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

<table>
<thead>
<tr>
<th>1.1 Provide the approval number of the ‘Netherlands Food and Consumer Product Safety Authority’.</th>
<th>50200</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 Provide the name of the licenced establishment.</td>
<td>Biomedical Primate Research Centre</td>
</tr>
<tr>
<td>1.3 List the serial number and type of animal procedure. Use the numbers provided at 3.4.3 of the project proposal.</td>
<td>Serial number 5 Type of animal procedure CoV antiviral efficacy study in NHP</td>
</tr>
</tbody>
</table>

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.

To evaluate the use of antiviral compounds to prevent or treat CoV infection we will use the following general study set-up for a 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 as well as bronchoalveolar lavages (BAL) 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. 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 or reduction of viral load in target tissues.

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 sensor 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.
Antiviral study set up:
At the start of the study, the animals will experimentally infected. The optimal route and inoculum dose are determined in an infection study with this inoculum (Appendix 1). At that same time point, a group of animals that will not receive the compound are also infected and will act as untreated infection controls in the study. Next, the animals will receive the antiviral compound. The route of administration and the dosage to be used are based on PK studies with this compound performed in NHP (Appendix 4), or they are based on studies performed by collaborating institutes. At the same time blood is collected for a zero-value determination. 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 haematology parameters. This intensive sampling is necessary because in this period significant and rapid changes in the virus load may occur in untreated animals. The daily anaesthesia may cause weight loss of the animals, to reduce this the animals will be given an adapted high calorie diet, or tube feeding during sedation. After this period, the frequency of sample collection will be reduced 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, typically 8 weeks after the start, but longer follow-up periods may be required (e.g. long Covid). At the end of the study, the animals will be killed 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. The application of CT or PET-CT scanning to measure lung infiltration will give us insight in the disease progression of the CoV infection, and how this is influenced by the antiviral compound. CT or PET-CT scanning will be performed when animals are already sedated for sampling of blood and swabs and will thus not cause additional discomfort.

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.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Maximum</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedation</td>
<td>40</td>
<td>15-60 min</td>
</tr>
<tr>
<td>Recorder in / out</td>
<td>2</td>
<td>30 min</td>
</tr>
<tr>
<td>Challenge</td>
<td>1</td>
<td>30 min</td>
</tr>
<tr>
<td>IV bolus</td>
<td>5</td>
<td>30 min</td>
</tr>
<tr>
<td>Blood sample</td>
<td>40</td>
<td>30 min</td>
</tr>
<tr>
<td>Gavage</td>
<td>15</td>
<td>30 min</td>
</tr>
<tr>
<td>Tube feeding</td>
<td>15</td>
<td>30 min</td>
</tr>
<tr>
<td>BAL</td>
<td>15</td>
<td>30 min</td>
</tr>
<tr>
<td>Nose and throat swabs</td>
<td>30</td>
<td>30 min</td>
</tr>
<tr>
<td>(PET-) CT</td>
<td>15</td>
<td>60 min</td>
</tr>
<tr>
<td>Killing</td>
<td>1</td>
<td>15 min</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Species</th>
<th>Origin</th>
<th>Life stages</th>
<th>Number</th>
<th>Gender</th>
<th>Genetically altered</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Rhesus or cynomolgus macaque</td>
<td>Purpose bred</td>
<td>adult</td>
<td>100</td>
<td>M/F</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

Provide justifications for these choices

**Species**
The experiments will be performed in rhesus macaques (*Macaca mulatta*) or cynomolgus macaques (*M. fascicularis*). 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. 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 (6-26). Mostly widely used in CoV research are rhesus macaques and cynomolgus macaques and their susceptibility for infection with coronaviruses is well established. This renders NHP an important preclinical animal model to investigate the therapeutic potential of anti-CoV compounds for human use.

**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**
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, and will be based on power calculations using reduction of virus load as primary outcome measure. 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 = maximally 100 animals. Since historical data are available on infection in untreated animals (Appendix 1), usually less animals can be used in the non-treated control group than in the groups treated with antiviral compound.

**Gender**
M/F

**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 wellie 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 compound and food administration via gavage.
3. Discomfort due to lung lavages.
4. Discomfort due to virus inoculation.
5. PET-CT.
6. Stress because of sedation.
7. Reduced food intake due to repeated daily sedations.
8. Disease symptoms due to the infection.

Explain why these effects may emerge.

1. The surgery needed for insertion of the telemetric temperature sensor will cause pain and some local inflammation.
2. Insertion of the tube may cause local irritation.
3. For the lung lavages a bronchoscope is used. Insertion will cause irritation.
4. Intravenous inoculation can cause mild irritation. 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 and virus inoculation. Nausea can sometimes be observed during recovery from the sedation.
7. Animals will be sedated daily during the first phase of the infection. This will have influence of the appetite.
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. Insertion of the tube will be done by experience caretakers. In case irritation occurs, this will be mild and no extra measures need to be taken.
3. For the lung lavages animals are first deeply sedated and receive a muscle relaxant.
4. If irritation occurs, this will be mild. It will therefore not be necessary to take additional measures.
5. The same procedure as described under 3 will be followed.
6. Recovery of the animals is monitored, and the veterinarian will intervene if animals do not recover fast enough.
7. Animals will receive tube feeding via gavage (this is applied during sedation for blood collection), or an adapted high calorie diet.
8. 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 killed and a full necropsy will be performed to establish the cause of the disease and viral distribution over the respiratory organs.

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 (24). 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.

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 experimental infection with CoV. 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.

**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**

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. Consequently, 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**

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 (27). Animals are monitored (twice) daily (and 24/7 by camera), and a weighed clinical scoring list is used to record the clinical symptoms (Brining, 2010 #2281). 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.

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 collaborate as much as possible voluntarily on invasive biotechnological actions such as giving anaesthesia or virus infection. In consultation with our collaborators, the
The number of blood samplings, and the collected volumes of blood will be reduced to a minimum.

The use of telemetric temperature sensors makes it possible to record the body 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 fever by some vaccine candidates (4). Such precise measurements are not possible with the traditional rectal temperature measurement. Placement of the telemetric temperature sensor will require a small surgery, which will be done under anaesthesia. Subsequently, animals will receive analgesics as long as required. The use of imaging (CT or PET-CT) will provide us with data regarding lung pathology.

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 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 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.
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 killed 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 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.

☑ 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.

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

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

References


Prescott MJ, Clark C, Dowling WE, Shurtleff AC. Opportunities for Refinement of Non-Human Primate Vaccine Studies. Vaccines [Internet]. 2021; 9(3).