

SCIENTIFIC OPINION

Scientific Opinion on field trials for bovine tuberculosis vaccination¹

EFSA Panel on Animal Health and Welfare (AHAW)^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

The opinion provides advice relating to the design of field trials to test the performance of a vaccine for bovine tuberculosis (bTB), along with a test to Detect Infected among Vaccinated Animals (DIVA). The objective of cattle vaccination is to use the vaccine in combination with presently applied control measures within the EU as an aid towards bTB eradication. The ideal field trials for the DIVA test will follow the OIE guidelines for test validation. Any deviations from the ideal trial design in relation to DIVA test performance should be justified, and the bias that may subsequently be introduced should be accounted for. The ideal field trial design for vaccination performance should implement a double-blind randomised test scenario, and allow for known risk factors in the field situation. Any deviations from the ideal trial design in relation to vaccine performance should also be justified and bias that may subsequently be introduced should be accounted for. Relevant risk factors and possible confounders that should be taken into consideration in the design of field trials are described in this opinion. The safety of a candidate vaccine is guaranteed in the registration of a vaccine medication by a competent authority. The field trials will need to fulfil these requirements to prove that the use of this vaccine in the field is safe for both public health and the environment. Some additional remarks regarding the safety of this specific vaccine are included in this opinion.

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bovine tuberculosis, vaccination, BCG, cattle, DIVA, field trial

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² Panel members: Edith Authie, Charlotte Berg, Anette Bøtner, Howard Browman, Ilaria Capua, Aline De Koeijer, Klaus Depner, Mariano Domingo, Sandra Edwards, Christine Fourichon, Frank Koenen, Simon More, Mohan Raj, Liisa Sihvonen, Hans Spoolder, Jan Arend Stegeman, Hans-Hermann Thulke, Ivar Vågsholm, Antonio Velarde, Preben Willeberg and Stéphan Zientara. Correspondence: AHAW@efsa.europa.eu

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Following a request from the European Commission, the Panel on Animal Health and Welfare (AHAW) was asked to deliver a scientific opinion on field trials for tuberculosis (bTB) vaccination in cattle.

The opinion provides advice regarding the design of field trials to test and validate a candidate bTB vaccine along with supporting tests to Detect Infected Vaccinated Animals (DIVA). The field trials should (also) aim for validation of the use of the vaccine as a contributory tool to assist in bTB eradication, when applied in combination with a test and cull program as currently conducted within the European Union (EU). The field trial should generate the information that is required to obtain marketing authorisation, but these aspects are beyond the scope of this opinion, and are not addressed in detail. The field trials may require exemption from the present bTB control measures, because the tuberculin skin test as applied in test and cull programs in the EU will likely always give a positive result in vaccinated animals. This constraint may present difficulties in implementing a double-blind study design. Any such exemption will require strong guarantees regarding the whereabouts and movements of all animals included in the field trial, especially with regard to export.

The ideal field trial for the DIVA test follows the OIE (World Organisation for Animal Health) guidelines for test validation. Any deviations from the ideal trial design should be justified and bias that may subsequently be introduced should be accounted for. Relevant risk factors and possible confounders that should be taken into account in the design of a field trial are described in the opinion. A complication here is that the test can only be validated in the field in the presence of field-based vaccination. Therefore, this analysis can only be performed in conjunction with work towards vaccine validation. Because of the lack of an optimal gold standard for detecting infection in bacilli Calmette-Guérin (BCG) vaccinated animals, a more thorough *post-mortem* examination of trial animals that is more thorough than the current routine abattoir post-mortem inspection, is recommended in order to increase the diagnostic sensitivity of bTB detection, and supplementary tests should be applied where appropriate. It is advisable to generate data relating to the test sensitivity as early as possible during the trial and to use this information to repeat the power analysis for the vaccination trial. This is a key issue, because if the vaccine does not provide complete protection in an animal, and this is combined with a test with sub-optimal sensitivity, there is the potential for implementing a less efficient set of control measures that includes vaccination. The adverse consequence of this is a less effective test and cull programme.

The ideal field trial for vaccination should implement a double blind randomised test scenario, taking account of known risk factors in the field situation. Any deviations from the ideal trial design should be justified and bias that may subsequently be introduced should be accounted for. Relevant risk factors and possible confounders that should be taken into account in the design of a field trial are described in the opinion. Modelling studies should be applied to optimise the design of the field trial and to estimate the minimal required numbers of animals and duration of the trial. These analyses should also take account of the highly uncertain wildlife exposure risks in the field. To facilitate a comprehensive analysis, full quantitative details of all tests applied during the field trials should be recorded. It is also recommended that modelling tools be developed to translate the individual animal test results into incidence data and transmission parameter estimates, which will help to obtain a more precise estimation of vaccine performance.

The safety of a new vaccine is assessed in the registration of new medication by the relevant competent medicines authority. The field trial will need to fulfil these requirements to prove that the use of this vaccine in the field is safe for public health and environment. Some additional remarks regarding the safety of this specific vaccine are described in this opinion, especially to secure safe trade of vaccinated animals.

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Vaccination of cattle against bTB is explicitly forbidden in the EU legislation on disease control (Council Directive 78/52/EEC) and implicitly also in intra-Union trade legislation, as vaccination is not compatible with the provisions for testing and herd qualification (Council Directive 64/432/EEC). EU legislation is fully in line with the relevant chapter of the OIE Terrestrial Animal Health Code.

One of the main reasons for the current vaccination ban is due to the possibility that vaccinated animals with existing candidate vaccines are not sufficiently protected against bTB infection. Due to the suboptimal protection induced by the available candidate vaccines (only live BCG vaccine), vaccinated animals may become infected if exposed to Mycobacteria species causing tuberculosis, might spread the disease agent and then they cannot be accurately distinguished from the non-infected vaccinated animals, due to the interference of vaccination with currently existing diagnostic methods (PPD-tuberculin skin test) or even with future adapted diagnostic tests.

The EU (research project FP7-KBBE-2007-212414 on Strategies for the eradication of bovine tuberculosis⁴) and also some Member States, notably UK⁵, have invested considerable resources to develop a candidate vaccine accompanied with diagnostic test(s) that would be compatible with the vaccine (DIVA tests).

A review of the scientific knowledge on available on bTB vaccination indicates that the hypothetical development of a policy allowing the use in the EU of the only candidate vaccine (live BCG vaccine) in cattle would need many knowledge gaps to be addressed.

Some of the key issues that need further attention as regards the generation of scientific knowledge and field experience are related to the performance of the candidate vaccine(s) (level and duration of protection from disease or infection), safety (possible shedding of the attenuated live pathogen by recently vaccinated animals, in particular milk-shedding), the conditions for use (age of animals, type of herd, frequency of re-vaccination) and the suitability of the associated candidate DIVA test(s).

Basic scientific information and preliminary field experience is not yet available on the reliability and feasibility of cattle vaccination under EU farming conditions accompanied by use of DIVA test(s) that is fundamental for a possible change in the current EU policy on the control and eradication of bTB and so-related trade consequences.

As regards a hypothetical practical application of vaccination the issue of booster vaccine and how the vaccine could be boosted after the initial inoculation that is related to the duration of immunity and protection is of major importance.

It becomes therefore essential that field trials are conducted and that these field trials are designed in a manner that its outcome enable the scientific community and decision makers to assess all the relevant information and decide on an eventual new bTB policy that incorporates vaccination of cattle as an additional element to combat bTB in the EU. The design of those field trials, considering also the randomisation of outcomes, is then critical as the trials must be comprehensive, sufficient to estimate all the impacts of vaccination but also affordable.

Taking into account the strategic importance of the design phase of the field trials is therefore appropriate to ensure that the preparation of the trials takes into account all the necessary elements that should be integrated in an optimised design.

⁴ <http://www.vigilanciasanitaria.es/tb-step/index.php>

⁵ <http://www.defra.gov.uk/animal-diseases/a-z/bovine-tb/vaccination/cattle-vaccination/>

In view of the above, and in accordance with Article 29 of Regulation (EC) No 178/2002, the Commission asks EFSA for a scientific opinion on the components, requirements, criteria, and specifications for the design of a field trial which outcome would be expected to fill the knowledge gaps related to the vaccination of cattle against bovine tuberculosis.

1.1. Background on bTB control

Bovine tuberculosis (bTB, caused by infection with *Mycobacterium bovis*)⁶ is a notifiable disease and is present in livestock and wildlife populations in a number of EU Member States or zones thereof (SANCO, 2012; EFSA, 2013).

As *M. bovis* is a zoonotic agent, control measures are in place to eradicate the disease in the EU. Council Directive 64/432/EEC defines that bovines can be moved from non-bTB-free regions to bTB-free regions if they originate from herds that have been declared officially bTB-free (OTF), and if the animals have tested bTB-negative in a tuberculin skin test (single intradermal test (SIT) or (single intradermal comparative cervical (SICCT) test). All positive reactors in a tuberculin skin test are subjected to removal from the herd, slaughter and *post-mortem* inspection.

Eradication of bTB has proven difficult in some areas using test and cull strategies, and other approaches, and cattle vaccination is being proposed. However, vaccination of cattle against bTB is currently prohibited by EU legislation. This is because vaccinated animals cannot be distinguished from infected animals by any validated test, but vaccinated animals can still be infected. The authorisation and use of a bTB vaccine in a Member State will require legislative changes, and any changes will need to be supported by an analysis of:

- (1) the performance of a diagnostic DIVA test,
- (2) the vaccine performance in its capacity to assist in the eradication of bTB when applied alongside a test and cull strategy
- (3) the safety of the candidate vaccine.

These three aspects, and how to achieve them in field trials, will be the main topic of this scientific opinion. In the assessment of vaccine performance, the vaccine efficacy as required for marketing authorisation will be included, but the main focus is on the analysis of the vaccine performance in the context of bTB eradication. This means that this opinion focuses on vaccine performance relating to susceptibility and infectiousness, and the value of the vaccine as an aid to bTB eradication when applied in combination with the existing test and cull strategy. The combined strategy could potentially be derived from modelling studies when it cannot directly be derived from the trial results.

The objective of this scientific opinion is to provide guidance for future field bTB vaccine trials in order that the best possible information is generated for assessing the use of the vaccine as a (additional) control option for the eradication of bTB. Data required for the marketing authorisation of the vaccine and the test are not specifically addressed in this opinion, but should also be gathered during the field trials.

1.2. Approach used in the scientific opinion

The background as provided by the EC states that only live BCG vaccine strains have to be considered in this scientific opinion. Several variants of BCG vaccine strains are available but differences in their genomic sequences may have an impact on their capacity to protect against infection and/or disease (Brosch et al., 2007). Furthermore, experiments in cattle suggest that BCG Pasteur induces significantly higher and more sustained IFN- γ responses in whole blood cells stimulated with purified protein derivative (PPD) obtained from *M. bovis* than does BCG Danish, although the level of protection is similar (Wedlock et al., 2007). Currently, the live attenuated BCG Danish strain 1331

⁶ Both *M. bovis* and *M. caprae* cause tuberculosis in bovines and other species, including humans. In the text, only *M. bovis* is mentioned, but any reference to *M. bovis*, unless the contrary is specified, also includes *M. caprae*.

(hereafter referred to as 'BCG Danish'; produced by the Staten Serum Institute in Copenhagen, Denmark) is the only licensed human TB vaccine and the only TB vaccine in the EU produced under Good Manufacturing Practices conditions. The UK Department for Environment, Food and Rural Affairs (Defra) and the Animal Health and Veterinary Laboratory Agency (AHVLA) intend to submit a dossier to the UK Veterinary Medicines Directorate (VMD) providing safety and vaccine performance data to obtain an Animal Test Certificate, required to start field trials using this strain as a candidate bTB vaccine. Other vaccine formulations, such as rationally attenuated *M. bovis* strains, recombinant viral vectored vaccines, or DNA vaccines, are described in the scientific literature (see review by Waters et al., 2012) but are not expected to be granted licences within the next five years. Therefore, this scientific opinion focuses only on BCG Danish and considers this strain as the only possible candidate bTB vaccine that could be used in field trials in the immediate future. This also implies that only the BCG Danish strain is available for a trial design in which an annual revaccination strategy would be applied. The scientific literature suggests that heterologous prime-boost strategies may be more promising (Buddle, 2010), but these methods are less developed.

The background as provided by the EC defines cattle as the target species for the candidate bTB vaccine to be tested in field trials. A 'field trial' is defined as a scientific investigation of a veterinary vaccine under field conditions and in target animals (in terms of animal species and categories), using the product as recommended (EMA, 2001). Within the framework of the pharmaceutical legislation, scientific guidelines do not have legal force and alternative approaches may be taken, provided that these are appropriately justified⁷. Therefore, if for valid scientific reasons the conditions of the field trials do not fully reflect the current field situation, this will not prevent the assessment of the studies and the future granting of a marketing authorisation.

The aim of this opinion is to give guidelines for an efficient design of field trials. The aim is not to actually carry out the design. The complicating aspect of combining field trials for DIVA test validation with vaccine evaluation is not covered in detail here, and both aspects (test and vaccine) are treated as separate questions. Supporting tests can be applied to obtain further knowledge of the disease status of the animals in the trials, and to gather additional data regarding the characteristics of the new test and the performance of the vaccine. Thus, vaccine performance is discussed on the assumption that a sufficiently good test is available for detecting infection in vaccinated animals (with as yet unknown characteristics).

The theoretical concepts for analysing the performance of a diagnostic test in field trials are described, taking into account an ideal trial design. This ideal design would be based on the OIE guidelines on test validation (OIE, 2013). Any deviation from the ideal trial design needs to be justified and accounted for since it may introduce bias that could influence estimates of test performance.

Current scientific knowledge and data gaps related to DIVA testing are presented. It is recommended that this information is considered when designing field trials, although it should be recognised that the background information provided is not exhaustive.

The theoretical concepts for analysing the performance of a bTB vaccine in field trials are described, taking into account an ideal trial design. This ideal design would be based on a double-blind randomised control trial (RCT) design. Any deviation from the ideal trial design needs to be justified and accounted for since it may introduce bias that influences the trial outcome.

⁷ http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004008.pdf

Current scientific knowledge and data gaps on bTB vaccination are presented. It is recommended that this information is considered when designing field trials, although it should be recognised that the background information provided is not exhaustive.

The analysis of DIVA test performance and vaccine performance can be performed at the same time. In general, a separate analysis would be preferred, with the test validation being concluded before the vaccine performance stage, allowing for better power estimates for the trials. However, to properly validate the test, vaccination in the field is required. Furthermore, in this specific case, information on the possible infectiousness of vaccinated animals in the field is required for test validation. Thus, combined trials for vaccine and test validation are a practical solution. The disadvantage is that, if the sensitivity of the DIVA test is low, then the results of the vaccine performance might be overestimated, and a test and cull programme will become less efficient. This could potentially lead to a less effective control programme that included vaccination. This potential risk should be taken into account during the design of the field trials and, because of this, the test validation analysis should be done as early as possible during the trials.

Data derived from laboratory studies involving the BCG vaccine and the DIVA tests, and data from tests on samples from the field, can be used throughout the design stage in order to estimate priors. These can be incorporated into models that are used, for example, to evaluate the power of the study and the numbers of farms and animals required.

Safety aspects related to medicinal products for veterinary use are specified in the Commission Directive 2009/9/EC⁸ which defines the content of the dossier necessary to grant a marketing authorisation in the EU. The field trials will need to provide data to comply with these legal requirements. In addition, the field trials shall provide data on some specific issues that may not be sufficiently covered by laboratory studies, for example, the risk related to trade in vaccinated animals.

2.1. Theoretical concepts of analysing the performance of a diagnostic test in field trials

To evaluate vaccination programmes, it is important that infected animals can be detected in vaccinated populations. For that reason, combinations of vaccines and accompanying tests have been developed for several diseases (including Aujeszky's Disease, Infectious Bovine Rhinotracheitis, Avian Influenza, among others), where the test can detect the immune response to wild-type pathogen and not to the vaccine. In the literature, these assays are usually referred to as DIVA tests (e.g. Uttenthal et al., 2010), with DIVA being an abbreviation for 'Differentiating Infected from Vaccinated Animals'. However, this definition is somewhat confusing in relation to this mandate on bTB vaccination, because the DIVA result in this context does not, in fact, differentiate infected from vaccinated animals. A DIVA-test negative result can originate from an uninfected animal irrespective of its vaccination status and a DIVA-test positive result can originate from an infected animal irrespective of its vaccination status. For this reason, throughout this opinion, the abbreviation DIVA refers to 'Detecting Infected among Vaccinated Animals'.

The bTB DIVA test (DIVA skin test and/or DIVA interferon-gamma (IFN- γ) test) uses antigens that are expressed and/or secreted by *M. bovis* field strains but not by the attenuated BCG Danish vaccine strain (**Error! Reference source not found.**; Vordermeier et al., 2009, 2011a, b; Jones et al., 2010). The most commonly used antigens include the mycobacterial proteins ESAT6 and CFP10 or peptides derived thereof. The ESAT-6 and CFP-10 proteins are encoded by genes absent from the BCG vaccine strain and from almost all environmental mycobacteria. Additional antigens can be included in the test

⁸ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:044:0010:0061:EN:PDF>

as stimulating antigen to increase test performance. One particular candidate antigen, Rv3615c, the gene of which is present in both BCG and *M. bovis* (but it is not secreted by BCG) has been shown to stimulate IFN- γ responses in a significant proportion of *M. bovis*-infected cattle, but not in naïve or BCG-vaccinated animals (Sidders et al., 2008). The same antigen stimulated IFN- γ responses in a significant proportion of infected cattle that did not respond to ESAT-6 and CFP-10. Therefore, inclusion of the Rv3615c epitope in combination with differential tests based on ESAT-6 and CFP-10 has the potential to significantly increase diagnostic sensitivity without reducing specificity in BCG-vaccinated populations. The current bTB DIVA test does not differentiate infected and vaccinated animals; it allows only identification of the status of the animals with regard to infection. Vaccinated and non-infected animals should give a negative result in the DIVA test, but infected animals should still be detected, independently of their vaccination status. The combination of a tuberculin skin test-positive reaction and a negative DIVA test may suggest that an animal is vaccinated. The vaccine-induced skin test sensitization (when using tuberculin) is present in all animals for up to 6 months post-vaccination (Whelan et al., 2011). Animals may become negative in the skin test nine months after vaccination, but a proportion remain sensitized for at least two years.

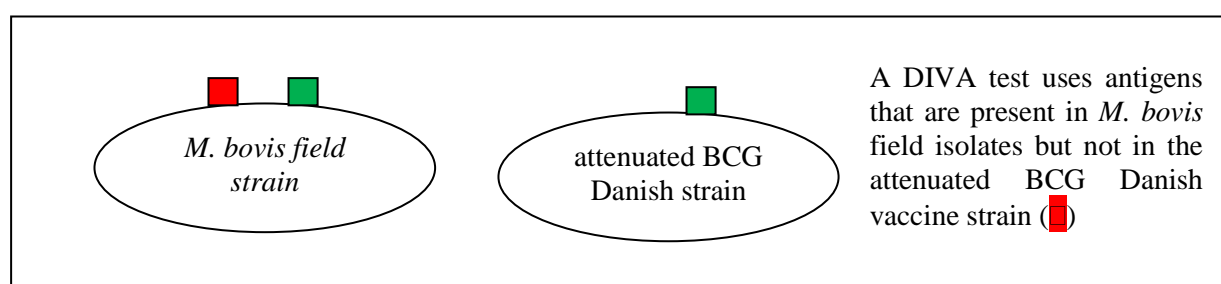


Figure 1: Antigens used in a DIVA test (adapted from Vordermeier et al., 2009)

DIVA test performance should be estimated before the vaccine performance trials are started, based on other laboratory studies that indicate that the DIVA test performance is at least in line with other currently available tests. The reason for this is that the vaccination trials will be ineffective if the test performance turns out to be poor at the end of the trials. More precise estimates can be made during the field trials. The analysis of DIVA test performance in the field trials should be based on the principles and methods to determine the diagnostic performance of an assay for infectious diseases (OIE, 2013). The estimates of sensitivity and specificity are the main performance indicators as they are the basis for the calculation of other parameters (e.g. predictive values). In order to overcome the absence of a gold standard test to define the true bTB status of an animal, a multiple model approach including latent class analysis could be developed. For this purpose, animal level information should be collected (e.g. optical density (OD) signals, or other continuous measures such as skin test readings). If *post-mortem* inspection is included in the multiple model approach, there will be a requirement to improve the positive detection rates over those detected at routine abattoir inspection. A more intensive *post-mortem* detection should be consistently performed (e.g., applying the protocol that is normally used for reactor animals to all animals enrolled in the trials), because *post-mortem* inspection is very insensitive and also has relatively low specificity in animals with early-stages disease. Further, there is currently evidence of variation in sensitivity and specificity of *post-mortem* inspection for bTB across different abattoirs (Frankena et al., 2007; Olea-Popelka et al., 2012). Furthermore, in a selected number of animals, the determination of the true disease status should be improved by slicing through and inspecting all pre-defined sets of lymph nodes where infection is likely to be detected and, where appropriate, by applying supplementary tests (e.g., collection of tissues for histology, bacteriology and polymerase chain reaction (PCR)). Procedures for detailed *post-mortem* inspection are available in the scientific literature (e.g., Corner et al., 1990; Corner, 1994).

Because the sensitivity of the available tests is influenced by the distribution of animals at various stages of infection and disease, the field trials (including analysis of DIVA test performance) should be conducted in herds with varying within-herd bTB prevalence.

The DIVA test performance should be compared in vaccinated and unvaccinated animals to assess the effect of vaccination on the assay characteristics through comparison of test results in both cohorts of animals: all raw data signals/readings should be available for analysis to determine if their range is altered as a result of vaccination. Although estimation of test characteristics in the vaccinated population is the main purpose of DIVA analysis within this mandate, the test characteristics in unvaccinated animals should also be determined, if the test is to be used beyond the field trials.

A standard operating procedure (SOP) for a clearly defined DIVA test and a quality control programme should be implemented in order to ensure harmonised implementation of the sampling protocols, and to facilitate analysis of the data collected throughout the trials. As the cut-off values used to interpret a test result can affect the test performance, the criteria for interpretation of the test should be justified based on best scientific evidence. The details should be described precisely and in accordance with the guidelines on good clinical practices (EMA, 2000) in order to prevent different implementation strategies and interpretation of the DIVA test results. If alternative DIVA test parameters are compared during the trials (e.g. commence antigen stimulation of whole blood 8 or 24 hours after sampling, with peptides or protein as antigen source), each alternative should be considered as a different DIVA test. In other words, separate SOPs should be made available for each alternative DIVA test, and test performance of each should be analysed in the field. The DIVA test used at the start of the trials should be used until the end of the trials. If, at any time, an alternative DIVA test is included in the trials, this test should be performed in addition to the original DIVA test. If the DIVA test is not used as part of a test and cull strategy, the quantitative test result can be used throughout the study and analysed retrospectively to estimate its performance. If, however, the test result forms the basis of a test and cull strategy, then the cut-off value needs to be determined as early as possible in the trial. This could prove difficult to achieve, given that the characteristics of the test when applied to vaccinated animals in the field are currently unknown.

Data collection should be informed by modelling statistics in the design phase of the trials, based on (a range of) a priori estimates of sensitivity and specificity. If stratification factors are required, they must be described as well as the sampling strategies that were used. In the case of a hierarchical population structure, simulations might be needed in order to estimate the sample size and to ensure the pre-specified level of confidence and power, which will heavily depend on the outcome of interest (primary and/or secondary). Data should be provided at the animal level and on a continuous scale (e.g. mm skin thickening for skin test readings and OD signals for the IFN- γ assay). These data could then be used to fit mixture models (given that no gold standard is available) in order to estimate test performance characteristics, such as sensitivity and specificity.

Animal characteristics should be reported in accordance with the OIE diagnostic assay validation guidelines (OIE, 2013). The variables to be noted include a unique identifier for the animal, date of birth, breed, sex, herd of birth, and movement history (herd identifiers). In addition, date(s) of vaccination, testing history and test results (signals/readings) should be provided. At the herd level annual official bTB status in the previous five years should be registered. A more detailed description of data collection is provided in Appendix A.

Confounders are aspects or variables that are associated with both vaccination and the probability of becoming infected. Where they are not equally distributed amongst the vaccinated and the unvaccinated groups, they may influence the outcome of the trials. If not included in the design and carefully incorporated in the control group, they can enhance uncertainty in the required estimates, because the impact of these aspects cannot (easily) be distinguished from the studied variables.

Examples of confounders include vaccination of cattle against Johne's disease (Coad et al., 2013), BCG Danish vaccination of animals that were already infected, and the influence of environmental mycobacteria on bTB testing (see Section 2.2.5).

Possible confounders should be identified before initiating field trials.

To obtain an unbiased estimate of the test performance, it is important that the samples used to validate the test have been randomly collected.

Any deviation from the ideal design is likely to introduce bias into the trials. The presence of bias on selection, performance, detection, attrition and reporting should be assessed (see EFSA guidance on systematic review methodology (EFSA, 2010a). Where a potential bias is recognized, it should be indicated how the bias may affect the outcome of the trials and how this is taken into account.

2.2. Current scientific knowledge and data gaps on DIVA testing

The available literature indicates that animals that are BCG vaccinated become reactors in the tuberculin tests (SIT test, SICCT test and IFN- γ assay) even if they are non-infected (Vordermeier et al., 2001; Sidders et al., 2008; Buddle et al., 2011; Whelan et al., 2011). Whelan and colleagues (2011) reported that all 20 calves vaccinated at 6 weeks of age remained SICCT skin test reactors at six months of age. Between six and nine months of age, the strength of the skin test responses decreased sharply, resulting in approximately 8% and 30% of the calves remaining positive at nine months according to the standard and severe interpretation of the SICCT, respectively. A significant proportion of calves (mean percentage of animals approximately 10% and 30% for standard and severe interpretation, respectively) remained sensitised to respond to SICCT for at least two years. Although these data should be generated and analysed in a larger population under field conditions, it is clear that the infection status of BCG-vaccinated animals cannot be reliably determined using the tuberculin tests alone as long as the vaccine-induced sensitisation lasts. In addition, if applied, the primary skin test readings of all animals, including unvaccinated, unvaccinated/infected, vaccinated and vaccinated/infected animals will need to be analysed to determine if any significant alterations in readings arise from vaccination and whether this compromises the standard and severe interpretation of the skin test. It appears, therefore, that the wider use of the candidate bTB vaccine can be considered only when a DIVA test is available (i.e. validated in the field) that can provide information on whether an animal that reacts in a tuberculin test has truly been infected with a virulent *M. bovis* field strain, since current legislation prescribes that all infected animals, based on failing the tuberculin skin test, need to be identified and culled in order to eradicate the disease (see Section 1.1).

The use of BCG in field trials raises legal problems since the vaccinated animals (or a proportion of) will become skin test positive and should be culled in order to comply with the current legislation. An option to consider is to seek derogation to allow retention of animals in herds involved in the field trials, to enhance the power of the study for quantifying transmission parameters, while recognizing the potential for increased risk of spread of bTB resulting from infected animals remaining in a herd for a prolonged period. The use of the DIVA test to identify infected vaccinated animals is not ideal because, without prior validation in the field, the determination of the cut off points would be arbitrary. Thus any test and cull program applied in the field trials will differ from the current control measures, since a different test will be applied.

Two types of DIVA test that could be used in field trials are described in the scientific literature: the DIVA IFN- γ assay and the DIVA skin test (Vordermeier et al., 2001, 2009, 2011; Cockle et al., 2002, 2006; Aagaard et al., 2003, 2006; Sidders et al., 2008; Ameni et al., 2010; Jones et al., 2010, 2012;

Whelan et al., 2010). There are relative merits associated with both testing platforms. The single visit and implementation of a quality assurance protocol associated with the laboratory-based DIVA IFN- γ assay could be advantageous relative to the SICCT test. However, the DIVA IFN- γ test will have significant logistical and laboratory resource implications that may prove difficult to address. In contrast, the skin test platform may be more practical and would likely have greater acceptance in the field. The fact that antigens would need to be injected into the animal could have important regulatory licensing implications for the skin test option and this may not be feasible in the immediate future. The consequences of the relative performance of each test may be highly dependent on how the tests are used. Ideally the trials would provide data on the performance of the DIVA test when used as a stand-alone test and if used in series with the skin test. Other DIVA systems are not considered since they are far from being validated and are unlikely to be tested in field conditions within the coming years (Sopp et al., 2006; Green et al., 2009; Whelan et al., 2010). In addition, test systems based on cellular immunity (e.g. skin test and IFN- γ assay) could be applied at an earlier time point following infection than serology-based assays, as the cellular immune response appears to precede the humoral immune response after a bTB infection (Ritacco et al., 1991; Whelan et al., 2010).

2.2.3.1. Tuberculin skin test and IFN- γ assay

Studies using experimental *M. bovis* infection have shown that calves infected with 10^4 *M. bovis* colony-forming units by the intranasal route become reactors to both the SICCT test (at standard interpretation) and the IFN- γ test as early as three weeks after challenge (Thom et al., 2006). Other experiments also conducted in the UK found no correlation between *M. bovis* infective dose and the time taken to develop a reaction to the intradermal injection of tuberculin (Dean et al., 2005). The precise dose and timing of the transmission event in natural infection is impossible to determine, but based on these results from experimental infections, it is likely that specific cell-mediated immunity (CMI) responses can be measured in the skin test or in peripheral blood within four weeks of natural infection.

2.2.3.2. DIVA test

The published literature contains no information on the minimum time between experimental infection of cattle with *M. bovis* and the detection of infection in these animals using a DIVA test. Moreover, in naturally infected animals, the timing and infective dose of *M. bovis* challenge is always an unknown, and the kinetics of IFN- γ secretion in the DIVA test may differ from those of experimental infection. Data from field trials could help in modelling the time between infection and detection.

Vaccination of calves with BCG Danish within the first four weeks of life has been shown to exert a significant protective effect against experimental challenge with *M. bovis* (Buddle et al., 2002, 2003; Hope et al., 2005, 2011; Wedlock et al., 2007). However, in most of these studies, there was a relatively short interval (e.g., typically three months) between vaccination and infectious challenge with *M. bovis*. A recent duration of immunity study has shown that BCG vaccination of calves induces a significant degree of protective immunity that lasts for at least 12 months but that this wanes by 24 months post vaccination (Thom et al., 2012). In that study, ESAT-6/CFP-10-specific IFN- γ secretion was observed in all animals post-challenge, irrespective of vaccination status. In the animals challenged at 12 months post vaccination, higher responses were observed in the control group from four weeks to eight weeks following infection. No differences in reactivity to ESAT-6/CFP-10 were observed between control and vaccinated animals challenged at 24 months.

Herds will be subjected to annual surveillance testing in the areas considered for the trial, with additional testing required when reactors are disclosed in the herd. There is currently no information on how vaccination will impact on test frequency. For example, there is a knowledge gap on the

appropriate timing of applying a DIVA test post tuberculin testing in vaccinated and unvaccinated cattle under conditions of natural exposure to *M. bovis*. The age of an animal at vaccination with respect to application of the first tuberculin test and DIVA test may need to be investigated, particularly if using the DIVA IFN- γ test, as the IFN- γ assay is not recommended as a diagnostic test in calves below six months of age (Jones et al., 2012). If it is considered in the trials that, as the initial immunity starts to wane, revaccination with BCG Danish is required to stimulate longer-duration protection, the influence of this revaccination on the long-term sensitisation to tuberculin, and whether it impacts on the optimal timing of the DIVA test, has to be determined.

If the skin test will be used as the main diagnostic test, then this should be established before the initiation of the field trials or at least during the field trials. The timing and intervals of tuberculin testing, DIVA testing and vaccination should be harmonised throughout the field trials (an SOP and quality control programme should be created and implemented).

The detection of *M. bovis* infection in cattle relies on the measurement of the CMI response, which dominates in the early stages of tuberculosis and involves recruitment and activation of a variety of T cells to the site of infection (Pollock et al., 2005). These responses can be measured peripherally and this has led to the development of several diagnostic assays that have proven to be effective in diagnosing tuberculosis in cattle (de la Rua-Domenech et al., 2006). The tuberculin used in cattle contains a crude mixture of predominantly secreted mycobacterial proteins derived from specified strains of *M. bovis* (Andersen, 1994; Inwald et al., 2003) and varies widely in both protein content and antigenic profile (Tameni et al., 1998). The reactive antigens are common to the members of the *M. tuberculosis* complex and tuberculin can be used to measure the CMI response as evidence of exposure to *M. bovis*. However, many of these antigens are also found in non-pathogenic environmental mycobacterial species and this cross reactivity to common antigens can result in a reduced specificity of the test, giving rise to non-specific reactors (false positives) (Francis et al., 1978; Monaghan et al., 1997). Where this problem occurs, a *M. avium*-derived tuberculin is included to perform a comparative test. Cross-reactivity can also potentially arise from concurrent infections with related pathogens e.g. *M. avium* subsp. *paratuberculosis*, or any microbe with shared epitopes, all of which potentially influence the specificity of this test. Defined mycobacterial antigens such as ESAT-6 and CFP-10 are included in the DIVA test as stimulating antigens, and serve to increase test specificity (Pollock and Andersen, 1997; Waters et al., 2004; Aagaard et al., 2006) as these proteins are encoded by genes absent from almost all environmental mycobacteria (Hughes et al., 2005). However, as any particular environmental cause is likely to vary both spatially and temporally, caution should be taken when extrapolating specificity estimates from one cohort of animals to another in a different environment or over time. In order to establish the true specificity of a test, it should be determined in unbiased cohorts of animals, i.e. after due consideration of those likely risk factors that might confound test specificity. Such factors might include age and breed of animal, environmental factors on the farm and surrounding area, concurrent infections, medications and the history of other diseases on the farm.

If a protective effect of BCG Danish is established (e.g. statistically significant reduction in the number and/or size of lesions), this may impact negatively on the ability to detect lesions at routine *post-mortem* detection. If animals are infected but without visible lesions, failure to detect this class of animals by the current routine *post-mortem* inspection may result in a failure to detect infected herds. As infected and potentially infectious animals may remain undetected, they pose a risk to susceptible animals, resulting in prolongation or recurrence of herd breakdowns. For this reason, a more exhaustive *post-mortem* inspection and sampling for bTB confirmation might be required in abattoirs that receive cattle from the trial areas (see Section 2.1.2). The impact of a positive vaccine effect on the performance of *post-mortem* detection will need to be established in the field trials.

In the initial period of the trials, there will be animals that are already infected when they get vaccinated. This is not the typical target group of the trials, which would aim to vaccinate animals before they become infected. Thus, an extended time period is required for thorough analysis of the tests. This initial stage of the experiment will supply further useful information that can be used to analyse the transition or 'run-in' period until a vaccination programme is being initiated.

Analysis of DIVA test performance and vaccine performance can be performed simultaneously. However, if the test sensitivity is insufficient, then the results of the vaccine performance will be more difficult to interpret. Furthermore, the added value of vaccination applied in conjunction with a test and cull strategy may be low or even negative, if the positive effects of test and cull are lessened owing to a lower sensitivity of the test. This risk should be taken into consideration in the design stage of the field trials.

Given all the above, and taking account of the fact that the analysis of the validation of the DIVA test and vaccine performance can be performed separately, it is suggested that a number of (infected and non-infected) farms are selected specifically for evaluation of the DIVA test, with regard to sensitivity and specificity. It would be of benefit during the vaccine trials if prior information about test performance were available. It is suggested that these farms will be partially vaccinated, with each animal being tested regularly using several tests, including, at least, the classical and the DIVA IFN- γ tests. It is difficult to foresee how long the trials will need to last, but three years will be the minimum, to allow collection of data on animals that became infected after vaccination, and to allow sufficient time for those animals to become positive to the test. The number of animals and farms involved will also influence the design of the trials.

Use of Bayesian techniques for test validation without a gold standard can be helpful, especially since a perfect test will not be available. The analysis will profit from a method that includes the latent period in the analysis. This may differ from the unvaccinated situation.

3.1. Theoretical concepts of analysing the performance of a vaccine in field trials

Although several aspects are relevant when considering vaccine performance, the focus in this opinion concerns the added value of vaccination for eradication purposes. Thus, the following aspects of vaccine performance need to be addressed.

1. Reducing within-herd transmission, which will help in achieving eradication of the infection from a herd. Two aspects are relevant here: the decline of susceptibility due to vaccination, and the impact of vaccination on infectiousness (be it delayed or reduced). Both aspects lead to lower within-herd transmission as demonstrated by a lower reproduction ratio of the infection. In order to estimate transmission, the prevalence of bTB-infected animals and the incidence of new infections needs to be established. Further, vaccination needs to be assessed in conjunction with a test and cull programme using the DIVA test, to evaluate the added value of vaccination in addition to culling based on test and removal.
2. Reducing the likelihood of introducing infection into free herds as a result of reduced susceptibility of the vaccinated animals (directly), and reduced susceptibility of the herd (indirectly). In this case, the performance of the vaccine partially depends on the risk and mode of introduction of infection.

These aspects cannot be measured directly, but follow from the results of regular testing of all animals in the field trial. The likelihood of an animals' infection status can be determined during the trial based

on the results of testing. This information can be used to estimate the transmission parameters addressed above.

The guidelines on good clinical practices related to the design and conduct of clinical studies of veterinary products (EMA, 2000) and the REFLECT statement on reporting guidelines for RCTs in livestock (O'Connor et al., 2010) should be implemented.

The double-blind RCT is accepted as the gold standard as it produces knowledge untainted by bias or confounding when ideally performed.

The unit of interest to determine vaccine performance could be the animal, the herd or the region (Table 1). The preferred unit depends to a large extent on the expected quality of the vaccine, but also on the way the vaccine is integrated in a control programme and the desired outcome measures. There are a number of advantages to using the animal as the experimental unit. It will probably provide the simplest design and least expensive trial, and would also require the lowest number of recruited animals. Furthermore, herd factors that affect the transmission of bTB would be expected to be the same for vaccinated and unvaccinated animals. A vaccine shown to be effective using this approach is also expected to be effective when applied at the herd or regional level (see VanNes et al., 1996 for explanation). Moreover, by varying the vaccination coverage within herds between 0% and 100%, the indirect effect of vaccination can also be estimated (Aznar et al., 2011). This approach also enables unvaccinated animals to act as 'sentinels', helping unexpected negative outcomes to be detected. However, there are also problems associated with a decision to use the animal as the experimental unit of interest. In situations where vaccine reduces transmission, but not to an extent that efficient transmission cannot occur (as might be the case for the BCG Danish vaccine), this design could result in an underestimation of the performance of the vaccine in a programme where vaccination is applied at herd or regional level. The reason is that the probability and size of an outbreak in a herd could be reduced to an extent that efficient transmission between herds is not possible. An example of the latter is the eradication of Aujeszky's disease virus by vaccination in several regions, despite the fact that major outbreaks can still occur in vaccinated herds (Stegeman et al., 1995).

Choosing the herd as the unit of interest could solve the afore-mentioned problem. A disadvantage, however, is that the cost of the trial would increase considerably and farm effects need to be taken into account (e.g., equal distribution of exposure across vaccinated and unvaccinated group, random allocation of herds and matching for equal distribution of risk factors and other control measures). If a vaccine is effective at this level, it would also be expected to be effective in a programme applied at the level of an entire region. If a vaccine does not prove to be effective in this setting, it might still be effective at the regional level (for example, Aujeszky's disease has been eliminated by vaccination in several regions in Europe, but major outbreaks in herds of vaccinated finishing pigs are still possible).

Choosing the region as the unit of interest best represents the way the vaccine will likely be implemented in a programme. In this case, exposure of vaccinated herds to *M. bovis* originating from unvaccinated farms is minimized and, consequently, the demands on performance of the vaccine are lowest. However, the costs will be highest in this scenario, and the number of regions that can be included is likely to be restricted, highlighting the need to assess confounding factors.

Table 1: Advantages and disadvantages of the different units of interest for a field trial

Unit of interest	Advantages	Disadvantages
Animal	<ul style="list-style-type: none"> • Simplest design • Least expensive • Lowest number of animals • Factors affecting bTB transmission will be the same for all groups • Possibility of estimating the indirect effect of vaccination • Positive result at this level might be extrapolated to the herd and regional levels 	<ul style="list-style-type: none"> • Possible underestimation of vaccine performance for vaccine that does not provide full protection • If vaccines proves inadequate at this level, it cannot be excluded that the vaccine would be effective when applied at herd or regional level
Herd	<ul style="list-style-type: none"> • No influence of unvaccinated animals on vaccinated animals within a herd. Especially relevant if ecological risk factors (at the farm level) have a different effect on vaccinated and on unvaccinated animals. • Positive result at this level might be extrapolated to the regional level 	<ul style="list-style-type: none"> • More complex design • Higher cost • Higher number of animals • Factors affecting bTB transmission should be taken into account • Negative result at the herd level cannot be extrapolated to the regional level
Region	<ul style="list-style-type: none"> • Best representation of situation after implementation of vaccination • Maximal indirect effect of vaccination • No underestimation of vaccine performance for imperfect vaccine 	<ul style="list-style-type: none"> • More complex design • Highest cost • The number of regions involved in the trial might be limited • Factors affecting bTB transmission should be taken into account

bTB: bovine tuberculosis.

A combination of different units (for example, animal and herd level) may provide the strongest experimental design (highest power).

To enable a proper analysis of the performance of the vaccine, data at the level of the individual animal should be provided for all animals present in the herds participating in the study. These include data normally registered in identification and registration systems, such as a unique identifier for the animal, date of birth, breed, sex, herd of birth and, movement history (herd identifiers). In addition, date(s) of vaccination, testing history and test results (signals of the assay) should be provided. At the herd level, annual official bTB status for the previous five (or more) years should be known and possible risk factors associated with the risk of bTB introduction and with within-herd transmission of bTB (e.g. herd type) should be registered. A more detailed description on data collection is provided in Appendix A. Preferably, the data collected would allow for different data analytical methods to be performed, since there is no accepted standard analysis method to assess vaccine performance at the moment.

Vaccine performance is best measured by double-blind RCTs. Such vaccine performance trials represent the “best case scenarios” of vaccine protectiveness under controlled conditions and are commonly required before a new vaccine is licensed by a regulatory authority (Clemens et. al., 1996). From the outcome data, vaccine performance can be estimated using different approaches. For example, vaccine performance could be expressed as vaccine efficacy as a proportionate reduction in disease/infection attack rate (also called risk) (AR) between the unvaccinated (ARU) and vaccinated (ARV) study cohorts and can be calculated from the relative risk (RR) of disease/infection among the vaccinated group and the unvaccinated one as followed:

$$VE = (ARU - ARV) / ARU \times 100 \quad (1)$$

More examples of frequently used methods are briefly described in Appendix B.

The advantages of a randomized clinical controlled trial include rigorous control for biases afforded by randomization, as well as prospective, active monitoring and careful tracking of vaccination status. Often there is, at least for a subset of the study population, laboratory confirmation of the infectious outcome of interest and a sampling of the immune response to vaccination. The major disadvantages of vaccine performance trials are the complexity and expense of performing them, especially for relatively uncommon infectious outcomes for which the sample size required is driven upwards to achieve clinically useful statistical power. From the collected data (e.g. test results of individual animals), different outcome measures can be estimated, such as transmission rate, which conditions of exposure to infection, or the incidence rate, hazard rate, or cumulative incidence (attack rate), which do not condition on exposure to infection (Halloran and Struchiner, 2010).

The number of animals that should be included in the vaccine trials is dependent on (1) the selected unit of interest, (2) the outcome of interest (incidence, transmission), (3) the error that is allowed in the estimation of vaccine performance and (4) the desired confidence interval for the estimates. It is recommended that the trial outcomes should allow estimation of vaccine effect on both the bTB incidence of bTB, and transmission of infection. The latter provides relatively straight forward insights with respect to the vaccine's potential for eradication. It is not within the scope of this mandate to define the minimum acceptable error or confidence interval. However, it is clear that the precision of the results should enable a meaningful conclusion. A modelling study to determine the size of the study required to achieve sufficient power is strongly recommended.

Analysis of results in the first phase of the vaccine trial (first infection, then vaccination) is different from analysis of results obtained in a second phase (first vaccination, then infection of animals) because initially, the infected animals will have been infected before vaccination, resulting in a possible overexposure to vaccinated uninfected animals. Depending on the performance of the vaccine, an effect is likely to be detected only in the second phase.

In addition to the parameters mentioned above that need to be taken into account in designing the field trials, it is necessary to stress that a further aim of the field trials is to confirm under field conditions the vaccine performance results obtained in laboratory conditions. In order to fulfil the requirements of Commission Directive 2009/9/EC with regard to the demonstration of efficacy, the vaccine used in the trials has to be administered in the target animals as recommended in the product information leaflet.

Confounders are aspects or variables that are associated with both vaccination and the probability of becoming infected. If they are not equally distributed amongst the vaccinated and the unvaccinated groups, they may influence the outcome of the trials, and if not included in the design and carefully incorporated in the analysis, they will enhance uncertainty in the required estimates, because the impact of these aspects cannot (easily) be distinguished from those of the studied variables. Often these aspects are referred to as risk factors.

Identification of 'possible confounders' should be done before initiating field trials since trial design is important to prevent confounding. In a perfect clinical trial, this is not an issue because the confounding factors will be equally distributed over the vaccinated and control groups. However, and particularly if the number of units is limited, there may be an unbalanced distribution. Consequently, it is important to identify the confounding factors and register them during the trials. Data analysis will show if 'possible confounders' are 'real confounders'.

Possible confounders could be for example, (1) vaccinated animals in regions with infected badgers and unvaccinated animals in regions with only uninfected badgers, or (2) differences in the presence of environmental non-tuberculosis mycobacteria.

Several methods are available to deal with confounders. Matched-pairing of herds is a good way to deal with the confounding effect of wildlife exposure.

Deviation from the RCT design is likely to introduce bias into the trials. The presence of bias on selection, performance, detection, attrition and reporting should be assessed (see EFSA guidance on systematic review methodology (EFSA, 2010a)). Where potential bias is recognized, it should be indicated how the effect of the bias may affect the outcome of the trials and how this is taken into account.

Although a joint analysis of the test and vaccine performance should be feasible, for simplicity of this description it is assumed that test validation has been completed successfully and that this test is available and was correctly applied during the trials. If test validation has not been completed prior to the vaccine trial, multiple model approach including latent class analysis will allow for a joint analysis. However, it will be hard to draw firm conclusions on vaccine performance if the DIVA test were subsequently found to perform poorly.

Quantifying reduced susceptibility of vaccinated animals, i.e. the lower probability of becoming infected, can be achieved by determining the fraction of vaccinated animals that becomes infected, compared with the fraction of unvaccinated animals. Obviously there are some complications, for example the age of the animals needs to be taken into account. Furthermore, at the start of the experiment, animals may already be infected before vaccination, but not yet detected: the influence on the results will be different for these animals than for 'normal' vaccinated animals. The duration of the trials should be long enough to take account of this aspect.

The relative infectiousness of vaccinated infected animals also needs to be evaluated. This can be delayed or reduced as compared to unvaccinated infected animals. Both delayed and reduced infectiousness can support an eradication programme. Quantifying this requires many long-term data from infected farms. An age-structured model for the evaluation is essential here.

Based on the results obtained regarding the within-herd transmission aspects, the probability of herds becoming free of infection can be evaluated. Determining the likelihood of (persistent) eradication should be based on the EU definitions regarding freedom of disease. The probability of becoming and remaining free can be evaluated, for example, by assuming a fully free cattle population and a constant infection pressure from wildlife (worst-case assumption).

3.2. Current scientific knowledge and data gaps on bTB vaccination

The implementation of an RCT design for assessing the vaccine performance is important in order to limit the introduction of bias. The text below highlights some aspects that should be taken into account when designing a trial. It is beyond the scope of the current mandate to design a study.

Blinding of treatments (vaccine and placebo) will be difficult to maintain if skin testing is performed on all the animals enrolled in the trials, since a high proportion of vaccinated animals may become skin sensitised to tuberculin for at least six months after vaccination (Whelan et al., 2011). The absence of blinding will introduce bias (e.g. reading of skin test and *post-mortem* detection). For that reason, the IFN- γ test using bovine (and avian) tuberculin is the preferred test to replace the tuberculin skin test which is required in the regulations, in order to obtain information on the immune response induced by vaccination. A previous EFSA opinion (EFSA, 2012) reported that the tuberculin IFN- γ assay could be a suitable alternative to the tuberculin skin tests in the unvaccinated animals, although in some populations the specificity may not be as high as the single intradermal test, the official test currently used with lowest specificity.

Tuberculin skin test reactors need to be removed from a herd based on the current legislation. This will make it extremely difficult to achieve the objectives of the field trial, as it will lead to removal of vaccinated uninfected animals (Whelan et al., 2011). Removal of animals based on a positive DIVA test would be the logical alternative in order to assess the performance of vaccination in conjunction with a test and removal strategy. Failure to remove DIVA-positive animals will result in over-challenge of susceptible animals, assuming that the infectiousness of animals increases as the disease progresses. This highlights the importance of having a reasonable estimate of the test performance of the DIVA test before the vaccine efficacy trials start.

On the other hand, if the vaccine is effective, the power of the study will increase if infected animals are not removed as this will result in more exposure to non-infected animals and thus less time will be needed to evaluate the performance of the vaccine. However, this does not necessarily reflect the way a vaccine would be applied in a control programme: thus, over-exposure of vaccinated animals to infection is a potential risk that needs to be considered.

Movement of animals during the field trials should be carefully documented since it can influence the conditions of the trial (introduction of vaccinated animals in unvaccinated herds and vice versa). Although cattle movement restrictions may limit the willingness of farmers to participate, movements of animals to farms not participating in the trials should be forbidden. If vaccinated animals are moved outside the vaccine areas, they are likely to test positive in the normal skin testing scheme, and blinding will be difficult. Moreover, these animals pose a risk because of the uncertainty of their bTB status, as the DIVA test has not been validated and vaccine performance is not known. Taking all of these issues into account, only cattle movements between farms included in the trial or to the abattoir should be allowed.

Experimental studies have shown that vaccination of calves with BCG as early as one day until six weeks after birth results in successful protection against challenge with *M. bovis*. Neonatal vaccination has been shown to induce significant reductions in bTB lesions and bacterial burden that were not observed in calves aged five to six months (Siddiqui et al., 2012; Thom et al., 2012). A key difference between neonatal calves and older animals that could be important for vaccination and immune response induction is the relatively high circulating numbers of innate T cells in young calves. The adaptive immune system is relatively immature in neonates and increased numbers of innate effector T cells present in young animals may enable effective immune responses to vaccination (Siddiqui et al., 2012). Notwithstanding the complex performance issue of the IFN- γ assay in young calves, an SOP will be required that defines the minimum age at which neonates will be vaccinated during the field trials. In addition, animal age at vaccination should be recorded and analysed to determine any age effect on generation of protective immunity. Although results from experimental studies suggest that pregnancy does not influence susceptibility to *M. bovis* challenge (Buddle et al., 1994), the effect of pregnancy on the response to BCG vaccination and bTB diagnosis has not, to date, been clearly elucidated. For this reason, the pregnancy status of cows vaccinated with BCG should be recorded and analysed to determine any impact on protection generated by BCG.

The effect of revaccination with BCG Danish on enhancement of protective immunity remains unclear. A number of human trial studies have failed to detect any protective effects of BCG revaccination (Sepulveda et al., 1992; Group KPT, 1996; Tala-Heikkila et al., 1998; Leung et al., 2001). In contrast, the authors of two other studies, conducted in Hungary (Lugosi, 1987) and in Poland (Kubit et al., 1983), concluded that BCG revaccination might be useful. A study conducted in calves in New Zealand showed that revaccination after a short interval (six weeks) had a detrimental effect on the pathology compared with a single vaccination, following experimental *M. bovis* infection

(Buddle et al., 2003). It was postulated that the time interval between vaccination and revaccination might be crucial in determining whether protection is reduced or enhanced. If applied in the field trials, the impact of revaccination on protection should ideally be addressed.

The eradication of bTB from cattle herds in the UK is compromised because infected wildlife species, such as Eurasian badgers (*Meles meles*), share the same environment and contribute to transmission of infection (Godfray et al., 2013). Quantification of the contribution is difficult to assess, as the nature of the interactions required for transmission is unknown. In the field trials, susceptible cattle will be under infection pressure from both infectious cattle and, where present, infectious badgers. The contribution of badgers to the local epidemiology of bTB will need to be considered in the analysis of field trial data if herd or region is taken as the unit of interest, as it may impact on the performance of the vaccine in cattle.

Since differences in the level of protection conferred by the different BCG vaccine strains have been reported in non-bovine species (Ritz et al., 2012; Venkataswamy et al., 2012; Zhang et al., 2013), differences in the protection conferred by the BCG Danish against different *M. tuberculosis* complex species and strains might be expected. Although a cross-protection is assumed, there is no strong scientific evidence to support the theory that similar levels of protection in cattle will be conferred against strains with different virulence, or against other mycobacteria causing bTB in cattle such as *M. caprae*. In this sense, it is important to characterise the isolates causing bTB in the herds included in the field trials in order to evaluate any significant difference in the levels of protection conferred by the BCG.

4.1. Theoretical concepts of vaccine safety

The use of a bTB vaccine may induce local or systemic reactions in the vaccinated animals. In addition, there may be an impact on animal health at the herd level if vaccinated animals are shedding the vaccine strain leading to infection among in contacts. There could also be a risk that a subset of the human population (e.g. immunocompromised persons) could become infected with BCG Danish, if in contact with vaccinated cattle, leading to the development of an associated pathological response (Murphy et al., 2008).

4.2. Current scientific knowledge and data gaps on the candidate bTB vaccine safety

The BCG Danish vaccine strain is an attenuated *M. bovis* strain and it is expected that it will be safer than a virulent field strain. Some safety data are available after the use of this vaccine in different animal species.

The BCG vaccine was administered to six captive badgers by either the subcutaneous or intramuscular route (Lesellier et al., 2006). Tracheal aspirates, saliva and urine were analysed by bacteriology. No clinical sample obtained during this study yielded a positive culture result for the BCG vaccine strain. A biopsy of skin and underlying subcutaneous tissue from one badger was taken from a site of high-dose subcutaneous BCG injection 371 days after administration. This biopsy revealed considerable granulomatous inflammation, including some necrosis, and numerous acid-fast bacilli at the site of injection. The large number of bacilli present and the fact that the lesion was not contained by fibrosis suggest that dissemination of BCG within this animal was a possibility.

White tailed deer were inoculated with by either the subcutaneous or oral route with a vaccine containing the BCG Danish strain (Palmer et al., 2010). The deer were examined 2 weeks and 1, 3, 5, 7, 9 and 11 months after vaccination. After subcutaneous administration, the vaccine strain was not isolated from any sample of muscle; or from mandibular, parotid, medial retropharyngeal or mediastinal lymph nodes; lung or liver. Although the latest date at which BCG could be isolated from the right superficial cervical lymph node (draining the site of vaccination) was three months after vaccination, BCG was isolated from sites such as the hepatic lymph node and mesenteric lymph node as late as nine months after vaccination. The BCG strain was isolated at the injection site in one deer one month after subcutaneous vaccination.

The BCG Danish vaccine was administered by the subcutaneous route to pregnant cattle in laboratory conditions (Glyn Hewinson, AHVLA, personal communication, 2013). The histopathology showed that acid fast resistant bacteria were present at the injection sites (left and right side) of one animal vaccinated during the first trimester of pregnancy (one out of six vaccinees) and four animals vaccinated during the last trimester of pregnancy (four out of eight vaccinees). The culture data indicate that the BCG strain was present in the left pre-scapular lymph node two days after the vaccination of a heifer. Saliva samples were taken from the BCG vaccinated heifers on days 0, 1, 3, 5, and 7 post-vaccination and thereafter weekly throughout the study. The BCG strain was not detected by culture in saliva (755 samples), in urine (50 samples), in faeces (734 samples) or in milk/colostrum (363 samples). The study is not sufficient to draw any conclusions with regard to the presence of the vaccine strain in milk, as it does not correspond to the worst case scenario, i.e. vaccination of lactating cows and milk sampling during the period immediately following the vaccination.

The vaccine was administered by the subcutaneous route to calves in laboratory conditions (Glyn Hewinson, AHVLA, personal communication, 2013). Faeces samples, saliva swab samples and a sample of the following organs/lymph nodes were taken for BCG culture: muscle adjacent to each injection site, right and left draining pre-scapular lymph nodes, a pool of the caudal mediastinal lymph node and the left bronchial lymph node, ileocaecal mesenteric lymph node, left and right kidney, liver and a sample of urine. The samples were taken 16 weeks after the first vaccination. Of the 584 clinical and *post mortem* samples cultured for BCG (obtained from 17 calves), two samples, the right pre-scapular lymph node from one calf and the left pre-scapular lymph node from another calf, gave low-level positive results for BCG and were confirmed by spoligotyping. A histopathological examination was performed on different organs and lymph nodes. Three animals showed acid-fast resistant bacteria at one of their inoculation sites when observed microscopically.

The field trials have to provide data to confirm the results observed in cattle vaccinated under laboratory conditions according to the requirements of Commission Directive 2009/9/EC. Taking into account the likely route of transmission of *M. bovis* and the potential risks to animals, the field trials should also provide data related to the potential shedding of the BCG vaccine Danish strain via the respiratory route and milk in order to allow risk assessment of trade in vaccinated animals.

The follow-up investigations of shedding should be conducted on a sufficient number of animals after the primary vaccination, and revaccination if performed. Nasal and milk samples should be taken from the day after vaccination/revaccination and for at least two weeks. If the microorganism is still detected after two weeks, analysis should be extended to define the duration of BCG Danish strain shedding. The samples should be tested for presence of BCG Danish using culture and/or PCR and the BCG isolates should be characterised (e.g. spoligotyping, variable-number tandem repeat (VNTR) analysis).

CONCLUSIONS

- The strategy of establishing and attempting to simultaneously evaluate DIVA and vaccine performance in the same field trial poses considerable risks, because only limited conclusions about vaccine performance can be made if test performance is later found to be poor.
- Evaluation of DIVA test performance (sensitivity and specificity in vaccinated animals) under field conditions should be based on a representative sample of the target population. Any proposed deviations from such a design should be justified and the bias that may subsequently be introduced should be accounted for.
- At slaughter, all trial animals should be examined in greater detail than is the case with the established routine *post-mortem* inspection, in order to increase the diagnostic sensitivity of the procedure and enable better estimation of the performance of the DIVA test. Furthermore, in a select number of animals, the determination of the true disease status should be improved by slicing through and inspecting all pre-defined sets of lymph nodes, and by applying supplementary tests.
- The DIVA test(s) should be standardised using an SOP and quality assurance that includes, but is not limited to, sampling, test setup, test data analysis and test interpretation. Training in the correct procedures for sampling should be provided to all relevant persons involved in the field trials. Variations in test parameter can be introduced during the trials, but any change in test format, including the use of new antigens, should be carried out in addition to the original DIVA test setup.
- The design of the trials for evaluating DIVA test and vaccine performance, with respect to numbers, duration and the sampling scheme of the animals, should be informed by modelling before the onset of the trials.
- The double-blind RCT design is recommended as it guarantees the lowest possible level of bias. Any proposed deviations from the preferred trial design should be justified and any biases that may subsequently be introduced should be accounted for.
- Estimates of the reproductive number should be sufficiently precise to enable conclusions to be drawn about the performance of bTB vaccination, in conjunction with test and cull using the DIVA tests, for bTB eradication.
- The risk posed by transmission of bTB infection from wildlife to cattle enrolled in the field trials should be considered and accounted for in any trial design.
- Data generated in the trials for DIVA and vaccine performance should include detailed quantitative test signals at the level of the individual animal and data normally recorded in identification and registration systems. At the herd level, annual official bTB status for the previous five years should be provided, as well as possible risk factors associated with both the risk of bTB introduction and within-herd bTB transmission.
- Data collected should enable estimation of vaccine-induced reduction of bTB transmission, and not just reduction of bTB incidence, in order to reach a conclusion regarding the contribution of vaccination to eradication.
- The field trials should provide safety data to confirm the results obtained in laboratory conditions according to the requirements of the Commission Directive 2009/9/EC. Specifically, data regarding the potential shedding of the BCG vaccine Danish strain via the

respiratory route and milk should be provided in order to allow risk assessment of trade in vaccinated animals.

RECOMMENDATIONS

- A simulation analysis of potential trial results should be performed prior to the start of the trials to ensure that sufficient data are collected during the trials, appropriate to the analytical methods to be used. There is also a need to ensure that the data required for test validation and vaccine performance can indeed be obtained, and that the DIVA test and BCG vaccine performs according to expectations based on the laboratory experiments, while taking account of the many factors that may generate additional variation in the field.
- DIVA test performance should be analysed as early as possible in the trials, while continuing to generate more data for analysis of vaccine performance in greater detail. Once the results from test validation are known, the analysis for the power of the field trials for vaccine performance can be repeated, using the updated results.
- The use of the tuberculin skin test should be avoided if possible as its use would limit the ability to double-blind during the field trials.
- Matched-pairing of herds should be considered if herd or region is included as the unit of interest, to deal with the confounding effect of wildlife exposure.
- For all animals included in the trials, a record of all possible confounding factors during the trial period should be collated, to allow for optimal analysis of the field trial data and to obtain maximal results for the test and vaccine validation.
- Practical measures should be put in place to guarantee that vaccinated animals cannot move out of the trial area or out of the country.

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Regions selected for inclusion in the study should be coded using the ‘Nomenclature of territorial units for statistics’. In addition, the OFT status, the between-herd bTB prevalence and, the presence of wildlife reservoirs, environmental factors and any other variables used for the selection process should be recorded in order to fully characterise the region. The herds included in the study should also be described in terms of herd size, annual official bTB status in at least the previous five years, bio security measures in place, records of animal movements, housing, management and access to pasture. The longitude and latitude of the herd location should be recorded using the WGS84 geodetic system to allow linkage with spatial datasets for environmental factors.

Descriptors for animals included in the study should include species, breed, age at start of study, pregnancy status during the study, history of vaccination and veterinary treatments, and herd of birth. For each animal the date of entry into the study should be recorded. If the animal is culled, the date and reason should be reported and all the animals considered to have completed the study should be clearly identified. For each animal vaccinated, the frequency and dates of vaccination should be reported.

For each diagnostic test included in the study, the timing, conditions and reagents used should be fully described (e.g. by providing the appropriate SOP). All antigens used should be specified including dose/concentration and potency. For skin tests, the type (SICCT or SIT) should be recorded and *post-mortem* analysis in terms of detection of macroscopic lesions, culture or microscopy should be described (e.g. by providing the appropriate SOP). In the case of laboratory samples, the sampling event and subsequent handling should be recorded; this would include the date and time, sampling point (on farm or at slaughter), type of tissue and storage conditions. The individual measurements for all diagnostic tests should be reported, where possible as continuous variables. For skin tests the measurement would be skin thickening in millimetres in addition to the evaluation using the standard and severe criteria

- Methods assuming equal follow-up

Short description: Is generally based on attack rate quantification (but it could be defined using other quantities, but always estimating risk based on sample units, not on sample units per time) and assume that sample units are followed over time equally and that sample units that are followed for longer period of time have the same weight in the calculation of the relative risk as those units followed for a shorter time period.

Advantages:

- Simplicity.
- Easy to explain and understand.

Disadvantages:

- For trials with staggered entry and a single closure date, the assumption of equal follow up is not likely to hold.
- Does not allow for covariate adjustment.

- Methods accounting for differential follow-up

Short description: risk in this case is measured as sample units per time, implying that the equation (1) is then modified to accommodate time as well. For this method several models could be used, of which Poisson and time to event models as the most common ones. If we consider the Poisson model (Agresti, 2013), the underlying assumption is that the number of cases is following a Poisson distribution, for which a model considering covariates representing the vaccinated and control group is used to estimate the vaccine performance.

Advantages:

- Allows inclusion of covariates in the model, as well as baseline prognostic factors.
- It could also accommodate characteristics of the sampling design used, such as clustering, stratification, etc.

Disadvantages:

- Distributional assumption need to be verified.
- Do not differentiate between sample units having the event of interest, having different time elapse to experience the event between sample units, i.e., ignores the fact that the time to experience the event could be prolonged in the vaccinated group with respect to the control.

- Time-to-event methods (e.g. breakdown at herd level)

Short description: If the measure of risk is considered to be for example the time to a breakdown at a herd level, different statistical techniques are needed, and these should correctly account for the fact that the outcome of interest is strictly positive, that the event of interest might have occurred before the sample units were enrolled in the trial or might not occurred during the trial period, or lost-to-follow up the sample unit could also occurs. Specifically, survival analysis techniques (Collett, 2003) have been developed to deal with

these data characteristics, the most common used ones being proportional hazard models, accelerated failure time models and frailty models (dealing with dependence between sample units).

Advantages:

- Model directly the ‘hazard’ function, which is the instantaneous potential of disease, at a specified moment in time, t , given that a subject has been disease free up to time that particular time. It could be interpreted as a conditional rate; similar in concept to velocity or speed.
- Allows inclusion of covariates in the model, as well as baseline prognostic factors.
- Can also accommodate characteristics of the sampling design used, such as clustering, stratification, etc.

Disadvantages:

- Distributional assumption as well as additional assumptions, such as proportionality in the proportional hazard models needs to be verified, although semi-parametric models could be implemented, such as the proportional hazard model, relaxing the distributional assumptions.

GLOSSARY

BCG Danish	A live attenuated <i>Mycobacterium bovis</i> BCG Danish strain 1331 produced by the Staten Serum Institute in Copenhagen, Denmark
Candidate vaccine	BCG Danish
Efficacy	A measure of the benefit of a vaccine in clinical trials or other controlled studies (as it is defined for marketing authorisation dossiers)
Revaccination	One or more administrations of a vaccine used to maintain its initial protective effects induced by the initial vaccination
Sensitivity	The ability of a diagnostic test to correctly identify a single infected animal
Specificity	The ability of a diagnostic test to correctly identify an uninfected animal
Severe interpretation	A positive bovine reaction that exceeds the avian reaction by more than 2 mm (or where a positive bovine and a negative avian reaction are measured)
Standard interpretation	A positive <i>M. bovis</i> tuberculin reaction >4 mm greater than a positive or negative avian tuberculin reaction are classified as reactors, and those with a <i>M. bovis</i> tuberculin reaction exceeding the avian reaction by 1 to 4 mm are deemed inconclusive results.
Tuberculin tests	SIT and SICCT tests and IFN- γ assay using tuberculin

ABBREVIATIONS

AHVLA	Animal Health and Veterinary Laboratory Agency
AR	attack rate
ARU	attack rate unvaccinated study cohort
ARV	attack rate vaccinated study cohort
BCG	bacille Calmette-Guérin
BCG Danish	BCG Danish strain 1331
bTB	bovine tuberculosis
CMI	cell-mediated immunity
CVMP	Committee for Medicinal Products for Veterinary Use
DIVA	detect infected among vaccinated animals
DEFRA	Department for Environment, Food and Rural Affairs (UK)
DIVA IFN- γ assay	gamma interferon assay to detect infected among vaccinated animals
DIVA skin test	skin test to detect infected among vaccinated animals
EFSA	European Food Safety Agency
EMA	European Medicines Agency
EU	European Union
IFN- γ assay	gamma interferon assay to detect bTB-reactors
OD	optical density
OIE	World Organisation for Animal Health
OTF	officially bTB-free
PPD	purified protein derivative of mycobacterium strain
RCT	randomized controlled trial
RR	relative risk
SICCT	single intradermal comparative cervical tuberculin
SIT	single intradermal tuberculin test
SOP	standard operating procedure
UK	United Kingdom
VE	vaccine efficacy

VNTR variable-number tandem repeats

VMD Veterinary Medicines Directorate