=y) indicated that *". ..In areas where COVID-19 virus is widely spread, a simpler algorithm might be adopted in which for example screening* ***by RT-PCR of a single discriminatory target is considered sufficient***....." (bottom of page 3) whereupon laboratories large-scale switched to analyzing only one target gene **and specialized only on the E-gene as group-specific valid PCR**, as e.g. explicitly described by the Augsburg laboratory on 03.04. (only available in the internet archive: https://www.oder-spree- piraten.de/wp- content/uploads/2020/05/Ge%C3%A4ndertes-Befundlayout-der-SARS-CoV2- PCR-Results- - Labor-Augsburg-MVZ-GmbH.pdf

The outstanding importance of the number of independent target genes analyzed by PCR in non-specific single tests as in the protocol of the Charite preferably recommended by the WHO (and very quickly applied worldwide via TIB MolBiol/Roche) results from the following exemplary calculation:

The three target regions E, RdRp and N gene originally specified in the WHO protocol for the detection of SARS-CoV-2 were rapidly used in many laboratory and commercial test systems. A first interlaboratory test from Institut Instant e.V. (https://corona-ausschuss.de/wp-content/uploads/2020/07/Instand-Interlaboratory-Test-Virus-Genome-Detection- SARS-CoV- 2.pdf) showed a mean specificity for these genes of:

Target gene of the SARS CoV-2 Genomes	Number of test kits checked	Specificity cell culture only (without viral RNA)	Specificity with related coronavirus (HCoV 229E)	%	Mean specificit y absolute	Mean error rate (1-abs. Spec.)
E-Gen	24	99,46%	95,17%	97,31	0,9731	0,0269
RdRp gene	13	97,80%	90,66 %	94,23	0,9423	0,0577
N gene	21	98,20%	87,95 %	93,08	0,9308	0,0692

In a mixed population of 100,000 tests, even no genuinely infected person would result because of the mean error rate:

For an E-only gene test: 100,000 x 0.0269 = 2690 false positives
For E and RdRp tests in sequence: 100,000 x (0.0269 x 0.0577) = 155 false positives
For all three genes (E, RdRp, N): 100,000 x (0.0269 x 0.0577 x 0.0692) = **10** false positives

This means that the WHO's requirement in March 2020, i.e. before the official pandemic declaration, to successively reduce the number of target genes to be tested while maintaining the original PCR requirements ("Corman/Drosten protocol") of SARS-CoV-2 from 3 to 1, resulted in an increase in the number of false positives in the above calculation example from 10 with 3 genes to almost 2700 with only the E gene per 100,000 tests performed.

If the 100,000 tests performed were representative of 100,000 citizens of a city/county within 7 days, this important question of the number of target genes used in one test would result in a difference of 10 versus 155 versus 2690 with respect to the "7-day incidence" and, depending on this, the severity of the restrictions on citizens' liberty taken.

Interim evaluation: The calculation example shows how the daily case numbers can be influenced by "playing to the specifications" regarding the target genes to be detected for the laboratories. In view of the immense impact on political decisions, which are determined by the absolute numbers of positive tests and the "7-day incidence" derived from them, the specification of the WHO (and also of the RKI) for the reduction of target genes has clearly been suitable for artificially inflating the "pandemic" by a factor of up to 300 through incorrect test specifications.
This is an evidence-free approach, which on the one hand entails enormous personal restrictions of quarantine/isolation, which the falsely "positively tested" persons must suffer, and on the other hand willingly accepts the enormous social and economic restrictions and damages via the "7-day incidence number" and ultimately also forms the basis for the "need for vaccination".
If the correct number of three or even better (as originally used in Thailand, for example) up to 6 genes had been consistently used for PCR analysis, the rate of positive tests and thus the "7-day incidence" would have been reduced almost completely to zero.

3.3. Number of cycles performed (CT value) in qPCR.

"***Infectious individuals typically have RNA viral loads of >10^6 genome copies per millilitre, which largely corresponds with a Ct of 25 in most RT-PCR assays.***"

This definition of a meaningful CT value is explicitly made in the review paper already cited in 2.1.G, which appeared in Nature Reviews Microbiology (https://doi.org/10.1038/s41579- 022-00822-w) on 02.12.2022, in the chapter "SARS-CoV-2 Diagnostics in public health" and thus usefully summarizes the aspects presented below.

3.3.1 Meaning of the CT value

In addition to the number of target genes detected, especially in the case of only one or a maximum of two genes, the number of cycles of amplification in the qPCR up to the "positive" result and the resulting CT value are decisive factors, regardless of the PCR design. The **smaller the CT value of a sample in a qPCR, the higher the initial amount of DNA in the sample**.
Under standardized conditions, this correlates with (in the case of viruses) the initial amount of viral genomes, the so-called viral **load,** which should ideally be expressed as "number of viral copies" per ml sample. This viral load also correlates in the case of SARS-CoV-2 with the cultivability of infectious viruses in cell culture as already published in March 2020. (Figure 1e in Wolfel et al., https://doi.org/10.1038/s41586-020- 2196-x) **Here,** a **minimum amount of 10^6 RNA copies/ml was necessary to be able to grow viruses from the sample accordingly**, in another work with the participation of C. Drosten from May 2021 even an average of 10^8 viruses in the sample were necessary for a positive cell culture necessary
(supplemental Figure S4 from https://pubmed.ncbi.nlm.nih.gov/34035154/).

In the latter work, it was also found that none of the 25,381 individuals tested had viral genomes per ml in the sample in the case of a determined viral load below 10^5 (Table S1), whereas RT-qPCR from the original protocol (Corman V et al., https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2020.25.3.2000045) can give a positive result already at about 4 copies per sample set (5µl corresponding to about 10^3 copies/ml), i.e. already by a factor of 1,000-10,000 earlier than in a sample with actually infectious virus load.

Also **commercial PCR test systems**, so-called kits, of which there are already about 630 different worldwide (as of May 2022) (https://www.finddx.org/covid-19/test-directory) sometimes show detection limits of less than 10 copies/reaction, e.g. kits of the company TIB-Molbiol (https://www.roche-as.es/lm pdf/MDx 53-0777 96 Wuhan-R-gene V200204 09155376001%20%282%29.pdf)- Point 5 “Specification". An example of the difference between detection capability and a reasonable detection limit is given in the **ANNEX 3** example.

In technical terms, a distinction must be made here between "contamination "* of the throat with a few individual viruses that do not cause infection and a genuine "infection". The latter is accompanied by viruses capable of multiplying, which then leads to a) symptomatic disease and b) infectivity, i.e. the ability to infect other persons. Note*: *on the term: "We have learned that, unlike with bacteria, there is no normal colonization with coronaviruses on the mucosa." (K. Henning in podcast 58 with C. Drosten)*

Christian Drosten already had described this **aspect of contamination versus true infection in** 2014 in an interview in the journal “Wirtschaftswoche” in connection with MERS (https://www.wiwo.de/technologie/forschung/virologe-drosten-im-gespraech-2014-die-who- can-only-recommend/9903228-2.html):
"*Yes, but the method* (note: PCR is meant*) is so sensitive that it can detect a single hereditary molecule of this virus. If such a pathogen, for example, flits across the nasal mucosa of a nurse for just one day* (note: this would be the above-mentioned "contamination") *without getting sick or noticing anything else, then suddenly she is a Mers case. Where previously deathly ill people were reported, now suddenly mild cases and people who are actually perfectly healthy are included in the reporting statistics."* [...] *"Because what is of interest first are the real cases* (note: these are the "infected"). ***Whether symptomless or mildly infected hospital workers are really virus carriers is, I think, questionable. Even more questionable is whether they can pass the virus on to others."***
The latter is a crucial statement also with respect to the SARS-CoV-2 viruses, which are very closely related to MERS.

Original text:

„Ja, aber die Methode (Anmerkung: gemeint ist die PCR*) ist so empfindlich, dass sie ein einzelnes Erbmolekül dieses Virus nachweisen kann. Wenn ein solcher Erreger zum Beispiel bei*

einer Krankenschwester mal eben einen Tag lang über die Nasenschleimhaut huscht (Anmerkung: das wäre die o.g. „Kontamination“)*, ohne dass sie erkrankt oder sonst irgend etwas davon bemerkt, dann ist sie plötzlich ein Mers-Fall. Wo zuvor Todkranke gemeldet wurden, sind nun plötzlich milde Fälle und Menschen, die eigentlich kerngesund sind, in der Meldestatistik enthalten.“* [....] *„Denn was zunächst interessiert, sind die echten Fälle* (Anmerkung: Das sind die „Infizierten“). ***Ob symptomlose oder mild infizierte Krankenhausmitarbeiter wirklich Virusträger sind, halte ich für fraglich. Noch fraglicher ist, ob sie das Virus an andere weitergeben können.“***

But it is precisely this point of virus transmission (and thus pandemic drift) that is the rationale for intervening measures such as quarantine/isolation orders that have "Lockdowns" and the so-called AHA rules.

3.3.2 Evidence for the relevance of the CT value

A) A Canadian study by Jared Bullard/Guillaume Poliquin in Clinical Infectious Deseases 2020, which can be read at the link (https://doi.org/10.1093/cid/ciaa638), came to the conclusion as early as May 2020 that no reproducible virus was found above a CT value of 24 - this means that the attempt to subsequently cultivate reproducible viruses from smear samples that only resulted in a positive test at a higher CT value failed. According to this study, above a CT value of 24, the amount of detectable viral genetic material is so low that the positive test could no longer be interpreted in terms of an active infection.

B) A large study by Jaffar et al. (Doi 10.1093/cid/ciaa1491) set the limit for the cultivability of SARS-CoV-2 from patient sample material at a CT value of 30.

C) In a study comparing antigen testing/RT-qPCR and virus culture from the CDC (https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciab303/6224406), successful virus culture was described for a CT range of 17.4-28.8), with only below a CT of 25 all specimens were from symptomatic individuals and associated with successful virus culture, and only 18.2% when the CT was between 25 and 29.

Quote: "*Virus was isolated from specimens with Ct values ranging from 17.4-29.8; virus was isolated from all specimens with a Ct value <25 and from 18.5% (5/27) of specimens with a Ct value >25* (center of page 9). However, irrespective of this check using virus cultivation, all samples that were positive in two target sequences from the "N gene" with a Ct up to 40 were considered "true positive".

D) In his NDR podcast of Feb. 16, 2021, C. Drosten explicitly named that an increase in CT from 25-27 across the border of 28 means that individuals from whom these smears were obtained with the higher CT are no longer infectious. "*And again, you see a Ct shift from 25 to 27*

approximately, 27, 28. And that's ***a range where****, in our estimation,* ***that's really where infectivity ends****. If you see such a patient sample and you would ask, is the patient still infectious, I would say:* ***No, this is now slowly no longer an infectious area****. You can correlate that.*" Page 4, top right column in:
https://www.ndr.de/nachrichten/info/coronaskript270.pdf

In original:

„und auch hier ist wieder eine Ct-Wertverschiebung von 25 auf 27 ungefähr, 27, 28 zu sehen. Und das ist ein Bereich, da ist nach unserer Einschätzung wirklich die Infektiosität zu Ende. Wenn man so eine Patientenprobe sieht und man würde fragen, ist der Patient noch infektiös, da würde ich sagen: ***Nein, das ist jetzt langsam nicht mehr ein infektiöser Bereich****. Das kann man korrelieren“*

With these CT data, C. Drosten presumably refers mainly to a study on vaccine efficacy in Israel, which was verified by RT-qPCR.
This study with the title "Initial report of decreased SARS-CoV-2 viral load after inoculation with the BNT162b2 vaccine (https://www.nature.com/articles/s41591-021- 01316-7) is also referred to in a letter from the RKI (AZ: ID3176 dated 31.03.2021) to the Federal Ministry of Health. In this study, PCR testing after vaccination (with BNT162b2) shows that in vaccinated subjects who became positive for SARS-CoV-2 in PCR from day 12 after the first vaccination, the CT for the three tested genes (E, N, RdRp using the Seegene Allplex test kit, which has a specificity of 96-98.4% according to Instant EQA 340) **increases** from a **mean CT of 25 to a mean CT of 27.**

Compared to a similarly SARS-CoV-2 PCR positive unvaccinated cohort, **this study establishes vaccination success based on a CT decrease of 1.64-2.33!!!**

In the original: "*Finally, applied on all infections (post-vaccination and unvaccinated, n=5,794), a multivariate linear regression model accounting for age, sex and vaccination quantify* ***Ct regression coefficients ranging from 1.64 (N gene) to 2.33 (RdRp) for vaccination after 12 days*** *or longer prior to infection sampling*", which is mathematically equivalent to a 4-fold reduction in viral load in vaccinated versus unvaccinated. In the original, *"As a difference of 1 Ct unit is equivalent to a factor of about 1.94 in viral particles per sample, these Ct differences represent a viral load ratio ranging from 2.96 to 4.68*."
It is also noteworthy that the CT values up to 40 were analyzed and evaluated in the PCR analyses (Extended Data Figure 4 of this publication).

E) Accordingly, the CDC also addresses the CT in SARS-CoV-2 PCR in a recommendation dated April 16, 2021, to the effect that it should have a value of no more than 28 in order to avoid PCR products from "vaccine breakthroughs" (i.e., RT-qPCR positive individuals after complete Vaccine) to be sent to the laboratory for sequencing (https://www.cdc.gov/vaccines/covid-19/downloads/Information-for-laboratories-COVID-vaccine-breakthrough-case-

investigation.pdf).

F) A study from South Korea mentions a CT of < 25 as the upper limit of clinically relevant "positive" and uses this value for comparison with the quality of antigen tests. Original quote: " *[....] based on a **clinically significant Ct value of < 25** [....]*" (p.3 in: https://jkms.org/DOIx.php?id=10.3346/jkms.2021.36.e101)

G) Unanimous scientific opinion (including from Dr. Fauci of the US CDC, but also from a number of scientists quoted in the New York Times in August 2020, https://www.nytimes.com/2020/08/29/health/coronavirus-testing.html) is **that all "positive" results, which are only detected from a cycle of 35**, **have no scientific (i.e.: no evidence-based) basis.** The RT-qPCR test for the detection of SARS-CoV-2, on the other hand, which was propagated worldwide with the help of the WHO, was (and following it all other tests based on it as a blueprint) set to 45 cycles without defining a CT value for "positive".

H) Also as early as May 2020, the National Center for Infectious Diseases in Singapore a Position paper (https://www.ncid.sg/Documents/Period%20of%20Infectivity%20Position%20Statementv2.p df), issued, which points out that

1. "*It is important to note that viral RNA detection by PCR does not equate to infectiousness or viable virus*".

2. The cycle threshold value (CT) of the PCR, as a **surrogate marker** for the viral RNA content, already detects viral RNA from a **CT of 30,** but no longer the presence of replicable viruses and the affected persons are not infectious.

Original text excerpt:
*"A surrogate marker of 'viral load' with PCR is the cycle threshold value (Ct). A low Ct value indicates a high viral RNA amount, and vice versa. As noted above, **detection of viral RNA does not necessarily mean the presence of infectious or viable virus**. In a local study from a multicenter cohort of 73 COVID-19 patients**,** when the Ct value was 30 or higher (i.e. when viral load is low), no viable virus (based on being able to culture the virus) has been found."*

I) Also the RKI explains on its homepage already to the status 11.08.2020

(https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Vorl_Testung_nCoV.html #doc13490982bodyText4)
"*Initial results from diagnostics at RKI show that loss of growability in cell culture was associated with an RNA amount of <250 copies/5 µL RNA determined by real-time PCR (*note: RT- qPCR*). This RNA- concentration corresponded to a Ct value >30 in the test system used.*"

Original quote: *"Erste Ergebnisse aus der Diagnostik am RKI zeigen, dass der Verlust der*

Anzüchtbarkeit in Zellkultur mit einer per real-time PCR (Anmerkung: ist die RT-qPCR) ermittelten RNA Menge von <250 Kopien/5 µL RNA einherging. Diese RNA-Konzentration entsprach im verwendeten Testsystem einem Ct-Wert >30.“

J) A study from South Korea (https://www.nejm.org/doi/full/10.1056/NEJMc2027040) defines the cutoff for virus cultivability at a CT value of 28.4.

K) And in a study from Frankfurt (https://www.mdpi.Com/2077-0383/10/2/328), it was shown that of 64 RT-qPCR positive patient samples (one gene tested), virus cultivation in cell culture was only possible from 33 (=52%). These infectious samples were already positive up to a mean CT value of 26 (Supplementary Figure 1), whereas virus cultivation was no longer possible from the samples with a higher CT.

L) The threshold CT 25 was already introduced in December 2020 by the English "Office of national statistics (ONS), here with CT above 25 as negative. Table sheet 2 (Data) in the linked Excel data sheet (link below). Results: *"The analysis shows: - People with a higher concentration of viral genetic material (positive cases with low Ct values; below 25) are more likely to be infectious in a household than those with lower concentrations (positive cases with high Ct values; above 25)."*
(https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/adhocs/12683coronaviruscovid19infectionsurveycyclethresholdandhouseholdtransmissionanalysis).

M) With reference to this ONS threshold, the authors of a large cohort study from Munster, Germany, (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8166461/), which was performed in summer of 2021 using RT-qPCR of the ORF-1ab and E genes in swab samples from 162,457 individuals, concluded: "*RT-PCR test positivity should not be taken as an accurate measure of infectious SARS-CoV-2 incidence.*"

This study showed that a total of 2.6% of the samples tested had a positive RT- qPCR result. The CT threshold above which samples were considered to be definitely negative was set very high at 40. The samples were also analyzed according to the number of samples, which became positive up to a threshold of 25 (always both genes, personal information on request to A. Spelsberg, a co-author).

The results showed that among **asymptomatic** individuals, a total of only 0.4% (68 of 16,874 individuals) had a positive RT-qPCR test with a mean CT of almost 29. Of these, only 27% (= 18 persons) had a CT up to 25, which *the* authors considered "*indicating a likelihood of the person being infectious*." This means that in only 18 of 16874 (=0.1%) asymptomatic (healthy) persons the PCR indicated a possible infectivity with respect to SARS-CoV-2.

Also, of 6212 **symptomatic** individuals from the peak periods of the first two "Corona waves", only 403 individuals (=6.5%) had a positive RT-qPCR for SARS-CoV-2 with a mean CT of 27.8 (1st wave) and 26.6 (2nd wave). Of these positives, a maximum (in the 2nd wave) of 40% (= 145/367) and in the first wave even only 26.5% (=10/36) individuals had a CT of up to 25 and could thus be classified as probably infectious. **Consequently, only 155 of 6212 symptomatic (ill) persons (=2.5%) could be assumed to be possibly infectious with SARS-CoV-2.**

N) In the Covid-19 Infection Control Study from England (https://elifesciences.org/articles/64683), the authors analyzed the results of SARS.CoV-2 PCR testing (3 genes, commercial test from Thermo Fisher) in 3.3 million cases. Overall, 0.83% test positive smears were found with a mean CT value of 29.2, whereby it was sufficient if one gene of the three target genes tested, the N gene, was positive alone and also without a defined CT limit. Here, "low evidence" for infection included those individuals who were asymptomatic and had only 1 gene with a CT of 34 or higher, whereas the "very likely" cases were scored as having symptoms and two or three positive target genes.
Interestingly, low CT (24.9) correlated with later antibody detectability in serum (indicating true infection) whereas test results with high CT values (above 33) were usually associated with no antibody response in affected individuals, indicating a lack of true infection.

O) A comparison of commercial PCR test kits (http://www.finddx.org/covid-19/pipeline/?section=molecular-assays#diagtab.), the enormous range of CT values even with highly standardized samples between the different test kits and also with regard to the different target genes becomes apparent. The results of the various interlaboratory studies of Instand e.V. would also be very exciting, but are largely not publicly available and are also kept under lock and key upon request according to the Freedom of Information Act (https://fragdenstaat.de/anfrage/herausgabe-der-auswertung-des-ringversuchs-der-gruppe-340-termin-4-2020/#nachricht-533736) Here, for example, in a submitted PDF of the test series from June/July 2020, the CT varies for the same defined diluted sample of SARS-CoV-2 (sample number 340061) for the WHO-recommended genes ranged from 15-40 (E gene), 20-40.7 (N gene) and 19.5-42.8 (RdRp gene). This impressively demonstrates an extreme lack of test standardization within the participating (and certified) laboratories.

P) In the evaluation of two standardized WHO controls for RT-qPCR by several laboratories and different RT-qPCR approaches, (Bentley E, WHO/BS/2020.2402 available: https://www.who.int/publications/m/item/WHO-BS-2020.2402) showed for the control sample 20/138 (a synthetic virus sample with the Wuhan1 sequence, black line in the figure) that a genomic quantity of $10^{6,73}$ (correlation to potentially infectious viral load) on average at a CT of 23-24, a genomic quantity of $10^{5,73}$ (below potentially infectious viral load) was considered positive at a CT of 25.5-26.5, so that with this approach the potentially infectious viral load of 10^6 viral genomes would lie in a range of CT23-26.5.

For an inactivated SARS-CoV-2 (sample 20/146, red line in the figure) with a defined amount of virus of $10^{7,7}$ in the stock solution, a positive RNA detection was already shown at a CT of below 20, at $10^{6,7}$ the CT was 22-23 and at $10^{5,7}$ the CT was 25.5 - 26, so that even for a defined sample with the SARS-CoV-2 viruses an equivalent to an infectious dose is already detected at a CT of 22 to 26.

The following is the authoritative figure from the publication page 63. The standardized RNA output amounts are given as 6.73 log10 IU/ml for sample 20/138 under point 3 (Unitage) on p. 66 and 7.7 log10 IU/ml for sample 20/146 on page 64 under point 3.

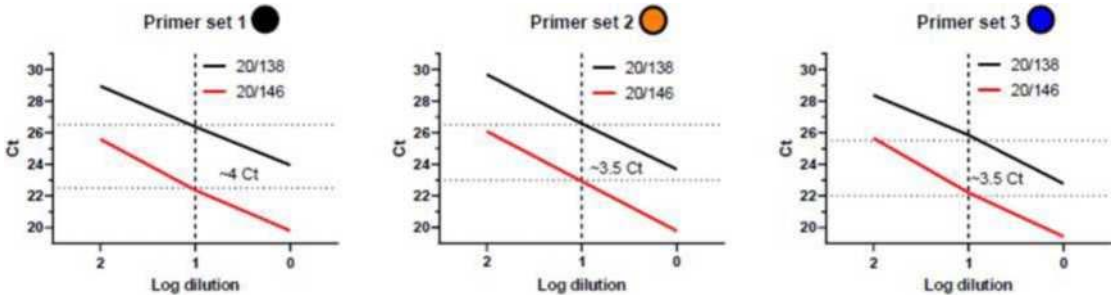


Figure 2. Relative potency of 20/138 compared to 20/146 by Real-time RT-PCR quantification using three primer sets. There is an approximate 0.5 Ct shift between the standard curves using primer set 1 which targets the region of lower coverage, in comparison to primer sets targeting the junction (primer set 2) and region of higher coverage (primer set 3).

3.3.3 Interim Evaluation:

Against this background of the points A-P listed above, it is disconcerting if RT-qPCR is still considered the "gold standard" by the RKI without defining the exact validations and external certification conditions and target values of CT (and without these apparently being fully monitored by the authorities).

In general, RT-qPCR cannot detect intact, replication competent (infectious) viruses, not even the complete intact viral genome, but only nucleic acid of the sought section, already due to the methodological procedures. It is generally possible to define a threshold value (CT) for well adjusted and correctly performed PCR tests **by validation with a parallel virus cultivation in cell culture,** above which a positive PCR signal no longer correlates with augmentable viruses. This has been a well-practiced routine in blood product monitoring for years.

This stringent validation then allows - as long as the test system is NOT changed - as a surrogate marker an estimation of the viral load and thus the possible infectivity of the tested sample, but never the definitive detection. As soon as a component of the PCR test system (be it chemicals, plastic goods, enzymes, protocol procedures or machines) is changed in one of the applied steps, it is mandatory to recalibrate the system.

From all the information published so far (see above), it can be concluded that any CT value

above 35 is no longer associated with the cultivability of infectious viruses and is thus the absolute cut-off value for the decision "positive", also independent of the test system used. The CT range 25-35 may still be valid test-dependently as a surrogate marker for "positive in the sense of a viral load potentially sufficient for infectivity" if, as described, it has been compared with a virus culture by adequate validation in the performing laboratory.

CT< 25 : positive genome detection high mRNA load in the sample
CT 26-35 : positive only when matched with virus cultivation
CT > 35 : negative

The strict evaluation of the CT value plays a role especially if only one gene segment is tested in the PCR, but generally applies to each individual target.

3.4. Adequate controls

In order to correctly assess the **sensitivity** and **specificity of** RT-qPCR, adequate samples must be included in each reaction run. This begins at the test site with "blank swabs" to reliably exclude contamination at the sample collection site, continues with extraction controls to ensure the correct isolation of reproducible RNA with all subsequent processing steps, i.e. an artificially produced defined RNA or - preferably - inactivated virus isolates of defined concentration, which is carried and processed in all work steps of sample preparation up to PCR and for which PCR is then also carried out with the aid of suitable primers. In this way, it can be ruled out that inhibitory substances or errors prevent the amplification of RNA during sample processing.

Such defined controls are available since December 2020 via WHO (see point 3.3.2P, source: https://www.who.int/publications/m/item/WHO-BS-2020.2402) and even since November 2020 via Instant e.V. From the booklet accompanying the mailing of the defined (https://www.instand-ev.de/fileadmin/uploads/userpload/documents/virology/20210118g supplementary booklet -.quantitative reference samples 1 and 2 - SARS-CoV-2.pdf.), the following aspectscan generally be identified:

- The strain BetaCoV/Munich/ChVir984/2020 was used as a control as a heat-inactivated sample with controlled viral counts corresponding to 10^6 and 10^7 RNA copies/ml, as this is the threshold for assessing patients as "probably contagious" was set (in: 2.2. Intended use). This strain was according to database (https://www.european-virus-archive.com/virus/human-2019-ncov-isolate) obtained in Munich on Jan. 28, 2020, and is sold through Charite.
- Depending on the tested genes and the performing laboratory, a wide range of CT values was shown despite defined sample in reference laboratories. For example, this CT value varied for the E gene for the sample with 10^6 copies/ml between 21.9

(laboratory 4) and 28.7 (laboratory 1); for the RdRp gene between 24.8 (laboratory 4) and 33.0 (laboratory 1). Across all tests from reporting laboratories, there was a spread of CT values of 12-38 for this sample (Figure 2) and a spread of CT values of 10-36 for the higher concentration sample. This example alone shows that each laboratory must always carry the defined samples in each test series in order to be able to convert the laboratory's own CT value to the viral load, which should be the actual reference sample for assessing the tested patient sample.

C.Drosten addressed the extreme variability of CT values in the different test systems in his NDR podcast 94 from 22.06.2021 (https://www.ndr.de/nachrichten/info/coronaskript306.pdf) as follows: "*And namely the Ct values that we have here, they are not readily comparable between test manufacturers.* "[...]. "*But only as long as we are in the same test system, we can compare them numerically. The differences are considerable in some cases. There are test manufacturers for whom a value of, say, 25 is nothing at all worrying, while the same value of 25 in another manufacturer's test shows that this is already a seriously infectious concentration.* ***That's simply because these test manufacturers don't standardize on the Ct value.***" (pg.17) He goes on to complain that this standardization is not happening with calibrations (made by him) (pg.18): "*What is not happening at the moment, however, is that nationwide recommendations are also made and applied by the state health offices or also by the Robert Koch Institute for certain areas of application on this technical laboratory basis that has now been created.*"

Original quote:
„Und zwar die Ct-Werte, die wir hier haben, die sind zwischen den einzelnen Testherstellern nicht so ohne Weiteres vergleichbar.“[....]“ *Aber nur so lange, wie wir uns in demselben Testsystem bewegen, können wir die zahlenmäßig vergleichen. Die Unterschiede sind da zum Teil erheblich. Es gibt Testhersteller, bei denen ist ein Wert von sagen wir mal 25 überhaupt nichts Besorg-niserregendes, während derselbe Wert von 25 in dem Test eines anderen Herstellers zeigt, dass das schon ernsthaft eine infektiöse Konzentration ist.* ***Das liegt einfach daran, dass diese Testhersteller nicht auf den Ct-Wert standardisieren***.“ (Seite 17) Weiter beklagt er, dass diese Standardisierung mit (von ihm) hergestellten Kalibrationen nicht stattfindet (S.18): „*Was aber im Moment noch nicht passiert ist, dass flächendeckend auf dieser jetzt geschaffenen technischen Laborbasis auch Empfehlungen von den Landesgesundheitsämtern oder auch vom Robert Koch-Institut für bestimmte Anwendungsbereiche ausgesprochen und angewendet werden*.“ [....]

Means: since autumn 2020, suitable controls for virus load determination would have been available and would have to be requested by the authorities for the laboratories to validate the test results, but this is obviously not happening (according to C. Drosten). "*We can even do it in such a way that this inherent problem of non-comparability of Ct values is already solved. Mind you, in the fall. The technique and the laboratory testing is not the catch here, but it is again the implementation and the regulation.* "
Quote:

„Wir können das eben sogar so, dass dieses inhärente Problem der Nichtvergleichbarkeit der Ct-Werte schon gelöst ist. Wohlgemerkt im Herbst. Die Technik und die Labortestung ist hier nicht der Haken, sondern es ist wieder mal die Umsetzung und die Regulation.“

3.4.1. Provide adequate controls:

Furthermore, each correct test series must include a series of external negative controls (i.e., carried in parallel as patient samples) and a positive control, ideally consisting of an inactivated defined SARS-CoV-2 virus strain. This would be an original task of the RKI (with the assistance of other suitable public institutions such as the Bernhard Nocht Institute or the Friedrich-Loffler Institute) or other national institutions like the CDC or the Institute Pasteuer etc., to isolate a sufficient number of SARS-CoV-2 viruses from patient samples in the laboratory facilities available there (safety level 4). Then to cultivate defined strains from these as controls, to inactivate these and to deliver them in defined virus numbers as controls to the testing laboratories via the local supervisory authorities. However, since this important service is still not offered routinely even after more than three years of the "pandemic", the positive control usually consists of a synthetic RNA that only encodes the target genes of the test system. This positive control can also be used to determine the lower detection limit of the PCR. This is specified by some commercial kits as 20 or fewer viral genomes per sample and thus (see point 1.3.2.) already detects a virus quantity in the smear that is below the infectious dose by a factor of 10^5, i.e. has no diagnostic/prognostic value whatsoever. An overview of the currently used commercial kits with their line data can be found at http://www.finddx.org/covid-19/pipeline/?section=molecular-assays#diag_tab.

3.4.2. Interlaboratory tests: abnormalities in the first batch

Correctly performed controls also include the participation of the laboratories performing the tests in so-called "**round robin tests**". In these, an anonymized panel of test samples is made available by an external provider. In the case of virus detection, these contain negative samples and samples with closely related viruses (inactivated) to check the specificity (these samples must not give a positive signal) and positive samples with different dilutions of the virus sought (inactivated) to determine the sensitivity (at what virus count does the PCR become positive, with what CT value).

Trial of Instant e.V.

In the case of SARS-CoV-2, the first EQA scheme "Virus Genome Detection - SARS-CoV-2 (340)" by the association "INSTANT e.V." was ready in April 2020. According to the report, 488 laboratories participated in this EQA scheme, of which 463 reported results. The results can be read in the published commentary (Zeichhardt M: *Kommentar zum Extra Ringversuch Gruppe 340 Virusgenom-Nachweis SARS-CoV-2*", available at: https://corona-ausschuss.de/wp-cpntent/uplpads/2020/07/Instand-Ringversuch-Virusgenpm-Nachweis-SARS-CoV-2.pdf. It shows two deviations from current Round robin test procedures, which already indicated laboratory problems with RT-qPCR for the detection of SARS-CoV-2: For example, on page 4 of the publication, it states, "*Important evaluation notice: only 4 of the 7*

samples tested in this extra EQA will be considered *for obtaining a certificate of successful participation*." The footnote on page 10 of the commentary states, "*In the April 17, 2020 interim evaluation, all participants in the Extra INSTAND EQA trial (340) Virus Genome Detection of SARS-CoV-2 April 2020 were notified ahead of time of the sample characteristics of samples 340059, 340060, and 340064. The results of these 3 samples will be disregarded for the purpose of granting a certificate* [...]."

Quote:
„*Wichtige Mitteilung zur Auswertung: Nur 4 der 7 Proben, die im diesem Extra-Ringversuch untersucht wurden, werden für die Erlangung eines Zertifikats über die erfolgreiche Teilnahme berücksichtigt*". In der Fußnote auf Seite 10 des Kommentars heißt es: „*In der Zwischenauswertung vom 17. April 2020 wurden allen Teilnehmern des Extra INSTAND Ringversuchs (340) Virusgenom-Nachweis von SARS-CoV-2 April 2020 die Probeneigenschaften der Proben 340059, 340060 und 340064 vorzeitig mitgeteilt. Die Ergebnisse dieser 3 Proben bleiben für die Erteilung eines Zertifikats unberücksichtigt* [....]"

The reason for this exclusion of certain samples is explained on page 4 of the commentary: "*While the extra ring test was still running, INSTAND e.V. received urgent requests from Germany and abroad to reveal the properties of the samples to be tested before the end of the extended submission period, i.e. before April 28, 2020, so that laboratories can improve their test method in the short term in case of possible incorrect measurements.*" (page 4 above in INSTANT e.V. report))

Quote:
„*Während der Extra-Ringversuch noch lief, erhielt INSTAND e.V. aus dem In- und Ausland dringliche Anfragen, noch vor Ende der verlängerten Abgabefrist, also vor dem 28. April 2020, die Eigenschaften der zu untersuchenden Proben aufzudecken, damit Laboratorien bei etwaigen Fehlmessungen ihre Testmethode kurzfristig verbessern können*."

This procedure is very unusual for a true interlaboratory comparison and thus no longer represents an independent external verification procedure of the participating laboratories.

Despite the samples already detected and the reduced testing scope, sample mix-ups occurred at a number of laboratories - as stated on page 18 of the commentary, "*For sample 340064 (SARS-CoV-2 positive diluted 1:100,000), the reduced success rate of only 93.2% is largely due to incorrect result assignments (**mix-ups) for** sample 340064 and sample 340065 (negative for SARS-CoV-2 and positive for HCoV 229E). The mix-ups for samples 340064 and 340065 involve 24 laboratories with a total of 59 results per sample. See also section 2.4.2.1* [...]".

Quote:

„*Bei Probe 340064 (SARS-CoV-2 positiv 1:100 000 verdünnt) beruht die reduzierte Erfolgsquote*

*von nur 93,2 % im Wesentlichen auf falschen Ergebniszuordnungen (**Verwechslungen**) bei Probe 340064 und Probe 340065 (negativ für SARS-CoV-2 und positiv für HCoV 229E). Die Verwechslungen bei den Proben 340064 und 340065 betreffen 24 Labore mit insgesamt 59 Ergebnissen je Probe. Siehe dazu auch Abschnitt 2.4.2.1.* […]".

Thus, a large number of laboratories mistakenly confused sample 340064 (slightly diluted SARS-CoV-2) with sample 340065 (negative for SARS-CoV-2 and positive for the closely related virus HCoV 229E).

Apart from the startling fact that a considerable number of samples were obviously interchanged even under highly standardized procedures in an interlaboratory test (which raises the question of the corresponding rate of sample interchanges and thus wrongly assigned swab samples under mass testing conditions), it is striking that all reported interchanges concerned only these two samples, but not the samples with the final number 61 (very highly diluted SARS-CoV-2) and 62 (negative), which were also evaluated.
The detailed results of a second round robin test from June/July 2020 (https://www.instand-ev.de/System/rv-files/summary%20of%20probe%20properties%20and%20setpoints%20virology%20340%20June%20July%2020200911a.pdf) are still not available for public review in detail.

3.5. Exclusion of contamination of reagents and "problems in the course of action".

3.5.1 Contamination within the laboratory due to errors in execution

The best PCR design can still lead to false positive results if either the underlying reagents / kits are contaminated with positive samples or, much more likely, **contamination** occurs in **the laboratory workflow.** Since PCR is an extremely sensitive method (exponential reaction course) that can detect few molecules of a DNA, laboratory contamination by PCR final products is a major problem in clinical diagnostics (described e.g. already in 2004 in Aslanuadeh J et al., http://www.an- nclinlabsci.org/content/34/4/389.full.pdf+html:
"*A typical PCR generates as many as 10^9 copies of target sequence and if aerosolized, even the smallest aerosol will contain as many as 10^6 amplification products [6]. If uncontrolled, within a relatively short time the buildup of aerosolized amplification products will contaminate laboratory reagents, equipment, and ventilation systems [6].*").

This extreme risk of contamination requires that diagnostic laboratories working with PCR take the utmost care in testing - highly skilled staff, contamination-proof environment, permanent independent control.

A problem with false positive results already emerged in the April round robin 340 already mentioned above, which was commented on as follows (page 20 below): "*In addition, in some cases, the assays with the SARS-CoV-2 negative control samples 340060, 340062, and 340065 indicate specificity problems that are independent of interchanges of samples 340064 and 340065. Clarification is needed as to whether these **false positives** are due to **a***

specificity problem with the tests used or to carryover of SARS- CoV-2 during test performance or to mix-ups with other samples *in this proficiency test at the laboratories in question.*" (Bottom of page 21 in https://www.instand-ev.de/System/rv-files/340%20DE%20SARS-CoV- 2%20Genome%20April%2020%20200502j.pdf). For confounding in this EQA scheme, see details above under "EQA schemes".

Quote:
„Zusätzlich weisen in einigen Fällen die Untersuchungen mit den SARS-CoV-2-negativen Kontrollproben 340060, 340062 und 340065 auf Spezifitätsprobleme hin, die unabhängig von Vertauschungen der Proben 340064 und 340065 sind. Es ist abzuklären, ob diese ***falsch positiven Ergebnisse*** *auf ein* ***Spezifitätsproblem der angewendeten Teste oder auf eine Verschleppung von SARS-CoV-2 bei der Testdurchführung bzw. auf Verwechselungen mit anderen Proben*** *in diesem Ringversuch in den betreffenden Laboren zurückzuführen sind.“*

If one sees against this background further, how e.g. after a BBC report in large test laboratories in England openly and extremely contamination-prone with untrained personnel one works (film up-to-date no longer openly accessible https://www.youtube.com/watch?v=Uk1VK1reNtE), it does not surprise, if also in Germany (where such contributions do not exist so far filmed) occasionally reports about "false positive cases" due to laboratory contamination can be found in the media (e.g. MVZ Augsburg - Link at the end of the section).

Even under controlled laboratory conditions, contamination due to the steps of PCR cannot be safely excluded in such a highly sensitive method. Thus, the problem of false positive PCR results in SARS-CoV-2 diagnostics due to laboratory procedures and **already** pointed out **in the first publication of** RT-qPCR (Corman et al., DOI: 10.2807/1560-7917.ES.2020.25.3.2000045): "*In four individual test reactions, weak initial reactivity was seen but they were negative upon retesting with the same assay*" [.....] ***mostproblably to handling issues*** "
Also a problem with contaminations, which led to false positive results in connection with genomes of SARS-CoV-2 in a study led by the Charite (among others with the authors Drosten and Landt from the above listed paper Corman et al, which described the first PCR for WHO already with contaminations), forced the authors even to withdraw a Science publication. Reason among others:
"*We found a* ***mixture of different SARS-CoV-2 genome fragments that contaminated some of the samples*** *"* In the original: *"We found a mixture of different SARS-CoV-2 genomic fragments contaminating some of the samples". ANNEX 5*

Even the "gold standard laboratories" of the Charite around C. Drosten have thus openly admitted and published clear problems with the contamination by Genome fragments of SARS-CoV-2, which can lead to false positive results in diagnostic PCR as well as genome (variant) analysis.

3.5.2 Contamination of the materials/reagents ex manufacturer

Even if the course of action in the laboratory functions optimally and is extremely monitored in order to greatly minimize laboratory-related **contamination**, an unexpected source of false positive results can **arise** here in the **contamination of the materials/chemicals used ex-manufacturer.** For example, the swab materials used to take samples may already be contaminated ex works - as in the case of the "Phantom of Heilbronn", in which the cotton swabs used to take DNA traces at the crime scenes were contaminated with the DNA of a packaging worker from the manufacturer's plant, thus hindering forensics with false traces for years (https://www.faz.net/aktuell/gesellschaft/kriminalitaet/dna-ermittlungspanne-das-phantom-von-heilbronn-ist-widerlegt-1925411.html).

In the case of SARS-CoV-2 diagnostics, a contamination problem due to PCR primers containing positive controls ex works was also published in June 2020 (Wernike et al., DOI: 10.1111/tbed.13684). Here, it was noticed that even pure water samples with several independent primer lots resulted in a clearly positive SARS-CoV-2 detection in RT-qPCR: "*However, there were also primers/sample sets that displayed very **low-level contaminations**, which were detected only during thorough internal validation.*"
Also, some false-positive results of SARS-CoV-2 RT-qPCR testing reported in the daily press in summer 2020 were attributed to material problems (e.g., http://web.archive.org/web/20210111010037/https://www.br.de/nachrichten/bayern/probleme-in-augsburger-lab-bringing-false-test-results,SEh5Qq4).

3.5.3 Interim evaluation:

Even with ideal RT-qPCR design and good laboratory practice with adequate validation, problems in daily handling procedures as well as from outside via already factory contaminated samples can significantly influence the result quality of RT-qPCR and lead to false positive results.

3.6. Commercial PCR test kits

Very early on, commercial PCR test systems, the "PCR kits" were used in routine laboratories for diagnostics, although a large proportion of them were declared for "RUO" ("research use only").
The first and therefore most prominent test manufacturer, the Berlin-based company TIB Molbiol, whose owner (Olfert Landt) was listed as author next to Christian Drosten at the WHO protocol recommendations. The kits, which are based on the WHO recommendations, are used by Roche on their large-scale automated testing machine "Cobas" and therefore, in addition to other suppliers (see https://www.vdgh.de/covid-19/sars-cov-2-und-die-industrie/hersteller/artikel16741 list of test manufacturers), probably still make up the majority of kits used for routine diagnostics in Germany. Interestingly, many test manufacturers also specify the "E gene" as the main detection target here and thus remain

close to the original WHO recommendation.

Exact numbers cannot be determined, however, TIB Molbiol has already delivered more than 60 million tests of these worldwide in 2020 according to its own information (https://www.tib-molbiol.de/en/covid-19), although these are still declared as "***Not tested for use in diagnostic procedures***" (e.g. header in https://www.roche-as.es/lmpdf/MDx 530777 96Wuhan-R- gene V200204 09155376001%20%282%29.pdf). The corresponding package inserts with the protocol information and kit descriptions of the company TIB Molbiol were surprisingly according to metadata of the originally available PDFs (can be provided electronically) already on **15.01.2020** (!!!) completely with ROCHE SAP number are still available unchanged (albeit with metadata analysis 06.02.2020) parallel to other test kits, which now have approval for in vitro diagnostics.

There is now (last updated June 2022) a wide range of PCR detection systems available (https://www.theglobalfund.org/media/9629/covid19_diagnosticproducts_list_en.pdf) many of which are also approved for in vitro diagnostics (IVD) of SARS-CoV-2.
In the exemplary Description of one of these kits (https://www.genesig.com/assets/files/Path_COVID_19_CE_STED_IFU_Issue_500.pdf),

”Intended use" reads: "***Positive results are indicative of the presence of SARS-CoV-2 RNA****. Positive results do not rule out coinfection with other bacteria or other viruses. Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions.* ***Positive and Negative results must be combined with clinical observations, patient history, and epidemiological information***".

4. Association of positive nucleic acid detection in RT-qPCR with disease and infectivity.

Only those actually infected with viruses that reproduce and are also released from the cells to the outside in sufficient "infectious" quantities can pass on the virus and carry the risk of disease and are thus to be used to determine the course of an infection rate and wave of disease

A) "*PCR detection is the standard test for diagnosing viral infections such as SARS- CoV-2.* ***The test detects individual pathogen genes but not intact pathogens.***" And, *"There is a possibility that the test will be positive beyond the duration of infection because "viral debris" is still present in the nose or throat.* ***Reliable proof of infectivity is only possible with elaborate tests that involve laboratory testing to determine whether the material from the swabs can kill living cells.***" This was written by the German journal “Dt. Ärzteblatt” on 02/01/2021. (https://www.aerzteblatt.de/nachrichten/120745).

Quote:
„*Der PCR-Nachweis ist die Standarduntersuchung zur Diagnose von Virusinfektionen wie SARS-CoV-2.* ***Der Test weist einzelne Erregergene, jedoch keine intakten Erreger nach***.“ Und: *„Es besteht die Möglichkeit, dass der Test über die Dauer der Infektion hinaus positiv ausfällt, weil noch „Virustrümmer“ in Nase oder Rachen vorhanden sind.* ***Ein sicherer Nachweis der Infektiosität ist nur mit aufwendigen Tests möglich, bei denen im Labor untersucht wird, ob das Material aus den Abstrichen lebende Zellen abtöten kann****.“*

B) Also the CDC points out under "Disadvantages" of NAATs (nucleic acid amplification tests = PCR) that "*A positive NAAT diagnostic test should not be repeated within 90 days, because people may continue to have detectable RNA after risk of transmission has passed*" (below in the summary table at: https://www.cdc.gov/coronavirus/2019-ncov/lab/resources/antigen-tests- guidelines.html#previous)

C) "*The PCR assay detects gene segments of SARS-CoV-2; it does not tell us whether they are infectious viruses or viral remnants after passed through infection. This would require pathogen culturing*." Was stated in a publication by the head of Frankfurt's Health Department from August 2020 (https://www.laekh.de/fileadmin/user upload/Heftarchiv/Einzelartikel/2020/10 2020/Die Covid-19-PandemieinFrankfurt amMain.pdf).

Quote:
„Der PCR-Test detektiert Genabschnitte von SARS-CoV-2; er sagt nichts darüber aus, ob es sich um infektionsfähige Viren oder um Virusreste nach durchgemachter Infektion handelt. Hierzu wäre eine Erregeranzucht erforderlich“

D) And in his expert opinion of April 21 for a court in Heidelberg (to be viewed anonymously here: https://www.corodok.de/wp- content/uploads/2021/05/Gutachten-Prof.-Drosten-v.-31.3.2021-anonymisiert.pdf), the expert C. Drosten confirms that an RT-PCR test can also become positive if "***at least the section of the virus genome to be detected is present in the tested sample*****. "**

Quote:
„..zumindest der nachzuweisende Abschnitt aus dem Erbgut des Virus in der getesteten Probe vorliegt.“

This means that genetic material fragments can also yield positive results in PCR without originating from an intact, interrogation-capable virus, thus also providing an alleged virus detection in non-infectious samples.

E) In a CDC publication dated 7/13/20 titled *"CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel For Emergency Use Only Instructions for Use",* (https://www.fda.gov/media/134922/download), on p. 38 under the heading "Limitations" (still found on p. 37) :

*"- **Detection of viral RNA may not indicate the presence of infectious virus or that 2019- nCoV is the causative agent for clinical symptoms."***

F) That a pure mRNA detection of SARS-CoV-2 does not necessarily correlate with disease and should not be used as the sole criterion for disease assessment, but is only an aid to confirm a clinical diagnosis, is also clearly stated in the WHO Information "*Notice for IVD Users 2020/05, Nucleic acid testing (NAT) technologies that use polymerase chain reaction (PCR) for detection of SARS-CoV-2*" dated 13/01/2021 (published on 20/01/2021 at https://www.who.int/news/item/20-01-2021-who-information-notice-for-ivd-users-2020-05): **"***Where test results do not correspond with the clinical presentation, a new specimen should be taken and retested using the same or different NAT technology.* "

"*Most **PCR assays are indicated as an aid for diagnosis**, therefore, health care providers must consider any result in combination with timing of sampling, specimen type, assay specifics, clinical observations, patient history, confirmed status of any contacts, and epidemiological information*"

G) Also in a publication in Lancet (https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(21)00425-6/fulltext#%20), the authors refer to the RT-qPCR test as follows: "*In our view, **current PCR testing is therefore not the appropriate gold standard for evaluating a SARS-CoV-2 public health test***" because, in their opinion, PCR still comes up positive even after those tested are no longer positive, since the RNA can continue to persist in the body for weeks and months even after the immune system has successfully fought it, without the person still being infectious. "*Once SARS-CoV-2 replication has been controlled by the immune system, RNA levels detectable by PCR on respiratory secretions fall to very low levels when individuals are much less likely to infect others. The remaining RNA copies can take weeks, or occasionally months, to clear, during which time PCR remains positive."*

H) .... In a May 2021 publication in Science led by C. Drosten (DOI: 10.1126/science.abi5273) examining the infectivity of SARS-CoV-2, the authors define the parameters for quantification and possible transmission of the virus in the very first sentence of the abstract as "... *are viral load and whether samples yield a replicating virus isolate in cell culture.*" They further state in the introduction that viral load is determined by viral RNA concentration and successful virus isolation in cell culture assays. "*While viral load and cell culture infectivity cannot be translated directly to in vivo infectiousness, and the impact of social context and behavior on transmission is very high, these quantifiable parameters can generally be expected to be those most closely associated with transmission likelihood.*"

I) In his NDR podcast 94 from 22.06.2021 page 16, C. Drosten addresses the relationship between Ct value and infectivity as follows: ".*that a case, just because the patient at this moment* ***has a high Ct value, so because he may not be infectious*** *right now, so he has little virus, he has virus, but he has little virus*, "
(https://www.ndr.de/nachrichten/info/coronaskript306.pdf)

Quote:
„....*dass ein Fall, nur weil der Patient in diesem Moment* ***einen hohen Ct-Wert hat, also weil er vielleicht nicht infektiös ist*** *jetzt im Moment, also der hat wenig Virus, der hat Virus, aber der hat wenig Virus*,....“

5. Conclusion: informative value of RT-qPCR tests for the detectability of infectivity with SARS-CoV-2 coronavirus.

1. In light of the problems and technical limitations outlined here, RT-qPCR is not a suitable reliable (and approved) diagnostic tool for the detection of infectious (replication-capable) SARS-CoV-2 viruses.

2. Furthermore, the pure RT-qPCR test result is only a laboratory value which, in view of the aspects outlined, never permits a valid statement about the presence of infectious viruses and may only be used at all in conjunction with a clinical symptom diagnosis (ascertained by healthcare providers, in Germany medical doctors) to estimate a possible viral infection.

Summary:

For the testing of asymptomatic and even symptomatic persons on the basis of a nasopharyngeal swab, as it is done uncritically in large numbers and predominantly by non-medical personnel WITHOUT (here decisive: contrary to the WHO requirement!) anamnesis and symptom collection from the tested persons, the RT-qPCR used is not suitable in any form to detect an infection and above all an infectivity with SARS-CoV-2.

ANNEX 1

Evaluation of the terms "asymptomatic patient" and "positive patient" in relation to RT-qPCR- tested individuals for SARS-CoV-2.

According to the definitions (see below), the term asymptomatic patient is nonsensical for healthy individuals who were only PCR positive without having any symptoms.

Since the term "patient" per se encompasses a medical condition that requires professional medical care, an RT-qPCR-positive person can only be defined as a patient if he or she has symptoms. The latter (symptoms) correspond to the term "infection" because a person can only be considered infected if his or her body reacts to an invading and multiplying pathogen.

CDC definition of Covid-19 patients (https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/symptoms.html)**.**

People with COVID-19 have had a wide range of symptoms reported - ranging from mild symptoms to severe illness. Symptoms may appear 2-14 days after exposure to the virus. Anyone can have mild to severe symptoms. People with these symptoms may have COVID-19:Fever or chills

- Cough
- Shortness of breath or difficulty breathing
- Fatigue
- Muscle or body aches
- Headache
- New loss of tasteor smell
- Sore throat
- Congestion orrunnynose
- Nausea or vomiting
- Diarrhea

Consequently, a person without any of the above symptoms is neither infected nor a patient, regardless of RT-qPCR results.

Infection:

Summary from the sources below:

An infection is defined as a situation in which at least the following three aspects occur together:

- Invasion of the body by microorganisms (germs) such as bacteria or viruses
- These invaded microorganisms multiply in the body
- And the body reacts to them (symptoms)

By definition, to speak of an infection or an infected person is only true if all three aspects mentioned above are present.

This means: without symptoms ("asymptomatic") - no infection can be defined in the affected person

Sources:

1. https://www.rxlist.com/infection/definition.htm:

The invasion and multiplication of microorganisms such as bacteria, viruses and parasites that are not normally present in the body.

2. Wikipedia:

An infection is the penetration of pathogens into the body tissues of an organism, their multiplication and the response of the host tissue to the infectious agents and their toxins produced.[1] An infectious disease, also called a communicable disease or transmissible disease, is a disease caused by an infection.

Infection

https://medical-dictionary.thefreedictionary.com/infection

Invasion and multiplication of microorganisms in body tissues, as in an infectious disease. The infectious process is similar to a circular chain with each link representing one of the factors involved in the process. **An infectious disease occurs only if each link is present and in proper sequence.**

These links are

(1) the causative agent, which **must be of sufficient number** and **virulence** to destroy normal tissue;

(2) reservoirs in which the organism can thrive and reproduce; for example, body tissues and the wastes of humans, animals, and insects, and contaminated food and water;

(3) a portal through which the pathogen can leave the host, such as the respiratory tract or

intestinal tract;

(4) a mode of transfer, such as the hands, air currents, vectors, fomites, or other means by which the pathogens can be moved from one place or person to another; and

(5) a portal of entry through which the pathogens can enter the body of (6) a susceptible host.

Open wounds and the respiratory, intestinal, and reproductive tracts are examples of portals of entry. The host must be susceptible to the disease, not having any immunity to it, or lacking adequate resistance to overcome the invasion by the pathogens. The body responds to the invasion of causative organisms by the formation of ANTIBODIES and by a series of physiologic changes known as INFLAMMATION.

https://www.cdc.gov/infectioncontrol/spread/index.html

How Do Infections Occur?

An infection occurs when **germs enter the body, increase in number, and cause a reaction of the body.**

Three things are necessary for an infection to occur:

- **Source:** Places where infectious agents (germs) live (e.g., sinks, surfaces, human skin)
- **Susceptible person** with a way for germs to enter the body
- **Transmission:** a way germs are moved to the susceptible person

Patient:

Definition as a summary from the sources below:

A patient is a person under the care of professional health care providers who exhibits symptoms of illness or injury or demonstrates other limitations of complete health.

By definition, a healthy person ("asymptomatic") without medical problems cannot be called a "patient."

German Wikipedia:

As a **patient** (from Latin *patiens* 'suffering, enduring', present participle active of *pati* 'suffer, endure') refers to a person who uses medical services or services of other persons who provide curative

treatment. This may be prevention, detection or medical treatment of diseases or consequences of an accident.

The word **patient** originally meant 'one who suffers'. This English noun comes from the Latin word *patiens*, the present participle of the deponent verb, patior, meaning 'I am suffering,' and akin to the Greek verb naoxeiv (= *paskhein*, to suffer) and its cognate noun naOoq (= pathos).

Medicine net:

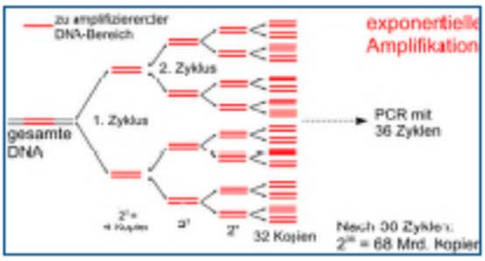
Patient: A person under health care. The person may be waiting for this care or may be receiving it or may have already received it. There is considerable lack of agreement about the precise meaning of the term "patient.

It is diversely defined as, for examples:

- A person who requires medical care.
- A person receiving medical or dental care or treatment.
- A person under a physician's care for a particular disease or condition.
- A person who is waiting for or undergoing medical treatment and care
- An individual who is receiving needed professional services that are directed by a licensed practitioner of the healing arts toward maintenance, improvement or protection of health or lessening of illness, disability or pain. (US Centers for Medicare & Medicaid Services)
- A sick, injured or wounded soldier who receives medical care or treatment from medically trained personnel. (US Army Medical Command)

From the Latin verb "patior" meaning "to suffer" both in the sense of feeling pain and in the sense of forbearance. Thus, the two uses of the word "patient" -- as a noun denoting "someone who suffers" and as an adjective meaning "to bear with forbearance" -- stem from the same origin.

Polymerase-Kettenreaktion

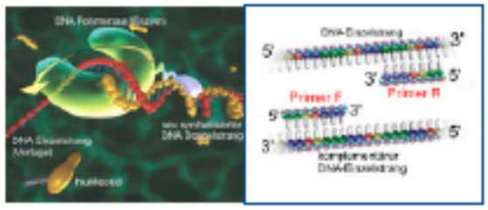


Polymerase-Kettenreaktion (PCR)?

Mit **PCR** (engl. polymerase chain reaction) wird ein vorbestimmter Abschnitt einer Erreger-DNA vervielfältigt. Als «Kopiermaschine» braucht es ein Enzym namens **Polymerase**, welches in einer Kettenreaktion aus sich wiederholenden Temperatur-Zyklen den gewünschten DNA-Abschnitt immer wieder verdoppelt und so eine messbare DNA-Menge generiert.

Vorteile: In 45 min können in einem Volumen von 20-50 µl bis zu zehn Erreger nachgewiesen werden.

Nachteile: Es können nur Erreger nachgewiesen werden, deren Gen-Sequenz bekannt ist. Ob ein Erreger infektiös (virulent, «lebendig») ist oder nicht bleibt unbekannt.



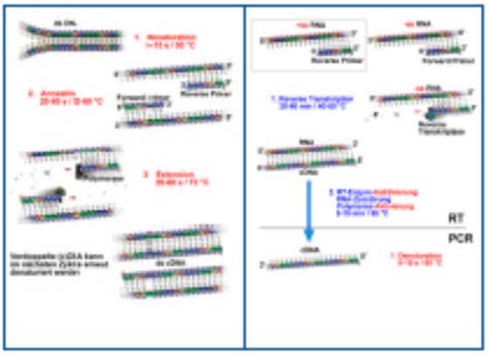
DNA-Polymerase und Oligonukleotide

Für die Vervielfältigung der DNA (**Amplifikation**) wird das Enzym **DNA-Polymerase** benötigt. Als Vorlage dient ein DNA-Einzelstrang des nachzuweisenden Erregers. Die passenden Nukleotide (dNTPs) werden durch die DNA-Polymerase zum Komplementärstrang verbunden.

Die DNA-Polymerase bindet aber nur dort an den DNA-Einzelstrang, wo eine kurze Doppelstrangstruktur als Startpunkt vorliegt. Dieser wird in der PCR mit der Zugabe von **Primer** bestimmt, die den zu amplifizierenden DNA-Abschnitt definieren.

Ein Primer ist ein kurzes DNA-Stück bestehend aus 15-30 Nukleotiden, ein so genanntes **Oligonukleotid**. Für beide komplementären DNA-Einzelstränge des Erregers wird je ein Primer (Forward (F) & Reverse (R)) mit Hilfe von Softwares festgelegt

Die dazu benötige DNA-Sequenz des nachzuweisenden Erregers wird im Internet aus öffentlichen Datenbanken kopiert. Die Oligonukleotide werden über Internet bei spezialisierten Firmen bestellt und synthetisiert.

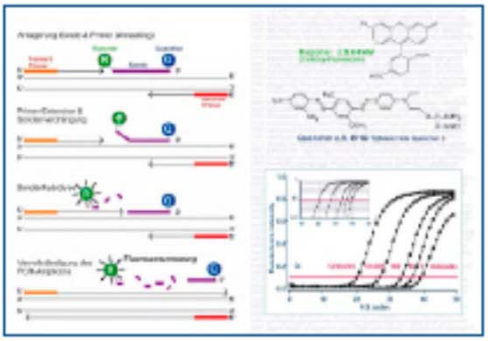


PCR & RT-PCR – Die Kettenreaktion

Die PCR besteht aus einer Serie (Kettenreaktion) von 30 bis 45 Temperatur-Zyklen und wird in einem Thermocycler durchgeführt. Jeder Zyklus besteht aus drei Schritten:

- **Denaturierung**: Im ersten Schritt wird die doppelsträngige DNA auf 95 °C erhitzt um die Stränge zu trennen. Es stehen zwei Vorlagen (Matrizen).
- **Annealing** (Anlagerung): Die Temperatur wird Primer-abhängig gesenkt damit sich die spez. Primer an die DNA-Einzelstränge anlagern können.
- **Extension** (Elongation): Die DNA-Polymerase bindet bei 60°C an die kurzen doppelsträngigen Bereiche und repliziert den komplementären Strang.

Da die Genome vieler Virenarten aus RNA bestehen, muss diese für die PCR zuerst in DNA umgeschrieben werden. Dieser Vorgang wird **Reverse Transkription (RT)** genannt und kann im selben Ansatz der PCR vorgeschalten werden.



Quantitative real-time PCR

Bei der real-time PCR findet zusätzlich eine **Quantifizierung** des amplifizierten DNA-Abschnittes statt. Dazu werden nach jedem PCR-Zyklus **Fluoreszenz**-Messungen durchgeführt, wobei die Zunahme der kopierten DNA mit der Zunahme der Fluoreszenz korreliert.

Als Fluoreszenz-Quelle können DNA-Farbstoffe (z.B. **SYBR Green**) verwendet werden, welche nur mit doppelsträngige DNA interkalieren (binden). Nach Anregung kann deren zunehmende Fluoreszenz gemessen werden. Es kann jedoch nicht zwischen spez./unspez. PCR-Produkten unterschieden werden.

Eine spezifischere Methode beruht auf den Zusatz einer zwischen den Primer liegenden Sonde (z.B. **TaqMan**-). Diese ist an den Enden mit einem Reporter-Fluoreszenzfarbstoff (R), und einem Fluoreszenz-absorbierenden Quencher (Q) markiert.

Durch Verwendung einer speziellen DNA-Polymerase mit **5'-3'-Exonuklease-Aktivität**, wird die Sonde während der Synthese des Gegenstranges vom 5'-Ende her abgebaut, wodurch Quencher und Fluorophor voneinander entfernt werden. Die steigende Reporter-Fluoreszenz kann gemessen werden.

ANNEX 3

VIASURE *SARS-CoV-2* Real Time PCR Detection Kit

It has a detection limit of ≥10 RNA copies per reaction for ***ORF1ab* and *N* genes** (Figures 1 and 2).

Dieser Test hat eine Nachweisgrenze von ≥ 10 RNA Kopien je Reaktion für die ORF 1ab und N Gene (Abbildungen 1 und 2)

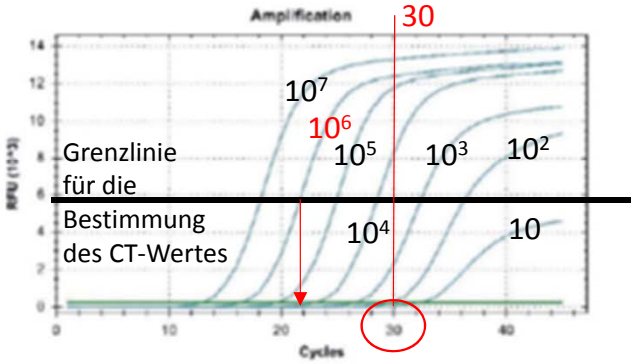


Figure 1.

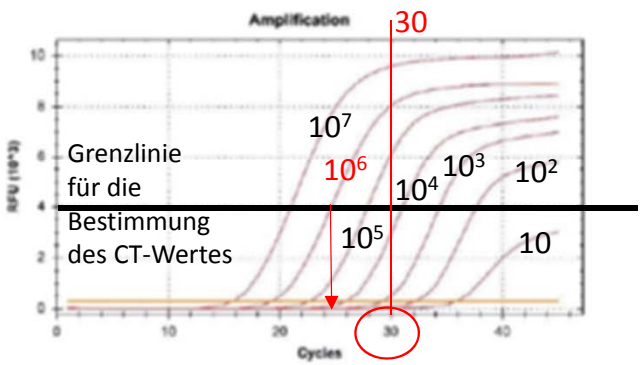


Figure 2.

Figure 1. Dilution series of ORF1ab gene (10^7-10^1 copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (FAM channel).

Figure 2. Dilution series of N gene (10^7-10^1 copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (ROX channel).

Für diesen Typ des Viasure-Testkits können bei einer CT von 30 (roter Kreis, Maximum des CT – Wertes gemäß den Angaben im schriftlichen Gutachten) weniger als 10^4 RNA-Kopien mit dem Zielgen ORF1ab (Abbildung 1) und weniger als 10^5 Genomkopien mit dem N-Gen (Abbildung 2) nachgewiesen werden.
Laut RKI entsprechen mindestens 10^6 RNA-Kopienl/ml einer plausiblen Korrelation mit einer Infektiosität (Nachweis von infektiösem Virus in Zellkultur).
Diese Kopienanzahl wird hier bei einem CT von weniger als 22 für das ORF1ab-Gen (Abbildung 1, Pfeil) und weniger als 25 für das N-Gen (Abbildung 2, Pfeil) gesehen.

Ein CT von 30 oder höher als Kriterium für "Infektiosität" ist also weit jenseits der realen Grenze UND der Testspezifikationen, dies ist bei allen kommerziellen Testkits vergleichbar

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Review article

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SARS-CoV-2 viral load and shedding kinetics

Olha Puhach [1], Benjamin Meyer [2] & Isabella Eckerle [1,3,4]

Abstract

SARS-CoV-2 viral load and detection of infectious virus in the respiratory tract are the two key parameters for estimating infectiousness. As shedding of infectious virus is required for onward transmission, understanding shedding characteristics is relevant for public health interventions. Viral shedding is influenced by biological characteristics of the virus, host factors and pre-existing immunity (previous infection or vaccination) of the infected individual. Although the process of human-to-human transmission is multifactorial, viral load substantially contributed to human-to-human transmission, with higher viral load posing a greater risk for onward transmission. Emerging SARS-CoV-2 variants of concern have further complicated the picture of virus shedding. As underlying immunity in the population through previous infection, vaccination or a combination of both has rapidly increased on a global scale after almost 3 years of the pandemic, viral shedding patterns have become more distinct from those of ancestral SARS-CoV-2. Understanding the factors and mechanisms that influence infectious virus shedding and the period during which individuals infected with SARS-CoV-2 are contagious is crucial to guide public health measures and limit transmission. Furthermore, diagnostic tools to demonstrate the presence of infectious virus from routine diagnostic specimens are needed.

Sections

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Introduction

At the end of 2019, a novel coronavirus emerged, later termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causative agent of coronavirus disease 2019 (COVID-19). SARS-CoV-2 primarily targets multiciliated cells in the upper respiratory tract (URT), but was also reported to infect cells outside the URT[1]. It can spread to the lower respiratory tract (LRT), where it infects alveoli, leading to reduced gas exchange, inflammation and pulmonary pathologies that are typical of COVID-19 (ref.[2]). Individuals who are infected shed the virus through the URT, with emission of infectious virus leading to secondary transmission and thus further spread of the virus.

Because of their nonspecific clinical presentation, precise diagnostic tools are needed to identify SARS-CoV-2 infections. Specific real-time PCR (RT-PCR) assays were quickly available after the emergence of the virus, later followed by antigen-detecting (rapid) diagnostic tests (Ag-RDTs) and serological assays. Although detection of viral RNA in respiratory specimens by RT-PCR is highly sensitive and specific, it does not distinguish between replication-competent virus and residual RNA. In the absence of a diagnostic test, infectiousness is often established using one of two proxies: the presence of viral RNA above a defined cycle threshold (Ct) value, or a positive Ag-RDT. RT-PCR is a useful tool for initial diagnosis, whereas Ag-RDTs can serve as an indicator for ending the isolation period. This is because viral RNA (which would be picked up by RT-PCR) remains detectable in the absence of infectious virus, whereas positivity of Ag-RDTs better correlates with the presence of infectious virus.

Aside from the respiratory tract, SARS-CoV-2 RNA has been detected in peripheral blood, stool, urine and ocular secretions[3–7]. Virus isolation from non-respiratory specimens was unsuccessful in most studies[4,8,9], with very few reported cases of infectious virus presence in non-respiratory specimens[10–13]. Furthermore, viral loads from respiratory tract samples were found to be much higher than from other materials, the latter often with RNA viral loads that are incompatible with the presence of infectious virus. Such specimens are not considered relevant for transmission and therefore, we concentrate on SARS-CoV-2 virus shedding only through the respiratory tract.

Here, we elucidate the relationship between SARS-CoV-2 viral load and infectious virus presence, the biological and host factors that determine infectious virus shedding, measurement of infectious virus and the role diagnostics can have as a proxy for infectious virus shedding.

Measuring SARS-CoV-2 viral load

The gold standard for laboratory diagnosis of a respiratory tract infection is demonstration of viral RNA with a virus-specific (semi-)quantitative RT-PCR from material collected from the respiratory tract. The most commonly used materials are swab specimens from the nasopharynx or oropharynx, but swabs of the nasal cavity, saliva or gargled liquid solution have also been suggested as alternative materials, with the advantage of being a less uncomfortable procedure for the participant. Viral load as determined by RT-PCR is either expressed as the number of viral RNA copies per millilitre of viral transport medium or per swab, or by the arbitrary test-specific Ct value. By contrast, infectiousness is determined by qualitative or quantitative assessment of infectious virus in a clinical specimen by replication of virus in cell culture. The limitations to measuring viral shedding are described in Box 1. In this Review, we refer to viral particles that can cause infection as infectious virus, and to viral RNA levels (which are widely used as surrogates for infectious virus) as viral load.

Detection of infectious virus

The gold standard for determining the presence of infectious (that is, replication competent) virus in respiratory specimens is the recovery of virus in cell culture, a procedure that is commonly termed virus isolation (Fig. 1).

In the case of SARS-CoV-2, various cell lines and primary cells can be used for virus isolation, including those that express angiotensin-converting enzyme 2 (ACE2; the receptor required for virus entry) or transmembrane protease 2 (TMPRSS2; which is also important for virus entry)[14]. A cell line derived from African green monkey kidney cells, Vero E6, is commonly used for virus isolation, propagation and titration[15]. Other human cell lines that have been successfully used for SARS-CoV-2 isolation are a colorectal adenocarcinoma cell line (Caco-2), a lung adenocarcinoma cell line (Calu-3), a lung adenocarcinoma cell line ectopically overexpressing ACE2 (A549) and a human hepatocellular carcinoma cell line (Huh7)[16,17].

The presence of infectious virus in the cell culture is qualitatively assessed using light microscopy, which can be used to identify cells undergoing the cytopathic effects (and death) caused by SARS-CoV-2 infection, consisting of syncytium formation, cell rounding, detachment and degeneration[17]. Infection is usually confirmed by a second method, either by a specific RT-PCR for viral RNA from the supernatant of infected cells, indicating virus replication by an increase of viral load over time in comparison to the baseline sample, or by immunostaining for viral proteins[15,18].

This qualitative measurement of virus presence cannot, however, quantify the infectious virions in the inoculated specimens, although samples with lower viral load commonly show delayed development of a cytopathic effect[19]. Instead, methods such as plaque assays, focus-forming assays or 50% tissue culture infectious dose ($TCID_{50}$) can be used to quantify infectious virus in a patient sample.

The above methodologies are reliable tools to detect infectious virus in clinical specimens of individuals who are infected with SARS-CoV-2, although there are limitations. Detection of viable virus particles is highly influenced by the quality of the sample, and infectious viral particles can quickly lose their infectiousness in unsuitable storage conditions. To preserve infectious virus in specimens, swab samples from patients infected with SARS-CoV-2 should be immediately submerged in a viral transport medium suitable for cell culture and stored at −80 °C as early as possible after collection. Prolonged exposure to higher temperatures or repeated freeze–thaw cycles can drastically influence the quality of the sample, leading to potentially complete loss of infectious viral particles. Therefore, many factors can influence the reproducibility of the results between different laboratories. Furthermore, cell lines used for isolation can show a high variability between laboratories even when they are presumably the same. Consumables used during cell culture, such as culture medium or additives such as fetal bovine serum and antibiotics, could potentially also impact virus isolation success. In human primary airway epithelial cells, which mimic the primary site of entry in the human respiratory tract, the probability of isolating infectious virus was reduced compared with that of Vero E6 cells, indicating that infectious virus determined using Vero E6 cells might be overestimated for assessing transmission risks in vivo[20].

Importantly, all cell culture work with SARS-CoV-2 is done under biosafety level 3 conditions, so only specially trained personnel in laboratories with advanced infrastructure can perform these experiments. Thus, detection of viable virus through virus isolation is not suitable for diagnostics and is restricted to research only.

Box 1

Limitations to measuring viral load

Specimen selection site

The anatomical site chosen to collect the swab specimen for detection of SARS-CoV-2 might influence viral load detection. Higher RNA viral load was reported from nasopharyngeal than oropharyngeal swabs[28,124,181]. As a result, nasopharyngeal samples show the highest diagnostic accuracy compared with other upper respiratory tract samples[182]. Similarly, higher virus isolation success was reported from nasopharyngeal swabs than from saliva, nasal or sublingual swabs[124]. However, another study found higher RNA viral loads in the throat and sputum than from nasal swabs[30]. Two studies indicate that virus can be detected earlier in the throat[29] or saliva[33], but reaches significantly higher levels and remains detectable for longer in the nose[29,33]. A meta-analysis, which evaluated different clinical sampling methods using nasopharyngeal swab as a reference, demonstrated that pooled nasal and throat swabs showed the best diagnostic performance[183]. Notably, this analysis revealed higher heterogeneity of results in studies using nasal or saliva specimens than using pooled nasal and throat swabs[183].

The effect of the swabbing method (self-administered or performed by trained person) on measured viral loads cannot be overlooked: the sensitivity of antigen-detecting (rapid) diagnostic tests achieved by health-care professionals was higher than for self-tests[57,184].

Impact of individual infection kinetics

To date, there is a limited number of studies that describe the longitudinal dynamics of SARS-CoV-2 shedding[29,33]. Most of the studies used only a single time point to collect respiratory swabs from individuals who were infected for measurement of viral load. As a result, different times from symptom onset can be a confounding factor when comparing viral load between different patients, which might also explain the variation of available data on viral load.

Influence of epidemic period

RNA viral loads across the specimens collected at single time points were found to indicate the trajectory of the epidemic, as a high proportion of individuals who were recently infected with low cycle threshold values correlates with a higher reproduction number, indicative of a growing epidemic[185]. Similarly, the rise and fall of RNA viral load correlated with the number of COVID-19 cases and hospital admissions across the population as it was identified using SARS-CoV-2 RNA measurement in wastewater samples[186]. Moreover, variation in estimates of the mean incubation period was shorter before the epidemic peak in China than after the peak[187]. Therefore, sampling at single time points can be biased by the epidemic period and might reflect more epidemiological dynamics than individual shedding kinetics.

Influence of SARS-CoV-2 variant of concern

Available data on SARS-CoV-2 variants of concern demonstrated that, although the overall pattern of viral load dynamics is conserved between the variants, infection with different SARS-CoV-2 variants of concern led to highly distinct infectious virus amounts and RNA viral loads[25,86,87,90,92,94] and variations in SARS-CoV-2 incubation period[113]. Therefore, extrapolation of our understanding from shedding of current or earlier SARS-CoV-2 variants to newly emerged variants may be of only limited value.

Detection of RNA viral load

Techniques for detecting viral RNA by RT-PCR were quickly established at the beginning of the pandemic[21,22] (Fig. 1). The high specificity and sensitivity of RT-PCR make it the gold standard for diagnosing SARS-CoV-2 infections. Quantitative RT-PCR assays provide a Ct value, which is inversely correlated with the concentration of the target viral RNA in the clinical sample (that is, the higher the value, the lower the target RNA in the sample). By using an external standard with a defined number of RNA copies, Ct values can be transformed into absolute viral RNA copy numbers or international units per millilitre of viral transport medium or per total swab.

Although RT-PCR cannot directly determine infectiousness owing to its inability to differentiate between replication-competent (infectious) virus and residual (non-infectious) viral RNA, a correlation between RNA viral load and the presence of infectious virus has been sought. Several studies have attempted to correlate the quantity of viral RNA with infectiousness by isolating virus across a range of Ct values. Indeed, there was a stepwise decrease in the probability of virus isolation with increasing Ct values in samples collected during the first 8 days post-onset of symptoms (dpos)[18,23]. However, other studies have found that the correlation between infectious virus and RNA viral load was low and that viral load (or Ct values as a proxy) is only a weak predictor of infectious virus presence in the first 5 dpos[4,20,24,25]. Furthermore, when taking a certain Ct value or RNA copy number as a threshold, it is not possible to determine whether the RNA viral load is increasing or already decreasing; therefore, a low viral load could be measured at the end of infection or in the early (pre-)symptomatic phase before reaching peak viral load.

In a routine diagnostic context, analytical sensitivity and limits of detection may vary between the tests and laboratories where they are applied. An analytical performance comparison between different RT-PCR assays showed variation between the measured Ct values and the detection rate[26]. Therefore, application of RNA standards and calculation of RNA genome copy number based on a standard curve can improve comparability between laboratories and assays. To facilitate easier calibration and control of nucleic acid amplification techniques, an international standard with assigned potency in the form of an inactivated SARS-CoV-2 isolate was introduced by the World Health Organization (WHO)[27].

As with the detection of infectious virus, several other parameters can influence whether viral load can be detected. The site of specimen collection can impact the findings on viral load; although some studies report higher RNA viral load in nasal or nasopharyngeal swabs[28,29], others show higher RNA viral load in throat samples[30]. Moreover, the transport

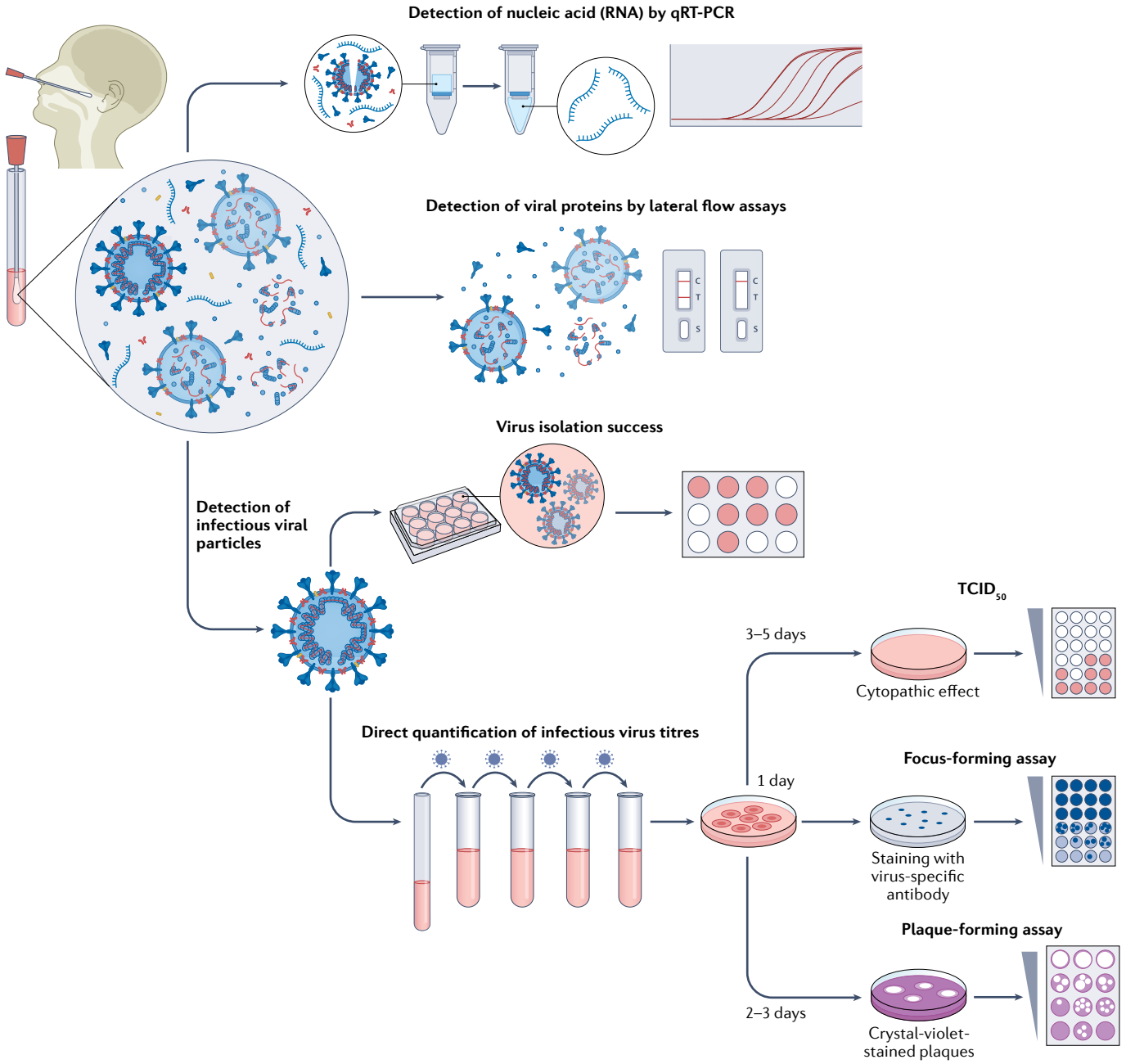

Fig. 1 | Methods to measure infectious virus and RNA viral load. Swab specimens from the nasopharynx or oropharynx are used for detection of SARS-CoV-2 viral loads. Detection of viral nucleic acids (RNA) is performed by quantitative real-time PCR (qRT-PCR). Viral RNA is extracted from lysed virus, reverse transcribed and amplified by qPCR using primers specific for one or more target regions in the viral genome. The amplification cycle at which samples cross the threshold (cycle threshold) defines the amount of viral RNA. RNA viral load can be expressed as the number of viral RNA copies per millilitre, or by the arbitrary test-specific cycle threshold value. Lateral flow assays detect the presence of specific viral proteins in the lysed viral particles. SARS-CoV-2 nucleocapsid is used in most antigen-detecting (rapid) diagnostic tests. The presence of infectious (replication-competent) virus in respiratory specimens can only be determined by the recovery of virus in cell culture by isolation or by quantification of infectious virus titres using 50% tissue culture infectious dose ($TCID_{50}$), focus-forming assays or plaque-forming assays. Virus isolation is performed by applying infectious medium on the monolayer of cells; isolation success is determined by the presence of a cytopathic effect approximately 3–5 days post-infection. White colour indicates the presence of a cytopathic effect in cells. For quantification of infectious virus titres, serial dilutions of respiratory samples are performed and used for inoculation on the monolayer of cells. In $TCID_{50}$, 3–5 days post-infection, viral-induced cytopathic effect is classically defined using microscopy. In focus-forming assays, cells are fixed 1 day post-infection and immunostaining with virus-specific antibodies is performed to detect groups of infected cells (foci). The foci, indicating the presence of infectious virus, are displayed in blue. In plaque-forming assays, plates are fixed 2–3 days post-infection and stained with crystal violet; wells with individual plaques are used to determine viral titres. The plaques, indicating the presence of infectious virus, are displayed in white.

media used for the sample, storage condition and quality of the sample may further influence the detection of viral RNA and their usefulness and limitations when extrapolating to potential infectiousness.

Although new variants have impacted some gene targets, in most instances, they did not have a major effect on molecular diagnostics, owing to the use of dual-target assays (in which at least two viral genes are detected simultaneously)[31].

Antigen-detecting rapid diagnostic tests

Most lateral flow tests are designed to detect SARS-CoV-2 nucleocapsid protein, as a proxy for infectious virus, in nasal or nasopharyngeal swabs[32–36] (Fig. 1). Indeed, most studies on Ag-RDT detection show good concordance with RT-PCR positivity when Ct values are below 25–30, a viral load compatible with the presence of infectious virus, whereas higher Ct values give less reliable results[34,37–41].

Early time points during infection often give negative results with Ag-RDT in individuals who have tested positive by PCR[29,42]. On average, the first positive Ag-RDT results are obtained about 1–2 days later than positive PCR results[37], whereas the highest sensitivity in patients was shown during the first 7 dpos in the studies with ancestral SARS-CoV-2 (refs.[42–44]). Antigen tests show highest sensitivity for specimens containing infectious virus and with Ct values below 25 (refs.[45–49]), and their positivity highly correlates with the presence of infectious virus[34,45,47,50]. By contrast, Ag-RDTs are less sensitive to low RNA viral loads (which have higher Ct values)[51]. Several studies have demonstrated a strong correlation between Ag-RDT positivity and the period in which infectious virus can be detected, indicating that Ag-RDTs can add an additional safety layer for deciding when to end isolation[29,39].

However, some inconsistencies between studies and tests have been noted. For instance, there have been reports (across a range of studies and Ag-RDTs) of failure to detect viral antigens in specimens with a low Ct value and/or containing infectious virus (beyond the early acute phase)[46,50]. Moreover, there are seldom reports of Ag-RDTs remaining positive after more than 10 dpos[42,46,50]. As most studies failed to isolate infectious virus after more than 10 dpos, it remains unclear whether Ag-RDT positivity beyond 10 dpos correlates with infectious virus shedding. One study showed that antigen tests predict infectiousness more accurately at 1–5 dpos, than at 6–11 dpos[52]. Notably, there was a good correlation between Ag-RDT positivity and infectious virus isolation within the first 11 dpos[52].

Conflicting results were found for sensitivity and specificity of Ag-RDTs for detection of SARS-CoV-2 variants, with large variations between manufacturers, the type of setting in which the Ag-RDTs were used (self-tests versus tests collected by a health-care professional) and the type of sample used for detection (nasal versus oral)[53–57]. With increasing hybrid immunity and the presence of mucosal antibodies, Ag-RDTs may further lose sensitivity[58].

Viral load and shedding dynamics

Viral loads are used as a proxy to characterize infectious viral shedding. The exact time for which individuals remain infectious is laborious to estimate and is likely to vary between patients. Viral factors, such as viral variant, and host factors, such as patient age and sex and immune status, influence shedding dynamics.

Viral load as a key determinant of viral shedding

After the emergence of SARS-CoV-2 in late 2019, the first details on viral load and infectious virus shedding were measured in a cluster of infections that occurred in January 2020 in Germany, assessing nine immunocompetent individuals with a mild course of disease[4]. Peak RNA viral loads were reached in the early symptomatic period at 5 dpos, a finding that was confirmed by other studies reporting peak viral loads at the time of symptom onset or even shortly before[4,7,28,59]. RNA viral loads gradually declined over the course of the disease in the nasopharyngeal and throat swabs, reaching low or undetectable levels 2 weeks after symptom onset[4,23,59,60] (Fig. 2). Declining RNA viral load is associated with resolution of clinical symptoms and gradual increase in antibody titres, for both binding and neutralizing antibodies[18,23]. However, ongoing detection of viral RNA has been described for prolonged periods up to 28 dpos in otherwise healthy individuals[61], and some studies have reported low-level detection of RNA by RT-PCR even for months[62]. Participants who continue to shed viral RNA for more than 4 weeks after initial detection by RT-PCR represent a minority of non-severe cases, estimated to be around 3%[63], 14%[64] or less than 20%[65].

Infectious virus shedding of the ancestral SARS-CoV-2 strain, as determined by virus isolation in cell culture, was reported to correlate with high RNA viral load in the early acute phase after symptom onset[23]. Importantly, daily longitudinal sampling of respiratory specimens from individuals with mild disease or asymptomatic infection revealed that infectious virus can already be detected before the onset of symptoms[33]. Successful infectious virus isolation was reported within the first 8–10 dpos, but culture probability after this time period rapidly declined[4,7,23,29,66,67]. Studies that assessed infectious virus quantitatively found that infectious virus titres declined over the first 10 dpos[25,29]. In addition, a reduced chance of virus isolation coincided with the time of seroconversion in hospitalized patients and, as a result, infectious virus could no longer be isolated from seroconverted patients with detectable antibody titres[18,68,69]. Although similar seroconversion studies performed on mildly symptomatic patients are missing, the number of immunologically naive individuals is declining and this broadly existing underlying immunity makes such an assessment more complex.

Most studies on infectious virus shedding in the acute symptomatic period were on immunocompetent patients that had mild-to-moderate disease, representing the majority of COVID-19 cases in the community. Therefore, the assessment of the presence of infectious virus in the URT from those studies was used to define the duration of the period of infectiousness and contributed to best public health practices for isolation and quarantine[62,70]. Although the pattern of infection is broadly similar in patients with mild and severe disease, key differences do exist. The first week of illness is comparable in terms of RNA viral load between patients with mild and severe disease. However, patients with severe disease have elevated RNA viral loads in the second week of illness, and RNA was detected for prolonged periods[71]. Moreover, infectious virus was recovered from hospitalized patients for prolonged periods of up to 32 dpos[18,72,73]; however, the median time from symptom onset to viral clearance in culture was similar to that of patients with mild or moderate disease[18,73]. Severe COVID-19 is also characterized by high and persistent RNA viral load in the LRT, whereas non-severe cases have similar viral loads in the URT and LRT[74].

Prolonged detection of viral RNA was also reported in immunocompromised patients; for example, 224 days after the beginning of the infection, virus was still detected in a man infected with HIV, including the detection of subgenomic RNA (sgRNA) indicating active viral replication[75]. Also, infectious virus was recovered up to 61 dpos in nasopharyngeal swabs collected from immunocompromised patients[76], and low RNA viral loads were still detected at 60 dpos in another study[77]. Infectious virus was isolated from bronchoalveolar fluids from patients receiving chimeric antigen receptor (CAR) T cell therapy

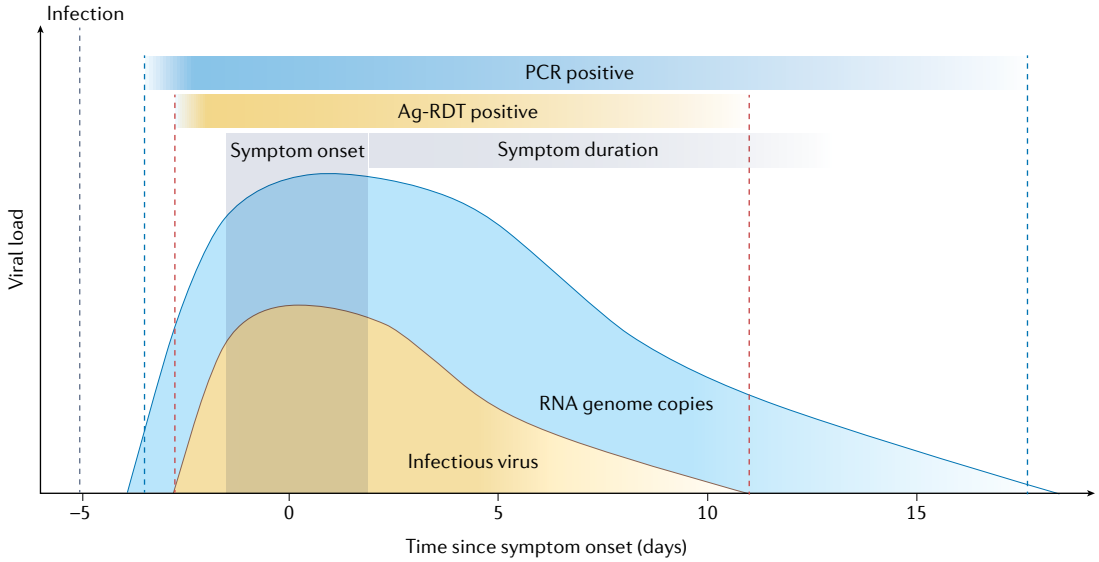

Fig. 2 | Kinetics of RNA viral loads and infectious virus for ancestral SARS-CoV-2 in patients with mild-to-moderate disease. According to different studies, the incubation period for ancestral SARS-CoV-2 was estimated to lie between 4.6 and 6.4 days. On average, symptoms continue to persist for 10 days. RNA can already be detected before the onset of symptoms; RNA levels peak around the onset of symptoms and then gradually decline. Median clearance for RNA viral load is 16 days post-onset of symptoms. Infectious virus titres are highest around symptom onset, and infectious virus can be isolated up to 8 or 10 days post-onset of symptoms. RNA can be detected for prolonged periods by real-time PCR, when infectious virus is no longer detectable, whereas virus detection by antigen-detecting (rapid) diagnostic tests (Ag-RDTs) was shown to be a better correlate for infectiousness. Gradients reflect variability between individuals (lighter shade towards the end of infection shows that viral loads continue to be detected in some but not all individuals). The grey dashed line marks the initial infection, the blue dashed lines mark the PCR-positive period and the red dashed lines mark Ag-RDT positivity. Details of the underlying studies used to generate Fig. 2 can be found in Supplementary Table 1.

up to 28 days after admission to an intensive care unit[78]. A case report on an immunocompromised patient showed isolation of infectious virus up to 78 dpos[79]. The reports of infectious virus isolation from severely ill or immunocompromised patients are limited (owing to the low number of patients), so it is difficult to define the proportion of cases with prolonged shedding.

The characteristics of viral shedding of other respiratory viruses are outlined in Box 2.

Viral shedding of SARS-CoV-2 variants

Viral evolution of SARS-CoV-2 over time has led to the emergence of numerous variants. Combined with increasing population immunity due to vaccination or natural infection, this has led to a need to reassess our knowledge of viral shedding patterns.

The WHO designated variants as variants of concern (VOCs) if they were associated with one or more of the following: elevated transmissibility or a detrimental change in COVID-19 epidemiology; increased virulence or a change in clinical disease presentation; or decreased effectiveness of public health measures or available diagnostics, vaccines or therapeutics[80]. To date, five VOCs are recognized: Alpha, Beta, Gamma, Delta and Omicron. In contrast to ancestral SARS-CoV-2, VOCs display some differences in evasion from immunity, viral loads, shedding period or even incubation period, resulting in drastically different levels of transmission[81–85] (Fig. 3).

All VOCs have shown changes in viral load compared with ancestral SARS-CoV-2. One study reported that infection with Alpha leads to approximately tenfold higher RNA viral load and an increased probability of cell culture isolation compared with the ancestral virus[86]. However, another study did not find a substantial difference in the infectious virus titre between Alpha and ancestral SARS-CoV-2 (ref.[33]). Delta reportedly led to an even higher increase in RNA viral load: one study reported a 1,000× increase relative to the ancestral virus[87], and other studies reported 1.7× (ref.[88]) or 6.2× higher[89] viral load than Alpha. Furthermore, Delta demonstrated elevated probability of cell culture isolation[90] and higher infectious virus titres than Alpha[91]. Although Omicron was shown to be highly transmissible, lower RNA viral loads[92], lower cell culture isolation probability[93] and lower infectious virus titres[25] were observed in patients infected with Omicron BA.1 than in those infected with Delta. Even within the Omicron clade, there are differences between sub-lineages, with infection with Omicron BA.2 leading to higher levels of RNA viral loads and longer time to viral clearance than with Omicron BA.1 (refs.[94–96]).

Similarly, VOCs have shown differences in the duration of viral shedding. Analysis of Ct values in respiratory specimens found that Delta showed longer persistence of viral RNA than ancestral SARS-CoV-2 (ref.[97]). Another study demonstrated that there was not significant difference in the mean duration of viral RNA presence in Delta and Omicron BA.1 infections[92]. The duration of infectious virus shedding appears to be similar to that observed with ancestral SARS-CoV-2, with culturable virus obtained at 5 dpos[85] and no replication-competent virus isolated beyond 10 dpos in patients infected with Delta and Omicron BA.1 (refs.[84,98]). It is important to note that pre-existing immunity to SARS-CoV-2, either from infection or vaccination, might influence the duration of infectious virus shedding (alongside immune status and disease severity, as discussed above), which may have driven some of these differences during the course of the pandemic.

Influence of age and sex on viral shedding

There is some evidence that age-associated and sex-associated differences in innate and adaptive immunity, as well as higher ACE2 expression in adults than in children, result in an increased risk for severe disease in older male patients[99–101]. Moreover, a few studies have found that age and sex influence viral loads and shedding dynamics. In cases of infection with ancestral SARS-CoV-2, resolution of RNA shedding was faster in participants <18 years of age and slower in participants >50 years of age[61]. According to one study, viral RNA can be detected for longer times in male patients infected with ancestral SARS-CoV-2 (ref.[102]), and RNA viral loads were elevated in male patients infected with either Alpha or Delta variants compared with female patients[88]. However, a possible association of viral load dynamics with age or sex is highly debated, as other studies demonstrated that they have no influence on infectious virus[25] or RNA viral loads[59].

Early studies in ancestral SARS-CoV-2 did not find a difference in virus isolation success[103] or RNA viral loads between children and adults[104–106], but sample sizes were small. Slightly lower RNA viral loads and a more rapid clearance of viral RNA was observed in children than in adults when analysing much larger cohorts, whereas the patterns of shedding curves over time were similar between children and adults[107]. Furthermore, large-scale analysis of viral loads across different age groups showed no differences of distribution of RNA viral load between children and adults[108] or only slightly lower viral loads (<0.5 $\log_{10}$ units) in children <5 years of age[86].

Symptoms as a correlate for shedding

One of the key epidemiological parameters for SARS-CoV-2 transmission is the incubation period, defined as the time from exposure or infection to the onset of symptoms. Studies on ancestral SARS-CoV-2

Box 2

Shedding of respiratory viruses

The dynamics of viral shedding differs between respiratory viruses, which influences their transmission and has an effect on diagnostics and measures applied to contain the outbreaks.

SARS-CoV

The epidemic of severe acute respiratory syndrome coronavirus (SARS-CoV) started in November 2002 in the Guangdong province of China and rapidly spread outside China. The virus was airborne and could also be spread via droplets of saliva, but is only moderately transmissible among humans[188]. Only low viral loads were detected in the early symptomatic period, generally peaking in the upper respiratory tract (URT) around 10–14 days post-onset of symptoms (dpos)[189,190], and then dropping to low levels at 3–4 weeks post-infection[191]. In patients infected with SARS-CoV, viral RNA was detectable for a maximum of 8 weeks in samples collected from the URT[191] and for 52 days in sputum samples[192], whereas infectious virus was isolated up to 28 dpos from stool and respiratory specimens and up to 36 dpos from urine samples[191,193]. SARS-CoV replicated less efficiently at low temperatures; thus, virus replication was more efficient in the lower respiratory tract (LRT) than in the URT[194]. Notably, asymptomatic or pre-symptomatic viral shedding and transmission were not recorded for SARS-CoV[190,195]; the peaks of transmission occurred around 2 and 10 dpos[195]. As a result, outbreaks were successfully contained through isolation of symptomatic patients infected with SARS-CoV, which reduced onward transmission[196].

MERS-CoV

Middle East respiratory coronavirus (MERS-CoV) was isolated from a patient with pneumonia in Saudi Arabia in 2012 and was shown to be the causative agent of a cluster of severe respiratory tract infections in the Middle East[197]. The disease caused by MERS-CoV is characterized by a wide range of clinical severities and by predominantly respiratory symptoms, such as acute viral pneumonia, with a high case fatality ratio[198]. The virus is capable of airborne transmission and has low transmissibility among humans, with a maximum estimated reproduction number below 1 (ref.[198]). Higher RNA viral loads were detected in the LRT than in the URT. Estimated mean shedding duration is 15.3 days in the URT and 16.3 days in the LRT[62]. Prolonged PCR positivity and higher RNA viral loads in the URT and LRT were associated with increased disease severity[62,199]. Viral RNA was also detected in the urine, stool and serum[200]. One study reported detection of viral RNA in the blood for 34 days and showed that presence of viral RNA in the blood is associated with higher mortality[201]; however, another study failed to isolate virus from PCR-positive serum samples[200].

Influenza virus

In symptomatic patients, RNA viral loads start to be detectable by real-time PCR 2 days before the onset of symptoms and peak at 1 dpos[202]. Human challenge trials with influenza A viruses show that viral loads already sharply increase at 1 day post-inoculation, reach a peak at 2 days post-inoculation and become undetectable at 8 days post-inoculation. The mean duration of viral shedding for influenza viruses is 4.8 days, and the maximum duration is between 6 and 7 days[203,204]. Kinetics of infectious viral titres were similar to the viral load trends detected by real-time PCR for different strains of influenza[205]. Lower RNA viral loads and shorter infectious viral shedding were noted in asymptomatic patients[202].

Human respiratory syncytial virus

This virus is the most frequent causative agent of LRT infections, leading to morbidity and mortality particularly in young children and older adults[206]. The virus is transmitted by contact with nasal secretions or large aerosols. Viral loads and symptoms increased simultaneously, reaching a peak at 5.4 days[207]. In human challenge trials, respiratory syncytial virus titres were detectable for an average of 4.6 days. Viral RNA could be still detected up to 9 dpos, whereas infectious virus titres could be detected from 1 to 8 dpos in adults[208] and up to 9 dpos in children[209].

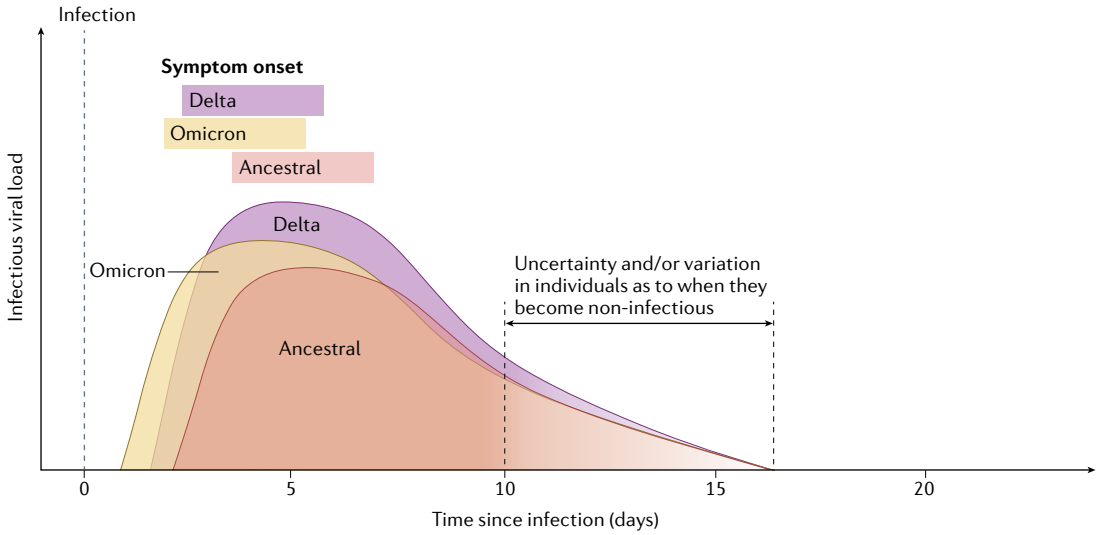

Fig. 3 | Infectious viral load and symptom onset in SARS-CoV-2 Delta and Omicron BA.1 variants of concern. Overall patterns of shedding dynamics are conserved between SARS-CoV-2 variants. In comparison to ancestral SARS-CoV-2, Delta and Omicron BA.1 have shorter incubation periods, estimated as approximately 3.7–4 days for Delta and approximately 3–3.4 days for Omicron BA.1. Higher infectious viral loads were detected in patients infected with Delta than in patients infected with Omicron BA.1 or ancestral SARS-CoV-2. Only a limited number of studies have determined when virus shedding for Delta and Omicron BA.1 ends, so this time point is not well defined. Owing to the low number of studies comparing the end of the infectious period between different SARS-CoV-2 variants of concern, the end point of infectivity is not well defined (shown as a colour gradient). Details of the underlying studies used to generate Fig. 3 can be found in Supplementary Table 2.

have estimated that the incubation period on average is between 4.6 and 6.4 days[59,109–111] (Fig. 2). A human challenge trial with ancestral SARS-CoV-2 demonstrated that symptoms start to appear 2–4 days after inoculation, and RNA viral loads reach their peak 4–5 days after inoculation[29]. Thus, artificial inoculation of the virus confirmed the timing of peak viral loads observed in naturally infected individuals, whereas onset of symptoms was faster in the human challenge cases. In contrast to natural infection, in artificial inoculation, virus-containing drops with high viral load are directly applied in the nose and therefore reach the nasal epithelium more quickly, which might lead to the more rapid appearance of symptoms. For Delta, the estimated incubation period was between 3.7 and 4 days[81–83,97], whereas infection with Omicron BA.1 was characterized by an even shorter incubation period of 3–3.4 days[83,112,113] (Fig. 3). However, as the time point of infection is rarely known outside of human challenge trials, dpos is most commonly used when analysing viral load and infectious virus.

Considering that high viral loads can be detected in the URT of infected individuals regardless of their clinical manifestations, the presence of symptoms is an unreliable indicator of infectiousness. Notably, individuals infected with SARS-CoV-2 can be infectious before the onset of symptoms[59], and it was estimated that about half of secondary transmissions take place in the pre-symptomatic phase[59,114]. Moreover, according to population surveys, asymptomatic cases represent around 40% of all SARS-CoV-2 infections with ancestral SARS-CoV-2 (refs.[115–117]), and tracing of close contacts of confirmed cases of SARS-CoV-2 found that up to 23% of infections were asymptomatic[118].

There are conflicting findings regarding viral shedding differences in symptomatic and asymptomatic patients. Comparison of viral loads between symptomatic and asymptomatic patients remains challenging, as the time of exposure cannot be clearly identified in asymptomatic individuals, and dpos cannot be used when comparing viral loads with symptomatic individuals. Furthermore, individuals who do not show clinical symptoms at the time of testing can represent either true asymptomatic individuals or pre-symptomatic individuals who will develop symptoms later. Thus, only well-controlled studies with a follow-up of assessed individuals can make a clear distinction between pre-symptomatic and asymptomatic individuals. A study on ancestral SARS-CoV-2, which followed COVID-19 confirmed cases hospitalized for isolation and recorded symptoms daily, found similar initial Ct values between asymptomatic and symptomatic individuals[119]. Similarly, no significant difference in RNA viral loads between symptomatic and asymptomatic patients was found in other studies in which patients were followed longitudinally and the presence of symptoms was either monitored by health-care professionals[120] or was self-reported[115]. By contrast, other studies, in which symptoms were also recorded by clinicians, reported lower RNA viral loads in asymptomatic participants[121,122]. In addition, one study found a faster clearance of viral RNA in asymptomatic than in symptomatic individuals[123], and another recorded a longer median duration of viral RNA shedding among asymptomatic patients[119].

There are limited data regarding the presence of infectious virus in asymptomatic patients. One study showed lower virus isolation success from asymptomatic patients[124], but only a small number of patients were included. Therefore, more studies evaluating infectious virus in asymptomatic patients would help to elucidate the differences in their infectivity compared with symptomatic patients.

SARS-CoV-2 transmission

Viral loads have a key role in the SARS-CoV-2 transmission. As previously discussed, host (role of vaccination or previous infection) and viral factors (SARS-CoV-2 variants) greatly influence viral load dynamics and therefore further influence viral transmission.

Influence of viral load on transmission

SARS-CoV-2 can be transmitted via larger droplets and aerosols produced when breathing, speaking, sneezing or coughing and to a lesser extend also by contaminated surfaces. As an infection can only be induced by infectious viral particles and not by remnant RNA or protein alone, the presence of infectious SARS-CoV-2 is required for secondary transmission. Although transmission is a multifactorial process that is also influenced, for example, by environmental and behavioural factors (such as humidity, air quality, exposure time or closeness of contact), the viral load of SARS-CoV-2 in the URT is considered to be a proxy for transmission risk.

An epidemiological study that included viral load analysis found that viral load of an index case strongly correlates with onward transmission, with higher viral loads for ancestral SARS-CoV-2 presenting a greater secondary attack rate risk[125]. In this study, viral load was identified as the main driver of transmission, with a more pronounced effect in household settings than in non-household settings (hospitals and nursing homes, among others). Transmission probability peaks around symptom onset, when infectious virus titres are estimated to be the highest during the course of infection. As viral load decreases with time, the probability of transmission also gradually declines in cases of infection with ancestral SARS-CoV-2 (ref.[126]). On this note, a study of health-care workers infected with ancestral virus documented no transmission from index cases later than 6 dpos, which is in line with findings showing reduced virus isolation success towards the end of week 1 of symptomatic disease[127].

However, there are limitations when using viral load of an index case as a proxy for transmission. To date, the infectious dose of SARS-CoV-2 required to lead to a secondary transmission is not yet known, and the association between presence of infectious virus in the respiratory tract and infectiousness of the same individuals is poorly understood. In the only available human challenge trial that was conducted with ancestral SARS-CoV-2, an initial infectious dose of 10 $TCID_{50}$ did not lead to an infection in 16 of 36 participants[29]. Other factors, such as symptoms, type of contact, protective measures, vaccination status and other host factors may have an additionally strong effect on transmission[128–133].

Viral load can markedly vary between individuals (as a result of individual susceptibility and of immunity from previous infections or vaccination), which leads to differences in their propensity to transmit the virus. Indeed, differences have been observed in the duration of infectious virus detection and in nasal and oral viral loads for both ancestral SARS-CoV-2 and Alpha[33]. Inter-individual variability was suggested to have a role in the observed heterogeneity of viral load dynamics, as some early immune signatures were significantly associated with higher oropharyngeal RNA viral loads in patients[134]. Therefore, observed heterogeneity between individuals has an important role in ongoing viral transmission[33].

Such differences can lead to heterogeneity in virus transmission. Modelling with ancestral SARS-CoV-2 and Alpha estimated that individuals who are highly infectious, known as superspreaders, shed 57-fold more virus over the course of infection than those with lowest infectiousness[33]. By contrast, most patients with COVID-19 do not infect other individuals as they expel few to no viral particles from their airways[135]. Indeed, only a minority (about 8%) of patients positive for SARS-CoV-2 infected with ancestral SARS-CoV-2 or Alpha have significantly higher infectious virus titres than the rest of the population (as shown in a study measuring virus isolation probability in a large cohort of patients)[86]. Moreover, only 15%[114] to 19%[136] of individuals that were infected led to 80% of secondary transmissions of ancestral SARS-CoV-2. Similar trends were confirmed for Omicron BA.1 and BA.2, for which only 9%[137] to 20%[138] of the infectious contacts were responsible for 80% of all transmissions.

Superspreading events are therefore characterized by infectious individuals having close contact with a high number of susceptible individuals and by a higher probability of transmission per contact. Aside from biological factors influencing these events, sociobehavioural and environmental factors contribute to the likelihood of superspreading (for example, large indoor gatherings with poor ventilation and no other infection prevention measures). Moreover, particular locations can represent a higher risk of transmission (for example, many superspreading events take place in crowded indoor settings, such as cruise ships, family gatherings, parties, elderly care centres and hospitals)[139].

The role of pre-existing immunity on viral shedding and transmission

All currently licensed SARS-CoV-2 vaccines are administered intramuscularly, leading to a rise in serum antibodies and protection from severe disease and death due to COVID-19, but not to long-term protection from infection[140–142]. The levels of circulating antibodies generated following vaccination decline over time, but can be elevated by a booster dose[143,144]. Furthermore, currently available vaccines were developed against the ancestral SARS-CoV-2 strain using the spike protein of the first sequenced virus, and the degree of protection from severe disease against other genetic variants was shown to vary[145]. Moreover, vaccination leads to limited induction of neutralizing antibodies on mucosal surfaces, which may have a role in mitigating virus replication and prevention of more pronounced disease[146,147]. For instance, secretory component antibodies, which are specific to mucosal surfaces, were detected in the saliva in 58% of participants 2 weeks post-vaccination with mRNA vaccines in one study, but the levels were significantly lower than in convalescent participants, and their neutralizing capacity significantly decayed 6 months post-vaccination[148]. A study on a small group of individuals uninfected or infected with Delta demonstrated that mucosal antibody responses induced by vaccination were low or undetectable, but breakthrough infections led to substantial increases of antibody titres in saliva[149]. However, the role of pre-existing mucosal immunity on infectious virus shedding and the possible correlation between the mucosal antibodies and viral loads in humans has not been elucidated.

As a result of waning antibodies and the emergence of VOCs with immune-evading properties, breakthrough infections have been increasingly reported among vaccinated individuals, mainly since the emergence of the Delta and Omicron VOCs. It has been debated whether vaccination with current SARS-CoV-2 vaccines impacts viral load (and therefore shedding) in breakthrough infections. The effect of vaccination on viral load and shedding is therefore of interest as it would mean that vaccination not only protects the vaccinee but can also help to mitigate virus spread by reducing infectious virus titres or shortening infectious shedding periods, thus having an impact beyond protection of the individual.

Overall, vaccination has been found to lead to reduced viral load (Fig. 4), although this decreases with time. Vaccination with ChAdOx1 vaccine (the Oxford–AstraZeneca vaccine) or BNT162b2 (the Pfizer/BioNTech vaccine) leads to lower RNA viral loads in individuals infected with Alpha, but the effect was weaker for breakthrough infections with Delta[150,151]. Immunization with BNT162b2 led to reduced RNA viral loads in Delta breakthrough infections, although this effect declined

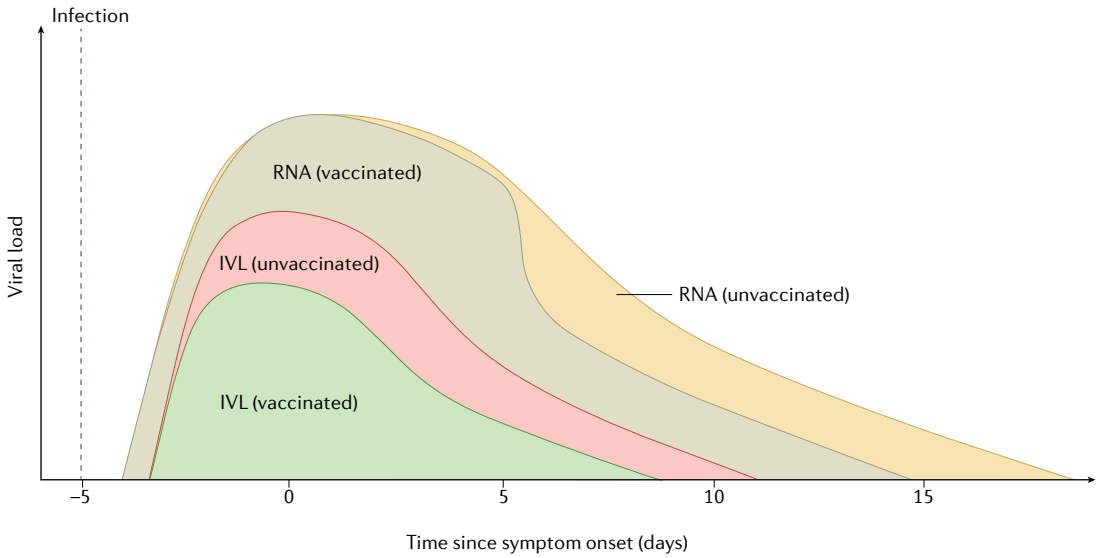

Fig. 4 | Influence of vaccination on viral load. Similar RNA viral loads were detected in vaccinated and unvaccinated patients infected with the Delta variant of concern during the first 5 days post-onset of symptoms. However, faster clearance of viral RNA was shown in vaccinated patients. Infectious viral loads (IVLs) were significantly lower in vaccinated individuals and declined faster than in unvaccinated individuals infected with Delta. Dynamics of viral loads in vaccinated individuals may vary widely in case of infection with another variant. Details of the underlying studies used to generate Fig. 4 can be found in Supplementary Table 3.

2 months after vaccination and ultimately faded 6 months after vaccination[152]. Immunization with ChAdOx1 vaccine also led to a reduction of RNA viral load in breakthrough infections with Alpha VOC[153]. Faster clearance of RNA viral loads was detected in the group of vaccinated patients who mostly received mRNA vaccines[154,155], and lower probability of isolation of infectious virus from patients vaccinated with mRNA or adenoviral vector vaccines was observed[156,157]. Even though not all studies could demonstrate a reduction of RNA viral loads in Delta breakthrough infections[150,154], infectious virus titres were reported to be lower in individuals vaccinated with mRNA or adenoviral vector vaccines despite similar levels of viral RNA[25,93,157]. Vaccination was also found to influence infectious virus isolation. Viable virus in cell culture was detected for significantly longer median time periods in unvaccinated patients infected with Delta than in vaccinated patients infected with Delta[155,158]. However, no significant differences in RNA viral loads were found between unvaccinated, fully vaccinated or boosted patients infected with Omicron BA.1 or BA.2 (refs.[93,159]), whereas infectious virus titres, measured quantitively at 5 dpos, were lower in Omicron BA.1 breakthrough infections only after a booster dose[25]. Other studies showed that vaccination status did not influence infectious virus isolation success[93] or the time from initial positive PCR assay to culture conversion in patients infected with Omicron BA.1 (ref.[85]). These studies indicate that triple vaccination reduces infectious viral load but not the time period during which infectious virus can be isolated from Omicron breakthrough infections.

There are limited data on the effect of previous infection on viral shedding. A study performed on ancestral SARS-CoV-2 demonstrated lower RNA viral loads among seropositive individuals than among seronegative individuals[160]. Although higher levels of reinfection with Omicron BA.1 were demonstrated among unvaccinated patients previously infected with other SARS-CoV-2 variants[161], there are no relevant data on the effect of previous infections on viral load dynamics.

Together, these findings suggest that vaccinated individuals are less infectious than unvaccinated individuals, although the duration of this effect has not been studied systematically. Nevertheless, there are some conflicting data on the effect of vaccination on onward transmission. An epidemiological study performed in the UK found that, despite RNA viral load declining faster among fully vaccinated than unvaccinated patients infected with Delta, the peak RNA viral loads were similar, and the secondary attack rate among household contacts exposed to fully vaccinated or unvaccinated index cases did not differ[151]. By contrast, data from Israel showed that less Delta transmission took place in households with vaccinated participants than with unvaccinated participants[130]. Another study from the UK showed that both BNT162b2 and ChAdOx1 vaccines led to the reduction of onward transmission from vaccinated index patients, although a stronger reduction was detected for Alpha than for Delta[129], probably owing to the higher viral loads in the case of infection with Delta, as shown previously[88,89,129]. Finally, another study found that vaccination was associated with reduced onward transmission of Delta breakthrough infection due to shorter duration of viable virus shedding[158].

Overall, even though the currently used vaccines are still based on the ancestral virus spike protein and elicit mainly a systemic rather than a mucosal immune response, some effect on viral load, infectious virus shedding and transmission has been observed[129,130,162]. Furthermore, with increasing rates of breakthrough infections in the Omicron waves since the end of 2021, many individuals display hybrid immunity consisting of vaccination combined with one or more natural infections before or after vaccination[163,164]. It is thought that such hybrid immunity may provide better control of virus replication in the mucosa[149,163,165].

With the constant emergence of novel variants that can evade existing immunity, our understanding of the effect of vaccination on viral shedding should be constantly updated[166]. Better understanding of the role of mucosal immunity, and potentially vaccines that elicit

Glossary

Chimeric antigen receptor (CAR) T cell therapy
A way to treat cancer by using T cells expressing genetically engineered receptors to target cancer cells.

Cycle threshold (Ct) value
The number of amplifications required for a target gene to cross the threshold determined by real-time PCR. Arbitrary test-specific Ct values inversely correlate with viral load.

Focus-forming assays
Assays that count the number of 'foci', defined as a cluster of adjacent cells expressing viral antigen stained by a specific antibody.

Immunostaining
A method for the detection of specific proteins in individual cells or tissues using antibodies. In the case of SARS-CoV-2, anti-nucleocapsid antibodies are used to detect virus in infected cells.

Index case
The infected individual who is triggering an outbreak or a cluster by transmitting an infectious agent to others. There might be multiple index cases in an outbreak or epidemiological study.

Plaque assays
Assays that quantify the number of infectious virions by counting plaques in a cell monolayer that correspond to single infectious particles.

Secondary attack rate
The probability that an infection spreads from an index case to susceptible people in a specific setting (usually, a household or close contacts). The term is used to evaluate the risk of onward transmission of pathogen within a population.

Seroconversion
The development of specific antibodies in the serum as a consequence of immunization by natural infection or vaccination.

sgRNA
Subgenomic RNA fragments that occur during viral replication.

$TCID_{50}$
A measurement of the presence of cytopathic effects in cells upon infection with serial dilutions of virus specimens, which indicates the dose needed to induce a cytopathic effect in 50% of the inoculated wells.

local rather than systemic immune responses, are needed to aim for viral load reduction as a means to control SARS-CoV-2 circulation[167–169].

Influence of SARS-CoV-2 VOCs on transmission

There are several possible underlying causes of increased transmissibility of newly emerging variants, which allow VOCs to quickly outcompete previously circulating strains, including increased viral loads, a lower infectious dose required to establish infection and prolonged period of infectiousness[170]. Furthermore, the immune-evading properties of new variants lead to higher susceptibility of infection for vaccinated and previously infected individuals and result in higher transmissibility, as was observed with Omicron[166,171].

The rapid emergence of SARS-CoV-2 variants with altered biological properties has shown that knowledge on viral loads, viral kinetics and infectious virus shedding is variant specific, and each emerging variant requires a reassessment. Although understanding of mutational profiles and associated phenotypes of SARS-CoV-2 variants has improved, reasons for enhanced transmissibility are manifold and not all understood yet. To date, shedding characteristics and transmission properties cannot be easily predicted based on sequences. Unlike immune-evasion mechanisms, shedding dynamics, such as kinetics of infectious virus titres or incubation periods of the SARS-CoV-2 variants, cannot be predicted from specific mutation patterns. With a still highly dynamic situation in terms of viral evolution of SARS-CoV-2, understanding viral kinetics and their effect on transmission remains of high public health interest.

SARS-CoV-2 diagnostics in public health

Our ability to define the presence of infectious virus is key to guiding public health measures, as it will enable the isolation of infectious individuals to limit secondary transmission. Unfortunately, no point-of-care diagnostic test currently exists to determine infectious SARS-CoV-2 in a patient sample[172], and virus culture as described above is not suited for diagnostic purposes. Thus, a range of approaches have been suggested to find a proxy for infectiousness to guide isolation periods.

One example is the detection of sgRNA transcripts, which are generated during virus replication, and specifically the synthesis of negative-strand RNA. Although sgRNAs are transcribed in infected cells, they are not packaged in the virions and can therefore serve as an indicator of active replication and thus of infectious virus. Specific RT-PCR assays were developed to detect sgRNAs in addition to the diagnostic detection of genomic SARS-CoV-2 RNA, but such assays have not made their way into routine diagnostic use owing to their lower sensitivity than conventional RT-PCR assays. Some studies found that detection of sgRNA correlates with detection of infectious virus[4,173,174], and that sgRNA was rarely detectable 8 dpos[67]. However, sgRNA was detected in diagnostic samples up to 17 days after initial detection of infection[175] or in culture-negative samples[176], probably owing to the stability and nuclease resistance of double-membrane vesicles containing sgRNAs. Thus, although the absence of sgRNA would indicate absence of viral replication, the presence of sgRNA does not necessarily indicate infectiousness[19].

Ct values have also been used as a proxy for infectiousness, as described above. However, as already discussed, low-quality specimens resulting from technical mistakes during the collection process can falsely indicate an absence of infectious virus. Furthermore, owing to the quick increase of RNA viral load at the beginning of the infection, a low viral load, especially in the absence of symptoms or in the early symptomatic period, does not preclude that an individual will not soon enter the infectious period with the highest transmission risk. At such a period, viral loads reach their peak levels, causing the majority of transmission events[59,126].

Even though Ag-RDTs are less sensitive than RT-PCR, they are less expensive, can be performed outside of laboratory settings and give faster results, and so are useful tools to guide isolation and limit transmission[177]. RT-PCR tests have a limit of detection of 10^2–10^3 genome copies per millilitre, whereas Ag-RDTs have a limit of detection corresponding to 10^4–10^6 genome copies per millilitre[177–180]. Infectious individuals typically have RNA viral loads of >10^6 genome copies per millilitre, which corresponds largely with a Ct of 25 in most RT-PCR assays[4], indicating that Ag-RDT is a good proxy for infectiousness[177]. However, the obvious limitations of Ag-RDT, such as lower sensitivity of infectious virus detection towards the end of infection[47,52], should not be neglected. Ag-RDTs have also shown variation in their sensitivity and specificity for detection of SARS-CoV-2 VOCs[53,54], which is a challenge as new variants emerge.

Overall, all of the currently available diagnostic methods have certain limitations for detection of infectious virus. However, even if these tests serve only as imperfect tools when used as proxies for infectiousness, their implementation as part of a public health strategy is not intended to prevent every single infection, but rather to reduce the

number of infectious people in the community and thus to decrease the number of secondary transmissions.

Conclusions

Entering the third year of the pandemic, much knowledge on SARS-CoV-2 viral loads, infectious virus shedding and windows of infectiousness has been gained, although emerging SARS-CoV-2 variants and an increasing population immunity add more complexity to the situation.

Although much progress has been made during the pandemic in the field of diagnostics, to date, no diagnostic tests exist that reliably determine the presence of infectious virus. Continuing evaluation of viral-shedding characteristics under these changing circumstances and understanding the biological properties of novel SARS-CoV-2 variants when it comes to viral shedding remain of importance to guide public health practices.

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O.P. and I.E. wrote the manuscript. B.M. created the figures. All authors contributed to the discussion of the content, and reviewed and edited the manuscript before submission.

Competing interests
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LETTERS

Edited by **Jennifer Sills**

Editorial Expressions of Concern

In 2001, *Science* published the Report "Binding of DCC by netrin-1 to mediate axon guidance independent of adenosine A2B receptor activation" by E. Stein *et al.* (*1*). In 2015, the authors made us aware of issues with Western blot images in Figures 1 and 3, which were caused by tiling with overlap of adjacent columns and combining blot images to standardize panel sizes. The authors provided corrected images, but due to an error on our part, *Science* never posted an Erratum. We regret this error and apologize to the scientific community. Both we and the authors are aware that additional concerns have been raised since 2015. We are therefore not proceeding with a correction at this point but are alerting readers to the concerns while the authors and the authors' institution investigate further.

H. Holden Thorp
Editor-in-Chief

REFERENCES AND NOTES

1. E. Stein, Y. Zou, M.-M. Poo, M. Tessier-Lavigne, *Science* **291**, 1976 (2001).

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In 2001, *Science* published the Research Article "Hierarchical organization of guidance receptors: Silencing of netrin attraction by Slit through a Robo/DCC receptor complex" by E. Stein and M. Tessier-Lavigne (*1*). In 2015, the authors made us aware of issues with Western blot images in Figures 4, 5, and 6, which were caused by tiling with overlap of adjacent columns and combining blot images to standardize panel sizes. The authors provided corrected images for those Western blots. In addition, images were duplicated in Figure 2D (micrographs) and in Figure 4B (blank Western blots), and an incorrect blank panel was used in Figure 5E (top right). The original data for these figures could not be accessed, so not all specific conclusions related to these panels are supported by these figures. *Science* agreed to the publication of an Erratum; however, due to an error on our part, it was not posted. We regret this error and apologize to the scientific community. In light of the delay, we are not proceeding with a correction at this point but are alerting readers to the concerns while the authors and the authors' institution investigate further.

H. Holden Thorp
Editor-in-Chief

REFERENCES AND NOTES

1. E. Stein, M. Tessier-Lavigne, *Science* **291**, 1928 (2001).

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Retraction

In the Research Article "Gradual emergence followed by exponential spread of the SARS-CoV-2 Omicron variant in Africa" (*1*), we reported data from retrospective characterization of viral genomes of putative ancestors of the SARS-CoV-2 Omicron variant from western Africa months before the first detection of Omicron. After several social media posts suggested that these putative early Omicron ancestor sequences may have been false positives, we reanalyzed our data and the residual samples. We found a mixture of different SARS-CoV-2 genomic fragments contaminating some of the samples and sequence data on which we based our analysis. The residual samples are now exhausted, and the reconstruction of evolutionary intermediates cannot be replicated. Therefore, we are retracting our Research Article. The epidemiological data are not called into question and will be made available.

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