# Pandemia de COVID - 19



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# Alterations in Immune Genes Make Bats Great Viral Hosts

Bat species use different strategies to dampen immune activation in response to viruses.

**Abby Olena**
*Oct 27, 2020*

Bats act as reservoirs for lots of viruses—including coronaviruses such as those that cause Middle East respiratory syndrome, severe acute respiratory syndrome, and possibly COVID-19—but they don't often get sick themselves. How they avoid viral illness has been an open question. Researchers reported in *PNAS* yesterday (October 26) that various species of bats have slightly different ways of suppressing inflammation, all centered on changes in genes responsible for triggering innate immune responses.

ABOVE: Cave nectar bat (*Eonycteris spelaea*)
FENG ZHU

The authors demonstrate a number of the mechanisms in bats that seem to support their capacity to tolerate viruses that make other mammals really sick, says Cara Brook, a postdoc at the University of California, Berkeley, who was not involved in the work. "This follows a series of other publications that really highlight a dampened inflammatory response in bats that suggests that they are uniquely resistant and resilient to the consequences of immunopathology . . . and don't experience the kind of autoimmune disease that we often incur against ourselves."

In a study published in 2013, Linfa Wang, an immunologist at Duke-NUS Medical School in Singapore, and colleagues compared the genomes of two bat species: the fruit bat (*Pteropus alecto*) and insectivorous bat (*Myotis davidii*). They found that both species had lost a gene called *AIM2,* which in other mammals encodes a protein that senses pathogenic DNA and triggers inflammasomes, protein complexes that activate proinflammatory signals that in turn promote the maturation of cytokines, small signaling proteins that can be released by immune cells and regulate inflammation and immunity.

In the current study, Wang's group followed up on *AIM2* to figure out what affect its loss has on cellular responses to pathogenic DNA. They compared macrophages, the innate immune system's primary effector cells, from mice and fruit bats. The mouse cells, which have a functional gene, make the aggregates of AIM2 and its protein binding partner, which together trigger the inflammasome pathway when cells are exposed to double-stranded DNA. None of this occurred in the fruit bat cells. When the researchers added in a copy of the human version of *AIM2* to fruit bat kidney cells aggregates still formed, but did not activate other inflammasome-related genes, including those that encode the effector enzyme caspase-1, which activates the proinflammatory cytokine IL-1β.

**What's nice about this paper is that it points to the fact that different species have evolved different mechanisms for achieving the same ends.**

—Vikram Misra, University of Saskatchewan

"We hypothesized that further downstream activation of the inflammasome pathway may be affected in bats and decided to investigate these signaling components in an effort to detect any alteration in their function," Wang writes in an email to *The Scientist.*

The researchers determined that the faulty caspase-1 response was due to bat-specific mutations in two sites within the fragment of the enzyme that must be cleaved in order for it to be activated. When they engineered the equivalent human amino acids back into the coding sequence, the bat enzyme worked just as the human protein does. The reverse experiment confirmed these mutations were responsible for the impaired enzyme function. Introducing both bat-specific mutations into the gene for the human protein resulted in a loss of function of human caspase-1.

Cave nectar bats (*Eonycteris spelaea*)
FENG ZHU

In contrast, they found, the *Myotis* genus of bats has functional caspase-1, but these animals' genomes instead contain mutations in IL-1β that prevent the cytokine's cleavage and subsequent for cellular secretion. A third species, the cave nectar bat (*Eonycteris spelaea*) had diminished, though not completely suppressed, function of both caspase-1 and IL-1β, resulting from a handful of mutations.

When people "find something about one species of bats, they assume that every bat species does the same thing, and that's not true," says Vikram Misra, a virologist at the University of Saskatchewan who did not participate in the study "What's nice about this paper is that it points to the fact that different species have evolved different mechanisms for achieving the same ends."

"It's very small changes in specific amino acids, where you have one change . . . that can completely change the function of a protein," Karen Mossman, a virologist at McMaster University who did not participate in the work, tells *The Scientist.* In the future, it will be "interesting to really understand how all of these subtle changes in these proteins work collectively to give the bats their immune system," she adds. "It's so similar to the human immune system; the components of the pathways are very similar. And yet, there're these vast, vast changes and differences in how they respond, say, to a viral infection."

Although many species of bats don't seem to get sick from viruses, inflammation in bats does exist, such as when they're exposed to fungal diseases, Misra says. "Even though inflammation because of the viral infection is dampened, there've got to be other pathways that bring out inflammation. That's something that I think we haven't, as a group of bat researchers, addressed completely at this point."

**G. Goh et al., "Complementary regulation of caspase-1 and IL-1β reveals additional mechanisms of dampened inflammation in bats," *PNAS*, doi:10.1073/pnas.2003352117, 2020.**

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bat, bats, cell & molecular biology, coronavirus, COVID-19, genetics & genomics, immunology, infectious disease, microbiology, News, pandemic, SARS-CoV-2, virus, viruses, zoonoses

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# SARS-CoV-2 Disables Key Components of Human Cells' Defense System

Researchers detail how viral proteins interact with host RNA to disrupt the cell's ability to fight back against infection.

**Catherine Offord**
*Oct 22, 2020*

Viral proteins encoded by SARS-CoV-2 disrupt critical components of human cells' molecular machinery and disable responses to infection, according to a study published October 8 in *Cell*. Researchers in the US describe how specific viral proteins bind to human RNAs involved in RNA splicing, protein translation, and protein trafficking, and in doing so suppress the host cell's coordination of a key antiviral defense known as the type I interferon response.

ABOVE: A mammalian cell infected with SARS-CoV-2, showing the formation of compartments where viral RNA is replicated (top left) and individual virions exiting the cell (right)
EMILY BRUCE

The study offers a possible mechanistic explanation for the blunted immune responses observed in some COVID-19 patients, says Benjamin Terrier, an immunologist at Cochin Hospital in Paris who was not involved in the work. The researchers "are clearly demonstrating how the virus is able to impair the production of proteins involved in [this defense]."

**See "The Immune Hallmarks of Severe COVID-19"**

In the type I interferon response, a cell that has detected foreign genetic material in its cytoplasm launches the production and secretion of interferons—signaling proteins that go on to trigger the expression of hundreds of antiviral genes.

Studies by Terrier and others have reported that patients with severe COVID-19 often show a huge reduction in interferon production, suggesting that the virus's success might in some cases be linked to an ability to disrupt this cellular defense.

**See "Cells' Response to SARS-CoV-2 Different from Flu, RSV"**

Caltech biologist Mitchell Guttman, along with Dev Majumdar of the University of Vermont's Larner College of Medicine and colleagues, discovered just how SARS-CoV-2 might be doing this while studying interactions between the virus's 27 known proteins and human RNA in vitro.

The researchers had inserted the genes for each of SARS-CoV-2's proteins into different sets of human cells, such that each cell would produce just one viral protein. Then, the team had extracted those proteins along with whatever RNAs they'd bound to and studied the interactions between the molecules.

The researchers found that 10 of the virus's proteins were bound to RNAs involved in critical cell processes such as RNA splicing, protein translation, and protein transport. To the team's surprise, Guttman says, as many as four proteins bound specifically to structural noncoding RNAs that make up the cell machinery in charge of coordinating those processes.

**We want to understand how the virus can disrupt host cell machinery and yet be able to engage ribosomes, be able to engage the trafficking machinery—all these things that have been shut off.**

The viral protein NSP16, for example, bound to a host RNA sequence in the spliceosome in a way that rendered the complex unable to recognize RNA transcripts, let

alone splice them properly. NSP1 bound to the ribosome in a position that made it impossible for mRNAs to enter —essentially "putting a cork" in the protein-translating structure, Guttman says. And NSP8 and NSP9 bound to and interfered with the signal recognition particle, a structure that helps transport newly translated proteins around the cell.

—Dev Majumdar, University of Vermont Larner College of Medicine

Based on these results, the team predicted that infection with SARS-CoV-2 would drastically reduce splicing, translation, and protein transport in human cells and would consequently suppress the interferon response, which relies on boosting these three processes. Indeed, lung epithelial cells infected with the virus showed just that, the researchers report in their paper.

**See "SARS-CoV-2 Protein Hampers Innate Immune Reaction In Vitro"**

Christian Münch, a cell biologist at Goethe University Frankfurt who was not involved in the work, praises the study's comprehensive description of the basic biological mechanisms underlying these effects. "This is one of these nice examples where very nice methods were [applied to] this new virus to look at what actually happens in the cell after infection," he says.

As the researchers note in their paper, Münch adds, the study did not rule out a role for other pathways in helping SARS-CoV-2 evade host defenses or examine potential interactions among the viral proteins themselves. He adds that future work could look more into how the effects develop over time from infection.

Guttman says the team is interested in possible therapeutic implications of the study's results. One route could be to target viral proteins such as NSP1 with drugs that stop them interacting with cellular apparatus.

The researchers are also investigating how the virus—which is dependent on the host to get its own RNA translated and its proteins transported—manages to replicate itself in such a severely compromised cell, says Majumdar.

"We want to understand how the virus can disrupt host cell machinery and yet be able to engage ribosomes, be able to engage the trafficking machinery—all these things that have been shut off," Majumdar explains. "What are the signals being presented by the viral mRNAs to basically give it a ticket to ride on all of those machines?"

Preliminary data indicate that distinctive loops in the structure of SARS-CoV-2 mRNAs could be acting as those signals, Majumdar says. Hitting the loops with small-molecule drugs might help block viral activity inside infected cells, he adds.

Terrier notes that most therapeutic strategies for SARS-CoV-2 focus on stopping the virus getting into cells, rather than dampening viral activity within already infected cells. "Since SARS-CoV-2 is really acute, I think the most important [thing] to do is to block the entry of the virus," he says. "If you are really efficient at that, you don't need to be as efficient" in targeting intracellular responses.

But the team's data offer crucial mechanistic information to inform future research on this and closely related viruses such as SARS-CoV (the virus responsible for the 2003 SARS outbreak) and Middle East respiratory syndrome coronavirus (MERS-CoV), he adds. "Trying to [understand] how the virus could determine the severity of the disease, the fact that it could paralyze the immune system—clearly that's very important."

**A.K. Banerjee et al., "SARS-CoV-2 disrupts splicing, translation, and protein trafficking to suppress host defenses," *Cell*, doi:10.1016/j.cell/2020.10.004, 2020.**

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cell & molecular biology, coronavirus, COVID-19, disease & medicine, interferon, mRNA, News, pandemic, protein trafficking, protein translation, ribosomes, RNA, SARS-CoV-2, splicing, virus

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# More SARS-CoV-2 Reinfections Reported, But Still a Rare Event

Repeat COVID-19 cases could offer clues about people's immunity to the novel coronavirus and how to vaccinate against it.

**Ashley Yeager**
*Oct 26, 2020*

At least 285 individuals in Mexico appear to have contracted the novel coronavirus twice, according to a preprint posted October 18 on *medRxiv*. The study, which has not yet been peer reviewed, is the largest to date to assess the possibility of SARS-CoV-2 reinfection. It fuels an ongoing discussion among researchers about how long immunity to the virus lasts after an initial infection and how that length of immunity may affect the way vaccines against the virus are administered in the future.

ABOVE: SARS-COV-2 virus particles (yellow) infect a cell (blue)
FLICKR, NIAID

"If we find that our immunity is poor, or nonexistent . . . this will be a big problem for vaccination policies," study coauthor Carlos Hernandez-Suarez, a researcher at Universidad de Colima in San Sebastian, Mexico, tells *The Scientist*, adding that no conclusions can be made from the current data about the strength of survivors' immunity or the protection offered by future vaccines. In his team's analysis of hospital records of 100,432 individuals infected with SARS-CoV-2 between March and July 2020, only 285 (0.26 percent) presented signs that they'd contracted the virus twice.

Hernandez-Suarez and colleagues set out to pinpoint the frequency of reinfections among the patients. They'd read several studies pointing to possible reinfections and also an August letter to the editor in the *Journal of Infection* by Julian Tang, a clinical virologist at the University of Leicester in the UK, and colleagues laying out criteria for defining reinfection. The definition included an initial PCR-confirmed infection with SARS-CoV-2, then clinical recovery and a negative SARS-CoV-2 PCR test, and then a confirmed SARS-CoV-2 PCR-positive test at least 28 days after the initial positive test. The suspected second infections need to be at least 28 days after the initial ones because evidence suggests it takes that long for viral shedding to begin to taper off and because certain SARS-CoV-2 antibodies start to drop off at that point, according to another study posted to *medRxiv* in July.

Following that definition, Hernandez-Suarez and colleagues dug into the hospital records and identified the 285 patients obtaining medical care after a second positive PCR-confirmed SARS-CoV-2 infection. For the majority of patients, "we didn't have the test in between," the negative test, Hernandez-Suarez notes.

The team did have that test for 14 individuals, and it was negative, but for the rest, the patients' second positive test came 28 days or more after the first, in some cases 60 or 70 days later, he explains. "The average was 66 days, with no complications, no problems, and then they went to the hospital again with [respiratory] problems." To the team, that was an indication of a second infection, rather than a prolonged initial illness.

It's hard to know without more data, such as negative tests in between positive ones, if the cases are in fact reinfections or long-lasting illnesses from a single infection, says Tang, who was not involved in the work. Still, the latest results align with other studies and case reports that indicate reinfection is rare.

"This reinfection thing is not new. It's not unusual, not dramatic, or scary, or whatever you want to call it. It's very normal," Tang tells *The Scientist*, noting that other viruses such as respiratory syncytial virus (RSV) can cause repeated reinfections. Whether it happens frequently with SARS-CoV-2, though, he says, is not yet clear.

## The genetic evidence for reinfection

The first indications that reinfection could occur came from case reports out of Hong Kong and Europe, and then Nevada. What distinguished some of these cases as clear reinfections rather than lingering initial infections is that the genomes of the viruses causing the first and second infections weren't identical.

"You can really only prove reinfection" if the viral variants from the first and second positive swabs are different, notes Angela Rasmussen, a virologist at Columbia University in New York, "because it's very unlikely that you would be infected with the exact same variants a second time."

SARS-CoV-2 has an RNA genome, which has a higher mutation rate than a DNA viral genome. The mutation rate is so fast, in fact, that "even within an individual host who's infected, you may have multiple variants that are just emerging as the virus replicates itself and makes mistakes," she explains. The genetic changes that SARS-CoV-2 has so far accumulated seem to be functionally inconsequential, and "in the population, when you think about how many millions of different coronavirus cases there are, different variants have emerged . . . [so] it's very unlikely that you would get the exact same one."

A study posted September 28 to *medRxiv* confirms four reinfections in Qatar using genetic sequencing data that found distinct viral variants between the first and second infections. These are among 133,266 confirmed SARS-CoV-2 cases that Roberto Bertollini, a doctor with the Ministry of Public Health in Doha, and colleagues analyzed. It's possible there were more reinfections, as the authors note that 243 individuals had positive PCR tests at least 45 days apart, but only 12 patients had sufficient viral genome data at both time points. Two of the cases had identical viral genomes on the first and second positive test, suggesting a lingering infection that never cleared in the weeks between the two positive tests, and the six others didn't have enough genetic changes to make a firm conclusion about reinfection.

Regardless, Bertollini and his colleagues conclude in their report that "the risk for documented reinfection was very rare at about 1-2 reinfections per 10,000 infected persons." The researchers further note that the "findings suggest that most infected persons do develop immunity against reinfection that lasts for at least [a] few months, and that reinfections (if they occur) are well tolerated and no more symptomatic than primary infections."

## Insights into immunity from reinfection cases

Contracting the virus twice within the span of a few months may be more common than the data collected so far suggest. That's if SARS-CoV-2 acts anything like seasonal coronaviruses, according to a paper published September 14. In that study, Arthur Edridge of the University of Amsterdam and colleagues analyzed stored blood samples of 10 healthy individuals that were taken, on average, every few months for the past 35 years. The team tested the samples for an increase in antibodies to a specific region of the structural capsid protein of each seasonal coronavirus.

Antibody levels spiked and dropped at regular intervals, showing "reinfections with all four seasonal coronaviruses are common and frequently occur after about a year," Edridge writes in an email to *The Scientist*. Reinfection is therefore a "common human coronavirus feature," he explains, and SARS-CoV-2 would most likely be no exception.

**See "Does the Common Cold Protect You from COVID-19?"**

Rasmussen, who was not involved with any of the studies, says that reinfection is certainly possible, but she's less convinced that SARS-CoV-2 will act like other coronaviruses that cause colds. Those other coronavirus reinfections may happen because those viruses only cause a mild infection and don't elicit a strong immune response with lots of long-lasting antibodies. SARS-CoV-2 could be very different, she says.

That immune durability should really be the focus of reinfection studies, Tang explains. Researchers need to look at the longevity of antibodies after infection or vaccination, because if they start to wane, then there may be a need for vaccine boosters several months after the initial shot is given. The case reports and preliminary studies contribute to that overall picture of understanding SARS-CoV-2 immunity and its implication for vaccination, he explains, "but there's a long way to go."

**Keywords:**

antibodies, coronavirus, COVID reinfection, COVID-19, disease & medicine, genetics & genomics, immune, immunology, News, news feature, pandemic, SARS-CoV-2, vaccination, vaccine, virology, virus, virus evolution

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# Monkeys Develop Protective Antibodies to SARS-CoV-2

A small study of macaques finds they don't develop a coronavirus infection the second time they are exposed, supporting the idea of using plasma from recovered patients as a treatment for COVID-19.

**Katarina Zimmer**
*Mar 17, 2020*

Whether people develop immunity to SARS-CoV-2 after being infected once is a pressing question for policymakers, public health professionals, and everyone affected by the spreading COVID-19 pandemic. It's of particular interest to several research groups and companies currently developing plasma therapies, whereby antibody-containing blood plasma is extracted from recovered patients and administered to patients with severe cases to help them fight off the infection.

ABOVE: © ISTOCK.COM, SELVANEGRA

Now, a study in monkeys provides some clues. Three rhesus macaques did not develop a second infection after recovering from a first exposure to the coronavirus and being reexposed to SARS-CoV-2, suggesting that primates are capable of developing at least some short-term immunity to the pathogen. The research, posted as a preprint to *bioRxiv* March 14, has yet to undergo peer review. To the authors, the results indicate that reports of some COVID-19 survivors being "re-infected" a second time can be explained by issues with testing rather than a failure to develop immunity.

"This is a really critical preliminary study," says Lisa Gralinski, a virologist at the University of North Carolina at Chapel Hill who wasn't involved in the study. Although many questions remain as to how the human immune system responds to the virus, "it's the start of an answer that's going to be really important to all of us."

The team's research began while the outbreak was mostly restricted to China. Around early February, reports surfaced that recovered COVID-19 patients who were discharged from hospitals later tested positive again, explains Chuan Qin, an experimental pathologist at the Institute of Laboratory Animal Sciences at the Chinese Academy of Medical Sciences. Qin and his colleagues, some of whom have previously studied monkey responses to MERS, wanted to investigate whether it was possible to become re-infected with the new coronavirus.

**Based on these results, the team questions the reports of COVID-19 survivors recovering and being discharged upon testing negative for the virus, only to then develop another infection.**

The team applied a dose of SARS-CoV-2 into the windpipes of four adult rhesus macaques. The researchers detected high concentrations of the virus in the animals' noses and throats, which peaked three days after the initial infection. This was accompanied by reduced appetite, weight loss, and an increased breathing rate. After sacrificing one of the monkeys and performing a necropsy a week after the initial exposure, the team found the animal had traces of the virus across many body tissues and had developed interstitial pneumonia—characterized by an inflammation of the lungs' alveoli, which is one of the key symptoms of COVID-19 that can occur in people.

The team collected the blood sera of the three other animals to track whether they were developing antibodies to the virus. Using a specialized assay, they found the monkeys' blood contained antibodies that targeted the spike protein of SARS-CoV-2, a surface protein the virus uses to enter human cells. Antibody levels were relatively low during the

first week after infection, but surged at three and four weeks. “Our study found that neutralizing antibodies are produced in the process of recovery after SARS-CoV-2 infection,” Qin writes in an email to *The Scientist*.

Roughly a month days after the initial exposure to SARS-CoV-2, the researchers verified that the animals had cleared the infection. They couldn’t detect the virus from swabbing the nose, throat, or anus, and there were no strikingly unusual features in the lungs based on a chest X-ray.

The team then exposed two of the remaining monkeys to a second dose of the coronavirus. Although the animals developed a slight fever, neither showed weight loss. In addition, the researchers couldn’t detect the virus based on nose, throat, or anal swabs. One of the two animals was euthanized and necropsied, which confirmed that there was no viral replication across body tissues.

## Support for plasma therapy for COVID-19

The researchers conclude that “neutralizing antibodies produced by SARS-CoV-2–infected monkeys can protect animals from reinfection,” Qin explains. Based on these results, the team questions the reports of COVID-19 survivors recovering and being discharged upon testing negative for the virus, only to then develop another infection. They suspect that this is probably the result of false-negative PCR tests in the hospital that missed lingering virus, and they note in the study that diagnostic techniques need to be further refined.

Columbia University virologist Angela Rasmussen agrees with this interpretation. “This study suggests that reinfection is likely not occurring frequently, if at all. It indicates that infection results in protective immunity against SARS-CoV, at least in the short term,” she writes in an email to *The Scientist*. Virologists on Twitter concur that reinfection with SARS-CoV-2 is unlikely, citing other recent preprints that describe a production of antibodies in people infected with SARS-CoV-2019.

It’s unclear how long this protective immunity lasts. Sheena Cruickshank, an immunologist at the University of Manchester, points out that the study is very short in duration. It’s too soon to say whether the researchers’ observations are due to the immediate, initial antibody response to the virus or to a long-lasting immune memory.

Typically, viral infection triggers plasma B cells to produce antibodies, but, eventually, memory B cells, which survive for long periods of time, are also stimulated to differentiate into plasma B-cells that generate more-targeted and more-effective antibodies upon re-encountering the same pathogen. It’s not clear whether the antibody surge the team observed is part of the “stage when [initial] antibody levels are still dropping off, rather than a true memory response,” Cruickshank writes to *The Scientist* in an email. In addition, the study only followed the immune responses of two macaques. “These are really tiny numbers to infer anything,” she adds.

Lengthier studies that follow animals or people at least three months after infection are critical to understanding long-term immune responses to SARS-CoV-2, she explains. Research on other coronaviruses suggest different long-term immune responses. For instance, studies in SARS patients find that antibody levels fade after a few years. Some cold-causing coronaviruses “tend to induce immunity that is very short-lived, at around three months,” notes Peter Openshaw, a professor of experimental medicine at Imperial College London, in a recent statement in response to recent discussions on herd immunity in the UK. (Last week, British officials seemingly presented a plan to combat the outbreak that was communicated as allowing the deadly virus to spread to build immunity among the general population and thereby slow transmission—an approach decried by many scientists. UK Health Secretary Matt Hancock later insisted to the *BBC*, “herd immunity is not our policy. It’s not our goal.”)

Despite the small numbers of animals observed in the study, “in these circumstances, this study is still informative and useful,” Rasmussen says. To her, the results underscore the possibility that plasma therapy approaches could be a useful treatment option. Administering antibodies from other people is thought to be one way to provide patients with immediate immunity. Some studies suggest this approach reduced the risk of death for flu patients during the 1918 influenza pandemic and improved the prognosis for certain SARS patients in the 2003 outbreak in Hong Kong. Several studies testing such therapies for COVID-19 are currently underway. “Convalescent plasma in particular is a therapy that could be undertaken fairly rapidly and might be a good therapeutic intervention until we can develop an effective vaccine,” Rasmussen says.

To Gralinski, the study underscores that non-human primates could be a useful animal model to study COVID-19, because their symptoms are similar to—but not exactly the same as—those in humans. “These animals certainly aren’t progressing to acute respiratory distress syndrome or anything like that, [which] you see in some people,” she says. Nevertheless, the animals may prove useful in studying whether vaccines, drugs, antivirals, and other therapies may be promising in people, she says.

“We’ve only known about this virus for ten, eleven weeks right now,” Gralinski says. “It’s great to see any type of data this quickly.”

**L. Bao et al., “Reinfection could not occur in SARS-CoV-2 infected rhesus macaques,” *bioRxiv*, doi:10.1101/2020.03.13.990226, 2020.**

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*Correction (March 18): A previous version of this article incorrectly stated that upon viral infection, plasma B cells produce antibodies on the spot. The article has been updated to reflect the fact that they take longer to generate.*

**Keywords:**

antibodies, B cell, blood plasma, coronavirus, COVID-19, immunity, immunology, macaque, monkeys, News, plasma therapy, SARS-CoV-2

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# US Primate Centers Work to Protect Animals from COVID-19

Rhesus macaques can be infected with SARS-CoV-2, leading primate center scientists to try to prevent outbreaks in their colonies, especially as experiments on coronavirus start.

**Ashley Yeager**
*Mar 19, 2020*

Earlier this month, the California National Primate Research Center at the University of California, Davis, canceled its tours, turned visitors away, and restricted students' access to many areas of the facility. Researchers even limited the movements of the animals in the colony, all with the goal of protecting the animals from SARS-CoV-2 infection.

ABOVE: ©ISTOCK.COM, DC_COLOMBIA

Like humans, monkeys are vulnerable to the virus behind the COVID-19 pandemic, although their symptoms are not entirely the same. This has opened up opportunities to study SARS-CoV-2, but has also meant facilities need to institute extensive precautions to prevent unintended contagion, particularly now as the researchers plan nonhuman primate experiments to aid in the development of coronavirus treatments and vaccines.

The level of risk primates face at these facilities is not well known. Investigators in China have shown that rhesus macaques can be infected with SARS-CoV2. "The animals did not demonstrate clinical signs [of infection] but were shown to have pneumonia," Jeffrey Roberts, the associate director of the California National Primate Research Center, writes in an email to *The Scientist*. "We have a population of 4,000 rhesus macaques and 100 titi monkeys and our concern is that, similar to the human population, a subset or our animal colony may become clinically ill and in the case of our aged rhesus colony, severely so." So far, initial antibody testing for signs of the virus in the California colony has been negative.

Keeping the virus out of primate colonies across the country will be essential for SARS-CoV-2 research, Roberts and other center officials say. Once an animal has been infected, it can't be used for research, so any colony outbreak would severely limit the number of animals available for COVID-19 experiments.

**If we wanted to test the efficacy of a vaccine and the animals have already been exposed [to the virus] and gotten over [an infection], you can't infect them, so you couldn't tell whether the vaccine's protective.**

—Skip Bohm, Tulane National Primate Research Center

At the Washington National Primate Research Center in Seattle, the standard protocol for researchers is to wear face masks, face shields, hair nets, two sets of protective gloves, Tyvek suits, and booties over their shoes whenever they go into an area where animals are housed. The intent is to prevent the monkeys there—rhesus macaques, pigtail macaques, long-tailed macaques, and squirrel monkeys—from contracting infections of any kind.

Because of the precautions, the animals at the Washington site are fairly well protected from accidentally contracting SARS-CoV-2 from humans, says Sally Thompson-Iritani, the interim director of the facility, but there's nothing known about how different primate species respond to the virus. Each species could be "differentially susceptible," she says. "That's something we are very aware of and we're really monitoring each species independently."

The facility is also taking additional precautions beyond the standard protocol of protective gear. "We're limiting the number of people who are coming to work every day because we want to ensure that we're limiting exposure

between people, which then limits exposure to the animals," Thompson-Iritani says.

Although all of the animals at the primate centers are at risk, some may be more vulnerable than others based on the different types of research programs run at each center, notes Skip Bohm, the associate director and chief veterinary medical officer at the Tulane National Primate Research Center. The California and Tulane sites, for example, have large, outdoor breeding colonies. At the California center, undergraduate and graduate students help with behavioral experiments, and until recently, were at the facility observing the animals and collecting data for behavior research projects. Because of the increased access to the animals there, they might have been at a higher risk of exposure compared with the Tulane facility where research focuses largely on infectious disease and so students and investigators from the university do not have access to the animals there.

At Tulane, similar to Washington, animal care workers, veterinarians, and behavioral management staff who come in contact with the outdoor colony wear protective gear when working with the animals, but the risk that the animals could contract SARS-CoV-2 remains. As a result, the facility may begin monitoring the health of its employees and will perform antibody testing for SARS-CoV-2 to detect if animals have unintentionally contracted the virus at any point.

This monitoring effort is principally important now, Bohm says, because next week the center will begin COVID-19 experiments in nonhuman primates. In the first experiment, the team will confirm that the animals—two rhesus macaques and two African Green monkeys—can be infected with SARS-CoV-2. "If you want to test treatments and vaccines, you have to be sure the animals can be infected," Bohm says.

**See "Monkeys Develop Protective Antibodies to SARS-CoV-2"**

A preliminary study from China shows rhesus macaques can be infected but don't develop the infection a second time they are exposed to it. Although the data are preliminary, it's a good sign that perhaps people are most likely protected from future SARS-CoV-2 infections, at least in the short term. It's not so great for running COVID-19 experiments in primates. "If we wanted to test the efficacy of a vaccine and the animals have already been exposed [to the virus] and gotten over [an infection], you can't infect them, so you couldn't tell whether the vaccine's protective," Bohm says. "You couldn't do drug treatment studies to see if you could cure the disease because you couldn't infect [the animals]." Maintaining the health of the colonies, he notes, is critically important now so those studies can be done.

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# Vector-Based Vaccines Come to the Fore in the COVID-19 Pandemic

Adenovirus vectors deliver the genetic instructions for SARS-CoV-2 antigens directly into patients' cells, provoking a robust immune response. But will pre-existing immunity from common colds take them down?

**Anthony King**
*Sep 8, 2020*

Six vaccine candidates in clinical trials for COVID-19 employ viruses to deliver genetic cargo that, once inside our cells, instructs them to make SARS-CoV-2 protein. This stimulates an immune response that ideally would protect recipients from future encounters with the actual virus. Three candidates rely on weakened human adenoviruses to deliver the recipe for the spike protein of the pandemic coronavirus, while two use primate adenoviruses and one uses measles virus.

ABOVE: Colored transmission electron microscopic image of adenovirus virions
CDC /DR. G. WILLIAM GARY, JR.

Most viral vaccines are based on attenuated or inactivated viruses. An upside of using vectored vaccines is that they are easy and relatively cheap to make. The adenovirus vector, for example, can be grown up in cells and used for various vaccines. Once you make a viral vector, it is the same for all vaccines, says Florian Krammer, a vaccinologist at the Icahn School of Medicine at Mount Sinai. "It is just the genetic information in it that is different," he explains.

Once inside a cell, viral vectors hack into the same molecular system as SARS-CoV-2 and faithfully produce the spike protein in its three dimensions. This resembles a natural infection, which provokes a robust innate immune response, triggering inflammation and mustering B and T cells.

But the major downside to the human adenoviruses is that they circulate widely, causing the common cold, and some people harbor antibodies that will target the vaccine, making it ineffective.

## Human adenovirus vectors

CanSino reported on its Phase II trial this summer of its COVID-19 vaccine that uses adenovirus serotype 5 (Ad5). The company noted that 266 of the 508 participants given the shot had high pre-existing immunity to the Ad5 vector, and that older participants had a significantly lower immune response to the vaccine, suggesting that the vaccine will not work so well in them.

"The problem with adenovirus vectors is that different populations will have different levels of immunity, and different age groups will have different levels of immunity," says Nikolai Petrovsky, a vaccine researcher at Flinders University in Australia. Also, with age, a person accumulates immunity to more serotypes. "Being older is associated with more chance to acquire Ad5 immunity, so those vaccines will be an issue [with elderly people]," Krammer explains. Moreover, immunity against adenoviruses lasts for many years.

**With vectors you are always trying to find the sweet spot. Too weak, and they don't work. Too strong, and they are too toxic.**
—Nikolai Petrovsky, Flinders University

"A lot of people have immunity to Ad5 and that impacts on how well the vaccine works," says Krammer. In the US, around 40 percent of people have neutralizing antibodies to Ad5. As part of her work on an HIV vaccine, Hildegund Ertl of the Wistar Institute in Philadelphia previously collected serum in Africa to gauge resistance levels to this and other serotypes. She found a high prevalence of Ad5 antibodies in sub-Saharan Africa and some West African

countries—80 to 90 percent. A different group in 2012 reported that for children in northeast China, around one-quarter had moderate levels and 9 percent had high levels of Ad5 antibodies. "I don't think anyone has done an extensive enough study to do a world map [of seroprevalence]," notes Ertl.

J&J's Janssen is using a rarer adenovirus subtype, Ad26, in its COVID-19 vaccine, reporting in July that it protects macaques against SARS-CoV-2 and in September that it protects against severe clinical disease in hamsters. Ad26 neutralizing antibodies are uncommon in Europe and the US, with perhaps 10–20 percent of people harboring antibodies. They are more common elsewhere. "In sub-Saharan Africa, the rates are ranging from eighty to ninety percent," says Ertl.

**See "COVID-19 Vaccine Frontrunners"**

Also critical is the level of antibodies in individuals, notes Dan Barouch, a vaccinologist at Beth Israel Deaconess Medical Center and Harvard Medical School. For instance, there was no neutralizing of Ad26-based HIV and Ebola vaccines in more than 80,000 people in sub-Saharan Africa, he says. "Ad26 vaccine responses do not appear to be suppressed by the baseline Ad26 antibodies found in these populations," because the titres are low, Barouch writes in an email to *The Scientist*. Barouch has long experience with Ad26-based vaccines and collaborates with J&J on their COVID-19 vaccine.

The Russian Sputnik V vaccine, approved despite no published data or Phase 3 trial results, starts with a shot of Ad26 vector followed by a booster with Ad5, both of which carry the gene for the spike protein of SARS-CoV-2. This circumvents a downside of viral vector vaccines, specifically, once you give the first shot, subsequent injections will be less efficacious because of antibodies against the vector. Ertl says she has no idea of the proportion of the Russian population with Ad26 or Ad5 antibodies, and there seems to be little or no published data from countries that have expressed interested in this virus, such as Venezuela and the Philippines.

## Simian adenovirus vectors

An alternative is look to our nearest relatives. Chimp adenoviruses were the focus of vaccine interest by Ertl for HIV and by Adrian Hill at the University of Oxford for malaria. "About one percent of people have antibodies to the chimp adenovirus, probably because of cross reactivity, which is why we use it," explains Hill, referring to the COVID-19 vaccine candidate ChAdOx1 nCoV-19, which has shown antibody and T cell responses in an early phase clinical trial. This candidate, which also encodes the instructions for producing SARS-CoV-2 spike protein, is now in Phase 3 trials in the UK, US, South Africa, and Brazil and is to be manufactured by AstraZeneca.

Unfortunately, says Ertl, use of the attenuated chimp virus in a COVID-19 vaccine means it cannot now be used for malaria, because those vaccinated for the coronavirus will have antibodies against the vector. But there are other simian vectors. In Italy, a Phase 1 trial of a COVID-19 vaccine with a gorilla adenovirus vector has begun recruiting healthy volunteers. Ertl says that having multiple adenoviruses from different species is "a good thing, because it broadens the range of diseases we could tackle." It could also allow animal virus vectors for COVID-19 vaccines to be used in places where human adenovirus immunity is high.

Not everyone is enthusiastic about vector-based vaccines. "Their reactogenicity profile is not great," says Petrovsky, meaning they stimulate a strong immune response. "Even [President Vladimir] Putin commented that his daughter had a fever [after taking Sputnik V]. Generally, fevers are a no-no for a vaccine." He says headache and fever have been relatively common in early results from vaccines based on viral vectors. Some people are prone to having convulsions from fevers, so extreme reactions cannot be ruled out, he adds.

Petrovsky says children generally react more strongly to vaccines than adults do, and that could be a huge drawback in countries with young populations such as India. "With vectors you are always trying to find the sweet spot," says Petrovsky, which is their Achilles's heel. "Too weak, and they don't work. Too strong, and they are too toxic." Petrovsky is involved in the development of Covax-19, a recombinant protein–based vaccine plus adjuvant that is in early clinical trials and was developed by his company Vaxine Pty in Australia.

So far, there is not much experience with vector-based vaccines on the market. The European Medicines Agency granted market authorization in May for a new Ebola vaccine that consists of a prime shot with an Ad26 vector, and a booster with an attenuated poxvirus (MVA). An HIV vaccine trial based on Ertl's research was to have started this fall, but has been delayed until next year due to COVID-19. "We don't have post-licensing experience," says Ertl, in relation to vector-based vaccines, "but these things have been in multiple trials, so we have a reasonably good idea about what doses are tolerated and about safety concerns."

## A measles vector

In August, a trial in France and Belgium began recruiting volunteers to test a COVID-19 vaccine based on a replicating measles vaccine virus. This so-called Schwartz strain was weakened in the 1960s by serial passaging on chicken cells. The virus expresses the full-length spike protein of SARS-CoV-2 and has been tested in mice, say scientists at the Pasteur Institute in France who licensed the vector technology to Themis in Austria. It was previously tested on mice for SARS and for MERS.

It was shown previously that pre-existing immunity to measles acquired by infection in the elderly or vaccination in young people did not dampen responses to a Chikungunya vaccine based on this same vector. The measles vector "goes into cells, then makes more measles vaccine. It will come out again, infect more cells, but after a few cycles it stops," says vaccine scientist Christiane Gerke of the Pasteur Institute who is leading the COVID-19 vaccine trial. That the measles strain replicates distinguishes it from the adenovirus vectors and could explain why pre-existing antibodies do not matter. "So long as measles antibodies at the start do not eliminate all of the vaccine, then the vaccine replicates itself," says Gerke.

The live nature of the measles vaccine strain means that it could not be given to immunocompromised individuals. However, the Swartz strain has about 50 mutations and measles vaccine strains have never escaped these attenuation shackles and caused disease in healthy people. "It is a promising candidate," says Krammer, though a little behind the others. The Pasteur Institute could not confirm whether volunteers had begun receiving the vaccine. In June, Themis was acquired by Merck, a company with a significant vaccine portfolio.

Success with viral vectors has implications for vaccine development overall. "It took a very long time for viral vectors to end up on the market, which they did with the Ebola vaccines," says Krammer. "The way I see it, this is going to speed up vaccine development in general." That is, as long as there is a successful outcome with a COVID-19 vaccine. Any misstep by a regulator with one of these vaccines could retard the potential of vector-based vaccines for multiple diseases, says Krammer.

**Keywords:**

adenovirus, antibody, coronavirus, COVID-19, disease & medicine, immunity, immunology, News, pandemic, pharma & biotech, SARS-CoV-2, spike protein, vaccine, viral vectors

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# COVID-19 Vaccine Trial Pauses After Adverse Reaction

The hold will likely delay trial results, and scientists say the pause is proof that safety protocols in clinical trials are working as they should.

**Amanda Heidt**
*Sep 9, 2020*

*Update (September 14): Oxford University and AstraZeneca announced Saturday, September 12, that they will be resuming their UK trials after a temporary pause following a case of transverse myelitis in a UK patient.*

ABOVE: © ISTOCK.COM, ZORANM

A series of late-stage clinical trials establishing the effectiveness and safety of a joint AstraZeneca and University of Oxford COVID-19 vaccine are on hold after one UK participant in a Phase 3 trial developed a severe and unexplained illness.

The incident was first reported on Tuesday (September 8) by *STAT*. The pause will delay results from one of the world's largest COVID-19 vaccine development efforts. A company spokesperson says the measure has been taken out of an abundance of caution.

"This is a routine action which has to happen whenever there is a potentially unexplained illness in one of the trials, while it is investigated," AstraZeneca shared in a prepared statement. "We are working to expedite the review of the single event to minimize any potential impact on the trial timeline. We are committed to the safety of our participants and the highest standards of conduct in our trials."

Details of the adverse event that initiated the pause, including how serious it is and when it happened, have not been shared publicly. A person familiar with the situation, speaking on condition of anonymity with *The New York Times*, says that the participant had been diagnosed with transverse myelitis, an inflammatory condition in the spinal cord that is often sparked by viral infections.

Gabriella Garcia, a neurologist at Yale New Haven Hospital, tells the *Times* transverse myelitis is often treatable with steroids. When asked to confirm the diagnosis, AstraZeneca declined to comment. "The event is being investigated by an independent committee, and it is too early to conclude the specific diagnosis," the company says.

Currently, there are nine vaccine candidates in Phase 3 trials, and AstraZeneca is the first to announce a pause. It is unknown how long the hold will continue. Marie-Paule Kieny, a vaccine researcher at INSERM, the national health research institute in Paris, tells *Nature* that if AstraZeneca can quickly rule out the vaccine as a source of the illness, the trial could be running again "in a matter of weeks."

One Phase 3 trial has been underway in the US since late August with plans to enroll up to 30,000 participants at 80 sites across the country. Other trials of the same vaccine are also progressing in other countries, including Brazil and South Africa, with additional trials planned for Japan and Russia. Money to fund the US trial came from Operation Warp Speed, a US federal initiative to quickly disperse funds to the most promising vaccine candidates. AstraZeneca received $1.2 billion dollars to produce 300 million doses, *Buzzfeed* reports.

The company's vaccine relies on an experimental method, based on an adenovirus vector from chimpanzees, that has yet to lead to any approved immunizations, although it was used previously in studies to treat influenza and Ebola, according to *STAT*. Scientists engineered the virus to smuggle the gene for the SARS-CoV-2 spike protein into human cells. Once inside, the protein prompts a natural immune response, priming immune cells to recognize SARS-CoV-2.

**See "Vector-Based Vaccines Come to the Fore in the COVID-19 Pandemic"**

The Phase 1 and 2 trials of the vaccine involved 1,077 patients, *Buzzfeed* reports, and the results showed that 90 percent of participants subsequently tested positive for neutralizing antibodies. While roughly 60 percent of patients reported mild or moderate side effects such as fever, headaches, and muscle aches, these symptoms resolved before the end of the trial.

Phase 3 trials, which assess efficacy, can also look for less common side effects only be detected when vaccines are tested on large numbers of people. Because of their large size, these studies are considered the most important for establishing safety, according to the Associated Press.

Some scientists are saying the pause is not unexpected, and in fact shows that the clinical trial protocol is operating as it should. On the same day that AstraZeneca announced the suspension, the company's CEO joined eight other pharmaceutical companies in announcing a commitment to integrity and safety moving forward with vaccine trials.

"This is the whole point of doing these Phase 2, Phase 3 trials," Phyllis Tien, an infectious disease physician at the University of California, San Francisco, tells the *Times*. "We need to assess safety, and we won't know the efficacy part until much later. I think halting the trial until the safety board can figure out whether or not this was directly related to the vaccine is a good idea."

Pauses in trials can happen for any number of reasons. Any unexplained illness that requires hospitalization, for example, could trigger a safety assessment to determine if it is linked to an injection with an experimental vaccine. "It does happen. It's not common, but it does happen," Paul Offit, a vaccine expert at Children's Hospital of Philadelphia, tells *BuzzFeed*. "The vaccine is to stop SARS-CoV-2. It's not designed to stop everything else that happens in life. You want to make sure it was related to the vaccine, especially if it happened a short period of time after the vaccine begins."

Joe Walters, one of the participants in the University of Oxford trial in the UK, spoke this week to *The Guardian* about his experiences, sharing that he developed a persistent fever after receiving his initial injection (although it's unknown whether he received the vaccine candidate or a control). He was briefly hospitalized and tested for COVID-19, and after his test came back negative, he learned that other participants had experienced similar side effects.

Walters heard about the pause the day he came to receive his second dose. For now, his participation is on hold, but Walters says he plans to continue "in the name of science," adding that the trial is "something positive to do in the face of something that rendered everyone rather helpless."

AstraZeneca is one of three companies testing vaccines in late-stage clinical trials in the US. The other two companies, Moderna and BioNTech, are developing vaccines using different methods than those used by AstraZeneca. A spokesperson for Moderna tells the *Times* the firm's work has not been affected by the latest pause.

**See "COVID-19 Vaccine Frontrunners"**

**Keywords:**

adverse reaction, AstraZeneca, clinical research, clinical trials, coronavirus, COVID-19, immunology, nutshell, pandemic, SARS-CoV-2, vaccine trials

# COVID-19 Vaccine Frontrunners

Stay up-to-date on the progress of dozens of vaccine candidates that are currently undergoing clinical testing.

**Jef Akst**
*Apr 7, 2020*

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In early March, National Institutes of Allergy and Infectious Diseases Director Anthony Fauci stated that it would take at least a year to a year and a half to get a COVID-19 vaccine approved for use in the US, and that estimate may be optimistic, according to some experts. There are many unknowns this early in the game. How the early candidates will perform, which will be advanced to later stages of clinical development, what safety issues might arise, and how a successful vaccine will be mass produced are among the questions that are now getting attention and funding.

**See "Newer Vaccine Technologies Deployed to Develop COVID-19 Shot"**

The Coalition for Epidemic Preparedness Innovations (CEPI), a nonprofit dedicated to the development of vaccines against emerging infectious diseases, was one early source of cash for this endeavor, with a total of nearly $30 million invested in several candidates by April. Another bolus of funds came from the Biomedical Advanced Research and Development Authority (BARDA), part of the HHS Office of the Assistant Secretary for Preparedness and Response, which is contributing hundreds of millions of dollars to two top vaccine candidates: one made by Johnson & Johnson's Janssen division and another developed by Moderna in collaboration with the US government. More recently, the US government's Operation Warp Speed has invested heavily, giving more than $1 billion each to Novavax and to AstraZeneca, which is collaborating on a vaccine developed by the University of Oxford.

**See "US Selects Two COVID-19 Vaccine Candidates for Huge Investments"**

Below, *The Scientist* rounds up those vaccine candidates that appear to be furthest along. But there are many more in preclinical development. "Nobody knows which vaccines are going to work," Moderna CEO Stéphane Bancel told *Science* in March.

**See "Clinical Trial of COVID-19 Vaccine Begins in Seattle"**

*Editor's note: This table was updated on October 26, 2020. New information in bold, red font.*

| DEVELOPER(S) | VACCINE METHOD | EVIDENCE | STATUS |
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| **Moderna and the US government**<br>*US* | Lipid nanoparticles containing mRNAs for the SARS-CoV-2 spike protein are injected into the arm. | Preliminary results from studies on older adults and those under age 55 suggest that the vaccine is safe and elicits higher levels of SARS-CoV-2 antibodies than does infection with the virus. A third study finds that vaccinated monkeys quickly cleared | Phase 1, Phase 2, and Phase 3 clinical trials underway across the US |

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| | | the virus. Moderna is developing similar vaccines against Zika and other viruses, but to date, no vaccine of this type has been approved for use. | |
| **CanSino Biologics and the Academy of Military Medical Sciences**<br>*China, Canada, Russia, and Saudi Arabia* | Nonreplicating adenovirus 5 (Ad5) vector carrying the gene for the SARS-CoV-2 spike protein is injected into the arm. | Preliminary results from the Phase 2 trial published in *The Lancet* in July suggest that the vaccine is safe and elicits an immune response, either a T cell response or an antibody response. Adenoviruses are well-established vaccine vectors, and CanSino produced an Ebola vaccine (approved in China in 2017) using the same Ad5 platform. | Phase 1 and Phase 2 clinical trials are underway in Wuhan, China. On June 25, following positive Phase 1 data published in *The Lancet* on May 22, China's Central Military Commission approved the vaccine's use by the country's military for a year. A Phase 1/2 trial has been approved to begin in Canada. Phase 3 trials have begun in Russia and in Pakistan, and a Phase 3 trial in Saudi Arabia will begin soon, according to Reuters. |
| **University of Oxford and AstraZeneca**<br>*UK, US, Brazil, and South Africa* | A chimpanzee adenovirus vaccine vector (ChAdOx1) carrying the gene for the SARS-CoV-2 spike protein is injected into the arm. | Preliminary results from the Phase 1/2 trial published in *The Lancet* in July suggest that the vaccine is safe and elicits strong antibody and T cell immune responses. Six macaques that had received a single dose of the vaccine candidate stayed healthy after being exposed to SARS-CoV-2. A Phase 1 trial using the same adenovirus vector to target MERS is ongoing in Saudi Arabia. | Phase 1/2 and 2/3 clinical trials are underway in the UK, and a Phase 3 trial has begun in the US. Researchers also began testing the vaccine in Brazil and South Africa in June, and additional trials are expected to soon begin in India and Japan. On September 9, all ongoing trials were placed on hold after a serious adverse event arose in a participant enrolled in the Phase 3 study in the UK. Three days later, UK trials resumed, **and in mid-October, the FDA authorized the restart of the US trial.** Researchers in the UK will also test an inhaled form in an early-stage trial. |
| **Inovio Pharmaceuticals** | A special device administers spike | Mice and guinea pigs mounted immune | Phase 1 clinical trial underway in Pennsylvania, Missouri, |

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| *US* | protein–encoding DNA molecules through the skin. | responses against the virus, according to a recent preprint, and the company announced interim results from the Phase 1 trial at the end of June that suggested the vaccine was safe and spurred immune responses in 94 percent of the 36 participants analyzed. | and Kentucky with plans to manufacture 1 million doses of its candidate this year |
| **Sinovac Biotech**<br>*China, Brazil, Bangladesh, and Indonesia* | Inactivated SARS-CoV-2 | In animal studies, the vaccine candidate provides protection against virus strains from different countries. Results from the Phase 2 trial announced in August suggest that the vaccine is safe and elicits an antibody-based immune response. Sinovac had used a similar platform to develop a vaccine against SARS in 2004 that showed promising results in early-stage human trials. | A Phase 1/2 clinical trial is underway in China, and Phase 3 trials are underway in Brazil, Bangladesh, Indonesia, and Turkey. |
| **Wuhan Institute of Biological Products and China National Pharmaceutical Group (Sinopharm)**<br>*China and United Arab Emirates (UAE)* | Inactivated SARS-CoV-2 | In mid-June, Sinopharm announced that nearly all of the more than 1,000 participants who had received two injections of the mid-dose vaccine tested positive for antibodies against SARS-CoV-2. In August, Phase 1/2 trial results published in *JAMA* indicated that the vaccine is safe and elicited neutralizing antibodies. | A Phase 1/2 clinical trial is underway in China. A Phase 3 trial is underway in UAE, and another has been approved to begin in Peru. |
| **Beijing Institute of Biological Products and China National Pharmaceutical Group (Sinopharm)** | Inactivated SARS-CoV-2 | The two-dose vaccine protects rhesus macaques against SARS-CoV-2, according to a paper published in *Cell* in early | Phase 1/2 clinical trial underway in China |

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| *China* | | June. In mid-October, Sinopharm published results from the early trials in *The Lancet* showing that the vaccine was safe and that participants receiving the vaccine had high titers of antibodies. | |
| **Symvivo**<br><br>*Canada* | Orally administered *Bifidobacterium* probiotic engineered to carry DNA encoding the SARS-CoV-2 spike protein | In addition to this vaccine currently in human testing, two other candidates for COVID-19 are being developed by Symvivo. | Phase 1 clinical trial underway in British Columbia and Nova Scotia |
| **BioNTech and Pfizer**<br><br>*International* | Four RNA vaccine candidates are being tested in parallel. | Preliminary results from the early stage trials suggest that the vaccine is safe and elicits higher levels of SARS-CoV-2 antibodies than infection with the virus, according to two preprints posted by the companies in July, one of which was later published in *Nature*. A second study published in *Nature* at the end of September reported that the vaccine generated T cell responses in addition to antibody responses. | Phase 1/2 clinical trial underway in Germany, China, and in Ohio, New York, and Maryland in the US. In July, the companies announced that they had picked their lead candidate and had begun a Phase 2/3 trial in the US, Germany, Brazil, and other countries, including a Phase 3 trial that has commenced in South Africa. The companies announced in early October that the vaccine was starting the process of rolling submission with the European Medicines Agency. |
| **Shenzhen Geno-Immune Medical Institute**<br><br>*China* | Immune cells (human dendritic cells and T cells, or artificial antigen presenting cells) are engineered to express a synthetic minigene based on SARS-CoV-2 proteins and injected or infused into the patient. | The research institute modifies cells using lentivirus vectors that it has used to develop CAR T cell therapies as well as gene therapies. | A Phase 1/2 clinical trial is underway in China for the dendritic cell and T cell–based vaccines, and a Phase 1 trial is underway for a vaccine using artificial antigen presenting cells. |
| **Clover Biopharmaceuticals** | The vaccine delivers pieces of the SARS-CoV-2 | The Trimer-Tag platform used is the basis for other | Phase 1 clinical trial underway clinical in Australia |

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| *Australia* | spike protein. | viral vaccines in development. | |
| **Novavax**<br>*Australia and South Africa* | Nanoparticles carrying antigens derived from the SARS-CoV-2 spike protein (with Matrix-M adjuvant) | In 2012, the company started development on a SARS vaccine that served as the basis for its new SARS-CoV-2 vaccine candidate. Data from the Phase 1/2 trial published in *The New England Journal of Medicine* in early September shows the vaccine candidate to be safe and elicit immune responses greater than those caused by treatment with COVID-19 convalescent serum. | A Phase 1 clinical trial is underway in Australia, and a Phase 2 trial is underway in South Africa. A Phase 1 clinical trial is underway in Australia, and a Phase 2 trial is underway in South Africa. A Phase 3 trial has begun in the UK, *The New York Times* reports, and late-stage trials are also planned to begin in the US and in India in October. |
| **Gamaleya Research Institute of Epidemiology and Microbiology, Health Ministry of the Russian Federation, Acellena Contract Drug Research and Development**<br>*Russia* | Adenovirus vector displaying the SARS-CoV-2 spike protein on its surface | Preliminary results from the early stage trial published in September in *The Lancet* point to the vaccine candidate's safety and ability to elicit an antibody response. Gamaleya Research Institute Director Alexander Gintsburg announced in May that he and other staff researchers had gotten the vaccine themselves, the AP reports. | Phase 1/2 clinical trials are underway in Russia to test liquid and powder forms of the vaccine. In August, Russian President Vladimir Putin said that the country approved the vaccine ahead of a Phase 3 trial. |
| **Immunitor**<br>*Canada and Mongolia* | Heat-inactivated plasma from donors with COVID-19 taken as a pill daily for a month | The initial safety test will give volunteers the pill for 15 days. | Phase 1/2 clinical trial underway in British Columbia and Mongolia |
| **Institute of Medical Biology at Chinese Academy of Medical Sciences, West China Second University Hospital, Yunnan Center for** | Inactivated SARS-CoV-2 | Data from the Phase 1 trial, posted as a preprint on October 6, suggests that the vaccine is safe and elicits an immune response, although levels of neutralizing antibody | Phase 1/2 clinical trial underway in China |

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| **Disease Control and Prevention**<br><br>*China* | | started to drop after just two weeks. | |
| **Imperial College London** | Self-amplifying RNA molecules are injected into the muscle. | The vaccine platform, which is designed to allow researchers to respond quickly to emerging pathogens, received $8.4 million from CEPI last December. “We cannot predict where or when Disease X will strike, but by developing these kinds of innovative vaccine technologies we can be ready for it,” CEPI CEO Richard Hatchett said at the time. | A Phase 1 trial is underway in the UK. Researchers in the UK will also test an inhaled form in an early-stage trial. |
| **CureVac**<br><br>*Belgium and Germany* | RNA vaccine; details not disclosed | CureVac reported in January that a Phase 1 trial of a comparable vaccine for rabies induced immune responses with just 1 microgram of mRNA, meaning it could be easy to scale up to produce mass quantities. | Phase 1 trial underway in Belgium and Germany; company says it could manufacture 10 million doses by that time. |
| **Medicago** | Virus-like particles that resemble SARS-CoV-2 are produced in a close relative of tobacco. | The company has a rotavirus vaccine in clinical trials that is based on virus-like particles, and another for norovirus in preclinical studies. | A Phase 1 trial has been approved |
| **Altimmune** | Undisclosed vaccination delivered intranasally | The company is using the same technology to develop a flu vaccine that is in clinical trials. | A Phase 2 trial has been approved |
| **AnGes, Japan Agency for Medical Research and** | Engineered circular DNA encoding the SARS-CoV-2 spike protein | The vaccine consists of two intramuscular injections. | Phase 1/2 trial underway in Japan |

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| **Development**<br>*Japan* | | | |
| **Aivita Biomedical**<br>*US* | A patient's own dendritic cells are modified to carry SARS-CoV-2 antigens and then reinfused. | Antigen-carrying dendritic cells triggered a response in the same patient's lymphocytes in vitro. | A Phase 1/2 trial has been approved to begin in California. |
| **Genexine**<br>*South Korea* | DNA encoding the SARS-CoV-2 spike protein | The vaccine was shown to produce neutralizing antibodies in nonhuman primates. | Phase 1/2 trial underway in South Korea |
| **Vaxine, Medytox**<br>*Australia* | Recombinant SARS-CoV-2 spike protein plus a polysaccharide adjuvant | Vaxine developed an experimental swine flu vaccine during the 2009 pandemic. | Phase 1 trial approved to begin in Australia |
| **Zydus Cadila**<br>*India* | Engineered DNA plasmid encoding a SARS-CoV-2 antigen | In a preclinical study, the vaccine neutralized SARS-CoV-2 in a virus neutralization assay. | Phase 1/2 trial underway in India |
| **Bharat Biotech**<br>*India* | Inactivated SARS-CoV-2 | In guinea pigs and mice, the vaccine was safe and elicited an immune response. | Phase 1/2 trial underway in India |
| **Janssen**<br>*International* | Nonreplicating adenovirus 26 (Ad26) vector carrying undisclosed genetic material of SARS-CoV-2 is delivered via intramuscular injection. | Janssen published preclinical data in *Nature* in July demonstrating that the vaccine protected monkeys against SARS-CoV-2 infection. In late September, the company posted a preprint with preliminary results from an early-stage trial that showed a single dose of the vaccine to be safe and to elicit neutralizing antibodies in nearly all study participants. The company is also | Early stage clinical trials are underway in the US, Belgium, and Japan. Meanwhile, a Phase 2 trial is scheduled to begin in early September in Spain, Germany, and the Netherlands, and an international Phase 3 trial is being launched in the US, Mexico, several South American countries, the Philippines, South Africa, and Ukraine. On October 12, the company announced that it had temporarily paused further dosing in all of these trials after a participant |

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| | | company is also developing other Ad26-based vaccine candidates, including its Ebola vaccine that was deployed in Democratic Republic of Congo in November 2019. | trials after a participant developed an unexplained illness, but less than two weeks later, it announced that it was preparing to resume these studies. |
| **Arcturus Therapeutics**<br>*Singapore* | Self-replicating mRNA encoding coronavirus proteins | The self-replicating mRNA platform is not the basis of any approved medicines, but preclinical results announced in April suggest the vaccine candidate triggers an immune response. | Phase 1/2 trial underway in Singapore |
| **University of Queensland**<br>*Australia* | Molecular clamp technology presents viral proteins to the immune system. | Preclinical work in cell culture showed that the vaccine candidate elicited an immune response that was capable of neutralizing SARS-CoV-2 infection. | Phase 1 clinical trial underway in Australia |
| **ReiThera**<br>*Italy* | Proprietary replication-defective gorilla adenoviral (GRAd) vector encodes the SARS-CoV-2 spike protein. | The vaccine candidate was safe and induced an immune response in animal models, according to the company. | Phase 1 trial underway in Italy |
| **Sanofi and GlaxoSmithKline (GSK)**<br>*US* | Antigen based on SARS-CoV-2 spike protein (with adjuvant) | Sanofi uses the same recombinant DNA technology in a flu vaccine and in a SARS vaccine candidate that never entered clinical trials. Meanwhile, GSK's adjuvant, AS03, was used in vaccines the company made against the H1N1 and H5N1 pandemic flu viruses. | A Phase 1/2 trial underway in the US |
| **State Research Center of Virology and Biotechnology "Vector"** | Peptide-based vaccine based on a platform developed for an Ebola vaccine candidate | Volunteers in the Phase 2 trial are "feeling good," the Russian consumer safety watchdog | Clinical testing was completed by the end of September, according to Reuters. In October, Russian |

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| vector<br>*Russia* | vaccine candidate | watchdog Rospotrebnadzor said in a statement, according to Reuters. | Reuters. In October, Russian President Vladimir Putin announced its approval ahead of a Phase 3 trial, NPR reports. |
| **Vaxart**<br>*US* | A pill containing different SARS-CoV-2 antigens | After testing five different vaccine candidates in animals, the company chose its lead candidate, which generated immune responses after a single dose, for clinical testing. The company has other oral recombinant vaccine candidates that have shown success in clinical trials. | Phase 1 trial underway in California |
| **ImmunityBio**<br>*US* | A human adenovirus (hAd5) vector delivers SARS-CoV-2 antigens, both the spike protein and the nucleocapsid protein found inside the virus. | The company says it is developing forms of the vaccine for oral, inhalational, and intranasal administration; the oral version will be tested in the initial Phase 1 trial. | Phase 1 trial approved to begin in the US |
| NOT YET IN CLINICAL TRIALS | | | |
| **University of Pittsburgh School of Medicine** | Microneedle patch delivers pieces of the spike protein through the skin. | Vaccinated mice produced antibodies specific to SARS-CoV-2 at levels that would likely neutralize the virus, according to a study published in *EBioMedicine* on April 2. | Expected to start clinical testing within a few months of that publication, according to the university |
| **Generex Biotechnology** | Undisclosed synthetic viral peptides are combined with proprietary Ii-Key immune system activation | The company has had success with the Ii-Key technology for other infectious diseases and for cancer in clinical trials. | Expected to start clinical testing "within 90 days," the company announced on February 27 |
| **Takis Biotech and Applied DNA Sciences** | The company is exploring five DNA-based candidates based on the | The vaccine candidates contain PCR-produced pieces of linear DNA, as | Expected to start clinical testing in the fall |

| | | | |
|---|---|---|---|
| | SARS-CoV-2 spike protein. | opposed to the more traditional circular plasmids, which could have several advantages including quick production. No vaccines using this approach have yet been tested in humans. | |
| **Merck** | A vesicular stomatitis virus (VSV) carries undisclosed viral components | The VSV vector is used for Merck's existing Ebola vaccine. | Expected to start clinical testing later this year |
| **Merck** | A weakened measles virus vector carries undisclosed viral components | Merck is purchasing Vienna-based Themis, which has an existing measles vaccine, to develop the COVID-19 vaccine. | Expected to start clinical testing later this year |

**In addition to vaccine candidates specific to SARS-CoV-2, several trials are underway testing vaccines against different pathogens as well as nonspecific formulations designed to stimulate an innate immune response.**

| DEVELOPER(S) | VACCINE METHOD | EVIDENCE | STATUS |
|---|---|---|---|
| **Multiple organizations**<br>*International* | The Bacille Calmette-Guerin (BCG) vaccine for tuberculosis consists of live attenuated *Mycobacterium bovis*. | Lower rates of COVID-19–related deaths in countries with mandatory BCG vaccination prompted the launch of several clinical trials to test whether the immune response triggered by the vaccine may protect against SARS-CoV-2. | Several Phase 3 and 4 trials are underway. |
| **Multiple organizations**<br>*International* | The measles-mumps-rubella (MMR) vaccine consists of live-attenuated strains of the three viruses. | Epidemiological data have revealed that places where the MMR vaccine is given as standard medical care have lower COVID-19 death rates than areas where MMR vaccination is not standard. Additionally, sailors aboard the *U.S.S. Roosevelt* who tested positive for COVID-19 had mostly mild symptoms, which some researchers suspect may have been due to administration of the MMR vaccine to all US Navy recruits. | A Phase 3 trial is underway in Egypt, led by researchers at Kasr El Aini Hospital. Separately, researchers at the Washington University School of Medicine are running an international Phase 3 trial of healthcare workers in the US, Canada, Europe, and Africa. |
| **Immunovative Therapies, Mirror Biologics**<br>*US* | An off-the-shelf living immune cell | The affiliated companies are currently testing the formulation as a therapeutic vaccine for chemotherapy-refractory metastatic cancers. | A Phase 1/2 trial for healthy older adults has been approved to begin in New York. |
| **Canadian Cancer Trials Group, others**<br>*Canada* | Heat-killed *Mycobacterium obuense* | The vaccine is intended to stimulate nonspecific innate immunity. The company is also testing the vaccine in clinical trials for cancer. | A Phase 3 trial has been approved to begin in Canada. |
| **Bandim Health Project**<br>*Guinea-Bissau* | Oral polio vaccine, an attenuated strain of the poliovirus | Researchers argue that the vaccine is safer and available in greater quantities than the BCG vaccine | A Phase 4 trial has been approved to begin in Guinea- |

| | | | |
|---|---|---|---|
| *Guinea-Bissau* | | against tuberculosis, which is also being tested as a possible COVID-19 preventive. | Bissau in West Africa. |
| **Inmunotek, BioClever**<br>*Mexico* | A mixture of inactivated bacteria | The vaccine is intended to stimulate nonspecific innate immunity. | A Phase 3 trial for healthcare workers has been approved to begin in Mexico. |
| **Pulmotect**<br>*US* | An inhaled combination of two synthetic Toll-like receptor agonists | The vaccine was originally developed as a potential therapeutic for cancer and has undergone early stage clinical testing. In mice, it provided protection against a range of respiratory pathogens, including MERS and SARS. | A Phase 2 trial for people with known SARS-CoV-2 exposure is underway in several US states. |

*Correction (June 11, 2020): An earlier version of this table stated that Janssen's adenovirus-based COVID-19 vaccine candidate is administered intranasally. In fact, the vaccine is administered via intramuscular injection.*

*Correction (July 27, 2020): An earlier version of this table stated that the Phase 1 and Phase 2 Moderna trials were taking place in Seattle. In fact, there have been multiple locations since the Phase 1.*

*Correction (September 9, 2020): A previous update to this table implied that the adverse event that caused the Phase 3 AstraZeneca trial to be put on hold occurred in the Phase 1/2 trial in the UK. In fact, the event occurred in a participant enrolled in the Phase 3 UK study.*

The Scientist *regrets the errors.*

**Keywords:**

coronavirus, COVID-19, disease & medicine, drug development, epidemic, infectious disease, outbreak, pandemic, SARS-CoV-2, vaccine, vaccine design, vaccine trials

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# FDA to Require 50 Percent Efficacy for COVID-19 Vaccines

Vaccine experts divided on whether that level of protection is too low or too demanding.

**Amanda Heidt**
*Jul 1, 2020*

The US Food and Drug Administration has released a set of guidelines outlining the approval process for future COVID-19 vaccines, stating that any product will need to prevent or decrease the severity of the disease by at least 50 percent.

ABOVE: © ISTOCK, MOUSSA81

The new guidelines were released during a June 30 briefing with the Senate Health, Education, Labor and Pensions Committee during which senators sought assurances from FDA Commissioner Stephen Hahn, National Institute of Allergy and Infectious Diseases Director Anthony Fauci, and other high-ranking health officials that the expedited speed of development wouldn't compromise the integrity of the final product.

"I want the American people to hear me when I say we will use the science and data from those trials, and will ensure that our high levels of standards for safety and efficacy are met," Hahn said during the briefing.

Currently, more than 145 vaccines are being tested worldwide, *The New York Times* reports, and a leading US candidate developed by the biotech company Moderna is slated to begin Phase III clinical trials this month.

**See "COVID-19 Vaccine Frontrunners"**

President Donald Trump announced in May the formation of Operation Warp Speed, a government initiative to hasten the development of a vaccine within the next 12 to 18 months, Reuters reports. In contrast, most vaccines can take more than a decade to be fully developed and brought to bear against a disease.

Responses to the FDA's report are mixed. Gregory Poland, the director of the Mayo Vaccine Research Group, tells Reuters the efficacy guidelines are standard compared to other vaccines. "They look pretty much like influenza vaccine guidelines," Poland says. "I don't think that's a high bar. I think that's a low to . . . appropriate bar for a first-generation COVID-19 vaccine." The effectiveness of the annual flu shot, for example, generally ranges between 40 percent and 60 percent, according to *The Washington Post*.

Peter Hotez, a vaccine expert at the Baylor College of Medicine, tells the *Post* the 50 percent threshold is low, a sign that the FDA recognizes "our first vaccine won't be our best." Ultimately, he says, vaccine developers should aim for 70–75 percent efficacy.

**See "US Selects Two COVID-19 Vaccine Candidates for Huge Investments"**

In contrast, Stephen Ostroff, a former acting FDA commissioner, says the 50 percent figure is too high. Given that the virus "is rampaging through a lot of parts of the United States, I would certainly consider a vaccine with less than 50 percent efficacy," he says.

During yesterday's briefing, Fauci discussed efforts to combat misinformation about vaccines, which many Americans view with distrust. Only 45 percent of Americans get the flu shot each year, *The Hill* reports, and Fauci claims that 75 percent to 80 percent of the public will need to be vaccinated to stop the spread of the coronavirus. Already, efforts are being made to engage with people at the community level.

"It is a reality. A lack of trust in authority, a lack of trust in government, and a concern about vaccines in general," Fauci said. "We need to engage the community by boots on the ground, and particularly those populations that have

not always been treated fairly by the government—minority populations, African Americans, Latinx, and Native Americans."

**Keywords:**

coronavirus, COVID-19, disease & medicine, FDA, FDA approval, nutshell, pandemic, SARS-CoV-2, vaccine, vaccine design, vaccine trials

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# The Immune Hallmarks of Severe COVID-19

Researchers are trying to make sense of immune systems gone haywire and develop biomarkers to predict who will become the sickest from a coronavirus infection.

**Katarina Zimmer**
*Sep 16, 2020*

One of the most striking features of SARS-CoV-2, the virus that causes COVID-19, is the exceptional breadth of symptoms it causes in people. Of the nearly 30 million recorded infections to date, the vast majority of people experienced mild or moderate disease—which itself can range from no symptoms at all to pneumonia or long-term, debilitating neurological symptoms. A minority ended up with severe respiratory symptoms but eventually recovered. And some—nearly 940,000 worldwide, of which 196,000 are in the US—took a turn for the worse and died.

ABOVE: Scanning electron microscope image of SARS-CoV-2 (gold) emerging from the surface of cells cultured in the lab. The virus shown was isolated from a patient in the US. Image captured and colorized at NIAID's Rocky Mountain Laboratories (RML) in Hamilton, Montana
FLICKR, NIAID

Why some people die while others recover is thought to depend in large part on the human immune response, which spirals out of control in severe disease. Over the past few months, researchers have developed a better understanding of this dysfunctional immune response. By comparing patients with varying degrees of disease severity, they've catalogued a number of dramatic changes across the human immune arsenal that are often apparent when patients first come into the hospital—from signaling cytokine proteins and first-responder cells of the innate immune system, to the B cells and T cells that confer pathogen-specific adaptive immunity.

The factors that trigger this immune dysregulation have so far remained elusive due to the complexity of the immune system, which consists of seemingly endless biological pathways that twist and turn and feed back on one another like a ball of spaghetti. But researchers—drawing on knowledge from other conditions such as sepsis, cancer, and autoimmune disease—are gradually building coherent theories of what puts patients en route to severe disease. Along the way, they're also uncovering signals that clinicians could use to predict disease prognosis and identify potential new treatment avenues.

"We don't have the clearest picture yet. Nor do we know why there's variability in this immune response," says Nuala Meyer, a critical care physician at the Hospital of the University of Pennsylvania who researches sepsis. While it's well-established that underlying conditions increase the risk for developing severe COVID-19, "I definitely see patients with diabetes, obesity, and high lipids that did not become severe [cases]," she says. "I think we have a lot of work to do to understand precisely what accounts for this differential response."

## Cytokine mayhem in severe COVID-19

Earlier this year, researchers learned that some of the body's very first defenses against SARS-CoV-2 seem to be perturbed in patients who develop severe disease. When SARS-CoV-2 begins to multiply in cells lining the respiratory tract, both infected and some bystander immune cells release interferons, cytokines that, in general, act to curtail viral replication. But in May, scientists at the Icahn School of Medicine at Mount Sinai reported strikingly low levels of interferons in infected human cells in culture, and in live ferrets infected with the virus. Instead of responding with interferons, the cells had boosted their inflammatory pathways. Then, in a July study of COVID-19 patients' blood, researchers in France found that the interferon response seemed to be blunted in patients with severe and critical disease, whereas interferon release was robust in those with mild and moderate symptoms.

Studies on mice infected with the coronaviruses SARS-CoV and MERS-CoV have shown that an early, strong interferon response is critical to resolving the infections, but if the response is delayed, the mice develop

inflammatory immune reactions instead. Some data suggest that SARS-CoV-2 is capable of blocking interferon production in the cells it infects, and it appears to be much more effective in doing so than its cousin SARS-CoV. But some patients also appear to be less capable of mounting an interferon response even in uninfected immune cells, notes Miriam Merad, who directs the Precision Medicine Institute at Mount Sinai. Either way, without a solid interferon response, the virus will persist, causing damage that activates inflammatory pathways. "The higher the damage is, the more the immune system is trying to get rid of the damage," says Merad, "so it gets activated and at some point … it goes completely crazy."

This over activation is clearly evident in the form of high concentrations of pro-inflammatory cytokines in patients' blood—the "cytokine storm" that COVID-19 has become known for. In a recent analysis of nearly 1,500 COVID-19 patients, Merad and her colleagues found that concentrations of IL-6, IL-8 and TNF-α in their serum upon admission correlated strongly with disease severity and death. Each cytokine could by itself predict whether a patient would survive. Interestingly, the nature of COVID-19's cytokine response is markedly different from the cytokine storm side effect observed in some cancer patients who receive cellular immunotherapies and certain other hyperinflammatory conditions. In COVID-19 patients, concentrations of certain cytokines tend to be much lower—King's College London immunologist Manu Shankar-Hari describes their increase as a "cytokine breeze" rather than a storm. But the increased cytokine levels are sustained over days and weeks, Merad says.

**See "Immune Biomarkers Tied to Severe COVID-19: Study"**

In a more comprehensive but smaller immunological study of moderate and severe COVID-19 patients, researchers documented a number of immune features that are relatively common in chronic viral infections, such as T cells bearing markers of exhaustion, which could mean they're less able to fight off pathogens. But in severe cases, three cytokines stood out and strongly correlated with the severity of the disease: IL-6, IL-10, and particularly IP-10. "Some of the changes are very similar to what people have reported in sepsis previously," notes Shankar-Hari, who is a coauthor on the study. But severely ill patients have the added complication of an impaired interferon response, which he says he sees as the "core mechanism" driving these changes in other kinds of cytokines.

Scientists at Yale University tracking the progression of COVID-19 patients found that the cytokine increase was followed by haphazard-seeming immune responses in severely ill patients. While people with moderate disease appeared to activate immune machinery designed to fight off viruses, those with severe disease seemed to recruit cells and proteins that are typically associated with combating parasitic worms as well as immune responses designed to go after fungi and bacteria that live outside of cells—an unusual response the team describes as "immunological misfiring," as if the immune system is failing to activate the right program. And while the immune responses of those who recovered faded gradually over time, the heightened activity was maintained in patients with severe disease. Ultimately, their frenzied cytokine response doesn't do much to stem the virus—based on swabs from the nose and throat, severe and moderate patients began with similar viral loads that only dropped off in the moderate group.

## Trouble in the innate immune system

Severe COVID-19 is also marked by dysfunction in the immune cells that are first at the scene of a viral infection, including myeloid cells such as neutrophils and monocytes. For instance, researchers from Germany recently analyzed the properties of these cells in the blood of 109 individuals with mild, moderate, and severe COVID-19. Although patients with severe disease seemed to be manufacturing larger quantities of such cells, the cells themselves seemed to be only partially activated and dysfunctional. The neutrophils were largely immature, a feature that is thought to have a suppressive effect on the immune system, while the monocytes tended to have an inflammation-promoting phenotype and often lacked a critical surface protein (HLA-DR) needed for presenting viral material to T cells. The researchers didn't spot these dysfunctional cells in mild or moderate cases.

Perhaps severe COVID-19 isn't a purely inflammatory disease, but rather a dangerous loop of ineffective human immune responses and continuous tissue inflammation, says coauthor Leif Erik Sander, an immunologist and

**It is, I think, really a myeloid-triggered disease.**

—Miriam Merad, Icahn School of Medicine at Mount Sinai

infectious disease specialist at the Charité hospital in Berlin.

**See "Neutrophil Extracellular Traps May Augur Severe COVID-19"**

Some of these myeloid cells in the blood of severely ill COVID-19 patients are indeed "functionally sluggish," explains Stanford University immunologist Bali Pulendran, who recently found that the cells didn't release cytokines when exposed to viral or bacterial debris in vitro. That suggested that the vast quantities of blood-borne cytokines weren't coming from these first-responders in the blood, but from the lungs, where researchers have spotted high abundances of inflammatory macrophages and other myeloid-derived cells, he says. "The immune response in the lung was excessive inflammation, lots of cytokines. [But the] immune response in the blood was the opposite—it was suppression." In contrast, moderately ill patients had significantly fewer suppressive cells in their blood. Sander notes that the precise sources of the cytokines, and their exact role in driving the severity of the disease, are yet to be elucidated.

But Merad suggests that the lung-dwelling inflammatory macrophages, the first immune cells to detect viral debris, could play an important role in driving the dysregulated cytokine response.

These cells have been implicated strongly in the deleterious effects of aging, obesity, and diabetes—the same comorbidities linked to risk for severe COVID-19. Perhaps they're "giving the wrong tone of inflammation in these patients" by over-activating T cells and other parts of the immune system, she says. "It is, I think, really a myeloid-triggered disease." As for the sluggish, suppressive cells in the blood, maybe the body is somehow shutting down other parts of the immune response to prevent it from becoming overwhelmed, Pulendran says. The simultaneous immune suppression and over-activation has also been observed in sepsis, where it's called "immunoparalysis."

Other innate immune cells, such as natural killer (NK) cells, are also altered in severe cases of COVID-19 compared to non-severe cases, research by scientists in Sweden has shown. NK cells sense stressed cells and kill those infected with pathogens, while also releasing pro-inflammatory cytokines and influencing T cell responses. NK cells come in different flavors, from less-differentiated ones fresh out of the bone marrow—which are good at proliferating and secreting cytokines—to highly differentiated super killers specialized for taking down virally infected cells. NK cells were more skewed toward the former group in moderate disease. But "a subpopulation of NK cells that are the most terminally differentiated, that are the most skewed towards killing, they were specifically expanded in the severely sick patients," explains senior author Niklas Björkström, an immunologist at the Karolinska Institute. Such killer cells have also been spotted in hantavirus and yellow fever virus infections, but it's not clear if they play a deleterious role in driving further immune dysfunction or a protective role. "I'm a positive person. So I would still be hoping that the fact that we see them is because we need them for something at that severe stage, and that they are rather trying to do something good," Björkström adds.

## An adaptive immune system out of kilter

The components that make up our adaptive immune system also undergo drastic transformations in severe COVID-19. Some of these changes are expected. Like some other infectious disease patients, those with COVID-19 almost always have unusually low numbers of lymphocytes such as B cells and T cells in their blood—perhaps because those cells are dying off for some reason, or because they're rushing into tissues to combat the infection, says Shankar-Hari. However, despite this shortage of lymphocytes, some patients' T cells seemed to be highly activated, says Meyer, speaking of her own recent investigation of 125 hospitalized patients in Pennsylvania.

The combination of depleted yet activated T cells "was not uniformly seen in all patients, but that was one of the features that did associate with the more severe course and more organ failure," Meyer adds. Other work in critically ill COVID-19 patients finds—somewhat counter-intuitively—that T cells specifically targeted to SARS-CoV-2 proteins are not associated with recovery. "Maybe it's not a

**Maybe it's not a numbers game with the T cells but rather has to do with the type of response**

numbers game with the T cells but rather has to do with the type of response that the ones present are exhibiting," Björkström says.

**that the ones present are exhibiting.**

—Niklas Björkström, Karolinska Institute

COVID-19 patients also have vast quantities of antibody-secreting plasmablasts—another unusual feature of the disease. While other viral infections can provoke such a response, the increase tends to be short-lived, whereas in COVID-19 it seems to persist, Meyer adds. The blood of COVID-19 patients is also flooded with antibodies. But interestingly, new data from a study of 22 hospitalized patients suggest that although the quantity of antibodies didn't differ between survivors and those who died, their function, and which viral proteins they targeted, correlated with severity.

**See "Differences in Antibody Responses Linked to COVID-19 Outcomes"**

Another peculiarity of the antibody response in severe COVID-19 patients is the apparent lack of a critical antibody creation process that takes place in the so-called germinal center of the lymph nodes and spleen. Ordinarily in viral infections, following a wave of initial virus-targeting antibodies that begins in the first few days of an infection, germinal centers form in the lymph nodes and spleen, where specialized B cells and T helper cells gather to produce a highly refined batch of antibody-producing cells that are crucial for lasting antibody immunity. But a study published last month demonstrated that those structures were absent in 11 COVID-19 patients who died from the disease. The germinal center formation may be stunted due to high levels of certain cytokines, the authors posit, or perhaps due to the defects of antigen-presenting cells that help drive that response, Shankar-Hari notes.

In line with those findings, data reported in a preprint by a team of Emory University researchers show that the abundance of two subtypes of B cells that create short-lived antibodies outside the germinal center correlated strongly with disease severity in 17 hospitalized patients, while those cells were barely present in healthy controls. The expansion of these B cell types has been primarily associated with flare-ups of autoimmune diseases such as systemic lupus erythematosus, where they're reflective of an overly inflammatory state. It's possible that this abnormal B cell response is the body's attempt to generate antibodies after being somehow hindered from making them in germinal centers. Yet findings from autoimmune diseases also hint that "it may be that some of these cells are actually worsening the inflammatory cascade," says Richard Ramonell, one of the study's coauthors and a fellow in pulmonary and critical care medicine at Emory.

Merad says she thinks these abnormalities of the adaptive immune system are driven by the dysregulated cytokine response, which may be ultimately rooted in myeloid defects. But the intimate dialog between the innate and adaptive immune systems makes it hard to tease out cause and effect—an issue that comes up in autoimmune diseases all the time, notes Matthew Woodruff, an immunologist at Emory. "You see an autoimmune patient in the clinic, and you've got this entire system that has already been thrown out of balance in some way. . . . You've already established a new homeostatic process, and you can describe it, you can intervene, you can do all of those things. But the chicken and egg questions continue to be very, very difficult to answer."

## Turning immunology findings into biomarkers and treatments

At the moment, clinicians use several biomarkers to assess COVID-19 patients' general inflammatory state, including D-dimer, which measures protein fragments that arise from blood clots, and the inflammatory indicator C-reactive protein (CRP), both of which correlate with disease severity. However, COVID-19–specific and earlier biomarkers would be needed to reliably identify patients on hospital admission or earlier who are destined to develop critical disease. "If we can somehow identify them before they come into the hospital, or while they're in the emergency room before they get intubated, we could potentially change their disease course," notes immunologist and pulmonologist F. Eun-Hyung Lee of Emory University.

Over the past few months, studies investigating immune features that correlate with COVID-19 severity have yielded a number of candidate biomarkers based on immune cells, specific antibody features, and cytokines. They include the IL-6, IL-8, and TNF-α cytokines that Miriam Merad and her colleagues found elevated in severely ill patients in a cohort of 1,500 individuals treated at Mount Sinai Health System. Merad's team validated those

cytokines in a second cohort of 231 patients, and demonstrated that they were indeed predictive of disease severity. Other research that has flagged IL-6, IL-10, and IP-10 as potentially predictive markers seems promising but would need to be validated in a larger group of patients, notes Nuala Meyer, a critical care physician at the Hospital of the University of Pennsylvania.

Some of Meyer's research has found that the ratio of neutrophils to leukocytes—which is easy to measure in the clinic—can predict dysregulated immune responses in COVID-19 patients. Other work has flagged other cytokines and serum bicarbonate, which reflects electrolyte imbalances and is a readily available lab test. But in that study, "it did not look like all COVID patients fit into [the] hyper-inflammatory group. So in a way I think we need to do more work to understand what are better plasma biomarkers for severe COVID," she says.

Findings from such studies are also providing insights into new treatment approaches for COVID-19. Several trials are underway that aim to administer interferon—which is already used to treat hepatitis C—to patients. However, timing is key when it comes to interferon treatment—if it's given too late, it could worsen the disease.

For later in the course of disease, immune-dampening steroid drugs such as dexamethasone and other corticosteroids—which can curtail the activity of multiple cytokines in tandem—appear to be effective in reducing the risk of death. But corticosteroid treatments often have side effects, especially for elderly patients, and more targeted therapies would be preferable.

So far, researchers haven't had any luck in tamping down levels of individual cytokines. Doing so for IL-6, for instance, is effective in dampening the cytokine storm that occurs in some cancer patients receiving cellular immunotherapy. The pharma giant Roche recently reported that its CONVACTA trial testing its IL-6–targeting drug Actemra did not improve the clinical status of hospitalized COVID-19 patients with severe pneumonia. And a smaller study testing the IL-6 blocking agent sarilumab in severe disease also proved disappointing to Merad, she says. A combination treatment that blocks multiple cytokines might be the way to go, she suggests. But COVID-19 is also a very heterogeneous disease, making it difficult to interpret clinical signals, she notes. "We need to tailor the treatment to the molecular effect."

Alternatively, it may not be the cytokine increase that's killing patients. Some researchers have recently expressed doubt that cytokines play a major role in driving the severity of the disease based on small studies suggesting that levels of pro-inflammatory cytokines are much lower in severe COVID-19 than in patients without COVID-19 who die of acute respiratory distress syndrome.

Perhaps the ultimate culprit is a coagulation problem driven by the raging inflammation of blood vessels, says immunologist Niklas Björkström of the Karolinska Institute in Sweden, noting that infusions of the anticoagulant heparin reduced the fatality rate in some patient groups. "The fact that a lot of our patients have problems with clotting raises interest perhaps in the complement pathway or novel anticoagulant strategies," Meyer says. In addition, "I do think we're interested to know if we can perhaps intelligently intervene on this T cell activation without tipping the balance too much towards immune suppression," she adds.

Half a year into a pandemic, "I understand that the public is frustrated. Even my family's like, 'Can't you figure it out?'" Merad says. However, answers are on their way, and at breakneck speed, in large part thanks to technological advances over the past decades. "We are [learning] much faster than any time in history where we've had a big disease [outbreak] like that. So I'm confident that the few months to come are going to be extremely informative."

**Keywords:**

coronavirus, COVID-19, cytokines, immunology, interleukin, monocyte, myeloid cells, neutrophils, news feature, pandemic, SARS-CoV-2

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# SARS-CoV-2 and the safety margins of cell-based biological medicinal products

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ABSTRACT

With the pandemic emergence of SARS-CoV-2, the exposure of cell substrates used for manufacturing of medicines has become a possibility. Cell lines used in biomanufacturing were thus evaluated for their SARS-CoV-2 susceptibility, and the detection of SARS-CoV-2 in culture supernatants by routine adventitious virus testing of fermenter harvest tested.

## 1. Introduction

A general concern in cell-based manufacturing of recombinant proteins, including vaccines, is the potential contamination of the cell culture with viruses, which has had severe consequences for patients and manufacturers [1,2]. With the pandemic emergence of SARS-CoV-2, the exposure of biomanufacturing cell lines to a new virus has become a possibility, and to safeguard biomedicines it is important to understand whether the virus is even capable of infecting bioproduction cell lines, and if so, whether the event would be detected by adventitious agent tests (AAT) that are part of biomanufacturing quality control.

This study investigated four widely used biomanufacturing cell lines, i.e. CHO and Vero, as well as the two human cell lines HEK-293 and HT-1080, for their susceptibility to infection with SARS-CoV-2.

In addition, adventitious virus testing (AAT) as routinely performed for in-process control testing per regulatory guidance [3,4], was evaluated for its ability to detect SARS-CoV-2 infection if it were to occur. As MRC-5 cells are part of the AAT detector cell line panel (Fig. 1), these cells were also tested for their susceptibility to SARS-CoV-2.

## 2. Materials and methods

### 2.1. Virus and cells

SARS-CoV-2 strain BetaCoV/Germany/BavPat1/2020 (kindly provided by Charité Universitätsmedizin, Institute of Virology, Berlin, Germany; EVAg 026V-03883) was used for all inoculations. Any work with SARS-CoV-2 was done under biosafety level 3 (BSL-3) containment conditions.

CHO cells (Chinese hamster ovary, ATCC CCL-61), Vero cells (African green monkey kidney epithelial, CCL-81, sourced from the ECACC 84113001), HEK-293 cells (Human embryonic kidney cell line, ATCC CRL-1573), HT-1080 cells (Human fibrosarcoma cell line, ATCC CCL-121), and MRC-5 cells (human lung fibroblast, ATCC CCL-171) were used.

### 2.2. Inoculation of cells with SARS-CoV-2 and adventitious agent testing

Cells (CHO, Vero, HEK-293, HT-1080 and MRC-5) in 6-well plates

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were incubated with virus for 14 days. Inoculations with virus were done at multiplicity of infection (MOI) = 0.01 and MOI = 1, that is, approx. $10^3$ and $10^5$ infectious virus particles per well, respectively, for 1 h. Then the respective medium was added to a total volume of 6 ml/well (CHO medium: Ham's F12 medium supplemented with 10% FCS [PAN-Biotech P40-2009], L-glutamine [2 mM], nonessential amino acids [1x], sodium pyruvate [1 mM] and Gentamycin sulfate [100 mg/ml]; Vero medium: TC-Vero medium supplemented with 5% FCS, L-glutamine [2 mM], nonessential amino acids [1x], sodium pyruvate [1 mM], Gentamycin sulfate [100 mg/ml] and sodium bicarbonate [7.5%]; HEK 293 medium: EAGLE-MEM (+Earles BSS) supplemented with 5% FCS and Gentamycin sulfate [100 mg/ml]; HT-1080 medium: McCoy 5A supplemented with 2% FCS, L-glutamine [2 mM], sodium pyruvate [1 mM] and Gentamycin sulfate [100 mg/ml]; MRC-5 medium: EAGLE-MEM (+Earles BSS) supplemented with 10% FCS, L-glutamine [2 mM], nonessential amino acids [1x], sodium pyruvate [1 mM], Gentamycin sulfate [100 mg/ml] and sodium bicarbonate [7.5%]). 6-Well plates were incubated for 14 days at 36 °C and 4.5% $CO_2$. The presence of CPE was assessed on days 2, 3, 6, 7 and 14 post-infection (p.i.).

The presence of infectious SARS-CoV-2 was tested by titration at various stages throughout the incubation period, on Vero cells using a $TCID_{50}$ assay (see below). Samples for titration were taken from the input inoculum (day 0 p.i.) and days 2 (Vero cells MOI = 1 only), 3, 6, 7 and 14 p.i.

For each cell line, inoculations with SARS-CoV-2 at the two MOIs was done in duplicate.

In addition to SARS-CoV-2 readout by CPE, after 14 days of inoculation, hemadsorption (HAD) and hemagglutination (HA) assays were done, as routinely performed during adventitious agent testing. Due to the CPE detected, Vero cells were evaluated for HAD and HA on day 3 of incubation.

For HAD, wells with MRC-5, Vero, CHO, HEK-293 and HT-1080 cells were covered with erythrocyte suspensions of two different species (human 0.5% [v/v] and guinea pig 1% [v/v]) and incubated at 4 °C for 30 min. The cell culture supernatant was then removed, and the cells washed twice with PBS before microscopic inspection for HAD.

For HA, supernatants of MRC-5, Vero, CHO, HEK-293 and HT-1080 cells were diluted with 0.9% [w/v] NaCl solution in twofold steps. Erythrocyte suspensions (human, 0.5% [v/v] and guinea pig 1.0% [v/v]) were added and separate V-shaped plates incubated for 35 min at 4 °C and 36 °C, before hemagglutination was inspected visually. The described AAT is essentially the routine procedure used for the testing of recombinant protein bulk harvests.

### 2.3. Infectivity assay ($TCID_{50}$)

Samples that potentially contained infectious virus were titrated by tissue culture infectious dose 50% ($TCID_{50}$) assay, that is, eightfold replicates of serial half-log sample dilutions were incubated with cells for 5–7 days and CPE was assessed microscopically. Virus concentrations were calculated according to the Poisson distribution and expressed as viral load ($log_{10}$ [$TCID_{50}$]).

## 3. Results and discussion

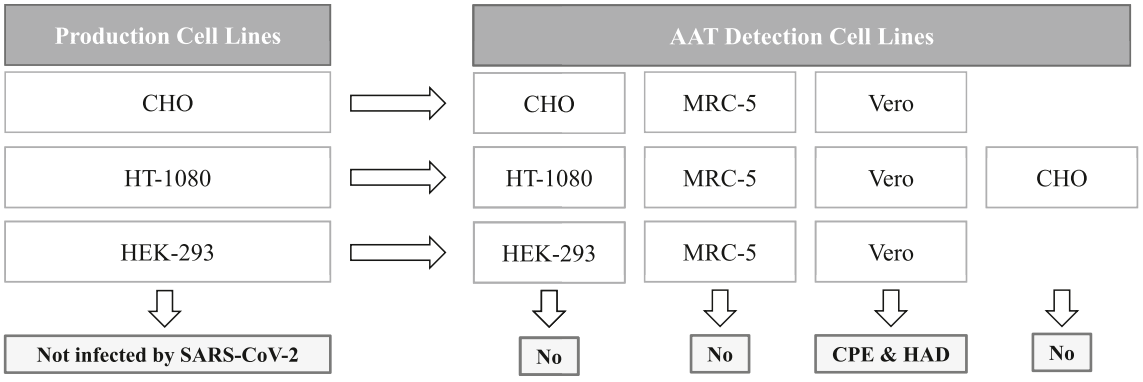
Five cell lines (CHO, Vero, HEK-293, HT-1080 and MRC-5) were inoculated in duplicate with SARS-CoV-2 at two different multiplicity of infection (MOI) values of 1 and 0.01, respectively, and incubated for 14 days. During the incubation period the cells were periodically observed for cytopathological effects (CPE), as an indicator for viral infection in the same way as performed during routine AAT.

No CPE was detected throughout the incubation period for CHO cells, HEK-293 cells, HT-1080 cells and MRC-5 cells. In contrast, Vero cells displayed a CPE already on day 2 (MOI = 1) or day 3 (MOI = 0.01) post infection (p.i.). The permissiveness of Vero E6 and CCL81 cells for SARS-CoV-2 infection and production of CPE has already been demonstrated [5], and thus Vero CCL81 cells were used in this study as positive control, as well as for quantitation of SARS-CoV-2 replication in the other cell lines evaluated: the presence of infectious virus in the cell culture supernatant was determined at regular intervals throughout the incubation period by tissue culture infectious dose 50% ($TCID_{50}$) assay, starting with the inoculum preparations and up to the last day of incubation (day 14).

For Vero cells inoculated with MOIs of 1 and 0.01, this analysis showed increasing SARS-CoV-2 titers in cell supernatants from day 2 and day 3 p.i., which peaked on days 6 and 7 p.i., at mean titers of 6.1 and 5.7 $log_{10}$ $TCID_{50}$/mL, and after 14 days p.i. slightly dropped by on average of 0.7 and 0.8 $log_{10}$, respectively (Table 1).

For the CHO, HT-1080 and MRC-5 cell lines, no SARS-CoV-2 infectivity was detected by titration on Vero cells throughout the entire incubation period, which indicates that SARS-CoV-2 cannot replicate in these cells.

For HEK-293 cells inoculated at MOI = 0.01, SARS-CoV-2 infectivity was never detectable in culture supernatants. After inoculation at MOI = 1, however, low and constantly declining SARS-CoV-2 infectivity titers were detected until day 7 p.i. (Table 1). Whether these declining levels of virus reflect residual inoculum which may be more effectively bound by the HEK-293 as opposed to the CHO, HT-1080 and MRC-5 cell layers, or even some limited yet abortive replication as observed for other HEK-293-derived cells [5] remains to be determined.




CPE, cytopathological effects; HAD, hemadsorption.

**Fig. 1. Overview of production cell lines evaluated for SARS-CoV-2 susceptibility and the corresponding detection cell lines that make up the respective AAT panels**. The information in boxes below indicate whether SARS-CoV-2 permissiveness was detected and for Vero cells the successful assay readouts.

**Table 1**
SARS-CoV-2 titers ($TCID_{50}$/ml) in supernatants of Vero and HEK-293 inoculated in duplicate at MOIs of 0.01 and 1, respectively. The value of 1.1 $TCID_{50}$/ml represents the detection limit of the assay.

| Cell line (MOI) | Days post infection | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 3 | 6 | 7 | 14 |
| Vero (0.01) | 2.0 | NT | 4.9/4.9 | 5.4/5.6 | 5.6/5.8 | 4.7/5.0 |
| Vero (1) | 4.1 | 5.1/ 5.2 | 5.1/5.4 | 6.1/6.1 | 6.0/5.9 | 5.4/5.3 |
| HEK-293 (0.01) | 2.4 | NT | ≤1.1/ ≤1.1 | ≤1.1/ ≤1.1 | ≤1.1/ ≤1.1 | ≤1.1/ ≤1.1 |
| HEK-293 (1) | 4.5 | NT | 3.2/3.1 | 2.2/2.5 | 1.4/≤1.1 | ≤1.1/ ≤1.1 |

MOI, Multiplicity of infection; NT, not tested.

In addition to the monitoring for the development of any CPE, the cells and supernatants were assayed at the end of the 14 days incubation period in the two standard AAT readouts, i.e., hemadsorption (HAD) to the cells, and hemagglutination (HA) activity in the supernatant, using human and guinea pig erythrocytes. This panel of altogether three assay readouts (CPE, HAD, HA) reflects exactly what is routinely performed during quality control testing of biomanufacturing fermenter harvests by using at least three different indicator cell lines (Fig. 1). The choice of detection cells lines for AAT includes per FDA guidance (i) the type of cells used for biopharmaceutical production, (ii) a human diploid cell line, e.g. MRC-5 cells, and (iii) a monkey kidney cell line, e.g. Vero cells [3].

For SARS-CoV-2 inoculated CHO cells, HT-1080 cells, HEK-293 cells and MRC-5 cells, virus was not detectable by any of the three AAT readouts, i.e. CPE as monitored throughout the two weeks of incubation and HAD/HA at the end of the incubation period. For Vero cells though, SARS-CoV-2 replication was, in addition to detection by CPE, detected by HAD with human erythrocytes (MOI = 1 only). These findings confirm that AAT would confidently detect a SARS-CoV-2 contamination in a biomanufacturing cell culture tested, so long as Vero cells are included as a detector cell line.

Entry of SARS-CoV-2 into susceptible cells is facilitated by the same receptor as for SARS-CoV, i.e. the Angiotensin-converting enzyme-2 (ACE 2) [6,7], which suggests that the spectrum of susceptible cells is similar for SARS-CoV and SARS-CoV-2. High abundance of ACE2 as a cellular surface protein on Vero cells [8] does correlate well with their susceptibility to infection with SARS-CoV-2. In contrast, the expression of ACE2 at low levels on CHO cells [9] might be the reason that SARS-CoV-2 replication in these cells was not observed. HEK-293 cells carry ACE2 at higher levels [9], which may facilitate binding and possibly entry of SARS-CoV-2 into HEK-293, followed by minimal and ultimately abortive replication (Table 1), or modest replication in HEK-293T cells [5]. However, the presence of a specific receptor may be the reason for virus entry in a target cell line but not the only requirement for productive virus replication. For SARS-CoV-2, 332 different protein-protein interactions have been identified [10], each providing for an interaction where virus proliferation might be blocked.

## 4. Conclusions

Permissiveness for proliferation of SARS-CoV-2 was confirmed for Vero cells, but was absent for CHO, HT-1080 and MRC-5 cells. HEK-293 cells did not show productive amplification of SARS-CoV-2 although some minimal and self-limiting replication may be possible. This study also showed that in CHO cells, HT-1080 cells and MRC-5 cells, no "silent" infection of SARS-CoV-2 did occur, i.e. virus replication but no development of detectable CPE, as a "silent" virus replication would have been detected by the titration of supernatants on Vero cells. In routine AAT assays, SARS-CoV-2 was confidently detected by CPE in Vero cells and HAD with human erythrocytes, i.e. two different readouts that revealed the presence of SARS-CoV-2, which underlines its reliable detection by AAT if it were to occur as a contaminant in a biomanufacturing cell culture process.

The detection of SARS-CoV-2 in AAT when Vero cells are part of the indicator cell line panel was reassuring, even in the hypothetical case that SARS-CoV-2 would replicate in the production cell line but would not produce a CPE and not be detected by cell parameter screening, for example by measurement of cell viability, oxygen consumption etc.

Furthermore, SARS-CoV-2 would represent a contaminant for which highly effective clearance capacities can be expected from widely used dedicated down-stream virus reduction steps: for example, as a lipid-enveloped virus SARS-CoV-2 would be robustly inactivated by solvent/detergent (S/D) treatment [11] and due to its large size of about 120 nm SARS-CoV-2 can be expected to be completely retained by nanofiltration, even when using larger pore sizes such as 35 nm [12].

## Declaration of competing interest

J.M., A.K., M.R.F., and T.R.K are employees of Baxter AG, Vienna, Austria, now part of the Takeda group of companies. J.M., M.R.F. and T.R.K. are Takeda stock owners.

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# Replacing the *in vivo* toxin challenge test with an *in vitro* assay for assessment of potency for diphtheria toxoid containing vaccines

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ABSTRACT

Replacement of the potency tests for diphtheria vaccines is a high priority for the international initiative to reduce, refine, and replace animal use in vaccine testing. Diphtheria toxoid containing vaccine products marketed in the US currently require potency testing by the United States Public Health Service (USPHS) test, which includes an *in vivo* passive protection test with a diphtheria toxin challenge. Here we describe an *in vitro* Diphtheria Vero Cell (DVC) assay which combines the immunization approach from the USPHS test and the use of a cell based neutralization assay for serological testing of vaccine potency. The DVC assay reduces the overall number of animals used compared to other serological potency tests and eliminates the *in vivo* toxin challenge used in the US test. The DVC assay can be used to test vaccine products with a low or high diphtheria toxoid dose. It has been optimized and validated for use in a quality control testing environment. Results demonstrate similar sera antibody unitage as well as agreement between the serum neutralization values determined using the USPHS test and the DVC assay and thus support the use of the DVC assay for routine and stability testing for diphtheria toxoid containing vaccine products.

## 1. Introduction

Diphtheria is an acute, toxin-mediated disease caused by the bacterium *Corynebacterium diphtheriae*. Diphtheria toxin is an exotoxin which inhibits cellular protein synthesis and is responsible for local tissue destruction and pseudomembrane formation. If left untreated, diphtheria can have a mortality rate of up to 50% [1]. With medical intervention, including the use of antitoxin and antibiotics, the overall fatality rate is reduced to 5–20%, depending on the age of the patients [2]. Diphtheria toxin can be inactivated by treating with formalin; the resulting toxoid is immunogenic and has been used successfully as a vaccine since the 1920s [2].

The Center for Biologics Evaluation and Research (CBER) regulates the potency testing of diphtheria vaccines marketed in the United States, where there are currently twelve such products approved for human use [3]. These vaccine products contain diphtheria toxoid at 15 or 25 Lf/dose ('high dose', used for primary immunization) and at 2 or 2.5 Lf/dose ('low dose', used for booster immunization). CBER recommends that the USPHS test be used to determine potency for these vaccines. The USPHS test is a two stage *in vivo* test that has been in use for more than 70 years [4,5]. In the first stage, the test vaccine is used to immunize a set of naïve guinea pigs and the resulting serum is collected and pooled. In the second stage the pooled serum is incubated with a lethal dose of diphtheria toxin prior to injection into a second set of naïve guinea pigs, and survival is monitored. This *in vivo* toxin challenge test determines the neutralizing titre of the sera, using an international standard antitoxin preparation as the reference. Vaccine specifications require a minimum level of neutralizing units/mL (U/mL) be induced in the guinea pig sera, according to the dose of diphtheria toxoid in the vaccine.

Potency tests which utilize animal models can measure the biological activity of vaccines with high specificity and sensitivity, however they are notorious for having low precision, high variability and poor reproducibility. Diphtheria vaccines in particular have been determined

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to be of high priority for the replacement of *in vivo* potency testing as part of the international 3R initiative to reduce, refine, and replace animal use in vaccine testing [6]. Although there are currently two partially *in vitro* diphtheria vaccine potency tests approved for use by the Eur Ph and WHO, including the titration of immune serum by ELISA or by toxin neutralizing Vero cell assay, these tests rely on a large number of immunized animals to determine relative potency by establishing dose response and parallelism compared to a reference vaccine [7]. Comparatively, the USPHS test requires fewer animals, as it uses a single dose of test vaccine as well as reference antisera rather than a reference vaccine. Nonetheless, due to the use of the *in vivo* toxin challenge, the USPHS test represents a prime opportunity for improvement, supporting the 3R initiative for potency testing of diphtheria vaccines marketed in the US.

Previous studies have been performed to develop an *in vitro* assay that measures toxin neutralization using Vero cells and that could be aligned with the USPHS test for diphtheria vaccines. Early studies were unable to establish a direct correlation to *in vivo* results [8]. Others were able to identify conditions where data correlated with *in vivo* results but could only be used to test high dose vaccine products, which induce 2 U/mL of antitoxin [9,10]. We describe here an *in vitro* assay, the DVC assay, which is a modification of these earlier methods and can be used to test serum from guinea pigs immunized with either high or low dose diphtheria toxoid-containing vaccine products. It uses considerably fewer animals than the WHO Vero cell assay, while removing the toxin challenge portion of the US *in vivo* test, and therefore results in reduction and replacement of animal use for quality control testing as mandated by the 3R initiative. Importantly, it aligns closely with the USPHS potency test in terms of the reportable value, could be used for all currently licensed vaccine products, and includes the use of the same standard reference antitoxin currently used in the USPHS test. The DVC assay has been optimized and validated for use in a quality control testing environment and is currently being assessed against the USPHS test for a number of diphtheria containing vaccines.

## 2. Materials and methods

### 2.1. USPHS potency test for diphtheria

All animal procedures were subject to ethical review and performed in accordance with Canadian Council on Animal Care guidelines. The compendial USPHS method for diphtheria toxoid potency testing [4] was performed for this study and is described here in brief. The USPHS test has two stages; first an immunization stage, then a toxin challenge stage that tests the resulting sera. For the immunization stage, vaccine formulations which contain diphtheria toxoid at either high dose (15 Lf/dose) or low dose (2 Lf/dose) were injected into five naïve female Hartley guinea pigs. Serum was collected at 4 or 6 weeks following immunization with high or low dose vaccines, respectively. Equal volumes of serum from each of the five immunized animals were pooled; this pooled serum is hereafter referred to as a 'serum sample'. In the toxin challenge stage, the serum sample was mixed with diphtheria toxin at predetermined doses and the mixture was injected into naïve male Hartley guinea pigs. The toxin dose used in the USPHS test is the L+/1 toxin dose, defined as the minimum lethal dose of diphtheria toxin that causes death in guinea pigs within 4 days in the presence of 1 U/mL US Standard Diphtheria Antitoxin (calibrated hyper-immune horse serum supplied by CBER). The reportable values for products tested in the USPHS test are the neutralization titres of the serum samples. High dose products are reported as either 2.5 U/mL, 2 U/mL, or below 2 U/mL, and low dose products are reported as either 0.5 U/mL or below 0.5 U/mL. The lower threshold acceptable limit for each product is defined as greater than or equal to either 2 U/mL for high dose products or 0.5 Units/mL for low dose products.

### 2.2. DVC assay method

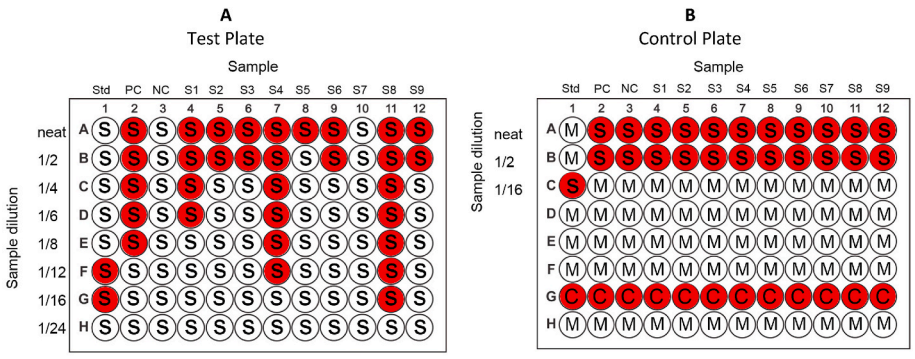
Serum samples (pooled sera from individual guinea pigs immunized with test vaccine) and US Standard Diphtheria Antitoxin (4 U/mL in assay medium (DMEM plus GlutaMAX (Gibco), 1% penicillin/streptomycin (Gibco), 1% gentamycin (Gibco), 10% FBS (Hyclone)) were each diluted for testing by preparing two independent serial dilutions. One set of two-fold serial dilutions started with undiluted serum sample or reference antitoxin at 4U/mL ('neat') and included 1/2, 1/4, 1/8, and 1/16 dilutions thereof. The second set started with a 1/6 dilution and included 1/6, 1/12, and 1/24 dilutions. Fig. 1 depicts a typical assay setup, wherein 200 μL of each dilution of each serum sample or reference antitoxin was added to a flat bottom 96-well tissue culture plate (Becton Dickinson) (Fig. 1A). A control plate was also prepared, containing 200 μL of the undiluted (neat) and 1/2 dilutions for each serum sample as well as the 1/16 dilution of the reference antitoxin, to serve as toxicity control wells (Fig. 1B). Remaining wells on the control plate received assay medium alone. Fifty μL diphtheria toxin (0.7 Lf/mL, see Section 2.3) was added to each well on the test plate, and 50 μL assay medium was added to wells on the control plate for a final volume in both test and control plates of 250 μL/well. Plates were incubated for 1 h at 37 °C, 5% $CO_2$. Next, Vero cells ($1 \times 10^4$ cells/50 μL per well in assay medium) were added to both test and control plates as indicated (Fig. 1A, B). Final volume in all sample and control wells was 300 μL per well. Plates were incubated for four days at 37 °C, 5% $CO_2$. Medium was removed, 200 μL/well neutral red (Sigma, 0.5 mg/mL in DMEM plus GlutaMAX) was added and the plates were incubated for 3 h at 37 °C, 5% $CO_2$. Plates were washed once with 200 μL/well D-PBS (Gibco) prior to addition of 200 μL/well 1% acetic acid (EMD) 50% ethanol (Commercial Alcohols) solution. Plates were incubated for 20 min at room temperature and then shaken at 600 rpm for 1 min prior to reading the absorbance of each well at $540_{nm}$ using a spectrophotometer. The cell viability threshold was calculated as a value of 50% of the average $A_{540}$ of the cell control wells; wells with $A_{540}$ above this value were considered positive for live cells. The most dilute sample well positive for live cells indicates the neutralizing titre of the sample (Fig. 1C; see Section 2.3 for detail on titre calculation). Note that in the case of the US Standard Diphtheria Antitoxin reference, which is provided in a 66% glycerol matrix, viable cells are not expected in wells containing antitoxin at higher concentrations (i.e. neat to 1/8 dilution) due to the toxic effects of glycerol on Vero cells. On the control plate only the 1/16 dilution of the reference antitoxin is tested to confirm that at this concentration (corresponding to 4 U/mL) there is no glycerol toxicity.

Validity criteria for the assay were (1) the prepared reference antitoxin must have a neutralizing titre of 4 U/mL (2) the Negative Control sera must have a neutralizing titre of <0.25 U/mL (3) the Positive Control sera must have neutralizing titres within the predefined expected range (4) serum sample and antitoxin wells on the control plate must be above the viability threshold (5) the average $A_{540}$ in media only wells on the control plate must be lower than half of the viability threshold.

### 2.3. Calibration of diphtheria toxin dose for DVC assay

Prior to performing the DVC assay to test serum samples, the optimal toxin dose for Vero cells in the assay was determined by performing a toxin calibration assay. The USPHS test uses the L+/1 dose of toxin, the lowest dose of toxin not neutralized by 1 U/mL antitoxin, and is confirmed by death of guinea pigs after four days. In contrast, the DVC assay uses the highest dose of toxin which is neutralized by 1 U/mL antitoxin and the readout is viable cells. We have found this approach to result in accurate and consistent titre determination using the *in vitro* method.

Calibration serves to identify the concentration of toxin that can be neutralized by the equivalent of 1 U/mL of antitoxin in the context of the DVC assay, i.e. $1 \times 10^4$ Vero cells in 300 μL total well volume incubated




C

| Highest sample dilution with viable cells | Neat | 1/2 | 1/4 | 1/6 | 1/8 | 1/12 | 1/16 | 1/24 |
|---|---|---|---|---|---|---|---|---|
| Corresponding neutralizing titre (U/mL) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 | 3.0 | 4.0 | 6.0 |

D

| Sample | STD | PC | NC | S1 | S2 | S3 | S4 | S5 | S | S7 | S8 | S9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Titre (U/mL) | 4.0 | 2.0 | <0.25 | 1.5 | 0.5 | 0.5 | 3.0 | 0.25 | 0.5 | <0.25 | 4.0 | 0.5 |

**Fig. 1. Representation of DVC assay test plate (A) and control plate (B) with correlation of serum sample dilution to neutralization titre (C) and representative results (D).** Nine serum samples (S1–S9) are depicted in addition to positive control serum (PC) of known titre, negative control serum (NC) from unimmunized animals, and US Standard Diphtheria Antitoxin (Std) prepared at a starting concentration of 4 U/mL ('neat'). End points for each sample are determined by the highest dilution with viable cells (indicated by the color red) and correspond to a neutralization titre in U/mL. "S" = wells with serum or Std plus cells, "C" = wells with Vero cells and medium only, "M" = wells with medium and no cells or sample. All wells on the test plate contain diphtheria toxin; toxin is not added to the control plate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for four days at 37 °C. Specifically, the toxin dose must be neutralized by 0.05 U antitoxin in the well; this amount of antitoxin in the assay well reflects a sample that is 1 U/mL when undiluted. For example, a sample with titre of 1 U/mL is diluted in series as described in Section 2.2 and 200 μL is added to the well at each dilution. At the well corresponding to the ¼ dilution (row C), there is 0.05 U antitoxin/well (0.2 mL/well of a ¼ dilution). If the toxin dose is correct, this well will be above the viability threshold, indicating neutralization of the toxin. For that same sample, the 1/6 and greater dilutions (rows D-H) would be below the viability threshold. Correspondingly, a sample with a titre of 2 U/mL would be above threshold at the 1/8 dilution (0.05 U antitoxin/0.2 mL x dilution factor of 8) and below threshold at higher dilutions.

The calibration assay compares results obtained with a range of different toxin doses. Antitoxin reference preparations at multiple concentrations are used as test samples, to confirm at which toxin dose the DVC assay will report an accurate titre for the antitoxin.

#### 2.3.1. Diphtheria toxin dose

To calibrate the toxin dose for use in the DVC assay, diphtheria toxin doses were tested in small increments above and below 1 Lf/mL. The method for the DVC assay was followed with one exception: instead of serum, US Standard Diphtheria Antitoxin prediluted in assay medium to 4.0 U/mL, 2.0 U/mL, 1.0 U/mL and 0.5 U/mL were tested as unknown samples. The toxin dose which consistently showed the expected titres for the prepared antitoxin samples in three separate assays was 0.7 Lf/mL (data not shown), and this was identified as the toxin concentration for use in the DVC assay thereafter. Specifically, 0.035 Lf diphtheria toxin in the well (50 μL at 0.7 Lf/mL) is neutralized by 0.05 U antitoxin in the well (200 μL at 0.25 U/mL).

### 2.4. Serum samples

Serum samples used to compare DVC assay and USPHS test results were generated through routine testing of vaccine products in the USPHS test, as defined in Section 2.1.

Serum samples used in validation experiments were prepared by combining multiple pools of sera generated from routine testing of vaccine products as described above (details in Table A.1).

Positive (PC) and Negative (NC) control sera were pooled from immunized and unimmunized guinea pigs, respectively, and were tested five times in the DVC assay to define the neutralizing titre. A low titre PC (0.5 U/mL) was used when testing low dose vaccine products and a high titre PC (2 U/mL) was used when testing high dose vaccine products.

### 2.5. Vaccine products

Multicomponent vaccine products were used (Sanofi Pasteur, Toronto Canada). These contained diphtheria toxoid at 2 Lf/dose (d) or 15 Lf/dose (D) in combination with any of the following: tetanus toxoid (T), acellular Pertussis antigens (aP), Inactivated Polio Vaccine components (IPV), Haemophilus influenzae type b (Hib) antigen, and/or Hepatitis B (HepB) antigen.

For heat treatment, vaccine products were exposed to 60 °C in a dry oven for periods of time from 24 h to 2 months in order to reduce potency.

### 2.6. Validation experiments

Specificity was assessed through the testing of naïve guinea pig serum and 'mock' serum samples, generated by immunizing guinea pigs with a formulation of tetanus toxoid, acellular pertussis antigens, Hib, HepB and IPV (no diphtheria toxoid). Serum samples were tested three times in total by two analysts (n = 3 measurements for each sample). Validity criterion was no detectable neutralizing activity.

Precision was assessed by repeated testing of eight serum samples (three low, three medium, two high titre serum samples, tested on the same assay plate). Each sample was tested by three analysts in one laboratory and two analysts in a second laboratory over multiple days (n = 18 measurements for each sample). The experimental results were analyzed for repeatability, intermediate precision and reproducibility. All precision data was assessed using the precision probability approach [11,12], with an allowable difference of one titre increment. The assay end point titre readouts are 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 6.0 U/mL, and the difference between each of the sequential titres is considered one titre increment.

Accuracy was assessed using the two high titre serum samples that were tested during precision testing. A two-fold serial dilution was prepared in naïve serum to create total of five samples (undiluted, 1/2, 1/4, 1/8, 1/16). The samples were all tested once per day on four days by one analyst in each laboratory (n = 8 measurements for each serum sample at each dilution). Theoretical titres of the undiluted sera were determined using the 18 measurements obtained during precision analysis, and from these values the theoretical titres for the diluted sera were mathematically calculated. Acceptance criterion was percent

recovery within 70%–130%. Average titre values from multiple results were calculated using the Spearmann Kärber (SK) method for assessing semi-quantitative endpoint titre data [13,14], using the following formula:

$$M = \sum_{i=0}^{k} (p_{i+1} - p_i)(x_i + x_{i+1}) / 2$$

where:

M = titre result calculated using the SK method

x1, x2, …,xk represent the log 2 transformed end point titres

p1, p2,…pk represent the proportion responding (no. responses/total no. of replicates) at each dose level.

Linearity was evaluated from the data obtained in the accuracy experiments, with the following acceptance criteria: the p-value of the significance of the slope is $< 0.05$; the coefficient of determination ($R^2$)$>$ 0.95; and the 95% Confidence Interval of the slope includes 1.

The limit of quantitation (LOQ) was based on the results from the accuracy experiments. Range was defined as the interval from the lower LOQ (LLOQ) to the upper LOQ (ULOQ), which represented the lowest and highest titre sera respectively that met validity criteria for accuracy and linearity and for which the precision probability was >90%.

## 3. Results

### 3.1. Validation

To assess specificity, titres were determined for naïve (unimmunized) sera and sera from guinea pigs immunized with mock vaccine preparations. No neutralization was detected (i.e. all wells were below the viability threshold) when sera were tested undiluted in the DVC assay; therefore all observed titres were determined to be $< 0.25$ U/mL, the lowest titre that can be determined in the assay.

Repeatability was assessed by testing eight serum samples, ranging from low to high titre, three times each within a period of eight days by the same analyst. This series of tests was repeated a total of six times by a total of five analysts in two laboratories; intermediate precision was assessed by analyzing the measurements from all of the repeatability experiments within each laboratory and reproducibility was assessed by analyzing the combined measurements of all of the repeatability experiments in both laboratories.

When results were compared for all the samples, titre determinations for some samples were seen to vary between one titre increment and the next (e.g. sample titre was either 1.0 U/mL or 1.5 U/mL over multiple tests), however there was no variation seen for any sample that exceeded one titre increment (see Table A.2). As a result, precision probabilities for repeatability, intermediate precision, and reproducibility were 100% for all assessments.

Results of the accuracy and linearity assessment are shown in Fig. 2. Serial dilutions of two high titre serum samples were tested four times each in two laboratories. Accuracy was assessed as % recovery and found to be between 84% and 120% for all samples. Linearity assessment showed that the p-value of the significance of the slope was $<0.0001$, $R^2$ was 0.99, and the 95% confidence interval of the slope was (0.873, 1.016).

Since the lowest and highest titre samples (0.25 and 4.0 U/mL respectively) tested met accuracy, linearity, and precision criteria, these values represent the LLOQ and ULOQ, and the range was defined as 0.25–4.0 U/mL.

### 3.2. Comparison of DVC assay and USPHS test results

The USPHS test and the DVC assay were used to test a number of vaccine products which were part of routine product quality testing (Table 1), as well as products which had been heat treated at 60 °C for increasing periods of time to produce reduced potency samples (Table 2). The vaccine products tested contained multiple antigens including diphtheria toxoid at either low or high dose (2 Lf/dose or 15 Lf/dose respectively). Results from both untreated and heat treated product testing are summarized in Table 3. USPHS test results of 0.5 U/mL meet product specifications for low dose products and are considered to be in agreement with DVC results of 0.5 U/mL or higher. USPHS test results of 2 U/mL or higher meet product specifications for high dose products and are considered to be in agreement with DVC results of 2 U/mL or higher. Overall, serum samples from 18/19 low dose and 30/36 high dose vaccine samples tested had neutralization titre results which were in agreement between the USPHS test and DVC assay (95% and 83% respectively).

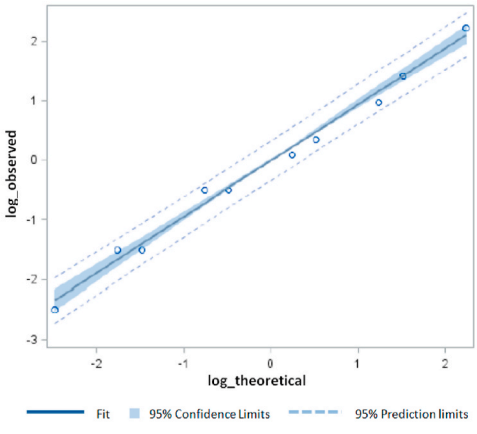
## 4. Discussion

The DVC assay performance was assessed with a validation study, which met all acceptance criteria across the serum neutralization titre range of 0.25–4.0 U/mL. The assay is precise (precision values of 100% for repeatability, intermediate precision, and reproducibility), accurate (84%–120% recovery), and linear (p-value for the significance of slope $<0.0001$, $R^2 = 0.99$, and 95% confidence interval for the slope of (0.873,

**A**

| Serum Sample lot# | Dilution | Theoretical Titre (U/mL) (n=18 for undiluted serum samples) | Observed Titre (U/mL) (n=8) | % Recovery |
|---|---|---|---|---|
| 2016-VH3 | undiluted | 4.71391 | 4.69128 | 100 |
| | 1/2 | 2.35696 | 1.97244 | 84 |
| | 1/4 | 1.17848 | 1.06759 | 91 |
| | 1/8 | 0.58924 | 0.70711 | 120 |
| | 1/16 | 0.29462 | 0.35355 | 120 |
| 2016-VH4 | undiluted | 2.85740 | 2.67119 | 93 |
| | 1/2 | 1.42870 | 1.27897 | 90 |
| | 1/4 | 0.71435 | 0.70711 | 99 |
| | 1/8 | 0.35718 | 0.35355 | 99 |
| | 1/16 | 0.17859 | 0.17678 | 99 |

**B**




**C**

| Parameter Estimate | Standard Error | Observations | t Value | Pr > \|t\| | 95% Confidence Limits | | $R^2$ |
|---|---|---|---|---|---|---|---|
| 0.944 | 0.03168 | 10 | 29.81 | <0.0001 | 0.873 | 1.016 | 0.99 |

**Fig. 2.** Accuracy and Linearity Assessments. Two serum samples were two-fold serially diluted in naïve serum for a total of five sample preparations per serum lot and each preparation was tested once per day on four days by each of two analysts (n = 8 measurements for each dilution of each sample). (A) Accuracy assessment: theoretical titre of the undiluted samples is the average of 18 values determined in precision testing. Observed titre is the average of 8 values determined in accuracy testing. % Recovery is observed/theoretical titre x 100 for each sample. Average values were calculated using the SK method for end point titres. (B) (C) Linearity assessment: log values of theoretical vs observed average titres for each sample (n = 10 samples) were plotted and analyzed using a linear model to assess dilutional linearity. $R^2$ = coefficient of determination, Pr=Probability.

**Table 1**
**Neutralizing titre of serum generated by vaccination of guinea pigs with routine diphtheria vaccines**. Immunization of guinea pigs was performed as per USPHS test (immunization portion) with low dose (2 Lf/dose) or high dose (15 Lf/dose) diphtheria toxoid containing vaccine products. The same sample of pooled serum from immunized guinea pigs was analyzed once by USPHS (toxin challenge test) and once by DVC assay. USPHS test results of 0.5 U/mL meet product specifications for low dose products and are considered to be in agreement with DVC results of 0.5 U/mL or higher. USPHS test results of 2 U/mL or higher meet product specifications for high dose products and are considered to be in agreement with DVC results of 2 U/mL or higher. Results which are not considered to be in agreement are italicized.

| Vaccine Product | Diphtheria Toxoid dose | Lot | Neutralizing titre determined by USPHS test (U/mL) | Neutralizing titre determined by DVC assay (U/mL) |
|---|---|---|---|---|
| Tdap | low (2 Lf/dose) | 1 | 0.5 | 1.5 |
| | | 2 | 0.5 | 1.5 |
| | | 3 | 0.5 | 1 |
| | | 4 | 0.5 | 1.5 |
| | | 5 | 0.5 | 1.5 |
| | | 6 | 0.5 | 1 |
| Td | low (2 Lf/dose) | 1 | 0.5 | 2 |
| | | 2 | 0.5 | 1 |
| DTaP | high (15 Lf/dose) | 1 | 2 | 2 |
| | | 2 | 2 | 2 |
| | | 3 | 2.5 | 2 |
| | | 4 | 2.5 | 2 |
| DTaP-IPV | high (15 Lf/dose) | 1 | <2 | 1.5 |
| | | 2 | 2 | 2 |
| | | 3 | ***2*** | ***1.5*** |
| | | 4 | 2.5 | 3 |
| | | 5 | 2 | 2 |
| | | 6 | 2.5 | 2 |
| | | 7 | ***<2*** | ***2*** |
| | | 8 | 2 | 2 |
| | | 9 | 2 | 2 |
| | | 10 | 2.5 | 3 |
| | | 11 | 2 | 2 |
| DTaP-IPV-Hib | high (15 Lf/dose) | 1 | 2.5 | 3 |
| | | 2 | ***2*** | ***1.5*** |
| | | 3 | 2.5 | 2 |
| | | 4 | 2.5 | 2 |
| | | 5 | ***2*** | ***1.5*** |
| DTaP-IPV-Hib-HepB | high (15 Lf/dose) | 1 | 2.5 | 2 |
| | | 2 | 2.5 | 2 |
| | | 3 | 2 | 2 |
| | | 4 | ***<2*** | ***3*** |

**Table 2**
**Neutralizing titre of serum generated by vaccination of guinea pigs with heat-treated diphtheria vaccines**. Low dose (2 Lf/dose) or high dose (15 Lf/dose) diphtheria toxoid containing vaccine products were incubated at 60 °C for up to two months prior to immunization. Immunization of guinea pigs was performed as per USPHS test (immunization portion). The same sample of pooled serum from immunized guinea pigs was analyzed once by USPHS (toxin challenge test) and once by DVC assay. USPHS test results of 0.5 U/mL meet product specifications for low dose products and are considered to be in agreement with DVC results of 0.5 U/mL or higher. USPHS test results of 2 U/mL or higher meet product specifications for high dose products and are considered to be in agreement with DVC results of 2 U/mL or higher. Results which are not considered to be in agreement are italicized. (h: hour, d: day, m: month, nd: not done).

| | | | Series 1 Neutralizing titre determined by USPHS test or DVC assay | | Series 2 Neutralizing titre determined by USPHS test or DVC assay | |
|---|---|---|---|---|---|---|
| Vaccine product | Diphtheria Toxoid dose | Heat treatment (time at 60°C) | USPHS (U/mL) | DVC assay (U/mL) | USPHS (U/mL) | DVC assay (U/mL) |
| Tdap lot #1[a] | low (2 Lf/dose) | 0 h | 0.5 | 0.5 | nd | nd |
| | | 24 h | 0.5 | 0.5 | 0.5 | 0.5 |
| | | 2 d | nd | nd | ***<0.5*** | ***0.5*** |
| | | 4 d | nd | nd | <0.5 | <0.25 |
| | | 7 d | <0.5 | 0.25 | <0.5 | <0.25 |
| | | 9 d | <0.5 | <0.25 | nd | nd |
| | | 11 d | <0.5 | <0.25 | nd | nd |
| | | 14 d | <0.5 | <0.25 | nd | nd |
| | | 28 d | <0.5 | <0.25 | nd | nd |
| DTaP-IPV lot #1[b] | high (15 Lf/ dose) | 0 h | 2 | 2 | 2 | 2 |
| | | 24 h | <2 | 1 | 2 | 2 |
| | | 2 d | <2 | <1 | ***2*** | ***1*** |
| | | 4 d | <2 | <1 | <2 | <1 |
| DTaP-IPV-Hib-HepB lot #1[c] | high (15 Lf/ dose) | 0 h | 2.5 | 2 | 2.5 | 2 |
| | | 1m | <2 | <1 | nd | nd |
| | | 2m | <2 | 0.5 | nd | nd |

[a] The same lot of Tdap was heat treated for varying times on two separate occasions to generate two sets of degraded material (series 1, series 2) which were used to immunize guinea pigs in two separate experiments.
[b] One lot of DTaP-IPV was heat treated for varying times once and then the same material was used to immunize guinea pigs in two separate experiments (series 1, series 2).
[c] One lot of DTaP-IPV-Hib-HepB was heat treated for varying times once and tested in one experiment (series 1) and the untreated sample was tested again in a second USPHS test (series 2).

1.016)). The assay is specific, as it does not detect anti-diphtheria activity in serum from animals that have been immunized with vaccines that do not contain diphtheria toxoid. The assay was validated in two laboratories, including the quality control laboratory, confirming that it is amenable to a routine quality testing environment.

In this study, a total of 37 serum samples from untreated vaccine products (Table 1 and untreated samples in Table 2) were tested in both the USPHS and DVC tests; results were in agreement for 32 (86%). Five of the untreated serum samples had results which were not in agreement; all five were from high dose vaccine products. Two had USPHS titres <2 U/mL and DVC titres ≥2 U/mL, and three were the reverse (USPHS titres ≥ 2 U/mL and DVC titres < 2 U/mL), indicating that there is not a trend for one assay to yield neutralization titres that meet specifications more often than the other. There were no disagreements between the two methods when testing low dose vaccine products. Additionally, the increased resolution of the DVC assay demonstrated that low dose vaccine products were frequently able to elicit a higher neutralizing response than the minimum product requirement of 0.5 U/mL, information which cannot be determined using the USPHS test.

To investigate results which might be obtained for subpotent vaccine products, 18 samples which had been purposely heat treated to reduce potency were tested in both assays (Table 2); results were in agreement for 16 (89%). The two assays are in agreement for products which had been exposed to heat for extended periods of time, however for two samples at the borderline where heat treatment was starting to reduce potency to below product specifications, some variability and disagreement was observed. Specifically, variation was observed in the *in vivo* results for the same material (DTap-IPV treated for 24 h at 60 °C) used to immunize animals in two separate USPHS tests; one USPHS test showed the product was below specifications of 2 U/mL, while the other USPHS test of the same material showed the product met product specifications. The DVC results were in agreement with the USPHS results in both instances. When the next heat treatment timepoint (DTap-IPV, 2d at 60 °C) was used to immunize animals in two separate USPHS tests, results showed once again that the material met 2 U/mL specifications in one USPHS test but not the other; in this case, however, the serum from both USPHS tests had neutralization titres below 2 U/mL in the DVC assay. In the case of Tdap heat treated for 24h, both USPHS and

**Table 3**
**Summary of USPHS test and DVC assay results for (A) low dose and (B) high dose diphtheria vaccine products.** 19 low dose and 36 high dose serum samples (serum pools from guinea pigs immunized with 2 Lf/dose and 15 Lf/dose diphtheria toxoid containing vaccines respectively) were tested once in the USPHS toxin challenge test and once in the DVC assay. Vaccines were either untreated or were heat treated at 60°C to reduce potency. Neutralization titres are summarized from Tables 1 and 2. Italicized are instances in which results from the two assays are not considered to be in agreement.

| A | Number of samples | B | number of samples |
|---|---|---|---|
| USPHS $\geq$ 0.5 U/mL, DVC $\geq$ 0.5 U/mL | 11/19 | USPHS $\geq$ 2 U/mL, DVC $\geq$ 2 U/mL | 22/36 |
| USPHS $<$ 0.5 U/mL, DVC $<$ 0.5 U/mL | 7/19 | USPHS $<$ 2 U/mL, DVC $<$ 2 U/mL | 7/36 |
| *USPHS $\geq$ 0.5 U/mL, DVC $<$ 0.5 U/mL* | 0/19 | *USPHS $\geq$ 2 U/mL, DVC $<$ 2 U/mL* | ***4/36*** |
| *USPHS $<$ 0.5 U/mL, DVC $\geq$ 0.5 U/mL* | ***1/19*** | *USPHS $<$ 2 U/mL, DVC $\geq$ 2 U/mL* | ***2/36*** |

DVC tests showed neutralization titres of 0.5 U/mL, but after 2d of heat treatment the UPSHS result fell below 0.5U/mL while the DVC results remained at 0.5 U/mL. Some variability in results of tests of the same or similar material is not unusual for *in vivo* immunization models and is generally addressed through a robust retesting policy which can account for this variability. Importantly, the level of agreement between the USPHS and the DVC assays was comparable to that seen when testing untreated material.

Overall, agreement between the two assays is consistent and similar whether testing untreated or heat treated vaccine products of low or high diphtheria toxoid dose, and shows no trend for one assay to yield neutralization titres above or below product specifications more often than the other.

Use of the DVC assay in place of the USPHS *in vivo* toxin challenge allows for accurate serum titre determination, while reducing the overall number of animals used compared to the complete USPHS test and eliminating the *in vivo* toxin challenge. Thus the assay is aligned with industry goals to reduce, refine, and replace animal testing for vaccine potency. In addition, the semi-quantitative DVC assay offers higher resolution in determining serum titres when compared to the USPHS test. DVC assay results include a wide range of values: 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 U/mL. The USPHS test is designed to report results as a lower threshold: at or below 0.5 U/mL for low dose vaccines and at or below 2 or 2.5 U/mL for high dose vaccines. Therefore the DVC assay offers increased resolution, which can be crucial during product development and manufacturing process changes.

The results presented here demonstrate similar unitage as well as preliminary concordance between the USPHS *in vivo* toxin challenge test and the *in vitro* DVC assay, and support the use of the DVC assay for routine and stability testing for vaccine products. In preparation for replacement of the USPHS toxin challenge with the DVC assay for vaccine potency testing, an extended study is currently being conducted. Multiple lots of many different diphtheria vaccine products are being tested to confirm comparability and assign appropriate product specifications for diphtheria potency acceptance criteria based on the DVC assay.

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## APPENDICES.

**Table A.1**
Sera tested in validation study
Serum samples generated through routine testing of vaccine products were pooled in different combinations to prepare serum pools of different neutralizing titre for use in validation experiments.

| Guinea pig serum pool Sample ID | Pooled serum sourced from animals immunized with one of the following vaccine products | Diphtheria toxoid concentration in immunizing vaccine product (Lf/dose) |
|---|---|---|
| 2016-VL4 | Tdap | 2 |
| 2016-VL5 | Td, Tdap, Tdap-IPV | 2 |
| 2016-VL6 | Tdap, Tdap-IPV | 2 |
| 2016-VM1 | DTaP | 15 |
| 2016-VM2 | DTaP-IPV | 15 |
| 2016-VM3 | DTaP-IPV-Hib-HepB | 15 |
| 2016-VH3 | DTaP-IPV-Hib-HepB | 15 |
| 2016-VH4 | DTaP-IPV-Hib-HepB | 15 |
| 2016-VMK1 | 'mock' (DTaP-IPV-Hib-HepB formulation without diphtheria toxoid) | 0 |
| 2016-VMK2 | 'mock' (DTaP-IPV-Hib-HepB formulation without diphtheria toxoid) | 0 |
| 2016-VMK3 | 'mock' (DTaP-IPV-Hib-HepB formulation without diphtheria toxoid) | 0 |
| 2016-N2 | Naïve serum (unimmunized guinea pigs) | N/A |

**Table A.2**
Validation study results
A set of guinea pig serum samples with a range of anti-diphtheria neutralizing titres were tested in the DVC assay by multiple analysts over multiple days at two locations. Assays were run with a single test plate or two independently prepared test plates. Neutralizing titre of sample determined in each assay is shown (U/mL). (A = Assay#, P = Plate#)

| Sample (lot#) | Lab 1 | | | Lab 1 | | | Lab 1 | | | Lab 2 | | | Lab 2 | | | Lab 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Analyst 1 | | | Analyst 2 | | | Analyst 3 | | | Analyst 4 | | | Analyst 4 | | | Analyst 5 | | |
| | A1 | A2 | A2 | A3 | A3 | A4 | A5 | A5 | A6 | A7 | A8 | A9 | A9 | A10 | A10 | A11 | A11 | A12 |
| | P1 | P1 | P2 | P1 | P2 | P1 | P1 | P2 | P1 | P1 | P1 | P1 | P2 | P1 | P2 | P1 | P2 | P1 |
| 2016-VL4 | 0.5 | 1.0 | 0.5 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.5 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 2016-VL5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.5 | 0.5 | 0.5 |
| 2016-VL6 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 1.0 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 2016-VM1 | 1.5 | 1.5 | 1.5 | 2.0 | 2.0 | 2.0 | 1.5 | 1.5 | 1.5 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| 2016-VM2 | 2.0 | 2.0 | 1.5 | 2.0 | 2.0 | 2.0 | 1.5 | 1.5 | 1.5 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| 2016-VM3 | 1.5 | 1.5 | 1.5 | 2.0 | 1.5 | 2.0 | 1.5 | 1.5 | 1.5 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| 2016-VH3 | 3.0 | 4.0 | 3.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| 2016-VH4 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 3.0 | 2.0 | 2.0 | 2.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 2.0 | 2.0 |

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# Animal testing for vaccines. Implementing replacement, reduction and refinement: challenges and priorities

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ABSTRACT

Transition to in vitro alternative methods from in vivo in vaccine release testing and characterization, the implementation of the consistency approach, and a drive towards international harmonization of regulatory requirements are most pressing needs in the field of vaccines. It is critical for global vaccine community to work together to secure effective progress towards animal welfare and to ensure that vaccines of ever higher quality can reach the populations in need in the shortest possible timeframe. Advancements in the field, case studies, and experiences from Low and Middle Income Countries (LMIC) were the topics discussed by an international gathering of experts during a recent conference titled "*Animal Testing for Vaccines – Implementing Replacement, Reduction and Refinement: Challenges and Priorities*". This conference was organized by the International Alliance for Biological Standardization (IABS), and held in Bangkok, Thailand on December 3 and 4 2019. Participants comprised stakeholders from many parts of the world, including vaccine developers, manufacturers and regulators from Asia, Europe, North America, Australia and New Zealand. In interactive workshops and vibrant panel discussions, the attendees worked together to identify the remaining barriers to validation, acceptance and implementation of alternative methods, and how harmonization could be promoted, especially for LMICs.

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**Abbreviations**

| | |
|---|---|
| 3Rs | Replace, Reduce, Refine |
| AEFI | Adverse Events Following Immunization |
| AGES | Austrian Medicine and Medical Devices Agency |
| AHI | Animal Health Institute (U.S.A.) |
| BET | Bacterial Entodoxin Test |
| BSP | Biological Standardization Programme |
| CBER | Center for Biologics Evaluation and Research (U.S.A.) |
| CEPI | Coalition for Epidemic Preparedness Innovations |
| cGMP | current Good Manufacturing Practice |
| CHO | Chinese Hamster Ovary cell |
| CPP | Critical Process Parameter |
| CQA | Critical Quality Attribute |
| DAFIA | Direct Alhydrogel Formulation ImmunoAssay |
| DCVMN | Developing Countries Vaccine Manufacturing Network |
| DTaP | Diphtheria Tetanus acellular Pertussis |
| DTP | Diphtheria Tetanus Pertussis |
| DTwP-HepB | Diphtheria Tetanus whole-cell Pertussis Hepatitis B |
| ECBS | Expert Committee on Biological Standardization (WHO) |
| EDQM | European Directorate for the Quality of Medicines & HealthCare |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EMA | European Medicines Agency |
| EPAA | European Partnership for Alternative Approaches to Animal Testing |
| EURL ECVAM | EU Reference Laboratory for Alternatives to Animal Testing |
| EVI | European Vaccine Initiative |
| FDA | Food and Drug Administration (U.S.A.) |
| FeLV | Feline Leukemia Virus |
| GAVI | Global Alliance for Vaccine Immunization |
| GMP | Good Manufacturing Practice |
| GSK | GlaxoSmithKline |
| GST | General Safety Test |
| HBV | Hepatitis B Vaccine |
| HIST | Histamine Sensitization Test |
| h-PBMC | human Peripheral Blood Mononuclear Cells |
| HPV | Human Papillomavirus |
| IABS | International Alliance for Biological Standardization |
| ICATM | International Cooperation on Alternative Test Methods |
| IBR | Infectious Bovine Rhinotracheitis |
| IBV | Infectious Bronchitis Vaccine |
| IMI 2 | Innovative Medicines Initiative 2 |
| IVRP | In Vitro Relative Potency Assay |
| LABST | Laboratory Animal Batch Safety Test |
| LMIC | Low- and Middle-Income Countries |
| MDCK | Madin-Darby Canine Kidney |
| MAT | Monocyte Activation Test |
| MIT | Mouse Inoculation Test |
| MNVT | Monkeys Neurovirulence Test |
| NC3Rs | National Centre for the Replacement, Refinement and Reduction of Animals in Research (UK) |
| NCL | National Control Laboratory |
| NEP | Non-Endotoxin Pyrogen |
| NGO | Non-Governmental Organizations |
| NIFDC | National Institute for Food and Drug Control (China) |
| NIH | National Institute of Health (U.S.A.) |
| NIIMBL | National Institute for Innovation in Manufacturing Biopharmaceuticals (U.S.A.) |
| NMPA | National Medical Products Administration (China) |
| NRA | National Regulatory Authority |
| OIE | Organization for Animal Health |
| OMCL | Official Medicines Control Laboratory Network |
| PCR | Polymerase Chain Reaction |
| Ph. Eur | European Pharmacopoeia |
| QC | Quality Control |
| RIVM | National Institute for Public Health and the Environment (Netherland) |
| RSE | Reference Standard Endotoxins |
| RPT | Rabbit Pyrogen Test |
| SRID | Single Radial Immunodiffusion |
| TABST | Target Animal Batch Safety Test |
| TBEV | Tick-borne Encephalitis Virus |
| TRS | Technical Report Series |
| VAC2VAC | Vaccine batch to vaccine batch comparison by consistency testing |
| VLP | Virus Like Particle |
| WHO | World Health Organization |

## 1. Introduction

The field of vaccines is experiencing significant momentum in the development of alternative methods to animal testing for quality control and release testing, leading to a technical progress in analytical methods and their application that offers the opportunity for Replacement, Refinement and Reduction (3Rs) implementation in specific animal-based tests, and opens the door to the implementation of the consistency approach as vaccine quality strategy [1]. However, scientific progress alone will not be enough to ensure acceptance of 3Rs by all stakeholders globally. Regulations need be updated to embrace alternative methods, and for this to happen, a concerted science driven effort of influencing and inclusion is called for. Influencing, in order to root and establish a perspective that is significantly different from the animal-based one that was ingrained in the sector for decades. Inclusion, so to ensure that the most advanced and newly developed methods do not remain prerogative of only those regions or countries already at the forefront of innovation, and that can be shared and implemented in as many regions as possible. Such an effort, now more necessary than ever, can only be carried out through the open cooperation of all the stakeholders involved. The end result of such a cooperation ought to be a more harmonized vaccine sector, where regulations are as aligned as possible between countries/regions, where obsolete animal tests are expunged from pharmacopoeias and regulations, and where alternative methods are recognized and accepted as quality control instruments, which will enable the reduction time to market of vaccines and increased global access reducing costs [2,3]. The IABS *Animal testing for vaccines - Implementing Replacement, Reduction and Refinement: Challenges and Priorities*, held in Bangkok, Thailand, on 3–4 December 2019, was organized specifically to discuss the current status of the field, and offer an opportunity to multiple stakeholders to share and gather information, to collectively identify the key hurdles and develop a roadmap that would lead to a wider acceptance of alternative methods in lot release for human and animal vaccines. Bangkok, Thailand, was chosen as the venue to further stress the importance of participation to the discussion of LMICs and Global Alliance for Vaccines and Immunization (GAVI) member countries including major stakeholders from Asia. Indeed, participants from many of these countries and from around the globe enriched the lively discussions that happened at the scientific sessions and workshops of this conference.

## 2. Opening remarks

Nakorn Premsri, Director of the National Vaccine Institute of Thailand, Joris Vandeputte, President of IABS, and Hilde Depraetere, European Vaccine Initiative (Germany), opened the conference welcoming all the participants and encouraged them to actively participating in order to learn more about everyone's perspective.

## 3. Animal use and 3Rs

This session was chaired by Coenraad Hendriksen, Intravacc, Netherlands, Koji Ishii, National Institute for Infectious Diseases, Japan, and Jim Webster, OIE Collaborating Center, New Zealand. The session collected interventions on ongoing and successful efforts to implement 3Rs, to remove animal testing from legislation, and on the importance of understating Reduction and Refinement when Replacement is still not possible.

### 3.1. Animals in batch testing: need for 3Rs

In the absence of **Dr Suresh Jadhav**, Executive Director of the Serum Institute of India Pvt. Ltd., his speech was conveyed by **Dr. Sunil Goel**, Additional Director also of the Serum Institute.

Goel's talk focused on a series of actions needed to proceed towards 3Rs, and on the analysis of the key hurdles to be dealt with: first, he suggested a particular attention be paid to pharmacopoeias' monographies, some of which contain reference to unnecessary tests, others imply indirectly that use of animals is allowed even when alternative methods exist, and others are mutually inconsistent – their inconsistencies leave the door open to animal testing. He was also clear in asking for an increased enactment of humane endpoints to improve animal welfare, especially for products like Diphtheria, Tetanus, Pertussis (DTP) and Rabies vaccines due to the severity of the diseases involved [4]. He then discussed key hurdles on the road to the implementation of 3Rs, in particular mentioning the *validation process*, often long and complex, suggesting that Pharmacopoeia Commissions should devise new strategies for validation aimed at ensuring batch-to-batch consistency of the most relevant parameters rather than seeking a correlation with animal methods, and *costs*, referring to costs incurred by manufacturers to submit a variation of a licensed product, which need be multiplied by every country in which the product is licensed. As a remedy to this one-sided burden, he proposed and advocated for a *fee amnesty* to process those license variations that would result in fewer animals being used in quality control for already licensed products.

The greatest hurdle identified is the lack of *international harmonization*, which forces vaccine manufacturers to meet the differing requirements of the different authorities of the countries they export to. While the obvious solution would be the harmonization of test requirements or mutual acceptance of test data, Goel advocated for the Pharmacopoeial Discussion Group to prioritize harmonization of monographs that describe challenge assays that are used as routine batch potency tests. Such result, he stated, would prevent unnecessary animal use and suffering, and would also permit the use of serological and in vitro methods of potency determination in all regulatory regions.

### 3.2. The (long) journey towards the implementation of the 3Rs – every step counts

**Eriko Terao,** scientific coordinator at the EDQM, the European Directorate for the Quality of Medicines & HealthCare (Council of Europe).

Her talk focused on the various European efforts towards the implementation of the 3Rs. Terao began by retracing the long-termed commitment of the Council of Europe to animal protection, which as early as the 1960s developed conventions on animals. In 1971, the Council began discussions on animals used for scientific purposes involving representatives from member states, observer states and non-governmental organizations (NGO), integrating the 3Rs principle, in an effort culminating in 1986 with the *Convention on vertebrate animals used for experimental and other scientific purposes*. A brief introduction was dedicated also to the European Directorate for Quality of Medicine & HealthCare's (EDQM) role in fostering scientific cooperation and exchanges towards a harmonized consensus on 3R approaches to the quality control of medicines (and on its other function of coordinating the Official Medicines Control Laboratory Network (OMCL)), briefly presented the European Pharmacopoeia Commission, and gave information on the Biological Standardisation Programme (BSP), a joint activity of the Council of Europe and the Commission of the European Union that supports standardized and harmonized quality control methods for biological medicines, that also coordinates international collaborative studies to generate scientific data corroborating the selection of the most suitable consensus alternative methods.

She showed the different strategies used by the Experts of the European Pharmacopoeia (Ph. Eur.) to apply the 3Rs principle to the elaboration and implementation of Ph. Eur.texts. For method refinement, she gave examples such as humane endpoints for challenge tests in many vaccine products, improvements of methods, and in vivo testing of lower severity replacing previous more severe tests (serology instead of challenge for tetanus, diphtheria, veterinary rabies etc., and the bacterial endotoxin test in place of the rabbit pyrogen test for vaccines and other products).

For reduction, she cited statistical evaluations that decrease animal cohorts by 20–50%, improvement of methods to avoid invalid tests and re-tests, and an official batch release reduction scheme for established products based on production history & pharmacovigilance.

For replacement, examples included the addition of a validated ELISA as alternative to serology for Hepatitis A vaccines, two Biological Standardisation Program (BSP) projects, one completed on veterinary Clostridium vaccines, and one ongoing on human rabies vaccine. She also touched the European Pharmacopoeia's 5.2.14 chapter, recently (2018) added, aimed at facilitating the substitution of in vivo method(s) by in vitro method(s) for vaccine quality control [5]. Terao also expounded on the idea of a fourth R, standing for Removal, that is the elimination from the European Pharmacopoeia of animal tests. Examples of significant successes are represented by the deletion the Abnormal Toxicity Test (2017) and the Target Animal Batch Safety Test (2013), the replacement of the residual pertussis toxin by an in vitro Chinese Hamster Ovary cell (CHO) assay and removal from the individual monographs of the test for irreversibility of pertussis toxoid and the requirement to test the final lot for residual toxin (2018, a result stemming from the BSP114 collaborative study). Promising ongoing activities were also reported by Terao, like the recently ended BSP130 collaborative study for the replacement of in vivo tests for the *Clostridium septicum* vaccine (in consequence of which the Ph. Eur. Group of Experts is currently revising Ph. Eur. texts), and the ongoing BSP136 collaborative study on Clostridium tetani human and veterinary vaccines, for the evaluation of an in vitro replacement for the residual toxicity test, which led in 2019 to the removal from the Ph. Eur. of the test for irreversibility of toxoid, for lacking scientific bases and due to the absence of batch release data on reversibility [6]. Such endeavor necessarily needs be based on an evaluation of the scientific rationale of the specific test to be removed and on a comprehensive risk assessment, based on data (historical data, including batch release), but it must also consider the context (alternative approaches, redundancy of tests in European Pharmacopoeia tests, regulations, Good Manufacturing Practices (GMPs), pharmacovigilance), and needs be carried out through a concerted effort of engagement of the key stakeholders to secure information (OMCLs, manufacturers, regulators, both European and global, and also taking advantage of discussion groups and workshops).

### 3.3. Towards deletion of general batch safety tests: recent progress and next steps

**Marlies Halder** of the European Commission, Joint Research Centre, Italy.

The presentation summarized recent progress and next steps towards the deletion of general safety tests. Halder gave a brief historical background on the abnormal toxicity test (ATT) used for human vaccines. More than 100 years ago, a test in mice was used to detect phenol in diphtheria antisera and a test in guinea pigs to detect contamination with tetanus toxin. Later, these two very specific safety tests were combined into a general safety test for detection of non-specific contaminants in vaccines and other pharmaceuticals for human use. The ATT is also known as General Safety Test (GST) or Innocuity Test. General safety tests for veterinary vaccines are the Target Animal Batch Safety Test (TABST) and Laboratory Animal Batch Safety Test (LABST). The scientific relevance of general safety tests has been questioned since more than 30 years and Halder highlighted the following points: lack of specificity, reproducibility, reliability, and suitability for the intended purpose. She further provided an overview on the progress achieved in various countries and regions; for example, the ATT was removed from European Pharmacopoeia monographs for batch release testing of human vaccines already in 1997 [7], but only recently also for the production step. The US-FDA revoked the GST in 2015, whereas the WHO ECBS announced in 2018 the discontinuation of the Innocuity Test in all future WHO recommendations, guidelines and manuals for biological products published in the Technical Report Series. Moreover, WHO ECBS emphasized that discontinuation should also be applied to previously published WHO Technical Report Series documents [8,9]. She mentioned that many other countries, for example, India, Brazil, Argentina and Africa, deleted the ATT over recent years or allow waivers after demonstration of consistency of production.

Halder reported on the progress achieved for veterinary vaccines [2,3]. Thus, European Pharmacopoeia deleted the LABST already in 1997 and the TABST in 2013. Furthermore, she mentioned the possibility of waiving the TABST if consistency of production was demonstrated as outlined in VICH GL50 and GL55 [10]. This is applicable for VICH regions (Europe, Japan, USA); however, the OIE refers to these VICH guidelines in its Terrestrial Manual. A comparable guideline on waiving possibilities for the LABST (GL59) is close to publication [11].

Next, Halder outlined possible steps and proposals to accelerate the deletion of general safety tests, involving international organizations, National Control Authorities and manufacturers, and underlined that collaboration is key. Referring to the recommendation of WHO ECBS 2018, she suggested that WHO should remove the Innocuity Test from all relevant documents, since it is still mentioned in most of WHO's recommendations. Halder further invited OIE to actively promote the deletion of TABST/LABST at national level, or at least underline the possibilities to grant waivers in the light of VICH GL50, GL55 and the upcoming GL59. National Control Authorities should remove the general safety tests from their requirements for human vaccines as recommended by WHO ECBS 2018. With regard to veterinary vaccines, they should promote the deletion of TABST/LABST or at least allow waivers as outlined in VICH GL50, GL55 and the upcoming GL59. Retrospective analyses of general batch safety data may help to facilitate deletion, as it had been the case in Europe [12,13]. Manufacturers should continue to ask for the deletion of general batch safety tests where they are still required, providing evidence on other safety measures, and referring to the WHO ECBS 2018 statement and VICH guidelines (GL50, GL55, GL59). Halder closed her talk by highlighting the importance of dedicated collaboration between stakeholders being a key element for success. She advocated for increasing the dialogue between authorities and manufacturers, for mutual learning and sharing of information between manufacturers, and called for collaboration of all stakeholders at a global level.

### 3.4. Monocyte activation test (MAT)

**Eliana M Coccia** from the *Department of Infectious diseases, National Health Institute of Italy (ISS)*.

She described her teams' effort – within the Vaccine batch to vaccine batch comparison by consistency testing (VAC2VAC project) – to investigate the possibility to apply the Monocyte Activation Test (MAT) to detect pyrogen contamination for a human vaccine against tick-borne encephalitis virus (TBEV). Coccia clarified how the currently prescribed test for TBEV, both in European Pharmacopoeia [14] and in the WHO Technical report series [15], is the Rabbit Pyrogen Test (RPT), but that in reality the MAT is a more **reliable** test. Reasons adduced by Coccia are the following: (1) MAT eliminates the need for animal testing and is considered a suitable (after product-specific validation) substitute for RPT by European Pharmacopoeia; (2) it is more appropriate for testing pyrogens for intramuscularly/subcutaneously administered vaccines (RPT is performed intravenously); (3) it allows testing a vaccine for human use in a human setting, and with its longer incubation time (22 ± 2 h, vs 3 h for RPT) allows the detection of delayed inflammatory response.

Currently, MAT is employed by both OMCLs and manufacturers for the batch release of the *Neisseria meningitidis* group B vaccine *(Bexsero®)* [16] while, by OMCLs only for the *Salmonella Typhi* vaccine (*Typhim Vi®*). RPT is state of the art for multivalent DTwP-HepB vaccine, vaccines against human rabies, pneumococcal and meningococcal polysaccharide vaccine, and TBEV.

Coccia proceeded then to expound on the methodological approach pursued. As the vaccine targeted contains TBEV inactivated by formaldehyde as active substance, and aluminum hydroxide, TRIS buffer, sucrose, traces of tetracycline, gentamicin, neomycin, and formaldehyde as excipients, but shows no intrinsic pyrogenicity, the choice fell on human peripheral blood mononuclear cells (h-PBMC) – for their ability to recognize a wide repertoire of pyrogens and release pro-inflammatory cytokines – and on the Interleukin-6 (IL-6) – chosen as a read-out for its robust production after PBMC stimulation with reference standard endotoxins (RSE) as well as with non-endotoxin stimuli, namely R-848 and FSL-1. The methods employed were MAT Method A (quantitative) and B (semi-quantitative), as described in European Pharmacopoeia chapter 2.6.30. The relative results highlighted the necessity to adapt the validity criteria of both methods to fulfill at best the Ph. Eur. requirements for a vaccine without intrinsic pyrogenicity.

Coccia observed that the experiences and results from her group's work demonstrate the suitability of MAT for product specific replacement for the RPT with the possibility of adjusting it to face a heterogenicity of vaccine formulations, both viral and bacterial, thanks to the possibility to select between primary cells or monocytic cells, and three different methods of analysis, and commenting that MAT could be a useful tool to rule out presence of endotoxins and non-endotoxin pyrogens (NEPs) in vaccines, both during the manufacturing process and in batch release (although changes in the application of Method A and B of the European Pharmacopoeia are probably to be expected for vaccines with no-intrinsic pyrogenicity) [17]. Coccia's final remarks were dedicated to the regulatory status of MAT, noting that while the test did receive good acceptance in Europe, the position of the Food and Drug Administration (FDA) and US Pharmacopoeia (USP) is not as well defined, but that luckily pharmacopoeia harmonization seems to be on a good track, with China having announced MAT implementation in its pharmacopoeia for 2020, and Health Canada and the Japanese National Institute of Health are on the way.

### 3.5. Rabies potency testing: glycoprotein assay

**Koraphong Pinyosukhee** of the Institute of Biological Products, Department of Medical Sciences of Thailand's Ministry of Public Health (National Control Laboratory for Biological Products). He presented Thailand's Institute of Biological Products efforts for the validation of a

method for potency testing and consistency of production of rabies vaccine based on the assay of Glycoprotein by ELISA [18] as an alternative to the National Institute of Health (NIH) test.

Pinyosukhee stressed the importance of the rabies vaccine for Thailand, and globally as a means for the World Health Organization's (WHO) *Zero by 30* project for the elimination of rabies by 2030 [19]. In Thailand, the potency of the rabies vaccine is tested by the National Control Laboratory with the NIH method, requiring 168 mice per sample, 30 days, with a cost of 3240 US Dollars, and being very poor from an animal welfare point of view. To overcome all these difficulties, the Institute of Biological Products worked on an alternative method for potency testing based on an ELISA assay to measure glycoprotein – the major protein playing a role in the host's immune response – content in the vaccine. The method was validated in 2015, and is extremely promising, significantly shortening the time needed for testing (30 days for the NIH, just 3 days with this method), being cheaper at 400 US Dollars, and could potentially replace the use of NIH potency test method for both human and veterinary rabies vaccines in the future. Pinyosukhee clarified that at the moment, rabies vaccines in Thailand are still being released with the NIH test by the Institute of Biological Products, which tests 1 every 5 lots. The institute is currently collecting data of glycoprotein content from every batch of every manufacturer, to amass enough information on the various vaccines to make it feasible to stop using the NIH and move to the glycoprotein assay for batch release (with NIH test remaining a fallback in case problems with out-of-range glycoprotein contents were to be identified).

### 3.6. Potency testing and 3Rs: general overview

**Sylvie Uhlrich,** Sanofi Pasteur, France, presented a general overview of vaccine Potency Testing and 3Rs. After a brief historical introduction on potency testing, showing the progression from the first challenge tests, to toxin neutralization tests and then to immunogenicity assays, finally to reach the stage where in vitro assays are established [4], Uhlrich proceeded to highlight the reasons that make transitioning to alternative methods important. In vivo models act as a "black box", with at times questionable relevance to humans, while at the same time suffering often from poor robustness and high variability inherent to the use of live individuals. Moreover, due to historical reasons, specifications and potency tests for human vaccine batch approval often differ for various parts of the world, resulting in either duplication of animal testing or partial implementation of 3Rs for some vaccines when distributed worldwide. A problematic issue, considering that 90–95% of the animals used by manufacturers is employed in batch control testing, with ulterior quantities employed in independent batch release testing by National Control Laboratories. Considerations other than those of scientific character also play an important role. Uhlrich mentioned (1) animal welfare considerations, with large quantities of animals subjected to severe pain, and a societal response increasingly concerned by the use of animals; (2) legal considerations, because in Europe, Directive 2010/63 clearly states the duty to use, wherever possible methods or testing strategies not entailing the use of live animals; and (3) economical, because in vivo tests are expensive, require long times, and their inherent variability can lead to the unjustified rejection of what are actually safe and efficacious vaccines and to delays in market release which may turn into shortages of vaccine. To showcase how alternative methods can influence the sector for the better, Uhlrich discussed the case of an ELISA as a potency assay for Hepatitis B vaccine. Comparing it to the Immunogenicity assay (described in WHO TRS 978), which requires 10 to 20 mice, at least 3 dilutions of vaccine, bleeding of the mice after 4–6 weeks, and then an ELISA assay for HBsAg antibodies, while the in vitro potency assay (IVRP) is based on a sandwich ELISA using 2 monoclonal antibodies H35 and H57 targeting the "a" determinant of HBsAg, and the IVRP of each formulation is then determined against a homologous reference. The in vitro assay proved to be much more consistent, and more discriminant to detect subpotent batches, than the immunogenicity assay [20]. The presentation moved then to discuss the current limitations to 3Rs implementation. They are of regulatory and scientific kinds. Regulatory ones are (1) lack of harmonization of regulatory requirements; (2) caution on the side of health authorities to accept deviations from established guidelines and monographs; (3) general risk-adverse attitude to forego in vivo assays used for decades and considered a "gold standard"; and (4) the complexity of regulatory changes that do not generate strong incentive to develop and implement alternatives to animal testing. Scientific ones are (1) historically, in vivo assays were not validated according to current ICH Q2(R1) principles [21], (2) change in assessment of product attributes very likely when switching from an in vivo to an in vitro method, (3) one-to-one comparison is usually very challenging and not necessarily justified (an example of this is the fact that challenge tests have limited discriminative power to detect sub-potent lots, so, for them, no concordance would be possible between in vivo and in vitro assays). Uhlrich shared her opinion that the time is ripe to consider a change in perspective, retiring the idea of a one-to-one replacement. In its place, an approach that permits an existing in vivo method be substituted by more than one in vitro method to control key qualitative and quantitative attributes, putting the focus on understanding the critical quality attributes of the product, and leveraging an integrated perspective on product quality that leads to the *consistency approach* as a methodology to ensure safety and efficacy of vaccines without need to use animals [1]. An approach of this kind is already implemented in the case of polysaccharide conjugate vaccines such as Haemophilus Influenza type b vaccine (Hib) for which appropriate control of conjugate composition, integrity, content and size at different manufacturing stages led to removal of in vivo test on final product. Uhlrich's conclusion expressed the need for a worldwide regulatory harmonization, and for the involvement all stakeholders (regulators, scientists, animal welfare organizations, the public and decision-makers) in the communication of best practices.

### 3.7. When animals are still needed for reduction and refinement

**Coenraad Hendriksen,** Chair of the Scientific Committee for this conference, from the Institute for Translational Vaccinology (Intravacc), the Netherlands, focused the attention on two components of 3Rs – reduction and refinement – that are less glamorous than replacement, but that are of pivotal importance, in those cases where animals remain essential, for improved animal welfare and for better science. Hendriksen noted how a variety of barriers (science, regulations, tradition, etc.) can slow down the implementation of non-animal methods, noting how efforts should be encouraged toward refinement and reduction, which represent low hanging fruits (less innovative, easier to develop, and relatively easy to implement in the regulatory setting) in those cases where scientific tools for replacement are not (yet) available, also in the light that about 15% of all animal use in Europe often undergo severe suffering [22]. Discussing reduction, Hendriksen noted that much can be achieved through improvements in standardization (an example was made of the high inter- and intra-laboratory variation leading to poor reproducibility and invalidity for the Kendrick Test [23], and of the influence of species and mouse strain on potency testing of the Tetanus toxoid), through use of Standard Operating Procedures, improved staff training, and richer information background on the animals (lowering the numbers of animals of sample sizes by correctly factoring in the animal related variability, physical and environmental noise, and the impact of husbandry and animal care). About refinement, Hendriksen stressed the importance of limiting pain and distress, highlighting the need for pain management – through anesthesia when needed, monitoring of animals, improved animal living conditions, and application of humane endpoints to be weighed against one or several markers (clinical, pathophysiological, behavioral, hormonal, haematological and micro-biological). An example of humane end-points application was supplied, regarding whole-cell Pertussis vaccine potency testing: clinical signs (through observation), body weight (daily), and body temperature

(monitored by temperature sensitive probe). In the conclusion, Hendriksen stressed again the fact that animal replacement has to be the ultimate goal, but that in the meantime, efforts must be made to implement reduction and refinement, as they lead both to better testing, better science, and better animal welfare.

### 3.8. Implementation of 3Rs in quality control testing of vaccines

**Sunil Goel**, Additional Director of the Serum Institute of India (SIIPL) Pvt. Ltd, described its organization's commitment to the development, introduction, validation, and implementation of 3Rs and consistency-based approaches, and how such activities are helped by the fact that Indian Pharmacopoeia always proved supportive and receptive to such endeavors.

The first progress described was related to the DTP group of vaccines, for which the Institute secured successes in both replacement and reduction. Replaced was the conventional lethal challenge on guinea pigs/mice for potency testing by two assays, a Vero cell assay for the potency testing of the diphtheria component, and a T-ELISA for the potency testing of the tetanus component. Both assays required about 3 years from development to final acceptance by National Control Laboratory (2006 and 2007 respectively), with a first approval requiring 1 in 10 batches potency testing, and since 2017 an approval requiring 1 in 25 batches to be tested (or once in six months, whichever was to be the earliest). The replacement reduced the number of guinea pigs used per batch from 232 to 30 (~85%). On the reduction front, the SIIPL secured authorization, by sharing data on a large number of batches, to switch from a multi-dilution assay to a single-dilution assay, receiving approval first from the National Control Laboratory (NCL)/National Regulatory Authority (NRA) and the WHO.

Refinement was achieved by securing approval in 2017 to switch from the lethal challenge test in guinea pigs for tetanus potency to a paralytic challenge test in mice. Goel proceeded to describe further results obtained in-house. For the Hepatitis B vaccine, leveraging WHO TRS 787 and 889 (which suggest the release of the final lot with a validated in vitro assay), data on in vitro and in vivo assays was submitted to the NCL for review and replacement of the in vivo assay and the in vitro assay was accepted by the NCL in 2006 (requiring in vivo testing for 1 in 5 lots). In 2017, the NCL approved a complete waiver, resulting in zero animals used for lot release.

Goel also described how SIIPL secured approval to discontinue the test for specific toxicity of the tetanus toxoid for two polysaccharide conjugate vaccines (Hib and Meningococcal Conjugate A), a result achieved in both cases by establishing consistency of a number of lots and sharing results with the National Regulatory Authority (NRA), and then receiving from that Authority (and later from WHO) authorization to discontinue the test. The elimination of the test spares 5 guinea pigs per bulk conjugate lot, and thus eliminates the need for the long (21 days) test. Discussing the Abnormal Toxicity Test, Goel first reminded of how the test causes substantial unjustified use of animals without any benefit with regards to demonstrating product safety, and how it clashes with animal welfare and the 3Rs principle, lacking as it is a sound scientific rationale and justification, and then shared that the SIIPL, following Indian Pharmacopoeia, implemented its deletion for most of its vaccines through the route of Post Approval Changes/Variations [24], although regulatory harmonization remains very much needed, as ATT is still required for product registration in different countries. Progress in 3Rs was met also with the replacement, for Hib and Meningococcal A vaccines, of the Rabbit Pyrogen Test (RPT) with the Bacterial Endotoxin Test (BET), with ongoing efforts to implement the same for Rabies and Hepatitis B vaccines.

Goel gave then a description of the significant results obtained in implementing 3Rs for the Rabies vaccine, for which alternative methods were used for characterization of the vaccine along with in vivo methods, suitable correlations were developed, and then monitored for a number of batches laying emphasis on data monitoring of critical parameters and trend analysis, which together allowed the implementation of non-animal methods. Specifically, in process in vivo tests were replaced by the Fluorescent Antibody Test, final bulk NIH potency test was replaced by the Single Radial Immunodiffusion (SRID) Test and in vivo Mouse Inoculation Test (MIT) challenge replaced with a Fluorescent Antibody Test, which led to a reduction in test duration (from 14 to 4 days), and of the animals used, from 32448 for 96 lots produced in a year to 0.

Also mentioned was the application of 3Rs in stability studies of various vaccines, replacing in vivo testing with in vitro methods for maximum time points, and performing the in vivo testing only at the terminal stability time point. In the case of Rabies vaccine, the SRID Test was implemented for the 3, 6, 9, 12, 18- and 24-months' time points, leaving the NIH potency only at the terminal (36 months) time point or either at annual time points, with a significant replacement and reduction of animal usage.

In closing the intervention, Goel touched on the importance of the consistency approach for routine lot release of vaccines, specifically noting how its approach, based on the identification of critical indicators of safety and efficacy and of parameters that indicate product consistency, can lead to the application of newer concepts, such as quality by design, and highlighting how the Rabies vaccine discussed before can represent an interesting case study of the combined outcome of 3Rs and consistency approach.

## 4. Product development and in vitro production/analysis

This session was chaired by Yeowon Sohn, Seoul National University, South Korea, and Robin Levis, FDA/CBER, U.S.A. The session was dedicated to ongoing and successful examples of non-animal approaches to product development and production.

### 4.1. Production of Japanese encephalitis vaccine using the vero cell-line

**Tuan Dat,** VABIOTECH, Vietnam, described its organization' success in shifting the production of Japanese Encephalitis vaccine from culture in mice to Vero cells. The previous vaccine, *Jevax*, was produced through virus inoculation in mouse and successive harvest from the mouse's brain, with about 1 million creatures needed to produce 6 million doses of vaccine per year, and a 3 months production process, for a vaccine that can cause allergy and acute disseminated encephalomyelitis in vaccinated subjects, and showing poor immunogenicity requires multiple doses and boosters. Thanks to a switch to Vero cell culture, Dat explained, the new vaccine, *Jecevax*, can be produced in only 2 months, replacing mice in production, while at the same time overcoming the disadvantages in terms of adverse reactions of the vaccine produced with mouse-brain tissue.

### 4.2. Development of cell-based pandemic influenza vaccine for national security

**Parichat Duangkhae,** from Thailand's Government Pharmaceutical Organization (GPO) – a State enterprise operating under the Ministry of Public Health and active in pharmaceutical products, including vaccines – described the development in Thailand's Government Pharmaceutical Organization of a cell-based influenza vaccine for national security and emerging preparedness, as Asia is at risk of becoming the epicenter of a future influenza pandemic. GPO began producing and egg-based Live Attenuated Influenza Vaccine including H1N1 strain in 2015, but recognizing the various limits of the egg-based technology (not last, limited egg supply at the time of pandemics), the decision was taken to create the next vaccine, targeting the H7N9 strain, with a cell-based process based on the Madin-Darby Canine Kidney (MDCK) cell line, for which 3Rs alternative quality control tests are being investigated (a plaque assay and quick real-time polymerase chain reaction (PCR) in lieu of eggs for infectivity, and high performance liquid chromatography in lieu of the single radial immunodiffusion (SRID) for the influenza

hemagglutinin quantity). The cell-based vaccine will offer increased possibilities to scale up production in case of national security needs.

### 4.3. HPV vaccine in vitro/in vitro release test history and current situation

**Rober Sitrin** (PATH) presented a case study on the implementation of an in vitro potency assay for a human papilloma virus (HPV) vaccine, Gardasil® (a Merck recombinant quadrivalent – HPV 6, 11, 16 and 18 – vaccine. Gardasil®, Sitrin explained, was specifically developed with in vitro potency [25] as the sole release testing in mind, a result achieved in 2006 when the product was licensed both in the US and in the EU with in vitro potency test only. The choice to pursue a non-animal potency test for release was motivated both by animal welfare considerations – 80 mice sacrificed per sample tested – and by practical ones – relying on mice adds cost, variability, lengthens the release cycle as much as 6 months and shortens the vaccine's shelf life. The HPV vaccine is based on the immunogenic properties of icosahedral virus like particles (VLP) produced by recombinantly expressing the major HP protein, L1, for each type of virus, in *saccharomyces cerevisae* yeast. The strategy was to use in vivo in early development, gather data and then transition to in vitro before approval. To measure potency, an in vitro relative potency (IVRP) assay was developed, a sandwich enzyme immunoassay measuring the amount of antibodies bound to neutralizing epitopes for each HPV type. The IVRP was used both in monovalent bulks and on final container samples. The assay proved able to provide a direct comparison between the antigen content of each VLP type in a given test sample and the content of a batch that was shown to be efficacious in humans, and its results also did show correlation with immunogenicity measured through a traditional mouse assay, so its result is considered predictive of immunogenicity in humans. Discussing the feasibility of in vitro only release testing, Sitrin stressed the importance of a good vaccine characterization and known monoclonal reagents as pre-requisites to develop in vitro potency assays that correlate with in vivo data, and the fact that human data can be leveraged to supplement existing data to gain additional concordance. Concluding the talk, Sitrin mentioned that in vitro potency was listed as an acceptable assay for HPV vaccines in the corresponding WHO TRS report (TRS 962), but also remarking that some countries, like China, still insist on an in vivo assay format, which is a negative byproduct of the uneven international regulatory framework.

**Li Shi,** of the Shanghai Zerun Biotechnology Co., Ltd, offered information on vaccine release using non-animal testing in China, focusing on HPV vaccines and the manufacturer's ongoing efforts. Shi reported a significant comment from an unnamed officer from China's National Institute for Food and Drug Control (NIFDC), dating 2019, stating that "in vitro testing is encouraged to replace in vivo testing", with the proviso that substitution can be granted in case of successful systematic verification of in vitro methods, and the fact that the Chinese Pharmacopoeia, in its 2015 edition, advocates the use of in vitro methods instead of animal experiments to identify the quality of biological products to reduce use of animals for experiments, while it also contains in vitro relative potency methods for recombinant Hepatitis B vaccine and for inactivated Hepatitis A vaccine. About those two vaccines, Shi explained, first a correlation between in vivo relative potency in mice and in vitro relative potency was fully established, national vaccines standards set, and highly valid testing kits were approved for both. Discussing the status of HPV non-animal vaccine release in the Country, Shi explained that there is no clear timeline for the transition to in vitro release, because, even though all the HPV vaccine releases for clinical studies are currently using both in vitro and animal testing, data on the correlation between the two methods is being collected (albeit not systematically yet), and while the country's NIFDC is working on establishing national vaccine standards in terms of antibodies and antigens, there is still no recognized or approved valid testing kit for HPV batch release test. Shi moved then to Zerun Bio's work on in vitro release for HPV vaccine, discussing both the work on the in vitro relative potency test and the correlation studies with the in vivo test (for strains 16 and 18), and then introducing the internal work on a Direct Alhydrogel Formulation ImmunoAssay (DAFIA) [26] developed internally (to determine antigen content, identity and integrity directly on the aluminum adjuvant). The DAFIA method was shown internally to produce maintain high specificity for the HPV16 and 18 strains, good repeatability, and correlation with both the mouse potency test and the ELISA.

## 5. Improved product characterization using non-animal methods

This session was chaired by Gautam Sanyal, Vaccine Analytics, LLC, USA, and Denis Lambrigts, GSK Vaccines, Belgium. The session was dedicated to ongoing efforts to characterize legacy vaccines to enable transition to alternative methods and to the successful implementation of the consistency approach.

### 5.1. Product characterization by non-animal methods: general overview and the VAC2VAC project

**Hilde Depraetere**, Director of Operations of the European Vaccine Initiative, spoke next, introducing the VAC2VAC (vaccine batch to vaccine batch comparison by consistency testing) project [27]. VAC2VAC is a wide-ranging collaborative research project funded by Innovative Medicines Initiative 2 (IMI 2) programme, formed by 22 partners in a public-private consortium involving experts from veterinary and human vaccine industry in a partnership with OMCLs, regulatory authorities, academia, translational research organizations, and vaccinology alliances. Its overall objective is to demonstrate proof of concept of the consistency approach for batch release testing of established vaccines by developing sets of in vitro and analytical methods and approaches. In describing the consistency approach, Depraetere underlined how its key tenet is not ensuring product quality through release testing only, but rather by ensuring that each batch produced is consistent with a (historical/clinical) batch already proven to be safe and efficacious. Such approach leads to a radical paradigm shift in vaccine quality control: from the current premise of the uniqueness of each produced batch and on the fundamental relevance of quality control testing on the final product, the consistency approach posits that each batch must be considered as one of a series, shifting quality control from testing on the final product to strict control of every step of the production process, and within this perspective, a vaccine is of demonstrable quality and efficacy if non deviation from consistency can be demonstrated [1]. Such approach, Depraetere added, increases the in-depth knowledge of the product, makes it possible to simplify the standardization of methods, which can lead to a global streamlining of batch release methods, and it can bring about beneficial consequences for animal welfare (with significative reductions of animals employed), plus overall savings of both time, and costs. These goals require the creation of new – or the optimization of – non-animal methods for consistency testing, which must be developed, pre-validated, and accepted by the regulatory authorities. VAC2VAC is currently focusing on a series of veterinary (Rabies, Canine Leptospira, Infectious Bovine Rhinotracheitis (IBV), Infectious Bovine Rhinotracheitis (IBR), Feline Leukemia Virus (FeLV), *C. Perfrigens*, *C. chauvoei*, Tetanus) and human (TBEV, and Tetanus, Diphtheria, acellular Pertussis in DTaP combinations) vaccines, for which work is ongoing on physicochemical, immunochemical and cell-based (notably, monocyte activation test for TBEV was pre-validated as replacement for the rabbit pyrogen test) methods. These activities, Depraetere added, do not happen in a vacuum: the consortium started, through a workshop in 2017 [28], an open discussion with all stakeholders – vaccine manufacturers of major human and animal health companies, competent authorities, OMCLs, EDQM etc. –, holds regular meetings with European regulatory agencies (with a strong interaction with EDQM), and crosses the European borders by executing outreach activities toward the international regulators and organizations, so that it results can be leveraged internationally.

### 5.2. Three samples of product characterization

#### 5.2.1. Tick borne encephalitis vaccine (TBEV)

**Dieter Pullirsch** from the Austrian Medicine and Medical Devices Agency (AGES), the Austrian Official Medicines Control Laboratory (OMCL). He discussed AGES activities within VAC2VAC for the prequalification of an ELISA test for potency testing of the Tick Borne Encephalitis vaccine (TBE). AGES had initiated the development of non-animal test methods already in 2011, and then proceeded within VAC2VAC as TBE vaccines were one of the topics selected for investigation in the project. Within VAC2VAC AGES initiated a cooperation with a manufacturer of TBE vaccine (only two registered vaccines in Europe for TBE, both based on inactivated whole virus, aluminum adjuvanted, produced by two manufacturers); since 2019, both manufacturers became project partners of VAC2VAC. In Europe, each vaccine batch must be tested for potency by the manufacturer and additionally by an OMCL – AGES is the only one performing it – and it currently consists of a lethal challenge assay on mice (Eu. Ph. 1375). Pullirsch explained that within VAC2VAC, immunochemical test methods were developed based on ELISA using structure specific monoclonal antibodies. TBEV antibodies were characterized with different methods (western blotting, pH treatment, freeze-thaw cycles, detergent treatment, thermal alterations) using ELISA plates coated with the inactivated virus non-adsorbed antigen, showing that one antibody is the most sensitive one in forced degradation experiments. Different ELISA formats and DAFIA were also tested, to demonstrate that all the antibodies are capable of recognizing the antigen in the presence of the aluminum hydroxide adjuvant. Currently recovery, specificity/selectivity, precision, robustness, structure/stability indications are qualified, while work is ongoing on the validation pertaining accuracy, response function/calibration cure, and intra-laboratory precision and transferability. Pullirsch also commented on the other ELISA method, developed autonomously (although with periodic scientific interactions with AGES) by the second manufacturer of TBEV vaccine. The method was transferred to the AGES laboratory and good interlaboratory precision was shown between the manufacturer and AGES. A comparison with the animal challenge test was initiated. Preliminary data show comparable mean results between the ELISA and the challenge assays performed by AGES and the manufacturer. In closing the intervention, Pullirsch expressed the conviction that these methods have the potential to accurately quantify the viral target antigens in TBE vaccines and to detect structural changes, but that to introduce these potency assays in the European Pharmacopoeia further work is needed, centered on implementing a small scale transferability study for both methods, on further investigations for stability testing, and on defining the specifications to replace the test for the Official Control Authority Batch release testing.

#### 5.2.2. DTaP vaccine

**Paul Stickings,** from the United Kingdom's National Institute for Biological Standard and Control (NIBSC), presented work on the development of monoclonal antibody immunoassays to measure the relative amount and quality of antigens in Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccines. This approach is based on the use of well characterized and relevant monoclonal antibodies to ensure the quality and consistency of vaccine batches. Such tests have the potential to play a key role in a control strategy no longer including an in vivo potency test [2].

The first part of this project focused on the thorough characterization of monoclonal antibodies (44 in total) directed against one of the antigens present in DTaP vaccines [29]. Antibodies were evaluated in terms of their ability to bind the native, detoxified and adsorbed antigen, and antigen that was altered following exposure to elevated temperature. For some antigens (D and T), neutralization tests were available and used to identify antibodies that target a relevant functional epitope on the antigen. Finally, affinity measurement and epitope competition studies were performed to identify pairs of high affinity antibodies that could be used in a sandwich ELISA format. Stickings presented the results obtained so far focusing on tetanus as an example. The developed monoclonal antibody capture ELISA for tetanus proved able to detect antigen in a wide range of tetanus vaccines for human and veterinary use, including antigen detection in the final lot in the presence of non-aluminum and aluminum-based adjuvants. It is specific, quantitative, and able to identify changes in antigen content for a vaccine that was deliberately formulated to contain a graded series of tetanus toxoid doses. The assay, is able to detect antigenic changes following exposure of non-adjuvanted toxoids to elevated temperature, although studies are still ongoing to determine whether similar changes will be detected in final lot vaccines containing adjuvant and whether the assays will be able to provide indications on stability. Further work will focus on desorption investigations to cover all aluminum containing vaccines available in the consortium to understand what proportion of the total antigen content is being detected by the ELISA when applied to final lot samples. The final part of the project will focus on validation studies according to ICH/VICH guidelines [21] and transferability studies, while efforts from other partners will focus on the development of a multiplex approach to measure of all the DTaP antigens in the same assay. In closing, Stickings shared some final considerations on what could affect the regulatory acceptance of an antigen immunoassay *in lieu* of in vivo potency testing. An antigen ELISA is a quantitative assay, but only in relative terms, which makes it unlikely that results could be expressed in units traceable to an International Standards, a fact that would give rise to the need to set product-specific specifications. Also, results obtained so far in the VAC2VAC project suggest that products would need be controlled with a specific reference vaccine (a common reference may work for some vaccines, but probably not for all). Lastly, for highly adsorbed aluminum containing vaccines a monoclonal antibody ELISA would detect only a proportion of the antigen present in the vaccine, and it will need to be demonstrated that the proportion of antigen being detected is representative for the quality of the vaccine as a whole.

#### 5.2.3. Towards the end of the NIH test for rabies vaccines

**Jean-Michel Chapsal,** from the European Partnership for Animal Alternative Approaches to Animal Testing (EPAA), made an overview of the current status of the validation of an ELISA for potency assay of human rabies vaccines within the Biological Standardisation Programme 148 (BSP148). Summarizing the current potency testing method, the NIH method, he noted its several criticalities (including a very high variability, and the safety factor of requiring use of a live virus [30]), and the extreme severity of the procedure's effects on the animals, to highlight the consensus on the need to find a suitable in vitro alternative, and then proceeded to trace back the steps that led to the current BSP148 project for the validation of a G-protein ELISA for potency testing [31]. He mentioned the 2012 EPAA workshop at Arcachon (France), where the decision to create an International Working Group for the creation of an G-protein ELISA replacement to NIH test was taken, to be based on the evaluation of three competing ELISA assays, a study which eventually produced a candidate ELISA (developed at Sanofi Pasteur) based on 2 monoclonal antibodies (accessible to all laboratories, and commercially available worldwide from two suppliers) specific to the conformational trimeric form of the glycoprotein G, which does not react with non-immunogenic soluble glycoprotein, that recognizes most rabies strains used worldwide for human vaccines, and is able to discriminate sub-potent vaccines altered by a variety of methods including over-inactivation by b-propiolactone, the viral inactivation agent. Based on these results [32], the international collaborative study BSP148 was launched by the Biological Standardisation Programme of the Council of Europe and the EU Commission to further validate the transferability and robustness of the selected ELISA, supported also by numerous stakeholders worldwide, including the World Health Organization, with a view to revise the relevant

European Pharmacopoeia to include a standardized ELISA, and propose ultimately a global replacement of the in vivo NIH test. The program is divided in three phases: Phase 1 of the study was focused on logistical support for the procurement and testing of additional vaccines, the study protocol preparation for participants and on reaching the commercial distribution of both the capture and the detection antibody, with production of batches for exclusive use of BSP148. Phase 2, which is foreseen in 2020, will see participants (about 30 laboratories worldwide, 9 manufacturers and 21 official control laboratories) use a standardized protocol to test a common set of samples covering various virus strains and potencies, and EDQM will be tasked with analyzing the generated data. Lastly, the Phase 3 (foreseen 2020-21) of the study will consist of testing of as many routine commercial batches as possible using the standardized ELISA protocol, with results reporting to EDQM, with the aim of using the results to support the evaluation of the applicability of the method to routine testing and of the potency requirements in view of the revision of compendial texts, including Ph. Eur. monograph 0216. In concluding the overview of the project, he mentioned that the BSP148 is expected to be able to produce its results in time for a revision of the Ph. Eur. monograph by the European Pharmacopoeia Group 15 in 2022-23.

## 6. Harmonization: challenges & opportunities

This session was chaired by Richard Hill, International Alliance for Biological Standardization (IABS), and Wassana Wijagkanalan, BioNet-Asia, Thailand. The session was dedicated to the discussion of the changes achieved, and those needed in the regulatory environment to make 3Rs implementation a concrete global achievement.

### 6.1. What did we learn from the past?

**Arnoud Akkermans**, from Netherland's National Institute for Public Health and the Environment (RIVM), talked of the lessons learned on the road leading to the replacement of in vivo testing. Animal testing, Akkermans reminded, had a crucial role in vaccine development and vaccine quality control at the initial developing stage of vaccine development, starting from the late XIX century when mice took the role of biological test tubes to identify phenol preservatives. But the developments of the recent decades in the applied technologies of vaccine production and also in the way vaccines can be controlled, and their application, contributed to a shift from traditional vaccine control technologies to more focused monitoring of critical quality attributes indicating consistency of vaccine production. Akkermans retraced some of these steps through few key articles [23,33–36], through activities, symposia and workshops which brought together and fostered the exchange of information between different stakeholders, and on how these activities produced tangible changes in the European Pharmacopoeia (like the revised 5.2.14 article of the Ph. Eur. on the substitution of in vivo methods by in vitro methods for the quality control of vaccines). The process of introducing innovative quality control tests for traditional vaccines took many years: replacing classical tests with an alternative test proved complex, with results often lacking correlation between in vivo and in vitro methods, while securing acceptance of an innovative test method by regulators (and industry) often proved difficult. Akkermans noted that the current highly defined vaccine production processes, online production controls, and critical quality indicating in vitro test methods enable extensive controlling of the production process for newly produced vaccine lots. But that alone won't bring about the change. What is needed, instead, is for a thorough scientific assessment of these in vitro models, and sharing of data between industry and regulators, and sharing of information on limitations, possibilities, focusing on the critical quality attributes for characterization and stability indication, and following these steps, success is possible. Akkermans showed examples of such successes, like the possibility to waive the in vivo potency test for poliomyelitis vaccine (inactivated), releasing the vaccine through an in vitro D-antigen ELISA, the introduction of a risk assessment as cornerstone of testing strategy for the test of extraneous agents in viral vaccines for human use *in lieu* of the test on adult mice and guinea pigs, which were deleted (Ph. Eur. 2.6.16), the replacement of the test for irreversibility of the toxoid for pertussis (acellular component) vaccine (adsorbed) with a CHO cell-clustering assay for residual pertussis testing (Ph. Eur. 2.6.33), deletion of test of tetanus specific toxicity for DTaP adsorbed vaccines, and the ongoing discussion on the removal of the test for specific toxicity of diphtheria from the General Provisions sections of the Ph. Eur., all successes, Akkermans concluded, made possible by successful interaction, cooperation and data sharing on the in vitro models between industry, National Control Laboratories and regulatory authorities.

### 6.2. Replacement of in vivo assays, from one to one replacement to the evolution of strategy on new products and beyond

**Jean-Francois Dierick**'s talks focused on Glaxo Smith Kline's (GSK) successes in enacting replacement for old and new products, on the internal strategy to reduce animals for quality control (QC) testing, and on how the company sees the replacement of in vivo tests for established commercial products through the consistency approach. First, Dierick described how the company tackled the replacement of in vivo potency test for four (unspecified) products. For three of them, parallel in vitro and in vivo testing were carried out during the various clinical phases, to accumulate a comprehensive set of data to demonstrate comparability (or superiority) of the in vitro assay, and the release packages included assays addressing antigen conformation, integrity and aggregation. The fourth product was an already marketed vaccine: in this case too, parallel in vivo and in vitro assays were run to create data sets demonstrating solid performance, and the replacement was performed in close collaboration with European authorities.

Dierick concentrated then on the strategy to replace in vivo potency testing for new products, which pivots on embedding replacement principles already at the beginning of development, on good characterization of the product, and on making the best use of clinical phases. Quality by Design is applied to identify critical quality attributes (CQA) and critical process parameters (CPP) supporting potency, while in vivo testing is used to confirm the identification of the critical quality attributes for potency. Relevant in vitro assays are developed that must be able to show solid performance both in assay validation and routine use, and superiority in detecting product evolution and alteration; making best use of clinical phases to support in vitro testing for the release and collect relevant data. Dierick moved then the focus to the legacy products, for which several exist (no in vitro potency assays addressing potency made at the time of clinical phases, products might be of low characterization and of high complexity) and key questions need be answered to transition to in vitro assays, examples of which being how to justify the relevance, and the comparability of an in vitro assay, how to establish a link to clinical data, and how to accumulate evidences required to justify replacement. Enablers and challenges were listed both for the removal of an in vivo assay from a control strategy, and for one-to-one replacements which is made possible when both assays measure the same CQA. In that case, key enabler would be the use of quality by design, and collaboration between manufacturers and release authorities towards harmonized solutions, while key challenges identified are product complexity – which can make it difficult to apply one in vitro assay to test potency at drug product level –, the need to have a deep understanding of the mechanisms of action of the product, difficulties in comparison and correlation between in vivo and in vitro due to the in vivo inherent variability and possible synergistic effects on the immune system, and, in the words of Dierick, the power still hold by the dogma that "in vivo assay sees it all", which can only be challenged by science-based approaches. Dierick moved then to discuss the Consistency Approach for legacy products, outlining different possibilities for replacement of in vivo potency testing at drug level: replacement by measurement of CQAs demonstrated to participate in potency (i.e.

antigenicity, antigen content, etc.) at Drug Product level, or with a mix of measurements at Drug Product and Drug Substance levels (in cases when the in vitro assay doesn't work at Drug Product level, and there is support from deep process understanding and solid data), and replacement with in vitro assays (at least one at Drug Product level) plus measurement of the CPPs that were demonstrated to ensure the delivery of a potent product. In closing the intervention, Dierick listed enablers and challenges specific to the consistency approach, listing as key enablers (1) Quality by Design principles and its repercussions on CPPs and quality attributes, (2) ICH's Q12 guideline allowing an evolution of regulatory approaches and design of control strategies, (3) sharing of knowledge between manufacturers and regulators on a same assay or product, (4) big data to transform historical data into product knowledge, while, as key challenges, (1) the need to take into account the process variability that will emerge by measuring CQAs and CPPs related to potency on legacy products, and that was not see in the in vivo testing, (2) how to establish product specifications considering that some assays were not applied on clinical batches, and (3) the fact that this approach will need to be proved in some successful applications before it can be widely applied. Still, Dierick concluded, the Consistency Approach is the best approach to date to deal with products for which attributes supporting potency are multiple, and complex [1].

### 6.3. Regulatory acceptance for the substitution of In vitro for In vivo vaccine potency and safety assays: science versus the fear factor

**Dean Smith**, Health Canada's Center for Biologics Evaluation, discussed the barriers to the development and authorization of in vitro assays for legacy vaccines, citing the European Pharmacopoeia (Ph. Eur.) General Chapter 5.2.14, as an important tool to address these barriers and drive the approval and implementation of alternative methods. Smith began the presentation illustrating a complex "catch-22" that regulators and manufacturers are locked in. As long as regulators are convinced of the superiority of in vivo methods, and are unwilling (or fearful of) changing long held (but unsupported) assumptions regarding the performance and value of animal assays, manufacturers will have no incentive to invest and develop innovative in vitro assay alternatives. However, Smith noted that Quality Control (QC) without in vivo testing for vaccines is already well established for several products. He gave the examples of two of highly effective types of human products: 1) Human Papilloma Virus (HPV) vaccines (based on recombinant viral-like particles), and 2) Meningococcal and Pneumococcal Bacterial Conjugate vaccines (based on polysaccharides conjugated to carrier proteins). With both groups of vaccines, the key quality attributes necessary to ensure the safety and efficacy profile of the products are accurately and robustly controlled using a combination physical/chemical and in vitro methods (i.e., without in vivo assays).

Smith emphasized that under cGMP, quality is built into the production process for all vaccines through in process controls and extensive consistency monitoring, which can best be achieved using more accurate, robust and rapid vitro methods, as noted above. Yet, for legacy products, such as Rabies and DPT vaccines, despite the demonstrated technical capability of manufacturers to successful implement vitro-based QC strategies, there is still a reluctance by some regulators to accept in vitro assays for these products. Smith asked, how can this be scientifically justified?

Importantly, Ph. Eur. 5.2.14 is explicit regarding the limitations of in vivo assays, when compared to appropriately in vitro alternatives. These include the inherent variability of in vivo assays, which typical lack of ICH Q2 (R1) [21] or VICH GL2 [37] validation. Such assay variability has resulted in failures of multiple international collaborative studies that required a one-to-one comparison between the vivo method (i.e., the in vivo NIH rabies potency test) and a more-consistent, validated in vitro method. These study failures were because of a lack of concordance between the two methods, due to the variability of the NIH test. Ph. Eur. 5.2.14 also recognizes that vivo methods for human vaccines "do not necessarily predict the actual responses in the target population". Smith notes that it is therefore more appropriate to consider in vivo assays as merely highly variable bioassays, with no special properties in a QC context, and several liabilities. Additionally, 5.2.14 notes that because in vivo and in vitro methods may assess the same quality attribute differently, one-to-one agreement between the two methods "is generally not scientifically justified and should not always be not always be expected".

Smith presented the assay substitution approach provided in Ph. Eur. 5.2.14, which is intended to facilitate the implementation of in vitro methods. Substitution is proposed "where a typical one-to-one assay comparison is not appropriate, unrelated to the suitability of one or more of the vitro methods". This approach came into effect in as of January 2018, through the work of the EDQM Groups 15 (human vaccines) and 15V (veterinary vaccines), which includes representatives from Health Canada and US/FDA CBER. Additional key statements in Ph. Eur. 5.2.14 note that "the inherent variability of in vivo assays can make them less suitable than appropriately designed in vitro assays for monitoring consistency of production and for assessing the potential impact of manufacturing changes. As a result, it is essential to continually challenge the scientific value and relevance of these in vivo test methods." "The use of appropriate in vitro methods … enhances the predictability of the release of safe and effective vaccine lots for use." Further considerations in 5.2.14 regarding the implementation of in vitro alternative methods include: (1) the importance of their scientific relevance, (2) that while international collaborative studies can be used to implement new methods, but this is not a requirement, and (3), in some cases, more than one in vitro method may be required to characterize a vaccine's key qualitative and quantitative attributes as measured by the existing in vivo test. Concluding, Smith commented how the new regulatory perspective described in Ph. Eur. 5.2.14 has provided additional support for industry to invest in in vitro assay development (e.g., the VAC2VAC Consortium). Additionally, it has greatly accelerated the discontinuation of longstanding animal-based tests, which are now understood to be scientifically unjustified. Examples include the recent discontinuation of the General Safety Test (GST)/Innocuity Test and the Histamine Sensitization Test (HIST) from the Ph. Eur., as well as the subsequent recognition by the WHO regarding lack of scientific justification for the GST. Many similar changes are now anticipated in the Ph. Eur [6].

### 6.4. 3Rs assessment of WHO guidelines and recommendations for biologics

**Anthony Holmes,** from the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), made a presentation on a new partnership between the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the WHO to review the animal testing requirements described in WHO guidance documents for biologics to identify opportunities for the integration of the 3Rs, with the aim of enabling vaccines manufacturers and regulators to apply the latest non-animal testing approaches and strategies to support faster access to cheaper vaccines by the global communities who need them most urgently. Holmes explained that no systematic review of established WHO guidelines for 3Rs had ever been made before, so there is no definite information on the amount of animal testing recommended or required by them for the manufacture and batch release testing of biologics. Due to this, non-animal methods already validated and approved within some regulatory jurisdictions are not yet included in the WHO recommendations; the opposite is also true, with non-animal methods present in the recommendations not being implemented by some regulatory authorities.

Holmes noted that there is a global movement – including cosmetics, pharmaceuticals and chemicals – for 3Rs driven by scientific, ethical, regulatory and economical rationales. To date there was little guidance on how to ensure a global harmonization of 3Rs tests and methods as they become available for biologics development. This results in missed

opportunities to embed the latest technologies into the development pipeline of biologics, leaving expensive and often poorly predictive animal tests in use. It is important, therefore, for the WHO to better understand the extent of animal testing requirements in their guidelines and recommendations and to assess where there are already opportunities to apply non-animal testing approaches.

The project envisioned in the NC3Rs and WHO's cooperation, Holmes explained, will be articulated to address three major areas: (1) what is the extent of animal testing included within the collection of WHO recommendations for biologics and, of those, are there alternative methods already validated and approved elsewhere that should be included in the recommendations; (2) evaluate whether a WHO guideline for the adoption of 3Rs principles into the quality control and lot release of licensed vaccines could be useful for harmonization of non-animal methods and for guidance to WHO member states; (3) analysis of the barriers hindering the adoption of 3Rs principles.

Holmes concluded extending an invitation to collaborate in the project, as a project so large and complex relies on the engagement of the global biologics' community.

### 6.5. Statements from various international organizations

**Eriko Terao** (EDQM) did not present the EDQM which activities and endeavors for the 3Rs were presented earlier during the meeting but highlighted 3 main challenges for the 3Rs: coordinated actions, data supported candidate methods and availability of samples & reagents for validation studies. She stressed the importance of coordinated actions and of a real global involvement of stakeholders in the dialogue, not only to increase confidence in harmonized alternative methods, but also to ensure wide implementation by ensuring applicability to a wide range of products and method accessibility.

**Jim Webster** (Ruakura Research Centre, Hamilton, New Zealand) reported on the World Organization for Animal Health's (OIE) effort in promoting and guiding animal welfare, good animal husbandry, good housing practices, and 3Rs (all contained in section 7 of the Terrestrial Animal Health Code) [38]. Webster mentioned also the organization's Global Animal Welfare Strategy to promote animal welfare and 3Rs, adopted by 145 countries in 2017, members of the organization, based on development of animal welfare standards, capacity building and education, communication between governments, organizations and public, and implementation of animal welfare standards and policies [39].

**Robin Levis** (FDA, U.S.A) introduced the United States' Center for Biologics Evaluation and Research (CBER) and the role of its scientists in supporting regulatory policy and their many collaborations in the last years, in particular with VAC2VAC. Levis traced an overview of the projects supporting alternative methods that were carried out in CBER, citing the deletion of the General Safety test (Revocation of general safety test regulations that are duplicative of requirements in biologics license applications, FDA Federal Register, 07/02/2015), refinement of the neurovirulence test in monkeys (MNVT) for the mumps vaccine [40].

**Laura Viviani** presented the efforts of the Humane Society International (HSI) and the activities initiated – promotion of global regulatory alignment for the deletion of the general safety test [3] and the use of non-animal based methods to replace the rabbit pyrogenicity test – through the engagement of key regulatory and industry stakeholders of various countries. Ms. Viviani also presented the project of a dedicated database for non-animal based methods, their implementation status and the current existing collaboration opportunities.

Speaking on behalf of the Bill & Melinda Gates Foundation (the foundation), **Gautam Sanyal** gave an overview of the foundation's strong commitment to delivery of life saving vaccines to the developing world at an affordable cost. The Foundation actively supports efforts to (a) accelerate development timelines, (b) reduce cost of manufacturing, (c) secure supply for GAVI, the Global Alliance for Vaccines and Immunizations, and (d) ensure appropriate product profiles, including new combinations and novel vaccine formulations, as needed for different geographies. The foundation's commitment to 3Rs is driven by several practical considerations, including: low precision and high variability of in vivo assays, long turn-around time, high cost, difficulty in sourcing and maintaining animals, and different requirements from different regulatory bodies. In a collaborative initiative with the National Institute for Innovation in Manufacturing of Biopharmaceuticals (NIIMBL) [41], the foundation has established a Global Health Fund to support development of in vitro assays aimed at replacing or reducing use of animals in potency and safety testing. Priority areas include replacement of monkey neurovirulence test, in vivo adventitious viral agents tests, the Kendrick test, etc. NIIMBL will also be looking for novel technologies that may potentially remediate manufacturing gaps in Developing Countries Vaccine Manufacturing Network (DCVMN). The foundation welcomes collaboration opportunities with private, public, governmental and regulatory groups across the globe in accelerating data driven reduction of animal testing.

**Gautam Sanyal** also spoke on behalf of the Coalition for Epidemic Preparedness and Innovations (CEPI), an innovative global partnership between public, private, philanthropic, and civil society organizations. CEPI's mission is to accelerate development of vaccines against emerging infectious diseases and to enable equitable and timely access to these vaccines for at risk populations, regardless of their ability to pay. The goal is to end an outbreak or curtail an epidemic. CEPI funds, coordinates and actively engages with partners in this process and builds capabilities as needed for rapid response to new or anticipated epidemic threats.

This presentation described CEPI's support of platform technologies in vaccine development to expedite delivery of "Just in time" vaccines. Shortly after this conference and as SARS-CoV-2 emerged, CEPI substantially expanded its investments in such platform technologies to accelerate the development of vaccines against this virus. Vaccine development efforts must be complemented with high quality in vitro or analytical assays that are precise, accurate, reproducible, sensitive and have short turn-around time. Analytical characterization based on critical quality attributes of vaccines are key to ensuring consistency and comparability between batches used in different clinical development phases, which often require transition from relatively small to large-scale manufacturing processes. Such analytical bridging is especially important in rapid response situations as it can eliminate the need for clinical comparability studies between batches, thereby reducing the development cost and timeline for regulatory approval. CEPI recognizes that pre-clinical safety and immunogenicity research often requires the use of animals, although results may not necessarily translate to response in humans. For CEPI-sponsored projects, CEPI will support animal studies if the potential health benefits are compelling, appropriate welfare standards are met, and where there are no alternatives. CEPI adheres to and requires its partners to fully comply with 3Rs as mandated by UK National Centre for the Replacement, Refinement, and Reduction of animals in research.

**Sunil Goel** described DCVMN's commitment to facilitate the implementation of 3Rs among its members. DCVMN is a voluntary, non-governmental, nonpartisan, not-for-profit, public health driven alliance of vaccine manufacturers, research and policy organizations from all over the world, representing 43 manufacturers from 14 countries. The current interest of the 3Rs Working Group from DCVMN is focused on DT-containing vaccines, whole-cell pertussis and rabies [42].

### 6.6. Collaboration and communication of regulatory bodies and industry: panel discussion

A panel discussion was dedicated to the collaboration and communication between regulatory bodies and industry, and it was kickstarted by two questions: (1) what the regulatory expectations are when a company wishes to change a testing method? and (2) what is the

effective interaction between industry and regulators?

**Robin Levis** cited the human rabies vaccine as an important showcase of the complexity of implementing change from the perspective of regulatory authorities. While a new method or assay must be able to secure the quality/safety/potency of a product, other guarantees must also be in place, including for example the proved availability of needed reagents for both manufacturers and regulatory, and an effective experience of manufacturers in the implementation of the consistency approach.

**Richard Hill** (International Alliance for Biological Standardization (IABS), U.S.A) commented on the need to encourage a change in the regulators' approach, which is often strongly favoring a conservative perspective based on old codified methodologies. As an example of the possible complexities involved in transitioning away from in vivo testing, Hill brought up the complex case of veterinary rabies vaccines [31] – a multi-species product that is tested and licensed separately for many species, each of which needs to be tested to validate efficacy. Veterinary vaccines comprise over 200 different antigens which are available in many combinations. An important scientific effort will be needed to define the best alternative testing strategy to address this complex issue, which can be simplified only through engagement of regulators and the biologics industry.

**Guang Gao** (PATH, China) reported personal experience of China's activities, noting the existence of delays in the application of 3Rs in vaccines due to some historical reasons related to product safety issues. Gao commented on the a very recently (June 2019) introduced, and very severe, new law on vaccines administration, which mandates stricter vaccine management and threatens grave penalties to ensure vaccine safety. This legislation, Gao commented, put a significant burden on regulators, which, together with the life-time consequences it threatens in case of emerging safety issues, could render enacting change a longer and more complex process, which will require engaging the regulators in dialogue as early as possible.

**Sunil Goel** voiced the effect of the absence of harmonized requirements for the testing strategies has on manufacturers, that is forcing them to continue to use very variable required tests instead of in vitro assays already established in the product's development (e.g. SRID, ELISA) to monitor the product before the release.

**William McCauley** (Animal Health Institute (AHI), U.S.A.) expanded on Goel's critical report, stating that the lack of harmonized difficulties also affects the U.S. veterinary vaccine manufacturers, and informed on an initiative of AHI to petition the US Department of Agriculture, expressing support for the Department's activities, but also to openly ask for more concrete actions.

**Dean Smith** commented on the perception that some manufacturers seem to have regarding the process of securing approval from regulators for a new method. Smith noted that that there is nothing mysterious about the process, which is in well and publicly articulated in ICH compliant regulatory environments. However, speaking as a regulator, Smith also made it clear, that in his opinion, there is still work to be done to overcome the misplaced value awarded to animal assays in a quality control context with some regulators, and their suspicion/fear of novel methods, even in Europe and North America is still an issue. Smith's comments raised a question by **Robert Sitrin**, who asked about what could concretely be done to change the current paradigm, for example in countries like India and in China?

**Sunil Goel** reported the current situation in India, and explained that a constant dialogue between the Indian Pharmacopoeia Committee, manufacturers and other organizations is in place and that 3Rs are getting into the agenda of the various Indian stakeholders.

**Li Shi** commenting on China, remarked that the country is inclined to follow the example of other key regulatory agencies and that if the US FDA were to approve an alternate to the NIH test, Chinese regulators would be under less pressure to stay with the status quo. Li Shi also noted that it is important for organizations like WHO and the Bill & Melinda Gates Foundations to be engaged and active to accelerate change.

**Jean-Michel Chapsal** put forth a suggestion that engaging more manufacturers from China in international collaborative studies ought to facilitate an exchange of information and data that could lead to regulatory changes.

**Gautam Sanyal** brought back the discussion on U.S. and Canada, asking what is effectively holding the respective regulators from accepting alternative methods.

Both **Robin Levis** and **Dean Smith** confirmed that some regulators showed openness to such changes for a number of years. However, manufacturers appear to be hesitant to make requests and submit the relevant data for assays that they propose as fit for purpose.

**Arnoud Akkermans** (RIVM) enquired on what the influence of EDQM working groups of experts on vaccines had in other countries.

**Sunil Goel** following indirectly Akkermans question commented on how manufacturers, in the presence of a too varied regulatory landscape (in which too many regulatory scenarios, including the WHO requirements, are to be considered), needs to take decisions based on the most efficient way to release its products.

**Guang Gao** brought the focus again on China, sharing information on the new version of the Chinese Pharmacopoeia expected in 2020 where no changes are expected in the vaccines' chapters, so she asked how Europe and U.S. could concretely help to rekindle discussion on alternative methods in China.

**Marlies Halder** commented that China is open to alternative methods and, in particular, for cosmetics testing. For example, the National Medical Product Administration (NMPA) approved two non-animal tests for the skin sensitization. China is observer of the International Cooperation on Alternative Test Methods, ICATM.

**Supaporn Phumiamorn** informed that also Thailand is interested in implementing alternative methods, surmising that a working strategy could be based on increased dialogue and alignment between the National Regulatory Authority and the National Control Laboratory (providing with scientific data).

The panelists were finally asked to conclude the session with key statements to summarize their position and the discussion. This resulted in a consensus on the fact that regulatory alignment and harmonization should be a priority from a global perspective, which should be enacted through the active participation of each stakeholder (manufacturers, regulatory authorities, international organizations and charities), common guidelines, and continuous collaboration.

## 7. Workshops

After the panel, two distinct, parallel workshops – on *Validation, acceptance, implementation & harmonization*, and on *Needs of emerging economies* – were organized to focus discussion on the specific themes, elicit direct contributions and foster exchanges between the participants, explore the possible consensus on different issues, and propose future actions.

### 7.1. Validation, acceptance, implementation & harmonization

Moderated by: Laura Viviani, Humane Society International (HSI), Switzerland, Marlies Halder, European Commission Joint Research Centre, EURL ECVAM, Italy, Jim Webster, OIE Collaborating Center, New Zealand, Hilde Depraetere, European Vaccine Initiative (EVI), Germany, Denis Lambrigts, GlaxoSmithKline, Belgium.

The aim of the workshop was discussion on the concrete meaning and the various aspects of validation, acceptance, implementation and harmonization of alternative methods.

The workshop moderators presented definitions of the four terms and invited participants to discuss the relevant drivers and barriers for each one; to foster participation, participants interacted through a live survey platform.

**Validation:** Defined, in agreement with the participants, as a process which should demonstrate that *a method is relevant and reliable for the*

*given purpose*. Further details were introduced about validation studies – defined as either as single-lab validation study for a given product (e.g. according ICH/VICH Validation guidelines, ICH Q2 R1 [22],VICH GL1, GL2 [37]) or as multi-lab validation study (e.g. ring trial or collaborative study to establish a new method to be used for a class of products). European Pharmacopoeia's new 5.2.14 chapter *Substitution of* in vivo *methods by* in vitro *methods for the quality control of vaccines* was mentioned since it outlines how the barrier of one-to-one replacement could be overcome, in particular, in light of the high variability of animal tests and the inherent fundamental differences of in vivo and in vitro tests.

Participants – all familiar with the concept and the dynamics of validation – expressed interest in participating to collaborative studies and in learning more about chapter 5.2.14 of the European Pharmacopoeia.

Participants considered the following as main barriers to the validation of alternative methods:

- risk averse attitude of regulators;
- lack of materials and/or reagents;
- how to design validation studies;
- business cost to plan with uncertain regulatory acceptance;
- lack of regulatory harmonization, and lack of innovation in investing in new approaches/products.

Their suggestion to overcome some of those difficulties were the following:

- more training, case studies and guidelines,
- but also, to be more proactive in creating or participating to international collaborative studies (only few of the workshop participants had participated in at least one collaborative study),
- and it was proposed creating a reagent bank, for consideration of future international conferences.

**Acceptance:** the group agreed upon that acceptance implies that a regulator considers a method appropriate to be used in a regulatory context (e.g. batch release testing).

Participants listed the following main barriers to the acceptance of 3Rs methods:

- the majority of alternative methods are not included in compendial text, i.e. not officially part of pharmacopoeias or regulatory requirements and participants identified this as the most important hurdle;
- manufacturers considered information on data requirements about alternative methods and documentation to be provided to the regulators as insufficient, and, therefore, the dialogue is perceived as inefficient.

Regarding suggestions on incentives and support regulators could provide to manufacturers to encourage them to invest in non-animal testing, the participants listed various possibilities:

- making license variations faster, easier, and less expensive.
- the introduction of a standardized dialogue between manufacturers and regulators (along the lines of the scientific advice procedure [43] introduced by European Medicines Agency (EMA)).
- national and international agreement on the type of data requested for the submission.
- rendering successful cases available for others to study/follow.
- increasing collaborations between manufacturers to approach regulators with a common agenda and more data.

Participants were then asked what actions a manufacturer could take to increase the acceptance of non-animal based methods. The answers once more confirmed that collaboration with other manufacturers, increase of scientific data sharing (validation data, trend/analysis of historical and stability data), openness with regulators, and early development of new methods, could all, if implemented, contribute and enhance the overall acceptance of 3Rs.

**Implementation:** agreed upon that implementation refers to the use of a validated and accepted method by a manufacturer or control authority for the quality control of a given product.

Most of the participants confirmed that 3Rs are routinely implemented within their institutions, although the process of securing their implementation usually proved challenging due to the lack of global harmonization of the testing requirements, technical difficulties in the product-specific validation, and its cost.

The following major difficulties related to implementation were pointed out by the participants:

- lack of interest of some regulatory authorities in alternative methods (still the case in some countries, although positive and encouraging examples were presented during the conference);
- the not-consistent and not-constant availability of reagents;
- the lack of interest from the manufacturers' management in investing in alternative methods (which can be the case both for manufacturers in developing countries and for multinational companies);
- and the lack of scientific and technical expertise.

**Global Harmonization:** participants were invited to discuss and define global harmonization of testing requirements through concrete cases, such as the deletion of the general safety test for human vaccines that was advanced by Europe, USA, Canada and recently recommended by WHO, and the international collaborative studies to replace the NIH test for Rabies (EDQM-BSP148), and the Vero cell based assays to replace tests on mice to determine toxicity of toxin/toxoid and antigenicity of *C. septicum* vaccines (EDQM-BSP130).The majority of the participants agreed on the key role international collaborative studies play as an instrument to promote harmonization.

- The discussion also focused on whether a universal assay would be preferable to product specific assays, and which of the two approaches would be more efficient in facilitating harmonization. No agreed position was reached.
- To promote harmonization, participants recommended the creation of a common process for submitting variations and the implementation of mutual acceptance of release data across regions.
- The participants were all interested and supportive of NC3Rs-WHO's project on reviewing and implementing alternative methods in the WHO requirements for vaccines and biologicals (which had been presented by A. Holmes on the 1st day of the conference), as a first crucial step to laying the basis for global harmonization and acting as an example for the stakeholders from the developing economies.

### *7.2. Needs of emerging economies (training, reagents, materials, etc.)*

Moderated by: Sunil Goel, the Serum Institute of India Pvt Ltd., India, Wassana Wijagkanalan, Bio-Net Asia, Thailand, Yeowon Sohn, Seoul National University.

The working group focused on understanding the difficulties of the implementation of 3Rs in the developing economies, with participants clearly stating the need for support to move ahead for implementation of 3Rs in their respective countries.

The group started the activity by laying out the situation about the 3Rs acceptance in some of the represented countries, with the general safety test (or abnormal toxicity test, or innocuity test) and some potency tests taken as examples, and then introducing country specific conditions.

- In Thailand, the National Control Laboratory welcomes in general the 3Rs approach and, specifically, the deletion of both the general safety test and of the pyrogenicity test is under discussion, based on the review of historical data; in addition, the single dilution assay for the acellular pertussis potency test is being considered.
- In India, the Indian Pharmacopoeia Committee has been allowing waivers for the general safety test [24] even before the recent WHO endorsement for its deletion [8]. To obtain the waiver, manufacturers have to submit consistency data (3 batches during pre-licensure), and in case of adverse events following immunization (AEFI) has the right to perform investigations on the safety of the product. The Indian regulatory authorities also accept the single dilution assay for the potency tests, as well as the specific toxicity for D and T Components with single dilution assay. Close communication and the regular meetings between manufacturers NCLs were reported. In Japan, ATT is still a requirement, but there is the interest in its removal, and there is a gradual implementation of 3Rs for some vaccines (e.g. Hepatitis B vaccine - (HBV)) based on data review in the framework of the consistency approach.
- In Indonesia, for the HBV the replacement of the in vivo potency test with the in vitro method was accepted by the national regulatory authorities and a change request to delete the general safety test was put forth by the local manufacturer.

After the exchange of information on the specific local conditions, participants discussed the key hurdles hindering transition to 3Rs in emerging economies, agreeing on the following list:

- lack of harmonization among pharmacopoeias, including the WHO requirements;
- different speed in acceptance and implementation of 3Rs;
- perceived lack of effective communication between manufacturers and regulators, and both suffer to resistance to the change;
- limited resources (equipment, funds, materials, personnel, knowledge on new method);
- lack of concrete case studies and scientific evidence that could be gained with investment on alternative methods in the early stage of development of the product;
- accessibility to IP protected methods, materials, reagents.

Discussing the hurdles, the group proposed the following activities as solutions to the above-mentioned difficulties:

- organization of a forum or conference dedicated to the harmonization of guidelines and/or pharmacopoeia on a regular base;
- increase of collaboration and effective communication (even at the early stage of a project) among stakeholders like NCL, industry, academia and global initiatives;
- inclusion of developing economies' stakeholders in collaborative study that could provide and serve as training and to improve their global network, knowledge, experience and also sharing of difficulties, problems but also solutions;
  - o allowing the possibility to have common protocol(s) and procedures, standards,
  - o favoring the interaction with large manufacturing companies for advising the new methods to other companies;
- definition of roadmaps for 3Rs implementation based sufficient scientific evidence;
- in interacting with WHO, establish small group to discuss about proposals for new methods;
- facilitation of access to methods, data (publication), critical reagents/reference standard;
- increase of the learning opportunities from other sectors;
- investments in communication, through IABS meetings for example, about WHO/pharmacopoeia changes toward the 3Rs implementation;
- promotion of the involvement of other NRAs when WHO or EU agencies begin the implementation of alternative methods.

In closing, the group agreed on additional discussion points that should be considered for future conferences, such as.

- reinforcing the concept and related evidences that in-vitro tests are not an addition to in-vivo tests, but valid replacements.
- the importance of common quality standards, ands of common methods with the same reference standards to remove inconsistency.
- application of 3Rs for stability testing (e.g. degradation testing to simulate the vaccine shelf life, stability indicating parameters).

## 8. Conclusion

The closing remarks to the congress were tasked to **Coenraad Hendriksen**, who began with sharing with the audience a prediction: that 25 years from now, animals will not be used anymore for the quality control of vaccines.

In his view, we are at a time when progress practically halted on animal methods, with all their issues with relevance and reproducibility unsolved, while non-animal models and techniques keep being developed and refined, proving fruitful and efficacious. Such a needed transition would, in his opinion, ferry vaccine quality control from its current empirical nature – where batches either pass or fail testing – to a more scientific approach that finally delves deeper into *why a batch is not doing what it should do.* Such transformation would free the sector from one of its most sedimented axioms, that a vaccine batch is to be considered a *unicum*, embracing a new perspective in which a batch is seen as *one* of a continuum in a series originating from the same master seed lot, which is the perspective embodied in the *consistency approach.*

But, he warned, this transformation will not happen without difficulty. A change of attitude in the community will be required, one that will make it possible to bring the role of animal testing in the context of facts, and away from the sedimented context of beliefs and tradition in which it has historically been enveloped. There's substantial reason to believe that vaccine quality was secured not thanks to animal testing, but because of our ability to consistently produce vaccine batches of high quality, but for this to become ingrained in the vaccine community much effort will be needed: routine, and long termed acceptance of the status quo desensitized the professionals working in the field to a state of conservatism, and, at times, even of ignorance of alternative methods.

And professionals must also be convinced that 3Rs cannot be marginalized merely as a question of ethics pertaining animal welfare. On the contrary, 3Rs represent first and foremost *better science*, overcoming the many issues (like poor relevance and reproducibility) of animal tests while also securing improved animal welfare.

Hendriksen proceeded then to list a series of remarkable changes in the field starting from the growing interest and commitment to 3Rs from many organizations; like DCVMN that set steps to start up activities in this direction; the global progress being made in the rabies project, with the involvement of NRA's, industries and NGO's; the Bill & Melinda Gates Foundation supports in this field; the EDQM that continues to be on the forefront with its Biological Standardisation Program; several vaccine manufacturers set up in-house centers to promote the 3Rs; and like the new initiatives from WHO that should started soon.

Another important project is VAC2VAC, which is a unique effort because it has a clear focus on replacement, it is based on a paradigm shift in vaccine quality control based on the principle of consistency testing and it involves all relevant stakeholders: manufacturers, academia, national control laboratories and regulatory authorities.

Other important progresses to highlights is how the vaccine quality control became an interdisciplinary collaboration, for example, in the field of bio-informatics, analytical techniques and in vitro methods.

In the conclusion of his speech, Hendriksen confirmed what was the *leitmotiv* of the conference: the need of collaboration between all the

stakeholders. Quoting Albert Einstein, that « *You can never solve a problem on the level on which it was created*», he proposed a new paradigm, based on the axiom that a vaccine batch is not unique, but one of several batches being produced of the same master seed lot. Such new paradigm would run in parallel to the development of new innovative technologies, which are the bases of the new paradigm. This change could take place under the aegis of what he defined as the 3Cs: *Commitment*, with every stakeholder accepting responsibility for 3Rs, *Common Sense*, as an appeal to being realistic on the prejudices affecting the field, with the view of overcoming them, and *Communication & Collaboration*, that is, defusing the "catch 22" situation that locks manufacturers and regulators in a difficult equilibrium, where manufacturers don't dare innovate for risk of rejection, and regulators unwelcoming of alternative methods as they have too little data to base their decision upon, something that might be addressed for example through a "safe harbour" mechanism, but also to include developing economies stakeholders in a constant dialogue and in concrete projects. Hendriksen closed the intervention with a quote by Mark Twain meant as an encouragement to look beyond the level of the problem, to a new level that can offer solutions: «They did not know it was impossible, so they did it».

## 9. A way forward

The conference *Animal testing for vaccines - Implementing Replacement, Reduction and Refinement: Challenges and Priorities* proved successful in engaging key global international stakeholders, including representatives from low- and middle-income countries. Many of the challenges faced the last 10 years by industry, regulatory authorities, public research institutions and not for profit organization in the implementation of 3Rs in the vaccines' field were discussed and acknowledged. But the attendees also reported on significant progresses being made, and on new multi-stakeholders'collaborations initiated that are producing meaningful results with regards to the development, validation and implementation of 3Rs opportunities.

The participants agreed that a way forward in the sector must rest on the cornerstone of a constant and continuous dialogue between the stakeholders, through more frequent conferences and meetings, more educational opportunities, more and better communication on the advancements in the field (including successful case studies), and an increase in the engagement in new collaboration opportunities of stakeholders from LMICs industry and regulatory authorities. Agreement also emerged on the welcoming of international organizations and non-for-profit organizations as facilitators and promoters of those initiatives.

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## Author contributions

Laura Viviani was responsible for writing the main draft. Coenraad Hendriksen was chair of the Scientific Committee of the conference. All the listed authors supplemented and revised, as needed, the manuscript sections specifically related to their own interventions and comments.

## Disclaimer

This manuscript and the views expressed herein are those of the authors and do not necessarily reflect the views or policies of the various regulatory authorities and organizations.

## Declaration of competing interest

None.

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