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'.	[redacted]
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: 1 — Type of animal procedure: Influenza vaccine evaluation 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.

We have developed a general study protocol for the evaluation of influenza vaccines in macaques. Temperature and potentially movement and heart rate will be measured by telemetry. A device that records and transmits these parameters is surgically placed in the abdominal cavity. When all mentioned parameters need to be monitored during the immunization as well as the infection phase then a relatively large device is needed that is capable of performing all these measurements and can be used during a long period. This larger device has the advantage that it can be temporarily put on stand-by to safe battery life and then be reactivated when needed, so that it will last through the entire study period. Such devices will be placed at least 4 weeks before the first immunization. In case only the temperature needs to be monitored during the influenza virus infection phase then a small device suffices. Such a device has however a shorter life-span (28 weeks) and has to be placed in the abdominal cavity four weeks before infection. Subsequently the animals are immunized either once or they receive a number of immunizations over a certain period of time. Although the vaccines that are used in these studies have already been extensively evaluated in other animals and are expected to give no or only very limited adverse effects, macaques will be monitored for possible changes in general behaviour and health. The site of immunization will be checked for local reactions and blood will be drawn to measure clinical chemistry and haematology parameters. Before, between and after immunizations, blood and occasionally nasal washes and lung lavages will be taken to measure induction of systemic as well as local immune responses. Both T-cell and antibody responses will be measured against several influenza viruses in order to establish the strength and breadth of the 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 influenza virus. A group of non-vaccinated animals will be included as infection controls. Usually only one virus will be used for experimental infection. The choice of the virus strain to be used will depend on the outcome of the evaluation of the immune responses and the stage of development of the vaccine. An entirely new vaccine concept may require that in first instance protection against infection by a homologous virus strain is tested. However, for most vaccine candidates protection against infection with a heterologous virus will be tested. All animals in a specific vaccine group will be challenged with the same virus. If protection against two different viruses needs to be established than two groups of animals are immunized with the same vaccine and subsequently group 1 will be challenged with virus 1 and group 2 with virus 2. If animals are protected against infection, then a second challenge with a different virus may be performed. In this case an additional challenge control group is needed. In this manner the breadth of the immune response is both measured *in vitro;* via the evaluation of the induced immune response and *in vivo* via experimental infection with different virus strains. The infection will be performed 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 multiplication 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 telemetry device is surgically placed in the abdominal cavity at least 4 weeks either before the first immunization or before the infection. This time frame is necessary for full recovery of the animals and to allow adequate recording of body temperature and/or heart rate and activity during a two to three-week period to establish normal values before immunizations start. Animals will receive one or more immunizations. If multiple immunizations are given then typically a 4 to 8-week time interval is used, although occasionally a longer time frame is needed between immunizations when different vaccine modalities are used for priming and boosting of the immune response. In rare occasions these limits may have to be exceeded, with a maximum of 6 immunizations. Specific rationale will then be presented to the animal welfare body (AWB). Immunizations will be done either by intradermal injection, intramuscularly, subcutaneously, intravenously, intranasally, intra-tracheally or via aerosol using a nebulizer. All vaccines have to be sterile and have to be given under aseptic conditions. At regular time intervals, usually two and four weeks after every immunization, blood is drawn to measure systemic adverse effects, which includes measurement of clinical chemistry and haematology, and to measure induction of cellular and humoral immune responses. The two-week interval after immunization is chosen, because this is optimal for measuring cellular immune responses, while four weeks is optimal for measuring humoral immune responses. In some studies, nasal washes and lung lavages (BAL) are taken after immunization in order to measure induction of local immune responses. BAL will be maximally collected three times after each immunization, resulting in total in 18 BAL collections if animals are immunized six times. Immune responses recorded after the final immunization will be used to decide whether protection against viral challenge can be reasonably expected. If these responses are inadequate then the study will be stopped and animals may be re-used in other non-influenza virus related experiments. Otherwise, experimental challenge will usually take place between 4 and 8 weeks after the last immunization. This time period is needed to allow immunological memory to form after the last immunization. Influenza infection may be done by intra-bronchial or intra-tracheal inoculation alone or in combination with oral, intranasal and intraocular inoculation or via aerosol using a nebulizer (as 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 multiplication in the upper airways. Lung lavages may be taken at selected time points (max 5 times) to measure virus multiplication in the lungs. Blood is taken simultaneously with the swabs, to monitor changes in clinical chemistry and haematology parameters, leucocyte subsets and cytokine production. At the same time points body weight and physiological parameters (for instance pulse rate, blood pressure) are recorded and imaging (for instance CT or PET-CT) is performed to measure lung infiltration. After the virus is cleared (usually at day 21 after infection) animals may be exposed to a second challenge virus. All handling will then be repeated. However, this will be rarely the case and specific argumentation will be presented to the AWB. In general animals are either returned to the experimental stock or they are euthanized and a full necropsy is performed in order to establish lung pathology. Euthanasia is only performed when assessment of lung pathology is required to establish vaccine safety and in case pathogenic influenza viruses are used that are known to cause persistent lung pathology. Some telemetric devices, that have been designed to measure multiple parameters, can be quite large and require a substantial operation. These will not be removed and the animal will be euthanized instead. A small telemetric device can be surgically removed and animals may be re-used. A specific rational has to be provided to the AWB for using these devices. In case an animal should reach the humane endpoint during the study it will be immediately euthanized and a full necropsy will be performed to establish lung pathology and virus multiplication in the respiratory tract.

The details of each study, regarding the interval between the immunizations, the number and time points of sampling, the specific criteria to proceed with a viral challenge, the time interval between the last immunization and viral challenge will depend on the actual type of vaccine that is being tested and this will be submitted to the AWB.

Table. Maximum number of repeats per procedure. Indicated is which procedure is performed under sedation and which procedure under deep anesthesia.

Procedure	Maximum	Duration	sedation	anesthesia
Recorder in/out	2	60 min		X
Vaccination	6	10 min	X	
Blood sample	44	10 min	X	
Bronchoalveola lavage (BAL)	28	30 min		X
Infection	2	10 min		X
Swabs	20	10 min	X	
CT-scan	10	15 min		X

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 or, in case different vaccines are compared, whether significant differences between the vaccine groups can be obtained. 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 vaccine groups and the challenge control group. Experience in previous influenza vaccine evaluation studies performed at our institute indicates that statistically significant reductions in virus multiplication can be obtained with six animals per group. However, in order to be able to measure differences between the vaccine groups themselves, more animals per group may be needed. Only the minimum number of animals required will be used. Since historical data are available on infection in non-vaccinated animals (appendix 2), usually less animals can be used in the non-vaccinated challenge control group than in the vaccine 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.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
1	Rhesus or cynomolgus macaque	Purpose bred	adult	90	M / F	no	Not applicable

Provide justifications for these choices

Species	Macaque species have been used in several influenza vaccine studies (1-6). The most frequently used species are the rhesus- (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*). Both species are semi-permissive to influenza infection. Therefore, both rhesus and cynomolgus macaques can be used for influenza vaccine evaluation 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 these allow larger volumes of blood to be collected.
Number	90 macaques in total (either cynomolgus or rhesus). 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 group size will be determined per experiment, based on power calculation specific for the experiment. As stated above, probably fewer animals will be needed in the non-vaccinated challenge control groups and also in the vaccine groups in some of the studies. In all, we anticipate performing 3 such studies over a 5-year period.
Gender	Adult male and female animals can be used. There are immunological differences between males and females (7, 8). However, for influenza vaccine induced responses these differences are only modest (9) and not observed in all reports (10) and are unlikely to affect study outcome in pre-clinical studies with relatively low number of animals. Therefore, both male and female animals can be used.
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 euthanized 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 PET-CTs
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. For the PET-CT animals are sedated, intubated and mechanical ventilation with a forced breathing pattern is applied. No adverse effects are expected from the scan itself
6. Animals will be repeatedly sedated for vaccine delivery, blood sampling, virus infection, collection of swabs, CTs and lung lavages. Nausea can sometimes be observed during recovery from the sedation.
7. Especially during daily sedation during the first 2 days after infection food intake might be reduced.
8. Influenza 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. The animals are first deeply sedated and then receive a local mucosal relaxant before intubation. The mechanical ventilation & forced breathing patterns will be monitored and adapted on each animals characteristics.
6. Recovery of the animals will be monitored and the veterinarian will be consulted in case of problems.
7. Animals will receive supportive feeding with dense "brokkenballen".
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 euthanized 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 (11). When a clinical score of 35 is reached this indicates that the maximum duration of severity is reached then the animal will be euthanized. Individual scores are added and the decision is based on the total daily score and veterinarian assessment of discomfort. Symptoms associated with score 35 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 likely incidence for reaching a clinical endpoint depends on the virus strain that is used for infection. Human influenza viruses only rarely cause severe disease (<1% of the animals). Highly pathogenic avian influenza viruses can cause lethal disease in up to 75% of the non-vaccinated control animals.

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 and the high maximum number of sedations.

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 influenza virus with different tissues and the role of local immunity in eradication of the virus, the efficacy of an influenza vaccine to protect against infection can only be adequately established in an animal model. Several animal species have been used as a model for human influenza virus infection (12). However, mice are not naturally susceptible to influenza and usually lethal infection models are used, that are based on extremely adapted human viruses. Ferrets are naturally susceptible to most human influenza viruses and recapitulate the natural course of infection. However, infection is often restricted to the upper airways and this model has 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 "universal" vaccine strategies. 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 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 so called "universal" influenza vaccines. For this type of vaccines new vaccination strategies as well as vaccine modalities are used that 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. 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	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. Since historical data are available on infection in non-vaccinated animals (appendix 2), usually less animals can be used in the challenge control group than in the vaccine groups. 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.

Refinement	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 (4). With this method we have observed a significant reduction in fever by influenza vaccine candidates (1). Such precise measurements are not possible with the traditional rectal temperature measurement. 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. Animals will be socially housed with a socially compatible animal. There is an extensive program for enrichment in our institute that consists of playing material and methods to present food. 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. (11). 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 supportive feeding with dense "brokkenballen". This is necessary, because animals have to fast the night before the sedation and since animals will be sedated daily during the first days after infection 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 influenza vaccine or influenza virus infection studies or that have pre-existing antibodies against influenza 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 euthanized 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.

References

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12. Bodewes R, Rimmelzwaan GF, Osterhaus AD. Animal models for the preclinical evaluation of candidate influenza vaccines. Expert Rev Vaccines. 2010;9(1):59-72. 10.1586/erv.09.148