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’.

1.2 Provide the name of the licenced establishment.

1.3 List the serial number and type of animal procedure

  Use the numbers provided at 3.4.3 of the project proposal.

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Type of animal procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Establishment of a new influenza infection model in macaques</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.
In order to establish the capacity of a vaccine to protect against influenza virus infection it is necessary to have a well-defined influenza virus infection model. Previously we have established a model for infection of macaques with pandemic H1N1 and highly pathogenic avian H5N1 viruses (1-3). For new influenza viruses that have not yet been tested at our institute it is necessary to establish infectivity and pathogenicity in macaques before they can be applied in influenza vaccine efficacy evaluation studies. The main objective is to obtain an infection model that is sufficiently robust to allow adequate evaluation of vaccine efficacy in terms of reduction in clinical symptoms, fever and virus multiplication. In cases viruses are used that are known to cause persistent lung pathology, this will also be a primary outcome parameter. In general, the study set-up is as follows: a small number of animals will be infected and monitored for clinical symptoms, fever, body weight and changes in blood parameters. Nasal and tracheal swabs will be taken to determine if the animals have become infected and determine the magnitude of virus multiplication. To evaluate a new virus, the virus is either inoculated by a combination of routes; for instance intra-bronchial, oral, intranasal and intraocular or by aerosol exposure using a standard dose. Proper application for vaccine evaluation requires that more than 80% of the animals become infected and that the amount of virus produced in the trachea 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 then 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 then a 10-100 times lower virus dose will be evaluated. The mode of exposure that will be tested will be identical to the method to be used in the vaccine evaluation study. Evaluation of new influenza viruses as described in this appendix is only performed when a vaccine evaluation study is already planned with the same virus.

Primary outcome parameters are:
Clinical symptoms, fever, virus multiplication.
Pathology in case viruses are used that are known to cause persistent lung pathology.

Secondary outcome parameters are:
Bodyweight, changes in leucocyte subset composition in peripheral blood.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.
Temperature and potentially activity and heart rate will be measured by telemetry. A device that records and transmits these parameters 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 and/or heart rate and activity during a two to three week period to establish normal values before infection. 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 delivery using a nebulizer. 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. 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 and leucocyte subsets. At the same time points body weight and physiological parameters (for instance pulse rate, blood pressure) are recorded and imaging (for instance (PET-)CT scan) 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 humanely euthanized and a full necropsy is performed in order to establish lung pathology and virus multiplication in the different parts of the respiratory tract. Euthanasia is only performed when assessment of lung pathology is required in case pathogenic influenza viruses are used that are known to cause persistent lung pathology. However, when animals are not yet virus negative at day 21 an extra tracheal swab will be taken at day 28. When that is also virus positive, which is very unlikely, the animals will be euthanized in order to preclude further discomfort. In case an animal should reach the humane endpoint during the study it will be immediately humanely euthanized and a full necropsy will be performed to establish lung pathology and virus multiplication in the respiratory tract. In case animals are returned to the experimental stock the recording devices are surgically removed and body temperature and/or heart 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 euthanized at the end of the study will be submitted for approval to the AWB.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Maximum</th>
<th>Duration</th>
<th>sedation</th>
<th>anesthesia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recorder in/out</td>
<td>2</td>
<td>60 min</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Blood sample</td>
<td>10</td>
<td>10 min</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Bronchoalveola lavage (BAL)</td>
<td>5</td>
<td>30 min</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Infection</td>
<td>1</td>
<td>10 min</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Swabs</td>
<td>10</td>
<td>10 min</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>CT-scan</td>
<td>5</td>
<td>15 min</td>
<td>X</td>
<td></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 initial experiment will be performed in four animals. Experience in the pandemic H1N1 and avian H5N1 influenza infection models have shown that with this number of animals an adequate assessment can be made on the reproducibility of infection (all 4 animals need to show virus multiplication in the trachea), the variability of virus production in the trachea and the amount of fever induction. On the basis of these data a power calculation can be made about 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 than a new experiment with 4 animals is needed with a higher virus dose. If also at a high virus dose the variation between the animals is still too high then it may be necessary to repeat the experiment in another macaque species. Experiments will preferentially be performed in cynomolgus macaques. However, if after virus inoculation the levels of virus production are too low (below the detection limit) or too variable (>10 animals needed/group) then the virus will subsequently be tested in rhesus macaques.

### 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>
</table>


Species
Macaque species have been used in several influenza vaccine studies (1, 2, 4-7). 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. Previous studies have shown that cynomolgus macaques are more susceptible than rhesus macaques for infection with pH1N1 influenza virus. Cynomolgus macaques show higher levels of virus replication and more fever than rhesus macaques (2). However, for other influenza viruses it is not known which of the two species is the most susceptible. Therefore, experiments will preferentially be performed in cynomolgus macaques. However, if after virus inoculation the levels of virus production are too low (below the detection limit) or too variable (>10 animals needed/group) then the virus will subsequently be tested in rhesus macaques.

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
24 macaques in total (either cynomolgus or rhesus). The number of animals requested is based on the assumption that each study will comprise two challenge doses, with 4 animals per group. We anticipate to perform 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 (8, 9). However, for influenza vaccine induced responses these differences are only modest (10), and not observed in all reports (11), 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 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 PET-CTs
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. 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.
5. 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.
6. Especially during daily sedation during the first 2 days after infection food intake might be reduced.
7. 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. For the lung lavages animals are first deeply sedated and then receive a local muscle relaxant.
3. The same procedure as described under 3 will be followed.
4. 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.
5. Recovery of the animals will be monitored and the veterinarian will be consulted in case of problems.
6. Animals will receive supportive feeding with dense “brokkenballen”.
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 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 (12). 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.

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**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.

<table>
<thead>
<tr>
<th>Replacement</th>
<th>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 infection model. Several animal species have been used to study influenza virus infection (13, 14). However, of these different species NHP have the advantage that they physiologically and immunologically most closely resemble humans. This has important implications, both for vaccine evaluation (explained in appendix 1), as well as for the interaction with influenza virus, since this is affected both by physiology and by the reaction of the innate and adaptive immune system. As explained in appendix 1, these aspects are especially important for the evaluation for “universal” influenza vaccines. The proper evaluation of these vaccines requires adequate infection models in NHP, which is the purpose of the studies proposed here.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction</td>
<td>Experience from previous experiments has shown that when the virus is inoculated by a standard combination of routes at a standard dose, four animals per test group is 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 another dose or in another NHP species. Only the minimum number of animals required, will be used.</td>
</tr>
</tbody>
</table>
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 (2). With this method we have observed a significant reduction in fever by influenza vaccine candidates (6). 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 animal caretakers. Should changes occur in behaviour, appetite or stool then a veterinarian will be informed and measures will be discussed with the investigator and implemented. During the infection animals will be observed twice daily and clinical symptoms will be scored using a well-established clinical scoring list adapted from Brining et al. (12). 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 disease. All handlings will be performed under sedation. On every time point when a handling is performed the animal will be weighed and closely examined. During the first 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.
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.

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


