



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

Serial number	Type of animal procedure
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1	RSV vaccine evaluation in macaques
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Use the numbers provided at 3.4.3 of the project proposal.

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

We have developed a general study protocol for the evaluation of RSV vaccines in macaques. Typically a recording device is surgically placed in the abdominal cavity before the start of the study to retrospectively evaluate body temperature (measured every 15 minutes) and/or heart rate, respiration and activity. Subsequently the animals are immunised either once or receive a number of immunisations over a certain time period. Although the vaccines to be used in these studies have already been extensively evaluated in other animals and therefore are expected to give no or only very limited adverse effects, macaques will be monitored for possible changes in general behaviour and health. The immunisation site will be inspected for local reactions and blood will be drawn to measure clinical chemistry and haematology parameters. Before, between and after immunisations, blood and occasionally nasal samples (either swabs or washes) or lung lavages will be collected to measure induction of systemic as well as local immune responses. Both T-cell and antibody responses will be determined against several RSV antigens in order to establish the strength and durability of the ensuing immune response. When adequate immune responses are induced that indicate that protection against infection might be achieved, the efficacy against infection will be tested by experimental infection with RSV. Whether an immune response is adequate depends on the nature of the vaccine under investigation; for antibody responses this can be assessed by comparison with data obtained in clinical trials, whereas for cell-mediated immune responses on few data are available and adequacy of the response will be assessed by comparison with published data where possible. A group of non-vaccinated animals will be included as infection controls. In addition, a group of RSV infected animals may also be included, such that the vaccine under evaluation can be compared with natural RSV infection. We will use a well-established RSV infection model using a macaque-adapted human RSV strain, as described in appendix 2 (1-3). In the event, the vaccine candidate requires testing with a different RSV strain, or aerosol-infection, the infection model will be established at BPRC as described in appendix 2. All animals in a specific vaccine group will be challenged with the same virus as described in appendix 2.

The primary outcome parameters are: 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 humoral immune responses. The type and strength of the induced responses will determine if the objectives of the vaccine strategy are achieved and whether immune responses are sufficiently strong to proceed with viral challenge. Efficacy: capacity to protect against viral challenge will be established in terms of: reduction in clinical symptoms, fever, virus replication and changes in blood parameters.

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

A recording device is surgically placed in the abdominal cavity at least 4 weeks before the first immunisation takes place. This time frame is necessary for full recovery of the animals and to allow adequate recording of body temperature and/or heart rate, breathing rate and activity during a two to three-week period to establish normal values before immunisations start. Animals will receive one or more immunisations, typically at 4 to 8-week time intervals. Occasionally a longer time frame is needed between immunisations when different vaccine modalities are used for priming and boosting of the immune response. Usually 3 immunisations suffice over a period of 24 weeks. However, in rare occasions these limits may have to be exceeded. Specific rationale will then be presented to the animal welfare body (AWB). Immunisations can be done by several routes (e.g. intradermal injection, intramuscularly, subcutaneously, intravenously, intra-nasally, intra-tracheally, or via aerosol using a nebulizer). The route of immunisation depends on the vaccine under investigation. All vaccines have to be sterile and have to be given under aseptic conditions. At regular time intervals, usually before, two and four weeks after every immunisation, 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 immunisation is chosen, because this is optimal for measuring cellular immune responses, while four weeks is optimal for measuring humoral immune responses. For new vaccine candidates aiming at the induction of mucosal responses, nasal washes and bronchoalveolar lavages (BAL) will be collected before and at time points (usually 2 and 4 weeks) after immunisation to determine the magnitude of local immune responses. Immune responses recorded after the final immunisation will be used to decide whether protection against viral challenge can be reasonably expected. If these responses are too low to realistically expect protection the study will be stopped and animals may be re-used in other non-RSV related experiments. Otherwise, experimental challenge will usually take place between 4 and 8 weeks after the last immunisation, but this may be longer when the longevity of protective responses is being evaluated. This time period is also required to allow immunological memory to form after the last immunisation. RSV infection may be done by intra-nasal, intra-tracheal or intra-broncheal inoculation alone or in combination with oral, intranasal and intraocular inoculation or via aerosol using a nebulizer (as described in appendix 2). The established infection routes for RSV infection using the macaque-adapted virus are intra-nasal and intra-tracheal using a challenge dose ranging between 10^5 and 10^6 TCID₅₀, at which all animals became infected (1, 3). Different vaccine concepts or research questions may, however, may require other challenge viruses or infection routes that need to be established before vaccine evaluation can be performed (described in appendix 2). Clinical symptoms will be monitored twice daily during the infection phase. 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 replication in the upper airways. Bronchoalveolar lavages (BAL) may be taken at selected time points (max 4 times post infection) to measure virus replication in the lungs, as previously described (1, 3, 4). Blood is collected simultaneously with the swabs, to monitor changes in clinical chemistry and haematology parameters, leucocyte subsets and cytokine production. At the same time points body weight and physiological parameters (e.g. pulse rate, respiratory rate, blood pressure) are recorded and imaging (CT or PET-CT) is performed to measure lung infiltration. After the virus is cleared (usually 21 days post infection) animals are either returned to the experimental stock or they are euthanised and a full necropsy is performed in order to establish whether persistent lung pathology occurred (1). Euthanasia is only performed when assessment of lung pathology is required to establish vaccine safety. 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 cause of death and investigate lung pathology and virus presence in the respiratory tract. In case animals will not be euthanised, the recording devices are surgically removed and body temperature, heart rate, respiratory rate and activity data are analysed, upon which, the animals may be re-used (within the limitations described in art 1e of the Wet op de Dierproeven). The details of each study, regarding the interval between the immunisations, the number and time points of sampling, the specific criteria to proceed with a viral challenge, the time interval between the last immunisation and viral challenge will depend on the actual type of vaccine that is being tested and this will be submitted to the AWB.

Table. Maximum number of repeats per procedure.

Procedure	Maximum Duration
Sedation	24 15-60 min
Recorder in / out	2 60 min
Vaccination	4 60 min
Blood sample	16 30 min
Bronchoalveolar lavage (BAL)	16 60 min
Infection	2 30 min
Virus load small BAL & Swabs	12 60 min
CT-Scan	8 30 min

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 take into account the number of animals needed to measure statistically significant induction of immune responses in relation to (unvaccinated) controls. In addition, calculations are performed to establish the number of animals needed to obtain a significant reduction in the primary outcome measure (throat or BAL virus load) between the vaccine groups and the challenge control group. Experience in RSV vaccine evaluation studies indicate that statistically significant reductions in virus replication can be obtained with five animals per group (3). In the event two different vaccines are compared or a vaccine is compared to previously RSV infected animals, group sizes may be larger. Only the minimum number of animals needed will be used.

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	90	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 (<i>Macaca mulatta</i>) and cynomolgus macaque (<i>Macaca fascicularis</i>). 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, 3, 5). 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, 3, 5)
Number	The number of animals requested is based on the assumption that each study will contain two vaccine groups and 1 control group, with 10 animals per group. The actual group sizes will be determined per experiment, based on a power calculation for each experiment. Probably fewer than the assumed 10 animals per group will be needed in most experiments. In all, we anticipate performing 3 such studies over a 5-year period, the total number of animals needed will be maximally 90

Gender	Adult male and female animals can be used. Since there are immunological differences between males and females (6, 7), 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 injection
3. Discomfort due to lung lavages
4. Discomfort due to virus installation
5. Discomfort due to CT-scans
6. Stress because of sedation and recovery
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 and removal of the temperature recording device will cause pain and some local inflammation.
2. When vaccines are given by injection, this can cause local pain and irritation.
3. For the lung lavages a bronchoscope is used. Insertion will cause irritation.
4. When virus is given intra-bronchially a bronchoscope is used and this 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.
5. Animals will be repeatedly sedated for vaccine delivery, blood sampling, virus infection, collection of swabs and lung lavages. Nausea can sometimes be observed during recovery from the sedation.
6. See 5.
7. Especially during daily sedation during the first 2 days after infection food intake might be reduced.
8. RSV infection can cause fever, coughing, sneezing, nose discharge, laboured breathing, increased breathing rate, 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. Animals will be sedated for vaccine delivery. Only rarely are strong adverse effects seen. Should granuloma formation be observed, then the animal will be sedated, the wound will be cleaned and analgesics are applied if necessary following veterinary consultation.
3. For the lung lavages animals are first deeply sedated and then receive a local muscle relaxant.
4. The same procedure as described under 3 will be followed.
5. Recovery of the animals will be monitored and the veterinarian will be consulted in case of problems.
6. See 5.
7. Animals will receive tube feeding. This is applied during sedation.
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 then the animal will be humanely 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 (8). 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 (3). 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 (see appendix 2). 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	<p>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.</p> <p>Several animal species have been used as a model for RSV infection (9). 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 (10). 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.</p>
Reduction	<p>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 respiratory tract between the vaccine groups and the challenge control group. Only the minimum number of animals needed will be used. Furthermore, we aim to evaluate multiple vaccine candidates in a single experiment, so that a single challenge control group can be used. This has to be weighed against the fact that in order to obtain significant differences in immune response between the vaccine groups, more animals per vaccine group may occasionally be required.</p>

Refinement	<p>The use of recording devices enables the monitoring of body temperature, heart- and breathing-rate every 15 minutes. We have designed a method that allows very precise calculation of fever induction caused by influenza infection (11). With this method we have observed a significant reduction in fever by influenza vaccine candidates (12). Such precise measurements are not possible with the traditional rectal temperature measurement. In addition, measurement of breathing rate may provide additional information on the RSV infection severity. Placement and removal of the temperature responders will require a small surgical procedure, 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.</p> <p>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/).</p> <p>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.(8). 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. The "Flora and Fauna wet" and "wet dieren" do not pose additional requirements that are needed for the type of studies proposed in this application.</p>
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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

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7. Klein SL, Marriott I, Fish EN. Sex-based differences in immune function and responses to vaccination. *Trans R Soc Trop Med Hyg*. 2015;109(1):9-15.
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