Centrale Commissie Dierproeven

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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0800-7890789).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50200
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 numbers provided at 3.4.3 of the project proposal.*	Serial number: 2 — Type of animal procedure: Coronavirus vaccine evaluation in NHP

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.

Animals will receive a number of immunizations and will thereafter be infected with a CoV. Immune responses will be monitored and following infection the protective effect of the vaccine will be evaluated (see below for a detailed description of the procedures).

Outcome parameters are the following:

- Absence of unexpected reactogenicity of the vaccine: effects of the vaccine on general behaviour, health, local reactions and blood parameters.
- Immunogenicity: Induction of cellular and or 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 suggest that protection is potentially achievable.
- 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.

A primary outcome measure will be selected from the above mentioned outcome measures based on the scientific hypothesis to be tested.
The following parameters are considered as a primary outcome measure:

- Virus load. The amount of virus RNA detected in the target organ (in life or at endpoint).
- Fever induction. The amount of change of body temperature during infection.
- Target organ pathology. Histopathological assessment of target organ.

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

A telemetric temperature sensor 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 2- 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. The 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, or spray device. Intravenous injection requires that an isotonic and pH neutral solution is used, under guidance of a veterinarian. 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 haematology, 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 in charge, based on the health status of the animal. Occasionally, and 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 local 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 halted 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 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 using a nebuliser or via a combination of the aforementioned methods (Appendix 1). Clinical symptoms will be monitored twice daily during the infection phase using a weighed clinical scoring list according to Brining et al 2010 (1). 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 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 (CTs or PET-CT) may be performed to measure lung infiltration. At the end of the study, the animals are killed 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 killed, 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. The maximum number of procedures as outlined in the table below is based on the current state of the art, future experiments, however, may require higher maximum numbers. The details of each future study, regarding the NHP species used, route of infection, dose used, etc., will be submitted for approval to the Animal Welfare Body (Instantie voor Dierenwelzijn; IvD).
Table. Maximum number of repeats per procedure.

Procedure	Maximum	Duration
Sedation	24	15-60 min
Recorder in / out	2	30 min
Immunization	6	60 min
Blood sample	16	30 min
Challenge	1	30 min
(PET-) CT scan	16	60 min
BAL	16	30 min

Nose and throat swabs	16	30 min
Killing	1	15 min

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

Based on the estimates obtained for the selected primary outcome measure, during the development of a coronavirus infection model in NHP as described in Appendix 1, a power calculation will be performed to define the minimum number of animals required in a vaccine evaluation study.

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
2	Rhesus or cynomolgus macaque	Purpose bred	Adult	150	M/F	Not applicable	Not applicable

Provide justifications for these choices

Species	Several research groups, including BPRC, have established nonhuman primate (NHP) models for infection with coronaviruses like SARS-CoV-1, SARS-CoV-2 and MERS CoV (2-22). Mostly widely used in CoV research are rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) and their susceptibility for infection with coronaviruses is well established.
Origin	All animals are purpose bred. They are either bred at our institute or obtained from a certified supplier
Life stages	Adult animals will be used
Number	150 macaques in total (either cynomolgus or rhesus). The number of animals requested is based on the assumption that each study will contain one vaccine group and 1 control group, with maximally 15 animals per group. The group size will be determined per experiment, based on power calculations specific for the experiment. Probably fewer animals will be needed in the non-vaccinated challenge control groups and also in the vaccine groups in some of the studies. In all, we anticipate performing 5 such studies over a 5-year period.
Gender	Adult male and female animals can be used. Since there are immunological differences between males and females (23, 24), we prefer that for each individual experiment either all animals are male or all are female, in order to minimise variation within the experimental test group, thereby increasing the probability of finding statistically significant differences between the experimental groups. This choice is also important regarding the amount of blood needed to perform all assays. Male animals are usually larger than female animals and therefore more blood can be drawn per time point from males. In case assays need to be performed that require large amounts of blood, male animals are preferred over females.
Genetic alterations	Not applicable
Strain	Not applicable

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☒ Yes

☐ No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☐ No

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

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

☒ 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 sensor 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 are expected not to experience pain 1 week after the operation.

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

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

Explain why these effects may emerge.

1. The surgery needed for insertion of telemetric temperature sensor 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. PET-CT requires extra sedation period for the animals
6. 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.
7. Especially during daily sedation during the first days after infection food intake will be reduced.
8. 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. The same procedure as described under 3 will be followed.
6. Recovery of the animals is monitored by the animal caretakers (and 24/7 by camera) and the veterinarian will intervene if animals do not recover fast enough.
7. Animals will receive a calorie-rich diet or tube feeding which is applied during sedation.
8. Animals are monitored (twice) daily (and 24/7 by camera), and a weighed clinical scoring list is used to record the clinical symptoms (1). When a clinical score of 35 is reached this indicates that the maximum duration of severity is reached then the animal will be killed and a full necropsy will be performed to establish the cause of the disease and viral distribution over the respiratory organs. Individual scores are added and the decision is based on the total daily score and veterinarian assessment of discomfort.

E. 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 F

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

Animals are monitored (twice) daily (and 24/7 by camera), and a weighed clinical scoring list is used to record the clinical symptoms (1). When a clinical score of 35 is reached this indicates that the maximum duration of severity is reached then the animal will be killed and a full necropsy will be performed to establish the cause of the disease and viral distribution over the respiratory organs. Individual scores are added and the decision is based on the total daily score and veterinarian assessment of discomfort. 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. The endpoint will be determined in consultation with the researcher and veterinarian.

Indicate the likely incidence.

Maximally 35%

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

Discomfort is caused by the implantation of the telemetric temperature sensor, the other procedures and symptoms due to infection with CoV. 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.

G. 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, MERS and SARS-CoV-2 (2, 3, 8, 10). 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.
Refinement	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 as per guidelines for macaques (25). The use of telemetric temperature sensor makes it possible to continuously record the temperature during the study-period. For our studies with respiratory viruses we have designed a method that allows very precise calculation of fever induction caused by the infection using this method (26). In influenza and SARS-CoV-2 studies, a significant reduction in fever by some of the vaccine candidates was observed Ref Mooij et al. (26). This method can also be applied in this project. Such precise measurements are not possible with the traditional rectal temperature measurement. Placement of the telemetric temperature sensor for body 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 procedures, such as receiving the sedation. The application of CT or PET-CT scanning to measure lung infiltration will give us insight in the disease progression of the CoV infection. CTs or PET-CT will be performed when animals are already sedated for sampling of blood and swabs and will thus not cause additional discomfort. 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.. Based on the scoring system a clinical endpoint is defined. When this endpoint is reached the animal will be immediately be humanely killed and a necropsy will be performed to determine the cause of disease. All procedures 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 or an adapted calorie rich diet. 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 an 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.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☒ No

☐ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☐ No > Continue with question I.

☒ 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 procedures. 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'?

☒ No

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

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Not applicable

J. Location where the animals procedures are performed

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

☒ No > Continue with question K.

☐ 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.

End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

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

Animals will be humanely killed 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 kill the animals at the end of the study. It was previously shown that for instance SARS-CoV-2 can stay present for a very prolonged time (27). Therefore it is not possible to reuse the animals for other studies or to rehouse and retire them.

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 > Will a method of killing be used for which specific requirements apply?

☒ No > Describe the method of killing.

Killing is done by injecting an anaesthetic dose of ketamine followed by intravenous overdose of barbiturate.

☐ Yes > Describe the method of killing that will be used and provide justifications for this choice.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

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