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: 3 — Type of animal procedure: Coronavirus vaccine evaluation under the "Animal Rule"

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.

The Food and Drug Administration (FDA) Animal Rule was devised to facilitate approval of candidate vaccines and therapeutics using animal survival data when human efficacy studies are not practical or unethical (1-5). This regulatory pathway is critical for candidates against pathogens with high case fatality rates that prohibit human challenge trials, as well as candidates with low and sporadic incidences of outbreaks that make human field trials difficult (1, 2). Important components of a vaccine development plan for Animal Rule licensure are the identification of an immune correlate of protection and immunobridging to humans (1, 2). The relationship of vaccine-induced immune responses to survival after vaccination and challenge must be established in validated animal models and then used to infer predictive vaccine efficacy in humans via immunobridging (1). In essence this implies that an animal model is used to establish the type and magnitude required to provide protection against infection or disease with a certain pathogen. The next step (immunobridging) will then take place in phase I and phase II clinical trials in humans, to demonstrate that the vaccine strategy is able to generate the same type and magnitude of responses in humans, assuming that these responses then provide the same level of protection... The steps under the Animal Rule are similar to what is described in Appendices 1 and 2. Because the Animal Rule applies to the licensure of a vaccine, the criteria are more stringent (i.e. require more animals) than those outlined in Appendices 1 and 2.
As a first step a virus challenge dose that reproducibly yields a pre-defined virus load needs to be established, where several challenge doses will be evaluated as described in Appendix 1. The challenge dose identification will, in most cases, be performed in iterative sequential experiments, i.e. the first experiment will be performed with a dose that is expected to yield the desired outcome. If the desired outcome is not achieved, the experiment will be repeated with an adapted virus dose until the desired outcome is achieved.

The virus challenge dose that yields the pre-defined virus load, as established in the initial step, then must be validated in an independent second challenge study as described in Appendix 1.
The next step is the identification of a correlate of protection (CoP), which will be done in two steps. In the initial step several vaccine doses (e.g. 4 and a placebo) will be tested in about half of the final group size as determined in the power calculation (as described in appendix 2) such that a range of immune responses are induced aiming at 50% protection (1). All animals are then infected using the validated virus challenge dose, and virus loads are determined in the target tissues. Following statistical analysis of the data obtained an initial CoP is then estimated. In the second step vaccine doses may be adapted, based on the results of the initial step, such that the desired 50% protection level is achieved, and the second half of the group size as determined in the power calculation will then be vaccinated using several vaccine doses and thereafter challenged with the validated virus challenge dose as described in Appendix 2, and virus loads are determined in the target tissues. The CoP is then established, with the pre-specified error probabilities, using the combined results obtained in steps 1 and 2.
The CoP thus established will then be used as a surrogate CoP in a clinical trial. When the vaccine is shown to be capable of inducing immune responses exceeding the established CoP in human volunteers the vaccine potentially qualifies for registration with the FDA.

Go / No-Go criteria for vaccine evaluation under the "animal rule":
The criteria to consider vaccine candidates for evaluation are:

- The safety, immunogenicity, and protective efficacy of vaccine candidate should have been demonstrated in a pre-clinical NHP study.
- Preliminary data on the CoP to be established needs to be available. These include the nature of the CoP (humoral or cellular immunity), and the availability of statistical parameters required to determine the number of animals required to establish the CoP with pre-specified error margins.
- Validated laboratory assays for establishing the CoP need to be available.
- GMP-grade vaccine needs to be available.

The decision trees with go/no go criteria for viruses and vaccines are shown graphically in Figures 1 to 2 below.

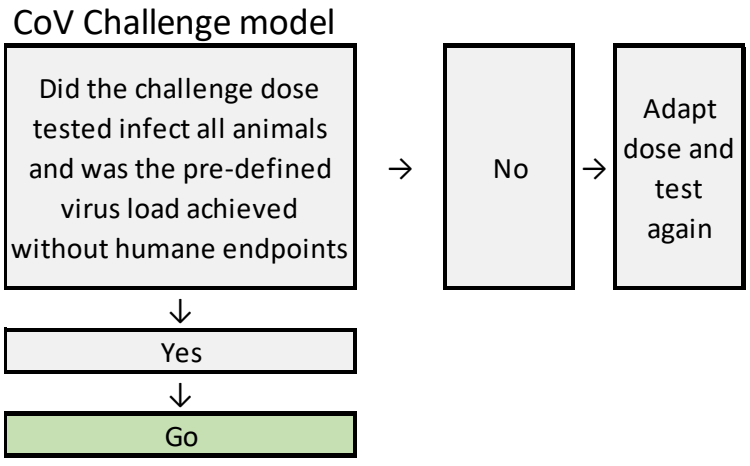

Figure 1. Decision tree for establishing the MERS-CoV challenge dose

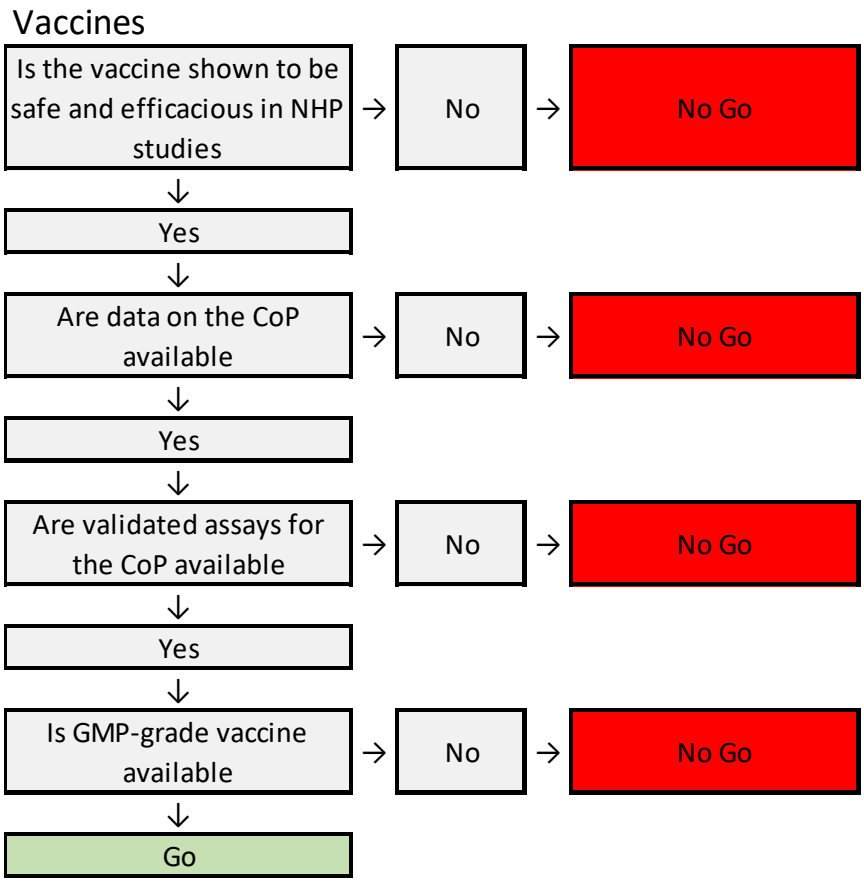

Figure 2. Go No Go decision tree for the MERS-CoV vaccine.

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

The proposed animal procedures to be used for the establishment of a validated challenge model are described in Appendix 1 (Development of a coronavirus infection model in NHP).
The proposed animal procedures to be used for the establishment of a Correlate of Protection (CoP) and validation of the CoP are described in Appendix 2 (Vaccine evaluation in NHP).

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

For the set-up and validation of the challenge model a maximum of 16 animals will be used (i.e. 4 doses tested in groups of 4 animals) and maximally 8 animals will be used for the validation of the challenge dose. The number of animals required for the establishment of the CoP will be done using the following three step approach: 1. The regulatory acceptable degree of precision of the CoP (i.e. confidence intervals for 75% and 90% protection) is estimated using results previously obtained for ebola (6). 2. The association between the envisaged CoP and outcome is estimated using results obtained in pre-clinical studies with the CoV under investigation. 3. Simulations using increasing group sizes (40 to 100) are run using the estimate obtained in step 2, and the precision of the CoP for each of the simulated group sizes are then calculated. The minimum group size achieving the pre-specified precision is then selected for the establishment of the CoP.
The total maximum number of animals required per experiment therefore is 24 + 100 = 124. In all, we anticipate performing maximally 2 such studies over a 5-year period.

A power calculation will be performed such that the EMA guidelines are fulfilled. The EMA states that: "The evaluation should include a detailed description of issues relating to the study design and statistical considerations, including error probabilities, hypotheses, and assumptions for recruitment and follow-up, sample size calculation and methodology" (20). Following discussions with the EMA the following strategy was chosen to calculate the number of animals:

The CoP needs to be established with pre-specified bandwidths for 75 and 90% protection. To this end the previously accepted P75 and P90 (i.e. at 75 and 90% protection) bandwidths for an Ebola vaccine were calculated (6). These bandwidths are 0.5 (=3.16-fold) and 0.65 (= 4.47-fold) log10 units for the P75 and P90, respectively.
Simulations (500 per condition) using estimates for the strength of association (actual estimate as well as lower and upper 80 confidences intervals) between the CoP obtained in a MERS-CoV NHP study (4) are then performed using group sizes ranging from 40 to 100 (increasing by 10) using an average protection rate of 50% in the simulations.
The resulting data from the simulations are then analysed using logistic regression with protection (yes / no) as a binary outcome measure and log10-transformed anti MERS-CoV Spike protein IgG as explanatory variable. The ensuing P75 and P90 CoP bandwidths are then calculated using the parameters obtained from the logistic regression.
The statistical power is defined as the fraction of P75 and P90 estimates that are within the pre-defined bandwidths. The power is set at 90% i.e. 9 out of 10 experiments should yield estimates that are within the desired bandwidths.

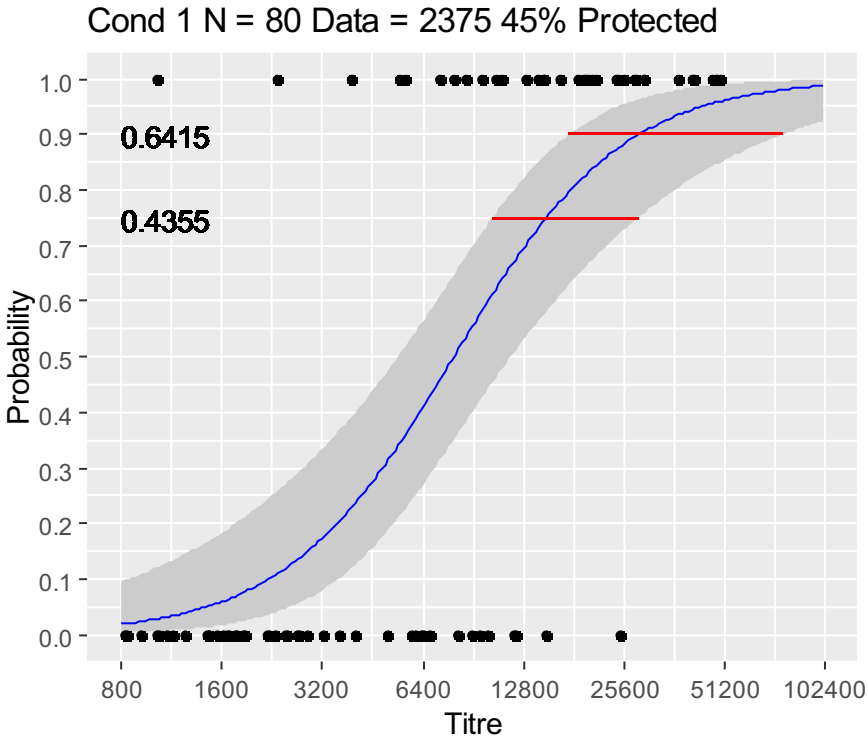

Figure 3. Representative example of a simulation with a group size of 80, P75 and P90 bandwidths are indicated by red lines and the numbers indicate the width in log10 units.

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
3	Rhesus or cynomolgus macaque	Purpose bred	Adult	248	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 (7-27). 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
Number	248
Gender	Adult male and female animals will be used, since there are immunological differences between males and females (28, 29). This is important because the CoP needs to be applicable to the human population.
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

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. 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 (30). 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 (30). 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 handlings and the experimental 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(7, 8, 13, 15). However, NHP have the advantage that their immune system most closely resembles that of humans. This is particularly relevant for evaluation of vaccine candidates for human use under conditions of animal rule. Direct translationability to the human situation is mandatory here. 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 group sizes requested by the FDA. Group sizes for experiments under the "animal rule" are substantially larger than those in regular vaccine evaluation experiments. This is because experiments under the "animal rule" are used as a substitute for clinical trials with the ultimate goal of obtaining an FDA product registration.
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 (31). 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 (32). In influenza and SARS-CoV-2 studies, a significant reduction in fever by some of the vaccine candidates was observed Ref Mooij et al. (32). 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. (30). 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.

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.

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

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

☒ No > Describe the method of killing.

☐ 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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