Format
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Onderzoek naar de werkzaamheid van vaccins en antivirale middelen tegen nieuwe coronavirussen
1.2	Looptijd van het project	01-04-2020 – 31-3-2025
1.3	Trefwoorden (maximaal 5)	Coronavirus, vaccin, niet-humane primaten, werkzaamheid, antiviral

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☐ Fundamenteel onderzoek
- X Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1	Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)	In de afgelopen 20 jaar zijn er drie uitbraken geweest van nieuwe coronavirussen bij de mens. Het SARS-coronavirus dat in 2002-2003 opdook in China, het MERS-coronavirus dat in 2012 opdook in Saoedi-Arabië, en het virus dat in 2019 ernstige luchtweginfecties veroorzaakte in China. Dit laatste virus heeft de naam SARS-CoV-2 gekregen vanwege de grote genetische verwantschappen met het SARS-Coronavirus, terwijl de ziekte COVID-19 (Coronavirus disease 2019) genoemd wordt.

		Alle drie de virussen zijn afkomstig van dieren (zgn. zoönose), en kunnen bij de mens zeer ernstige luchtweginfecties veroorzaken. De SARS uitbraak resulteerde in 8096 ziekenhuisopnames, waarvan 774 patiënten overleden (9.5%), terwijl van MERS 2499 ziektegevallen bekend zijn, met 861 doden als gevolg (34.5%). De huidig uitbraak van SARS-CoV-2 heeft al geleidt tot 73.328 gevallen met 1.873 doden (2,5 %), en heeft zich al over 28 landen wereldwijd verspreid (dd. 18/2/2020). Vanwege de ernst van de uitbraak heeft de Wereldgezondheidsorganisatie deze coronavirus-uitbraak een volksgezondheidprobleem van internationaal belang genoemd. Op dit ogenblik zijn er nog geen geregistreerde vaccins of antivirale middelen tegen coronavirussen. In dit project zullen wij de beschermende werking van nieuw-ontwikkelde coronavirus-vaccins en antivirale middelen onderzoeken in een model voor coronavirusinfecties in apen
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Wij willen vaccins en antivirale middelen evalueren die gericht zijn tegen het coronavirus dat in december 2019 in China een uitbraak onder mensen veroorzaakte, het SARS-CoV-2. Het grote doel is vaccins en antivirale middelen te ontwikkelen die bescherming kunnen bieden tegen verschillende bekende en nog onbekende coronavirussen. Afgaande op de recente coronavirus uitbraak, maar ook die van het SARS-coronavirus en het MERS-coronavirussen, kunnen dergelijke vaccins en antivirale middelen veel levens redden bij een toekomstige uitbraken van coronavirussen.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Maximaal 382 resusapen, cynomolgus makaken, en/of penseelapen
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	De dieren ondervinden ongerief door biotechnische handelingen, en het plaatsen van een temperatuurtransponder in de buikholte. Daarnaast kunnen de dieren ziek worden door de virusinfectie
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Door toepassing van een humaan eindpunt wordt de welzijnsaantasting beperkt tot matig.
3.6	Wat is de bestemming van de dieren na afloop?	De dieren worden aan het einde van het experiment geëuthanaseerd.

4 Drie V's

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Het is nog niet mogelijk om de beschermende werking van vaccins en de werkzaamheid van antivirale middelen te bepalen zonder gebruik van proefdieren. Het afweersysteem is dermate ingewikkeld dat de beschermende werking van een vaccin tegen coronavirusinfectie nog niet in het laboratorium kan worden nagebootst. Vanwege hun grote immunologische overeenkomsten met de mens zijn apen het meest geschikt voor dit vaccinonderzoek. De grote gelijkenis in metabolisme, en fysiologie met dat van de mens maakt hen ook het meest geschikt voor het onderzoek

		naar de veiligheid en werkzaamheid van antivirale middelen voordat deze in klinische studies in de mens worden getest.
4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Alleen vaccinkandidaten en antivirale middelen die eerst in andere proefdiersoorten veilig en veelbelovend zijn getest zullen in apen worden getest op hun werkzaamheid. Het aantal benodigde dieren wordt per experiment bepaald aan de hand van statistische analyses. Waar mogelijk zullen meerdere vaccins of middelen tegelijk getest worden, waardoor maar één controlegroep nodig is. Bij vaccins wordt gebruik gemaakt van een twee-fase benadering: als het vaccin geen immuunreactie opwekt, of als het nadelige invloed heeft op de gezondheid van de dieren, zal niet worden overgegaan op het infecteren met coronavirus. In de farmacokinetiek studies voor antivirale middelen zullen meerdere doseringen worden getest in hetzelfde dier, waardoor het totale aantal dieren in deze studies zo beperkt mogelijk zal zijn. In de effectiviteitsstudies wordt gebruik gemaakt van een twee-fase benadering. Na de eerste fase vindt statistische analyse plaats van de tot dan toe bereikte resultaten. Op basis van deze analyse wordt dan besloten om wel of niet door te gaan
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	Onderzoek naar de werkzaamheid van coronavirusvaccins kan in diverse dieren worden uitgevoerd. Alleen in de laatste fase van de vaccinontwikkeling is uittesten in apen nodig omdat deze dieren wat betreft hun fysiologie en afweersysteem het meest op de mens lijken. Andere proefdieren, zoals knaagdieren zijn in deze fase van het onderzoek niet geschikt omdat deze virussen in muizen vaak niet een goede infectie geven en wat betreft hun afweersysteem op diverse punten afwijken van de mens. Daarom is in apen de kans het grootst dat eventuele onverwachte nadelige effecten alsnog opgespoord kunnen worden en een goede voorspelling gedaan kan worden wat betreft werkzaamheid bij de mens.
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	De verwachte ziekteverschijnselen zijn te vergelijken met een ernstige griep. De dieren worden intensief geobserveerd zodat wanneer ziekteverschijnselen optreden zeer snel actie kan worden ondernomen. Alle biotechnische handelingen worden uitgevoerd onder verdoving. Waar nodig wordt pijnstilling gegeven. De dieren worden getraind om zoveel mogelijk vrijwillig mee te werken aan de toediening van de verdoving. Om de dieren zo veel mogelijk natuurlijk gedrag te laten vertonen is op het onderzoeksinstituut een uitgebreid programma voor diertraining en kooiverrijking opgezet.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	Provide the title of the project.	Evaluation of vaccines and antiviral compounds against emerging coronavirus infections

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☐ Basic research
- X Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Coronaviruses (CoVs) are a group of enveloped viruses with non-segmented, single-stranded, and positive-sense RNA genomes. CoVs are important pathogens for human and other vertebrates. They infect a variety

of economically important vertebrates, such as pigs and chickens, but have also been described in birds, bats, mice, and various wild animals. However, CoVs can also infect the respiratory, gastrointestinal, hepatic and central nervous system of humans. Until the end of 2019, six human coronaviruses (HCoV) were described; HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, severe acute respiratory syndrome (SARS)-CoV, and the Middle East respiratory syndrome (MERS)-CoV (2,5,8).

Until 2002, CoVs were only known to cause mild respiratory infections in humans. However, in November 2002, a viral respiratory disease first appeared in southern China and quickly spread to other countries, leading to over 8,000 confirmed cases at the end of the epidemic in June 2003, with a mortality rate of~9.6% (10). The causative agent was identified as SARS-CoV, a zoonotic coronavirus. SARS-CoV originated in horseshoe bats, and had later adapted to infect the intermediate host, the palm civet, and ultimately infected humans (7). After an incubation period of 4–6 days, SARS patients developed flu-like symptoms and pneumonia, which in severe cases led to fatal respiratory failure, and acute respiratory distress syndrome (9). SARS-CoV can infect multiple organs and causes systemic disease, but symptoms worsen as the virus is cleared, suggesting that aberrant immune response underlies the pathogenesis of SARS-CoV (10). No cases of SARS have been reported since 2004, but a rich gene pool of bat SARS-related coronaviruses was discovered in a cave in Yunnan, China, highlighting the necessity to prepare for future re-emergence (6).

Eight years after the SARS epidemic, in June 2012, MERS-CoV emerged in Saudi Arabia as the causative agent of a SARS-like respiratory disease (4). Although human-to-human transmission is considered limited, MERS-CoV has caused two major outbreaks in Saudi Arabia (2012) and South Korea (2015), with the global confirmed cases exceeding 2,000 and a mortality rate of ~35% (1). Similar to SARS-CoV, MERS-CoV originated in bats, but it later adapted to dromedary camels as intermediate hosts (3). Currently, no vaccines or specific antiviral drugs are available for either SARS-CoV or MERS-CoV.

For their potential to cause global epidemics, and the abovementioned lack of vaccines and antivirals, SARS and MERS are prioritized by the WHO on their R&D blueprint (11). The WHO R&D Blueprint focuses on severe emerging diseases with potential to generate a public health emergency, and for which insufficient, or no preventive (vaccines) and curative solutions (antiviral compounds) exist. The 2018 WHO list of diseases to be prioritized is:

The 2018 annual review determined that given their potential to cause a public health emergency and the absence of efficacious drugs and/or vaccines, there is an urgent need for accelerated research and development for:[10]

- Crimean-Congo Hemorrhagic Fever
- Ebola Viral Disease and Marburg Viral Disease
- Lassa Fever
- MERS and SARS
- Nipah and henipaviral diseases
- Rift Valley Fever
- Zika disease
- Disease X

[10] The order of diseases on this list does not denote any ranking of priority.

In December 2019, a cluster of 27 patients were hospitalized in Wuhan, China, with respiratory symptoms of unknown etiology. Cases showed symptoms such as fever, dry cough, dyspnea, and radiological findings of bilateral lung infiltrates. The causative agent was quickly identified as a coronavirus, initially called 2019-nCoV (2019 new coronavirus)(12), and on 10 January its genome sequence was made publicly

available (GenBank acc. no. MN908947). On 11 February, the virus was officially named SARS-CoV-2, and the disease caused by the virus, COVID-19 (Coronavirus disease 2019).
Since the first publications, the epidemic rapidly spread: on February 18, 2020, 8:00 CET, 73.328 lab-confirmed cases were known, with 1.873 deaths, including 2 outside China. World-wide 28 countries have reported cases of COVID-19, including nine European countries (source: ECDC). International public-health authorities consider the respiratory virus a significant threat beyond China and therefore, on 29 January, the World Health Organization declared the SARS-CoV-2 outbreak a public-health emergency of international concern.
The sporadic emergence and outbreaks of new types of CoVs in the last 2 decades remind us that new CoVs are a severe global health threat. It is highly likely that new CoV outbreaks are unavoidable in the future due to the increased interactions of human with animals. Thus, there is an urgent need to develop effective therapies and vaccines against CoVs

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The **aim of this research project** is to test the immunogenicity, efficacy, and absence of pathogenicity (safety) of novel coronavirus vaccines, and the pharmacokinetics, safety and efficacy of anti-viral compounds that are developed for use in humans in nonhuman primates (NHP). The **main focus** will be on coronavirus vaccines and antivirals that are designed to protect against infection with the recently emerged SARS-CoV-2.
The institute has extensive and long-standing expertise in conducting vaccination studies using nonhuman primates. Since 2012, researchers at the institute have been working on other respiratory infections, like influenza virus infection in macaques and marmosets, and on the evaluation of vaccines against influenza, and are thus skilled in working with pathogenic, airborne viruses. The institute has the appropriate facilities and experience to work with airborne pathogenic viruses at DM-III and ML-III biosafety conditions. In addition, they have the appropriate virological and immunological assays for assessment of vaccine efficacy, and to determine absence of pathogenicity. The experience with respiratory viruses guarantees that these animal studies will be adequately performed.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

The ongoing outbreak of SARS-CoV-2, as well as the outbreaks of pathogenic SARS- and MERS-CoVs in the last two decades, have shown the urgency of developing vaccines and antiviral compounds against these emerging coronaviruses, but also points towards the need for preparedness for future outbreaks of yet unknown CoVs. Since beginning of January 2020, over 73.000 infections with SARS-CoV-2 have been reported, with a 2-2.5% case fatality rate (dd. 18-02-20). Human-to-human transmission of the virus, in combination with an asymptomatic period during which infected people can already spread the virus to others, likely guarantee further spreading of the epidemic. Because of this concern the World Health Organization declared the coronavirus outbreak a public-health emergency of international concern.
The WHO put SARS and MERS on the 2018 list of priority diseases. For the first time WHO also included 'Disease X' on this list. Disease X is a placeholder name given by the WHO for any new unknown pathogen that may cause disease and potentially an epidemic in the future. Clearly, SARS-CoV-2 has all these characteristics of Disease X, and as such can join the MERS- and SARS-CoVs on the list.
For the listed agents, there is an urgent need for research and development of vaccines and antiviral treatments. As the main goal of this project is the evaluation of vaccines and antiviral compounds against emerging coronaviruses, with a focus on SARS-CoV-2, this project perfectly fits within the scope of the WHO strategy.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

Other research groups have established nonhuman primate (NHP) models for infection with emerging coronaviruses like SARS and MERS CoV (13-17,19-21). Mostly widely used in CoV research are rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*), but their susceptibility for infection with the recently emerged SARS-CoV-2 is not yet known. Currently, the first macaque studies are ongoing in other research institutes, and our choice for a specific macaque species will probably be based on the outcome of these studies. Depending on the virus strain used, slight differences were seen in virus replication, clinical signs, and pathology, indicating that both can be used in SARS-CoV-2 studies (13,14).

Common marmosets (*Callithrix jacchus*) have also proven a suitable model for infection with the SARS- and MERS-CoVs (15,21), and generally show more severe clinical signs than macaques. However, the small size of the marmoset complicates sampling of blood, but also collection using swabs. For this reason, common marmosets will not be the preferred species of choice in this project, but may be used when macaque species do not meet the necessary requirements for CoV vaccine evaluation or antiviral testing, like reproducible infection. At this moment we cannot predict which of the NHP species will be used in our project, but the first results of the SARS-CoV-2 infection studies will become available within a few weeks. Only if these studies show the susceptibility of NHP to this virus, the current project will be initiated.

In order to evaluate that a new coronavirus vaccine candidate is immunogenic, has the capacity to protect against infection, and that no adverse effects occur, a vaccine evaluation experiment will be performed according to well-established procedures, as described in Appendix 2. In case the evaluation of the capacity of a vaccine to protect against infection requires that a virus, or virus stock, has to be used that has not been tested before in NHP at our institute, then this virus will first be tested in a number of animals to determine if all animals become infected and what the amount of virus production is (type 1 experiment).

The evaluation of the efficacy of antiviral compounds to protect against infection (prophylactic use), or to interfere with an ongoing CoV infection (therapeutic use) requires that the virus inoculum stock of each new strain/serotype is tested for its infectivity and the course of viremia in the nonhuman primate species used, prior to efficacy testing (Appendix 1).

The efficacy of a specific antiviral compound is determined using two different study protocols: a prophylactic efficacy study, and/or a therapeutic efficacy study protocol, depending on the research aims set for a specific compound. In both protocols a standard viral inoculum (determined in Appendix 1), and an established antiviral compound dose (determined in Appendix 2) will be used. In both protocols, the decrease in viral load will be measured as primary indicator of antiviral activity (Appendix 4). Before starting an efficacy testing, the compound will be tested in a pharmacokinetics (PK) study, in order to assess the bioavailability and half-life of the test compound

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Coronavirus infection in NHP. In order to establish infectivity and pathogenicity of a new virus, or virus stock that has not been tested previously in NHP at our institute or at other institutes, animals may be infected and monitored for clinical symptoms, fever, body weight and changes in blood parameters. Nasal and tracheal swabs will be taken to determine if the animals have become infected and what the amount of virus production is. To evaluate a new virus, the virus is inoculated via (a combination of routes); i.e. intra-bronchial, oral, intranasal, or via an aerosol. Proper application in vaccine evaluation requires that in these infection studies > 80% of the animals become infected and that the amount of virus produced in the trachea over the infection period is clearly measurable to allow measurement of reduction in virus load in vaccinated animals with less than 10 animals per group. In case these parameters are not achieved, the experiment will be repeated with a 10-100 times higher virus dose. In case any of the animals reaches the clinical endpoint within the first four days after infection then a 10-100 times lower virus dose will be evaluated.

Vaccine evaluation in NHP. For this type of experiment, animals will be immunized either once or they will receive a number of immunizations over a certain time period. During the study, animals will be monitored for adverse effects of the vaccine, including monitoring of general behavior and health. Blood and

occasionally nasal washes and lung lavages will be taken to measure induction of systemic as well as local immune responses. When adequate immune responses are induced that indicate that protection against infection might be achieved, the efficacy against infection will be tested by experimental infection with coronavirus. A group of non-vaccinated animals will be included as infection controls.
Pharmacokinetics (PK) of CoV antiviral compounds in NHP. For this type of study, animals will be administered with different dosages of an antiviral compound. If necessary, the compound may be given at multiple time-points over a certain time period. At regular time-points after administration, blood samples will be collected for measurement of the concentration of the compound, or active component thereof. In parallel, blood chemistry and hematology will be performed to monitor for adverse effects of the compound. General behaviour, health, body weight, and body temperature will also be monitored.
CoV antiviral efficacy study in NHP. The efficacy of a specific compound against CoV may be determined using two different study protocols: a prophylactic efficacy study, or a therapeutic efficacy study protocol, depending on the research question. In both protocols a standard viral inoculum, and an established antiviral compound dose will be used. In the prophylactic efficacy study protocol animals will first be given an antiviral compound, followed by experimental infection with the virus. In the therapeutic efficacy study protocol, the animals will first be infected, and at a later time-point be given the antiviral compound. In the protocols, the absence or decrease in viral load in nasal and tracheal swabs will be measured as an indicator of antiviral activity. Additionally, during the studies the animals will be monitored for adverse effects of the compound, including monitoring of general behaviour and health. A group of non-treated animals will be included as infection controls.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

Only vaccine candidates and antiviral compounds that have previously been tested for safety and efficacy in other test species will be tested for their efficacy in NHP.
Candidates that fulfil these criteria for evaluation in NHP may be directly tested in a vaccine evaluation study (Appendix 2) if the coronavirus that is to be used for establishing capacity of the vaccine to protect against infection has already been used in NHP at our institute. If this is not the case then the virus has to be tested first in a coronavirus infection study (Appendix 1). Requirements to proceed from coronavirus infection study to vaccine evaluation or antiviral efficacy studies are: a) more than 80% of the animals have become infected, b) variation between animals is such that in a vaccine evaluation study less than 10 animals per test group suffice to obtain statistically significant results, c) no animals reach the clinical endpoint within 4 days after infection.

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3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Coronavirus virus infection in nonhuman primates
2	Vaccine evaluation in nonhuman primates
3	Pharmacokinetics (PK) of CoV antiviral compounds in nonhuman primates
4	CoV antiviral efficacy study in nonhuman primates
5	
6	
7	
8	

9	
10	

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 — Type of animal procedure: Coronavirus virus infection in nonhuman primates

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In order to establish the capacity of a vaccine to protect against coronavirus infection it is necessary to have a well-defined coronavirus infection model. For new emerging coronaviruses, or viral strains that have not yet been used in NHP, it is necessary to establish infectivity and pathogenicity in NHP before they can be applied in vaccine efficacy evaluation studies. The main objective is to obtain an infection model that is sufficiently robust to allow adequate evaluation of vaccine efficacy in terms of reduction in clinical symptoms, fever and virus replication.

Typically, the study set-up is as follows: a small number of animals will be infected and monitored for clinical symptoms, fever, changes in body weight and changes in blood parameters. Nasal and tracheal swabs will be taken to determine if the animals have become infected and what the amount of virus production is. To evaluate a new virus, the virus can be inoculated by various (and combinations of) routes, like intravenous, intratracheal, oral, intranasal, intraocular, and aerosol. Proper application for vaccine evaluation requires that more than 80% of the animals become infected and that the amount of virus produced in the respiratory tract over the infection period is clearly measurable and that the variation between the animals is sufficiently low to allow measurement of reduction in virus load in vaccinated animals with less than 10 animals per group. In case these parameters are not achieved then the experiment will be repeated with a higher dose.

The **primary outcome parameters for virus infection** are:

1. Clinical symptoms, fever, virus replication.
2. Pathology in case viruses are used that are known to cause persistent lung pathology.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

At least four weeks before infection, a telemetric temperature transponder will be implanted in the abdominal cavity of the animals that will allow continuous monitoring of body temperature. This time frame is necessary for full recovery of the animals and to allow adequate temperature recording during a two to three-weeks period to establish normal values before infection.
Then, the animals will be infected by intravenous, intradermal, intranasally, intra-tracheally, intra-bronchially using a bronchoscope, via aerosol, or a combination of these routes. At the same time, blood is collected for a baseline-value determination. The animals will be monitored daily during the study period for general behaviour, appetite, faeces, etc., and at each time-point when the animals are sedated, body weight will be measured. Typically, shortly before, and after infection of the animals, nasal and tracheal swabs and/or washes will be taken to measure virus replication in the upper airways. Lung lavages may be taken at selected time points (max 6 times) to measure virus replication in the lungs. Blood is taken simultaneously with the swabs, to monitor changes in clinical chemistry and haematology parameters and leucocyte subsets, as well as to monitor the development of plasma viremia. At the same time points body weight is recorded and imaging (X-ray or PET-CT scan) may be performed to measure lung infiltration. After the animals have become virus-negative in the PCR on swab/wash samples for the first time, they may be followed for 4 weeks to confirm absence of the virus and to monitor for potential re-activations of virus replication. Thus, the length of an infection study will be maximally 6 weeks. At the end of the study, the animals will be humanely euthanized and necropsy will be performed for the collection of tissue samples for histopathological and virological tests. The latter will be done to investigate tissue and organ distribution of the virus, and to identify potential viral reservoirs. Viral reservoirs may contribute to virus re-activation, and are therefore of particular interest.
The details of each study, regarding the NHP species used, route of infection, dose used, etc., will be submitted for approval to the AWB.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The initial experiment, in which the NHPs are inoculated with the virus, will be performed in four animals. Experience by other researchers in the SARS and MERS NHP infection models has shown that with this number of animals an adequate assessment can be made on the reproducibility of infection (all 4 animals need to show virus replication in the trachea), the variability of virus production in the trachea and the amount of fever induction. On the basis of these data a power calculation can be made about the number of animals needed in a vaccine evaluation study. Should the result of these calculations be that more than 10 animals are needed per group, or should not all four animals have become infected, then a new experiment with 4 animals is needed with a higher virus dose. Also, if at a high virus dose the variation between the animals is still too high then it may be necessary to repeat the experiment in another NHP species.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The experiment will be performed in macaques or common marmosets, adult, M/F, n= 60.
Both rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) have been shown to be susceptible to emerging coronaviruses, like SARS and MERS (2-4,7-11), but their susceptibility for infection with the recently emerged SARS-CoV-2 is not yet known. Currently, the first macaque studies are ongoing in other research institutes, and our choice for a specific macaque species will probably be based on the outcome of these studies. Both macaque species have been widely used in emerging coronavirus pathogenesis and vaccine research. Depending on the virus strain used, slight differences were seen in virus replication, clinical signs, and pathology, indicating that both can be used in SARS-CoV-2 studies (4,9).
Common marmosets (*Callithrix jacchus*) have also proven a suitable model for infection with the SARS- and MERS-CoVs (2,12), and generally show more severe clinical signs than macaques. However, the small size of the marmoset complicates sampling of blood, as well as collection using swabs. For this reason, common marmosets will not be the preferred species of choice in this project, but may be used when

macaque species do not meet the necessary requirements for CoV vaccine evaluation, like reproducible infection, or clear signs of disease.
Thus, at this moment we cannot predict which of the NHP species will be used in our studies to establish the infection model.
In the literature, SARS and MERS CoV have been used at a dose of 10^6 to 10^7 $TCID_{50}$ in NHP (4). However, it may be necessary to evaluate an extra dose in some cases. Assuming 4 animals per group, the evaluation of 3 new viral strains, at 3 doses, the number of animals needed will be 36. In case it is necessary to use another NHP species, or another route of infection we calculate the use of 2 x 12 = 24 additional animals. Thus, the total maximum number of animals needed for setting up infection models for new coronaviruses is expected maximally 60 over a period of 5 years.
All animals are purpose bred. They are either bred at our institute or obtained from a certified supplier. Both male and female animals can be used.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

X Yes > Explain why re-use is considered acceptable for this animal procedure.

Animals that will be used in these experiments, have possibly been used in previous experiments. Animals that have pre-existing antibodies against recently emerged coronaviruses are not suitable. In view of the long life of the animals of this species re-use of animals will take place within the limitations described in art 1e of the Wet op de Dierproeven.

Are the previous or proposed animal procedures classified as 'severe'?

X No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement
Several animal species have been used to study coronavirus infection (2-4,7-10). However, of these different species, NHP have the advantage that they physiologically and immunologically most closely resemble humans. This has important implications, both for vaccine evaluation (Appendix 2), as well as for the interaction with coronaviruses, since this is affected both by physiology and by the reaction of the innate and adaptive immune system. As explained in Appendix 2, these aspects are important for the evaluation for vaccines. The proper evaluation of these vaccines requires adequate infection models in NHP, which is the purpose of the studies proposed here.
Reduction
Based on the extensive experience with other viral infection models within the institute where this research will be performed, it is expected that four animals per test group are sufficient in order to determine whether a suitable infection model has been achieved and to perform a power calculation to determine the number of animals needed in a vaccine evaluation study. In case the criteria, as outlined under A are not met, a second experiment may be needed with another dose or in another NHP species. On the basis of the outcome of the first study the number of animals needed in follow up experiments can be calculated and less animals may be needed. Only the minimum number of animals needed, will be used.
Refinement
The use of telemetric temperature recording devices makes it possible to continuously record the temperature during the study-period. For our studies with the H1N1 influenza virus, we have designed a method that allows very precise calculation of fever induction caused by the infection using this method (6). In influenza studies, a significant reduction in temperature by some of the vaccine candidates was observed (5). This method will also be applied in this project. Such precise measurements are not possible with the traditional rectal temperature measurement. Placement of the telemetric device for temperature measurement will require surgery, which will be done under anaesthesia. Subsequently animals will receive

analgesics as long as required. Animals are trained to cooperate as much as possible for the invasive handlings, such as receiving the sedation. The application of X-ray or PET-CT scanning to measure lung infiltration will give us a better insight in the disease progression of the CoV infection.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals will be socially housed with a socially compatible cage mate. There is an extensive program for enrichment in our institute that consists of playing material and methods to present food. During the study animals will be observed daily by qualified animal caretakers. Should changes occur in behaviour, appetite or stool then a veterinarian will be informed and measures will be discussed with the investigator and implemented. During the infection animals will be observed twice daily and clinical symptoms will be scored using a well-established clinical scoring list adapted from Brining et al. (1; see below). On the basis of the scoring system a clinical endpoint is defined. When this endpoint is reached the animal will be immediately euthanized and a necropsy will be performed to determine the cause of disease. All handlings will be performed under sedation. On every time point when a handling is performed the animal will be weighed and closely examined. During the first days of the infection the animal will receive tube feeding. This is necessary, because the daily sedations of the animals necessitate fasting of the animals, and the food intake during this period would otherwise be very limited.
The "Flora and Fauna wet" and "Wet Dieren" do not pose additional requirements that are needed for the type of studies proposed in this application.

Reference List

1. Brining, D. L., Mattoon, J. S., Kercher, L., LaCasse, R. A., Safronetz, D., Feldmann, H., & Parnell, M. J. (2010). Thoracic radiography as a refinement methodology for the study of H1N1 influenza in cynomologus macaques (Macaca fascicularis). *Comparative Medicine*, *60*(5), 389–395.
2. Carrion, R., Jr, & Patterson, J. L. (2012). An animal model that reflects human disease: the common marmoset (Callithrix jacchus). *Current Opinion in Virology*, *2*(3), 357–362.
3. Fouchier, R. A. M., Kuiken, T., Schutten, M., van Amerongen, G., van Doornum, G. J. J., van den Hoogen, B. G., et al. (2003). Aetiology: Koch's postulates fulfilled for SARS virus. *Nature*, *423*(6937), 240.
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5. Marriott, A. C., Dove, B. K., Whittaker, C. J., Bruce, C., Ryan, K. A., Bean, T. J., et al. (2014). Low Dose Influenza Virus Challenge in the Ferret Leads to Increased Virus Shedding and Greater Sensitivity to Oseltamivir. *PloS One*, *9*(4), e94090.
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7. Prescott, J., Falzarano, D., de Wit, E., Hardcastle, K., Feldmann, F., Haddock, E., et al. (2018). Pathogenicity and Viral Shedding of MERS-CoV in Immunocompromised Rhesus Macaques. *Frontiers in Immunology*, *9*, 1814.
8. Rowe, T., Gao, G., Hogan, R. J., Crystal, R. G., Voss, T. G., Grant, R. L., et al. (2004). Macaque Model for Severe Acute Respiratory Syndrome. *Journal of Virology*, *78*(20), 11401–11404.
9. Sutton, T. C., & Subbarao, K. (2015). Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology*, *479-480*, 247–258.
10. van Doremalen, N., & Munster, V. J. (2015). Animal models of Middle East respiratory syndrome coronavirus infection. *Antiviral Research*, *122*, 28–38.

11. Yao, Y., Bao, L., Deng, W., Xu, L., Li, F., Lv, Q., et al. (2013). An Animal Model of MERS Produced by Infection of Rhesus Macaques With MERS Coronavirus. *The Journal of Infectious Diseases*, *209*(2), 236–242.
12. Yu, P., Xu, Y., Deng, W., Bao, L., Huang, L., Xu, Y., et al. (2017). Comparative pathology of rhesus macaque and common marmoset animal models with Middle East respiratory syndrome coronavirus. *PloS One*, *12*(2), e0172093.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

X No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

X No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

X Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

X Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

After placing the telemetric temperature recording device in the abdomen, the animals will receive analgesics for as long as necessary.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Discomfort because of insertion of the temperature recording device.

2. Discomfort due to lung lavages
3. Discomfort due to virus installation
4. Stress because of sedation
5. Reduced food intake during the first days after infection
6. Disease symptoms due to the infection

Explain why these effects may emerge.

1. The surgery needed for insertion of the temperature recording device will cause pain and some local inflammation.
2. For the lung lavages a bronchoscope is used. Insertion will cause irritation
3. When virus is given intra-bronchially a bronchoscope is used and this will cause irritation.
4. Animals will be repeatedly sedated for blood sampling, virus infection, collection of swabs and lung lavages. Nausea can sometimes be observed during recovery from the sedation.
5. Especially during daily sedation during the first days after infection, food intake will be reduced.
6. Coronavirus infections can cause fever, coughing, sneezing, nose discharge, laboured breathing, loss of appetite, loss of weight, inactivity.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

1. Surgery will be done under anaesthesia and after surgery analgesics will be applied.
2. For the lung lavages animals are first deeply sedated and receive a muscle relaxant.
3. The same procedure as described under 2 will be followed.
4. Recovery of the animals is monitored and the veterinarian will intervene if animals do not recover fast enough.
5. Animals will receive tube feeding. This is applied during sedation.
6. Animals are monitored twice daily and a weighed clinical scoring list is used to record the clinical symptoms. When a certain pre-determined clinical score is reached the animal will be humanely euthanized and a full necropsy will be performed to establish the cause of the disease and viral distribution over the respiratory organs

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals are monitored twice daily and a weighed clinical scoring list is used to record the clinical symptoms. When a certain pre-determined clinical score is reached the animal will be humanely euthanized. Individual scores are added and decision is based on the total daily score. Symptoms that lead to an immediate endpoint are respiratory problems (convulsive breathing, flank contraction) or lack of breathing, lethargy as defined by minimal response to human approach, and excessive loss of body weight of more than 15% in two days or 20% from the start of the infection.

Indicate the likely incidence.

100%

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Discomfort is caused by the implantation of the telemetric device. By using this device, the animals can be continuously monitored for changes in body temperature. In combination with frequent observations by animal caretakers, this will facilitate the appropriate intervention by veterinarians at the earliest time-point, and will preclude progression to serious disease caused by coronavirus infection. Therefore, the cumulative discomfort will be moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

X Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be euthanized in case they show serious signs of disease in order to avoid severe discomfort. To investigate the presence of virus in tissues and organs, and for the investigation of possible tissue damage caused by CoV, it is necessary to euthanize the animals at the end of the study

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

X Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: Vaccine evaluation in nonhuman primates

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

We will make use of a general study protocol for the evaluation of CoV vaccines in NHP. Typically, a telemetric temperature recorder/transponder is surgically placed in the abdominal cavity before the start of the study to evaluate body temperature in real-time. Subsequently, the animals are immunized either once or they receive a number of immunizations over a certain time period. Although the vaccines that are used in these studies have already been extensively evaluated in other animal models, and are expected to give no or only very limited adverse effects, animals will be monitored for possible changes in general behaviour and health. The site of immunization will be checked for local reactions and blood will be drawn to measure clinical chemistry and hematology parameters. Before, between, and after immunizations, blood and occasionally nasal and tracheal swabs or washes, and lung lavages will be taken to measure induction of systemic as well as local immune responses. When adequate immune responses are induced that indicate that protection against infection might be achieved, the efficacy against infection will be tested by experimental infection with coronavirus. A group of non-vaccinated animals will be included as infection controls. The infection will be performed as described in Appendix 1.

The **primary outcome parameters** are:

Absence of unexpected reactogenicity of the vaccine: effects of the vaccine on general behavior, health, local reactions and blood parameters.

Immunogenicity: Induction of cellular and humoral immune responses. The type and strength of the induced responses will determine if the objectives of the vaccine strategy are achieved and whether immune responses are sufficiently strong to proceed with viral challenge.

Efficacy: Capacity to protect against viral challenge will be established in terms of reduction in clinical symptoms, fever, virus replication, and changes in blood parameters.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

A telemetric temperature recorder/transponder is surgically placed in the abdominal cavity at least 4 weeks before the first immunization takes place. This time frame is necessary for full recovery of the animals and to allow adequate temperature recording during a 2- to 3-weeks period to establish normal values before immunizations start.
Animals will receive one or more immunizations, typically at 4- to 8-week time intervals, although occasionally a longer time frame is needed between immunizations when different vaccine modalities are used for priming and boosting of the immune response. Usually 3 immunizations suffice over a period of 20 weeks. However, in rare occasions these limits may have to be exceeded. Specific rationale will then be presented to the animal welfare body (AWB).
Immunizations will be done either by intradermal injection, intramuscularly, subcutaneously, intravenously, intranasally, intra-tracheally, intra-bronchially using a bronchoscope, via jet-injection, or via aerosol using a nebulizer. Intravenous injection requires that an isotonic and pH neutral solution is used, under guidance of a veterinarian with a shock set ready. All vaccines will be sterile and will be given under aseptic conditions.
At regular time intervals, usually two and four weeks after every immunization, blood is drawn to measure systemic adverse effects, which includes measurement of clinical chemistry and hematology, and to measure induction of cellular and humoral immune responses. The two-week interval after immunization is chosen because this is optimal for measuring cellular immune responses, while four weeks is optimal for measuring humoral immune responses. The total amount of blood will be less than 1% of the body weight per month and less than 0,7% of body weight per bleeding. This amount can only be exceeded if the specific study requirements leave no other options, specific permission is obtained from the AWB, and the veterinarian agrees, based on the health status of the animal. Occasionally, usually before the start of the study and after the last immunisation, a nasal wash and lung lavage is taken in order to measure induction of local immune responses. Lymph node biopsies may be taken to study locak immune responses in lymphoid tissue. Immune responses recorded after the final immunization will be used to decide whether protection against viral challenge can be reasonably expected. If these responses are inadequate then the study will be stopped and animals may be re-used in other non-coronavirus related experiments. Otherwise, experimental challenge will usually take place between 4 and 8 weeks after the last immunization. This time period is needed to allow immunological memory to form after the last immunization. CoV infection may be done intravenously, intradermally, intranasally, intra-tracheally, or intra-bronchially using a bronchoscope, or via aerosol (Appendix 1). Clinical symptoms will be monitored twice daily during the infection phase. Nasal and tracheal swabs will be taken before infection and at regular time points post-infection to measure virus replication in the upper airways. Lung lavages may be taken at selected time points (max. 6 times) to measure virus replication in the lungs. Blood is taken simultaneously with the swabs, to monitor changes in clinical chemistry and haematology parameters, leucocyte subsets and cytokine production. At the same time points body weights are recorded and imaging (X-ray or PET-CT scan) may be performed to measure lung infiltration. At the end of the study, the animals are humanely euthanized and a full necropsy is performed in order to establish (lung) pathology. In case an animal should reach the clinical endpoint during the study, it will be immediately humanely euthanized and a full necropsy will be performed. Also, tissues will be taken to determine virus dissemination.

The details of each study, regarding the interval between the immunizations, the number and time points of sampling, the specific criteria to proceed with a viral challenge, the time interval between the last immunization and viral challenge will depend on the actual type of vaccine that is being tested and this will be submitted to the AWB.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of animals will be based on statistical power analysis. Calculations account for the number of animals needed to measure statistically significant induction of immune responses in relation to unvaccinated controls or, in case different vaccines are compared, whether significant differences between the vaccine groups can be obtained.

In addition, calculations are performed to establish the number of animals needed to obtain a significant reduction in virus load between the vaccine groups and the challenge control group. Only the minimum number of animals needed will be used. Since historical data are available on infection in unvaccinated animals (Appendix 1), usually less animals can be used in the non-vaccinated challenge control group than in the vaccine groups.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The experiment will be performed in rhesus macaques, cynomolgus macaques and/or common marmosets, adult, M/F, n=150.

Macaque species have been extensively used in coronavirus research (6,7). The most often used species are the rhesus macaque (*Macaca mulatta*) and cynomolgus macaque (*Macaca fascicularis*). Both species are susceptible to an array of (emerging) coronaviruses. The decision to use rhesus or cynomolgus macaques will be based on currently ongoing infection studies with the recently emerged SARS-CoV-2, and studies already performed with SARS and MERS CoVs in NHP. Equally, common marmosets (*Callithrix jacchus*) may be used as they have proven a suitable model for infection with the SARS- and MERS-CoVs (2,8), and generally show more severe clinical signs than macaques. All animals are purpose bred at our institute or obtained from a certified supplier. Both male and female animals can be used. The number of animals calculated assumes that each study will contain two vaccine groups and 1 control group, with max. 10 animals per group. The group size will be determined per experiment, based on power calculation specific for the experiment. As stated above, probably fewer animals will be needed in the non-vaccinated challenge control groups. In all, we anticipate performing 5 such studies over a 5-year period.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

X Yes > Explain why re-use is considered acceptable for this animal procedure.

Animals that will be used in these experiments have possibly been used in previous experiments. Animals that have pre-existing antibodies against recently emerged coronaviruses are not suitable. In view of the long life of the animals of this species re-use of animals will take place within the limitations described in art 1e of the Wet op de Dierproeven.

Are the previous or proposed animal procedures classified as 'severe'?

X No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement

The immune system is very complex and the *in vivo* interactions between virus and/or vaccine and host are not completely understood. At present there is no *in vitro* model available that can mimic the human immune system sufficiently to study the potential of a vaccine to induce protective immune responses. Due to the complex interaction of coronaviruses with different tissues and the role of local immunity in eradication of the virus, the efficacy of a CoV-vaccine to protect against infection can only be adequately established in an animal model. Several animal species have been used to study infection of emerging coronaviruses, like SARS and MERS (3,6,7). However, NHP have the advantage that their immune system most closely resembles that of humans. In addition, the availability of many cross-reactive reagents makes it possible to study in detail the contribution of the innate immune system and to analyse vaccine induced immune responses and evaluate their role in control of infection. These aspects are essential for the evaluation of vaccines, especially for vaccines designed to protect to a range of CoVs. The latter type of 'universal' CoV vaccines will aim for the induction of cross-protective cellular immune responses or

induction of broadly cross-neutralizing antibody responses or non-neutralizing antibody responses that become effective through interaction with innate immune cells. Here, the close homology between the immune system in NHP and humans is essential for proper induction and adequate analysis of these responses, allowing a better extrapolation to the human situation. Evaluation in NHP is therefore needed before clinical evaluation in humans can start.

Reduction

The number of animals needed per experiment will be based on statistical power calculation for achieving statistically significant induction of immune responses and a significant reduction in virus load in the trachea between the vaccine groups and the challenge control group. Only the minimum number of animals needed will be used. Since historical data are available on infection in unvaccinated animals (Appendix 1), usually less animals can be used in the challenge control group than in the vaccine groups. Furthermore, we aim to evaluate multiple vaccine candidates in a single experiment, so that a single challenge control group can be used. This has to be weighed against the fact that in order to obtain significant differences in immune response between the vaccine groups, more animals per vaccine group may be needed.

Refinement

The use of telemetric temperature recording devices makes it possible to record and monitor the temperature in real-time. We have designed a method that allows very precise calculation of fever induction caused by the infection (5). With this method we have observed a significant reduction in temperature by some of the vaccine candidates (4). Such precise measurements are not possible with the traditional rectal temperature measurement. Placement of the temperature responders will require surgery, which will be done under anaesthesia. Subsequently, animals will receive analgesics as long as required, typically 3 days. Animals are trained to cooperate as much as possible for the invasive handlings, such as receiving the sedation. The application of X-ray or PET-CT scan to measure lung infiltration will give us a better insight in the disease progression of the CoV infection.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals will be socially housed with a socially compatible cage mate. There is an extensive program for enrichment in our institute that consists of playing material and methods to present food.

During the study animals will be observed daily by qualified and competent animal caretakers. Should changes occur in behaviour, appetite or stool then a veterinarian will be informed and measures will be discussed with the investigator and implemented. Possible local reactions on the injection site of the vaccine will be recorded at multiple time points using a scoring system that includes redness, swelling and induration. In case substantial induration is seen, then the wound will be treated and analgesics will be applied. During the infection phase, animals will be observed twice daily and clinical symptoms will be scored using a well-established clinical scoring list adapted from Brining *et al.* (1) On the basis of the scoring system a clinical endpoint is defined. When this endpoint is reached the animal will be immediately euthanized and a full necropsy will be performed to determine the cause of clinical disease. All handlings will be performed under sedation. On every time point where a handling is performed the animal will be weighed and closely examined. During the period after infection when animals are sedated daily, they will receive tube feeding. This is necessary, because animals have to fast the night before the sedation and the food intake during this period would otherwise be very limited.

The "Flora and Fauna wet" and "Wet Dieren" do not pose additional requirements that are needed for the type of studies proposed in this application.

Reference List

1. Brining, D. L., Mattoon, J. S., Kercher, L., LaCasse, R. A., Safronetz, D., Feldmann, H., & Parnell, M. J. (2010). Thoracic radiography as a refinement methodology for the study of H1N1 influenza in cynomologus macaques (Macaca fascicularis). *Comparative Medicine*, *60*(5), 389–395.
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6. Sutton, T. C., & Subbarao, K. (2015). Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology*, *479-480*, 247–258.
7. van Doremalen, N., & Munster, V. J. (2015). Animal models of Middle East respiratory syndrome coronavirus infection. *Antiviral Research*, *122*, 28–38.
8. Yu, P., Xu, Y., Deng, W., Bao, L., Huang, L., Xu, Y., et al. (2017). Comparative pathology of rhesus macaque and common marmoset animal models with Middle East respiratory syndrome coronavirus. *PloS One*, *12*(2), e0172093.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

X No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

X No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

X Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

X Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

After placing the temperature recording device in the abdomen, the animals will receive analgesics for as long as necessary, typically 3 days. In previous studies we have observed that animals can experience some fever during the first days after insertion of the temperature recording device, but have recovered very well within 1 week after the operation.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Discomfort because of insertion of the temperature recording device.
2. Discomfort due to injection
3. Discomfort due to lung lavages
4. Discomfort due to virus installation
5. Stress because of sedation
6. Reduced food intake during the first days after infection
7. Disease symptoms due to the infection

Explain why these effects may emerge.

1. The surgery needed for insertion of the temperature recording device will cause pain and some local inflammation.
2. When vaccines are given by injection, this can cause local pain and irritation.
3. For the lung lavages a bronchoscope is used. Insertion will cause irritation
4. When virus is given intra-bronchially a bronchoscope is used and this will cause irritation.
5. Animals will be repeatedly sedated for blood sampling, virus infection, collection of swabs and lung lavages. Nausea can sometimes be observed during recovery from the sedation.
6. Especially during daily sedation during the first days after infection food intake will be reduced.
7. Coronavirus infections can cause fever, coughing, sneezing, nose discharge, laboured breathing, loss of appetite, loss of weight, inactivity.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

1. Surgery will be done under anaesthesia and after surgery analgesics will be applied.
2. Animals will be sedated for vaccine delivery.
3. For the lung lavages animals are first deeply sedated and receive a muscle relaxant.
4. The same procedure as described under 3 will be followed.
5. Recovery of the animals is monitored and the veterinarian will intervene if animals do not recover fast enough.
6. Animals will receive tube feeding. This is applied during sedation.
7. Animals are monitored twice daily and a weighed clinical scoring list is used to record the clinical symptoms. When a certain pre-determined clinical score is reached the animal will be humanely euthanized and a full necropsy will be performed to establish the cause of the disease and viral distribution over the respiratory organs.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals are monitored twice daily and a weighed clinical scoring list is used to record the clinical symptoms. When a certain pre-determined clinical score is reached the animal will be humanely euthanized. Individual scores are added and decision is based on the total daily score. Symptoms that lead to an immediate endpoint are respiratory problems (convulsive breathing, flank contraction) or lack of breathing, lethargy as defined by minimal response to human approach, and excessive loss of body weight of more than 15% in two days or 20% from the start of the infection.

Indicate the likely incidence.

100%

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Discomfort is caused by the implantation of the telemetric temperature transponder. By using this device, the animals can be continuously monitored for body temperature or changes in activity. In combination with frequent observations by animal caretakers, this will facilitate the appropriate intervention by veterinarians at the earliest time-point, and will preclude progression to serious disease caused by coronavirus infection. Therefore, the cumulative discomfort will be moderate

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

X Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be euthanized in case they show signs of disease symptoms in order to avoid severe discomfort. To investigate the presence of virus in tissues and organs, and for the investigation of possible tissue damage caused by CoV or by the vaccines, it is necessary to euthanize the animals at the end of the study.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

X Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: Pharmacokinetics (PK) of CoV antiviral compounds in nonhuman primates

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In order to evaluate the efficacy of an antiviral compound in an NHP model for CoV infection the pharmacokinetics of the compound are tested using different dosages in order to determine the effective compound concentration in plasma after administration (the 'bioavailability'). If the results of such a PK study are positive, then, studies are planned to test the efficacy of the compound in macaques, for use as a prophylactic, as well as a therapeutic drug (Appendix 4).

In general, the study set-up is as follows: a group of animals will receive the compound in different doses. Typically, one group of animals will be intravenously given the compound. This group will act as control group, and will provide baseline data of the main PK parameter, like clearance of compound from the body, volume of distribution, and half-life of the compound. Then, experimental groups will receive the compound in different dosages. After administration, the animals will be bled at regular time points in order to determine the concentration of the compound in the blood.
The primary outcome parameters for the PK are:

1. the maximum concentration of the active compound in plasma, and
2. the total amount of the active compound in plasma in time

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

A typical PK study consists of groups of animals that are given the antiviral compound at different dosages and/or different routes of administration. One group will first receive the compound by intravenous bolus

in order to derive the main PK parameters (clearance, volume of distribution, half-life). Then, other groups will be given the compound at different dosages. The used dosages will be calculated on basis of PK studies in other animal models. Typically, blood samples will be collected at regular time points until 24 h post-administration, and will then be performed daily until the end of the study (maximally 7 days post-administration).
The PK study may be designed as a study in which the animals are first given a low dose of compound. Then, after a wash-out period during which the compound is cleared from the body, the animals can be administered a second, higher dose of the compound, and blood will be collected at regular time points. Plasma from blood samples will then be analyzed for PK parameters.
As a follow-up to the single-dose PK study, animals may also receive antiviral compounds by repeated administration to determine if this prolonged bioavailability leads to either unwanted accumulation of the substance in the blood, or to an equally unwanted decrease because repeated-dosing influences the body metabolism. A prolonged bioavailability is essential when anti-CoV compounds are used as a prophylaxis, i.e. they should protect against CoV infection for a certain period. Essentially, blood sampling will be similar to a single-dose study, with multiple samplings the first day after administration, followed by daily blood collection until the next drug administration.

The details of each study, the number of animals, the route of administration, dose used, and the time points of blood sampling, will be submitted for approval to the AWB.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

A group size of 3 animals is the standard group size for PK studies. From previous PK studies in macaques, it was concluded that experimental groups of three animals are sufficient to obtain a pharmacokinetic profile in blood of the compounds. This group size is sufficiently large to allow insight in the variance between the individual animals and allows us to identify possible outliers.
Block randomization will be used for the group composition. The animals will be randomly allocated to treatment groups according to a randomized block design based on the sex and weight of the animals.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The experiments will be performed in 72 nonhuman primates: rhesus macaques (*Macaca mulatta*), cynomolgus macaques (*M. fascicularis*), or common marmosets (*Callithrix jacchus*). All animals are purpose bred at our institute, or incidentally they will be obtained from a certified supplier. Both mature male and female animals can be used. In contrast to other animal species that are used in virus research, i.e. rodents, NHP have the advantage that their body surface area/mass ratio, drug metabolism, pharmacokinetics, and anatomical structure are highly comparable to that of humans (1-5). As a consequence, drugs are metabolized in a similar way in NHP as in humans, and also exert their mode of action similarly. This renders NHP an important preclinical animal model to investigate the pharmacokinetics of potential anti-CoV compounds for human use.

In a period of 5 years we expect to test a maximum of five antiviral compounds. On basis of *in vitro* studies, and studies done in rodent models, a dosage range will be set that will then be evaluated in NHP. For each compound, we plan to evaluate maximally five dosages in a single-dose PK study. The exact number of dosages that will be tested per compound will be decided per study, as this depends on the outcomes from in vitro studies and studies in rodents, as well as on experience from studies with related compounds.

The exact design of the study, including the NHP species used, and number of dosages, will be submitted for approval to the AWB.

In each PK study, 2 groups of 3 animals will receive 3 different dosages of the drug, as described in section A. For the single-dose PK studies we plan to test maximally eight compounds. Per compound we

will test 6 different dosages (5 dosages+ 1 standard control dose given) in 3 groups of 3 animals = 9 animals per compound. For 8 compounds we will need 72 animals. For each compound, a repeated-dose administration study is planned with groups of 3 animals. These animals will be selected from the group of animals already used in the single-dose PK study.

Thus, over a 5-year period, maximally 72 animals will be needed for the PK studies.

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4. Chan, J. F.-W., Yao, Y., Yeung, M.-L., Deng, W., Bao, L., Jia, L., et al. (2015). Treatment With Lopinavir/Ritonavir or Interferon-β1b Improves Outcome of MERS-CoV Infection in a Nonhuman Primate Model of Common Marmoset. *Journal of Infectious Diseases*, *212*(12).
5. Orsi, A., Rees, D., Andreini, I., Venturella, S., Cinelli, S., & Oberto, G. (2011). Regulatory Toxicology and Pharmacology. *Regulatory Toxicology and Pharmacology*, *59*(1), 19–27.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

X Yes > Explain why re-use is considered acceptable for this animal procedure.

Animals that will be used in these experiments, have possibly been used in previous experiments. In view of the long life of the animals of this species reuse of animals will take place within the limitations described in art 1e of the Wet op de Dierproeven.

Are the previous or proposed animal procedures classified as 'severe'?

X No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

All compounds have been tested extensively for efficacy and toxicity in *in vitro* assays. Also, *in vivo* toxicity studies have been performed in rodents. Only when no side effects or signs of toxicity are found in the rodent model, the compounds will be evaluated in nonhuman primates.
In this animal procedure, the pharmacokinetic profile of anti-CoV compounds is determined in NHP, before their use in antiviral compound efficacy testing in the same animal species. A group size of 3 animals is the standard group size for PK studies. From previous PK studies, it was concluded that experimental groups of three animals are sufficient to obtain a pharmacokinetic profile of the compounds in blood of macaques. This group size allows us insight in the variance between the individual animals, and at the same time allows us to identify possible outliers. Only the minimum number of animals needed, will be used.

Supplementary nutritious and calorie-rich diet is administered (via a gavage) when the animals are daily sedated for blood sampling or compound administration. This eliminates the possible negative effects of fasting for the purpose of frequent sedation. Compound administration and blood sampling take place under sedation, and at the same time the animals will be weighed and examined. The animals are trained to work as much as possible voluntarily on invasive biotechnological actions such as giving anaesthesia or compound administration. In consultation with our collaborators, the number of blood samplings, and the collected volumes of blood will be reduced to a minimum.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

For the PK studies using anti-CoV compounds, no safety and/or environmental risks can be envisioned. All compounds have been tested *in vitro* and *in vivo in rodents* for their potential to inhibit CoV virus, but also for presence/absence of toxicity, before their evaluation in nonhuman primates.
Animals will be socially housed with a socially compatible animal, whenever possible. There is an extensive program for environmental enrichment in our institute. During the study animals will be observed daily by qualified animal caretakers. Should changes occur in behaviour, appetite or stool then a veterinarian will be informed and measures will be discussed with the investigator and implemented. All handlings will be performed under sedation. On every time point when a handling is performed the animal will be weighed and closely examined. During the period that an animal is bled daily, the animal will receive tube feeding. This is necessary, because animals have to fast the night before the sedation and since animals will be sedated daily after infection the food intake during this period would otherwise be very limited.
The studies will be performed according to Dutch laws, and it is not expected that the experiments will have adverse effects on the environment

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

X No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

X No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

X Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

X Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

In case an animal suffers pain, oral or parenteral analgesia may be administered after consultation with the veterinarian.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Stress because of recovery from sedation
2. Reduced food intake due to repeated daily sedations for blood collection and compound administration
3. Discomfort due to administration of compound via gavage

Explain why these effects may emerge.

1. Animals will be repeatedly sedated for blood sampling and compound administration. Nausea can sometimes be observed during recovery from the sedation.
2. Animals will be sedated daily during the PK study for blood collection. Therefore, the animals will have to fast during that period.
3. Insertion of the tube may cause local irritation

Indicate which measures will be adopted to prevent occurrence or minimise severity.

1. Recovery of the animals is monitored and the veterinarian will intervene if animals do not recover fast enough.
2. Animals will receive tube feeding via gavage. This is applied during sedation.
3. Insertion of the feeding tube will be done by experience caretakers. In case irritation occurs, this will be mild and no extra measures need to be taken

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

It is not expected that animals will reach a humane endpoint due to the PK study. However, through intensive monitoring of the animals, deviations in behavior, appetite etc. will be noted quickly. In addition, the animals are weighed at each blood collection. A major weight loss of > 20% relative to the weight at the start of the study is seen as humane endpoint. In case an animal is seriously ill, as judged by the veterinarian, it will be humanely euthanized.

Indicate the likely incidence.

it is not expected that the animals reach the humane endpoint criteria. Therefore, the percentage is zero.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative discomfort is estimated as moderate. This is mainly caused by the daily sedations after compound administration

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

X No

☐ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☐ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 4 Type of animal procedure: CoV antiviral efficacy study in nonhuman primates

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In order to evaluate the use of antiviral compounds to prevent or treat CoV infection, we will use the following general study set-ups:

1. Prophylactic treatment: a group of animals will receive the compound. Then, the animals will be challenged with CoV (Appendix 1), and nasal and tracheal swab samples are collected at regular time points to determine if the animals have been protected against infection, or if the virus load is influenced by the prophylactic administration of the compound. A group of animals will not receive the compound, and will be used as controls.
2. Therapeutic treatment: a group of animals will be experimentally infected with CoV (Appendix 1). Then, the animals will be administered the compound, and nasal and tracheal swab samples are collected at regular time points to determine if the virus load is influenced by the therapeutic administration of the compound. A group of animals will not receive the compound, and will be used as controls.

When a compound is evaluated for its therapeutic potential, i.e. to cure infection, animals will first be infected with the virus, and subsequently the compound will be administered. During the study, nasal and tracheal swabs are collected at regular time points and tested for the presence or absence of virus.

The primary outcome parameter for antiviral efficacy will be the reduction of viral load in nasal and tracheal swabs.
Secondary outcome parameters for CoV infection that may be evaluated are:

1. Absence or reduction of fever caused by CoV infection
2. Absence or reduction of clinical symptoms caused by CoV infection
3. Absence or reduction of lung pathology caused by CoV infection

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

A telemetric temperature transponder is surgically placed in the abdominal cavity at least 4 weeks before the first compound administration takes place (prophylactic treatment), or before experimental infection (therapeutic treatment). This timeframe is necessary for full recovery of the animals from the surgery, and to allow adequate body temperature recording during a two to three-week period to establish normal values before the administration/infection start.

Prophylactic antiviral study:
At the start of the study, the animals will receive the antiviral compound. The route of administration and the dosage used are based on PK studies with this compound performed in NHP (Appendix 3). At the same time blood is collected for a zero-value determination. Then, the animals are experimentally infected. The optimal route and inoculum dose are determined in an infection study with this inoculum (Appendix 1). Also, at that time point, a group of animals that did not receive the compound will also be infected, and will act as untreated controls in the study. Typically, after infection of the animals, swabs and blood will be collected daily for a period of maximally 14 days to monitor the progress of the viral infection and to control for changes in clinical chemistry and hematology parameters. This intensive sampling is necessary because in this period significant and rapid changes in the amount of virus in the blood may occur in untreated animals. During this period of daily sampling the animals will be given liquid foods by means of a probe because the daily anesthesia necessitates fasting of the animals. In this way, the wellbeing of the animals is affected as little as possible. After this period, the frequency of sample collection will be brought down to maximally once every two days. After the untreated control animals have become virus-negative in the PCR for the first time, the groups may be followed for an extra period of 3-4 weeks to confirm absence of the virus and to monitor for sudden re-activations of virus replication in any of the animals. At the end of the study, maximally 6 weeks after the start, the animals will be humanely euthanized and necropsy will be performed for the collection of tissue samples for histopathological and virus tests. The animals will be monitored daily during the study period for general behaviour, appetite, faeces, etc., and at each time-point when the animals are sedated, body weight and will be measured.

Therapeutic antiviral study:
The set-up of a therapeutic study using the antiviral compound is essentially similar to the prophylactic study. However, in such a study the animals are first infected and then treated with the antiviral compound.

The details of each study, regarding the route of infection, dose used, number of animals used, will be submitted for approval to the AWB.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of animals will be based on statistical power analysis. Calculations account for the number of animals needed to measure statistically significant reduction in virus load in relation to untreated controls. In addition, calculations are performed to establish the number of animals needed to obtain a significant reduction in virus load in the trachea between the treated groups and the untreated control group. Only the minimum number of animals needed will be used. Since historical data are available on infection in untreated animals (Appendix 1), usually less animals can be used in the control group than in the antiviral-treated groups.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The experiments will be performed in NHP (rhesus macaques, cynomolgus macaques and/or common marmosets), adult, M/F, n=100.
All NHP are purpose bred at our institute, or incidentally they will be obtained from a certified supplier. Both mature male and female animals can be used.

Rhesus macaques (*Macaca mulatta*), cynomolgus macaques (*Macaca fascicularis*), and common marmosets (*Callithrix jacchus*) are susceptible to an array of (emerging) human coronaviruses (5-9), and macaques have already been used in coronavirus antiviral research (1-4). The decision to use a specific NHP species will be based on currently ongoing infection studies with the recently emerged SARS-CoV-2, and studies already performed with SARS and MERS CoVs in NHP. The decision will be submitted for approval to the AWB.

The calculated number of animals assumes that each study will contain 1 treatment group and 1 control group, with max. 10 animals per group. The group size will be determined per experiment, based on power calculation specific for the experiment. As stated above, probably fewer animals will be needed in the non-vaccinated challenge control groups. In all, we anticipate performing 5 such studies over a 5-year period with 5 x 20 = 100 animals

References:

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C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

X Yes > Explain why re-use is considered acceptable for this animal procedure.

Animals that will be used in these experiments, have possibly been used in previous experiments. Animals that have been involved in previous CoV studies or that have pre-existing antibodies against CoVs are not suitable because of possible immunological cross-reactivity between the different CoVs. In view of the long

life of the animals of this species reuse of animals will take place within the limitations described in art 1e of the Wet op de Dierproeven.

Are the previous or proposed animal procedures classified as 'severe'?

X No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement

Although animal models for CoV infection, other than nonhuman primates (NHP), are also in use in research for CoV, NHP are the animal model that best mimic infection and pathogenesis in humans. Equally, in contrast to other animal species that are used in biomedical research, like rodents, NHP have the great advantage that their body surface area/mass ratio, drug metabolism, pharmacokinetics, and anatomical structure are highly comparable to that of humans. As a consequence, drugs are metabolized in a similar way in NHP as in humans, and also exert their mode of action similarly. This, in combination with the fact that CoV infection of NHP mimics infection and pathogenesis in humans, renders them preclinical animal models of choice to investigate the efficacy of potential anti-CoV compounds for human use.

Reduction

This study involves the efficacy testing of antiviral compounds in the CoV infection model in NHP. Because the variability in viral replication kinetics in the NHP will only become available after the completion of the infection studies, the exact number of animals to be used in the studies cannot be provided at this point. Under A we have described the statistical analyses that will be performed on basis of the infection studies. Only the minimum number of animals needed will be used. If possible, studies will be combined. In such a case, one control group will suffice and the total number of animals will be reduced.

Refinement

Supplementary nutritious and calorie-rich diet is administered when the animals are daily sedated for blood sampling. This eliminates the possible negative effects of fasting for the purpose of frequent sedation. Infection and bleeding take place under sedation, and at the same time the animals will be weighed and examined. The animals are trained to work as much as possible voluntarily on invasive biotechnological actions such as giving anaesthesia or virus infection. In consultation with our collaborators, the number of blood samplings, and the collected volumes of blood will be reduced to a minimum.

The use of telemetric temperature recording/transponder devices makes it possible to record the temperature 24/7, and to monitor the body temperature in real-time. We have designed a method that allows very precise calculation of fever induction caused by the infection (5). With this method we have observed a significant reduction in temperature by some vaccine candidates (4). Such precise measurements are not possible with the traditional rectal temperature measurement. Placement of the telemetric temperature devices will require a small surgery, which will be done under anaesthesia. Subsequently, animals will receive analgesics as long as required. The use of imaging (X-ray or PET-CT scan) will provide us with data regarding lung pathology.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals will be socially housed with a socially compatible animal, whenever possible. There is an extensive program for environmental enrichment in our institute.
All experimental procedures will be performed under sedation. Each time an animal is sedated, the animal will be weighed, and the animal will be closely examined. Our institute uses a customized database that documents all individual animals in the institute. General observations like behaviour, appetite and stool are part of this database. This database thus facilitates early recognition of minor changes in these general parameters. During the study, care will be taken to avoid pain. In case an animal suffers from pain, a veterinarian will be informed, and the animal will receive analgesics to relief the pain, if necessary.
During the first 2 weeks of the infection the animal will receive tube feeding. This is necessary, because the daily sedations of the animals necessitate fasting of the animals, and the food intake during this period would otherwise be very limited.
Regular analysis of haematological and clinical chemistry parameters is part of the experiment. During these experiments, the virus load in plasma will also be analysed as primary indicator of infection. These data will also be consulted to determine if changes in behaviour, appetite or stool are clinically relevant. If necessary, judged by the veterinarian, measures will then be taken to treat the animal.
The studies will be performed according the Dutch laws, and will cause no adverse effects on the environment.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

X No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

X No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

X Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

X Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

In case of symptoms caused by CoV infection, this can result in pain. For this purpose, oral or parenteral analgesia will be administered after consultation with the veterinarian.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Discomfort due to virus inoculation
2. Stress because of sedation
3. Reduced food intake due to repeated daily sedations

Explain why these effects may emerge.

1. Intradermal inoculation can cause mild irritation
2. Animals will be repeatedly sedated for blood sampling and virus inoculation. Nausea can sometimes be observed during recovery from the sedation.
3. Animals will be sedated daily during the first phase of the infection. This will have influence of the appetite

Indicate which measures will be adopted to prevent occurrence or minimise severity.

1. If irritation occurs, this will be mild. It will therefore not be necessary to take additional measures.
2. Recovery of the animals is monitored and the veterinarian will intervene if animals do not recover fast enough.
3. Animals will receive tube feeding via gavage. This is applied during sedation for blood collection.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

X Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals are monitored twice daily and a weighed clinical scoring list is used to record the clinical symptoms. When a certain pre-determined clinical score is reached the animal will be humanely euthanized. Individual scores are added and decision is based on the total daily score. Symptoms that lead to an immediate endpoint are: open mouth breathing or cyanosis, lethargy as defined by minimal response to human approach.

Indicate the likely incidence.

After CoV infection each animal may become seriously ill. Thus, the percentage is 100%

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Discomfort is caused by the implantation of the telemetric device. By using this device, the animals can be continuously monitored for body temperature. In combination with frequent observations by animal caretakers, this will facilitate the appropriate intervention by veterinarians at the earliest time-point, and will preclude progression to serious disease that may be caused by coronavirus infection. Therefore, the cumulative discomfort will be moderate

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

X Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be euthanized in case they show signs of disease symptoms in order to avoid severe discomfort. To investigate the presence of virus in tissues and organs, and for the investigation of possible

tissue damage caused by CoV or by the compounds, it is necessary to euthanize the animals at the end of the study
Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?
☐ No > Describe the method of killing that will be used and provide justifications for this choice.
X Yes