		Inventaris Wob-verzoek W17-18								
			wordt verstrekt				**weigeringsgronden**			
nr.		**document NTS 2016731**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
1		Aanvraagformulier				x		x	x	
2		Projectvoorstel			x					
3		Niet-technische samenvatting	x							
4		Bijlage beschrijving dierproeven 1			x					
5		Bijlage beschrijving dierproeven 2			x					
6		Bijlage beschrijving dierproeven 3			x					
7		Bijlage beschrijving dierproeven 4			x					
8		Bijlage beschrijving dierproeven 5			x					
9		DEC-advies				x		x	x	
10		Ontvangstbevestiging				x		x	x	
11		Advies CCD		x						x
12		Beschikking en vergunning				x		x	x	

09 NOV. 2016

Centrale Commissie Dierproeven

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☒ Ja > *Vul uw deelnemernummer in* 22400
☐ Nee > *U kunt geen aanvraag doen*

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	Boehringer Ingelheim Animal Health Operations bv	
Naam van de portefeuillehouder of diens gemachtigde	[redacted]	
KvK-nummer	55530133	
Straat en huisnummer	J.C. van Houtenlaan	36
Postbus	postbus 36 (1380AA Weesp)	
Postcode en plaats	1381CP	Weesp
IBAN	NL52DEUT0265175240	
Tenaamstelling van het rekeningnummer	Boehringer Ingelheim AHO	

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☐ Dhr. ☒ Mw.
Functie	[redacted]	
Afdeling	Animal Investigations Group	
Telefoonnummer	[redacted]	
E-mailadres	[redacted]	

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters		☐ Dhr. ☐ Mw.
Functie		
Afdeling		
Telefoonnummer		
E-mailadres		

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☒ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☐ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☐ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1 - 11 - 2016
		Einddatum	1 - 11 - 2021
3.2	Wat is de titel van het project?	Development of a vaccine against avian pathogenic E. Coli (APEC)	
3.3	Wat is de titel van de niet-technische samenvatting?	Ontwikkeling van een vaccin tegen E.coli bij kippen	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC BIAHO
		Postadres	postbus 36 (1380AA Weesp)
		E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1727 Lege

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☒ Melding Machtiging

☒

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted]
Plaats	Weesp
Datum	28 - okt - 2016
Handtekening	[redacted]

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix t this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see 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'.	**22400**
1.2	Provide the name of the licenced establishment.	**BIAHO bv**
1.3	Provide the title of the project.	**Development of a vaccine against avian pathogenic E. Coli (APEC)**

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☐ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

[**201609**]
Avian Pathogenic E. Coli (APEC) is a bacterium causing inflammations in multiple organs in

chickens and has accounted for a large quantity of applied antibiotics in the field. Typically around 5-week old broilers and around 30-week old layers are affected. Although all organs may be affected through septicaemia of APEC, clinical signs of the respiratory apparatus stand on the foreground. Today many strains are multiresistant, due to applied antibiotics against this widespread disease. Especially flocks infected by Infectious Bronchitis-virus are susceptible to APEC infections. The use of a vaccine against APEC can improve animal welfare and production, as few antibiotics are effective. To date only one commercial APEC vaccine is available; therefore there are still opportunities in this market.

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The main objective is the development of a safe, effective vaccine against APEC with a genetically attenuated E coli strain, which is severly impaired in its replication metabolism. As this is an important virulence-factor, the attenuated strains are promising vaccine candidates. By following the stepwise strategy in selecting the candidates (see 3.4) it is ensured that the final candidates indeed provide safe yet effective immunity against APEC.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

APEC is a bacterium causing significant losses in production and increased death rates in both broilers and laying chicken flocks due to a range of inflammations, predominantly in the air sacs and lungs. As the use of antibiotics needs to be reduced to decrease antibiotic resistance, a vaccine is a valuable alternative asset in decreasing morbidity and mortality. At the same time this would improve animal welfare and prevent the associated economic loss caused by the decreased production, increased mortality and treatment costs.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

The project is divided into 5 separate steps. In the first study, challenge models for the three most common virulent serotypes O1, O2 and O78 are developed by testing challenge candidates from these serotypes in multiple doses on non-vaccinated animals. In the second study the genetically attenuated vaccine candidates are evaluated by determining their persistence (amount of time they are detectable, an indication whether the vaccine candidate facilitates adequate time for an immune response to develop in the animal) and virulence. In the third study, the efficacy (protection against challenge) of the selected vaccine candidate(s) that is/were proven appropriate in the second study is/are tested. In the fourth study the minimum immunising dose (MID) of these vaccine candidate(s) is/are determined, and in the fifth study the extent of cross-immunity they provide is tested.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

In the first study, groups of non-vaccinated chickens will be infected with different APEC strains (from each of the 3 serotypes) in different doses to determine a sufficient challenge dose. In the second study, chickens will be vaccinated with different vaccine candidates, and the residual pathogenicity (virulence) and persistence will be evaluated. In the third study, chickens will be vaccinated with the remaining vaccine candidates and will be challenged thereafter to evaluate efficacy. In the fourth study, groups of chickens will be vaccinated with different vaccination doses, to determine the MID. In the fifth and final study of the project animals will be vaccinated and challenged with the selected 3 different challenge strains (one for each serotype), to determine the extent of cross-immunity.

3.4.3 Describe the coherence between the different components and the different steps of the project. If

applicable, describe the milestones and selection points.

The developed challenge models in the first study are used to test the efficacy of the vaccine candidates. The candidates are selected based on the results from the second study (those with no residual virulence). Only the vaccines that are efficacious (study 3) will be enrolled for the MID finding and cross-immunity studies. If, in study 201609.2, only 1 or 2 vaccines are selected, the testing for efficacy and the MID (studies 201609.3 and 201609.4) will be combined.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	**201609.1 Development of challenge models for 3 APEC serotypes**
2	**201609.2 Virulence and persistence of 2 wildtype and 6 modified APEC strains to be used in vaccine formulation**
3	**201609.3 Efficacy of vaccine candidates against APEC infections compared to a commercially available vaccine**
4	**201609.4 Minimum immunising dose finding study for vaccine candidates against APEC**
5	**201609.5 Evaluation of the cross-protection of candidate APEC-vaccines using the minimum immunising dose**
6	
7	
8	
9	
10	

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, see 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'.	**22400**
1.2	Provide the name of the licenced establishment.	**BIAHO bv**
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: **3.4.4.1** Type of animal procedure: **201609.1 Development of challenge models for 3 APEC serotypes**

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.

[201609.1 Development of challenge models for 3 APEC serotypes]
Avian Pathogenic E. Coli (APEC) is a bacterium causing a wide spectrum of inflammations in chickens, making it a major problem for which antibiotics are applied. To prevent the use of large quantities of antibiotics, vaccines against APEC are desired. To be able to test the efficacy of vaccine candidates, a challenge model which can reliably represent field disease conditions is needed. The objective of this study is to select E. Coli strains of the most common serotypes (O1, O2 and O78) that are capable of inducing clinical or in necropsy visible colibacillosis in non-vaccinated chickens, making them suitable to be used in a challenge model. Also the required challenge doses are determined.

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

Maximally 6 E. coli challenge strains (from all 3 serotypes) will be tested in 3 doses (10^8, 10^9 and 10^{10} cfu), leading to 18 groups. Two time points will be investigated, meaning that maximally 36x16 animals will be used. Sixteen animals per time point (and 32 animals per group) results from the statistical considerations as detailed in the next section. Therefore, maximally 576 animals are used. The animals will be 35 days (5 weeks) old when they are challenged intra-tracheally with the challenge dose (procedure according to Antao et al., 2008, The chicken as a natural model for extraintestinal infection caused by avian pathogenic Escherichia coli (APEC)). 24 hours post-challenge, 16 animals per group will be euthanized and necropsied. 48 hours post-challenge, the remaining 16 per group will be euthanized and

necropsied. In the study of Antao et al. it was found that the lesions caused by E. coli after 48 hours are less than those found at 24 hours; yet this varies between strains and is not exactly known for the candidate challenge strains. Therefore the effects of the strains must also be assessed at 48h post-challenge. All animals will receive a clinical score based on disease symptoms (ordinal scale, described by Antao et al, 2008). The internal organs will each be given a score (ordinal scale) according to the system as also described by Antao et al. 2008. Depending on the results of the clinical and necropsy scores, additional tissue samples of the organs may be taken to determine whether the challenge bacterium can be re-isolated. The goal is to create a challenge model generating morbidity in 50% of non-vaccinated animals.

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

In the given reference (Antao et al, 2008), a significant difference in mean organ lesion score between a pathogenic and non-pathogenic E. coli strain was found using 20 animals per group (15 sacrificed 24 hours post-challenge, and 5 animals 48 hours post-challenge). The average lesion score of the animals treated with the pathogenic strain was 7,5 (μ); the standard deviation was 2,2(σ). In our study, we wanted to be able to detect a difference of at least 2,5 between average lesion scores (δ). The significance level α was set on 0,05; the power (1-ß) was set on 80%. The standardized difference then becomes 2,5/2,2=1,14. Given these values, a power analysis based on a t-test as used in Antao indicates a sample size of 14 to be adequate (see, for example, http://biomath.info/power/ttest.htm). Since the Antao score is not a continuous variable but yields a range of integer values, a non-parametric test such as a Mann-Whitney or Wilcoxon test is preferable; for these tests, a sample size of 14 is still adequate. It was decided that 16 animals per time point, per group will be included. Maximally 10 animals can be housed in 1 isolator, and groups of 8 give an equal distribution over two isolators. This optimizes the odds of finding significant results while minimally wasting isolator capacity and simultaneously minimizing animal numbers. Therefore, per group maximally 32 animals are used.
16 animals per challenge strain will initially be tested (8 at 24 hours, 8 at 48 hours). If there are no effects of the challenge, that strain is eliminated from further testing. Those strains that do give an effect are tested again with 2 more groups of 8 (again 8 at 24 hours, 8 at 48 hours). This way, there is no unnecessary animal use on non-suitable strains. At the same time the suitable strains are tested with the calculated number of animals (32 per strain, 16 per time point).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Maximally 576 SPF animals of 35 days old will be used in the study to ensure their immunocompetency yet APEC susceptibility. Chickens are the best model to be used as they are the target species for the vaccine. SPF birds are not vaccinated or treated against E. coli, eliminating the risk that the study is influenced by maternally derived immunity in the animals. The project is a proof of concept study, for which a reproducible model is desired. Once this model is established, it will later be adapted to the target animal (commercial broilers).

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

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.

D. 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.

The in-vivo chicken model is most suitable for investigating the challenge model, as the chicken is the target species. The interaction between the challenge, the immune system and other organ systems is vital to obtain a reliable challenge model; therefore replacement by an in vitro model is not possible.
Once a suitable E. coli challenge strain for a serotype is found, other candidates of the same serotype will not be tested. The number of animals is based on the average and standard deviation found in the study of Antao et al., 2008, which found significant results using 20 animals per group, sacrificing 15 animals 24 hours after challenge and 5 animals 48 hours after challenge. As only the 24-hour statistic was significant, the minimal group size was calculated through a power analysis using the results found in this 24h group.
Because standardized animals are used and these are housed in a controlled environment, a negative control group is not enrolled.
Refinement is achieved by selection of the infection model. This is based on a short observation time, focusing on the effects of septicaemia (visible through laboratory analysis and necropsy) and not waiting for severe clinical symptoms. Frequent observation (described below) during the observation periods of 24 and 48 hours ensures timely application of a humane endpoint where required. The animals are handled by experiences animal technicians, ensuring that stress is minimized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals exhibiting severe clinical signs, including:
- **the production of serous exudate upon breathing;**
- **heavy breathing for over 6 hours;**
- **or other signs of severe illness or severe discomfort as noted by the animal technician or veterinarian,**

which lead to unnecessary suffering as physiological behaviour is impaired, will be euthanized as the humane end point has been reached.
All animals will be inspected clinically every 6 hours, starting 12 hours after challenge (no signs are expected before this point) to obtain the most accurate clinical scores and humane end points. Care will be given by experienced animal technicians. The same experienced animal technicians will handle the animals and do all administrations, ensuring that stress is kept to a minimum.
The animals are housed in isolators to prevent adverse effects due to pathogen spreading.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

E. coli strains from recent field cases will be used for the development of the challenge models to ensure that an up to date vaccine profile and challenge are developed.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. 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 H.

☐ 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.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

To determine the most accurate clinical score and reliable organ bacterial counts, the animals must exhibit the natural clinical symptoms. Therefore the use of anaesthesia or analgesia will interfere with the study. If, however, it appears that the chickens experience severe discomfort during the intra-tracheal challenge procedure, outweighing the effects of anaesthesia, the animals will be anaesthetized by inhalation during the challenge procedure. To minimize suffering, the animals will be euthanized as soon as the humane end point is reached.

☐ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

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

Stress due to handling and identification measures, stress due to challenge administration.

Explain why these effects may emerge.

Fixation to place identification wing-tags; fixation for intra-tracheal challenge administration; intra-tracheal administration.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

The animals will be observed every 6 hours, from 12 hours after challenge and onwards (as before this no effects are expected) to ensure accurate clinical scores and a precise humane end point determination. All observations are noted in the study report. Handling, fixation and administration will be done by experienced animal technicians who ensure the least stress for the animal as possible. If necessary, the intra-tracheal inoculation occurs under inhalation anaesthesia.

J. 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 K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals exhibiting severe clinical signs, including:

- **the production of serous exudate upon breathing;**

- **heavy breathing/severe respiratory distress lasting 6 hours;**
- **or other signs of severe illness or severe discomfort as noted by the animal technician or veterinarian,**

which lead to unnecessary suffering as physiological behaviour is impaired, will be euthanized as the humane endpoint has been reached.
All animals will be inspected clinically every 6 hours (starting 12 hours post-challenge) to obtain the most accurate clinical scores and humane end points.

Indicate the likely incidence.

50% of 576 animals = 288 animals

It is expected that the groups receiving the highest doses will suffer the most severe clinical symptoms, as the E. coli infection will generate a systemic illness through septicaemia. Therefore these animals are most expected groups to reach a humane endpoint before termination of the study. The other groups may display clinical symptoms, yet morbidity will be relatively less (10-30%). On average, severe illness/discomfort may be expected in 50% of the animals.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Severe 288 animals. This is the average expected discomfort of the groups receiving the highest challenge doses and the animals reacting on the lower doses. It is based on the worst case scenario (developing clinical illness of the highest category, 4, as described by Antao et al.).
Moderate 288 animals. As described by Antao et al (2008), lower doses may also give clinical symptoms, yet this will be to less animals and the symptoms are mostly restricted to the respiratory apparatus, giving lower clinical scores (up to category 2).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

To obtain lesion scores for the organs and the bacterial content, necropsy must be 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

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, see 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'.	**22400**
1.2	Provide the name of the licenced establishment.	**BIAHO bv**
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: **3.4.4.2** Type of animal procedure: **201609.2 Virulence and persistence of 2 wildtype and 6 modified APEC strains to be used in vaccine formulation**

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.

[201609.2 Virulence and persistence of 2 wildtype and 6 modified APEC strains to be used in vaccine formulation]
Avian Pathogenic E. Coli (APEC) is a bacterium causing a wide spectrum of inflammations in chickens, making it a major problem for which antibiotics are applied. To prevent the use of large quantities of antibiotics, vaccines against APEC are desired. To be able to test the efficacy of vaccine candidates, a challenge model is needed, which will be developed in another study in this project (201609.1). The objective of this study is to select from a panel of 6 modified E. Coli strains the most suitable vaccine candidates based on organ lesions induced in the vaccinated animals. The panel of modified E. coli strains have been attenuated using recombination technology severely affecting replication, and are expected to induce few or mild lesions in comparison to wildtype strains.

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

6 recombinant strains will be tested; 3 recombinants derived from wildtype strain 51 (wt51), and 3 recombinants derived from wildtype strain 52 (wt52). All belong to the serotype O78. The level of attenuation will be measured by comparing the recombinant strains with their corresponding wildtype strain, which are expected to give lesions that are visible on necropsy. The chickens will be vaccinated by coarse spray as day-old chicks, corresponding to the

vaccination scheme of the available commercial vaccine.
On 6 consecutive time points, 3 animals from each group will be euthanized and necropsied to evaluate the vaccine lesion effects (virulence) and bacterial count in the internal organs (persistence). 6 time-points are evaluated as it is unknown for how long colonisation by the vaccine candidates will last. 3 of the time-points are located in the first week, as the initial colonisation period is the most important and therefore requires more information. The scoring protocol is based on Antao et al, 2008 (The chicken as a natural model for extraintestinal infections caused by avian pathogenic Escherichia coli).

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

18 animals are included for each APEC strain. It provides a sufficient number of animals to view multiple time-points, yet is the minimum to be able to compare individuals at each time-point even if some animals are lost during the course of the study due to reasons unrelated to colibacillosis (3 animals for each time-point; compare whether there are lesions at necropsy and whether APEC bacteria can be re-isolated).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

8 groups of 18 (total 144), day-old SPF chicks will be vaccinated by coarse spray (study day 0), with an approximate droplet size of 100 µm and a dose per animal of approximately $9{,}1x10^9$ cfu (the upper limit of the range registered for the available commercial vaccine). On days 2, 4, 7, 14, 21 and 28, 3 animals from each group will be euthanized and necropsied to assess the virulence (by gross lesions) and persistence (through re-isolation of the APEC in tissue samples) of the vaccine in the course of time. On day 28 the last 3 birds of each group are therefore euthanized and necropsied, ending the study. The procedure is summarized in the table below.

SD: study day; wt: wild type; RC: recombinant; n: number of animals

Group	n	SD0	SD2	SD4	SD7	SD14	SD21	SD28
1: wt51	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds
2: wt52	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds
3:wt51 RC1	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds
4: wt51 RC2	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds
5: wt51 RC3	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds
6: wt52 RC1	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds
7: wt52 RC2	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds
8: wt52 RC3	18	Vacc by spray	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds	Necropsy 3 birds

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

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.

D. 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.

As the chicken is the target animal species, it is also the best model in this study. As the aim is to measure the effect of the vaccine on the animal itself, no replacement of the in-vivo model is possible. Vaccine candidates displaying high residual virulence will not be investigated further (study is a decision point). The minimum number of animals that is necessary is used. Using 18 animals per group provides a sufficient number of animals to view multiple time-points, yet is the minimum to be able to compare individuals at each time-point even if some animals are lost during the course of the study due to reasons unrelated to colibacillosis.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals exhibiting severe clinical signs of illness or discomfort which leads to impaired physiological behaviour and/or the inability to maintain homeostasis will be euthanized, as the humane endpoint has therefore been reached. All animals will be inspected every 6 hours to ensure that clinical signs are not missed and therefore the humane endpoint is accurately set and the animal is euthanized timely, preventing unnecessary suffering. All animals are handled by experienced animal technicians, ensuring that stress is minimized.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The tested vaccine candidates were developed recently and have not yet been tested.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. 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 H.

☐ 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.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

To accurately determine the residual virulent effect of the vaccine candidates and their persistence, the immune system must not be iatrogenically influenced. Therefore, no analgesics will be used as this will interfere with the study results.
The animals will be clinically inspected every 6 hours to ensure accurate determination of the humane endpoint and timely euthanasia.

☐ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

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

Stress due to handling and identification measures; stress due to vaccine administration.

Explain why these effects may emerge.

Fixation to place identification wing-tags; vaccination by spray.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

All fixation and administration procedures will be performed by experienced animal technicians, ensuring fast and accurate administration minimizing stress for the animals.

J. 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 K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals exhibiting severe clinical signs of illness or discomfort which leads to impaired physiological behaviour and/or the inability to maintain homeostasis will be euthanized, as the humane endpoint has therefore been reached. Respiratory signs or circulatory defects may be possible due to colibacillosis vaccination with high residual virulence. In general, severe respiratory distress lasting 6 hours is considered a humane endpoint. All animals will be inspected every six hours to ensure that clinical signs are not missed and therefore the humane endpoint is accurately set and the animal is euthanized timely, preventing unnecessary suffering.

Indicate the likely incidence.

25% - Two groups of 18 birds (total 36/144 → 25%) receive the wildtype strains, which are not attenuated. The strains are, however, administered by coarse spray, meaning that the wildtype strains are unlikely taken up in the chicken's respiratory system in the concentrations required to cause clinical disease. The lesions are expected to only be visible during necropsy.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures

are assigned ('non-recovery', 'mild', 'moderate', 'severe').

As the degree of attenuation of the vaccine candidates is not known, the expected level of discomfort is therefore considered as moderate for 75% of the animals, and as severe for 25% of the animals (assuming the worst case scenario that the attenuated strains still cause mild clinical disease and the wildtype bacteria, which have no attenuation, cause moderate or severe clinical disease).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

To determine the extent of the vaccine virulence, all organs must be assessed for gross lesions. Samples from the organs are used to determine the persistence of the vaccine.

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

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, see 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'.	**24400**	
1.2	Provide the name of the licenced establishment.	**BIAHO bv**	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.3	**201609.3 Efficacy of vaccine candidates against APEC infections compared to a commercially available vaccine**

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.

[201609.3 Efficacy of vaccine candidates against APEC infections compared to a commercially available vaccine]
Avian Pathogenic E. Coli (APEC) is a bacterium causing a wide spectrum of inflammations in chickens, making it a major problem for which antibiotics are applied. To prevent the use of large quantities of antibiotics, vaccines against APEC are desired. To be able to test the efficacy of vaccine candidates, a challenge model is developed in the first study in this project and suitable vaccine candidates are selected in the second study. In this third study the efficacy of the selected vaccine candidates will be tested with a homologue challenge and compared to the only available commercial APEC vaccine. Efficacy will be based on clinical and necropsy evaluation and organ bacterial count as described by Antao et al, 2008, The Chicken As a Natural Model for Extraintestinal Infections Caused by Avian Pathogenic E coli (APEC).

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

A maximum of four vaccine candidates will be tested for efficacy. For this purpose the chickens will be vaccinated as day-old chicks (study day (SD) 0) by coarse spray, with an approximate droplet size of 100 µm and a dose per animal around the lower limit of the dose range registered for the available commercial vaccine. The animals will all be challenged with the homologous challenge strain of the commercial and candidate vaccines (serotype O78) on

day 35 (5 weeks of age) according to the challenge model developed in 201609.1. To assess the correct application of the challenge, a positive control group (receiving only the challenge on day 35) is also enrolled in this study. To compare the vaccine candidates with the commercially available vaccine, a group that is vaccinated with the available commercial vaccine and subsequently challenged is also enrolled. The animals will be observed for 24 or 48 hours and then euthanized and necropsied. The choice for 24 or 48 hours post-challenge depends on the results of 201609.1: if the challenge is hard to detect at 48 hours post-challenge, the end of study time is put at 24 hours post-challenge.

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

In the given reference (Antao et al, 2008), a significant difference in mean organ lesion score between a pathogenic and non-pathogenic E coli strain was found using 20 animals per group (15 sacrificed 24 hours post-challenge and 5 animals 48 hours post-challenge). The average lesion score of the animals treated with the pathogenic strain was 7,5 (μ); the standard deviation was 2,2(σ). In our study, we wanted to be able to detect a difference of at least 2,5 between average lesion scores (δ). The significance level α was set on 0,05; the power (1-ß) was set on 80%. The standardized difference then becomes 2,5/2,2=1,14. Given these values, a power analysis based on a t-test as used in Antao indicates a sample size of 14 to be adequate (see, for example, http://biomath.info/power/ttest.htm). Since the Antao score is not a continuous variable but yields a range of integer values, a non-parametric test such as a Mann-Whitney or Wilcoxon test is preferable; for these tests, a sample size of 14 is still adequate. It was decided that 16 animals per group will be included. Maximally 10 animals can be housed in 1 isolator, and groups of 8 give an equal distribution over two isolators. This optimizes the odds of finding a significant result, while minimally wasting isolator capacity and simultaneously minimizing animal numbers.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

A maximum of 96 SPF chickens will be used for this study. 80 will be vaccinated as day-old chicks; 16 of these will receive the commercially available vaccine, another 16 will be vaccinated with the first vaccine candidate, 16 more will be vaccinated with the second vaccine candidate, another 16 with the third candidate and 16 with the fourth candidate. The number of vaccinated groups depends on the number of vaccines selected in study 201609.2. The last 16 animals will be the positive control group, to confirm that an adequate challenge was given. The positive control group can be reduced to 10 animals, depending on the robustness (the size of the dose/effect relationship) of the challenge model developed in study 201609.1. The goal of the challenge is to achieve 50% morbidity by challenge in non-vaccinated animals. All animals (vaccinated and positive control) are challenged intra-tracheally at 35 days of age with a challenge dose that will be determined in study 201609.1. Efficacy will be evaluated by comparing morbidity between the vaccine groups (the candidates and the commercially available vaccine).

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

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.

D. 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.

Because the chicken is the target species, these are best used as the study model. Testing the efficacy of a vaccine can only be done in vivo. The required number of animals was calculated using a power calculation (see section A).
If only 1 or 2 vaccines candidates are selected in study 201609.2, the efficacy and minimum immunising dose (MID) finding study (201609.4) are put together in 1 study, to reduce the number of animals (only 1 positive control group needed). Testing more vaccine candidates for both efficacy and MID in one study is not wanted as the risk becomes too great that non-efficacious vaccine candidates are tested for the MID (leading to unnecessary animal and resource use). If 3 or 4 vaccine candidates are selected in study 201609.2, a selection through the efficacy test must be done first. All animal handling and vaccine and challenge administrations are performed by experienced animal technicians, minimizing stress and discomfort for the animals.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals exhibiting severe clinical signs of illness or discomfort which leads to impaired physiological behaviour and/or the inability to maintain homeostasis will be euthanized, as the humane endpoint has therefore been reached. All animals will be inspected every 6 hours to ensure that clinical signs are not missed and therefore the humane endpoint is accurately set and the animal is euthanized timely, preventing unnecessary suffering. To reduce environmental effects, the animals are housed in isolators with HEPA filters.
The standard procedure for intra-tracheal inoculation is swift, and it appears that the effects of anaesthetizing the animal do not outweigh the limited stress of the procedure. If, however, the circumstances become unfavourable towards the animal's welfare, it will be decided to put the animal under inhalation anaesthesia during intra-tracheal inoculation.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The investigated vaccine candidates have been newly developed and have not yet been tested for efficacy.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. 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 H.

☐ 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.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

Applying analgesia will interfere with the immune reaction of the animals and therefore cannot be applied without interfering with the study results. The animals will be clinically inspected every six hours to ensure timely application of the humane endpoint.
The standard procedure for intra-tracheal inoculation is swift, and it appears that the effects anaesthetizing the animal do not outweigh the limited stress of the procedure. If, however, the circumstances become unfavourable towards the animal's welfare, it will be decided to put the animal under inhalation anaesthesia during intra-tracheal inoculation.

☐ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

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

Stress due to handling and identification measures; stress due to vaccine administration; stress due to challenge administration.

Explain why these effects may emerge.

Fixation to place wing tag identification; vaccination by spray; intra-tracheal challenge; fixation for intra-tracheal challenge.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

All fixation and administration procedures will be performed by experienced animal technicians, ensuring fast and accurate administration minimizing stress for the animals. Animals exhibiting severe clinical signs of illness or discomfort which leads to impaired physiological behaviour and/or the inability to maintain homeostasis will be euthanized, as the humane endpoint has therefore been reached. All animals will be inspected every 6 hours to ensure that clinical signs are not missed and therefore the humane endpoint is accurately set and the animal is euthanized timely, preventing unnecessary suffering.
Inhalation anaesthesia will be applied in case unforeseen circumstances make the intra-tracheal inoculation welfare-compromising.

J. 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 K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals exhibiting severe clinical signs, including:

- **the production of serous exudate upon breathing;**
- **heavy breathing/severe respiratory distress lasting 6 hours;**
- **or other signs of severe illness or severe discomfort as noted by the animal technician or veterinarian,**

which lead to unnecessary suffering as physiological behaviour is impaired, will be euthanized as the humane end point has been reached.
All animals will be inspected clinically every six hours to obtain the most accurate clinical scores and humane end points.

Indicate the likely incidence.

These severe effects are only expected for the positive control group = 16 of 96 animals

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

16/96 animals (17%): severe discomfort (the positive control group, receiving only challenge)
80/96 animals (83%): moderate discomfort (the animals receiving a vaccination prior to challenge)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

To confirm that also the animals that did not display clinical symptoms are not affected by the vaccine or challenge, necropsy must be performed on all animals.

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

Centrale Commissie Dierproeven

Appendix
Description animal procedures

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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	**22400**	
1.2	Provide the name of the licenced establishment.	**BIAHO bv**	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number **3.4.4.4**	Type of animal procedure **201609.4 Minimum immunising dose finding study for vaccine candidates against APEC**

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.

[201609.4 Minimum immunising dose finding study for vaccine candidates against APEC] Avian Pathogenic E. Coli (APEC) is a bacterium causing a wide spectrum of inflammations in chickens, making it a major problem for which antibiotics are applied. To prevent the use of large quantities of antibiotics and diminish APEC antibiotic resistance, vaccines against APEC are desired. In the project challenge models, virulence and persistence and efficacy of the vaccine candidates are tested. This study continues with investigating the minimum immunising doses (MID) of the selected vaccine candidate strains.

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

A maximum of two candidate vaccine strains will be tested for the MID. SPF chickens will be vaccinated as day-old chicks; each group of 14 animals will receive a different dose of a candidate vaccine. 4 doses are tested: 10^6, 10^7, 10^8 and 10^9 cfu per bird. Alongside the vaccinated animals a positive control group (to validate the challenge) will be enrolled. Challenge will be performed intra-tracheally at 5 weeks according to the challenge model developed in 201609.1. The birds will be observed for 24 or 48 hours for onset of disease and/or general performance. The time-point of euthanasia will depend on the developed challenge model – if lesions do not manifest themselves at 48 hours in study 201609.1, the birds will be euthanized and necropsied at 24 hours post-challenge. All animals will be given a clinical score, and at necropsy the organs are scored as described in Antao et al, 2008 (The chicken as a natural model for extraintestinal infections caused by avian pathogenic

Escherichia coli (APEC)) to evaluate the effect of vaccination and challenge per dose. Depending on the results of the clinical and necropsy scores, additional tissue samples of the organs may be taken to determine whether the challenge bacterium can be re-isolated.

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

In the given reference (Antao et al, 2008), a significant difference in mean organ lesion score between a pathogenic and non-pathogenic E. coli strain was found using 20 animals per group (15 sacrificed 24 hours post-challenge and 5 animals 48 hours post-challenge). The average lesion score of the animals treated with the pathogenic strain was 7,5 (μ); the standard deviation was 2,2(σ). In our study, we wanted to be able to detect a difference of at least 2,5 between average lesion scores (δ). The significance level α was set on 0,05; the power (1-ß) was set on 80%. The standardized difference then becomes 2,5/2,2=1,14. Given these values, a power analysis based on a t-test as used in Antao indicates a sample size of 14 to be adequate (see, for example, http://biomath.info/power/ttest.htm). Since the Antao score is not a continuous variable but yields a range of integer values, a non-parametric test such as a Mann-Whitney or Wilcoxon test is preferable; for these tests, a sample size of 14 is still adequate. It was decided that 16 animals per group will be included. Maximally 10 animals can be housed in 1 isolator, and groups of 8 give an equal distribution over two isolators. This optimizes the odds of finding a significant result, while minimally wasting isolator capacity and simultaneously minimizing animal numbers.
4 candidate vaccine doses are tested to be able to create a clear dose-response curve. Both candidates are tested at the same time, so only 1 positive control group is required.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

A maximum of 144 SPF chickens will be used. 128 will be vaccinated by spray as day-old chicks (day 0). On day 35 (5 weeks) all 144 animals (the vaccinated and positive control chickens) are intra-tracheally challenged according to the developed challenge model of study 201609.1. After 24 or 48 hours (depending on the results of 201609.1) of observation all animals are necropsied to evaluate efficacy.

Group	N	Vaccination	Challenge
1: challenge control/positive control	16	NA	Day 35
2: Vaccine 1, 10^6 cfu	16	Day 0, coarse spray	Day 35
3: Vaccine 1, 10^7 cfu	16	Day 0, coarse spray	Day 35
4: Vaccine 1, 10^8 cfu	16	Day 0, coarse spray	Day 35
5: Vaccine 1, 10^9 cfu	16	Day 0, coarse spray	Day 35
6: Vaccine 2, 10^6 cfu	16	Day 0, coarse spray	Day 35
7: Vaccine 2, 10^7 cfu	16	Day 0, coarse spray	Day 35
8: Vaccine 2, 10^8 cfu	16	Day 0, coarse spray	Day 35
9: Vaccine 2, 10^9 cfu	16	Day 0, coarse spray	Day 35

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

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.

D. 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.

As the chicken is the target animal species, these are also the best model for this study. As the aim is to measure the dose response of the vaccine on the animal itself, no replacement of the in-vivo model is possible. By combining the testing of vaccine candidates, only 1 positive control group is needed. The latter is reduced to 10 animals if this is possible, based in the results of 201609.1. Refinement will be fulfilled by monitoring the animals to precisely set and act upon the humane endpoint.
Further reduction will take place if only 1 or 2 vaccine candidates are selected in study 201906.2. In that case the testing for efficacy will be combined with testing the MID, leading to the use of 1 positive control group for both instead of a positive control group per study (a reduction of 16 animals).

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Animals exhibiting severe clinical signs of illness or discomfort which leads to impaired physiological behaviour and/or the inability to maintain homeostasis will be euthanized, as the humane endpoint has therefore been reached. All animals will be inspected every 6 hours to ensure that clinical signs are not missed and therefore the humane endpoint is accurately set and the animal is euthanized timely, preventing unnecessary suffering. All handling will be done by experienced animal technicians, ensuring the minimization of stress.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The tested vaccine candidates were developed recently and have not yet been tested for their dose-response relationship.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. 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 H.

☐ 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.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

To accurately determine the dose-response relationship, the immune system must not be iatrogenically influenced. Therefore, no analgesics will be used as this will interfere with the study results. The animals will be clinically inspected every 6 hours to ensure accurate determination of the humane endpoint and timely euthanasia.
The standard procedure for intra-tracheal inoculation is swift, and it appears that the effects of anaesthetizing the animal do not outweigh the limited stress of the procedure. If, however, the circumstances become unfavourable towards the animal's welfare, it will be decided to put the animal under inhalation anaesthesia during intra-tracheal inoculation.

☐ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

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

Stress due to handling and identification measures; stress due to vaccine administration; stress due to challenge administration.

Explain why these effects may emerge.

Fixation to place identification wing-tags; vaccination by spray; fixation for challenge; administration of intra-tracheal challenge.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

All fixation and administration procedures will be performed by experiences animal technicians, ensuring fast and accurate administration minimizing stress for the animals.

J. 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 K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Animals exhibiting severe clinical signs of illness or discomfort which leads to impaired physiological behaviour and/or the inability to maintain homeostasis will be euthanized, as the humane endpoint has therefore been reached.
Respiratory signs or ciculatory defects may be possible due to colibacillosis. In general, severe respiratory distress lasting 6 hours is considered a humane endpoint. All animals will be inspected every 6 hours to ensure that clinical signs are not missed and therefore the humane endpoint is accurately set, meaning the animal is euthanized timely, preventing unnecessary suffering.

Indicate the likely incidence.

48/144 animals (The animals of the challenge control groups and, reasoning from the worst case scenario, the groups receiving the lowest dosages of vaccine)

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

48/144 animals: severe discomfort (the animals of the challenge control groups and,

reasoning from the worst case scenario, the groups receiving the lowest dosages of vaccine) 96/144 animals: moderate discomfort (the other vaccinated animals)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

To determine the dose-response relationship, all organs must be assessed for gross lesions indicative of colibacillosis.

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