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 (0900-2800028).

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: Establishment of a new RSV infection model in macaques

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 protective efficacy of an RSV vaccine, a well-defined RSV infection model is required. We will use a well-established RSV macaque infection model using a macaque-adapted human RS-virus (1-3). When a different new challenge virus is used, or the administration route is changed, it is necessary to establish the infectivity and potential pathogenicity of these changes in macaques before the model can be applied in RSV vaccine efficacy studies. To evaluate a new virus or alternative infection route, the virus is inoculated using a standard dose (10^5 – 10^7 $TCID_{50}$). 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, the experiment will be repeated with a 10-100 times higher dose. In case any of the animals reaches the clinical endpoint within the first four days after infection, a 10-100 times lower virus dose will be evaluated. The challenge virus under investigation will be dropped if the required group size is more than 10 animals. Infant, young and adult macaques are all equally sensitive to RSV infection and mount local and systemic immune responses that protects from re-infection (4).

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

A recording device is surgically placed in the abdominal cavity at least 4 weeks before the infection. This time frame is necessary for full recovery of the animals and to allow adequate recording of body temperature, respiratory rate and activity during a two to three week period to establish baseline values before infection. For the established RSV infection model with the macaque-adapted virus two infection routes have been used, namely intra-nasal (2) and intra-tracheal (just below the larynx) (1) using a dose of 10^5 – 10^6 TCID50. Using the aforementioned routes and doses all (control) animals became infected (1, 2). In the event a new challenge virus is required for vaccine evaluation, this model needs to be validated: i.e. route of infection and challenge dose need to be established. Clinical symptoms will be monitored twice daily. Nasal and tracheal swabs will be taken before infection, frequently during the first days after infection (daily and then every other day) and subsequently less frequently until day 21, to measure virus multiplication in the upper airways. Small volume (3 mL) bronchoalveolar lavages (BAL) may be taken at selected time points pre- and post-infection (max 6 times) to determine virus replication in the lungs. Blood is collected simultaneously with the swabs, to monitor changes in clinical chemistry and haematology parameters and leucocyte subsets. At the same time points body weight and physiological parameters are recorded and imaging (CT or PET-CT, max 4 times per infection) is performed to measure lung infiltration. After the virus is cleared (usually at day 21 after infection) animals are either returned to the experimental stock or they are euthanised and a full necropsy is performed in order to investigate lung pathology and virus replication in the different parts of the respiratory tract. However, when animals are not yet virus negative at day 21 an additional tracheal swab or BAL will be taken at day 28. When that is also virus positive, which is very unlikely, the animals will be euthanised in order to preclude further discomfort. In case an animal should reach the humane endpoint during the study it will be immediately euthanised and a full necropsy will be performed to establish lung pathology and virus replication in the respiratory tract. In case animals are returned to the experimental stock the recording devices are surgically removed and body temperature and/or respiratory rate and activity data are analysed. The details of each study, regarding the route of infection, dose used, species and whether animals are to be euthanised at the end of the study will be submitted for approval to the AWB.

Table. Maximum number of repeats per procedure.

Procedure	Maximum	Duration
Sedation	12	30-60 min
Recorder in / out	2	60 min
Infection	1	30 min
Blood sample	8	30 min
Virus load small BAL & Swabs	6	60 min
CT-Scan	4	30 min

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

Experience in the RSV macaque infection model has shown that with a number of four animals an adequate assessment can be made on the reproducibility of infection (2) (all 4 animals need to show virus replication in the respiratory tract). The variability of outcome parameters like: virus production and physiological parameters (fever induction, breathing frequency) needs to be limited. The primary outcome measure will be throat or BAL virus load in most experiments. On the basis of these data a power calculation can be made concerning 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, a new experiment with 4 animals is needed with a higher virus dose.

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
1	Rhesus or cynomolgus macaque	Purpose bred	adult	16	M / F	Not applicable	Not applicable

Provide justifications for these choices

Species	Macaque species have been used in several RSV vaccine studies (1-3). The most frequently used species are the rhesus monkey (*Macaca mulatta*) and cynomolgus macaque (*Macaca fascicularis*). Both species are semi-permissive to RSV infection. It was shown that both rhesus and cynomolgus macaques (infant, young and adult) are equally susceptible to (macaque-adapted) RSV infection (1, 2, 4). Therefore, both rhesus and cynomolgus macaques can be used for RSV vaccine evaluation studies. There is no specific preference for rhesus or cynomolgus macaques. As both species are suitable, the choice of species depends on availability. In some cases the choice of species is determined by other factors (e.g. availability of specific reagents or comparison with other studies).
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 because infant, young and adult animals are equally susceptible to (macaque-adapted) RSV infection (1, 2, 4)
Number	Assuming group sizes of 4 animals, evaluation of 2 new viral stains, which 2 have to be tested at two doses, the total number of animals needed will be maximally 16 over a period of 5 years.
Gender	Adult male and female animals can be used. Since there are immunological differences between males and females (5, 6), we prefer that for each individual experiment either all animals are male or all are female, in order to minimise the variation within the experimental test group, thereby increasing the probability of finding statistically significant differences between the experimental groups. This choice is also important with regard to the amount of blood needed to perform all assays. Male animals are usually larger than female animals and therefore more blood per time point can be drawn from males. In case assays need to be performed which consume large amounts of blood, male monkeys 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 placement of the recording device in the abdomen and after removal, which is needed in case the animal will not be euthanised after completion of the experiment, animals will receive analgesics for as long as necessary, typically 3 days. In previous studies we have observed that animals can experience some body temperature elevation during the first days after insertion of the recording device, but have recovered very well within 1 week after the operation. Pain relieve will also be applied when substantial induration is seen at the site of vaccine injection. In case of the latter, analgesics known not to interfere with the induction of the vaccine response will be used.

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

1. Discomfort because of insertion or removal of the temperature recording device
2. Discomfort due to lung lavages
3. Discomfort due to virus installation
4. Discomfort due to CT-scans
5. Stress because of sedation and recovery
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 and removal 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 combined with the inoculum volume will cause irritation. Aerosol application implies that animals have to be sedated and forced to breathe via a nebulizer device and this can give irritation.
4. Animals will be repeatedly sedated for vaccine delivery, blood sampling, virus infection, collection of swabs, imaging and lung lavages. Nausea can sometimes be observed during recovery from the sedation.
5. See 4.
6. Especially during daily sedation during the first 2 days after infection food intake will be reduced.
7. RSV infection can cause fever, coughing, sneezing, nose discharge, laboured breathing, loss of appetite, 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 then receive a local muscle relaxant.
3. The same procedure as described under 2 will be followed.
4. Recovery of the animals will be monitored and the veterinarian will be consulted in case of problems.
5. See 4.
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 then the animal will be euthanised and a full necropsy will be performed to establish the cause of the disease and viral distribution over the respiratory tract.

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 a weighed clinical scoring list is used to record the clinical symptoms (7). When a clinical score of 35 is reached this indicates that the maximum duration of severity is reached then the animal will be humanely euthanised. 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: open mouth breathing or cyanosis, lethargy as defined by minimal response to human approach.

Indicate the likely incidence.

The percentage of animals reaching the end point will depend on the virus and the challenge dose used. Most RS viruses, including the macaque-adapted human-RSV strain, will only cause minimal disease and typically resolve within 14-21 days (1-4, 8-10). In the event another than the macaque-adapted human-RSV strain will be used or when challenge route and / or dose require adaptation, this will first be evaluated in a small number of animals. Weight will be recorded during the study, but a weight loss of 10% because of the infection has not been observed in any study described thus far and therefore cannot serve as a suitable end point.

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).

The total amount of discomfort is estimated as moderate. This is mainly caused by the surgical implantation and removal of the recording device and development of disease due to infection.

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 RSV with different tissues and the role of local immunity in eradication of the virus, the efficacy of an RSV vaccine to protect against infection can only be adequately established in an animal model. Several animal species have been used as a model for RSV infection (11). However, mice are only susceptible to high RSV challenge doses. Cotton rats and ferrets are also semi-permissive to RSV and recapitulate the natural course of infection. However, these models have the disadvantage that limited immunological tools are available, especially to study innate and cellular adaptive immune responses, which are especially important in the evaluation of vaccine strategies (12). NHP have an immune system that most closely resembles that of humans. Also 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 (local) immune responses and evaluate their role in control of infection. In addition, vaccine strategies that aim to trigger immune responses through targeting of specific cell surface molecules or innate immune receptors have either limited cross reactivity to other animal species or trigger different cell types or different responses because of differences in receptor expression pattern or cell signalling pathways. These aspects are essential for the evaluation of RSV vaccines. For these type of vaccines new vaccination strategies as well as vaccine modalities are used that aim for the induction of protective cellular immune responses or induction of neutralising antibody responses or non-neutralising 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. Vaccines that will be tested in NHP have to be in the final stages of preclinical development and require this last validation step in order to assure that there are no adverse effects that were missed in the preliminary studies in other species and that they are effective in an animal species that has an immune system closely related to humans.

Reduction	Experience from previous experiments has shown that when the virus is inoculated by a standard route at a standard dose, 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 an adapted dose. On the basis of the outcome of the first study the number of animals required 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 recording devices makes it possible to record physiological parameters, (temperature, heart- and respiratory-rate etc.) every 15 minutes. For influenza infection, we have designed a method that allows very precise calculation of fever induction caused by the infection (13). With this method we observed a significant fever reductions in influenza vaccinated animals (14). Such precise measurements are not possible with the traditional methods. RSV infection may, unlike influenza, not cause fever, but an increase in respiratory rate. As currently very limited information is available on these parameters in the macaque RSV infection model, we will evaluate their suitability as exploratory outcome measures. Placement and removal of the recording devices will require a small 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. Animals will be socially housed with a socially compatible animal, whenever possible. There is an extensive program for enrichment in our institute that consists of playing material and methods to present food (http://www.bprc.nl/en/welfare/). 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.(7) . On the basis of the scoring system a humane endpoint is defined. In addition, a sudden strong decrease in body temperature is taken as a humane endpoint. 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 first 2 days of the infection the animal will receive tube feeding. This is necessary, since animals will be sedated daily during the first days after infection and the food intake during this period would otherwise be very limited.

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 RSV vaccine or RSV virus infection studies or that have pre-existing antibodies against RSV are not suitable. In view of the long life-span 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.

In most studies the animals will be re-used after the virus is cleared (usually at day 21 after infection). However, in cases where possible adverse effects of the vaccine have to be studied animals are humanely euthanised and a full necropsy is performed.

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.

Euthanasia is done by injection of an anaesthetic dose of ketamine followed by an overdose of barbiturate intravenously

☐ 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.

Not applicable

References

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3. de Waal L, Wyatt LS, Yuksel S, van Amerongen G, Moss B, Niesters HG, et al. Vaccination of infant macaques with a recombinant modified vaccinia virus Ankara expressing the respiratory syncytial virus F and G genes does not predispose for immunopathology. Vaccine. 2004;22(8):923-6.
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12. Albrecht RA, Liu WC, Sant AJ, Tompkins SM, Pekosz A, Meliopoulos V, et al. Moving Forward: Recent Developments for the Ferret Biomedical Research Model. mBio. 2018;9(4).
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14. Impagliazzo A, Milder F, Kuipers H, Wagner MV, Zhu X, Hoffman RM, et al. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. Science. 2015;349:1301-6.